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How azobenzene photoswitches restore visual responses to the blind retina

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Summary

Azobenzene photoswitches confer light sensitivity onto retinal ganglion cells (RGCs) in blind mice, making these compounds promising candidates as vision-restoring drugs in humans with degenerative blindness. Remarkably, photosensitization manifests only in animals with photoreceptor degeneration and is absent from those with intact rods and cones. Here we show that P2X receptors mediate the entry of photoswitches into RGCs where they associate with voltage-gated ion channels, enabling light to control action potential firing. All charged photoswitch compounds require permeation through P2X receptors, whose gene expression is upregulated in the blind retina. Photoswitches and membrane-impermeant fluorescent dyes likewise penetrate through P2X receptors to label a subset of RGCs in the degenerated retina. Electrophysiological recordings and mapping of fluorescently-labeled RGC dendritic projections together indicate that photosensitization is highly selective for OFF-RGCs. Hence P2X receptors are a natural conduit allowing cell type-selective and degeneration-specific delivery of photoswitches to restore visual function in blinding disease.

Keywords

Blindness; vision; retina; retinal ganglion cell; retinitis pigmentosa; retinal degeneration; photoswitch; azobenzene; ion channel; P2X receptor

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Author contributions

I.T., V.M., M.T., B.D. and J.M. performed electrophysiological experiments. R.B.F. performed qPCR experiments. V.M. and Z.H. performed YO-PRO imaging experiments. K.V. and E.F. performed retinal immunohistochemistry experiments. I.T. and R.H.K. designed the experiments and wrote the manuscript. R.H.K. supervised the project.

Introduction

Retinitis pigmentosa (RP) and age-related macular degeneration (AMD) are blinding diseases caused by the progressive loss of rod and cone photoreceptors. Downstream neurons in the retinal circuit survive but no longer respond to visual input. We have developed a vision restoration strategy that employs azobenzene photoswitches to impart light sensitivity onto voltage-gated ion channels in these neurons. We showed that photoswitches can restore electrophysiological and behavioral light responses in mutant strains of blind mice (Polosukhina et al., 2012). One promising photoswitch, named DENAQ, acts primarily on retinal ganglion cells (RGCs). Intraocular injection of DENAQ enables moderate intensity white light to trigger action potential firing in RGCs, restoring visual signaling through neural circuits in the brain (Tochitsky et al., 2014).

Our studies on DENAQ-treated retinas revealed a surprising feature. While the compound photosensitizes RGCs from mutant mice with degenerated rods and cones, it has almost no effect on RGCs from wild-type (WT) mice with healthy retinas (Tochitsky et al., 2014), suggesting that the death of rods and cones leads to changes in RGCs that allow DENAQ to bestow light sensitivity. Previous studies have characterized structural and functional changes in the retina that occur subsequent to photoreceptor degeneration, collectively known as retinal remodeling (Jones et al., 2012). Over time, synaptic connectivity is altered and neurons gradually migrate, disrupting the highly layered architecture of the retina. The electrical excitability of retinal neurons is also altered (Sekirnjak et al., 2011) perhaps as a result of changes in gene expression of ion channels and neurotransmitter receptors (Cheng and Molday, 2013; Uren et al., 2014). Despite the many changes in the retina during remodeling, RGC connections to the brain remain intact, encouraging the idea that photosensitizing these cells might restore light perception and perhaps image-forming vision to blind animals. The aspects of retinal remodeling which lead to photoswitch degeneration-specificity are an intriguing puzzle, but the phenomenon is also of potential therapeutic significance because it implies that photosensitization will be constrained to local degenerated regions of the retina (e.g. geographic atrophy) while having minimal effects on regions that are still healthy.

Two events must occur for a photoswitch compound to photosensitize a cell. First, the compound, which carries a permanent positive charge, must cross the surface membrane into the cell. Second, it must bind to a voltage-gated ion channel, allowing the channel to be controlled with light (Banghart et al., 2009). Biophysical features of photoswitching and its persistence for hours after the compounds are removed from the extracellular saline indicate that ion channel control is exerted from the intracellular side (Banghart et al., 2009). We have shown that Hyperpolarization-activated, Cyclic Nucleotide-gated (HCN) channels are the crucial type of channel photosensitized by DENAQ in RGCs (Tochitsky et al., 2014), but here we explore how DENAQ and related photoswitches cross the plasma membrane in the first place, and ask if this process is also altered during retinal remodeling following photoreceptor loss.

Specifically, we focus on P2X receptors, ionotropic receptors that are activated by extracellular ATP (Burnstock and Kennedy, 2011). ATP is elevated in many tissues in

response to traumatic injury, inflammation, and degenerative disease processes (Tewari and Seth, 2015). Long-term activation of some types of P2X receptors triggers pore-dilation, allowing permeation of large (up to ~900 Da) molecules that are ordinarily membrane-impermeant. We have previously shown that activation of large-pore Transient Receptor Potential (TRPV1) channels in nociceptive neurons from dorsal root ganglia enables the entry of QAQ, a divalent cation photoswitch that is normally excluded from cells (Mourot et al., 2012). Here we report that photosensitization of RGCs by DENAQ, QAQ, and other photoswitches is mediated by P2X receptors. Hence, these receptors serve as a conduit that allows these compounds to enter into RGCs, but this only occurs after retinal remodeling.

In healthy retina, RGC light responses are heterogeneous, with some cells firing action potentials at light onset (ON-RGCs), some at light offset (OFF-RGCs) and some responding to both light onset and offset (ON/OFF-RGCs). In degenerated retina, chemical photosensitization could conceivably be indiscriminate, for example causing all RGCs to fire at light onset. Having disparate types of RGCs simultaneously respond to light might be a confusing perceptual signal for visual regions in the brain, whereas selective photosensitization of only one class of RGCs might be easier to interpret. Remarkably, we find that P2X-receptor-mediated photoswitch photosensitization is not indiscriminate, but rather selective for OFF-RGCs in the blind retina. The mechanisms of disease-specific and cell-selective photosensitization provide important insights into the pathology of retinal degeneration and may be advantageous for the use of photoswitch compounds as vision-restoring drugs.

Results

Retinal ganglion cell photosensitization requires photoreceptor degeneration

In pursuit of compounds suitable for treating human blindness, we have developed a family of photoswitches with different photochemical properties and different specificities for voltage-gated ion channels (Fig. 1A, B). AAQ blocks hyperpolarizing K^+ channels and depolarizing HCN channels (Fortin et al., 2008; Ko et al., 2016). Short wavelength light (380 nm) photoisomerizes AAQ from *trans* to *cis,* relieving channel block and longer wavelength light (500 nm) promotes *cis* to *trans* photoisomerization, restoring block. Channel blockade by AAQ can produce either a net hyperpolarization (Fortin et al., 2008) or depolarization (Ko et al., 2016), depending on which ion channel predominates in a cell. DENAQ and BENAQ convert from *trans* to *cis* with longer wavelengths of light (Mourot et al., 2011) (e.g. 480 nm) and quickly relax back to *trans* in darkness. In RGCs from degenerated retina, the effect of DENAQ and BENAQ is primarily on HCN channels, such that light leads to RGC depolarization and increased firing (Tochitsky et al., 2014). Finally, QAQ, which operates at the same wavelengths as AAQ, blocks a broader array of voltage-gated channels including Na^+, Ca^+, and K^+ channels, similar to local anesthetics (Mourot et al., 2012). For QAQ, Na^+ channel blockade predominates, such that 380 nm light suppresses firing and 500 nm light restores firing.

We used a multielectrode array (MEA) to record RGC activity and compare the photosensitization of the WT and *rd1* mouse retina by DENAQ, BENAQ, AAQ and QAQ. We eliminated contributions from rod- and cone-mediated phototransduction with a cocktail

of excitatory and inhibitory neurotransmitter receptor antagonists, blocking synaptic transmission throughout the retina (Tochitsky et al., 2014). All 4 photoswitches supported direct light-induced RGC firing (Figures 1C–F) in *rd1* RGCs. Therefore, at least a large part of the photoswitch-mediated light-response is autologous to RGCs.

We calculated the Photoswitch Index (PI) (see Experimental Procedures) to quantify the effect of light on RGC firing. DENAQ selectively photosensitized synaptically-isolated RGCs from *rd1* mice but not from WT mice (Figures 1C, 1G, 1H). BENAQ, a closely related derivative of DENAQ (Mourot et al., 2011), also photosensitized RGCs from *rd1* but not WT mice (Figures 1D, 1G, 1H). Degeneration-specific photosensitization also applied to QAQ (Figures 1E, 1G, 1H) and AAQ (Figures 1F, 1G, 1H). Thus, all of the compounds photosensitized RGCs in *rd1* retina, but not in WT retina.

We next asked whether the degeneration-dependence of photosensitization is limited to rapidly degenerating *rd1* mice or if it extends to other animal models that degenerate more slowly during postnatal life, as in human RP. We first compared the photosensitization of postnatal day 90 WT and transgenic S334-ter rats (Ray et al., 2010). In this rat strain, photoreceptors degenerate over several months after birth, owing to a rhodopsin mutation that is also found in some cases of human RP. BENAQ photosensitized the S334-ter but not the WT rat RGCs (Figures S1A–C). Likewise, DENAQ photosensitized RGCs from slowly-degenerating blind dogs (*rcd1* strain) but not from sighted dogs with healthy retinas (William Beltran, University of Pennsylvania, personal communication). These results, together with our previous studies on other mouse models of RP (Tochitsky et al., 2014) suggest that the degeneration-selective action of photoswitches might also occur in humans with degenerative blindness.

P2X receptors mediate photoswitch entry into RGCs

Our photoswitch compounds are permanently charged, which should make them relatively membrane-impermeant. We were especially surprised to find that QAQ, a divalent cation (Mourot et al., 2012), can enter into *rd1* mouse RGCs and photosensitize firing activity. In nociceptor neurons, photosensitization by QAQ requires large-pore ion channels, such as TRPV1 channels or P2X receptors (Mourot et al., 2012). Immunohistochemical studies have suggested that these channels are expressed in many types of neurons, including RGCs (Ho et al., 2016; Sappington et al., 2009).

To test whether large-pore channels mediate photoswitch entry in the degenerated retina, we applied photoswitches together with pharmacological antagonists. After washing with normal saline, we measured the amount of RGC photosensitization that had been induced. The non-selective P2X receptor antagonist TNP-ATP (North and Jarvis, 2013) prevented photosensitization by QAQ, AAQ, DENAQ and BENAQ (Figures 2A, 2B). PPADS, another non-selective P2X receptor blocker (North and Jarvis, 2013) also eliminated photosensitization. The selective P2X7 receptor blocker, A740003 (North and Jarvis, 2013), reduced photosensitization by ~50% (Figure 2B). By contrast, the selective TRPV1 antagonists AMG517 and capsazepine (Vriens et al., 2009) had no effect on photosensitization (Figure 2B).

These results implicate P2X receptors and not TRPV1 channels as the crucial conduit that enables photoswitches to enter into *rd1* RGCs *in vitro*. To confirm that P2X receptors also mediate photoswitch entry *in vivo*, we injected DENAQ alone or together with P2X receptor antagonists into the vitreous cavity of the eye. One hour after injection, we sacrificed the mouse, removed the retina, and recorded from synaptically-isolated RGCs with the MEA. We found that intravitreal injection of DENAQ selectively photosensitized *rd1* RGCs but not WT RGCs (Figures S3A–C). P2X receptor antagonists TNP-ATP or PPADS significantly reduced the photosensitization of *rd1* RGCs when injected intravitreally together with DENAQ (Figure S3C). Hence P2X receptors also mediate photoswitch entry *in vivo*. TNP-ATP had no effect when added after DENAQ had already been loaded into RGCs (Figures S3D, S3E). Hence P2X receptors are necessary for photoswitch loading, but they are not necessary for photoswitch action.

P2X receptors must be activated by ATP to enable photoswitch entry

P2X receptors are activated by extracellular ATP. Prolonged activation of certain P2X receptors enables the intracellular accumulation of large cations (Virginio et al., 1999). Having discovered that P2X receptors are necessary for photoswitch entry, we asked whether activation by extracellular ATP is also required. Treatment with the ecto-ATPase apyrase (Cohn and Meek, 1957) prior to and during DENAQ application reduced the photosensitization of *rd1* RGCs (Figure 2C). However, photosensitization was preserved when apyrase was applied together with ATPγS, a non-hydrolyzable ATP analog (Figure 2C). The rescue of photosensitization by ATPγS supports the conclusion that P2X receptor activation is necessary for photoswitch entry.

Intraocular ATP is known to be elevated in some retinal diseases, including neovascular AMD (Notomi et al., 2013) and diabetic retinopathy (Loukovaara et al., 2015). If ATP is also elevated in the RP retina, it could chronically activate the normal complement of P2X receptors, mediating enhanced DENAQ photosensitization. To test this possibility, we asked whether activating P2X receptors in WT retina with an exogenous agonist is sufficient to enable DENAQ photosensitization in WT RGCs. We found that bzATP, a selective P2X receptor agonist, did not enable the photosensitization of WT RGCs by QAQ, AAQ, DENAQ or BENAQ (Figure 2D). BzATP also had no effect on photosensitization of *rd1* RGCs by a sub-saturating (300μM) concentration of DENAQ. Hence while some ATP is necessary for photoswitch entry, elevating ATP alone is not sufficient to induce or enhance RGC photosensitization. This conclusion points to intrinsic changes in the *rd1* RGCs as critical for degeneration-specific photosensitization.

Some, but not all, of the photoswitches target HCN channels to photosensitize RGCs

All of the photoswitches enter RGCs through a common pathway, involving activated P2X receptors. But once the compounds are in RGCs, do they act on the same, or different, types of ion channels to mediate light-elicited firing? Our previous studies established that DENAQ-elicited photosensitivity in RGCs is mediated primarily by HCN channels (Tochitsky et al., 2014). We now find that photosensitization by BENAQ, like DENAQ, is eliminated by the highly selective HCN antagonist ivabradine (Postea and Biel, 2011) (Figures 3A, 3D), which also induces burst-like firing in RGCs (Bemme et al., 2014). By

contrast, ivabradine did not impair the ability of QAQ to photosensitize RGC firing (Figures 3B, 3D). This is consistent with our previous finding that QAQ does not block HCN channels, but instead has as its primary target voltage-gated Na^+ channels (Mourot et al., 2012).

For AAQ, we found a more complex effect of ivabradine. Rather than blocking photoswitching, ivabradine reversed its polarity, such that 380nm light reduced rather than enhanced RGC firing rate (Figures 3C, 3D). This result suggests that the primary target of AAQ in RGCs is again the HCN channel, which tends to depolarize the cells and enhance firing. However, when the HCN channels are pharmacologically blocked, photocontrol of K^+ channels begins to dominate, and these channels tend to hyperpolarize the cell and reduce firing. The reduced firing is similar to previously observed effect of AAQ on hippocampal and cerebellar neurons (Fortin et al., 2008). Taken together, these data indicate that DENAQ and BENAQ require HCN channels to render RGCs light sensitive, whereas QAQ and AAQ strongly affect other types of channels, and therefore do not require HCN channels.

Genes encoding photoswitch conduits and targets are up-regulated in degenerated retina

We next tested if the expression of P2X receptor genes is altered in *rd1* mice, as has been shown in mouse strains with other types of retinal degeneration (Cheng and Molday, 2013; Franke et al., 2005). Analysis by qRT-PCR (Figure 4A) showed a significant increase in expression of P2X4 (1.7 fold) and P2X7 receptors (2.5 fold) in *rd1* retina as compared to WT. These two isoforms form large conductance channels that can conduct organic cations (Browne et al., 2013). Expression of P2X2 and P2X3 receptors was reduced in the *rd1* retina, but since these isoforms are ordinarily expressed in cholinergic amacrine cells (Kaneda et al., 2004; Shigematsu et al., 2007), down-regulation of these genes in *rd1* may not involve changes in RGCs. P2X5 and P2X6 receptor expression was not significantly altered in *rd1* retina and mRNA for P2X1 was not detected, consistent with previous WT expression studies (Brandle et al., 1998). Immunofluorescent labeling showed that P2X7 receptors co-localized with RPBMS (Figures S3A–H), an RGC-selective marker (Rodriguez et al., 2014). We could resolve no significant difference in antibody labeling of P2X7 receptors between *rd1* and WT RGCs (Figures S3A–H), but immunohistochemistry may be insufficiently sensitive and specific to accurately report relatively small changes of P2X7 at the protein level.

We also analyzed the expression of genes encoding HCN channel subunits, the primary electrophysiological targets of DENAQ and BENAQ (Figure 4B). HCN1 is expressed in rod photoreceptors (Della Santina et al., 2012), so it is not surprising that the HCN1 transcript was greatly reduced in *rd1* retina (0.3 fold), similar to previous findings (Cheng and Molday, 2013; Uren et al., 2014). However, HCN3 and HCN4 were significantly up-regulated (increased by 2.7 and 2.1 fold, respectively). Immunolabeling has shown that these two HCN channel subtypes are found primarily in the inner retina, with HCN4 expressed in the dendrites, soma, and axons of RGCs (Stradleigh et al., 2011). These results, together with our previous characterization of HCN channel function in *rd1* RGCs (Tochitsky et al., 2014), demonstrate that both the channel conduits for photoswitch entry (P2X receptors) and

channel targets for photoswitch action (HCN channels) are up-regulated in the *rd1* retina, explaining, at least in part, the molecular basis of degeneration-specific photosensitization.

Finally, we measured the expression of several other RGC markers. We detected almost no rhodopsin (*Rho*) expression in the *rd1* retina (8×10^{-5} fold of normal) (Figure 4C), consistent with nearly complete loss of rods. We also found no significant change in the expression level of 3 RGC-selective marker genes (*Sncg*, *Pou4f1*, and *Thy1*), consistent with maintenance of a normal number of RGCs in *rd1* retina. Expression of all transcripts was normalized with respect to cellular housekeeping genes compensating for the decrease in total cell number in the *rd1* retina.

DENAQ exhibits no toxicity on healthy or degenerated retina

We previously showed that DENAQ injected into the eye has no apparent deleterious effects on the retinas of WT mice. However, now that we have established that photoswitches only enter into RGCs if the retina is degenerated, it is important to also evaluate toxicity in blind mice. Therefore we compared neuronal survival after injecting either DENAQ-containing or DENAQ-free saline in both WT and *rd1* mice. We counted cell nuclei at 1 and 7 days after injection, standard times for measuring acute and chronic retinotoxicity by drug candidates (Puthussery and Fletcher, 2009). In WT retina (Figs. S4A–B) the density of cell nuclei was the same in DENAQ- and vehicle-injected eyes at both 1 and 7 days. Likewise, the TUNEL assay showed no significant cell death after DENAQ injection (DENAQ-treated, (0.21±0.11 TUNEL vehicle-treated: 0.04±0.05, positive (green) cells/mm^2 retina, p=0.122) (Figures S4C–D), (vehicle = 0.06±0.06 vs WT, DENAQ 0.00±0.00 TUNEL positive cells/mm^2 retina, p=0.391). DENAQ caused no change in the thickness of individual retinal layers (IS/OS, ONL, INL, IPL, GCL; p>0.05) (Figures S4E–F) or thickness of the entire retina (vehicle-treated 110±4 vs DENAQ-treated 118±5 μm, p=0.26) in *rd1* and WT mice, measured at 1 and 7 days (Figures S4G–H) (*rd1* vehicle 69±5 vs DENAQ 71±4 μm, p=0.70). The degree of microglia activation, a sign of chronic retinotoxicity (Puthussery and Fletcher, 2009), was unaffected by DENAQ injection (Figures S4J–L). Finally, Müller glial cell morphology was unaffected by DENAQ in WT (Figure S4M) or *rd1* (Figure S4N). Müller glia cells in *rd1* retina display gliosis, but there was no additional effect of DENAQ (Figures S2O–P).

P2X receptor-permeant dyes label a subset of RGCs in a degeneration-dependent manner

To corroborate the increase in membrane permeability of RGCs in degenerated retina, we tested fluorescent molecules that can enter cells only through large-pore channels. YO-PRO-1, a dye similar in size (376Da) and charge to DENAQ (403Da), can enter cells through some types of activated P2X receptors to label cell nuclei (Virginio et al., 1999). Consistent with previous studies (Innocenti et al., 2004), in WT retina there were very few YO-PRO-1 labeled neurons in the ganglion cell layer (GCL). We did observe sparse labeling of some vascular cells or pericytes (Figure 5A), which are known to express a high density of P2X7 receptors (North and Jarvis, 2013). By contrast, in the *rd1* retina, YO-PRO-1 clearly labeled many cells within the GCL (Figure 5B). Hence there is a population of highly fluorescent cells present in *rd1* retinas and not in WT retinas, corresponding to cells that are permeable to YO-PRO-1 (Figures 5C and 5D).

To quantify the difference in labeling in WT vs. *rd1* retina, we first determined the total number of cells in a given field of view by counter-staining with Nuclear-ID, which labels nuclei of all cells. We then measured the fraction of cells labeled with YO-PRO-1 by setting a threshold of +2 standard deviations over the auto-fluorescence observed in unlabeled retina. This analysis showed a significantly higher fraction of cells that were YO-PRO-1 labeled in *rd1* retina than in WT retina (Figure 5E). The GCL contains cell bodies of displaced amacrine cells in addition to RGCs. The cell bodies of the two cell types can be of similar size, but only the RGCs have axons. To determine specifically whether the RGCs show enhanced dye permeability, we used FITC, a dye that distributes evenly throughout the cytoplasm of cells, including in axons, instead of concentrating in the nucleus. Like YO-PRO-1, FITC loading into cells requires the activity of large-pore ion channels (Browne et al., 2013). We observed many FITC labeled axons in the nerve fiber layer (NFL) in *rd1* but not WT retinas (Figures S5A–C) indicating the dye labeled RGCs.

TRPV1 channels and P2X receptors have pores of sufficient size to pass YO-PRO-1 into cells (Browne et al., 2013; Virginio et al., 1999). To determine which of these mediates YO-PRO-1 loading into *rd1* RGCs, we treated retinas with pharmacological blockers of these channels prior to and during dye-treatment. For this analysis, Student's t-tests were employed for pairwise comparisons only as the data set is undercomplete for an ANOVA analysis. The non-selective P2X receptor antagonist TNP-ATP completely eliminated YO-PRO-1 staining ($p<0.001$), reducing the fraction of YO-PRO-1 positive RGCs to the level found in WT retina ($p=0.590$) (Figure 5E). The selective P2X7 receptor antagonist A740003 also reduced staining ($p<0.001$), whereas capsazepine, a TRPV1 antagonist, had no effect ($p=0.46$). YO-PRO-1 labeling was also reduced by treating the retina with apyrase ($p<0.05$). Taken together, these results indicate that P2X receptors are the main conduits allowing entry of both YO-PRO-1 and photoswitch compounds into RGCs.

Genetic perturbation of P2X receptor expression alters RGC permeability to dyes and photoswitches

As an independent test of whether P2X7 receptors can support permeation of dyes and photoswitches into RGCs, we over-expressed the P2X7 receptor in the WT mouse retina by intravitreal injection of an AAV2 viral vector, with GFP as a co-expression marker (Figure S6). YO-PRO-1 failed to enter and label virally-transduced RGCs, but application of a P2X7R agonist after P2X7R overexpression promoted YO-PRO-1 loading, similar to that of *rd1* RGCs (Figures 6A, B). In non-transduced RGCs, the P2X7R agonist failed to promote YO-PRO-1 loading (Figures 6A, 6B). Taken together, these results show that WT RGCs can admit YO-PRO-1, but only upon overexpression and activation of P2X7Rs.

Does P2X7R over-expression also enable photosensitization? We found that neither DENAQ nor BENAQ, which act preferentially on HCN channels, photosensitized virally-transduced WT RGCs, even when a P2XR agonist was added. However, QAQ, a photoswitch that acts preferentially on voltage-gated Na^+ channels, photosensitized RGCs when co-applied with the agonist (Figure 6C, D). QAQ was ineffective when either the over-expressed P2X7 receptor or the agonist were absent. These results are consistent with the requirement of both

an active conduit (P2XRs) and an appropriate target ion channel to enable the photocontrol of RGC activity.

To further test the involvement of P2X7Rs in degeneration-specific photosensitization, we crossed *rd1* mice with P2X7R knock-out mice, to obtain a homozygous strain of blind mice that also lack P2X7Rs. While DENAQ-mediated photosensitization of RGCs was not eliminated, retinal MEA recordings showed that it was significantly reduced as compared to *rd1* retinas (PI=0.31 for P2X7R-KO; 158 RGCs in 3 retinal samples; PI=0.38 for *rd1*; 695 RGCs in 8 retinal samples; p=0.032; Mann-Whitney U test). Considerable photoswitching remained in the P2X7 receptor knock-out, consistent with other P2XR isoforms also contributing to DENAQ permeation. It is also possible that genetic removal of P2X7R leads to compensatory changes in the expression of the other isoforms.

Dye-labeling is specific for OFF-RGCs

Different types of RGCs play different roles in visual perception making it important to determine which types of RGCs are affected by photoswitches. Classifying ON, OFF, and ON/OFF RGCs based on physiological light responses is impossible in the fully blind *rd1* retina. However, the stratification of RGC dendrites to ON vs. OFF sublamina of the inner plexiform layer (IPL) is preserved until very late stages of degeneration (Mazzoni et al., 2008). This provides a morphological basis for RGC classification.

We used this approach to ask which RGCs accumulate YO-PRO. To visualize RGC dendrites we used a mouse line expressing a genetically-encoded fluorescent protein in a sparse assortment of cells, specifically, by expressing YFP under the control of the *Thy-1* promoter (O'Brien et al., 2014). This line was crossed with *rd1* mice to produce a *Thy1*-YFP-*rd1* strain, allowing visualization of the dendritic morphology of individual RGCs in a degenerated retina (Fig. 7A, B). We treated these retinas with YO-PRO-3 and determined which particular RGCs accumulate the dye. YO-PRO-3 binds to DNA and is therefore concentrated in the nucleus, whereas YFP is dispersed uniformly throughout the cytoplasm (Fig. 7C). We found that nearly all of the RGCs with YO-PRO-labeled nuclei had dendrites ramifying exclusively in the outer, presumptive OFF-sublamina (n=11 of 13) and none exclusively in the inner, presumptive ON-sublamina (n=0 of 17) (Fig. 7D, E). None of the 9 RGCs with bistratified dendrites were YO-PRO-labeled. Hence YO-PRO labeling in the *rd1* retina appears to be specific for OFF-RGCs.

Photosensitization is selective for OFF-RGCs

Since the same membrane conduits underlie photoswitch-loading and dye-entry, we wondered whether photoswitching, like dye-labeling, occurs selectively in OFF-RGCs. To answer this we obtained patch clamp recordings from individual RGCs to measure photosensitization by extracellular bath application of BENAQ and injected a fluorescent dye (AlexaFluor 488) through the patch pipette to map each cell's dendritic stratification. We first recorded RGC activity in the absence of synaptic blockers to determine whether upstream neurons were photosensitized and then applied synaptic blockers to measure RGCs' intrinsic light responses. We found a light-elicited increase in action potential frequency in RGCs with dendrites in the OFF-sublamina (n=5 of 6; Fig. 8A–C) which

persisted after we blocked synaptic inputs. RGCs with dendrites in the ON-sublamina did not respond to light either in the absence or presence of synaptic blockers (Fig. 8A–C; 1 out of 7; p=0.029, Fisher Exact Test), indicating that the ON visual pathway was not photosensitized. On average, light increased firing rate by 6.4 Hz in OFF-RGCs (n=6), but only 0.2 Hz in ON-RGCs (n=7), a significant difference (p=0.016) (Fig 8B). Light had no effect on firing in bistratified cells, classified as presumptive ON-OFF RGCs (n=6). Hence photosensitization is highly selective for OFF-RGCs.

Trans-DENAQ and *trans*-BENAQ block HCN channels (Tochitsky et al., 2014) and HCN channel function is necessary to photosensitize RGCs (Figures 3A, 3D). BENAQ's apparent selectivity for OFF-RGCs vs. ON-RGCs could be due to a difference in the light-elicited HCN current (I_h). To test this, we treated *rd1* retinas with BENAQ in the bath and measured I_h using whole-cell patch clamp recordings. Once again, dye was injected into the RGCs to visualize their dendritic stratification. We found that the light-elicited change in I_h was significantly larger in OFF-RGCs than in either ON-RGCs or in bistratified ON-OFF RGCs (Figures 8D, 8E, S7A). This larger light-elicited current is presumably sufficient to depolarize OFF-RGCs and cause them to fire, while being too small to trigger firing in ON-RGCs. The larger light-elicited current might be caused by a biochemical change that alters HCN channel sensitivity to photoswitches or may simply reflect an increased number of active HCN channels in OFF-RGCs. To test this possibility, we measured the current density of I_h in ON-RGCs and OFF-RGCs. We found that the I_h current density is higher in OFF-RGCs than in ON-RGCs (Figures S7B, C), suggesting a higher expression of these channels in OFF RGCs, which may be associated with the high level of spontaneous activity exhibited by OFF-RGCs in the blind retina (Sekirnjak et al., 2011).

To determine whether cell-type selective entry contributes to selective photosensitization of OFF *rd1* RGCs, we bypassed membrane conduits and included BENAQ in the patch pipette during whole-cell recording, allowing it to diffuse into the cytoplasm. Direct intracellular dialysis of BENAQ resulted in robust photosensitization of I_h in OFF *rd1* RGCs but not in ON *rd1* RGCs, (Figures 8F, 8G). This indicates a functional difference in the channels underlying I_h in ON vs OFF RGCs in the *rd1* retina. In contrast, BENAQ dialysis failed to photosensitize I_h in ON and OFF RGCs in WT retina (Figures 8F, 8G). Hence degeneration leads to a change in I_h, but only in OFF RGCs, enabling photoswitch compounds to impart light responses with cell-type selectivity.

Discussion

Photoswitching is degeneration-dependent and cell-type selective

Neurons can be photosensitized with optogenetic or optopharmacological tools (Kramer et al., 2013). Optogenetics involves the genetic expression of an exogenous protein (e.g. an opsin), which associates with an endogenous photoswitch (11-*cis* retinal). Optopharmacology involves the delivery of an exogenous photoswitch (e.g. BENAQ), which associates with an endogenous protein (a voltage-gated ion channel). For optogenetic control, the exogenous protein can be targeted to a particular cell type with a viral vector that incorporates a cell-selective promoter. Until now, there was no obvious way to target a particular retinal cell type with optopharmacological tools. Without cell-type specificity,

retinal neurons would indiscriminately respond to light, conceivably degrading the ability of the visual system to process and perceive images.

Our new findings overturn these assumptions about the optopharmacological approach. Surprisingly, photoswitch compounds are targeted to a particular cell type, not by a rationally designed engineering method, but by natural processes that occur upon rod/cone degeneration. In the degenerating retina, BENAQ nearly exclusively photosensitizes OFF-RGCs, sidestepping ON-RGCs and ON/OFF-RGCs. The preference for OFF-RGCs is also seen with normally cell-impermeant dye molecules such as YO-PRO.

All of our photoswitches enter RGCs through a common P2X receptor-dependent mechanism that is set in motion in degenerated retinas. However, different photoswitches act preferentially on different types of voltage-gated ion channels. Hence the primary target for DENAQ, BENAQ and AAQ is the HCN conductance, whereas for QAQ, it is the Na^+ conductance (Mourot et al., 2012). The net consequence of optical stimulation is an increase in firing rate in RGCs treated with any of the four photoswitch compounds, as *cis* photoisomerization relieves channel blockade in each case, depolarizing RGCs.

Our results do not exclude the possibility that photoswitches also have effects on presynaptic neurons in the retinal circuit. Pharmacological experiments show that the dominant effect of AAQ is on inhibitory amacrine cells, with bipolar cells and RGCs playing a less important role (Polosukhina et al., 2012). However, for all of the other photoswitches, neurotransmitter antagonists that isolate RGCs from synaptic inputs cause no reduction in the strength of light-elicited firing. Furthermore, the selective photosensitization of OFF-RGCs was observed in both the presence and absence of synaptic blockers. Hence for DENAQ, BENAQ, and QAQ, RGC-autologous effects dominate over synaptic-mediated effects.

Photoswitches provide new insights into retinal remodeling

These findings reveal new aspects of retinal remodeling that occur as a consequence of degenerative disease. The loss of rods and cones leads to progressive structural, functional and signaling changes that begin in the outer retina and spread gradually to the inner retina (Jones et al., 2012). The dendritic projections of RGCs in the IPL remain remarkably unchanged for >6 months after the loss of rods and cones in *rd1* mice (O'Brien et al., 2014) and their axonal projections to the brain also remain intact (Mazzoni et al., 2008). However, functional changes in RGCs occur much earlier (within the first 3 months), as revealed by photoswitches and dye molecules.

Degeneration-dependent remodeling must be initiated by a cue from the outer retina. The nature of the signal is unclear, but our findings suggest that photoreceptor death *per se* is necessary for the changes in RGCs to occur. Mutations that disrupt phototransduction and light-dependent synaptic signaling without leading to rod and cone degeneration do not enable DENAQ photosensitization (Tochitsky et al., 2014). Possible initiation cues include the loss of neurotrophic factor(s) (Leveillard et al., 2004), release of cytoplasmic constituents by degenerating photoreceptors, and disruption of the outer limiting membrane, which could allow retinal neurons to be exposed to RPE-derived retinoids (Lin et al., 2012) and other signaling molecules. Once the degeneration cue reaches RGCs, changes ensue that

allow permeation of photoswitches, dyes, and perhaps natural extracellular and cytoplasmic constituents. Genes encoding P2X4 and P2X7 receptor subunits are up-regulated, and the resulting channels mediate heightened membrane permeability to large molecules.

Remodeling also changes the electrical excitability of RGCs. Spontaneous firing increases in OFF-RGCs and decreases in ON-RGCs, attributable, at least in part, to cell-autologous changes (Sekirnjak et al., 2011). We find that I_h is larger and more sensitive to photoswitching in OFF-RGCs than in ON-RGCs. HCN channels contribute to spontaneous activity in many neurons and HCN blockers prevent the actions of DENAQ and BENAQ in RGCs (Fig. 3D and (Tochitsky et al., 2014)), leading us to propose that these channels mediate both the hyperexcitability and the photoswitch-sensitivity of RGCs. Thus, photoswitches inhibit spontaneous RGC activity in darkness when HCN channels are blocked, and photoisomerization increases RGC activity in the light when HCN channel blockade is relieved. To summarize our model, we propose that P2X receptor functional upregulation allows degeneration-dependent photoswitch entry and HCN channel functional upregulation allows degeneration-dependent photoswitch action. Both of these phenomena are more prominent in OFF-RGCs compared to other types of RGCs allowing for their selective photosensitization. The discovery of the mechanism for the disease-selective and cell-type selective photoswitching identifies new drug targets for potentially treating retinal degenerative disease and informs efforts to optimize photoswitch compounds for clinical use.

Implications for vision restoration

The goal of the photoswitch strategy is to develop a safe and effective drug that can restore some degree of visual perception to patients suffering from photoreceptor degeneration. A number of alternative methods for vision restoration are currently being developed both in the laboratory and in the clinic. Electronic retinal prosthetics such as the Argus II, the only FDA approved treatment for end-stage RP, have been able to restore some visual function to blind patients (Yue et al., 2016). Gene therapy approaches either focus on correcting the inherited defects that cause photoreceptor degeneration (Trapani et al., 2015) or seek to convert surviving neurons in the blind retina into artificial photoreceptors via the expression of optogenetic tools using viral vectors (Busskamp et al., 2010; Lagali et al., 2008). Stem cell-based treatments aim to deliver photoreceptor progenitors or other cells into the blind retina to replace cells lost due to disease (Jayakody et al., 2015). Neither gene therapy nor stem cell based treatments for vision restoration have yet been approved by the FDA, although some of them are currently in clinical trials (Kimbrel and Lanza, 2015).

The photoswitch approach offers a more straightforward path to the clinic than that offered by gene and stem cell based therapeutics, due to a more established FDA regulatory process for testing the safety and efficacy of small molecules. A drug-based treatment would be less invasive than implanting optoelectronic devices (Yue et al., 2016) and more reversible than delivering genes encoding optogenetic tools (Busskamp et al., 2010; Lagali et al., 2008). The degeneration-dependence of photoswitches is another advantage, raising the possibility that photoswitches may act selectively on diseased retinal tissue. Most RP or AMD patients exhibit regional degeneration, with some retinal regions exhibiting loss of rods and cones

and other regions remaining healthy for years. In RP, degeneration slowly progresses from the periphery to the center (Hartong et al., 2006), whereas AMD affects the macula, the central 2–3% of the retina, potentially causing a blind spot in the center of the visual field (Holz et al., 2014). Ideally, a vision restoring therapy would target these blind retinal areas without affecting healthy areas that are functioning normally. If the degeneration-dependence of RGC photosensitization in mice extends to humans, photoswitches might be useful for delivering light-sensitivity only where it is needed.

Likewise, selective photosensitization of particular types of RGCs might be advantageous for visual perception. The simultaneous activation of ON and OFF RGCs generates potentially conflicting signals, a possible factor in limiting the acuity of blind patients implanted with electronic chip prosthetics (Cottaris and Elfar, 2005). Optogenetic tools can be targeted with a cell-selective gene promoter to one cell type, for example ON-bipolar cells (Lagali et al., 2008), or remnant cones (Busskamp et al., 2010). Now we know that photoswitches can also be targeted to a particular cell type, OFF-RGCs, but by a natural process. It may seem imperfect that the compounds generate an ON-like response in these cells, eliciting activity at light onset, not offset. However, this sign reversal may be obviated with an optical display that projects negative images of visual scenes onto the retina. Reanimation of only the OFF-RGCs should, in principle, be sufficient to restore relatively normal vision, as patients with a dysfunctional ON pathway due to congenital stationary night blindness (Dryja et al., 2005) or Duchenne Muscular Dystrophy (Barboni et al., 2013) retain nearly normal photopic visual perception.

Broader translational implications

Our discovery of a disease-associated and cell type-selective change in P2X receptor function also has potential implications for treating other neurological disorders. Changes in P2X receptor expression or function accompany a wide variety of neurodegenerative disorders and P2X receptor ligands are being explored as potential treatments thereof (Burnstock and Kennedy, 2011). In this paper, we highlight an alternative potential approach to treating diseases involving purinergic receptor dysfunction – exploiting the large-pore property of P2X receptors to deliver membrane impermeable drugs to diseased cells and tissues for therapeutic benefit. This strategy may find broader application in treating other diseases of the nervous system.

Experimental Procedures

Chemicals

Photoswitch compounds were synthesized as previously described (Fortin et al., 2008; Mourot et al., 2012; Mourot et al., 2011). All other chemicals were purchased from Sigma-Aldrich or Tocris Bioscience.

Animals

Retinas from 3–6 month old mice and rats were used for most experiments. All animal use procedures were approved by the UC Berkeley Institutional Animal Care and Use Committee. Also see Supplemental Experimental Procedures (SEP).

MEA Recordings

For extracellular recordings, a flat-mounted retina was placed ganglion cell layer down onto an MEA system (MEA 1060-2-BC, Multi-Channel Systems). The MEA electrodes were 30 μm in diameter, spaced 200 μm apart, and arranged in an 8 × 8 rectangular grid. Photoswitches were added to the retina on the MEA for 30 min, followed by a 15 min wash. Extracellular spikes were high-pass filtered at 200Hz and digitized at 20kHz. A spike threshold of 4 SD was set for each channel. Typically, each electrode recorded spikes from one to three RGCs. Principal component analysis of the spike waveforms was used for sorting spikes generated by individual cells (Offline Sorter, Plexon). Also see SEP.

Light Stimulation

Light stimulation was done as previously described (Polosukhina et al., 2012; Tochitsky et al., 2014). Also see SEP.

Data Analysis and Statistics

We calculated the average RGC firing rate for individual retinas in light and dark for DENAQ and BENAQ treated retinas and RGC firing rate in 380nm and 500nm light for QAQ and AAQ treated retinas. In order to normalize light-elicited changes in firing rate of individual mouse and rat RGCs, we calculated the Photoswitch Index (PI). For DENAQ and BENAQ treated retinas, PI = (mean firing rate in light − mean firing rate in dark)/(mean firing rate in light + mean firing rate in dark). For QAQ and AAQ treated retinas, PI = (mean firing rate in 380nm light − mean firing rate in 500nm light)/(mean firing rate in 380nm light + mean firing rate in 500nm light). Unless otherwise stated, all statistical significance (p-value) calculations were performed using two-tailed unpaired Student’s t test. Results with $p < 0.05$ were considered significant. P-values for all experiments are: *<0.05, **<0.01, **<0.001.

P2X permeant dye loading, imaging, and analysis

Three month old *rd1* retinas were isolated and mounted onto windowed nitrocellulose filters. Isolated retinas were treated with 100nM YO-PRO-1, 100nM YO-PRO-3 (Life Technologies) or 5μM fluorescein isothiocyanate (FITC; Sigma Aldrich) in oxygenated ACSF for 15 minutes. Retinas were then transferred to a bath containing Nuclear-ID at a 1:2000 dilution for 3 minutes. Image analysis was performed using ImageJ. A threshold for YO-PRO loading was established by measuring the level of autofluorescence in each channel and finding a baseline value with +2SD being the threshold for a YO-PRO positive cell. Nuclear-ID was used to count the total number of cells within a field of view. The percentage of cells above the threshold was then calculated for comparison. Also see SEP.

Confocal microscopy

RGCs were filled 40μM Alexa Fluor 488 dye (Life Technologies) via diffusion from the recording pipette, and cell nuclei were stained with Nuclear-ID Red DNA (10μM) (Enzo Life Sciences) for the discrimination of inner nuclear layer (INL) and ganglion cell layer (GCL). RGCs were identified as ON, ON/OFF (bi-stratified) or OFF types based on the depth of their dendritic stratification in the IPL. RGCs stratifying in the outer 50% of IPL

were considered OFF RGCs, whereas the ones that stratified in the inner 50% of IPL were considered ON RGCs. Also see SEP.

Patch-Clamp Electrophysiology

Patch clamp recordings were made with a Multiclamp 700B amplifier (Molecular Devices). Cell-attached recordings were performed under voltage clamp at 0 mV and transient capacitive currents generated by action potentials across the somatic membrane were recorded from RGCs. Stimulus delivery and data acquisition were performed using pClamp 10.4 (Molecular Devices). Patch-clamp data were analysed with Clampfit 10.5 (Molecular Devices) and Origin 7.0 (OriginLab Corporation). Also see SEP.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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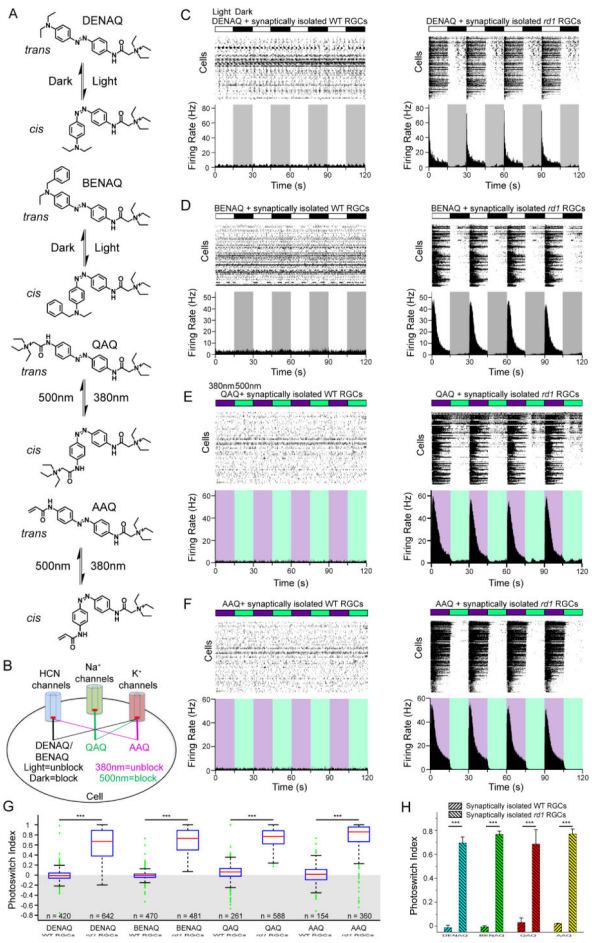

Figure 1. Photoswitch compounds photosensitize RGCs from *rd1* but not WT mice

A) Photoswitch structures. Visible light converts DENAQ and BENAQ from *trans* to *cis*; the compounds then quickly relax back to *trans* in the dark. 380nm light converts AAQ and QAQ from *trans* to *cis* and then the compounds can be converted back to *trans* by 500nm light.

B) Ion channels targeted by the photoswitches. DENAQ, BENAQ and AAQ block HCN channels and K^+ channels, while QAQ blocks Na^+ and K^+ channels but not HCN channels.

C–F) MEA recordings of RGC activity from synaptically isolated WT (left) and *rd1* (right) mouse RGCs treated with DENAQ **(C)**, BENAQ **(D)**, AAQ **(E)** or QAQ **(F)**. Light stimuli (top), raster plots of RGC activity (middle), average firing rate plots (bottom).

G) PI value box plots - median (red), $1^{st}/3^{rd}$ quartiles (blue) with outliers (green) for RGCs from photoswitch treated WT and *rd1* mouse retinas. Ns are RGCs. P-values obtained using rank sum test.

H) Average PI values from photoswitch treated synaptically isolated WT and *rd1* mouse retinas. Data are mean±SEM.

See also Figure S1, Table S2.

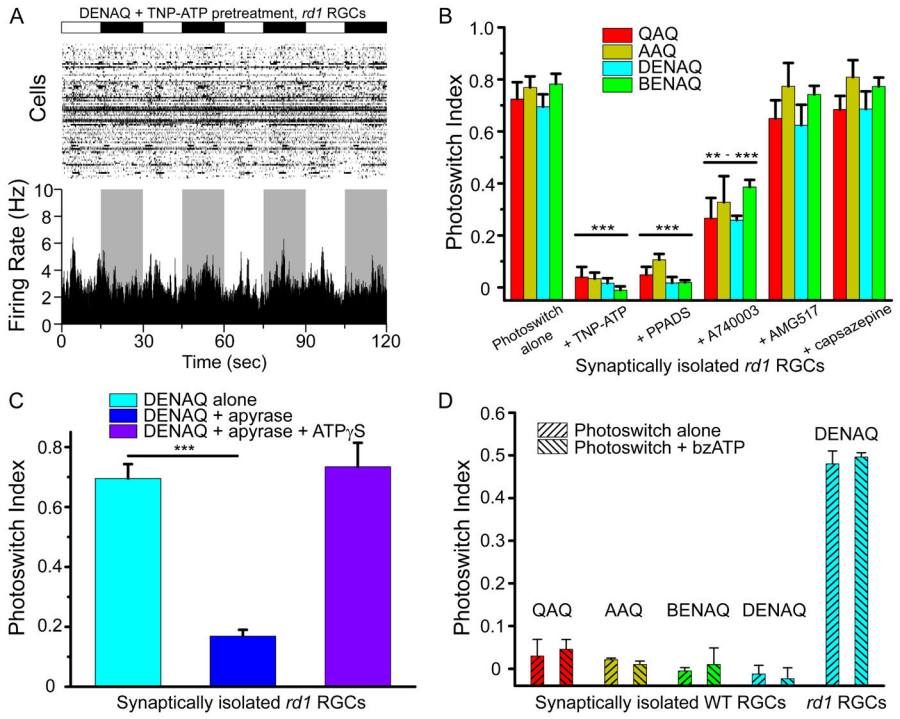

Figure 2. Photoswitches enter *rd1* RGCs through activated P2X receptors

A) Representative MEA recording from *rd1* RGCs treated with DENAQ and the P2X receptor antagonist TNP-ATP. Photoswitching is absent.

B) PI values from *in vitro* photoswitch treated *rd1* retinas after pretreatment with P2X and TRPV1 receptor antagonists.

C) PI values from *in vitro* DENAQ treated *rd1* retinas after pretreatment with apyrase with or without the addition of ATPγS.

D) PI values from *rd1* retinas treated *in vitro* with a photoswitch alone or with bzATP.

P values in panels **B–D** compare PI values for *rd1* retinas treated with photoswitch alone vs photoswitch + specified compound(s). All data are mean±SEM. See also Figures S2, S3, Table S2.

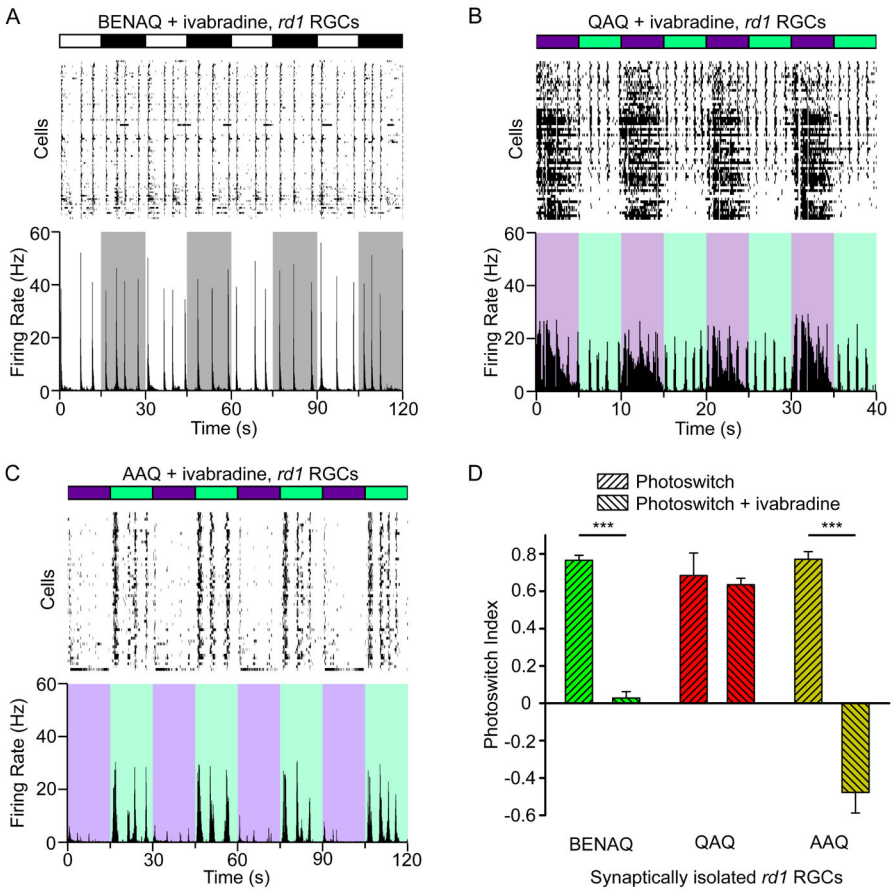

Figure 3. HCN channels are necessary for RGC photosensitization by some, but not all, photoswitches

A–C) MEA recordings from synaptically isolated *rd1* RGCs treated with BENAQ **(A)**, QAQ **(B)** or AAQ **(C)** and the HCN channel blocker ivabradine. BENAQ-treated RGCs **(A)** are no longer light sensitive after HCN channel block. Both QAQ-treated **(B)** and AAQ-treated **(C)** RGCs remain photosensitive even after HCN channel block, but the polarity of photoswitching is reversed in AAQ-treated RGCs **(C)**.

D) PI values from *rd1* retinas treated with various photoswitches alone or with photoswitches followed by ivabradine. Data are mean±SEM.

See also Table S2.

Figure 4. Genes encoding photoswitch conduits and targets are up-regulated in WT and *rd1* mouse retinas

A–C) Differences in gene expression in *rd1* retinas relative to WT retinas based on the ddCt values from RT-qPCR, normalized to β-actin (*Actb*) expression. Data are mean±SEM, n=5 retinas.

See also Figure S4, Table S1.

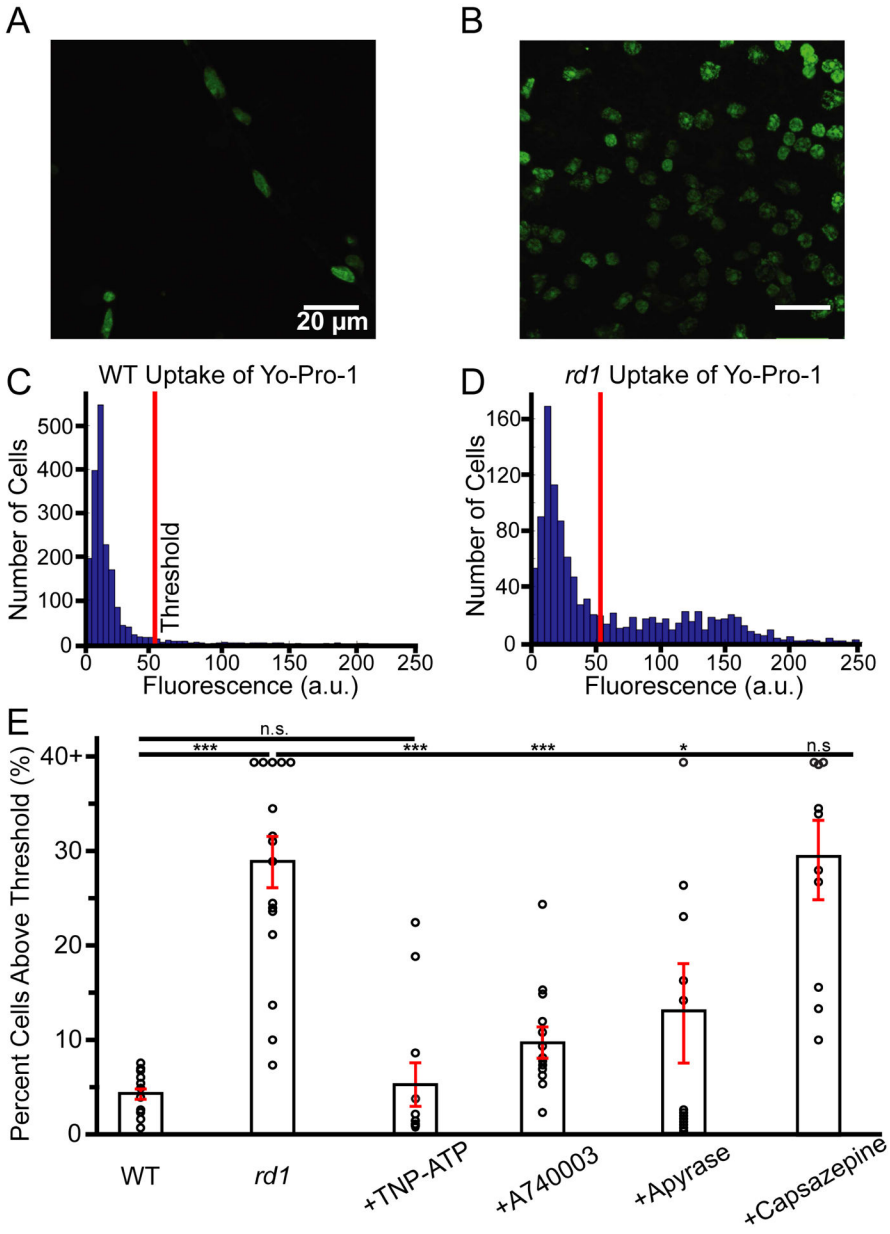

Figure 5. P2X receptors are necessary for dye entry into RGCs in the *rd1* retina

A) WT retina loading of YO-PRO-1 (green) shows loading primarily in vasculature with neuronal populations being almost completely absent of YO-PRO-1 labeling.

B) By contrast, a subpopulation of *rd1* RGCs are readily labeled with YO-PRO-1.

C–D) Fluorescence histograms of YO-PRO-1 labeling. The threshold for labeled cells was set at +2SD above autofluorescence values. YO-PRO-1 labels a larger fraction of *rd1* RGCs **(C)** compared to WT RGCs **(D)** ($p<0.001$).

E) Percentage of YO-PRO labeled cells in *rd1* and WT RGCs. YO-PRO labeled WT RGCs=3.7±1.3%, n=1854; *rd1*=29.0±6.9%, n=1106, $p<0.001$, two-tailed unpaired Student's t-test. P2X channel blockers TNP-ATP (5±2%) and A740003 (9±1.5%); and breakdown of

extracellular ATP with apyrase (12±5%) all reduced YO-PRO labeling while capsazepine had no effect. Data are mean±SEM.
See also Figure S5.

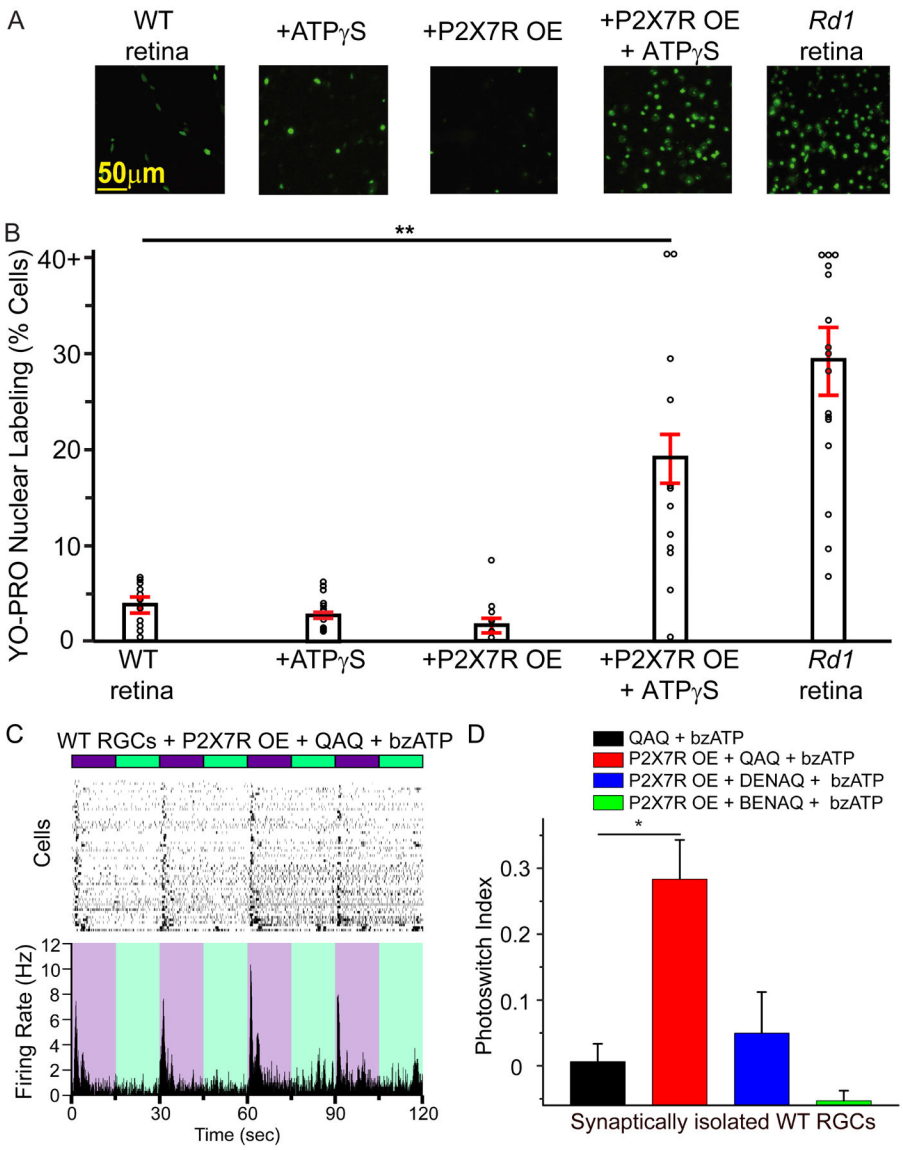

Figure 6. Activation of over-expressed P2X7R enables permeation of dye and photoswitch into WT RGCs

A–B) Confocal images (**A**) and quantification (**B**) of YO-PRO-1 loading (green) into WT RGCs. WT RGCs are not labeled with YO-PRO after addition of exogenous ATPγS or P2X7R overexpression (OE) alone. P2X7R OE together with the addition of ATPγS enable dye loading into WT RGCs (2×2 ANOVA $p<0.0001$, TukeyHSD Interaction $p<0.01$). By comparison, YO-PRO-1 robustly loads into *rd1* RGCs without any genetic or pharmacological manipulation.

C) Sample MEA recording from synaptically isolated WT RGCs overexpressing P2X7 and treated with QAQ+bzATP.

D) PI values from photoswitch treated WT mouse retinas overexpressing P2X7 and treated with bzATP. Data are mean±SEM.

See also Figure S6, Table S2.

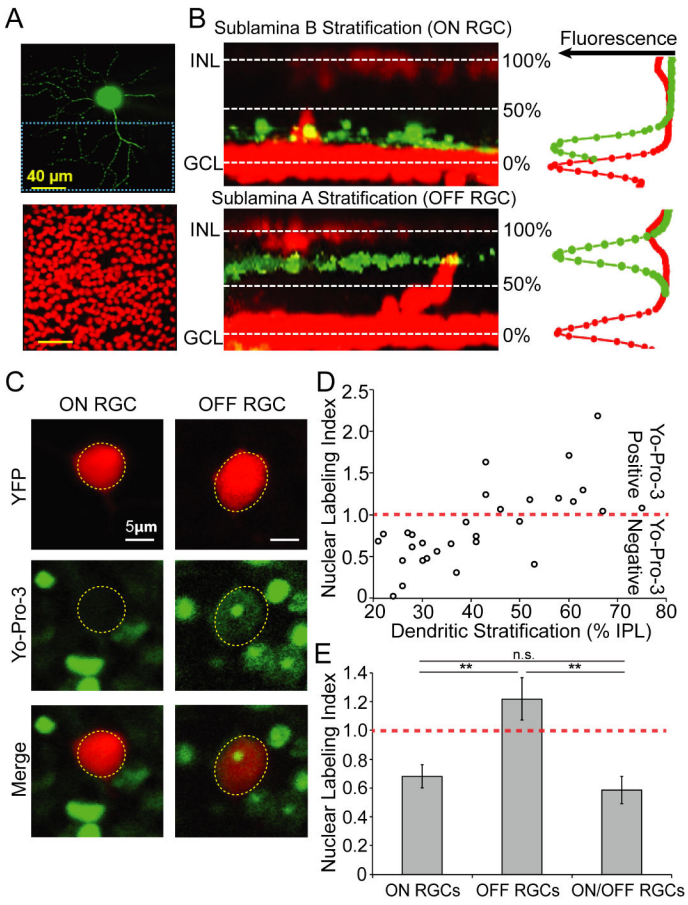

Figure 7. P2X receptor permeant dyes selectively enter OFF-RGCs in *rd1* retina

A) (Top) *Rd1* RGC filled with a cytoplasmic fluorophore (Alexa 488). The outlined area was aggregated to a single plane for analysis (Bottom) Nuclear-ID labeling of RGCs (red).

B) Orthogonal views of aggregated fluorescence data show the GCL and INL. The location of the peak of the dendritic fluorescence was used to differentiate between putative ON and OFF RGCs.

C) RGCs with dendrites stratifying in the ON sublamina lacked the nuclear labeling that is present in those cells whose dendrites lay in the OFF sublamina.

D) RGC YO-PRO nuclear fluorescence plotted as function of RGC dendritic stratification in the IPL. Cells with nuclear labeling index greater than 1 (red dashed line) are above threshold for labeling. Cells with dendrites deeper in the IPL (above black dashed line) are readily labeled by YO-PRO.

E) Nuclear labeling index for putative ON (n=17) and OFF (n=12) RGCs as well as bistratified ON/OFF RGCs (n=9) shows preferential loading in ON RGCs. Data are mean ±SEM. (1×3 ANOVA $p<0.001$, TukeyHSD $p<0.001$)

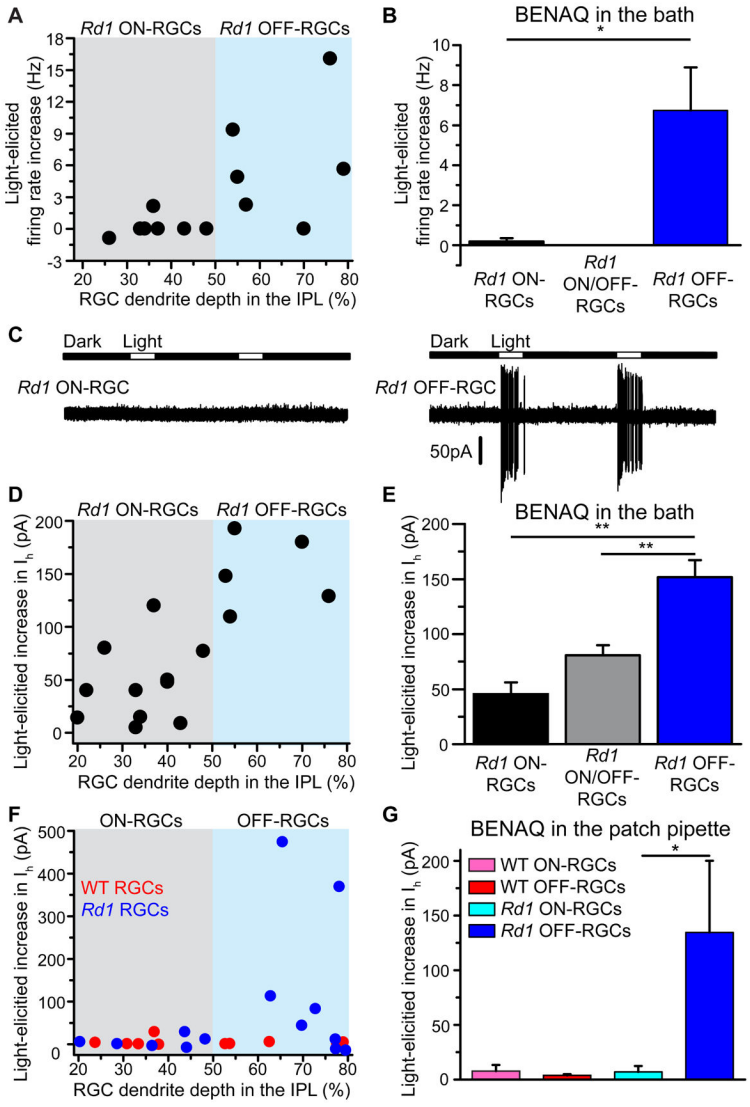

Figure 8. BENAQ selectively photosensitizes OFF-RGCs in *rd1* retina

A) Light-elicited increase in firing rate in BENAQ-treated *rd1* RGCs plotted in relation to the depth of the RGC dendritic stratification in the IPL. Bistratified ON/OFF RGCs are not shown. Putative ON-RGC/OFF-RGC classification shown in gray/blue.

B) Light elicits a larger firing rate increase in BENAQ-treated OFF-RGCs (mean=6.4 Hz, n=6) compared to ON-RGCs (0.2 Hz, n=7, p=0.016). Light evoked no response in ON-OFF RGCs (n=6).

C) Example traces from BENAQ-treated ON (left) and OFF (right) RGCs. Bar above the trace represents the presence or absence of light.

D) Light elicited increase in I_h in extracellular bath BENAQ-treated *rd1* RGCs plotted in relation to the depth of the RGC dendritic stratification in the IPL. Putative ON/OFF-RGC classification shown in gray/blue.

E) Average light elicited increase in I_h in extracellular bath BENAQ-treated OFF-RGCs (mean=151.8±15.5 pA, n=5), ON/OFF-RGCs (mean=80.6±9.3 pA, n=6, p<0.001), and ON-

RGCs (mean=45.3±10.7 pA, n=11, p<0.001, Bonferroni post hoc test). Data are mean ±SEM.

F) Light elicited increase in I_h in intracellular patch-pipette BENAQ-treated WT (red) and *rd1* (blue) RGCs plotted in relation to the depth of the RGC dendritic stratification in the IPL.

G) Average light elicited increase in I_h in intracellular patch-pipette BENAQ-treated WT ON-RGCs (mean=7.8±5.6 pA, n=5), WT OFF-RGCs (mean=3.9±1.0 pA, n=5), *rd1* ON-RGCs (mean=7.0±5.4 pA, n=6), and *rd1* ON-RGCs (mean=135±65 pA, n=8, p=0.046). Data are mean±SEM.

See also Figure S7.