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Photopharmacology for vision restoration

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Abstract

Blinding diseases that are caused by degeneration of rod and cone photoreceptor cells often spare the rest of the retinal circuit, from bipolar cells, which are directly innervated by photoreceptor cells, to the output ganglion cells that project axons to the brain. A strategy for restoring vision is to introduce light sensitivity to the surviving cells of the retina. One approach is optogenetics, in which surviving cells are virally transfected with a gene encoding a signaling protein that becomes sensitive to light by binding to the biologically available chromophore retinal, the same chromophore that is used by the opsin photodetectors of rods and cones. A second approach uses photopharmacology, in which a synthetic photoswitch associates with a native or engineered ion channel or receptor. We review these approaches and look ahead to the next generation of advances that could reconstitute core aspects of natural vision.

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Approaches to vision restoration

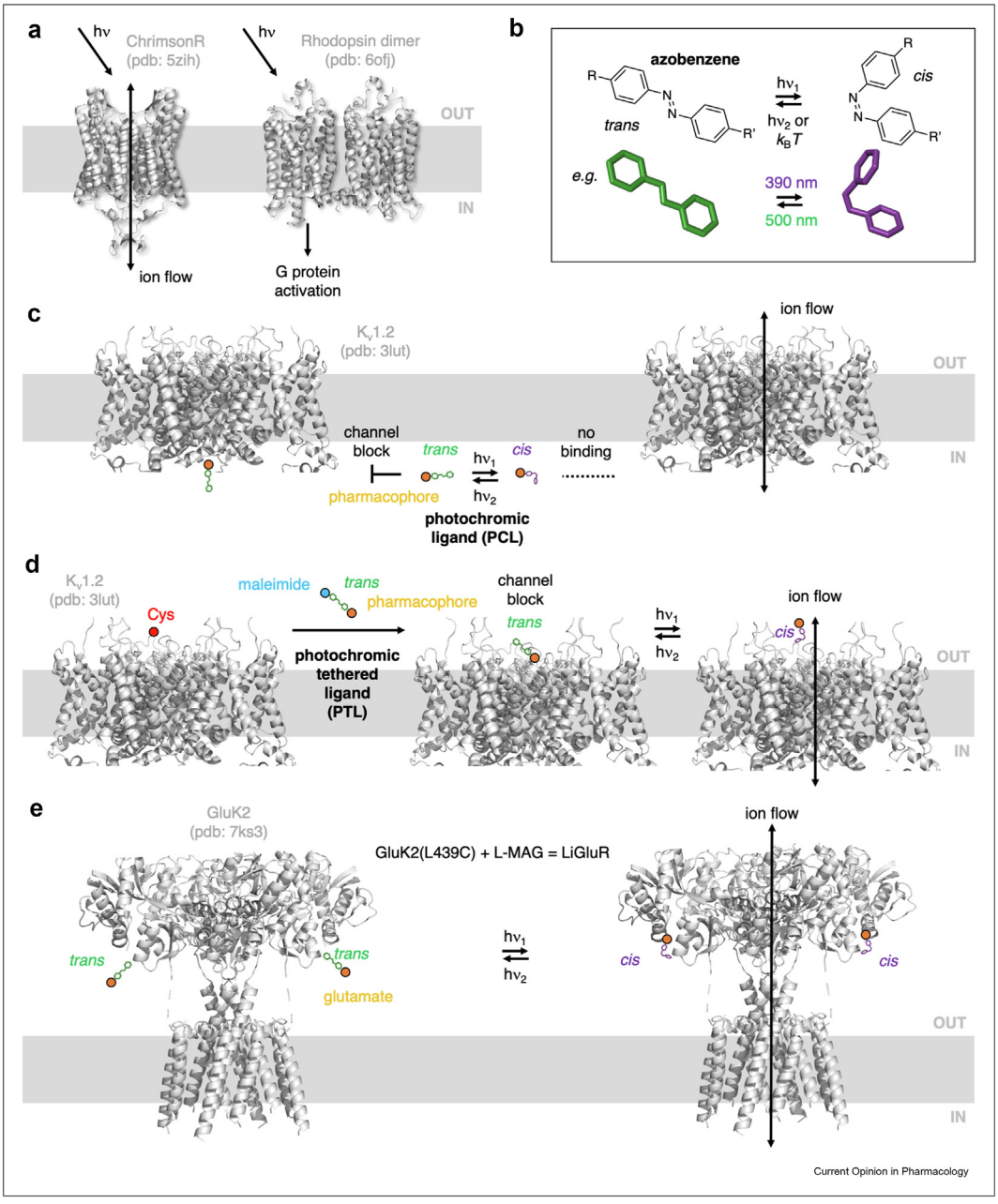
Inherited retinal degenerations, such as retinitis pigmentosa (RP), result in blindness due to progressive loss of rod and cone photoreceptor cells. However, the disease process spares other retinal cells, providing a potential target for therapy. There's hope that installation of light-sensitivity into the surviving cells could restore vision. Three approaches have been used to restore aspects of vision in models of RP. These include: 1) a 1-component gene therapy "optogenetics" approach, which employs either a microbial ion channel opsin (channelrhodopsin) or a G protein coupled receptor opsin from rods, cones or intrinsically photosensitive ganglion cells (ipRGCs) (Figure 1a), [1–9] 2) a 1-component small molecule "photopharmacology" approach, which introduces a synthetic light-sensitive drug that modulates native channels of surviving retinal cells (Figure 1b, c) [10–15], and 3) a 2-component "chemical optogenetics" approach, which combines gene therapy with photopharmacology (Figure 1d, e; Fig. 2), the focus of this review.

The pure gene therapy optogenetics approach has the appeal of being a "one-and-done" solution. In contrast, the 1-component and 2-component photopharmacology approaches combine gene therapy with pharmacology and require regular injection of the photoswitch to maintain a reservoir of the photochemical in the eye. This extra burden is counterbalanced by several advantages: the photochemical can be dosed, it can be upgraded as new photoswitches are developed, and the system can be turned off, if needed, by withholding the photoswitch.

1- and 2-component photopharmacology

The 1-component and 2-component photopharmacology approaches replace the native chromophore retinal with synthetic photo-isomerizable molecules. These engineered chemicals are designed to control classes of channels or GPCRs that have not evolved light sensitivity of their own (Figure 1c–e; Figure 2; Figure 3). The mechanisms of action rely on two main features: (1) the ability of the molecule to assume two photo-isomeric configurations with substantially different structures, robustness, reversibility and ease of incorporation with the ligand of interest. (2) The ability to bind and alter the function of the target protein that is restricted to only one photo-isomer state (Figure 3). The photo-isomer moiety that shares these

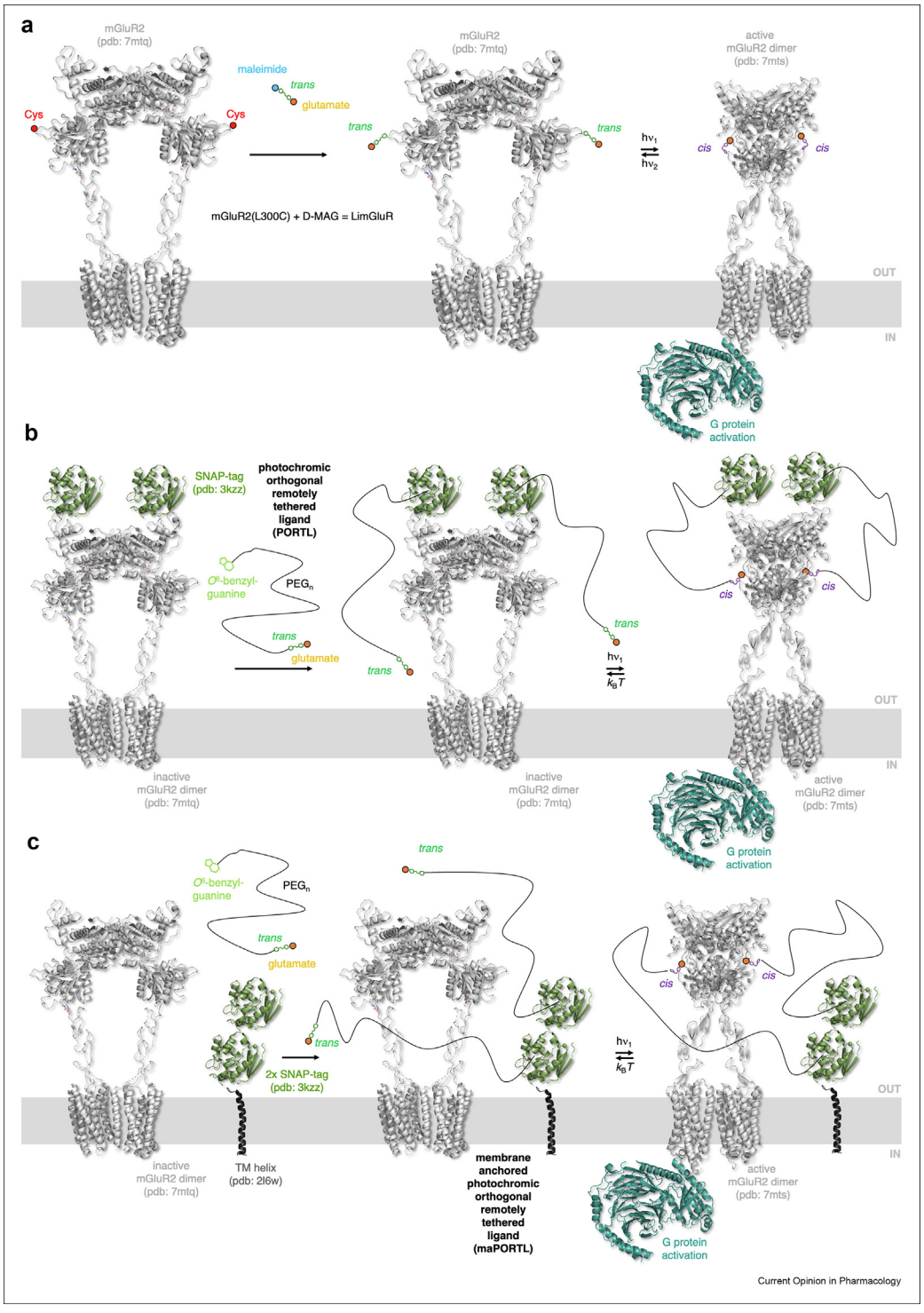
Figure 1



Naturally and endowed light-sensitive proteins. **(a)** Proteins that sense light to be translated into ion flows across the membrane or G protein activation comprise of ion channels (e.g. chrimsonR, pdb: 5zih [70]) or GPCRs (e.g. rhodopsin, pdb: 6ofj [71]), respectively (**b)** Small-molecule photoswitches, for example, azobenzenes, may serve as a scaffold that can be reversibly isomerized between its cis- and trans-state with orthogonal wavelengths of light or by thermal relaxation. (**c**). Photochromic ligands (PCLs) are soluble small-molecule photoswitches that act by diffusion and are attached to a pharmacophore. They can block an ion channel from the inside (e.g. the voltage-gated Kv1 potassium channel, pdb: 3lut [72]) in one state (trans) but not the other (cis). (**d, e**) Genetic precision can be achieved by labelling the protein of interest with a photochromic tethered ligand (PTL) *via* thiol-maleimide chemistry through an introduced cysteine with an appropriate photo-blocker of a Kv channel (**d**), or a photo-agonist or photo-antagonist of a kainate-type, dubbed LiGluR (**e**) (pdb: 7ks3 [73]).

features and has been the focus of most vision restoration efforts is azobenzene (Figure 1b). In the 1-component system, the therapeutic agents that have been developed are azobenzene-ligands (Figure 1c; Figure 3a, photochromic ligands or "PCLs"), where the ligand is a blocker that obstructs native ion channels in one permissive photo-isomer. A detailed review of 1-component photopharmacology was published recently [16]. In the 2-component system, a ligand-gated channel or GPCR is engineered to possess a unique

Figure 2



Photocontrol over metabotropic glutamate receptors (mGluRs). **(a)** A PTL approach using D-MAG on an cysteine-engineered mGluR2 subtype (pdbs: 7mtq, 7mts [74]) achieved optical control over G protein signalling. (**b)** SNAP tag fused proteins can be endowed with light sensitivity with photochromic orthogonal remotely tethered ligands (PORTLs). A self-labelling protein tag (e.g. SNAP, pdb: 3kzz(DOI: 10.2210/pdb3KZZ/pdb)) that is fused to the target protein and covalently reacts in a specific manner with O^6-benzylguanines to anchor a photoswitch on a linker that is long enough to permit the ligand to reach the ligand binding site. Upon illumination, the switch can activate the receptor and engage a G protein that amplifies downstream signaling. Such "photochromic orthogonal remotely tethered ligands" (PORTLs) have been applied to glutamate and dopamine GPCRs. (**c)** Native receptors can be optically controlled by outsourcing the SNAP-tag onto a membrane anchored alpha-helix (pdb: 2l6w [75]) (termed maPORTL or MP), which can be reacted with BGAGs to activate the receptor with light, similar to (**a**).

anchoring site to which an azobenzene-agonist tethers. The anchor can be a cysteine residue introduced near the agonist binding site, which attaches with chemical selectivity to the photoswitchable agonist (Figure 1d, e; Figure 2a; Figure 3b), photoswitchable tethered ligand or "PTL"). Alternatively, the anchor can be a protein tag, like SNAP, which is fused to the N-terminus of the receptor. The protein tag attaches with bioorthogonal selectivity to a PCL that contains a long linker to enable the photoswitchable ligand to reach from the attachment site to the agonist binding site (Figure 2; Figure 3c,d, Photochromic Orthogonal Remotely Tethered Ligand or "PORTL"). Photo-isomerization puts the agonist into a conformational state, which permits binding to activate the receptor. Genetic targeting enables the engineered receptor to be expressed in a specific cell type, localizing photosensitivity just as with optogenetics. This approach has been applied to ionotropic glutamate receptors (iGluRs), including kainate and NMDA receptors [17–23], and to metabotropic (GPCR) glutamate receptors (mGluRs) [24–26] a dopamine [27] and serotonin receptor [28].

The PTL photo-agonized kainate receptor, LiGluR (Figure 1e), allows neurons to be depolarized by gating the flow of non-specific cations in a light dependent manner [29,30]. LiGluR works in cultured neurons [31], assembled 3D neural networks [32] and cultured glia [33], was successfully used *in vivo* for the dissection of a behavioural module in the zebrafish spinal cord [34] and to reveal a form of fast retrograde control of presynaptic strength in Drosophila [35].

In the degenerating (*rd1*) retina, LiGluR expressed in retinal ganglion cells (RGCs) or ON bipolar cells (ON-BCs) introduced a robust light-evoked increase in spiking activity, resembling ON-type responses observed in wild type mouse [36,37] and healthy human retina [38,39]. This restored light sensitivity, provides light-guided navigation, as well as pattern discrimination to the RP mouse model [40,41]. LiGluR also successfully restored photosensitivity to a canine model of inherited blindness (*Rcd1*), suggesting that PTLs hold therapeutic value in large animal models [41].

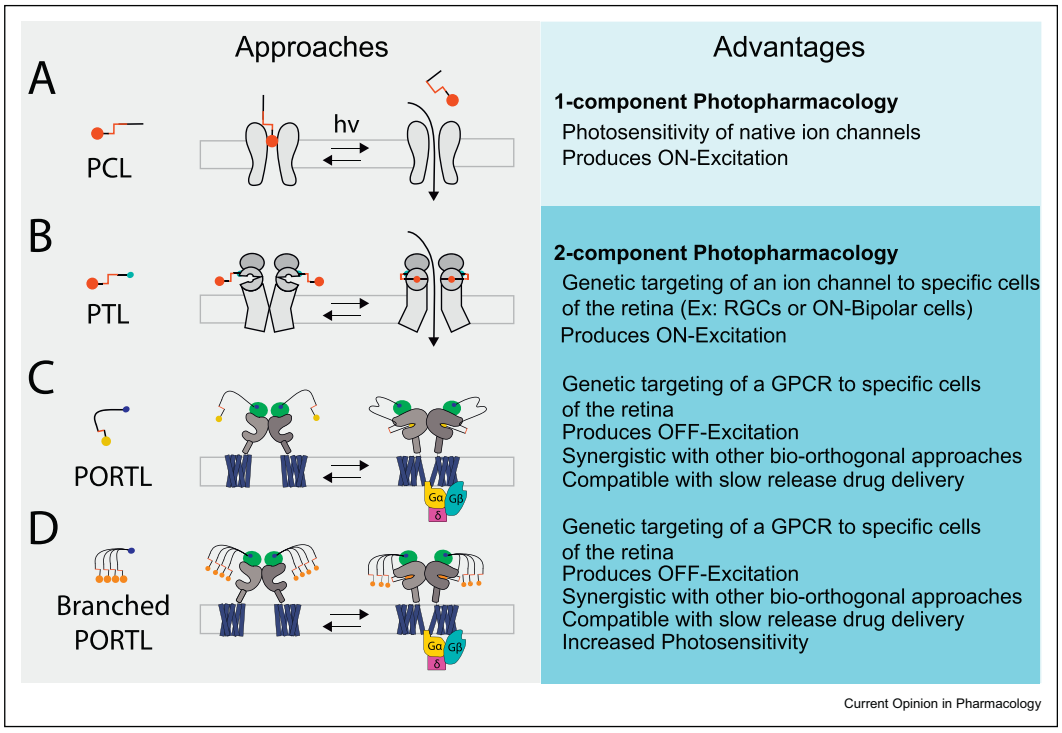
These studies provided the first direct comparison of vision restoration in two different retinal cell types (RGCs vs ON-BCs) using the same photopharmacological tool [41]. This is relevant as the interneurons (such as ON-BCs) situated below the photoreceptors of a healthy retina perform a substantial amount of image computation. Signals are summated in RGCs forming multiple distinct subtypes [38,42] each encoding different aspects of the visual environment. This diversity is presumably lost during the process of retinal degeneration. One motivation for restoring photosensitivity upstream of the RGCs, within the ON-BCs, is an attempt to rescue more of the retinal circuitry. When light responses from LiGluR expressed in RGCs or ON-BCs were compared in RP mouse models, it was observed that restoration of vision at the level of the ON-BCs produce a more diverse signal output. Furthermore, this diversity was enhanced when LiGluR was expressed in the ON-BCs of a RP model with a slower course of retinal denervation [41]. These studies suggest that photopharmacological vision restoration that involves more of the retina circuit may enhance the diversity of signal encoding. However, synaptic reorganization in the RP retina has a bigger impact on BCs [43,44], suggesting that targeting RGCs may be the safer bet, especially in late-stage disease.

Though LiGluR restores robust photoresponses, it has a drawback. Because free cysteines are found in the ectodomain of many proteins, PTLs that rely on cysteine interaction will produce off-target attachment. Moreover, the three most widely used moieties for cysteine attachment—maleimide, iodoacetamide and methanethiosulfonate—are all subject to hydrolysis, meaning that photoswitch conjugation to the target can only occur for some tens of minutes before the PTL loses reactivity [45–47]. To achieve a high labeling efficiency across the entire retina in this short period of time, the PTL needs to be injected into the eye at a high concentration, outcompeting hydrolysis [40,41], but risking toxicity. After the pulse of labeling that results from intra-ocular injection, the response to light will fade as the photoswitch attachment moiety hydrolyzes, the photoswitch washes out of the eye, and photoswitch-conjugated receptors are turned over. As a result, the photoswitch either needs to be injected at short intervals or more stable thiol-reactive groups need to be used (e.g. vinylphosphonothiolates) [48] or one would need a slow-release formulation that protects the thiol-reactive moiety from water until release.

The PORTL photoswitch: A PLC on a leash that conjugates to a protein tag fused to the target receptor

To circumvent the problems of off-target labeling and hydrolysis, a new chemical approach was employed in which the photoswitch is instead attached to a protein tag, such as SNAP and CLIP, *via* bioorthogonal chemistry. SNAP and CLIP are self-labeling small enzymes, about ~3/4 the size of the green fluorescent protein [49,50]. SNAP reacts its active site cysteine with O^6-benzylguanine (BG) to form a covalent bond and CLIP conjugates in the same way to O^2-benzylcytosine (BC). SNAP, CLIP, BG, and BC do not react with any other biological molecule and BG and BC are highly resistant to hydrolysis. Importantly, SNAP and CLIP can be fused to a target protein, most effectively at its N-terminus, to enable selective labeling. The labeling is highly specific in intact neural tissue [51]. SNAP or CLIP was fused to

Figure 3



Summary of photopharmacological approaches to vision restoration. (**a**) Relaxed (left) and photo-activated (right) conformations of 1-component and (**b–d**) 2-compoenent photopharmacological approaches in the retina. (**a**) photochromic ligands (PCL) on native receptors [10–15], **(b)** photochromic tethered ligand (PTL) on virally expressed LiGluR [16,40,41], **(c,d)** photochromic orthogonal remotely tethered ligands (PORTLs) and branched PORTLs on SNAP-mGluR2 [58,62].

the N-terminus of mGluR2, the mGluR with the slowest turnover rate [52].

To complement this addition to the mGluR2, photoswitches were synthesized to contain either BG or BC (for chemical attachment) at one end and the PCL azobenzene-glutamate (AG) (for photoisomerization) at the other. These were connected *via* a linker made of 0–28 polyethylene glycol (PEG) repeats to generate "BGAG," a PCL version of glutamate [53] that tethers to SNAP-mGluR2 *via* an adjustable linker. The purpose of the linker is to enable the AG to extend from the SNAP conjugation site to the ligand-binding site (LBD) of mGluR2. These PCLs on a long tether that attach bioorthogonally to the receptor are called Photoswitchable Orthogonally Remote Tethered Ligands or in short PORTLs.

BGAG and BCAG with a classical (unmodified) azobenzene [26,54] photoisomerize under near-UV light (380 nm) from the dark-stable *trans* state, in which the glutamate is obstructed (unable to bind to the receptor's ligand binding pocket) and the receptor is inactive, to the *cis* state, in which the glutamate is exposed and binds and activates the receptor (Figures 2b and 3c). BGAG is selective for SNAP-mGluR2 and BCAG for CLIP-mGluR2 [26]. Following photo-isomerization to *cis*, BGAG and BCAG return to the *trans* state very slowly (hours) in the dark but can be rapidly (in ms) photoisomerized to *trans* by blue–green light (488–532 nm). The nomenclature for these photoswitches is $BGAG_{n,380/532}$, where n = the number of PEGs and 380/532 are the preferred wavelengths (nm) for photoswitching.

This PORTL approach is generalizable and has also been demonstrated with direct attachment to SNAP fused to a dopamine receptor with a D1 receptor PCL agonist, BGAP [55] as well as SNAP fused to the serotonin receptor with a 5-HT_{2A} agonist, BG-Azo5HT [28]. The PORTL approach also works on unmodified "native" mGluR or dopamine receptors when the PORTL is attached to a separate membrane anchor protein that contains an extracellular SNAP and is genetically expressed in select target cells [55,56] (Figure 2c). This is termed a membrane anchored PORTL. Receptor specificity depends on the selected cell type and the choice of ligand. This approach works in cultured cells and in the brain of behaving animals.

For vision restoration, one wants a system that turns on in response to changes in light, rather than a system that needs to be pushed on and off by different wavelengths of light. One also wants a system that responds to visible light at intensities that range from indoor to outdoor light. To address this a modified push−pull azobenzene was developed that photoisomerizes to *cis* under blue light (460 nm) and returns spontaneously and rapidly to *trans* in the dark [57]. A light-ON/dark-OFF characteristic is best suited to vision restoration as it recapitulates some aspects of natural vision encoding. We found that a 12 PEG linker version of BGAG provides maximal photo-activation of SNAP-mGluR2 [54]. Therefore, for vision restoration we first used $BGAG_{12,460}$ in combination with SNAP-mGluR2.

Intravitreal viral delivery reconstitutes $BGAG_{12,460}$:SNAP-mGluR2 in RGCs and restores aspects of vision

SNAP-mGluR2 under the control of the human synapsin promoter (hSyn) was packaged into the 4YF capsid version of adeno-associated virus 2 (AAV2-4YF) and delivered *via* intravitreal injection to the eyes of the *rd1* mouse [58]. The synapsin promoter was chosen because, in both mouse and human, synapsin is expressed at much higher levels in RGCs than in other retinal cell types and because it is expressed relatively evenly across RGC subtypes of mouse [59] and human [60]. Intravitreal injection of a SNAP-reactive BG-fluorophore $\geq$ 6 weeks following injection of AAV2(4YF)-hSyn-SNAP-mGluR2 stained the cell bodies and dendrites of the RGCs of the *rd1* retina, and staining was even across the retina, confirming both the selectivity of the BG to SNAP conjugation and the anticipated pan-RGC targeting [58].

Multi-electrode array (MEA) recordings of RGC activity in the retina of *rd1* mouse with complete photoreceptor loss, showed that $BGAG_{12,460}$:SNAP-mGluR2 introduces a large and fast light-evoked inhibition of firing followed by a transient after-excitation at the cessation of illumination. These responses resemble that of OFF-RGCs of the wildtype mouse [36,37] and human retina [38,39]. Pharmacological analysis suggested that the inhibitory response to light is mediated by KCNQ (K_v7) channels, which are known targets of $G_{\beta\gamma}$ from G_i-coupled receptors like mGluR2. The after-excitation appears to be mediated by hyperpolarization-activated HCN channels as a rebound from the inhibitory action of the light-triggered inhibitory phase.

In vivo, $BGAG_{12,460}$:SNAP-mGluR2 in RGCs restored natural light aversion and supported a learned visual discrimination task in which animals discerned parallel (||) *versus* intersecting (+) lines or pairs of vertical parallel lines separated by different distances. *Rd1* mice with $BGAG_{12,460}$:SNAP-mGluR2 in RGCs (producing inhibitory ON/excitatory OFF response) out-performed those with L-MAG_{460}:GluK2(L439C) (= LiGluR), the light-gated kainate type ionotropic glutamate receptor that produces excitatory ON type responses. These results suggest that some visual tasks may benefit from restoration of OFF-RGC like functions. However, natural vision employs populations of both ON and OFF type RGCs in order to navigate the visual world efficiently.

Because BGAG attaches selectively to SNAP and MAG (maleimide-azobenzene-glutamate) to an introduced cysteine, SNAP-mGluR2 and GluK2(L439C) could be co-expressed and separately labeled, each with its own photoswitch. When AAVs that deliver SNAP-mGluR2 and LiGluR to RGCs were mixed and injected intravitreally together, RGCs expressed varying amounts and ratios of the two engineered receptors. This resulted in a diverse array of light responses. Instead of the uniform inhibitory ON/excitatory OFF characteristic of $BGAG_{12,460}$:SNAP-mGluR2 or the sustained excitatory ON response of LiGluR, the mixture generated light responses that ranged from sustained ON to transient ON to various sized versions of transient ON/excitatory OFF to inhibitory ON/excitatory OFF. Behaviorally, animals with this restored diversity of ON and OFF type responses displayed enhanced acuity compared to either construct alone. This suggests that RGC response diversity improves the quality of the restored vision. It is not known if the diversity of light response is solely determined by random uptake of capsids and expression of the engineered receptors, or if it also depends on the transfected RGC subtypes, which may differ in excitability or expression or localization of native channels that are activated by mGluR2.

Overcoming a major barrier for 2-component vision restoration: Poor sensitivity to light

A major limitation of both $BGAG_{12,460}$:SNAP-mGluR2 and L-MAG_{460}:GluK2(439C) is that they have low sensitivity to light. This places these 2-component systems in the sensitivity range of channelrhodopsin and implies that vision restoration would only work in bright outdoor light or, if indoors, would require intensifying goggles, as seen in a first human patient with RGC expression of the channelrhodopsin variant chrimsonR [61]. To address this, we redesigned a 2-component photoswitch that relies on the same SNAP-mGluR2 receptor but restores vision with enhanced sensitivity [62]. $BGAG_{12,460}$ bears one light-activated azobenzene-glutamate (AG) for each receptor attachment moiety (BG), so that each of the subunits of the SNAP-mGluR2 dimer has one photoswitchable ligand (Figure 2b; Figure 3c, PORTL). We synthesized a BGAG in which one BG branched to four PEG linkers, each with its own AG (Figure 3d). This $^{4x}BGAG_{12,460}$ dangles four AG ligands in front of each glutamate binding site. Native rod and cone photoreceptors densely pack opsin proteins into

membrane discs in close proximity to the abundant supply of retinal chromophores. Analogously, the increase in the number of AGs in $^{4x}BGAG_{12,460}$ molecule is expected to increase both photon capture and the probability that both LBDs in the dimer will be liganded at the same time. These effects are each expected to boost sensitivity and the degree of receptor activation, since receptor activation is supra-linearly dependent on liganding: that is, liganding of only one mGluR2 subunit yields ~20% of maximal activation, whereas liganding of both gives full (100%) activation [63]. Indeed, in HEK293 cells, $^{4x}BGAG_{12,460}$ increases both sensitivity and efficacy [64]. As hoped, $^{4x}BGAG_{12,460}$:SNAP-mGluR2 in RGCs restores vision to *rd1* mice in dim light. Maximal photo-aversion was seen when the avoidant side of a light/dark-chamber was illuminated at only 25 μW/cm^2 by an iPad-mini at ~30% of its maximal brightness of 500 nits.

A key practical issue for therapeutics is that intra-ocular injection cannot be done very frequently. For example, in age-related macular degeneration, the current treatment is repeated intravitreal injections of *Lucentis* or *Avastin,* typically at monthly intervals. The restoration of light aversion by the unbranched $BGAG_{12,460}$ injected in saline washes out of the eye in 1−2 weeks, but this can be extended to 6 weeks by formulation in β-cyclodextrin [58]. Formulation in β-cyclodextrin also extends the action of the 4-branched $^{4x}BGAG_{12,460}$ to 6 weeks [62]. Given the stability of SNAP-reactive PORTL photoswitches, additional longer-term slow-release formulas or implantable reservoirs are likely possible.

Our recent study of $^{4x}BGAG_{12,460}$:SNAP-mGluR2 in RGCs [62] shows that it: 1) provides sufficient sensitivity to support object exploration under incidental illumination from above by a standard LCD tablet (iPad-mini) display, 2) supports line pattern discrimination (again using an iPad-mini display) with an acuity that approximates what has been reported for wildtype mouse vision, and 3) has a sufficiently fast retinal refresh to discriminate between line patterns that are in motion at up to 36° of arc per second. In contrast, chrimsonR does not support pattern discrimination in motion at one third the speed. This performance of $^{4x}BGAG_{12,460}$:SNAP-mGluR2 is striking given that its installation in RGCs, the output cells of the retina, bypasses the upstream circuitry and retinal processing used for direction selectivity in visually intact *wildtype* mice [65].

What next for photo-pharmacological vision restoration?

A key advance in sensitivity has come from moving to branched photoswitches that bear several light-sensitive ligands, allowing ultra-efficient optical control over mGluR2 with blue 460 nm light and mGluR3 with UV light [64]. Further sensitivity gains may be achieved by a redesign that increases light capture and/or photoswitching quantum yield. The higher the sensitivity, the lower the dose of AAV and/or photoswitch, reducing the risk of inflammation and toxicity. Enhanced sensitivity could mean that vision would be restored at regions with lower transfection peripheral to the foveal ring. In addition, when multiple photoswitches surround the receptor, molecular degradation is less of an issue, since it is rescued by the high effective concentration of remaining switches. Apart from optimizing the photoswitch, protein-engineering could be another avenue, especially since the mGluR2 ligand binding domain can be transplanted to other signaling proteins to create chimeras between mGluR2 and mGluR1 or mGluR5 [64,66,67]. In this way, calcium signals could be triggered by light and may amplify activity changes in retinal neurons. An additional, equally exciting avenue for photopharmacology is the development of photoswitch-fused nanobodies [68]. This approach allows for immune-targeting of photoactivatable ligands to native receptors, avoiding gene therapy in the manner of 1-component vision restoration but obtaining some of the target selectivity of 2-component vision restoration.

For now, pure optogenetic gene therapy lacks control elements that disengage or adjust the function of the introduced gene. Although it adds an extra layer of complexity by requiring repeated injection, the exogenous photoswitch of photopharmacology enables upgrade as new photoswitches are developed and provides patient choice in that treatment can be discontinued in case of an undesired experience or in case an alternative therapy becomes available. Given the stability of PORTL photoswitches, they may be compatible with slow-release from aqueous devices [69], which could extend delivery for months and reduce the frequency of eye injections in patients. Added to the unique combination of high sensitivity, high acuity and motion vision, these advantages make a compelling case for further development of 2-component photopharmacology for vision restoration.

Credit author statement

J.B. created the figures. M.H.B. and E.Y.I. wrote the manuscript. All of the authors edited the manuscript.

Conflict of interest statement

E.Y.I and J.G.F. are founders of Vedere Bio II, Inc. and serve on its scientific advisory board. A.H. and J.B. are consultants for Vedere Bio II, Inc.

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