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Restoring Vision to the Blind with Chemical Photoswitches

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Abstract

Degenerative retinal diseases such as retinitis pigmentosa (RP) and age-related macular degeneration (AMD) affect millions of people around the world and lead to irreversible vision loss if left untreated. A number of therapeutic strategies have been developed over the years to treat these diseases or restore vision to already blind patients. In this Review, we describe the development and translational application of light-sensitive chemical photoswitches to restore visual function to the blind retina and compare the translational potential of photoswitches with other vision-restoring therapies. This therapeutic strategy is enabled by an efficient fusion of chemical synthesis, chemical biology, and molecular biology and is broadly applicable to other biological systems. We hope this Review will be of interest to chemists as well as neuroscientists and clinicians.

Graphical Abstract

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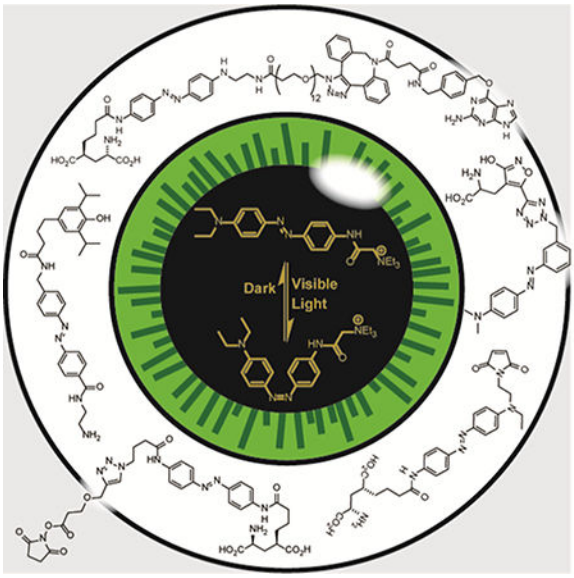
The authors declare the following competing financial interest(s): Isacoff and Kramer are forming a company for vision restoration using some of the methods described in this review.



1. INTRODUCTION

This Review will cover the use of chemical photoswitches—reversibly photoisomerizing small molecules—to improve or restore visual function in degenerative retinal diseases such as retinitis pigmentosa (RP) and age-related macular degeneration (AMD). We will specifically focus on azobenzene-containing photoswitches that have been tested in blind animal models of retinal degeneration. Chemical photoswitches will also be compared with other treatments for degenerative retinal diseases, including gene-replacement therapy, stem cell therapy, electronic prosthetics, and optogenetic gene therapy. The properties and the development of chemical photoswitches will be described in their historical context. Finally, the Review will evaluate the translational potential of existing chemical photoswitches and explore possible improvements to these compounds that might be made in the future.

2. MAMMALIAN RETINA: OVERVIEW

2.1. Structure and Function

The vertebrate retina has evolved over hundreds of millions of years to be a marvelously intricate light-sensing organ necessary for all visually guided behaviors and other physiological functions, like synchronization of the circadian rhythm with the environmental light–dark cycle. The retina is a complex tissue made up of different types of cells that receive and process visual information. Starting from the back of the eye, the vertebrate retina is composed of layers of neurons and support cells that acquire, process, and transmit visual information through the retina and onward to the brain (Figure 1). The retina is separated from the vascular tissue in the back of the eye by the retinal pigment epithelium (RPE), a layer of pigmented cells that nourish retinal neurons and provide them with the ability to sense light by replenishing their supply of the chromophore retinaldehyde. In the neural retina, two types of photoreceptor cells, the rods and cones, interdigitate with the retinal pigment epithelium. Three different chromatic types of cones respond to bright light

of different wavelengths and mediate high-resolution photopic color vision under daylight conditions (luminance level 10^1–10^6 cd/m^2).[1] Rods respond to dim light and mediate lowresolution scotopic vision under low-light conditions (luminance level 10^{-6}–10^{-3} cd/m^2).[1] After photons are absorbed by the retinaldehyde-conjugated opsin in rods and cones, an intracellular signaling cascade leads to a change in the release of the neurotransmitter glutamate from photoreceptor terminals onto cells in the next layer of the retina—bipolar cells. Horizontal cells with processes that project laterally across many photoreceptors help distribute signals and regulate the release of neurotransmitters from photoreceptors to bipolar cells. Bipolar cells respond to the glutamate released from the photoreceptors either by depolarizing their membrane potential (ON bipolar cells) or by hyperpolarizing their membrane potential (OFF bipolar cells). This establishes two parallel pathways for the transmission of visual information. Bipolar cells then transmit visual information by releasing glutamate onto retinal ganglion cells (RGCs), with ON-center RGCs transiently increasing their firing of action potentials when the light is turned on and OFF-center RGCs transiently increasing their firing of action potentials when the light is turned off. Amacrine cells are inhibitory interneurons projecting across many bipolar cells and RGCs. They help shape and process the visual response by releasing GABA and glycine neurotransmitters. Finally, a very small subset of retinal ganglion cells are intrinsically photosensitive (ipRGCs), which can also directly sense light. These ipRGCs are important for entraining the intrinsic circadian clock in the hypothalamus in the brain to light. For a more detailed discussion of retinal structure and function, see refs 2 and 3.

The retina is an intricately complex tissue that does far more than simply relay visual information to the brain in an analog manner. While a full discussion of the structure and function of retinal circuits is outside the scope of this Review, these circuits also process visual information and perform important computations necessary for normal vision. For example, the retina adapts to different light levels such that RGCs generate an appropriate signal in response to visual stimuli varying up to ~9 log orders of background light intensity, a process that begins in the photoreceptors and involves horizontal cells and bipolar cells.[4] Retinal circuits also sharpen visual images in both space and time by a process of lateral inhibition, which involves center-surround antagonism in the receptive fields ON and OFF bipolar cells, and again in RGCs.[5] The retina also plays an important role in motion detection, with certain retinal neurons preferentially responding to moving stimuli of different directions.[6] For a more detailed discussion of these and other retinal circuits, see ref 7.

2.2. Photochemistry of Vision

Rod and cone photoreceptors contain proteins called opsins that allow them to respond to light. Opsins are members of a large class of cell-signaling proteins called G-protein coupled receptors (GPCRs). The chromophore in all mammalian opsins is 11-*cis*-retinaldehyde, which is covalently bound to the opsin as a protonated Schiff base via a lysine residue (Lys296).[9] Absorption of a photon isomerizes 11-*cis*-retinaldehyde to all-*trans*-retinaldehyde, which changes the conformation of the opsin (Figure 2), leading to a signal transduction cascade that causes the closure of cyclic-nucleotide-gated cation channels in photoreceptors and hyperpolarizes the membrane potential of these cells (i.e., drives the

inside of the cell to have a more negative voltage with respect to that outside the cell). The wavelength to which each opsin best responds (e.g., blue, green, and red cones) depends on the local environment of the retinaldehyde in the opsin-binding pocket.[10]

After photoisomerization, all-*trans*-retinaldehyde is released from the opsin GPCR and is transported into the retinal pigment epithelium, where a series of enzymes convert it back to 11-*cis*-retinaldehyde, which can then reenter photoreceptors and associate with the opsin. For more detail, the interested reader may consult the recent review on the chemistry of the visual cycle by Palczewski and co-workers.[9] Photoreceptors are depolarized in the dark, which leads them to continually release glutamate onto bipolar cells. The glutamate released from photoreceptors causes OFF-bipolar cells to depolarize, so that they in turn release more glutamate at their nerve terminal, and ON-bipolar cells to hyperpolarize, so that they release less glutamate. Rod and cone photoreceptors are impressively sensitive to light and can even generate a visually perceptible response to single photons.[11] This sensitivity is the result of the enormous number of opsin molecules (~10^8) present in each rod (providing an extremely high probability of photon absorption) as well as signal amplification caused by the second messenger cascade.

3. DEGENERATIVE RETINAL DISEASES: OVERVIEW AND CURRENT THERAPIES

3.1. Retinal Degeneration

Degenerative retinal diseases including retinitis pigmentosa (RP) and age-related macular degeneration (AMD) affect millions of people worldwide.[12,13] These disorders are characterized by the progressive loss of rod and cone photoreceptors from the retina, which results in permanent visual deficits and even blindness (Figure 3). RP is a set of inherited diseases characterized by the progressive loss of rod and cone photoreceptors that starts in the peripheral retina and progresses to the central retina, eventually leading to complete blindness in advanced cases[14] (Figure 3D). AMD is also a progressive degenerative disease that begins with the accumulation of soft drusen—protein and lipid deposits—in the central retina, followed by the degeneration of retinal pigment epithelium cells[15] (Figure 3C). In geographic atrophy (dry AMD), these early stages are followed by the degeneration of photoreceptor cells in the macula, the central part of the retina, leading to localized vision loss.[16] In neovascular (wet) AMD, abnormal blood vessels grow under the retina, which can then leak and damage the macula.[17] While treatments exist for wet AMD,[14,17] there is yet no treatment for patients with geographic atrophy.[18] Without a means of restoring visual function, patients with RP and geographic atrophy face the prospect of irreversible vision loss.

3.2. Retinal Remodeling

The pathological changes that take place in the retina during retinal degeneration are not solely limited to photoreceptors. In fact, an extensive program of retinal remodeling takes place after photoreceptor degeneration in RP and AMD retinas.[20,21] Retinal remodeling in RP consists of several different aspects and occurs in different stages, advancing with the progression of disease. Phase I of retinal remodeling is the predegeneration period that is

primarily characterized by the appearance of markers of photoreceptor stress. Phase II is the period of photoreceptor loss accompanied by glial remodeling of the outer nuclear layer, which leaves a glial seal between the remnant neural retina and the remnant RPE/choroid. Phase III is a protracted, life-long period of neural, glial, and vascular remodeling of the surviving retina that involves a host of different molecular and cellular changes. Some of these changes include neuronal cell death, changes in neuronal morphology and aberrant migration of retinal neurons, de novo neuritogenesis, microneuroma formation, network rewiring, changes in glial structure and metabolism, and invasion of the neural retina by RPE.[20,21] During phase III, progressive cell death of bipolar, horizontal, and amacrine cells also takes place, whereas RGCs are largely spared even in the end-stages of retinal degeneration.[20,21] In addition to these structural changes, the latter stages of retinal degeneration are accompanied by functional changes in surviving retinal neurons, such as the hyperactivity of RGCs.[22,23] This phenomenon is clinically relevant because many vision-restoring treatments create stimuli that are then superimposed on a higher background level of retinal activity, which may reduce the sensitivity or spatial acuity of resultant vision.[24] The final stage of retinal remodeling is a retina bereft of many types of neurons and unable to transmit coherent visual information to downstream brain processing centers.

3.3. Therapeutic Strategies for Vision Restoration

A number of different treatments for degenerative retinal diseases are currently in clinical trials or already being used in the clinic (Figure 4). Inherited genetic mutations causing photoreceptor degeneration can be corrected via the use of gene-replacement therapy, where a copy of the wild-type gene is expressed in cells harboring the mutation, thus slowing down or even stopping the progression of disease. Some mutations corrected using gene therapy include those responsible for Leber's congenital amaurosis, Usher syndrome, and X-linked juvenile retinoschisis.[25–28] Transplantation of stem-cell derived photoreceptors can restore retinal light responses to blind mice, and retinal pigment epithelium transplants have improved visual function in some patients with AMD or Stargardt disease.[29–32]

In principle, gene and cell replacement therapies will be most beneficial in less advanced cases of retinal degeneration where some visual function remains intact, but several other methods are being developed to treat more advanced retinal degeneration, after rods and cones have been lost completely. Surgically implanted multielectrode retinal prostheses like the Argus II (an epiretinal chip) and Alpha-IMS (a subretinal chip) can electrically stimulate the activity of the retinal ganglion cells (RGCs) and other retinal neurons, restoring light perception to blind RP patients.[33,34] Instead of stimulating the retina, an electronic retinal prosthesis can also be implanted in the visual cortex directly, bypassing much of the circuitry for visual-information processing.[35] Although one of these cortical implants, the Orion, was recently approved for clinical trials in the United States, it is still unclear whether cortical stimulation will prove advantageous over retinal stimulation. As an alternative to electrical stimulation, viral expression of light-sensitive microbial opsins (optogenetics) in retinal neurons that survive after the death of photoreceptors can also restore visual responses in blind animal models of RP.[36–38]

All of these strategies have shown promise for either preventing vision loss or restoring visual function in animal models of retinal degeneration and in some cases even human patients, but each strategy requires significant improvement to overcome its shortcomings. Gene-replacement therapy offers a large potential therapeutic benefit because the treatment can be started even before visual function is lost. However, gene replacement may be less effective in more advanced cases of retinal degeneration, when photoreceptor cell death has already taken place. Additionally, each gene-replacement therapy needs to be tailored to a specific gene and tested in separate long-term clinical trials, complicating the translational path. The use of stem cells to restore or prevent vision loss is complicated by potential side effects like immune rejection and teratoma formation[39] and will require new standards in quality control and long-term monitoring of treated patients.[40] The potential permanence of stem cell and gene therapies could be beneficial in the absence of any complications, but the possibility of irreversible adverse effects necessitates careful and deliberate implementation in humans. Even more so than gene therapies, stem cell treatments face a complicated translational path, with none yet approved by the FDA.

Retinal implants, although effective at restoring some visual function to blind patients, face a different set of challenges. These implants require invasive surgery and rely on extracellular electrical stimulation of RGCs, which can be cytotoxic at high stimulus intensities.[41] Furthermore, the spatial acuity of retinal implants is limited by the number of stimulating electrodes, which is 60 for the Argus II implant and 1500 for the Alpha-IMS,[33,34] although newer photovoltaic retinal implants with more dense electrodes are currently being developed.[42] The viruses used for optogenetic gene therapy can have off-target effects[43] and may elicit inflammatory responses.[44] Additionally, the long-term safety of microbial opsins in the human eye is yet unknown and will need to be carefully studied.

3.4. Translational Status of Current Vision-Restoring Therapies

The various vision-restoring therapies discussed above are in different stages of translational development. The Argus II and Alpha-IMS retinal implants are FDA/CE Mark approved and are currently used in the clinic in the United States and/or Europe.[33,34] Suprachoroidal implants, which are simpler and less surgically invasive than eipretinal or subretinal implants, are currently in clinical trials.[45] A number of gene-replacement therapy clinical trials are underway for a variety of inherited retinal-degeneration diseases.[46] The first of these therapies, a treatment for inherited retinal degeneration caused by mutations in the *RPE65* gene,[47] has recently received FDA approval and is currently being used in the clinic.[48] A number of stem cell clinical trials for treating RP or AMD are currently underway, with some promising preliminary results.[40,49] The first clinical trial of an optogenetic (channelrhodopsin) gene therapy is also currently underway (NCT02556736), although it may be some time before the results are known. Overall, the field of vision restoration has undergone a dramatic transformation over the past decade, with several different therapeutic strategies now being used in the clinic and yet more being evaluated in clinical trials. While great progress has been made, there is still room for further improvement. To that end, this Review will focus on a novel small-molecule treatment modality for vision loss that offers some advantages as compared to existing therapies.

3.5. Therapeutic Implications of Retinal Remodeling

The extent or stage of retinal degeneration and accompanying retinal remodeling is an important consideration for identifying the therapeutic strategy most relevant to a particular patient.[50] In early stages of retinal degeneration, before photoreceptors have been lost, gene-replacement therapy might be most appropriate, because it can potentially prevent any future vision loss. Because retinal prostheses and optogenetic proteins stimulate surviving retinal neurons indiscriminately, they may interfere with remaining photoreceptor-mediated visual function in early stages of retinal degeneration, making them a less desirable treatment option for these patients. In later stages of retinal degeneration, when some photoreceptors have been lost but the extent of retinal remodeling is not too severe, stem cell therapies might still work well if these cells can integrate into the remaining retinal circuits.[32] In advanced cases of retinal degeneration, once most or all photoreceptors have been lost and the subsequent retinal remodeling is well underway, gene-replacement therapy may no longer be beneficial. Likewise, stem cells may have greater difficulty differentiating into the missing cell type(s) in vivo and establishing the appropriate synaptic connections in advanced cases of retinal degeneration.[51] In these patients, electronic retinal prosthetics, optogenetic gene therapy, and chemical photoswitches may be more suitable.

The later stages of retinal degeneration and retinal remodeling offer some unique opportunities for optogenetic gene therapy and chemical photoswitch therapy. These therapies primarily target the remaining bipolar cells and/or retinal ganglion cells,[36,52] both of which survive after photoreceptors have been lost.[20,21] Of these two cell types, the choice of target is somewhat dependent on the extent of retinal remodeling. In earlier stages of retinal degeneration, bipolar cells may be an attractive target for photosensitization because doing so may preserve more of the intrinsic signal-processing ability of the retina. However, in advanced cases of retinal remodeling, bipolar cells become a less suitable target due to their synaptic remodeling and cell death.[20,21] Retinal ganglion cells remain an attractive target throughout the course of degeneration because they are the output cells of the retina and are the least affected by retinal degeneration.[53] Finally, recent evidence suggests that chemical photoswitches might be able to fill in the gaps in the visual field in earlier stages of retinal degeneration by selectively photosensitizing RGCs in parts of the retina undergoing photoreceptor cell death. This disease-selectivity is enabled by exploiting molecular and functional changes in retinal neurons that take place during retinal remodeling.[54,55] The unexpected degeneration selectivity of some chemical photoswitches and its translational implications will be discussed in greater detail in section 9 of this Review.

4. CHEMICAL PHOTOSWITCHES

As discussed earlier, the various vision-restoration therapies currently in development or in clinical use each suffer from different drawbacks and translational challenges. Some of these challenges can be overcome by using chemical photoswitches to photosensitize retinal neurons in a blind retina lacking photoreceptors. This Review will discuss the relevant chemistry of photoswitches, the history of the development and preclinical testing of chemical photoswitches for vision restoration, and their future translational potential.

Because this strategy was enabled by the power of synthetic chemistry, we will begin by describing the physicochemical properties of chemical photoswitches.

4.1. Properties of Biocompatible Photoswitches

Of the many different types of known chemical photoswitches, a handful have been successfully tailored for use in biological applications[56,57] and, more specifically, for use in neuroscience.[58–60] A selection of common photoswitches is presented in Figure 5, featuring spiropyrans, diarylethenes, fulgides, azobenzenes, naphthopyrans, and stilbenes. All of these compounds can repeatedly and reversibly interconvert between photoisomers when irradiated, but there are significant differences in their chemical properties. An important classification to consider when selecting a photoswitch is p-type versus t-type systems. P-type (photochemically reversible) photoswitches are defined as being driven by absorbance of light. In contrast, t-type (thermally reversible) photoswitches can thermally relax back to their lower energy isomer over time. It is important to stress that t-type and p-type photoswitching are not mutually exclusive, and in fact many photoswitches (the majority in Figure 5) are capable of both.

While many properties of photoswitches can change upon light absorption (e.g., dipole, charge separation, and solubility), the majority of photoswitches used to date in biological contexts rely on a change in shape. Generally, these photorearrangements are due to two types of reactions, electrocyclic ring opening/closing (e.g., diarylethenes and spiropyrans) and double-bond isomerizations (e.g., azobenzenes and stilbenes). They are not mutually exclusive, as some photoswitches can do both (e.g., the fulgides and naphthopyrans); however, this could be a complication, due to the additional light-sensitive isomers generated.

Other desirable properties for photoswitches in biological environments include water solubility, a lack of toxicity, and stability toward hydrolysis, reduction/oxidation, and photobleaching. Finally, the synthetic tractability of the photoswitch is important not only for the convenient synthesis of the core photoswitch but also for generating derivatives with desirable properties like light absorption spectra.[61] Because azobenzenes—colorful dyes first reported over 180 years ago[62]—possess nearly all of these desired traits, they are the photoswitches employed most frequently for biological applications, including vision restoration.

4.2. Vision-Relevant Physicochemical Photoswitch Properties

In addition to general biocompatibility, two other properties specifically relevant to vision need to be considered in prospective azobenzene photoswitches. First, the photoswitch must absorb visible light (~400–700 nm). The three types of human cone opsins have maximum absorbances at 420, 534, and 564 nm, while rhodopsin has an absorption peak at 498 nm. Moreover, if multiple wavelengths of visible light are to be discriminated across the visible spectrum, then different photoswitches with nonoverlapping absorption spectra must be identified and used in tandem. So-called regular azobenzenes have a peak absorbance for their cis to trans isomerization in the ultraviolet (UV) range,[63] which is problematic for vision restoration, because UV light is normally filtered out by the human lens[64] and can

damage the retina.[65] However, “push—pull” azobenzenes, which are formed by installation of conjugated donor and acceptor groups onto the azobenzene scaffold, show significant red-shifting of the key π–π^* electronic transition,[63] bringing the absorption maximum into the visible range.

The second consideration is the thermal stability of the light-activated (generally cis) isomer of the photoswitch. Because azo compounds are t-type photoswitches, they thermally revert from their higher-energy cis isomer back to the trans isomer. The rate of a photoswitch’s thermal relaxation can directly affect the resulting visual function. If too much of the cis isomer builds up with repeated light stimulation, light sensitivity will decrease, although a second wavelength of light can be used to drive the cis → trans photoisomerization of some photoswitches. Conversely, if the cis isomer dissipates too rapidly to act on retinal neurons, light detection will require extremely bright light in order to maintain a sufficient amount of the cis isomer. The rate of thermal relaxation depends on many factors—e.g., solvent, temperature, and the particular azobenzene structure, with the corresponding rate varying with many orders of magnitude, from milliseconds to days.[66] This tunable t-type behavior can be an asset depending on the application and is desirable here because it can mimic the light response of an opsin. The half-life of activated rhodopsin is 80 ms,[67] and so a similar rate of thermal relaxation for a vision employed photoswitch is a reasonable starting point. Push–pull azobenzenes have significant contributions from hydrazone resonance structures, which decreases the bond order of the nitrogen–nitrogen double bond (Figure 6A). Intriguingly, these two considerations, red-shifting and rate of thermal relaxation, are not independent of each other, and this relationship is an active area of research.[68,69] A small sample of visible-light-active azo compounds showing the wide variance of cis isomer relaxation times is presented in Figure 6B. Tetraortho substitution of azobenzenes generally increases the half-life of the cis isomer.[70] For example, tetra-*ortho*-fluoroazobenzene has a trans absorption peak at 450 nm and a cis half-life of ~700 days in acetonitrile.[71] While this is much too slow to be directly useful for vision, applying the same concept to an azobenzene that is usually much faster could be of use. Tetra-*ortho*-methoxyazobenzene is protonated in water at pH 7.5 to the corresponding azonium and has a trans absorption peak at 560 nm and a cis half-life of ~10 s.[72] A similarly wide range in cis half-lives is evident in heteroaryl azobenzenes.[73,74] The azobenzene–pyrazole (Figure 6B) absorbs blue light and has a cis half-life of ~1000 days in acetonitrile, yet the azopyridinium absorbs the same blue light but has a time constant of ~3 ms in acetonitrile.[75] As our understanding of what drives the photochemical properties of azobenzene derivatives to these extremes continues to improve, so too will our ability to precisely design and synthesize azobenzene photoswitches with the desired spectral and thermal relaxation properties.

5. PHOTOCHEMICAL CONTROL OF PROTEIN FUNCTION

5.1. Overview

Having established certain translationally desirable photopharmacological and physicochemical properties of reversible chemical photoswitches, we next present several methods for utilizing chemical photoswitches to reversibly control neuronal function with

the ultimate goal of using these compounds to restore visual function to blind patients in the clinic.

Over the past several decades, a number of different methods for photosensitizing cellular proteins such as enzymes, neurotransmitter receptors, and ion channels have been developed by using light-sensitive ligands.[58,76–78] One major class of light-sensitive ligands are so-called caged compounds, where the active ligand is released by the irreversible photolysis of a protective group.[60] However, because the photoactivation of these compounds is irreversible, they are not well-suited for long-term vision restoration. This Review will thus solely focus on reversible light-sensitive ligands, which usually contain a previously described small-molecule ligand of the target of interest (e.g., agonist, antagonist, or pore blocker), covalently conjugated to a photoswitchable moiety such as azobenzene. The photoisomerization of azobenzene then causes a significant change in the molecule's shape, with the desired effect being that only one of the two isomers is able to bind its target receptor or binds at a much higher affinity than the other.

Reversible light-sensitive ligands can be broadly divided into two categories—freely diffusible photochromic ligands (PCLs) (Figure 7) and ligands that are covalently attached to their molecular target, including photoswitchable tethered ligands (PTLs), photoswitchable affinity labels (PALs), and photoswitchable orthogonal remotely tethered ligands (PORTLs) (Figure 7). Genetic engineering is often used to introduce the desired attachment site to the target receptor for specific tethered ligands, but suitable endogenous attachment sites also exist in some proteins.

5.2. Types of Photoswitchable Ligands

5.2.1. Photochromic Ligands.—Photochromic ligands take advantage of existing ion-channel pharmacology by adding photoisomerizable moieties to known or predicted ligand-gated or voltage-gated ion-channel agonists, antagonists, or pore blockers.[59,76] Adding a photosensitive azobenzene group to a known ion-channel blocker, for example, enables the activity of that ion channel to be controlled by light, because one of the two PCL isomers often has a higher affinity for its target, resulting in the channel being blocked by one but not the other isomer (Figure 7A). By controlling the photoisomer of the PCL and the function of its target endogenous ion channel using light, it is possible to control the activity of neurons expressing that ion channel. Depending on their physicochemical properties, PCLs can diffuse throughout cells or tissues and bind to their target receptor wherever they are present. Certain types of charged PCLs can enter into cells through large pores, then bind to and modulate the function of voltage-gated ion channels in response to light.[55,83] This property of certain PCLs is especially relevant for their translational use in restoring visual function, as discussed in detail in this Review.

The first described azobenzene PCL, bis-Q (Table 1), is a photoswitchable acetylcholine receptor agonist that can control muscle-type nicotinic acetylcholine receptors (nAChRs) in different cell types.[79–81] Optical control of nAChRs is a powerful tool for controlling muscle function, but acetylcholine primarily plays a modulatory role in mammalian central nervous system (CNS) signaling[96] and is less suited to directly drive CNS neuronal activity. Voltage-gated ion channels, including potassium (K_v), sodium (Na_v), and calcium (Ca_v) channels,

are ubiquitously expressed throughout the mammalian CNS, including the retina, where they directly mediate neuronal activity,[97] making them an attractive target for photopharmacological manipulation. To explore this possibility, a series of voltage-gated ion-channel blocker PCLs were developed, which include the compounds AAQ,[86] QAQ,[83] QENAQ,[84] DENAQ, BENAQ, and PHENAQ[89] (Table 1). These PCLs have somewhat overlapping but distinct sets of molecular targets, consisting of K_v, Na_v, Ca_v, and HCN channels,[54,59,91] all of which are broadly expressed throughout the nervous system. In addition to nAChRs and K_v channels, PCLs affecting a wide variety of neurotransmitter receptors and ion channels were designed, synthesized, and characterized in vitro and in vivo. Prominent recent PCLs include GluAzo,[94] ATA-3,[98] and ATG[92] (Table 1), which are, respectively, light-sensitive agonists of the kainate, AMPA and NMDA subtypes of glutamate receptors that mediate excitatory neurotransmission in the mammalian nervous system.

These and other PCLs have been demonstrated to confer robust light sensitivity onto neurons and animals in vitro and in vivo and have proven capable of manipulating animal behavior with light. However, PCLs are ultimately dependent on the potency and selectivity of their constituent ligand component. Thus, some PCLs are fairly selective for certain receptors, such as the NMDA subtype of glutamate receptors;[92] while others such as QAQ exhibit a lower degree of selectivity and affect a broad variety of voltage-gated ion channels, including sodium, calcium, and potassium channels.[83] Additionally, the potency of PCLs is quite variable, ranging from micromolar to millimolar concentrations[82] depending on the ligand and target receptor.

5.2.2. Photoswitchable Tethered Ligands.—Another way to achieve selective optopharmacological manipulation is to tether the ligand to the receptor of interest in order to minimize the possibility of undesirable off-target interactions as well as increase the effective local concentration of the ligand. These ligands, called photoswitchable tethered ligands (PTLs), were developed in parallel with the PCL approach (Figure 7B). nicotinic receptor agonist similar to bis-Q that could be The first synthetic PTL was QBr (Table 2), a muscle-type covalently tethered to the receptor via alkylation of the thiol group of a cysteine residue following the reduction of the endogenous disulfide bonds with dithiothreitol (DTT).[79] *trans*-QBr was a considerably more potent agonist of the receptor than cis-QBr, allowing for the photocontrol of nicotinic receptor function following QBr attachment.[79,99] Despite its technical innovation, the original PTL approach was not immediately translated to other classes of receptors. The need to use a reducing agent may have contributed to the slow initial adoption of the PTL method because these reducing agents can interfere with normal protein function and are difficult to use in vivo.

With the development of molecular biology techniques in the 1990s and the emergence of the first ion-channel crystal structures, it became easier to rationally design new PTL systems by replacing specific endogenous amino acids with cysteine residues in locations adjacent to a receptor's ligand-binding site. Functional screening of these mutant residues could then identify sites where PTL attachment would result in the ligand being able to bind the receptor and either activate it in the case of an agonist or close/block it in the case of an antagonist or pore blocker. Empowered by the tools of molecular biology and molecular

modeling, the PTL strategy was quickly extended to a number of ion channels that are important in neurobiology.

The first such engineered ion channel was SPARK, a light-sensitive voltage-gated potassium channel.[100] SPARK was created by introducing a cysteine mutation (E422C) into the Shaker voltage-gated potassium (K_v) channel. A PTL named maleimide azobenzene quaternary ammonium (MAQ, Table 2) was designed in combination with the mutant receptor. Maleimide reacts with cysteine residues to form a covalent bond, while quaternary ammonium acts as an external K_v channel pore blocker. After tethering MAQ to the engineered Shaker K_v channel, light can be used to reversibly control the state of the channel —switching it from blocked to unblocked. After the expression of SPARK in hippocampal pyramidal neurons, their activity could be controlled by light, with blue—green light driving activity when hyperpolarizing K_v channels were blocked and UV light blocking activity when K_v channels were open.[100] The ability to make structure and function guided mutations in ion channels was also later exploited to make a version of SPARK that depolarized neurons upon illumination with UV light, enabling bidirectional optical control of neuronal activity.[110]

Shortly thereafter, the technique was extended to ionotropic glutamate receptors, which are ion channels with a high single-channel conductance that mediate fast excitatory synaptic transmission in the retina and other parts of the nervous system. Direct photoactivation of glutamate receptors offers the possibility for much faster photocontrol of neuronal activity, and any engineered glutamate receptors can potentially replace their endogenous counterparts with minimal disruption to neuronal function. A cysteine residue was introduced into a mammalian kainate ionotropic glutamate receptor (GluK2), which was then combined with a PTL named maleimide azobenzene glutamate (MAG, Table 2).[101,102] In its cis form under UV light, tethered MAG could bind to the ligand-binding site of GluK2 and activate the channel, while the trans form of MAG was not able to bind to or activate the receptor. This light-sensitive glutamate receptor (LiGluR) was then expressed in hippocampal neurons, where it could generate light-elicited responses that resembled glutamatergic excitatory postsynaptic potentials as well as drive neuronal activity under UV illumination.[101] Furthermore, brief pulses of UV light were sufficient to elicit action potentials in hippocampal neurons, and these neurons could also follow a high-frequency stimulus of up to 50 Hz. Finally, expression of LiGluR in zebrafish could also control neuronal activity and resultant zebrafish behavior in vivo, indicating that LiGluR could also work in live animals.[101]

Other types of common neurotransmitter receptors that have recently been photosensitized via PTLs include neuronal nAChRs[90] and GABARs.[111,112] These PTLs can produce photoagonism or photoantagonism of their target receptors in vitro and in vivo and are particularly well-suited for the dissection of neuronal circuit and synaptic function. For a more detailed discussion of these and other PTLs, see refs 58, 60, and 77.

5.2.3. Photoswitchable Orthogonal Remotely Tethered Ligands.—

It is also possible to use other bioorthogonal chemical-attachment methods to tether a ligand to a receptor. One such strategy, PORTL, involves the use of a genetically encoded self-labeling

protein called SNAP-tag[113] that is subsequently used to tether a photoswitchable ligand on a long, flexible linker (Figure 7C). Using PORTL offers a number of advantages over the traditional cysteine-based PTL chemistry—for example, the addition of a long, flexible linker makes it substantially easier to tether a ligand such that it will be able to bind the receptor without time-consuming screening of cysteine point mutations. The benzylguanine moiety recognized by SNAP-tag is resistant to reduction and hydrolysis, making it stable for months in solution and aiding its use inside cells in addition to plasma membrane surface proteins. Most importantly, PORTL offers the ability to control the function of distinct types of ion channels or receptors in the same cells by using different attachment methods, such as SNAP,[113] CLIP,[114] or Halo[115] -tags for different receptors of interest. Together with ligands responding to different wavelengths of light, this method enables the interrogation of biological systems with even greater power and precision.

Recently, the power of the PORTL strategy was used to independently control different subtypes of metabotropic glutamate receptors with light.[107,108] Metabotropic glutamate receptors from different families could be activated by light upon conjugation of different photoswitches via either a SNAP-tag, CLIP-tag, or traditional cysteine-maleimide PTL (Table 2). Given the diversity of metabotropic glutamate receptors and their various important functions in the brain,[116] this toolset should further the understanding of the function of individual receptors even when more than one type is present in a particular cell type or brain area.

6. PTLS FOR VISION RESTORATION

With the development of PTLs and the demonstration of their ability to control neuronal activity with light in vitro and in vivo, it became clear that these tools could also potentially be used to restore visual function to retinas with photoreceptor degeneration. Recent advances in gene therapy that enable cell-specific targeting of retinal neurons together with the ability to optimize the properties of PTLs for vision restoration make PTLs a promising new approach to treating diseases such as RP.

6.1. First-Generation Light-Regulated Ionotropic Glutamate Receptors

Because of their prevalence in the retina, glutamate receptors were selected as candidate channels capable of mediating direct neuronal photoactivation (Figure 8).[101,102] To determine whether LiGluR could be expressed in the blind retina to restore visual function, the gene encoding LiGluR was packaged into an adeno-associated virus (AAV) vector and injected into the vitreous cavity of the eye, a common means of administering ophthalmic drugs. Intravitreal injection of LiGluR into the eyes of blind *rd1* mice,[103] a common animal model of RP, resulted in expression of LiGluR in most RGCs but not bipolar or amacrine cells. Blind mouse retinas expressing LiGluR alone did not exhibit any response to UV or blue—green light ex vivo before MAG treatment.[103] Addition of MAG ex vivo conferred retinal light responses as measured by extracellular electrophysiological recordings, with UV light causing an increase in RGC activity and blue–green light turning RGC activity off (Figure 8A and B). As was previously observed in hippocampal neurons, brief 50 ms UV light pulses were sufficient to drive robust light responses, suggesting that high-frequency

stimulation should be possible. When MAG was injected intravitreally into the eyes of mice expressing LiGluR, it was able to restore the pupillary light reflex in blind mice as well as drive a simple light-avoidance behavior in a five-arm water maze.[103] These results indicate that formerly blind mice were now able to sense light and use their novel visual perception to guide their behavior.

6.2. Second-Generation LiGluRs

While the original MAG with LiGluR was able to drive neuronal activity and even restore simple visual behaviors in blind mice, it was not well-suited for clinical use. Photoswitching of MAG requires high-intensity UV light, which can damage the retina.[65] Additionally, *cis*-MAG has a half-life on the order of minutes and so requires a second wavelength of light to turn off activation. To overcome these shortcomings, improved glutamate receptor PTLs were developed and tested as potential drugs for vision restoration. A red-shifted version of MAG, called MAG_{460} (Figure 9A), was designed by the principles outlined in section 4.2 to have a red-shifted maximal absorption at blue (460 nm) rather than UV light wavelengths.[104] Upon illumination with blue or white light, MAG_{460} isomerized to its cis-form and in the dark quickly converted back to its more stable trans-form by a process of thermal relaxation within several hundred milliseconds.

The same cysteine mutant ion channel (LiGluR, described earlier) was expressed either in RGCs or ON-bipolar cells of *rd1* mice by intravitreal virus injection (Figure 9B and C).[105] Several weeks after virus injection, the activity of LiGluR expressing retinas was again recorded ex vivo, where no light response was observed prior to MAG_{460} treatment. Following MAG_{460} application, robust responses to blue light were present and quickly decayed when the light was turned off.[105] The light intensity required to drive a robust response was approximately equal to that of bright daylight and similar to that required for stimulation of channelrhodopsin expressing retinal neurons. As predicted earlier, LiGluR enabled relatively high-frequency retinal responses, with RGCs able to follow a stimulus of up to 10 Hz. LiGluR expression in RGCs from blind *rcd1* dogs also restored visual responses similar to those observed in those from blind *rd1* mice.[105]

There was a key difference in the light responses of *rd1* mouse retinas expressing LiGluR in RGCs versus ON-bipolar cells. Expression of LiGluR in RGCs resulted in uniform light-on responses, with virtually all RGCs synchronously responding to light onset by increasing their firing rate (Figure 9D and F).[103] However, expression of LiGluR in ON-bipolar cells resulted in heterogeneous light-on and light-off retinal responses, with some RGCs increasing and some decreasing their firing rate in response to light (Figure 9E and G).[105] In addition to ex vivo retinal light responses, LiGluR also enabled light-avoidance behaviors and enabled light detection in a forced two-choice task in a modified water maze, indicating that MAG_{460} worked in vivo (Figure 9H).[105]

6.3. Light-Regulated Metabotropic Glutamate Receptors

Metabotropic glutamate receptors (mGluRs), in contrast to the ion channels of iGluRs, are a class of GPCRs that allow for signal amplification, because they recruit second messenger signaling molecules upon activation.[116] mGluRs belong to several different groups—

mGluR1–8—and also play an important role in retinal visual signal transmission where mGluR6 mediates glutamatergic signaling from photoreceptors to ON-bipolar cells.[117] Light-regulated metabotropic glutamate receptors (LimGluRs) were engineered via the tethering of the PTL agonist D-MAG0 or the antagonist D-MAG1 to the mGluR2, mGluR3, and mGluR6 receptors.[118] If LimGluR6 was to be expressed in *rd1* mouse ON-bipolar cells, then heterogeneous ON and OFF retinal light responses might be restored, similar to those observed after channelrhodopsin or LiGluR expression in *rd1* mouse ON-bipolar cells.

Second-generation PTLs, activated by white light and affecting more diverse receptors and cell types, made significant improvements on the first iterations but still suffer two significant drawbacks. First, rapid hydrolysis of the maleimide of the MAG PTLs requires application of the compound at concentrations high enough to potentially interact in undesired places. Second, while LiGluR-MAG_{460} established that robust retinal light responses could be elicited at clinically relevant light intensities (equivalent to sunlight), many vision situations routinely encountered occur under orders of magnitude lower light intensities (indoors, moonlight night). To address these concerns, further refinements were necessary.

These refinements came in the form of another tethered photoswitch solution, specifically a SNAPtag-mGluR2 fusion protein photosensitized by labeling with a PORTL named $BGAG_{12,460}$ (Table 2).[107] The mGluR2 GPCR, which is expressed in many mammalian neurons, functions by activating downstream signaling cascades that amplify signals received. By using a PORTL to render mGluR2 photosensitive (this PORTL/GPCR complex is named SNAG-mGluR2), less intense light should then be required to generate a response in the retina, compared to ion channels like LiGluR. At the same time, by switching ligands from MAG_{460} to $BGAG_{12,460}$, the rapidly hydrolyzed maleimide used to tether the MAG is replaced with the water-stable benzylguanine tethering moiety of the $BGAG_{12,460}$, which is maximally effective at a very low dose, providing a therapeutic window of at least 500-fold.

When the SNAG-mGluR2 system is present in the RGCs of *rd1* blind mice, a stereotypical OFF response (suppression of RGC firing under illumination, followed by a burst of RGC firing immediately after the light stops) is observed in MEA recordings.[106,119] Blind mice treated with SNAG-mGluR2 were able to discriminate between different images, e.g. between a pair of parallel versus perpendicular lines (Figure 10). These mice could also perform a close-line discrimination task, down to a line spacing indicating ~20/200 vision, [106,119] an impressive visual acuity given that the sighted mouse retina normally has a low visual acuity similar to that of human peripheral retina.

The excitatory (ON response) LiGluR and inhibitory (OFF response) mGluR2 systems were combined to simulate a more natural light response that has a mixture of ON and OFF responses by mixing AAV vectors encoding the two and capitalizing on the orthogonal reactivities of the LiGluR/MAG and SNAG-mGluR2 systems. The random expression of each AAV led to a range of expression levels of the two proteins in RGCs, leading to a wide variety of light responses, ranging from ON to OFF and various ratios of ON/OFF light responses.[106] Critically, this dual system creates even better visual acuity than either system alone (Figure 10F).

6.4. Labeling Endogenous Proteins with Photoswitchable Affinity Labels

While the genetically engineered receptors described earlier provide exceptional selectivity by targeted expression of exogenous mutant proteins, photoswitches can also be tethered to endogenous (wild-type, WT) ion channels or receptors. Notably, the first described PAL, QBr (Table 2), was covalently tethered to endogenous cysteine residues after reductive treatment.[79] More recently, a series of photoswitches have been described that contain amino acid reactive groups like acrylamide, epoxide, and chloroacetamide.[86] These photoswitches were designed as photoswitchable affinity labels (PALs) that would covalently react with endogenous cysteines and eliminate the need for genetic engineering and gene therapy. One of these compounds, acrylamide azobenzene quaternary ammonium (AAQ, see section 8.1) initially seemed to show excellent photoswitch affinity labeling of K_v channels; however, subsequent experiments instead showed AAQ to be acting at an internal channel pore-blocking site.[82] Photoswitch affinity labeling was successfully demonstrated with MAG and LiGluR,[120] where in this case, the maleimide of *cis*-MAG conjugated to its target cysteine at a faster rate than the trans isomer.

Recently the concept was revisited, now in the context of iGluRs.[109] In this instance, a series of PALs consisting of glutamate, azobenzene, and an N-hydroxysuccinimide (NHS) ester were constructed. Because of the potential reactivity of the glutamate portion of the PAL with the NHS ester tether, these two portions of the molecule were combined directly before use with a Cu-catalyzed click reaction (forming TCP-9, Figure 11A). The resulting “hot” TCP-9 labeled WT GluK1 expressed in cultured cells, rendering them photoactivatable with UV light.[109] When degenerate retinas from *rd10* mice were treated with the PAL and then rinsed to remove excess PAL, robust light-dependent currents were recorded from the RGCs that lasted for hours (Figure 11B and C).

7. PTL PROSPECTS AND FUTURE DIRECTIONS

Persistent effort has considerably improved the properties of tethered glutamate photoswitches to make them more suitable for vision restoration. The second-generation MAG_{460} photoswitch enables rapid retinal light-response kinetics and restores simple visual behaviors in blind animal models of RP. It is not clear, however, whether these visual responses are rapid enough to enable perception of moving objects or complex visual scenes. Ultimately, these tests will need to be performed in large animals with eyes more similar to human eyes, especially those possessing foveas, which are absent in mice. As with optogenetic tools, PTLs need to at least be sensitive to light similar in intensity to ordinary daylight in order to minimize potential phototoxicity due to long-term exposure. While the current MAG_{460} ligand satisfies this requirement, there is still room for optimization of the PTL. With further rational design, both the light sensitivity and the thermal relaxation kinetics of MAG-type PTLs can be improved further. Additionally, long-term intraocular safety studies will need to be performed on the glutamate receptor PTLs.

Another interesting feature of the LiGluR and SNAP-m2 mediated retinal light responses is the ability to generate RGC responses of different polarities, both light-ON and light-OFF, either by expressing LiGluR in bipolar cells or by expressing both LiGluR and SNAP-m2 in RGCs where these two receptors have opposite effects on neuronal activity. These responses

may be advantageous for restoring more complex visual perception in human patients, although this hypothesis will first need to be tested in large animal models. In the future, it will be important to explore the full range of sophisticated visual perception that might be possible with such a combinatorial treatment together with an appropriately optimized visual stimulus. Given the different nature of the light-response properties of PTLs as compared to rod and cone photoreceptors, their potential clinical use may require additional image processing and intensification hardware and software, for example, in the form of goggles similar to those being developed for optogenetic therapies.[121] These devices will need to be developed in collaboration with hardware and software engineers in the future.

7.1. Alternative PTL Targets in the Mammalian Retina

7.1.1. AMPA/NMDA Receptors.—In addition to kainate receptors, two other types of ionotropic receptors are present in the mammalian retina—AMPA and NMDA receptors. Both of them have been made light-sensitive via the addition of azobenzene photoswitches.[93,122] NMDA receptors have also been photosensitized by the incorporation of unnatural light-sensitive amino acids.[123] NMDA receptors in particular are an interesting potential means of photosensitizing retinal neurons due to their larger single channel conductance as compared to AMPA or kainate receptors.[124] However, NMDA receptors are also permeable to calcium and excessive stimulation of NMDA receptors can lead to excitotoxicity and cell death.

7.1.2. P2X Receptors.—ATP-activated ionotropic P2X receptors have also been photosensitized via the addition of light-sensitive azobenzene “molecular tweezers” that force the pore of the channel open in response to light.[125,126] These receptors are expressed in many retinal neurons, including RGCs, and could, in principle, be used to control neuronal activity. However, some P2X receptors, especially P2X7 receptors, are also calcium-permeable, and their prolonged activation can be cytotoxic. Ultimately, all the alternative tools described earlier will need to be evaluated for their efficacy and safety in restoring visual function to the blind retina in animal models of retinal degeneration.

7.2. PTL Translational Potential

As tools for vision restoration, PTLs share a common translational path with optogenetic therapies such as channelrhodopsin and halorhodopsin, with some important differences. Both optogenetic and PTL therapies benefit from the power of genetic targeting, which enables the targeted expression of the transgene in the cell type of interest, such as RGCs or bipolar cells. Optogenetics requires only the delivery of an exogenous gene to the retina, because the chromophore necessary for opsin function, retinaldehyde, is present at sufficient concentrations in the mammalian retina to render the transgenic channels light-sensitive. Exogenously delivered opsins such as rhodopsin can cause experimental autoimmune retinitis,[127] and microbial opsins will need to be evaluated to eliminate the possibility of this potential side effect. Commonly used optogenetic tools such as channelrhodopsin and halorhodopsin also have a small single-channel conductance,[128,129] requiring high expression to drive neuronal activity, which could present issues if the gene therapy vector cannot drive sufficiently high expression or if very high protein expression proves toxic or alters neuronal function.[130] By contrast, engineered mammalian receptors such as LiGluR

require an additional exogenous chromophore such as MAG in order to photosensitize neurons. In vivo delivery of MAG is somewhat complicated by its short lifetime in the eye, but longer-lasting ligands (e.g., $BGAG_{12,460}$) and slow-release polymer formulations are under development. While repeated intraocular delivery of PTLs in the clinic may be a potential translational challenge, it would also allow clinicians to optimize PTL dosage and stop treatment if adverse effects appear. Another advantage of glutamate receptors over channelrhodopsin is their higher single-channel conductance,[131] which suggests that lower levels of expression may be sufficient to control neuronal activity.

The most important next step on the translational pathway for PTLs is to demonstrate the safety of both the gene-therapy vector as well as the photoswitchable ligand itself in animals with large eyes similar to humans. Recent ongoing gene-replacement therapy clinical trials involving adeno-associated viral vectors similar to those used to deliver PTLs suggest these vectors are fairly well-tolerated in human patients,[132] which is encouraging for the translation of PTLs. The long-term effects of intravitreal MAG treatment will also need to be evaluated, given potential concerns about excitotoxicity. MAG is an agonist of kainate receptors and may affect other types of glutamate receptors depending on the concentration used in vivo, which can potentially be cytotoxic. The SNAG ligands offer a potential solution to this problem because they are functional at much lower effective concentrations compared to maleimide ligands like MAG.[133] The proposed therapeutic use of a PTL would involve a single intraocular virus injection to express the engineered receptor followed by monthly or even less frequent injections of MAG, and this proposed dual-treatment paradigm will need to be further characterized in animal models prior to beginning clinical trials. If evidence suggests that MAG or other glutamate photoswitches are safe, then a clinical trial combining gene therapy and intravitreal ligand injections can take place after FDA approval. Although much more work lies ahead, we are hopeful that, with the right combination of chemical design and hardware and software engineering, PTLs may yet someday help restore visual function to blind patients suffering from retinal degeneration.

8. PCLS FOR VISION RESTORATION

Optogenetic or engineered ion channel (PTL) gene therapy offers a number of features potentially advantageous for disease treatment, the main one being the ability to target the expression of the channel to a genetically defined cell type or population. Additionally, gene therapy is very long-lasting and even potentially permanent, meaning a single treatment may be sufficient to ameliorate the disease. However, caution is warranted, since the irreversibility of gene therapy means that any undesirable side effects are likely to be permanent. Although viral vectors have demonstrated a favorable safety profile in early-stage clinical trials,[132] questions about the long-term safety of gene therapy still linger following previous clinical failures.[134] For each different introduced gene, a unique gene therapy vector needs to be created, and the safety and efficacy of each of these vectors then needs to be characterized in separate clinical trials. To eliminate the need for gene therapy, an alternative method for vision restoration was developed that confers light sensitivity onto endogenous retinal ion channels, namely, light-sensitive drug-like small molecule (PCLs).

8.1. First-Generation Cationic PCLs

AAQ (acrylamide azobenzene quaternary ammonium, Table 1), the first PCL characterized as a potential tool for vision restoration, is a light-sensitive cationic ion-channel blocker that acts on endogenous voltage-gated potassium (K_v) channels and hyperpolarization-activated cyclic nucleotide-gated (HCN) channels.[86,87] AAQ contains a quaternary ammonium (QA) moiety that can bind to either an internal or external site on K_v channels, with the internal binding site having a higher affinity for QA.[135] In the dark or when stimulated with 500 nm light, AAQ blocks ion channels in its trans configuration, whereas 380 nm UV light converts AAQ to its cis configuration and unblocks ion channels.[86] AAQ was first tested in HEK cells expressing a variety of K_v channels, most of which were robustly blocked by *trans*-AAQ but not after photoisomerization to *cis*-AAQ.[86] Subsequently, AAQ treatment was found to also photosensitize HCN channels.[87] Studies showed that AAQ can photosensitize many types of neurons, including mouse hippocampal neurons and cerebellar basket cells, where light can stimulate neuronal firing.[86,87]

AAQ treatment restored robust light responses to blind *rd1* mouse retinas, as measured by extracellular and intracellular ex vivo electrophysiological recordings[88] (Figure 12). AAQ photosensitized multiple types of retinal neurons, including bipolar cells and amacrine cells, such that light regulated both excitatory and inhibitory synaptic potentials. The combined effects of light on synaptic inputs and intrinsic voltage-gated channels in RGCs was to recapitulate center-surround antagonism of RGC light responses. Pharmacological block of synaptic inhibition from amacrine cells reversed the polarity of AAQ-mediated RGC photoswitching, further supporting the hypothesis that upstream retinal neurons are dominant in mediating the light response.[88] A single intravitreal injection of AAQ was sufficient to restore the pupillary light reflex and light-avoidance behaviors in blind mice in vivo,[88] indicating that these blind animals were now able to sense light.

Despite its ability to restore visual function to blind mice, several properties of AAQ complicate its therapeutic potential. Because the thermal relaxation of AAQ is slow, photoswitching requires alternating pulses of UV and green light to convert the molecule from trans to cis and back to trans. If AAQ were to be used in the clinic, a dual-wavelength auxiliary light-stimulation system (i.e., goggles) would most likely be required to restore useful visual function. Additionally, acrylamide is reactive and neurotoxic,[136] although it is not clear whether this is also true of AAQ. AAQ's effect in vivo lasts for less than a day after an intravitreal injection. Given AAQ's short half-life (4 h), long-term treatment in blind patients would require frequent injections of the compound into the eye—an untenable therapeutic regimen. To overcome these shortcomings, several improved photoswitch compounds were developed and tested as potential drugs for vision restoration.

8.2. Second-Generation Cationic PCLs

To eliminate the need for UV light, a series of photoswitches, including DENAQ, BENAQ, and PHENAQ, were rationally designed using photochemical principles described in section 4.2 and synthesized.[89] All of these compounds are cationic ion-channel blockers containing a permanently charged quaternary ammonium moiety. DENAQ, BENAQ, and PHENAQ block voltage-gated potassium channels as well as HCN channels.[54,89] When exposed to

blue—green or white light, these photoswitches quickly convert to the cis state and, at light offset, quickly return to the trans state via thermal relaxation (Figure 13A).

Of these compounds, DENAQ and BENAQ proved to be the best at restoring visual function. Treatment with either photoswitch conferred robust light responses onto blind mouse, rat, and dog retinas from several different models of retinal degeneration[54,55,91,137] (Figures 13B and C and 14A and B). White light in the midphotopic range, similar in intensity to ordinary daylight, was sufficient to stimulate strong retinal responses. After the light was turned off, the response terminated within 100 ms, allowing for the detection of moderate-frequency visual stimuli.[54] Blocking synaptic inputs onto RGCs treated with DENAQ or BENAQ did not alter the polarity of the light response, suggesting that, unlike AAQ, these photoswitches primarily target RGCs.[54,91] Direct photosensitization of RGCs by DENAQ and BENAQ enabled spatially precise light responses, allowing for the independent stimulation of individual illuminated RGCs and suggesting that high-acuity vision might be possible (Figure 15). A single in vivo intravitreal injection of DENAQ photosensitized blind *rd1* mouse retinas for up to 7 days, with no measurable toxicity. In addition to restoring innate visual behaviors in blind mice, DENAQ also enabled a learned visual fear conditioning behavior, suggesting that formerly blind mice were able to acquire and process visual input[54] (Figure 15).

Both DENAQ and BENAQ display improved spectral response profiles, light sensitivity, and lifetime in vivo as compared to AAQ. Of the two photoswitch molecules, BENAQ may be more suited for eventual clinical use because it is 20-fold more potent compared to DENAQ[91] (Figure 16). BENAQ-mediated retinal photosensitization is species agnostic, working equally well in blind mouse, rat, and dog models of RP.[54,91] Intravitreal injection of BENAQ is safe in both mice and rabbits, which are large animals with eyes similar to human eyes that are often used for ophthalmic drug testing.[91] Finally, BENAQ has a much longer lifetime in vivo compared to DENAQ and AAQ, photosensitizing *rd1* mouse RGCs for up to 1 month following a single intravitreal injection[91] (Figure 16). A recently developed slow-release formulation of BENAQ extends the photosensitization even further, for up to 10 weeks after injection (Richard Kramer, unpublished data). Although PHENAQ,[89] possesses similar spectral properties to DENAQ and BENAQ and is also capable of photosensitizing retinal neurons,[138] it has a low solubility in the vitreous humor of the eye, making it less suitable for clinical use (Russell van Gelder, University of Washington, unpublished data).

These features make BENAQ a favorable photoswitch candidate for preclinical development as a potential therapeutic for human use. It is likely that BENAQ or other photoswitches would be delivered in the clinic via regular intravitreal injections, which are standard in clinical practice. Monthly intravitreal injections of antiangiogenesis drugs such as Lucentis (rabinizumab) and Avastin (bevacizumab) are commonly used in the clinic to treat wet AMD,[139] a type of retinal degeneration characterized by abnormal growth and leakage of blood vessels under the retina.[139] When injected intravitreally in the same formulation used for Lucentis, BENAQ has a 10× greater lifetime in the rabbit eye as compared to Lucentis and 5× greater lifetime as compared to Avastin,[140,141] suggesting that BENAQ or related photoswitches may be effective with even less frequent injections. A slow-release,biodegradable polymer formulation may extend the release lifetime even further.[142]

BENAQ's favorable safety profile and long lifetime in the eye may make it the first photoswitch compound suitable for clinical testing in blind patients

8.3. Uncharged (Protonatable) PCLs

DENAQ and BENAQ are permanently charged cationic molecules. While the permanent charge makes these compounds good pore blockers, it also makes the molecules membrane-impermeant, unless they encounter a large-pore ion channel that allows the molecule to cross. DAD, a protonatable derivative of DENAQ containing a tertiary amine rather than a quaternary ammonium (Figure 17), was synthesized in order to explore the properties of uncharged photoswitchable channel blockers. Some well-studied ion-channel blockers such as lidocaine exploit the lack of permanent charge to easily cross biological membranes, then become protonated inside neurons where the charged form can block channels, particularly voltage-gated sodium channels.[143] We reasoned that protonatable photoswitches may exhibit a similar behavior.

Like DENAQ and BENAQ, DAD is a photoswitchable K_v channel blocker that responds to blue—green or visible light.[85] DAD was first tested on mouse cortical neurons, where it blocked voltage-gated potassium currents in a light-dependent manner. DAD treatment also conferred a robust light response onto blind mouse retinas (Figure 17). Unlike BENAQ and DENAQ, the photosensitizing effect of DAD on RGC light responses was almost completely eliminated by synaptic blockers, suggesting that presynaptic retinal neurons are the main target of DAD.[85] Bipolar cells from retinas treated with DAD exhibited light-sensitive currents, indicating bipolar cell photosensitization. DAD restored both ON and OFF RGC light responses in many RGCs in the blind mouse retina as well as light–dark preference behaviors in blind mice.[85] The lifetime of DAD in vivo was quite short, with the photosensitizing effect wearing off 24 h after intravitreal injection. Like AAQ, DAD may thus require a slow-release formulation for potential clinical use.

8.4. Other PCLs

8.4.1. AMPA Receptors.—AMPA receptors are excitatory ionotropic glutamate receptors expressed in multiple types of retinal neurons, including horizontal cells, bipolar cells, amacrine cells, and RGCs.[144] A series of photochromic glutamate receptor agonists were synthesized and evaluated for their ability to control the activity of endogenous glutamate receptors as well as retinal neurons. One compound, ATA (Table 1), is a selective red-shifted AMPA receptor agonist that activates AMPA but not NMDA receptors in the dark and can be turned off by blue light stimulation.[145] When applied to a blind retina, ATA restored robust RGC responses to alternating light and dark stimuli. Experiments with synaptic blockers revealed that ATA affects RGCs and amacrine cells, which is consistent with the pattern of AMPA receptor expression in the retina.[145] These results suggest that it is possible to use a PCL-gated ion-channel agonist to directly drive neuronal activity rather than modulating neuronal activity by blocking voltage-gated ion channels in a manner similar to DENAQ and BENAQ.

8.4.2. $GABA_A$ Receptors.—$GABA_A$ receptors are inhibitory ionotropic GABA receptors expressed in RGCs where they modulate RGC activity in response to the release of

GABA from amacrine cells. Thus, photoregulation of $GABA_A$ receptor function in RGCs may allow for control of their activity. MPC088 (Table 1) is a light-sensitive allosteric $GABA_A$ receptor agonist that activates $GABA_A$ receptors in the dark or under 440 nm light in its trans isomer.[95] Illumination with UV light converts MPC088 to its cis isomer and terminates GABA receptor activation. Stimulation of dissociated rat RGCs with trans-MPC088 triggered an outward hyperpolarizing GABAergic current while cis-MPC088 had no effect.[95] Thus, MPC088 may potentially be used to photoregulate the activity of RGCs in animal models of RP and in human patients.

9. CELL-TYPE AND DEGENERATION-SELECTIVE PCLS

While the first cohort of patients for clinical photoswitch testing might be end-stage RP patients with no light perception (i.e., patients who would also be candidates for electronic retinal prosthetics[146]), recent evidence suggests that photoswitches may also be appropriate for less-severe visual impairment. This is important because the vast majority of patients with retinal degeneration are not completely blind, with RP patients gradually losing peripheral vision,[14] while patients with advanced dry AMD primarily lose central vision.[147] If photoswitches are to be used in the clinic to treat partially sighted patients, it is critical to ensure that they do not impair normal photoreceptor-mediated visual function. To test this, the effects of PCL treatment were measured on healthy retinas from sighted WT mice and rats. Surprisingly, DENAQ and BENAQ had almost no effect on the normal, photoreceptor-mediated light responses of wild-type mouse retinas, in contrast to their robust effect on retinas from a variety of animal models of RP.[54,55,91] Similarly, while DENAQ and BENAQ robustly photosensitized *rd1* mouse RGCs, they did not photosensitize wild-type mouse RGCs.[55,91]

Earlier studies with another photoswitch, named QAQ, showed that it can selectively enter into cells that express large-pore ion channels, including P2X receptors, ionotropic receptors for extracellular ATP.[83] Because DENAQ and BENAQ are similar to QAQ, we asked whether degeneration-dependent photosensitization is a result of chronic activation of a large-pore channel in the degenerated retina. To test this hypothesis, the activity of large-pore channels in RGCs was first measured by imaging the loading of YO-PRO, a cationic dye similar in size to DENAQ and BENAQ.[54] Because YO-PRO is also not normally membrane-permeable, it has been used in the past to probe the functional state of large-pore channels such as TRPV1 and P2X receptors.[149,150] YO-PRO loaded much more robustly into RGCs from *rd1* mouse retinas compared to WT mouse retinas. By a combination of various pharmacological and genetic manipulations, P2X receptors, especially the P2X7 receptor, were found to be the main large-pore channel mediating YO-PRO entry into *rd1* RGCs.[55] P2X receptors have been implicated in a range of neurodegenerative disorders, including retinal diseases.[151,152] Elevated ATP is found in the eyes of patients suffering from proliferative diabetic retinopathy[153] and wet AMD,[154] and the expression of P2X receptors is elevated in retinas from mouse models of RP.[155,156] Hence, purinergic signaling is altered in several retinal-degenerative diseases.

Additional experiments showed that loading of DENAQ and BENAQ into *rd1* mouse RGCs was also mediated by P2X receptors (Figure 18). Treating the retina with an ATPase reduced

the photosensitizing effect of DENAQ in *rd1* mouse RGCs, while overexpression and pharmacological activation of the P2X7 receptor enabled DENAQ to photosensitize wild-type RGCs.[55] This result suggests that P2X receptors are both upregulated and chronically activated in RGCs in animal models of RP after photoreceptor degeneration. If these receptors are also upregulated or activated in the retinas of patients with midstage RP or geographic atrophy, it is possible that intravitreally injected photoswitches would selectively enter and photosensitize retinal neurons in regions of the retina undergoing photoreceptor degeneration while leaving neighboring healthy regions unaffected. This hypothesis is currently being tested in mice and rats surgically implanted with a subretinal chip, resulting in local photoreceptor degeneration.[157]

Gene therapy can be targeted to introduce an optogenetic tool to a particular cell type, for example, remnant cone photoreceptors,[38] bipolar cells,[36] or ganglion cells.[37] Targeting a specific cell type might be beneficial for vision restoration because more of the intrinsic retinal circuitry for image processing may be utilized by stimulation of remnant photoreceptors or bipolar cells, for example, which may lead to improved visual function. Similarly, it may be advantageous to selectively target and stimulate either the ON or the OFF pathway for the transmission of visual information in the retina, as such selective stimulation may be more similar to normal visual input and thus easier for the brain to process and interpret. Some PCLs display cell-type selective effects on the retina. Photoswitches such as DENAQ and BENAQ selectively photosensitize OFF *rd1* RGCs, but not ON or ON–OFF *rd1* RGCs or any type of WT RGCs[55] (Figure 18). DAD selectively affects bipolar cells in retinas with photoreceptor degeneration.[85]

Together, the studies on the effects of various types of PCLs on the retina uncovered important functional changes associated with photoreceptor degeneration, which appear to be a part of the retinal remodeling discussed earlier.[21] The disease-selective action of DENAQ and BENAQ revealed that retinal degeneration leads to a functional upregulation of P2X receptors, likely caused by a combination of elevated extracellular ATP and a change in P2X receptor expression. PCLs have not only proved to be capable of restoring visual function to the blind retina but also identified important novel aspects of retinal remodeling that have furthered the understanding of this complex process and may yet lead to drugs that slow down or prevent retinal degeneration. Additionally, while certain aspects of retinal remodeling can potentially impair the effectiveness of retinal implants or optogenetic therapies,[158–160] they seem to only facilitate the action of PCLs. Thus, PCLs may be uniquely suited to treat the blind retina suffering from retinal remodeling.

10. PCL PROSPECTS AND FUTURE DIRECTIONS

Ultimately, vision is a product of our brains as much as the retina. The prolonged absence of visual input from the retina to the brain in advanced RP patients may lead to structural or functional changes in the lateral geniculate nucleus or the visual cortex, where visual information is processed. Even after light-elicited retinal signals are restored, there may be heightened neural background noise or underperforming attention circuits in the brain that limit the complexity of perceived visual scenes. On the other hand, plasticity in the cortical circuitry may allow the brain to learn to make sense of retinal signals that are quite different

from those generated by normal visual signaling pathways. The clinical data from patients implanted with the Argus II retinal prosthesis are encouraging in that regard, as several patients with bare or no light perception prior to the treatment could identify and distinguish different objects after the surgery. Likewise, early experiments with direct electrical stimulation of the visual cortex in blind patients resulted in visual percepts sufficient to read letters, supporting the idea that the cortex is highly plastic and can learn to interpret novel and unusual stimuli.

10.1. Alternative PCL Targets in the Mammalian Retina

10.1.1. Voltage-Gated Sodium Channels.—

Voltage-gated sodium (Na_V) channels are a potentially attractive target for modulating neuronal activity because they are ubiquitously expressed throughout retinal neurons, including bipolar cells and RGCs.[161] QAQ (Table 1) is a light-sensitive Na_V channel PCL that unblocks Na_V channels upon UV illumination and depolarizes different types of neurons.[83] QAQ treatment restored light sensitivity to RGCs from blind *rd1* mouse retinas, and QAQ-mediated photosensitization exhibited the same disease selectivity as DENAQ and BENAQ.[55] However, like AAQ, QAQ suffers from an important drawback—the requirement for UV illumination, which is potentially damaging to the eye. Recently, QENAQ (Table 1), a red-shifted derivative of QAQ, was synthesized to overcome this limitation. QENAQ was capable of photosensitizing mouse somatosensory neurons to 480 nm blue light, similar to that used to photoisomerize DENAQ and BENAQ.[84] In the future, we expect to test the action of QENAQ in animal models of RP to compare its efficacy and lifetime in vivo with that of DENAQ and BENAQ.

10.2. PCL Translational Potential

As drug-like small molecules, PCLs have several potential advantages over optogenetic and PTL strategies for vision restoration. In contrast to gene-therapy-based vision-restoration approaches, the effect of PCLs is reversible, which would allow clinicians to adjust drug dosage to maximize efficacy and minimize toxicity. While other vision-restoration technologies such as electronic retinal implants and gene therapy commit a patient to a specific product for potentially their entire life, PCL treatment can be easily terminated if necessary and improved photoswitch compounds with higher light sensitivity and faster response kinetics can be deployed into the clinic once they are developed. The disease selectivity of DENAQ and BENAQ may also prove to be useful in the clinic if these compounds target blind regions of the retina without adversely affecting any remaining photoreceptor-mediated visual function. The potential ability to fill in gaps in the visual world would be quite valuable to patients suffering from localized forms of degeneration, including midstage RP and dry AMD.

In addition to their reversibility, recent work has shown that PCLs also possess a remarkable degree of cell-type selectivity, almost rivaling that of gene-therapy-based approaches. The effect of compounds like DENAQ and BENAQ is highly selective for RGCs,[55] while DAD preferentially photosensitizes bipolar cells.[145] In the future, clinicians may eventually choose a PCL therapy that is most suited for an individual patient’s retina, depending on the severity of retinal degeneration, with bipolar cell photosensitization being more useful for patients in the early and middle stages of retinal degeneration while RGC photosensitization

would be more suitable for very advanced RP patients suffering from extensive retinal remodeling. The well-established regulatory approval process for small-molecule ocular therapeutics may also make it easier to obtain FDA approval for PCL clinical trials, assuming they prove to be safe in preclinical animal testing.

10.2.1. Charged Ion-Channel Blockers.—At present, several charged PCLs have been tested in rodent models of retinal degeneration. Ideally, a vision-restoring therapy would also be tested in primates prior to clinical trials, but no good primate models of inherited retinal degeneration are currently available. Instead of primate models, it may be possible to validate the vision-restoring power of PCLs in blind dogs or pigs,[162] although these large animal models are expensive and require long generation times. The toxicity and pharmacokinetics of PCLs such as BENAQ will also need to be carefully measured in large animal models such as rabbits in the future. Ultimately, the translatability of retinal photosensitivity into useful vision will need to be evaluated in blind human patients in a clinical trial, which will only be possible if the FDA confers an Investigational New Drug designation.

10.2.2. Uncharged Ion-Channel Blockers.—Like the charged PCLs, DAD has been evaluated in rodent models of retinal degeneration. The lack of a permanent charge may have contributed to its short lifetime in vivo (half-life = 9 h),[85] which would be undesirable for clinical use because it would require daily intravitreal injections. However, it is possible that novel uncharged photoswitches with improved in vivo pharmacokinetic profiles could be developed. The solubility of both DAD and charged PCLs in human vitreous will need to be measured in the clinic, and it may be one factor limiting the in vivo therapeutic effectiveness of PCLs, as was the case for PHENAQ. Newer and more-soluble azobenzene derivatives can be prepared in the future if vitreal photoswitch solubility proves a major problem. Alternatively, slow-release polymer formulations of photoswitch compounds that are currently being developed[142] may obviate potential solubility limitations as well as significantly extend the lifetime of ocular therapeutics, as has been demonstrated for similar formulations of Lucentis.[163]

10.2.3. Other PCLs.—MPC088 has not yet been evaluated for in vivo safety or its ability to restore visual perception in blind animal models of retinal degeneration. These studies would need to be completed prior to any clinical testing. Additionally, the photochemical properties of MPC088 could be improved further via a strategy similar to the one used to design DENAQ and BENAQ.

Like MPC088, ATA faces translational challenges. Prolonged activation of AMPA receptors is cytotoxic and is thought to contribute to RGC cell death in glaucoma.[144] ATA requires continued blue light illumination to prevent the molecule from activating AMPA receptors in the dark. Thus, while stimulation of endogenous AMPA receptors is an intriguing potential strategy for vision restoration, new photoswitch compounds that are inactive in the dark would need to be developed and their in vivo safety thoroughly characterized prior to any clinical testing.

11. CONCLUDING REMARKS

While the preliminary studies of PTLs and PCLs in animal models of retinal degeneration are promising, these tools are still far from being used in the clinic. Even if it is possible to restore retinal light sensitivity in blind patients, it is unclear what extent of visual function can be restored, due to remapping of synaptic connections in the visual cortex as well as the potential difficulty of processing the very different form of retinal output generated by PTL/PCL treatment as opposed to normal vision. Thus, these early studies, while promising, need to be evaluated cautiously, and their clinical utility will only be proven once their safety is established. These caveats aside, PTLs and PCLs offer an intriguing novel means of potentially restoring visual function to blind patients that might be a useful complement to existing treatments such as retinal implant as well as future gene and stem cell therapies.

Chemical photoswitches are an excellent example of the power of chemistry and the value of collaborations between chemists and neuroscientists both from a basic science and a translational standpoint. Even if the compounds described in this Review do not end up being used in the clinic to restore vision, they remain valuable tools for investigating both basic neuroscience as well as disease mechanisms, as they allow for the targeted photochemical manipulation of different molecular receptors and cell types both in vitro and in vivo. Optopharmacology also has great potential for translational applications beyond vision restoration, because it can draw on the essentially infinite variation in structure and function of drug-like small molecules enabled by both rational chemical design as well as high-throughput screening. This power enables the development of compounds with properties uniquely tuned to the relevant molecular target and disease, which have only begun to be explored in the pioneering studies presented in this Review. Because synthetic chemistry has produced virtually all of the drugs currently used in the clinic, it is likely that optopharmacology will likewise make an important contribution to the development of novel therapies in the future, making it an exciting new field of study with tremendous potential.

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Biographies

Ivan Tochitsky obtained his B.S. in Biochemistry at the University of California, Los Angeles, followed by a Ph.D. in Molecular and Cell Biology at the University of California, Berkeley, with his advisor, Dr. Richard Kramer. During his Ph.D., Ivan Tochitsky developed PTL and PCLs as tools for basic neuroscience research and potential therapies for vision restoration together with Drs. Kramer and Isacoff. He is currently a research fellow in neurobiology at Boston Children’s Hospital and Harvard Medical School. His current

research is focused on the study of neuropathic pain and other neurodegenerative diseases as well as the development of novel therapeutics for these disorders.

Michael Kienzler obtained his B.S. in chemistry from Rensselaer Polytechnic Institute in 2005 and his Ph.D. in organic chemistry from U.C. Berkeley in 2010. His Ph.D. research included the total synthesis of marine natural products and, after moving with his advisor Dirk Trauner to Munich's Ludwig Maximillian University in 2008, the synthesis of new azobenzene photoswitches. He then returned to U.C. Berkeley as a postdoctoral fellow, and he developed visible-light activated photoswitches for glutamate receptors and other neuroscience applications. Since 2016, he has been an assistant professor of chemistry at the University of Maine in Orono. His research interests include organic synthesis, chemical biology, and molecular neuroscience.

Ehud Isacoff obtained his B.Sc. and Ph.D. at McGill University, was a postdoctoral fellow at UCSF, and has been a professor at U.C. Berkeley since 1993, where he is currently Rauch Professor of Neurobiology and Director of the Helen Wills Neuroscience Institute. He has worked on the mechanisms of ion channel and neurotransmitter receptor function, the molecular basis of synaptic transmission and plasticity, and the development of neural circuits. With Richard Kramer and Dirk Trauner, Isacoff developed PTLs that provide optical control of neural firing and synaptic transmission by activating or blocking ion channels and ionotropic and metabotropic neurotransmitter receptors. This approach has opened a novel branch of optogenetics that enables synaptic connections to be probed in real time in intact circuits at multiple scales and has led to an effort to create a treatment for blindness by installing light sensitivity into surviving retinal layers following photoreceptor cell degeneration.

Richard Kramer obtained his B.Sc. at the State University of New York, Albany, and his Ph.D. in Neurobiology at the University of California, Berkeley. He is currently the C. H. and Annie Li Chair in Molecular Biology of Diseases and Professor of Neurobiology at the University of California, Berkeley. He has worked on the mechanisms of ion-channel and neurotransmitter receptor function, the origin of repetitive activity in neurons, mechanisms of sensory transduction, and synaptic physiology in the retina and brain. With Ehud Isacoff and Dirk Trauner, Kramer developed PTLs that enable optical control of neural firing and synaptic transmission by activating or blocking ion channels. Most recently, he has led the development of PCLs and their translational application to restore vision to blind patients suffering from retinal degeneration.

ABBREVIATIONS

AAQ	acrylamide azobenzene quaternary ammonium
AAV	adeno-associated virus
AMD	age-related macular degeneration
AMPA receptor	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor

ATA	azobenzene tetrazolyl AMPA
ATP	adenosine triphosphate
BENAQ	benzyl ethyl amine azobenzene quaternary ammonium
Bis-Q	bis-quaternary ammonium azobenzene
CNS	central nervous system
DAD	diethylamino azobenzene diethylamino
DENAQ	diethyl amine azobenzene quaternary ammonium
FDA	Food and Drug Administration (of the United States of America
GABA	gamma-aminobutyric acid
GPCR	G-protein coupled receptor
GluR	glutamate receptor
HCN channel	hyperpolarization-activated cyclic nucleotide-gated channel
K_v channel	voltage-gated potassium channel
LiGluR	light-gated ionotropic glutamate receptor
LimGluR	light-gated metabotropic glutamate receptor
MAG	maleimide azobenzene glutamate
MAQ	maleimide azobenzene quaternary ammonium
nAChR	nicotinic acetyl choline receptor
NMDA	*N*-methyl-D-aspartate
P2X receptor	ATP-gated cation channel
PAL	photoswitchable affinity label
PCL	photochromic ligand
PORTL	photoswitchable orthogonal remotely tethered ligand
PTL	photoswitchable tethered ligand
QAQ	quaternary ammonium azobenzene quaternary ammonium
QBr	quaternary ammonium azobenzene bromomethyl
RGC	retinal ganglion cell
RP	retinitis pigmentosa

RPE	retinal pigment epithelium
rd1	retinal degeneration 1, a mouse strain with a mutation in the phosphodiesterase (Pde6b) gene, leading to retinal degeneration; a common animal model of RP
SNAP-tag	self-labeling protein that recognizes and covalently binds to molecules with an O^6-benzylguanine moiety
SPARK	synthetic photoisomerizable azobenzene-regulated K^+ channel
TRPV1 channel	transient receptor potential cation channel subfamily V member 1
YO-PRO	cyanine-based fluorescent dye

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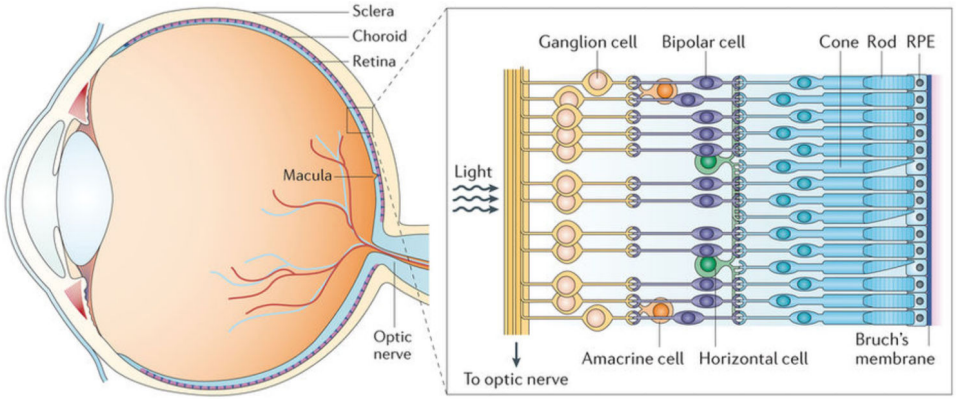


Figure 1.

Overview of the eye and retina. Light enters the eye and hits the retina, the neural structure that converts visual stimuli into signals that are sent to the brain through the optic nerve. The retina is composed of several layers of neurons, including rod and cone photoreceptors; various interneurons, including horizontal, bipolar, and amacrine cells; and retinal ganglion cells (RGCs), which transmit visual information to the brain. Adapted with permission from ref 8. Copyright 2015 Springer Nature.

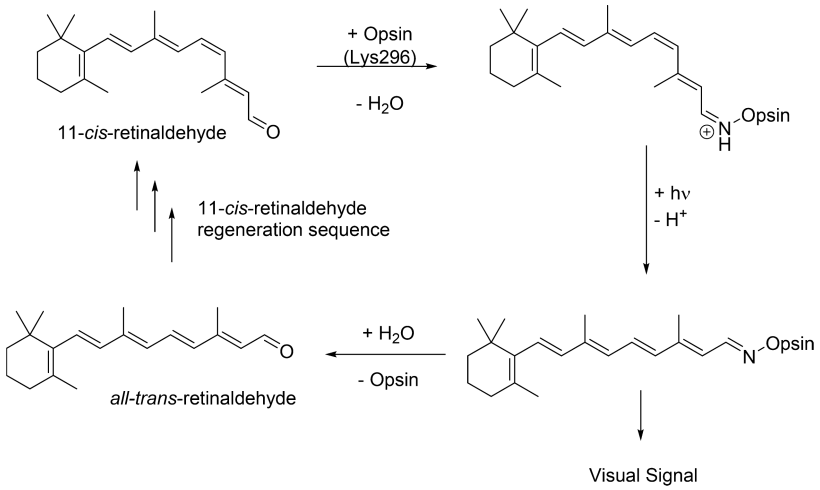


Figure 2.

Visual (retinoid) cycle. Retinal photoreceptors contain membrane proteins (opsins) that absorb light. Within each opsin protein, a molecule of 11-*cis*-retinaldehyde absorbs visible light to photoisomerize into the all-*trans*-retinaldehyde, propagating the signaling cascade that eventually results in vision. Simplified scheme of retinal photoisomerization and regeneration in the retina.

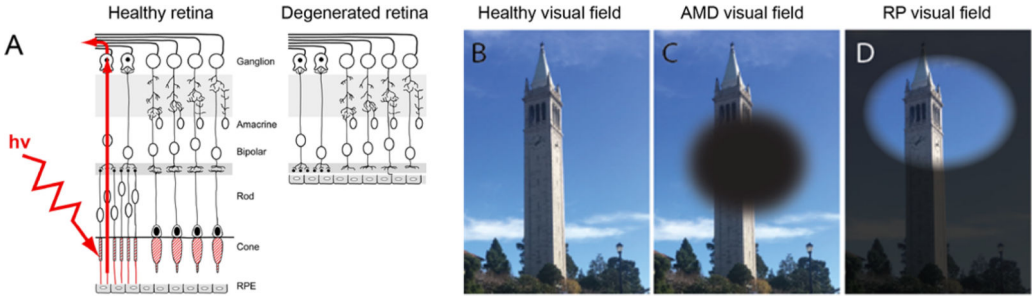


Figure 3.

(A) Retinal circuitry and retinal degeneration. In the wild-type retina (left), incident light is absorbed by the photoreceptors (highlighted in red), where it is converted to an electrical signal (straight red arrow). The signal is processed and transmitted by inner retinal neurons to the retinal ganglion cells before being passed to the brain via the optic nerve. Because photoreceptors are absent in the degenerated retina (right), there is no electrical response to illumination. Adapted with permission from ref 19. Copyright 2012 Elsevier, Inc. Representative visual scenes as perceived by a healthy sighted person (B), a person with advanced age-related macular degeneration (AMD) (C), and a person with retinitis pigmentosa (RP) (D). (B–D) Images copyright Michael Kienzler.

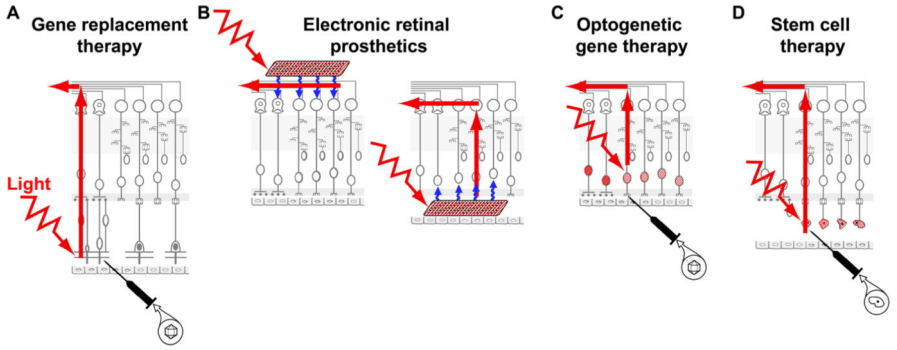


Figure 4.

Therapeutic strategies for vision restoration. Each diagram shows the light-activatable cell type or device (red hatches) and the pathway of the light-generated electrical activity (straight red arrows) from the retina to the brain. (A) Gene-replacement therapy uses viral vectors to replace mutant genes, preserving or restoring visual function. (B) Electronic retinal prosthetics (epiretinal, left; subretinal, right) electrically stimulate surviving retinal neurons after photoreceptor degeneration, thus transmitting visual information to the brain. (C) Optogenetics uses gene therapy to express light-sensitive opsins in surviving retinal neurons, making those cells light-sensitive. (D) Stem cell therapies can potentially replace degenerated cells, thus restoring the retinal circuits for transmission of visual information. Adapted with permission from ref 19. Copyright 2012 Elsevier, Inc.

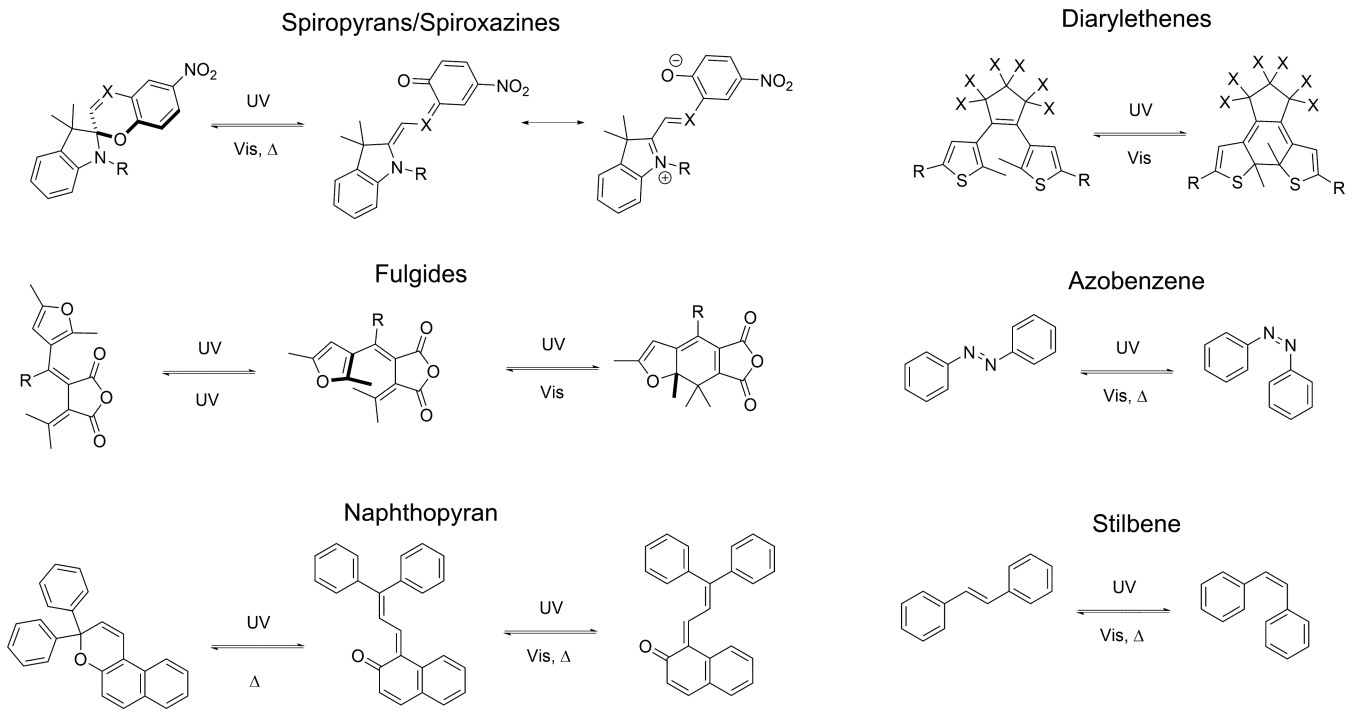


Figure 5.

Chemical photoswitches. A selection of synthetic photoswitches with a wide range of photochemical and physicochemical properties: spiropyrans, diarylethenes, fulgides, azobenzenes, naphthopyrans, and stilbenes. Ease of synthesis and stability in physiological environments are important considerations in selecting photoswitches for biological applications.

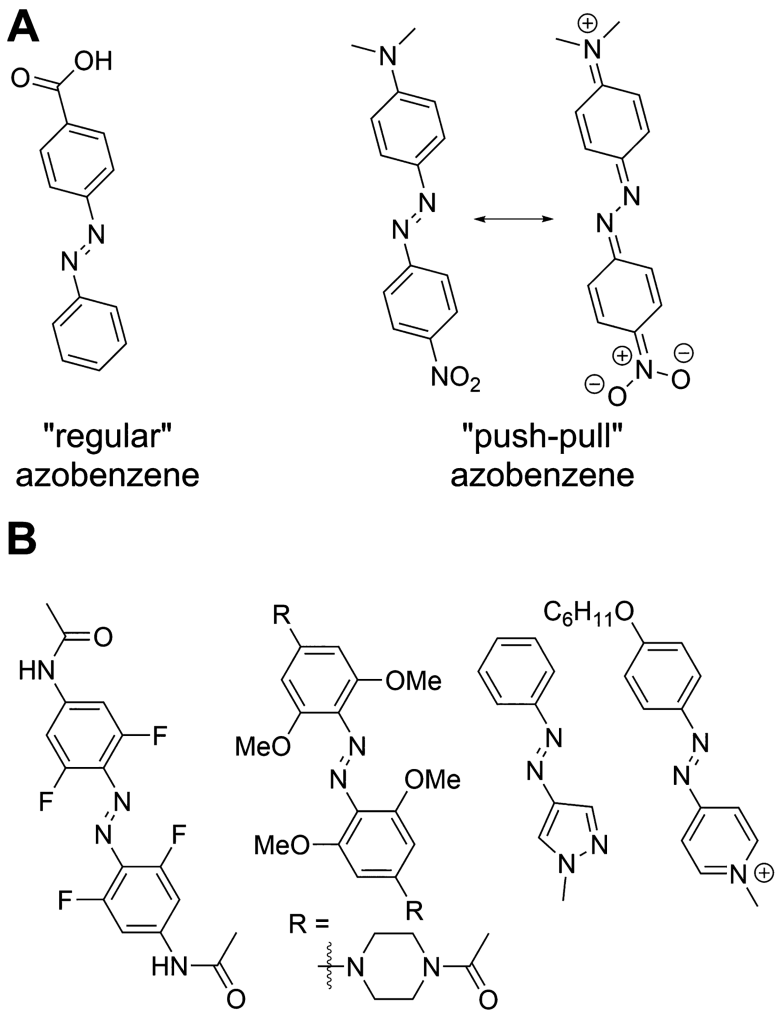


Figure 6.

Azobenzene photoswitch properties. The type and location of functional-group substitution on an azobenzene affects the optimal wavelengths of photoisomerization as well as the thermal stability of cis isomers. (A) Regular azobenzenes photoisomerize from trans → cis with UV light, and the cis isomer is relatively stable; push–pull azobenzenes have red-shifted absorptions and short half-lives due to weakening of the azo double-bond character. (B) Recent methods for modulating azobenzene photochemical properties include tetra-ortho and heteroaryl substitutions.

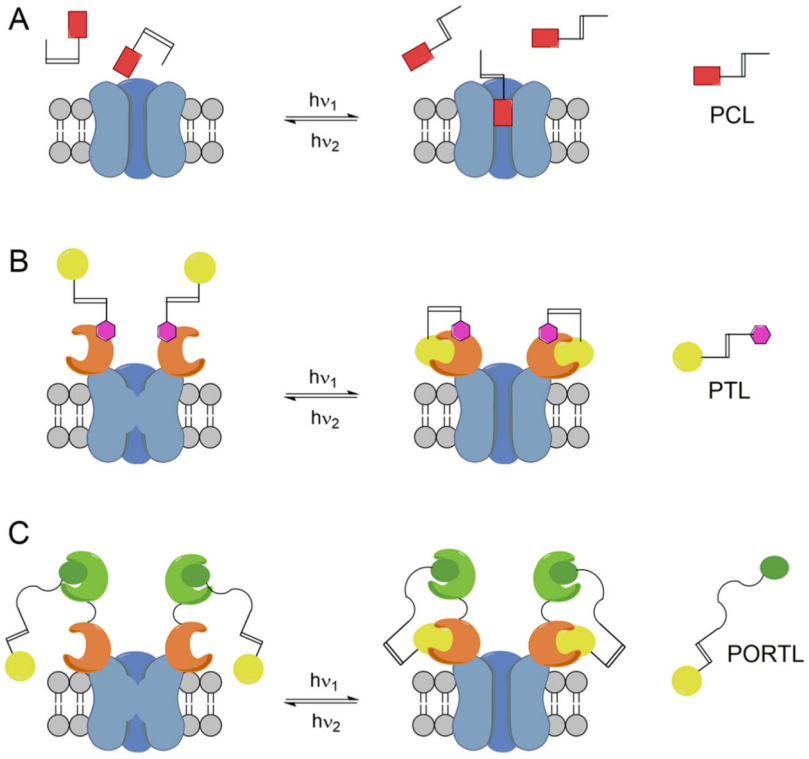


Figure 7.

Diagram of methods for photocontrol of protein function using reversible photoswitchable ligands. (A) A photochromic ligand (PCL) is freely diffusible, with one active photoisomer and the other inactive. (B) A photoswithable tethered ligand (PTL) is covalently attached to the protein of interest, again such that only one photoisomer is active. (C) A photoswitchable orthogonal remotely tethered ligand (PORTL) is a PTL variant that uses self-labeling protein chemistry for the covalent tether and possesses a much longer linker.

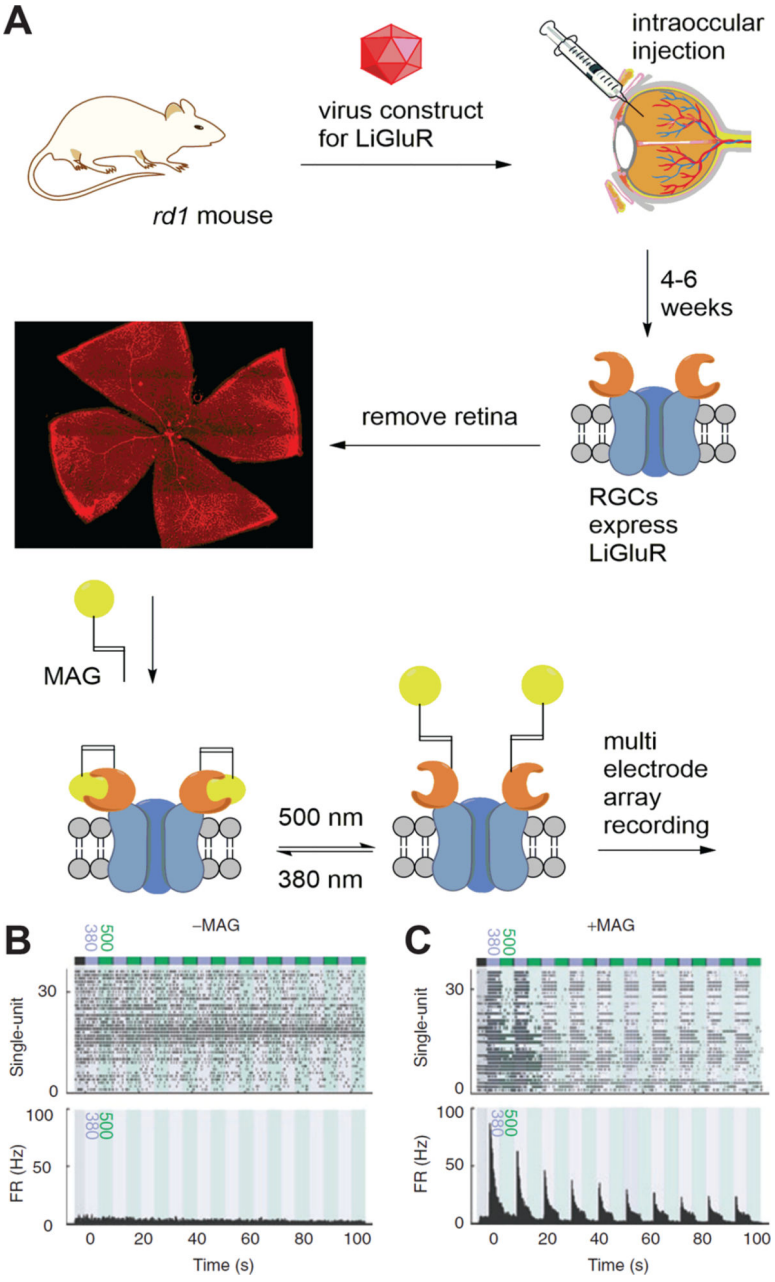


Figure 8.

Using a PTL approach on a mouse model of blindness. (A) Workflow for making blind mouse RGCs light-sensitive: create virus with LiGluR gene and cell-type promotor, inject intravitreal virus, wait 4–6 weeks for LiGluR to express in RGCs, remove retina, label with MAG, and record with a multielectrode array. (B) Microelectrode array (MEA) recording of blind mouse retina expressing LiGluR, but without MAG labeling. (C) Same retina after labeling LiGluR with MAG. Adapted with permission from ref 103. Copyright 2011 The American Society of Gene & Cell Therapy.

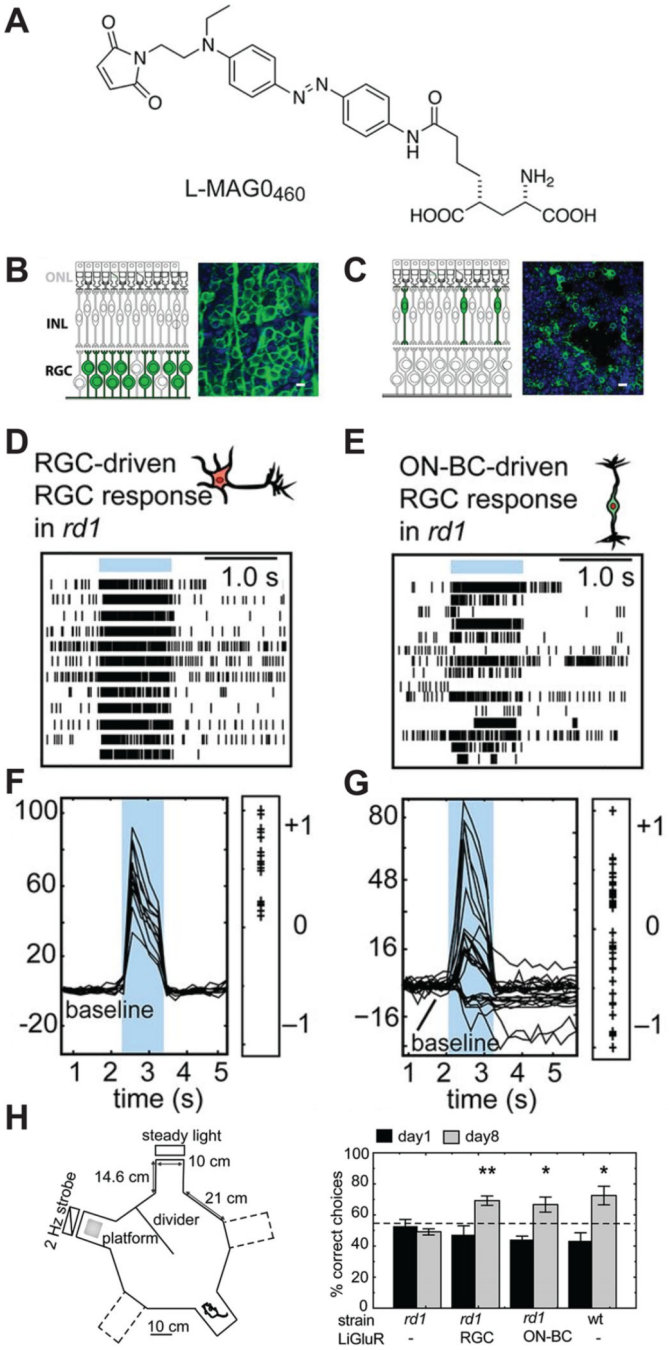


Figure 9.

Vision restoration in blind mice using PTLs sensitive to visible light. (A) The red-shifted MAG_{460} has a peak at 460 nm and responds to white light. (B–C) Cartoons depicting viral targeting of LiGluR to different retinal cell populations: (B) RGCs and (C) ON-Bipolar cells (ON-BCs). (D–G) MEA recordings from RGCs and ON-BCs that have been photosensitized with LiGluR + MAG_{460}. Note the different firing patterns seen in RGCs (D, F) and ON-BCs (E, G). (H) (left) Water maze forced choice setup; (right) mice expressing LiGluR in either

RGCs or ON-BCs show significant improvement in finding the submerged platform. Adapted with permission from ref 105. Copyright 2014 National Academy of Sciences.

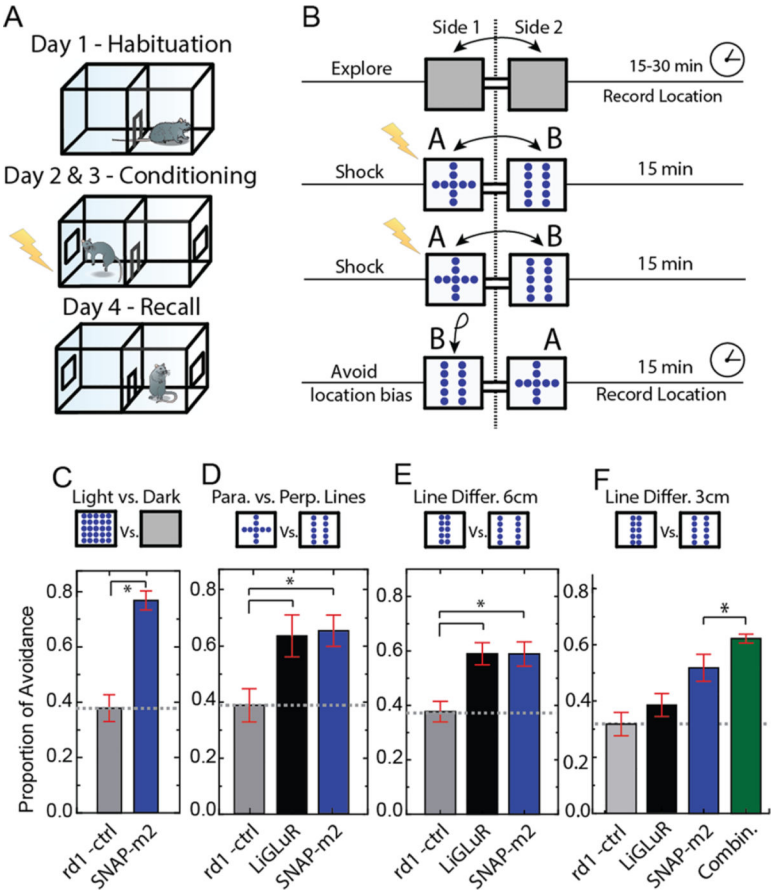


Figure 10.

Establishing higher-order vision restoration in blind mice, pattern recognition, and visual acuity. (A, B) Cartoon schematic describing the vision experiments: mice were conditioned to associate electric shocks with different visual patterns. (C–F) Quantification of learned behaviors: (C) light avoidance, (D) pattern differentiation, and (E) line-spacing differentiation; (F) the cumulative effect of both LiGluR and SNAP-m2 is greater than either alone. Adapted with permission from ref 106. Copyright 2017 Springer Nature.

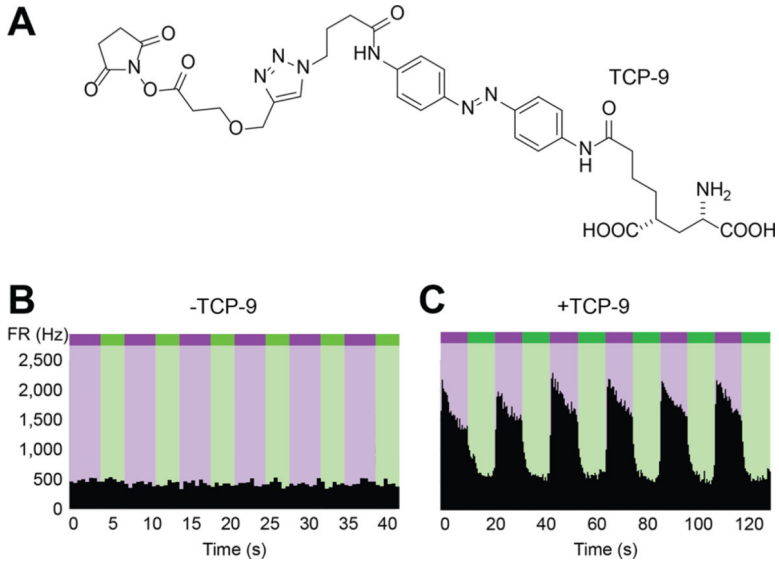


Figure 11.

TCPs restore light responses in the retina of *rd10* mice. (A) Structure of TCP-9, which uses the reactive NHS ester to label wildtype receptors after the glutamate portion binds. (B) Blind *Rd1*0 mouse retinas do not respond to UV or visible light; (C) after addition of TCP-9, robust light responses are recorded from the retina. Adapted with permission from ref 109. Copyright 2016 Springer Nature

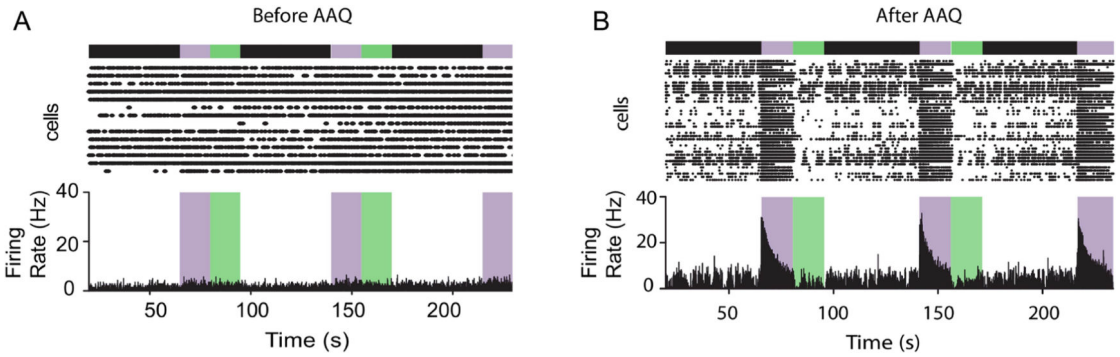


Figure 12.

AAQ confers UV light sensitivity to blind *rd1* mouse retinas ex vivo. (A, B) Multielectrode array recording of *rd1* mouse RGC activity in response to UV light stimulation (purple) before (A) and after (B) treatment with AAQ. AAQ enables robust retinal light responses. Adapted with permission from ref 88. Copyright 2012 Elsevier, Inc.

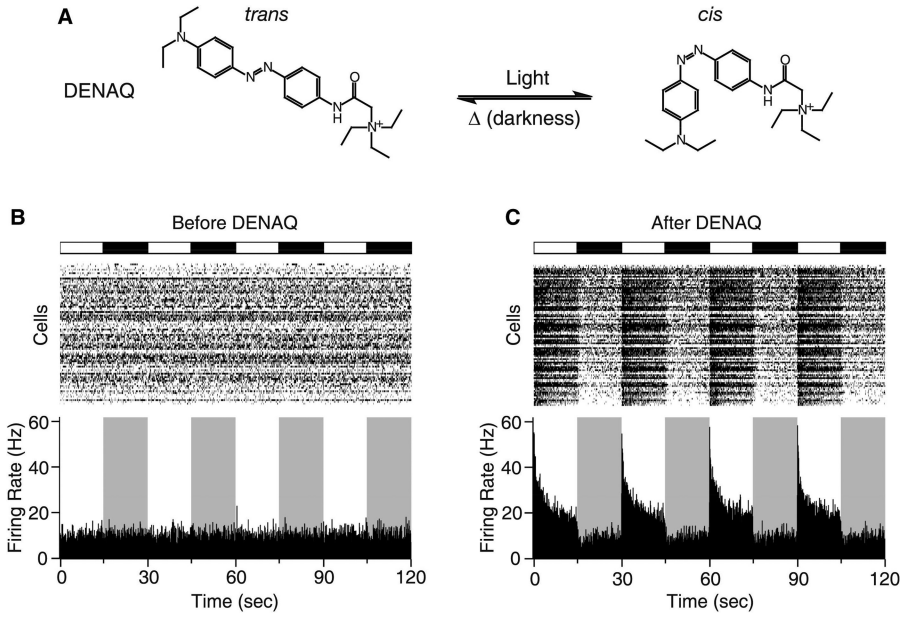


Figure 13.

DENAQ restores visible light sensitivity to blind mouse retinas ex vivo. (A) Molecular structure of DENAQ. Visible light converts DENAQ from the trans to the cis form, and the compound quickly relaxes back to the trans form in the dark. (B, C) Multielectrode array recording of *rd1* mouse RGC activity in response to white light stimulation before (B) and after (C) treatment with DENAQ. DENAQ restored robust retinal light responses. Adapted with permission from ref 54. Copyright 2014 Elsevier, Inc.

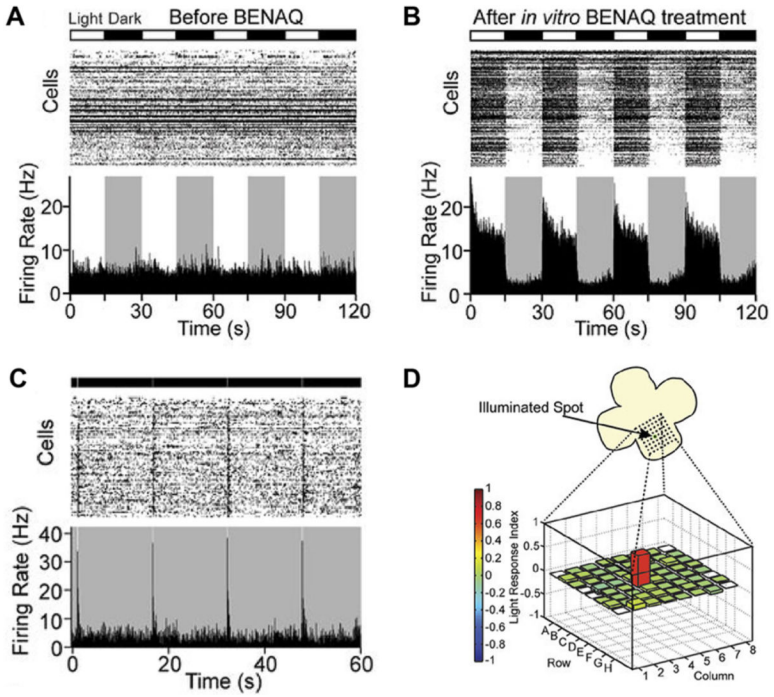


Figure 14.

BENAQ restores robust, spatiotemporally precise light responses to blind mouse retinas ex vivo. (A, B) Multielectrode array recording of *rd1* mouse RGC activity in response to white light stimulation before (A) and after (B) treatment with BENAQ. (C, D) BENAQ-treated *rd1* retinas respond robustly to brief, 50 ms light flashes (C) and small spot light stimuli (D). Adapted with permission from ref 91. Copyright 2017 Springer Nature.

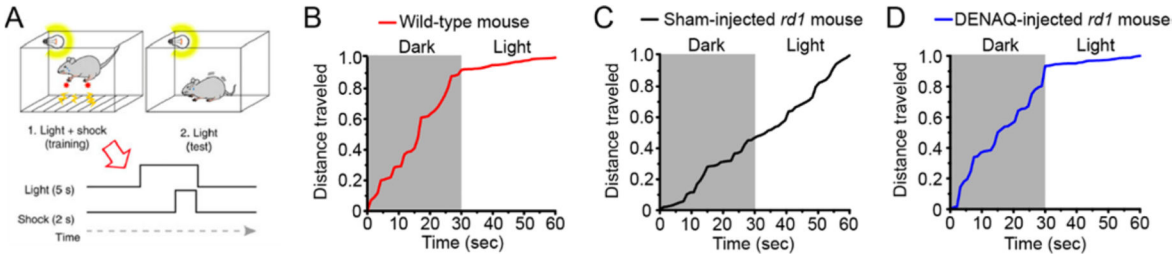


Figure 15.

DENAQ injection restores visual cued conditioned fear in blind mice in vivo. (A) Experimental design: light is turned on before a mild foot shock for several cycles during the conditioning day. Mice normally freeze (stop moving) in response to the foot shock. The following day (test day), mice are exposed to light alone and their movement is measured. (B) Sighted mice learn to associate the light with the foot shock and freeze in response to the light after conditioning. (C) Saline (sham)-injected blind *rd1* mice do not respond to light because they cannot see it and keep moving at the same rate. (D) DENAQ injected *rd1* mice are able to see the light and learn to associate the light with the foot shock, freezing when exposed to light after conditioning. Adapted with permission from ref 54. Copyright 2014 Elsevier, Inc.

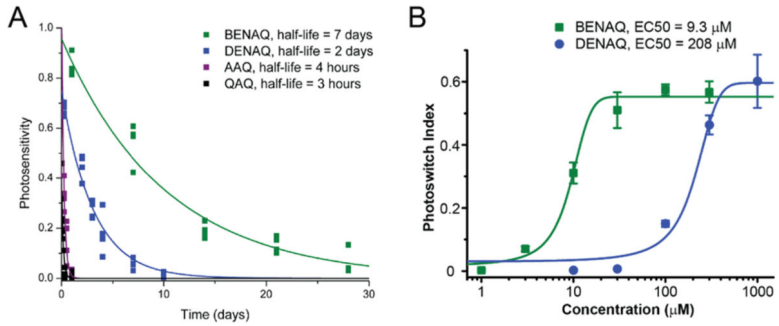


Figure 16.

PCL potency and lifetime in vivo. (A) PCLs exhibit different lifetimes in vivo after a single intravitreal injection, ranging from hours (AAQ) to weeks (BENAQ). (B) BENAQ is the most potent charged PCL tested in the retina. Adapted with permission from refs 54 and 91. Copyright 2014 Elsevier, Inc. (A) and 2017 Springer Nature (B).

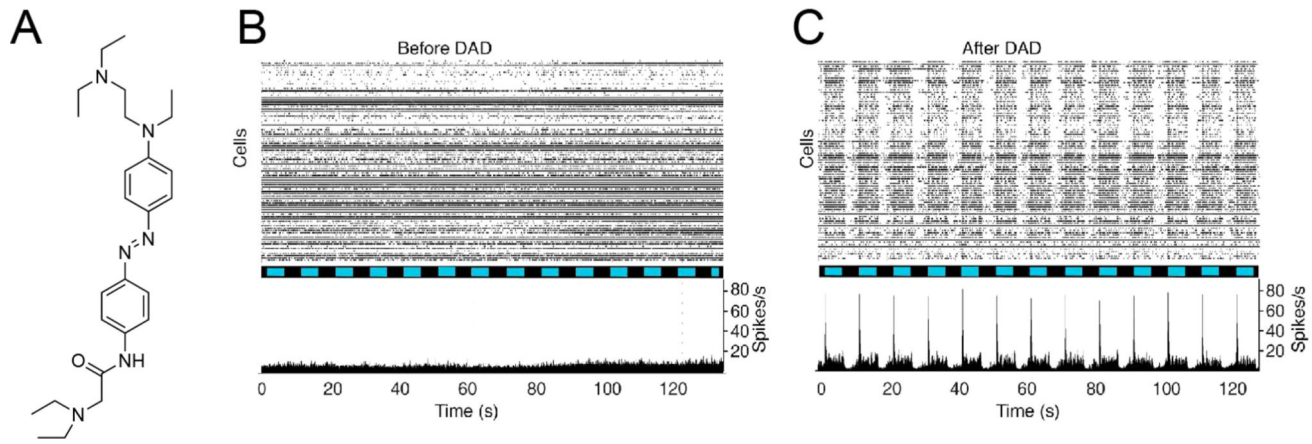


Figure 17.

(A) Structure of DAD, an uncharged (protonatable) voltage-gated ion-channel PCL. (B, C) Multielectrode array recording of blind mouse RGC activity in response to blue light stimulation before (B) and after (C) treatment with DAD. DAD enables robust retinal light responses. Adapted with permission from ref 85. Copyright 2017 The American Society for Clinical Investigation

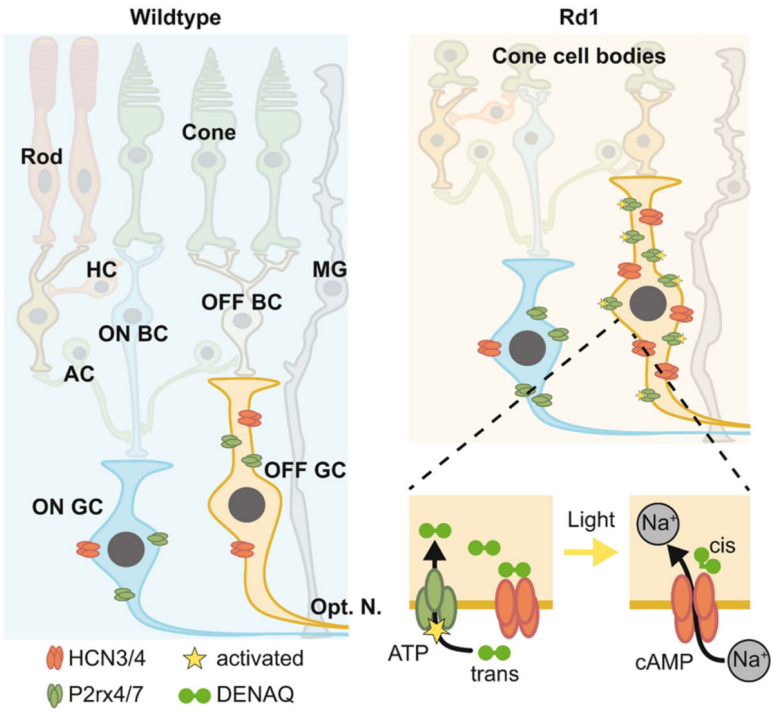
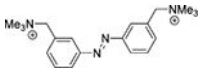
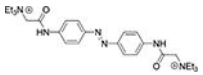
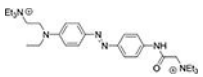
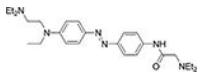
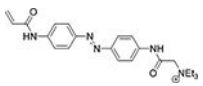
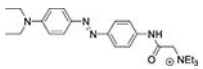
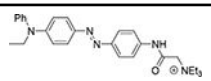
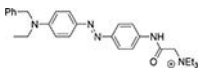
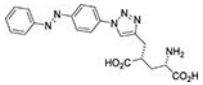


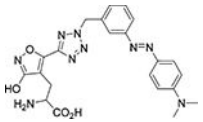
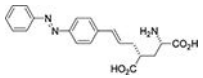
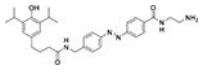
Figure 18.

PCL degeneration-dependence and cell-type selectivity. DENAQ and BENAQ selectively photosensitize OFF-RGCs in retinas suffering from photoreceptor degeneration. Large-pore P2X receptors enable permeation of cationic PCLs selectively into RGCs in retinas suffering photoreceptor degeneration, like those from *rd1* mice (right) but not healthy mouse retinas (left). Once inside RGCs, PCLs such as DENAQ selectively photosensitive HCN channels in OFF but not ON RGCs, modulating the activity of OFF RGCs in response to light stimuli. Adapted with permission from ref 148. Copyright 2016 Elsevier, Inc.

Table 1.

Photochemical and Pharmacological Properties of the Freely Diffusible Photochromic Ligands (PCLs) Discussed in This Review[a]

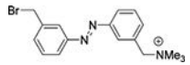
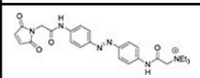
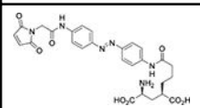
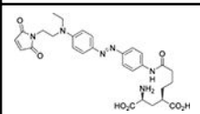
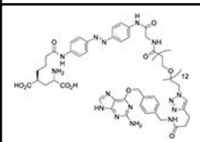
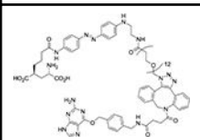
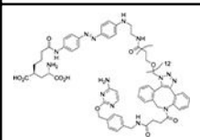
Photoswitch (common name)	Chemical structure	Molecular target(s)	Ligand type	Optimal wavelengths of light for photoswitching	Thermal stability	Active conformation	Key references (where used on retina)
Bis-Q		Nicotinic acetylcholine receptors	Agonist	320 nm (*trans*→*cis*) 420 nm (*cis*→*trans*)	bistable	*trans*	79, 80,81
QAQ		Voltage-gated sodium, potassium and calcium channels	Channelblocker	360 nm (*trans*→*cis*) 500 nm (*cis*→*trans*)	bistable	*trans*	82, 83, 55
QENAQ		Voltage-gated sodium and potassium channels	Channelblocker	460 nm (*trans*→*cis*) Dark (*cis*→*trans*)	fast-relaxing	*trans*	84
DAD		Voltage-gated potassium channels	Channelblocker	460 nm or white light (*trans*→*cis*) Dark (*cis*→*trans*)	fast-relaxing	*trans*	85
AAQ		Voltage-gated potassium channels, HCN channels	Channelblocker	360 nm (*trans*→*cis*) 500 nm (*cis*→*trans*)	bistable	*trans*	86, 82, 87, 88, 55
DENAQ		HCN channels, voltage-gated potassium channels,	Channelblocker	470 nm or white light (*trans*→*cis*) Dark (*cis*→*trans*)	fast-relaxing	*trans*	89, 90, 55
PhENAQ		Voltage-gated potassium channels	Channelblocker	460 nm or white light (*trans*→*cis*) Dark (*cis*→*trans*)	fast-relaxing	*cis*	89
BENAQ		HCN channels, voltage-gated potassium channels	Channelblocker	460 nm or white light (*trans*→*cis*) Dark (*cis*→*trans*)	fast-relaxing	*trans*	89, 55, 91
ATG		Ionotropic glutamate receptors, NMDA type	Agonist	360 nm(*trans*→*cis*) 500 nm(*cis*→*trans*)	bistable	*cis*	92

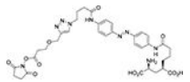
Photoswitch (common name)	Chemical structure	Molecular target(s)	Ligand type	Optimal wavelengths of light for photoswitching	Thermal stability	Active conformation	Key references (where used on retina)
ATA-3		Ionotropic glutamate receptors, AMPA type	Agonist	360 nm (*trans*→*cis*) 500 nm (*cis*→*trans*)	bistable	*trans*	93
GluAzo		Ionotropic glutamate receptors, kainate type	Agonist	360 nm (*trans*→*cis*) 500 nm (*cis*→*trans*)	bistable	*trans*	94
MPC088		Ionotropic GABA receptors	Agonist	360 nm (*trans*→*cis*) 470 nm (*cis*→*trans*)	bistable	*trans*	95

[a]The data presented in this table include photoswitch common names, their chemical structures, molecular target(s), type of pharmacological effect, optimal wavelengths of light for photoisomerization, thermal stability of the cis isomer (categorized as bistable photoswitches, with a cis isomer halflife of >1 min, or fast-relaxing photoswitches, with a half-life of <1 s), photoswitch conformation that acts on protein target, and key references for the reader (vision-related photoswitch references are in bold and underlined).

Table 2.

Photochemical and Pharmacological Properties of the Photoswitchable Tethered Ligands (PTLs) Discussed in This Review[a]

Photoswitch (commonname)	Chemical structure	Channel/receptor(s) regulated	Construct(s) Used	Optimal wavelengths of light for photoswitching	Thermal stability	*Cis* or *Trans* Agonist	Key references (where used on retina)
QBr		Nicotinicacetylcholinereceptors	AChRs (wild type)	320 nm (*trans*→*cis*) 420 nm (*cis*→*trans*)	bistable	*Cis*	79, 99
MAQ		Shaker voltage-gated potassium channels	SPARK(ShakerE422C)	380 nm (*trans*→*cis*) 500 nm (*cis*→*trans*)	bistable	*Cis*	100
MAG		Ionotropic glutamate receptors, kainate type 2	LiGluR(GluK2L439C)	380 nm (*trans*→*cis*) 500 nm (*cis*→*trans*)	bistable	*Cis*	101, 102, 103
MAG_{460}		Ionotropic glutamate receptors, kainate type 2	LiGluR(GluK2L439C)	460 nm or white light (*trans*→*cis*) Dark (*cis*→*trans*)	fast-relaxing	*Cis*	104,105,106
$bgag_{12}$		Metabotropic glutamate receptors 2, 6, 7, 8	SNAP-mGluR2SNAP-mGluR6SNAP-mGluR7-N74KSNAP-mGluR8	380 nm (*trans*→*cis*) 500 nm (*cis*→*trans*)	bistable	*Cis*(SNAP-mGluR2) *Trans*(SNAP-mGluR6,mGluR7,mGluR8)	107, 108
$BGAG_{12,460}$		Metabotropic glutamate receptor 2	SNAP-mGluR2	460 nm or white light (*trans*→*cis*) Dark (*cis*→*trans*)	fast-relaxing	*Cis*	108,106
$bcag_{12}$		Metabotropic glutamate receptor 2	CLIP-mGluR2	380 nm (*trans*→*cis*) 500 nm (*cis*→*trans*)	bistable	*Cis*	107

Photoswitch (commonname)	Chemical structure	Channel/receptor(s) regulated	Construct(s) Used	Optimal wavelengths of light for photoswitching	Thermal stability	*Cis* or *Trans* Agonist	Key references (where used on retina)
TCP-9		Ionotropic glutamate receptor, kainate type	GluKl (wild type)	380 nm (*trans*→*cis*) 500 nm (*cis*→*trans*)	bistable	*Cis*	109

[a]The data presented in this table include photoswitch common names, their chemical structures, molecular target(s), the mutation or tag engineered to create an attachment site, optimal wavelengths of light for photoisomerization, thermal stability of the cis isomer (categorized as bistable photoswitches, with a cis isomer half-life of >1 min, or fast-relaxing photoswitches, with a half-life of <1 s), photoswitch conformation that acts on protein target, and key references for the reader (vision-related photoswitch references are in bold and underlined).