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Journal

BioEssays, 45(9)

ISSN

0265-9247

Authors

Hong, John D

Palczewski, Krzysztof

Publication Date

2023-09-01

DOI

10.1002/bies.202300068

HHS Public Access

Author manuscript

Bioessays. Author manuscript; available in PMC 2023 October 30.

Published in final edited form as:

Bioessays. 2023 September ; 45(9): e2300068. doi:10.1002/bies.202300068.

A short story on how chromophore is hydrolyzed from rhodopsin for recycling

John D. Hong[1,2], **Krzysztof Palczewski**[1,2,3,4]

[1]Gavin Herbert Eye Institute, Department of Ophthalmology, University of California Irvine, Irvine, CA 92697, USA

[2]Department of Chemistry, University of California Irvine, Irvine, CA 92697, USA

[3]Department of Physiology and Biophysics, University of California Irvine, Irvine, CA 92697, USA

[4]Department of Molecular Biology and Biochemistry, University of California Irvine, Irvine, CA 92697, USA

Abstract

The photocycle of visual opsins is essential to maintain the light sensitivity of the retina. The early physical observations of the rhodopsin photocycle by Böll and Kühne in the 1870s inspired over a century's worth of investigations on rhodopsin biochemistry. A single photon isomerizes the Schiff-base linked 11-*cis*-retinylidene chromophore of rhodopsin, converting it to the all-*trans* agonist to elicit phototransduction through photoactivated rhodopsin (Rho*). Schiff base hydrolysis of the agonist is a key step in the photocycle, not only diminishing ongoing phototransduction but also allowing for entry and binding of fresh 11-*cis* chromophore to regenerate the rhodopsin pigment and maintain light sensitivity. Many challenges have been encountered in measuring the rate of this hydrolysis, but recent advancements have facilitated studies of the hydrolysis within the native membrane environment of rhodopsin. These techniques can now be applied to study hydrolysis of agonist in other opsin proteins that mediate phototransduction or chromophore turnover. In this review, we discuss the progress that has been made in characterizing the rhodopsin photocycle and the journey to characterize the hydrolysis of its all-*trans*-retinylidene agonist.

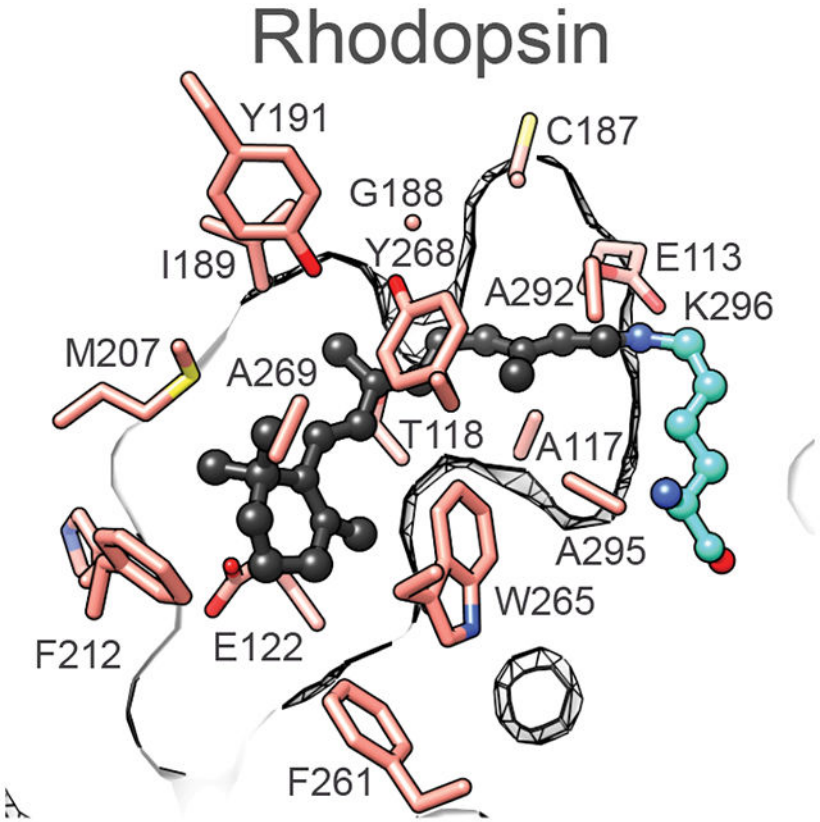
Graphical Abstract

[*]**Corresponding Author:** Krzysztof Palczewski, Department of Ophthalmology, Gavin Herbert Eye Institute, UCI, Irvine, CA 92697; kpalczew@uci.edu; phone (949) 824-6527.

AUTHOR CONTRIBUTIONS

Both authors contributed to the writing of the manuscript.

Conflict of interest: K.P. is a consultant for Polgenix Inc. J.D.H. has declared that no conflict of interest exists.



Keywords

rhodopsin; 11-*cis*-retinal; all-*trans*-retinal; chromophore; retinal hydrolysis; dark adaptation; retinylidene phospholipids; visual cycle

Introduction

The mysteries of vision become resolved at the molecular level with the characterization of a pigment that changes color in response to light. This pigment was discovered to be the opsin protein in close interaction with its 11-*cis*-retinylidene chromophore (Fig 1). These pigment molecules densely fill the outer segment region of photoreceptor cells (Fig 2), providing our retinas with the ability to sense light [29,31,126]. The photochemistry of the color change that communicates the presence of light is characterized by the immediate isomerization of the 11-*cis*-retinylidene chromophore of opsin to the all-*trans*-retinylidene agonist upon absorption of a photon of light [32,34,120,127]. The light-triggered formation of the agonist induces a series of conformational changes in rhodopsin leading to its active signaling state [28,34,84,111]. This photochemistry and the biochemical response of opsin proteins serve as the foundation to perception of light and the consequent behavior of organisms. The key role of opsins to provide organisms with the ability to perceive and respond to light was aptly demonstrated by Salom and Cao using transgenic *Caenorhabditis elegans* modified to express bovine rhodopsin or human melanopsin in neural tissue [17,97-98]. The wild-type worms are naturally blind, lacking eyes and opsins, and are non-responsive to light. In

contrast to transgenic worms with bovine rhodopsin or human melanopsin, light completely arrested movement or enhanced locomotion, respectively. These results suggest application of rhodopsin as a light detector in many systems including optogenetics [1,36,43,61].

The opsin best characterized for its biochemistry is rhodopsin, an opsin that is fundamental to nocturnal vertebrate vision. To sustain light sensitivity of the vertebrate retina, the crucial step of the hydrolysis of the Schiff base to release the agonist from photoactivated rhodopsin (Rho*) must occur to bridge two important cycles that maintain vertebrate vision: the rhodopsin photocycle and the visual cycle (Fig 3) [55,91,126]. This hydrolysis not only diminishes ongoing phototransduction but also generates apo-opsin, permitting the entry of fresh 11-*cis*-retinal ligand to re-form the chromophore Schiff-base linkage and regenerate the rhodopsin pigment [67]. Hydrolysis also supplies the visual cycle with all-*trans*-retinal to be reconverted to 11-*cis*-retinal that regenerates the pigment. Without hydrolysis, both rhodopsin and its chromophore would, in principle, be only usable once, and sensitivity to light would dramatically and quickly decline under constant illumination.

One of the earliest recorded observations on the photochemistry of rhodopsin came from Franz Böll, who reported the color changes that occurred when the visual red/purple pigment of the retina was exposed to light [16]. Fascinated by Böll's study of photosensitive retinal pigment, Friedrich Wilhelm Kühne described light-induced changes of the red pigment, converting to orange, then yellow, and eventually colorless [58]. This pigment within rods gradually became restored in the dark, with a corresponding decrease in the amount of golden yellow oil droplets inside the neighboring hexagonal epithelial cells, or retinal pigment epithelial (RPE) cells [16]. These early investigations corresponding to the photobleaching of rhodopsin and its regeneration set forth more than a century's worth of major breakthroughs in the biochemistry of opsin proteins and insights into the physiology of vision [37,39,54-55,84-86,100].

Identification of the chromophore and its relation to opsin

In the 1930s, George Wald discovered the identity of the chromophore of the opsins to be retinal, the aldehyde form of vitamin A [119] (historical perspective by Ripps in reference [92]). This finding aligned with the ability of retinal to form a Schiff base with amines, causing a bathochromic shift in the absorption maximum from 380 nm to 440 nm. The additional shift toward 500 nm, the absorption maximum of rhodopsin, was later attributed to the effects of interactions of residues within the chromophore-binding pocket of the opsin protein with the polyene chain of the bound retinal chromophore [46,75]. The hydrolyzable nature of the Schiff base also aligns with the observations of visual pigment bleaching in response to light, corresponding to the release of retinal. Notably, the released retinal does not rebind to apo-opsin to reform pigment, suggesting a chemical structural difference between the species that binds to form pigment and the species that is released. The isomeric identity of the released species was found to be all-*trans*-retinal; but the identity of the pigment-forming species, 11-*cis*-retinal, was not discovered until 1956 in the Wald laboratory [81].

The nature of the covalent linkage between retinal and opsin, suspected to be a Schiff base bond according to the work of Collins, Morton, and Pitts, was later confirmed by Bownds in the 1960s [13-14,70,90]. His use of borohydride reduction documented the conversion of the hydrolyzable Schiff base adduct between retinal and opsin to a nonhydrolyzable secondary amine linkage [13-14]. The resultant reduced product was characterized as the retinyl moiety attached to the ε-amino group of an opsin-Lys residue, after exhaustive proteolysis of the borohydride-treated rhodopsin pigment by pronase [13]. The location of this chromophore-binding Lys residue was later determined to be Lys^{296} by the studies of Ovchinnikov and Hargrave [40,82].

Later studies by Sakmar, Khorana, Oprian, and Nathans demonstrated that the retinylidene Schiff base linked to Lys^{296} also formed a non-covalent salt bridge interaction with Glu^{113}, stabilizing the ground-state rhodopsin pigment [76,95-96,132]. Glu^{113} was also shown to facilitate the transition to the metarhodopsin II (MII) signaling state by acting as a proton acceptor in the deprotonation of the retinylidene Schiff base [48]. Furthermore, Glu^{181} in extracellular loop 2 also participates in this transition by acting as a partial counterion switch for relaxation of the Rho* structure to metarhodopsin I (MI) [62,125].

Eventually, the crystal structure of chromophore-bound rhodopsin was achieved, representing the first 3-dimensional characterization of a G protein-coupled receptor (GPCR) [79,86]. However, pursuit of the structure of Rho* served as an even greater challenge, given the transient nature of the all-*trans*-retinylidene agonist being susceptible to hydrolysis [12,70]. Recent methodological advances have allowed the capture of specific Rho* species, including a very early intermediate and potentially the MII signaling state [22,37,100,109].

Properties of Rho*

The visual pigment, appearing purple when concentrated, and red upon dilution, was observed by Kühne and his associates Ewald and Ayres to change to a more persistent “visual yellow” color before eventually becoming colorless, or “visual white”, which was later determined to contain free retinal [58,119]. Further investigations by Lythgoe of rhodopsin kept on ice and exposed to light showed evidence of a “transient orange” form that was not the result of a mixture of visual yellow and unbleached visual red pigments [63]. The orange color was more stable when the rhodopsin preparation was maintained on ice rather than when warmed to 30°C, which led to a pale-yellow color. Upon attempted extraction of retinal, the transient orange and visual yellow forms were found to still contain bound retinal. Furthermore, the visual yellow form was observed to change spectral properties in response to changes in pH; therefore it became more aptly named “indicator yellow” [63]. Lythgoe’s observations were further followed up by Collins, Morton, and Pitts, who observed analogous pH-dependent spectral changes with a model Schiff base adduct of retinal with a primary amine, mimicking the epsilon amine of a Lys residue [70,90]. They also reported the hydrolysis of the retinylidene Schiff base occurring fastest at a mildly acidic pH of 5.7, close to its pKa [70,90].

Expanding upon Lythgoe's observation of "transient orange," the use of absorbance measurements at very low-temperatures allowed for the capture of each major photo-intermediate of Rho*, prior to the eventual hydrolysis and release of retinal, in the following order: bathorhodopsin (λ_{max} = 543 nm, at < −150°C), lumirhodopsin (λ_{max} = 497 nm, at −140°C to −40°C), MI (λ_{max} = 478 nm, at −35°C to −10°C), and MII (λ_{max} = 380 nm, at −10°C to 0°C) [121]. Metarhodopsin III (MIII, λ_{max} = 465 nm) was later identified with its all-*trans*-retinylidene Schiff base in the *syn*-configuration, distinct from the *anti*-configuration of the previously identified species [6,118]. This *anti*-to-*syn* conversion has been proposed to occur either through photic or thermal energy [6,93]. All three metarhodopsin species are in thermal equilibrium, shifting under higher pH conditions counterintuitively towards the protonated states of MI and MIII, and under lower pH conditions towards the deprotonated state of MII [6]. MII, the signaling state of Rho*, exists as the dominant species under physiological pH and has been shown to be the species from which the hydrolysis of the all-*trans*-retinylidene Schiff base largely proceeds (Fig 4) [22,45,100,103].

An extensive water network in rhodopsin confers structural integrity and plasticity

In 1977, Downer and Englander discovered an usually vast proportion of rhodopsin of around 70% of amide groups was interacting with water based on hydrogen-tritium exchange experiments performed on native ROS membrane suspensions; in contrast, this proportion is typically around 20-40% in other proteins [26]. This water network spanning rhodopsin was better characterized later with X-ray crystallographic structures and fourier transform infrared (FTIR) spectroscopy. Water plays an indispensable and integral role within the H-bonding networks of the interhelical spaces of rhodopsin to stabilize structure [4-5,72]. These structural waters as well as those present within the chromophore binding pocket are essential to the spectral tuning of rhodopsin by solvatochromic interactions with the retinylidene polyene chain [3,24,35,46,51,77,106-107]; additionally, water adjacent to the retinylidene Schiff base influences the pKa of Glu^{113} to shift the proton towards the retinylidene Schiff base along the salt bridge for the bathochromic shift to absorb longer wavelengths of light [15]. Furthermore, this Schiff base in ground-state rhodopsin is protected against hydrolysis by an intradiscal "plug" preventing the entry of bulk water into the chromophore binding pocket [131]. Therefore, perturbations to the water network and protein structure of rhodopsin lead to instability of ground-state rhodopsin to retain chromophore, leading to retinitis pigmentosa [49,53]. Similar perturbations in other GPCRs could also affect the stability of their inactive resting state, given the highly conserved nature of structural waters across GPCRs [9,80].

Structural waters within rhodopsin not only provide stability but also plasticity to facilitate protein conformational changes upon the photoisomerization of the 11-*cis* chromophore to the all-*trans* agonist [4-5,11,128]. Changes in the water network were observed in the transitions through different photointermediates with the greatest change occurring upon reaching the MII active state, whereby the tilting of transmembrane helices 5 and 6 opens a solvent pore on the cytosolic face of rhodopsin to allow a large influx of bulk water

that ultimately hydrolyzes the Schiff base of the all-*trans* agonist for the release of all-*trans*-retinal [18,50,65,102,113,116,128-129]. A significant amount of this bulk water is then displaced upon MII binding of transducin to elicit the phototransduction cascade [18]. The hydrolysis detailed above exhibits stereoselectivity for the all-*trans* configuration as demonstrated by the lack of hydrolysis of the 11-*cis*-locked retinal analogue that photoisomerizes to an 11,13-*dicis*-agonist that remains bound and thermally reverts to the mono-11-*cis* configuration, re-establishing ground-state rhodopsin [38].

The water network changes in the photoactivation of rhodopsin are highly conserved across GPCRs [10,60,128]. The binding of agonist to the ligand-binding site of GPCRs is analogous to the production of the all-*trans* agonist by photoisomerization. Agonist binding induces concerted protein conformational and water network changes very similar to those observed in rhodopsin, including the influx of bulk water that occurs in the active state conformation despite not requiring hydrolysis to release the agonist, given a reversible binding to the ligand-binding site [10,60,94]. Aberrancies in the water network and protein structure that cause abnormally fast or slow decay of Rho* to apo-opsin and all-*trans*-retinal are implicated in the stability of the active state of other GPCRs and release of their agonist [49].

Early kinetics studies on the decay of Rho*

One of the earliest measurements of the rate at which Rho* decays to retinal and apo-opsin was done by Baumann, using absorbance spectroscopy to study dissected and re-perfused retinal tissue of frogs and humans [7-8]. He determined the rate constants for decay of MII and MIII, at pH 7.5 and 36°C, to be about $k = 0.003$ sec^{-1} and $k = 0.004$ sec^{-1}, respectively, which correspond to half-lives of about 4 min and 3 min, respectively [8]. However, a thorough understanding of the nature of the metarhodopsin species had not been established, especially for MIII, which is now understood to decay much more slowly than MII [6,59]. Notably, it is not known currently whether MI undergoes hydrolysis to release retinal, and capturing this process is especially challenging given its rapid conversion within milliseconds to the longer-lasting MII form.

Tryptophan fluorescence measurements of MII decay

The broad overlapping nature of the absorption spectra of the ground-state rhodopsin pigment, its photo-intermediates, and free retinal presents challenges to tracking the hydrolysis event, given the difficulty of definitively distinguishing each species in the complex mixture after light exposure. This problem was circumvented through use of fluorescence spectroscopy to study Rho* hydrolysis and release of retinal. The intrinsic fluorescence of the five tryptophan residues of rhodopsin is quenched by bound retinal; therefore, increase in tryptophan fluorescence emission provides a precise measurement of the overall process of Schiff base hydrolysis and release of retinal from Rho* [30,105]. The half-life of Rho* decay was found to be on the order of minutes at 20°C, with an activation-energy barrier for the combined events of hydrolysis and retinal release being ~20 kcal/mol [30]. The timescale of Rho* decay (minutes) highlights the importance of rhodopsin kinase and arrestin in halting ongoing phototransduction long after the light stimulus. This

role is especially evident in mice lacking rhodopsin kinase or arrestin, where light-induced photoreceptor degeneration occurs very early in life [19,122].

Monitoring intrinsic tryptophan fluorescence has provided the most precise way to track Rho* decay; however, it cannot distinguish the two processes of Schiff base hydrolysis and subsequent release of retinal. Furthermore, as typically done to study membrane protein biochemistry, mild detergents were used to solubilize rhodopsin to allow for more precise spectroscopic measurements. The detergents could alter protein properties relative to their native state in the membrane, potentially producing misleading results especially in the case of rhodopsin, which resides in membranes rich in polyunsaturated acyl chains, such as docosahexaenoic acid [2,87].

Native MS to capture first steps of vision

Recent advances have led to the development of mass spectrometry (MS) techniques that can detect whole membrane proteins in their native membranes, along with their complexes with small molecules, lipids, nucleic acids, and other proteins. Using a state-of-the-art native MS system, the Robinson group characterized the very first steps of the visual cycle and phototransduction in rod outer segment (ROS) membranes, avoiding the reductionist approach of studying proteins or complexes in isolation [21]. The hydrolysis of Rho* was tracked in its native environment of natural lipids and proteins, providing the most accurate and precise method to monitor Rho* decay into apo-opsin and retinal. The hydrolysis was found to have a rate constant $k'_{hyd} = 3.20 \pm 0.17$ (x 10^{-3}) sec^{-1} at pH 7.0 and 28°C, corresponding to a half-life of 3.61 ± 0.19 min, in agreement with previous results (described above) [21]. Despite its astonishing capabilities in capturing the kinetics of the phototransduction cascade as well as Rho* decay, native MS comes with several limitations. The method is not widely accessible to many laboratories. Furthermore, the method cannot truly discriminate between ground state and Rho*; despite having different isomeric configurations of the retinylidene adduct, both forms have the same molecular weight. Additionally, the method does not allow for non-volatile buffers and salts, including NADPH, which is the required cofactor for reduction of retinal by retinol dehydrogenases (RDHs), which may interact with Rho* and potentially affect the rates of Schiff-base hydrolysis and/or retinal release [104].

LC-MS/MS-based method of tracking Rho* decay

A method complementary to native MS was recently developed that allows for the investigation of Rho* decay in native membranes, using reagents and instrumentation more likely to be present or readily accessible in laboratories. The method involves the use of borohydride to trap the opsin-bound chromophore as described by Bownds [13-14], followed by proteolysis and LC-MS/MS analysis (Fig 5). The $NaBH_4$ is dissolved in isopropanol (*i*PrOH) to facilitate both Schiff base reduction and rapid protein denaturation of Rho or Rho* simultaneously [45]. *i*PrOH also induces the separation of proteins from lipids in the ROS membranes by solubilizing the lipids and precipitating the proteins. Retinal liberated from Rho* by hydrolysis can be quantified in the lipid-soluble fraction, while retinal that remains bound to Rho* as a Schiff base can be measured in the protein precipitate. With

this approach, the hydrolysis kinetics were found to be comparable to those determined with native MS. An Arrhenius plot for Rho* hydrolysis was produced in this study [45] given the temperature dependency of the hydrolysis rate. The plot can be interpolated to calculate the Rho* hydrolysis rate at the temperature that had been used in the native MS study (28°C) [21]. This calculation yields a value of $k = 2.90 \pm 0.11$ (x 10^{-3}) sec^{-1} or a half-life of 3.99 ± 0.15 min, in agreement with the values reported above.

Furthermore, chromatographic separation of the retinyl isomers obtained from the N^{ε}-retinyl-Lys proteolysis product demonstrated the ability to directly determine the stereochemistry of the retinylidene isomers in an aliquot of Rho exposed to light, showing the extents of photoisomerization as well as hydrolysis, after subsequent analysis of the lipid soluble fraction. Additionally, *i*PrOH/$NaBH_4$ also trapped retinal that subsequently went on to form Schiff base adducts with membrane-derived phosphatidylethanolamines (PE) [123]. These PE-adducts were identified from LC-MS/MS analysis of the lipid soluble fraction and were found to form at a rate that was an order of magnitude faster than that of their hydrolysis from Rho*, as measured in isolation by the addition of exogenous all-*trans*-retinal to ROS membranes (Fig 5G) [45]. Notably, the LC-MS/MS based method described above does not directly measure the kinetics of the release of all-*trans*-retinal from the chromophore binding pocket after Rho* hydrolysis. Since N-retinylidene-PE formation occurs with all-*trans*-retinal that had been released after Rho* hydrolysis, the rate of this release can be inferred from the rate of appearance of the PE adducts, because the limiting rate for PE-adduct formation would be the release of endogenous all-*trans*-retinal from Rho*. Accordingly, the rate of release of all-*trans*-retinal was determined to be $k = 3.13 \pm 0.30$ (x 10^{-3}) sec^{-1}. The process of this release could potentially be influenced by a variety of factors, including the binding of 11-*cis*-retinal to the entry site of rhodopsin followed by its incorporation into the chromophore binding pocket to drive the release of all-*trans*-retinal [42,44,104]. This mechanism of facilitated release of all-*trans*-retinal by 11-*cis*-retinal should be further explored experimentally.

The substitution of $NaBH_4$ by $NaBD_4$ allowed for monitoring of RDH-mediated reduction of retinal by NADPH in the outer segments. Thus, retinal not reduced by RDHs was distinguished by deuterium incorporation *via* $NaBD_4$-mediated reduction. Accordingly, the presence of active RDH was found not to affect the rate or extent of Rho* hydrolysis, and RDH-mediated reduction of retinal was found to occur at a rate two orders of magnitude faster than hydrolysis of Rho* in native membranes. The method described in Hong *et. al.* [45] therefore captures and quantifies all of the major steps of the visual cycle occurring in the ROS membranes, without any requirement for purification or isolation of any of the proteins or complexes, in a manner similar to native MS in its non-reductionist approach to studying multiple complex processes occurring simultaneously.

Mono-stable vs. bistable opsins

Rhodopsin expressed by rod photoreceptor cells is one of the nine opsin gene products encoded in the human genome, as listed in Table 1. Of the nine, rhodopsin and the three cone opsins are known to be mono-stable or “bleaching opsins,” losing their photoisomerized all-*trans*-retinylidene agonist through hydrolysis and release of all-*trans*-

retinal. The hydrolysis of cone opsins is understood to be significantly faster than that of rhodopsin [20,47]. However, the study of cone opsins has presented several challenges; namely, low yields from native sources as well as difficulties with efficient expression of stable recombinant protein. Expression of the stable protein has been improved by using insect cell line systems or through fusion with stabilizing protein motifs [52,83,99].

Encephalopsin, melanopsin, and neuropsin are non-bleaching opsins that also bind 11-*cis*-retinal to form pigments [56,108,112]. Following light activation, these opsins do not undergo hydrolysis of their all-*trans*-retinylidene photoisomerization product, which has a new absorbance maximum whereby a second photon of light induces reverse isomerization back to the *cis* configuration; thus, they are known to be "bistable opsins". The bistable property of these opsins has in part been explained by the lack of the prototypical Glu^{113} counterion that emerged in vertebrates to stabilize the protonated retinylidene Schiff base in the mono-stable opsins [57,114]. Instead, in the bistable opsins, Glu^{181} serves the primary role of counterion for the protonated Schiff base in either the ground-state opsin pigment or the photoisomerized product [57,115]. Notably, encephalopsin contains two aspartate residues in place of Glu^{113} and Glu^{181}; therefore, either position could potentially serve as counterion for the protonated retinylidene Schiff base, but the extent to which, if at all, each aspartate serves in that role has yet to be determined. It also remains to be investigated thoroughly whether the photoactivated conformation of encephalopsin, melanopsin, or neuropsin undergoes eventual hydrolysis of the retinylidene adduct, and if so, how fast. Furthermore, melanopsin has been reported to form a 7-*cis*-retinylidene photoisomerization product, an isomeric configuration that should be further explored given the exceedingly rare, twisted, and unstable nature of the 7-*cis* isomer [68]. Therefore, all three bistable opsins are great candidates to be studied using the *i*PrOH/$NaBH_4$, LC-MS/MS-based method [45]. A better understanding of the biochemistry of these bistable opsins would elucidate how they influence a variety of physiological processes.

The retinal G protein-coupled receptor (RGR) provides an alternative route from the classical visual cycle to supply 11-*cis*-retinal to the photoreceptors [25,130]. RGR resides in the RPE and Müller glia, where it binds all-*trans*-retinal to form a pigment that involves a photochemistry that is the reverse of that of the photoreceptor opsins, producing 11-*cis*-retinylidene that is hydrolyzed to generate 11-*cis*-retinal [23,69,130]. As a mono-stable opsin, the rate at which RGR hydrolyzes its 11-*cis* product to resupply photoreceptors with 11-*cis*-retinal is not known [130]. Notably, the rate at which opsins consume 11-*cis*-retinal in the photoreceptors under daylight conditions is much faster than the rate at which the classical visual cycle generates 11-*cis*-retinal through RPE65, as demonstrated by the slow rate of dark adaptation [41,64,66]. This mismatch in rates would correspond to a continual decline in light sensitivity over time in the absence of an alternative supply of 11-*cis*-retinal; however, RGR provides an alternate mechanism, using the same daylight conditions that bleach the photoreceptor opsins to instead generate 11-*cis*-retinal [23]. Determining the rate of hydrolysis of the RGR-11-*cis*-retinylidene adduct would afford a more comprehensive and quantitative understanding of how photoreceptors maintain light sensitivity.

Though peropsin also engages in photochemistry analogous to RGR, it is understood to be bistable [71]. Whether hydrolytic turnover of the 11-*cis* photoproduct of peropsin occurs is

not known. Furthermore, since formation of the peropsin pigment and its photochemistry have been studied mainly with the invertebrate homolog [71], it is unknown whether the same reactions occur with the vertebrate form. Pigment formation and kinetics of hydrolysis of the vertebrate peropsin homolog and RGR are both suitable to be studied by the *i*PrOH/$NaBH_4$, LC-MS/MS-based method [45], given the ability to chromatographically separate the isomers of retinylidene bound to the respective opsins while also measuring the amount of retinal hydrolyzed from the photoisomerized opsin pigments.

Conclusions

The fundamental and first step of vision begins at the molecular level with rhodopsin. The photocycle of rhodopsin is essential to maintain the light sensitivity of the retina. An extensive breadth of knowledge about the photocycle has been gained from thorough investigations conducted over a century. These studies have led to the identification of the chromophore and characterization of its interaction with rhodopsin; and solution of the 3D molecular structure of the ground-state rhodopsin pigment, and the structures of several key photointermediates exceedingly difficult to capture. Importantly, major progress has been made to precisely and accurately measure the key step of hydrolysis of the all-*trans* agonist that bridges the rhodopsin photocycle with the visual cycle to sustain light sensitivity. Recent advancements with mass spectrometry-based methods have now characterized the specifics of Rho* hydrolysis within its native membrane environment, documenting a pseudo first-order process with a half-life of 7.70 ± 0.69 min at 20°C and a half-life of 1.73 ± 0.29 min at 37°C [45]. These methods are aptly suitable for the study of the photochemistry and hydrolysis of retinylidene adducts of the many other opsin proteins whose physiological roles in mediating phototransduction or chromophore turnover are not well understood.

ACKNOWLEDGMENTS

We thank Dr. Sahil Gulati for preparing Figure 1, Drs. Philip D. Kiser, Kota Katayama, Vladimir J. Kefalov, Oliver P. Ernst, Roxana Radu, and members of the Palczewski laboratory for helpful comments on this project.

Funding:

This research was supported in part by NIH research grants EY034519 and EY09339 (NEI) to KP; and NIH training grants 1F30EY033659-01 and T32-GM08620 for JDH. The authors acknowledge support from a Research to Prevent Blindness Unrestricted Grant to the Department of Ophthalmology at UCI.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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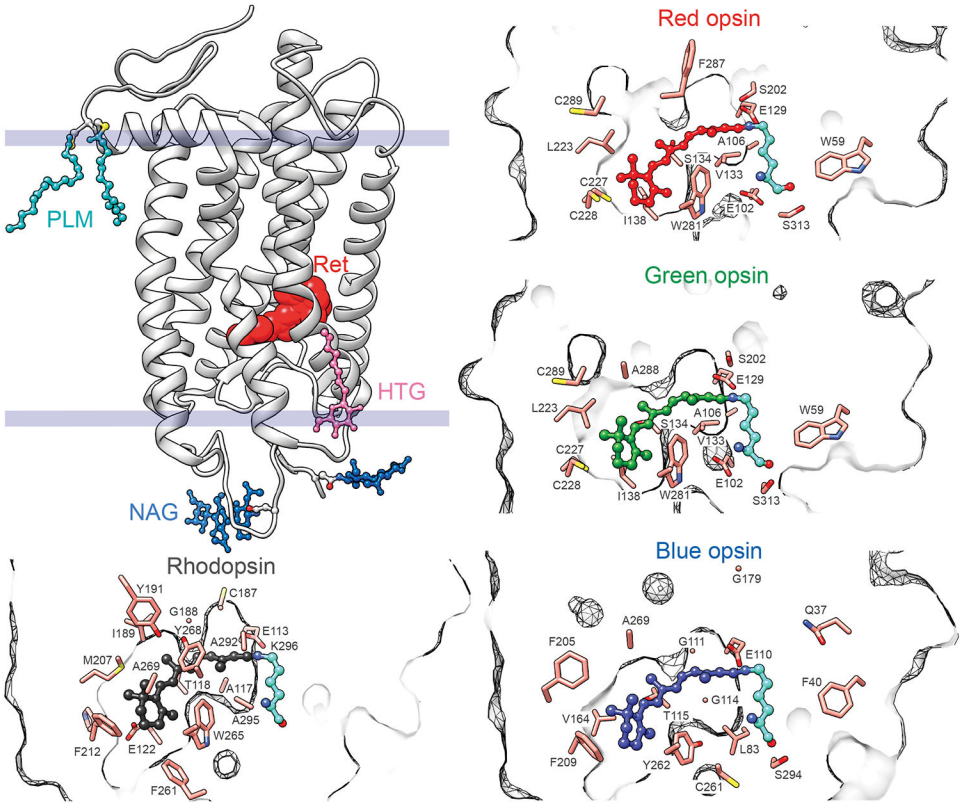

Figure 1. Three dimensional models of rhodopsin and cone opsins.

Residues surrounding the chromophore are depicted for rhodopsin and three human cone opsin pigments. The rhodopsin model is based on PDB 1U19, and the homology models of the cone opsin pigments are deposited in the PDB (identifiers 1KPN, 1KPW, and 1KPX for the blue, green, and red cone pigments, respectively) [110]. Each pigment has a characteristic absorbance maximum (λ_{max}): rhodopsin (500 nm), red (560 nm), green (530 nm), blue (420 nm). The absorbance maximum of the protonated retinylidene Schiff base (λ_{max} = 440 nm) undergoes a bathochromic shift by interaction of the polyene chain with key residues in the chromophore binding pockets of rhodopsin, red opsin, and green opsin; whereas for blue opsin, a hypsochromic shift occurs.

Abbreviations: Ret, retinylidene; PLM, palmitoyl; NAG, N-acetylglucosamine; HTG, Heptyl 1-thio-β-D-glucopyranoside

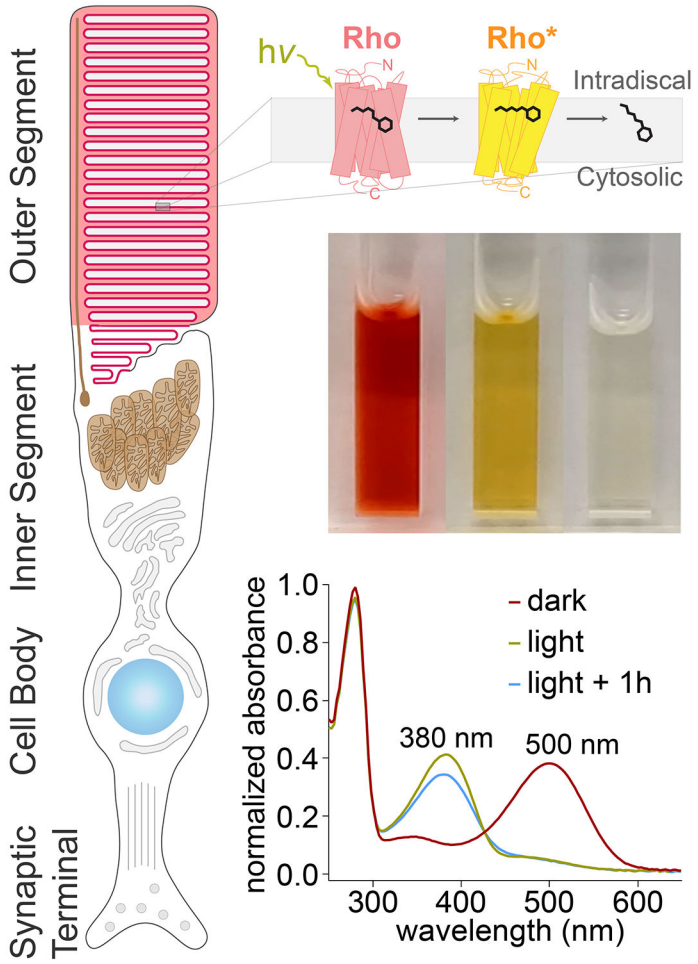

Figure 2. Rod photoreceptor with rhodopsin photochemistry.

Rhodopsin is abundant and tightly packed in rod outer segment discs for high sensitivity to light, needed for optimal capture of photons under dim light conditions. Light induces the characteristic color changes that were observed originally by Böll and Kühne, with gradual bleaching of the red pigment of rhodopsin to the visual yellow form of Rho*, and eventually to the visual white (or colorless) form of apo-opsin with free retinal [16,58]. The broad absorbance spectrum with a λ_{max} of 500 nm results in the apparent red color of rhodopsin. The change in absorption maximum to 380 nm from light exposure results in the apparent yellow color of Rho*. The fading of the yellow pigment is due to the hydrolysis of Rho*, releasing all-*trans*-retinal with a lower extinction coefficient than the deprotonated retinylidene Schiff base adducted to Rho*.

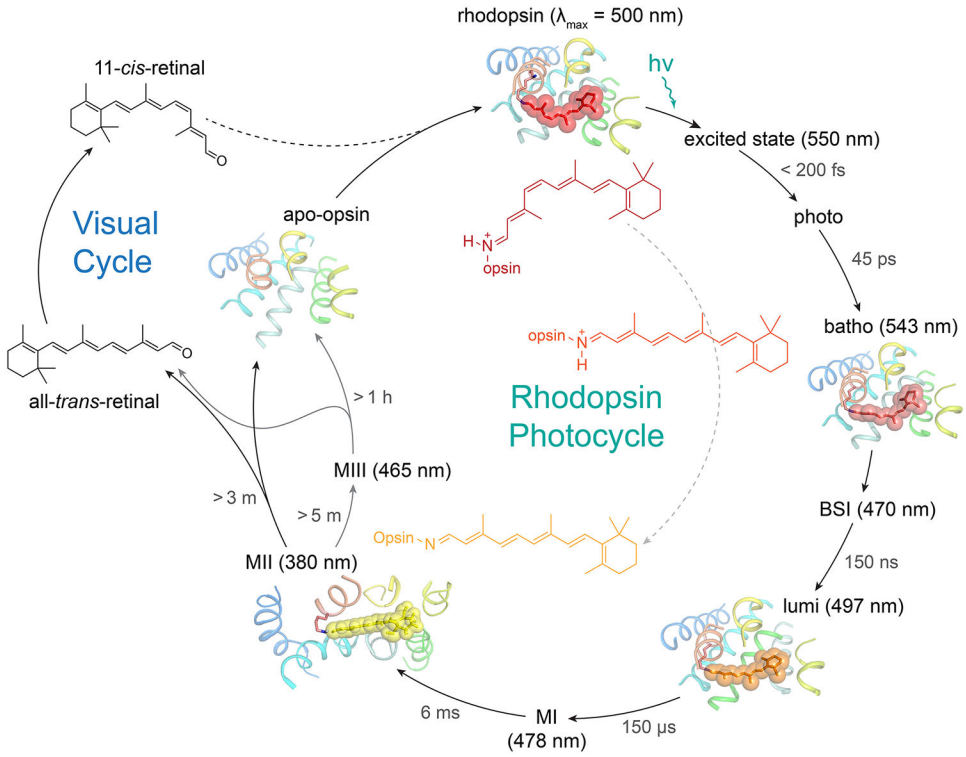

Figure 3. Rhodopsin photocycle.

The photocycle is initiated upon photoisomerization of the 11-*cis*-retinylidene chromophore of ground-state rhodopsin to the all-*trans* configuration. The substantial change in retinylidene ligand structure from the *cis*-to-*trans* configuration induces a series of conformation changes in rhodopsin. Exceedingly transient key photointermediates are formed in the process of relaxation of the protein structure to accommodate the all-*trans* agonist in route to the conformation of the MII active signaling state. The subsequent step of hydrolysis is essential not only to the rhodopsin photocycle but also to the visual cycle. Hydrolysis of the retinylidene Schiff base in MII releases all-*trans*-retinal, resulting in apo-opsin that can be regenerated to ground-state rhodopsin by binding fresh 11-*cis*-retinal from the visual cycle. MII can alternatively convert to MIII which possesses the *syn*-configuration of the retinylidene Schiff base, which hydrolyzes significantly slower. PDB IDs used were: bathorhodopsin, 2G87 [73]; lumirhodopsin, 2HPY [74]; MII, 3PXO [22], 3PQR [22], 2I37 [100]; opsin, 3CAP [88]; rhodopsin 1F88 [86]; 1L9H [78].

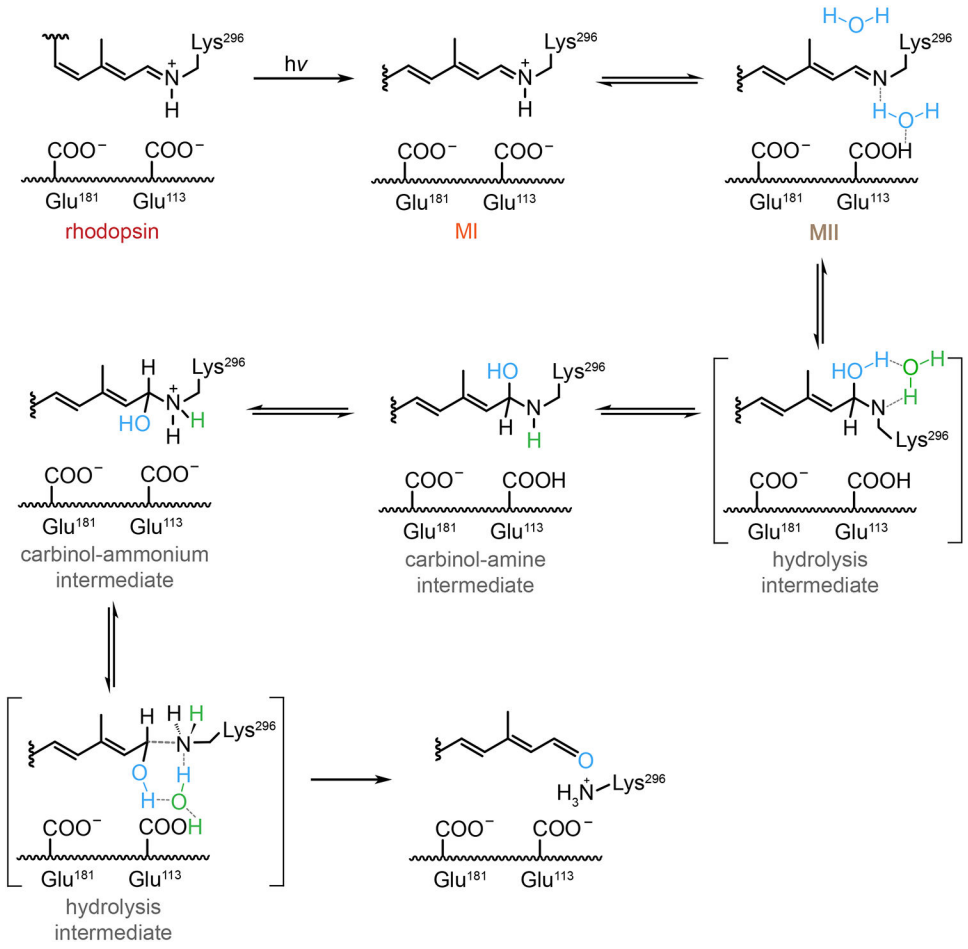

Figure 4. Mechanism of the hydrolysis of the all-trans-retinylidene agonist of Rho*.

The following diagram was adapted from Palczewski 2006 [84] and updated with mechanistic descriptions from Hong, *et al.* 2022 [45]. Light exposure of rhodopsin momentarily forms MI, which quickly deprotonates to form MII, the active signaling state for phototransduction. The extensive water network within rhodopsin provides an aqueous environment [116] which can mediate the hydrolysis process through MII [50,116]. The protic environment provided by water allows for quick transfer of protons as well as H-bonding interactions to help stabilize each intermediate. Nucleophilic attack by water on C15 of the retinylidene Schiff base forms an intermediate stabilized by H-bonding interactions with water [50]. A series of proton transfers mediated by the protic environment of water produces the carbinol-ammonium intermediate, which is primed for the completion of hydrolysis, with the protonated Lys residue serving as a favorable leaving group. The elimination of the protonated Lys residue leads to formation of the all-*trans*-retinal hydrolysis product. The mechanistic route as well as the protonation states of E113 and E181 shown for MII were based on quantum chemical calculations [45]. The actual protonation state of E181 is unclear, with different conclusions having been reported in previous studies; however, more recent studies have corroborated a deprotonated E181 [33,62,89,101,125]. If and how E181 protonation status affects the hydrolysis mechanism requires further experimental investigation to potentially enrich the description above; nevertheless, the presence of

E181 in extracellular loop 2 serves to stabilize the Schiff-base-bound chromophore against hydrolysis [124].

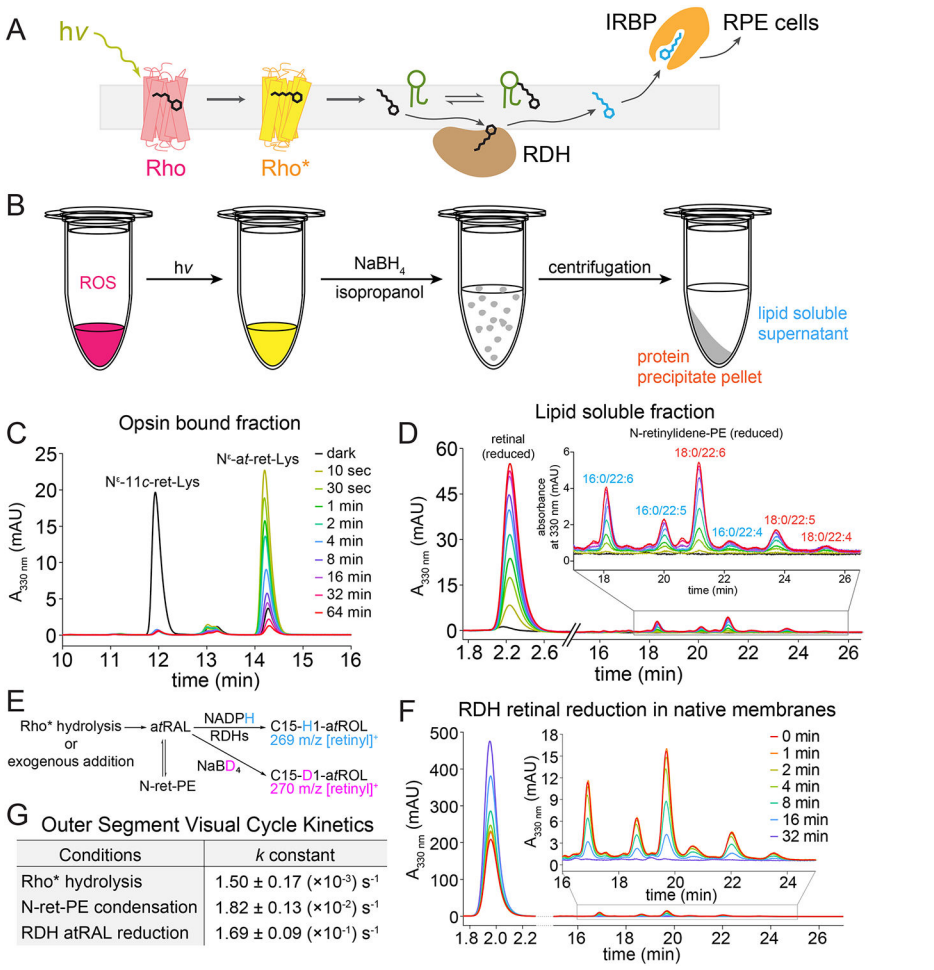

Figure 5. Characterizing visual cycle kinetics in the rod outer segment

(A) Schematic diagram depicting the visual cycle processes of the ROS, initiated upon light exposure. In black: retinal, in blue: retinol. (B) Experimental diagram depicting *i*PrOH/$NaBH_4$ treatment of ROS membranes for separation of the protein-precipitate pellet, which is proteolyzed and analyzed by LC-MS/MS for measurement of opsin-bound retinal; in turn, the lipid soluble supernatant is analyzed by LC-MS/MS to measure retinal produced by Schiff base hydrolysis in Rho* hydrolysis, and subsequent N-retinylidene-PE adducts. (C) Progressive chromatographic traces reflecting the status of the retinylidene Schiff base bound to rhodopsin, as a function of time after light exposure. The 11-*cis* to all-*trans* photoisomerization is captured by the pronase digestion of *i*PrOH/$NaBH_4$-treated ROS membranes. The N^{ε}-all-*trans*-retinyl-Lys signal decreases over time, corresponding to the hydrolysis of the all-*trans*-retinylidene Schiff base of Rho*. (D) A corresponding increase in free all-*trans*-retinal product from Rho* hydrolysis is observed over time, as well as production of all-*trans*-retinylidene Schiff base adducts with phosphatidylethanolamines from the outer segment membrane. (E) Scheme depicting the tracking of NADPH-dependent reduction of retinal by outer segment RDHs as follows: deuterium-isotope labeling of retinal by $NaBD_4$ (used in place of $NaBH_4$) distinguishes the retinal that is yet to be reduced by RDHs. (F) Addition of NADPH to the outer segment membranes leads to reduction of retinal to retinol by native RDHs, with a corresponding gradual decline of

phosphatidylethanolamine adducts. (G) Kinetics of each major process occurring after the exposure of the rod outer segment membranes to light at 20°C and at physiologic pH 7.4, showing Rho* hydrolysis as the rate limiting step of the visual cycle within the rod outer segment. Panels B-E were adapted from: Hong, J. D., Salom, D., Kochman, M. A., Kubas, A., Kiser, P. D., and Palczewski, K. (2022) Chromophore hydrolysis and release from photoactivated rhodopsin in native membranes, *Proc Natl Acad Sci U S A 119*, e2213911119 [45].

Table 1.

Characteristics of opsin gene products[a].

Opsin	Gene Name	Chromophore ligand	Ground-state λ_{max} (nm	Photoisomerized product λ_{max} (nm)	Mono-stable or Bistable	Expression Location	References
Rhodopsin	*RHO/OPN2*	11-*cis*-retinal	500	380	mono-stable	retina (PRCs)	Katayama, *et al.* 2019 [52]
Blue cone opsin	*OPN1SW*	11-*cis*-retinal	415	380	mono-stable	retina (PRCs)	Katayama, *et al.* 2019 [52]
Green cone opsin	*OPN1MW*	11-*cis*-retinal	530	380	mono-stable	retina (PRCs)	Katayama, *et al.* 2019 [52]
Red cone opsin	*OPN1LW*	11-*cis*-retinal	560	380	mono-stable	retina (PRCs)	Katayama, *et al.* 2019 [52]
Encephalopsin	*OPN3*	11-*cis*-retinal	465 *	<465 *	bistable	bodily; sparse in eye	Sugihara, *et al.* 2016 [112]
Melanopsin	*OPN4*	11-*cis*-retinal	470 *	480 *	bistable	retina (GCs)	Matsuyama, *et al.* 2012 [68]
Neuropsin	*OPN5*	11-*cis*-retinal	380	470	bistable	retina (GCs), brain, spine, testes	Kojima, *et al.* 2011 [56]
RGR	*RGR*	all-*trans*-retinal	470, 370	470, 370	mono-stable	RPE	Zhang, *et al.* 2019 [130]
Peropsin	*RRH*	all-*trans*-retinal	536 **	460 **	bistable	RPE	Nagata, *et al.* 2010 [71]

[a]The human genome possesses nine opsin genes, each forming pigments by Schiff base linkage to either 11-*cis*- or all-*trans*-retinal. Each opsin pigment exhibits a characteristic absorption maximum measured in the dark. Following light exposure, the photoactivated state of each opsin exhibits a new absorption maximum. Photoactivated opsins that undergo hydrolysis to release the photoisomerized retinal product are known as mono-stable or bleaching opsins, whereas those that do not undergo hydrolysis but instead produce the ground-state pigment by absorption of energy from a second photon are known as bistable opsins. Each opsin is expressed in various parts of the body, with all opsins dominantly present in the eye, except for encephalopsin.

*Measurements are from non-human vertebrate homologs: zebrafish encephalopsin and mouse melanopsin. Melanopsin reportedly exhibits tristability with an extramelanopsin state (446 nm) containing an elusive 7-*cis*-retinylidene adduct [27,68]. In a different study involving human melanopsin, the dark-state pigment had a λ_{max} ~ 470 nm in agreement with prior studies; however, mono-stability was observed [117]. Therefore, the biochemical behavior of melanopsin remains controversial.

**Measurements are from an invertebrate peropsin homolog

Abbreviations: PRCs, photoreceptor cells; GCs, ganglion cells; RPE, retinal pigment epithelium