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New insights into retinoid metabolism and cycling within the retina

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Abstract

The retinoid cycle is a series of biochemical reactions within the eye that is responsible for synthesizing the chromophore, 11-*cis* retinal, for visual function. The chromophore is bound to G-protein coupled receptors, opsins, within rod and cone photoreceptor cells forming the photosensitive visual pigments. Integral to the sustained function of photoreceptors is the continuous generation of chromophore by the retinoid cycle through two separate processes, one that supplies both rods and cones and another that exclusively supplies cones. Recent findings such as RPE65 localization within cones and the pattern of distribution of retinoid metabolites within mouse and human retinas have challenged previous proposed schemes. This review will focus on recent findings regarding the transport of retinoids, the mechanisms by which chromophore is supplied to both rods and cones, and the metabolism of retinoids within the posterior segment of the eye.

Keywords

visual cycle; retinoid; photoreceptors; opsin; visual pigment; retinal

1. Introduction

Retinoid cycling and metabolism within the eye have been studied extensively for decades. Indeed, George Wald received a Nobel Prize in 1967 for his recognition that the photoreceptive proteins in the eye are “chromoproteins” containing the essential 11-*cis* vitamin A retinaldehyde (11-*cis* retinal; see Figure 1 for retinoid structures) as its chromophore (Wald, 1935, 1951). Research has focused on elucidating the mechanisms by which 11-*cis* retinal is synthesized, transported between the photoreceptors and the retinal pigment epithelium (RPE), and incorporated within the rods and cones, as well as the process by which the product of pigment photobleaching, all-*trans* retinal, is metabolized. Although much is known and will be discussed in detail within this review, numerous mechanisms still remain to be clarified. Our recent findings that RPE65, the proposed

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isomerase in the RPE, is present within cones (Tang et al., 2011a; Tang et al., 2011b), and the unexpected distribution of the retinoid metabolite N-retinylidene-N-retinylethanolamine (A2E) ((Ablonczy et al., 2012a) and see Fig. 12) in the human RPE pose challenges to previously accepted mechanisms.

This review will focus on recent findings regarding the transport of retinoids, the mechanisms by which visual pigments are regenerated, and retinoid metabolism within the retina and RPE. Key to the function of visual pigments is the supply of the essential chromophore, 11-*cis* retinal. There is growing evidence to show that two separate retinoid cycles exist, one that supplies both rods and cones with chromophore (classic visual cycle) and another that specifically supplies cones (cone visual cycle), possibly as a supplemental source. Furthermore, studies regarding retinoid analogues have shown that the binding constraints of the various rod and cone opsins are quite different. Contrary to early dogma, cones are not exclusively dependent upon 11-*cis* retinal for visual pigment formation, but can also utilize the alcohol form 11-*cis* retinol. Finally, we will discuss the fate of the retinoid as it is released from the opsin after the absorption of light as well as its regeneration back to the 11-*cis* form. This area is currently of great interest due to the toxic nature of the metabolites and the implications for further understanding and treatment of retinal degenerative diseases.

2. Visual pigments and chromophore binding

2.1 Visual photoreceptors

The retina is the anatomical component of the eye responsible for encoding light into neuronal signals for vision, a process collectively known as phototransduction. Photoreceptors are highly specialized neurosensory cilia within the retina that initiate this cascade of events and are classified into two major families, rods and cones, to mediate low- and bright-light vision, respectively. Humans are more reliant upon cones to carry out daily functions, but the health of both families of photoreceptors is essential for normal vision, as massive death of rods leads to cone degeneration (Hicks and Sahel, 1999; John et al., 2000). The highest concentration of cones within the human retina is found in the macula, the region that is responsible for sharp central vision, while rods compose the majority of photoreceptor cells in the surrounding (peripheral) retina.

Photoreceptors are structurally compartmentalized into regions located within distinct layers of the outer retina (Figure 2; for review, see (Mustafi et al., 2009)) and make synaptic associations with secondary neurons such as bipolar cells in the outer plexiform layer (OPL). The nuclei of the photoreceptor cells are located in the outer nuclear layer (ONL); the majority of organelles involved in cellular metabolism are present in the inner segment (IS); and the phototransduction components reside in the outer segment (OS). In rod-dominant retinas such as in mice and humans, rods comprise over 94% of the total photoreceptor population (Carter-Dawson and LaVail, 1979). The majority of the rod phototransduction proteins are associated with disc membranes isolated within the OS, whereas cone phototransduction proteins are embedded in the OS plasma membrane that is arranged into stacked invaginations (Arikawa et al., 1992). This open OS structure allows cones to have a much larger surface area in the OS region than rods, facilitating rapid exchange of retinoids and ions between the cell and its surrounding environment (Fu and Yau, 2007).

2.2 Characteristics of visual pigments

Light-sensitivity in the human eye is mediated by the rod pigment rhodopsin and three cone pigments, all of which contain a protein component (opsin) and the chromophore 11-*cis* retinal. Rod and cone opsins are members of the super-family of G-protein-coupled

receptors (Shichida and Imai, 1998). The ligand (chromophore) for opsins is covalently linked to the strictly conserved lysine residue in the seventh transmembrane helix to form the visual pigment. The chromophore is the same for all of mammalian visual pigments, and thus it is the interaction between the protein and 11-*cis* retinal that gives each pigment its distinct spectral properties. There have been extensive studies on defining the various residues that control the “tuning” of the visual pigments, that is the control of the absorption properties of those visual pigments (Hunt et al., 2009).

11-*cis* Retinal serves as an inverse agonist to the opsins (Ala-Laurila et al., 2009) resulting in a reduction of the photoreceptor resting current. Light isomerizes the 11-*cis* chromophore to an all-*trans* form (Figure 1), leading to a conformational change of the opsin to an intermediate Metarhodopsin II (Meta II) that activates its G-protein, transducin. The pigment remains in an active conformation for some time, but further transducin activation is inhibited by phosphorylation of the opsin C-terminal tail and subsequent arrestin binding. Eventually, all-*trans* retinal is released from the opsin, the bound arrestin dissociates, and the protein is dephosphorylated. To fully deactivate the receptor and prepare it to respond to another photon, the opsin binds additional 11-*cis* retinal to regenerate visual pigment. The sources of chromophore can come from the RPE as well as Müller cells, at least for cone photoreceptors (see below).

High sequence homology exists among the vertebrate opsins. For human opsins, there is roughly 40-45% sequence homology, except for red and green cone opsins, which are 96% homologous (Figure 3). Phylogenetic analyses of opsin sequences demonstrate that opsins can be divided into 5 classes correlating roughly with absorption properties (Ebrey and Koutalos, 2001; Yokoyama, 2000). Rhodopsin falls into the RH1 class of opsins; the human blue cone opsin is one form of a short wavelength-sensitive (SWS1) opsin that includes pigments that absorb maximally from about 350 to 440 nm; and the human red and green cone opsins are both medium/long wavelength-sensitive (M/LWS) opsins, which account for their high sequence identity. The other two classes of opsins are not found in mammalian retinas and are a rhodopsin-like blue-shifted opsin (RH2) and a second class of short wavelength-sensitive pigments (SWS2).

A structural feature that has received some attention is the palmitoylation of the opsins. Rhodopsins are modified at two adjacent cysteines in the C-terminal region, by fatty acid acylation, the majority of which is palmitoylation. Human M/LWS cone opsins have no cysteines at corresponding positions. SWS2 cone opsins have a single corresponding cysteine, and partial palmitoylation of salamander SWS2 cone opsin has been shown (Ablonczy et al., 2006). Human SWS1 cone opsins have one corresponding cysteine, but no acylation of that cysteine was observed for mouse or salamander variants (Ablonczy et al., 2006). Several studies have shown that removal of the palmitate groups on rhodopsin has little effect on the functional properties except, surprisingly, that excessive light greatly accelerates retinal degeneration compared to wild-type animals (Maeda et al., 2010; Wang et al., 2005).

2.3 Opsin specificity and photoreceptor type

In non-mammalian retinas, opsins are not necessarily unique to a single type of photoreceptor. The salamander green rod has been shown to have the same SWS2 opsin as the blue cone of that species (Ma et al., 2001). Interestingly, the two cells express distinct G-protein transducin alpha-subunits: rod alpha-transducin in green rods and cone alpha-transducin in blue-sensitive cones, demonstrating that this opsin can activate both transduction processes.

The question has been addressed as to if the class to which the opsin belongs controls the physiology of the receptor. From studies where the human rod RH1 pigment has been expressed in *Xenopus* M/L cones, and the human or salamander M/L cone pigment has been expressed in *Xenopus* rods, it has been clearly demonstrated that the respective cells have the same amplification and kinetics with either the rod or the cone opsin, thus eliminating the possibility that the signaling properties of the cell are controlled by the particular opsin that is expressed (Kefalov et al., 2003).

A further confounding issue is that cones can express more than one opsin. This was first shown for the guppy (Archer and Lythgoe, 1990) and the salamander (Makino and Dodd, 1996). Further studies have shown that several species of rodents, notably the well characterized and genetically-modified mouse, contain a mixture of the two cone opsins with a well-defined dorsal-ventral gradient across the retina, even in the wild-type animals (Applebury et al., 2000; Lukáts et al., 2005).

2.4 Chromophore binding to visual pigments

Despite the similarities of the opsins, there are key differences between rhodopsin and the cone pigments. The rates of sensitivity recovery (Perry and McNaughton, 1991) and visual pigment regeneration (Schnapf et al., 1990) are greater in cones than in rods (Baylor et al., 1979; Baylor et al., 1984). Recent *in vitro* studies of retinal release from cone opsins after bleaching compared to rhodopsin have shown that cone pigments release retinal at a much faster rate (Chen et al., 2012b; Das et al., 2004; Hunt et al., 2009). In addition, 11-*cis* retinal is generally not as tightly bound in cone pigments as it is in rhodopsin. Hydroxylamine, which can disrupt the Schiff base linkage of 11-*cis* retinal by forming an oxime, has greater accessibility to the chromophore in the dark in most cone pigments than in rhodopsin (Fukada et al., 1990; Wald et al., 1955). Furthermore, chromophore has been demonstrated to be exchanged in the dark with cone pigments, but not rhodopsin (Crescitelli, 1984; Matsumoto et al., 1975). The loss of chromophore was also shown to take place in cones as well as in expressed and purified cone pigments; however, this did not occur with rods and rhodopsin (Kefalov et al., 2005).

Studies using retinoid analogues have been instrumental in demonstrating that cone opsins are quite different from one another, both in their ability to bind to various analogues and in the effect of the analogue on the conformation of the proteins, as measured by their ability to activate transducin and thus activate the phototransduction process. These properties have been thoroughly studied in the salamander, with both *in vitro* studies of transducin activation and single-cell experiments measuring pigment formation and activation (Ala-Laurila et al., 2009; Isayama et al., 2006). Table 1 summarizes a portion of these data. Studies with 11-*cis* 9-demethyl retinal, where a single methyl group is lacking, have been particularly informative. Salamander M/LWS cone pigments generated with 11-*cis* 9-demethyl retinal resulted in a longer-lived Meta II intermediate (i.e., slower release of all-*trans* retinal from the opsin) (Das et al., 2004). The other two salamander cone pigments (SWS1 and SWS2) did not display any alterations in activation or deactivation rates with 11-*cis* 9-demethyl retinal (Das et al., 2004). These results suggested that the 9-methyl group of retinal was critical in the fast deactivation of only M/LWS cone pigments. Consistent with these results, studies have demonstrated that a prolonged light-activated salamander red cone occurs in isolated salamander red cone cells regenerated with 11-*cis* 9-demethyl retinal (Estevez et al., 2006; Estevez et al., 2009).

Compounds representing a fraction of the structure of retinal have been known for some years to fit into the binding site of rhodopsin and to inhibit the binding of the native chromophore (Matsumoto and Yoshizawa, 1975; Nelson et al., 1970). Unexpectedly, it was found that such small molecules could also act as agonists or inverse agonists to opsins

(Kono and Crouch, 2010, 2011). The first observations were made using salamander rods and cones with measurements on isolated photoreceptors (Isayama et al., 2006; Jin et al., 1993; Kefalov et al., 1999). Table 2 presents results from *in vitro* studies on human opsins. The use of these small molecules is being explored as a means to control cone degeneration observed in situations where the supply of 11-*cis* retinal has been compromised.

2.5 Generation of the visual pigment chromophore

Intrinsic to the ability of rods and cones to detect light is the availability of chromophore (Figure 1). The stable form of vitamin A (all-*trans* retinol) is an essential fat-soluble vitamin acquired through the dietary consumption of either β -carotene from plants or retinyl-esters from animal products. After β -carotene is absorbed by the intestinal lumen, it is symmetrically cleaved by β , β -carotene 15,15'-monooxygenase into two molecules of all-*trans* retinal and eventually reduced to all-*trans* retinol by retinoid dehydrogenases (RDHs) (Redmond et al., 2001; von-Lintig and Vogt, 2000). Retinyl-esters are composed of all-*trans* retinol bound to a fatty acid through an ester linkage that must be hydrolyzed for absorption to occur (Rigtrup and Ong, 1992). The most common ester found in the retina is retinyl-palmitate (Trehan et al., 1990). The generation of 11-*cis* retinal and retinol has been the subject of much study and debate. Current data suggest there are two systems for producing these critical retinoids, the classic visual cycle and the cone visual cycle, both of which are discussed below.

3. Retinoid processing for the rod photoreceptors

3.1 Classic visual cycle

Generation of 11-*cis* retinal from all-*trans* retinol occurs in the RPE in a process referred to as the classic visual cycle (Figure 4) (Travis et al., 2007). After absorption of light by visual pigments, 11-*cis* retinal is destroyed through isomerization to the all-*trans* configuration. To regenerate 11-*cis* retinal, all-*trans* retinol is released from opsin and reduced to all-*trans* retinol by the combined action of RDH8 and RDH12 (Chen et al., 2012a). All-*trans* retinol is transported to the RPE where it is esterified by lecithin retinol acyl transferase (LRAT) (Batten et al., 2004). This retinyl-ester is the substrate for the proposed isomerohydrolase, RPE65 (Moiseyev et al., 2003). RPE65 converts all-*trans* retinyl-ester to 11-*cis* retinol (Jin et al., 2005; Moiseyev et al., 2005; Redmond et al., 2005), which is then oxidized to 11-*cis* retinal and transported to photoreceptors to regenerate visual pigments to complete the "visual cycle" (see Section 3.2). The shuttling of all-*trans* retinol from the photoreceptors to the RPE and 11-*cis* retinal from the RPE to the photoreceptors is presumably aided by a retinoid binding protein, interphotoreceptor retinoid binding protein (IRBP) (see Section 3.3) (Edwards and Adler, 1994, 2000). Both rods and cones apparently receive 11-*cis* retinal from the RPE, and we are referring to this as the classic visual cycle to distinguish it from a more recently identified cone visual cycle involving the Müller cells that appears to be exclusive to cones (see Section 4).

3.2 Role of RPE65

RPE65 is an abundant protein in the RPE that was first identified and cloned from the RPE by the Redmond group (Hamel et al., 1993). This 61 kDa protein undergoes post-translational modification with palmitates to facilitate association with membranes (Takahashi et al., 2006) and is homologous to beta-carotene oxygenases (Redmond et al., 2001). A crystal structure for RPE65 has been determined at 2.14 Å resolution (Kiser et al., 2009). Studies are underway in several laboratories probing the RPE65 mechanism of action.

In 2005, three groups independently concluded that RPE65 was the isomerohydrolase, most probably acting in complex with other retinyl-binding protein (s) and dehydrogenase(s), responsible for converting all-*trans* retinyl-ester to 11-*cis* retinol within the RPE (Jin et al., 2005; Moiseyev et al., 2005; Redmond et al., 2005). The mechanism of action is still unclear, but recent studies support a carbocation mechanism (Redmond et al., 2010) as previously proposed (McBee et al., 2000). Surprisingly, the RPE65-based complex is not specific for the 11-*cis* isomer, but may be driven by a mass action effect of the binding protein(s) (Redmond et al., 2010). Mutations to RPE65 are associated with Type 2 Leber congenital amaurosis (LCA2), an early onset congenital blindness that reduces or abolishes the innate synthesis of 11-*cis* retinal (Harris EW, 2001; Marlhens et al., 1997). Mice whose RPE65 gene has been knocked out (*Rpe65*^{-/-}) do not generate 11-*cis* retinal (Redmond et al., 1998) and exhibit rapid cone loss and cone opsin mistrafficking (Fan et al., 2008; Tang et al., 2010; Znoiko et al., 2005). Gene therapy studies on mouse (Pang et al., 2006) and canine (Acland et al., 2001; Acland et al., 2005) models, as well as LCA2 patients (Bainbridge et al., 2008; Maguire et al., 2008; Maguire et al., 2009), have shown that inserting a functional gene for RPE65 into RPE cells holds great promise for restoring visual function.

3.3 Role of IRBP in rods

IRBP is the most abundant soluble protein in the sub-retinal space (Gonzalez-Fernandez, 2003) and is known to bind retinoids (Edwards and Adler, 1994; Pepperberg et al., 1993). *In vitro* data from several laboratories have shown that IRBP facilitates clearance of all-*trans* retinol from photoreceptor cells and uptake of 11-*cis* retinal from the RPE (Edwards and Adler, 2000; Jones et al., 1989). In addition, IRBP preserves the isomeric state of retinoids in the sub-retinal space (Crouch et al., 1992). The specific role of IRBP is still not clear. Knocking out IRBP in mice (*Irbp*^{-/-}) did not affect recovery of sensitivity by the rods as might have been expected if IRBP were critical for the transport of 11-*cis* retinal to photoreceptors (Palczewski et al., 1999; Ripps et al., 2000); however, *Irbp*^{-/-} mice do exhibit rod degeneration (Liou et al., 1998), indicating that IRBP's absence is not benign.

4. Retinoid processing for the cone photoreceptors

4.1 Cone visual cycle

Recent evidence suggests the existence of a second retinoid visual cycle within the vertebrate retina functioning in parallel with the classic visual cycle to specifically support rapid cone pigment regeneration (Figure 5; for reviews, see (Muniz et al., 2007; Wang and Kefalov, 2011; Wolf, 2004)). Salamander cones maintained within an isolated retina free of the RPE recover light sensitivity after bleaching; however, this recovery disappears in bleached cones disassociated from the retina (Wang et al., 2009). Previous work in the isolated frog retina has also implicated the regeneration of 11-*cis* retinal by the retina independent of the RPE (Goldstein, 1968; Hood and Hock, 1973).

Isomerization of all-*trans* retinol has been proposed to occur in the Müller cells through an RPE65-independent manner. Cultured chicken Müller cells were shown to convert all-*trans* retinol to all-*trans* and 11-*cis* palmitates and 11-*cis* retinol, similar to reactions in the classical visual cycle of the RPE (Das et al., 1992). Recently, the Travis group identified the presence of dihydroceramide desaturase-1 (DES1) in Müller cells and reported that this protein mediates a direct isomerization of all-*trans* retinol to 11-*cis* retinol (Kaylor et al., 2012). *In vitro* studies using the expressed protein demonstrated that DES1 has isomerase activity. Interestingly, like RPE65 (Redmond et al., 2010), DES1 is not specific in forming 11-*cis* retinol, but also generates 13-*cis* and 9-*cis* retinol as well. Müller cells contain CRBP and CRALBP, both of which are retinoid binding proteins found in the RPE, with the latter preferentially binding 11-*cis* retinoids (Bunt-Milam and Saari, 1983; Eisenfeld et al., 1985).

Zebrafish express two forms of CRALBP, one in the RPE and another in the Müller cell. Knocking down the expression of CRALBP in the Müller cells of zebrafish larvae by using antisense morpholinos results in a substantial decrease in 11-*cis* retinal and reduction in light sensitivity, supporting the key role of this protein (Collery et al., 2008; Fleisch et al., 2008). DES1 is reported to bind CRALBP, supporting its role as an isomerase (Kaylor et al., 2012). Unlike the LRAT-dependent retinol esterification in the RPE, microsomes of chicken retina generated 11-*cis* retinyl-ester from all-*trans* retinol through a mechanism requiring palm-CoA as an acyl donor, suggesting this isomerase is dependent on acyl CoA-retinol acyltransferase (ARAT) (Muniz et al., 2009). Within the intact isolated salamander retina, lesioning Müller cells with a gliotoxin inhibited the ability of bleached cones to recover light sensitivity (Wang et al., 2009).

Thus, Müller cells are capable of converting all-*trans* retinol to 11-*cis* retinol similar to what is observed in the RPE with the exception of the final oxidation reaction to generate 11-*cis* retinal. It has been shown that cones are capable of carrying out 11-*cis* retinol oxidation, whereas rods cannot. Bleached cones, but not bleached rods, were first demonstrated to regenerate resting dark current and light-sensitivity after the addition of 11-*cis* retinol in single salamander cell electrophysiological recordings (Jones et al., 1989). More recently, it has been shown directly that 11-*cis* retinol can indeed regenerate cone pigments within the cells by microspectrophotometry (Ala-Laurila et al., 2009). Further demonstration of oxidase activity in cones was shown in eyecups of mice that lacked endogenous 11-*cis* retinal from which 11-*cis* retinal could be generated after incubation with 11-*cis* retinol (Parker et al., 2011). Interestingly, 11-*cis* retinol is now known to act as an agonist to rod opsin, activating the rod opsins in a light-independent manner (Kono et al., 2008). The compound was originally proposed to be toxic to bleached rods due to increased desensitization, but now has been shown to be unable to form pigment in rods (Ala-Laurila et al., 2009).

Müller cells and cones contain the necessary machinery for completion of the cone visual cycle. By not performing the final oxidation step in the Müller cells, 11-*cis* retinol becomes a source of chromophore usable only by cones. Using suction electrodes to mask the OS of isolated salamander rods and cones, 11-*cis* retinal presented to the cell bodies of bleached photoreceptors restored light sensitivity in cones, but had no effect on rods (Jin et al., 1994). This finding is particularly interesting considering the topology of Müller cell processes, as they make numerous associations with cones at this region (Sarantis and Mobbs, 1992; Wang et al., 2009). These data suggest that the site of entry of the chromophore may be different for rods and cones. A cone-specific visual cycle involving the Müller cells (Kaylor et al., 2012) has been proposed and an isomerase identified which is localized in Müller cells. In the RPE, RPE65 is the accepted isomerohydrolase that converts all-*trans* retinyl-ester to 11-*cis* retinol (Jin et al., 2005; Moiseyev et al., 2005; Redmond et al., 2005). There is no evidence for the presence of RPE65 within Müller cells; however, the absence of RPE65 abolishes the generation of 11-*cis* retinoids in the mammalian eye (Redmond et al., 1998). In *Rpe65*^{-/-} mice, the absence of 11-*cis* retinal also results in rapid degeneration of cones (Rohrer et al., 2005; Znoiko et al., 2005). RPE65 has been shown to be essential in cone function of the neural retina leucine zipper knockout (*Nrl*^{-/-}) mouse (Wenzel et al., 2007), a model for a pure-cone retina (Mears et al., 2001). The level of 11-*cis* retinal generated by the cone visual cycle synthesis appears to be insufficient to promote cone survival. Data to support this come from *Lrat*^{-/-} mouse studies (Fan et al., 2008; Zhang et al., 2008), where the lack of esterification of all-*trans* retinol in the RPE, the substrate for RPE65-dependent isomerization (Moiseyev et al., 2003), leads to a similar degeneration of cones as observed in the *Rpe65*^{-/-} mouse.

4.2 RPE65 within cones

4.2.1 Identification of RPE65 in cones—RPE65 has been well-documented to be abundant in the RPE of many species (Hamel et al., 1993; Tsilou et al., 1997) and was originally proposed to be specific to that tissue. However, the mRNA of RPE65 has been reported in the iris (Kociok et al., 1998), and the protein has been found in transformed kidney cells (Ma et al., 1999), human epidermis (Hinterhuber et al., 2004), and non-melanocytic skin tumors (Hinterhuber et al., 2005). We have localized RPE65 to cones although, interestingly, there does not appear to be a common location within cones for RPE65 among the cones of different species that we have studied.

RT-PCR analysis of isolated salamander photoreceptors first demonstrated the presence of RPE65 mRNA in cones, but not rods (Ma et al., 1998). Using a polyclonal anti-RPE65 antibody generated from a synthetic peptide corresponding to residues 150-164 of bovine RPE65 (NFITKVNPELETIK) (Redmond and Hamel, 2000), we showed localization of RPE65 in the OS of cones of the wild-type mice (Figure 6) (Tang et al., 2011b). Further support for the existence of RPE65 in mouse cones comes from the *Nrl*^{-/-} mouse, which contains elevated levels of RPE65 within the retina when compared to the wild-type mouse (Wenzel et al., 2007). Immunohistology showed RPE65 to be present in the OS of these cone-like photoreceptors (Figure 6, B-C) (Boyer et al., 2012b). Interestingly, the level of RPE65 within cones across different strains of mice appears to be inversely related to the level of RPE65 present within the RPE (Tang et al., 2011b). This last finding is likely the basis of some controversy over the presence of RPE65 in cones (summarized in (Tang et al., 2011b)) as RPE65 is highest in the *C57BL/6* mice (low levels of RPE65 in the RPE) and undetectable by immunostaining in *BALB/c* and *129Sv* mouse eyecups where RPE65 is high in the RPE. Furthermore, the affinity of the antibody proved to be critical for its immunodetection (Tang et al., 2011b).

We have recently found RPE65 to also be localized in the OS of human cones (Figure 7) (Tang et al., 2011a). The RPE65 antibody labels the red/green cones in a punctate appearance with the majority of the fluorescence between the IS/OS interface and in an apical pattern extending along the length of the OS. In humans, RPE65 appears to be selectively expressed in the OS regions of cones that detect green/red light, but not in blue cones (Tang et al., 2011a). This may be due to a requirement of green and red cones for a supplemental source of retinoids for rapid chromophore turnover, as they are the only subtypes that appear within the fovea. Further studies are required to explore these findings.

While much of the data gathered about RPE65 in cones come from the rod-dominant mouse and human retina, little is known about the presence of the protein in cones of the cone-dominant retina. We have conducted a preliminary evaluation of RPE65 staining in cones of the cone-dominant *Anolis* lizard and found, surprisingly, that RPE65 is localized to the IS, specifically within the paraboloid (Figure 8). This structure is known to be composed of smooth ER and glycogen (Amemiya, 1975; Braekevelt, 1990); however the implications for this varied appearance of RPE65 compared to cones of the rod-dominant retinas will require further investigation.

4.2.2 Role of RPE65 within cones—Unlike in the RPE, the role of RPE65 in cones remains unclear. As isomerization of the retinal/retinol does not occur within the cones, RPE65 is not functioning as an isomerase within the cone. It is possible that RPE65 has a role as a retinol binding protein or perhaps as a hydrolase. Because processes from the Müller glia make numerous associations with the cone cell body (Uehara et al., 1990), and 11-*cis* retinol is thought to be delivered to this region of the cell (Wang et al., 2009), a retinoid binding protein within cones would be useful as new opsins are being synthesized. The cone-derived 661W cells (Tan et al., 2004) are known to have retinol esterase activity

(Kanan et al., 2008), and we found that 11-*cis* retinol can be esterified in these cells (Tang et al., 2011a). The role of RPE65 may be to bind the ester and mediate the ester hydrolysis, perhaps occurring at the OS interface at the connecting cilium in the mouse which could explain the localization of the protein in this region of the mouse cone (Tang et al., 2011b). RPE65, in a role as a retinoid binding protein, may shuttle 11-*cis* retinol along the OS, thus explaining the punctate appearance of RPE65 immunostaining in the apical regions of the mouse cone OS (Tang et al., 2011b). Higher resolution imaging with techniques such as immuno-electron microscopy will be critical to determining the exact subcellular localization of RPE65 within cones. Another possibility is that retinol binding function of RPE65 may have a role in the 11-*cis* retinol oxidation by RDHs (Hemati et al., 2005; Kiser et al., 2009). We have found that mouse strains with higher levels of cone RPE65 are more efficient at synthesizing cone pigments than those with less of the protein (Tang et al., 2011b). We thus propose that RPE65 is acting as a retinol binding protein, and the distribution within the cones of various species may vary depending on the localization of the hydrolysis/oxidation reactions. The design of animal models that specifically lack the cone RPE65 while the RPE65 remains intact within the RPE and vice versa is needed to address these questions. Thus, the role of RPE65 in cones remains unresolved, but there is no evidence for this protein acting as an isomerase in the cone.

4.3 Role of IRBP in cone pigment regeneration

The role of IRBP for shuttling 11-*cis* retinol from Müller cells to cones is unclear. Its involvement in promoting the cone visual cycle is supported by evidence that IRBP binds 11-*cis* retinol endogenously (Saari et al., 1985), is co-localized with Müller cell processes surrounding the cone cell body (Uehara et al., 1990), and is found in high concentrations around the cones (Carter-Dawson and Burroughs, 1992a, 1992b). Cones in *Irbp*^{-/-} mice were found to be retinoid-deficient under photopic conditions in two studies (Parker et al., 2009), leading to the proposal that 11-*cis* retinol supplies are disrupted in the absence of IRBP. Using isolated *Nrl*^{-/-}*Rpe65*^{-/-} retinas, IRBP was shown to deliver 11-*cis* retinol for oxidation in cones and to enhance the efficiency of the oxidation reaction (Parker et al., 2011). The isoform of RPE65 appears to affect the phenotype of *Irbp*^{-/-} mice as Jin et al. (Jin et al., 2009) reported cones in *Irbp*^{-/-} mice with the Leu450 variant of RPE65 also had reduced outer segment length, partially mislocalized cone opsin, and underwent progressive degeneration. Parker et al. (Parker et al., 2009) found a reduction in cone ERG responses in *Irbp*^{-/-} mice having the Met450 variant of RPE65 but observed no change in cone density or cone opsin levels and distribution. The studies are in agreement that retinoid deficiency is a likely cause for the impaired cone function in the absence of IRBP. As IRBP protects the isomeric state of 11-*cis* retinol, even in the presence of light (Crouch et al., 1992), the protein is likely to have a critical role in the delivery of 11-*cis* retinol to cones and to facilitate cone function.

5. Products of retinoid metabolism

There is a constant supply of all-*trans* retinol from the blood stream and from the supplies of retinyl ester stored in the RPE. STRA6, the recently identified membrane receptor for plasma retinol binding protein (RPB), mediates the cellular uptake of *trans* retinol into the RPE (Kawaguchi et al., 2007; Sun, 2012). An additional supply of all-*trans* retinol comes from the stores of the retinyl esters within the RPE itself. There are two sources of retinoids resulting from the visual process: the all-*trans* retinal generated from the absorption of light by the visual pigments and the 11-*cis* retinal linked to unbleached visual pigments. Under light conditions, the all-*trans* retinal produced from the absorption of light is assumed to be reduced to all-*trans* retinol and then transported to the RPE where it is stored as the ester. The portion of visual pigments that are not bleached will contain the 11-*cis* retinal and could contribute a minor source of retinoids to the visual process. The rod photoreceptors are

phagocytized by the RPE at a rate of about 10% per day for humans (Anderson et al., 1978). Much less is known about phagocytosis of the cones; however, as the cone pigments only represent 1-5% of the total visual pigment, this retinoid source is likely to be minor in rod-dominant mammalian eyes.

5.1 Reduction of all-*trans* retinal

Due to its relation to the toxicity of non-reduced all-*trans* retinal, the formation of lipofuscin precursors, and the recycling of the retinoid chromophore, the reduction of all-*trans* retinal to retinol has been examined in detail. Imaging the fluorescence of retinol has permitted the study of its formation kinetics in isolated photoreceptors from several vertebrate species, including salamander (Ala-Laurila et al., 2006; Tsina et al., 2004), frog (Chen et al., 2005; Wu et al., 2007), gecko (Kolesnikov et al., 2007), and mouse (Blakeley et al., 2011; Chen et al., 2009). Figure 9 shows the kinetics of retinol formation after bleaching in mouse rod photoreceptors and in the cone-like photoreceptors from the *Nrl*^{-/-} mouse retina. In *Nrl*^{-/-} photoreceptors as well as cones, formation of retinol after bleaching occurs more rapidly than in rods. This is in agreement with the faster release of retinal from the photoactivated visual pigment, and the higher retinol dehydrogenase activity in cones (Miyazono et al., 2008). It would allow cone photoreceptors to recycle the chromophore faster than rods, so they can support faster visual pigment regeneration. However, the faster formation of retinol in cones is not simply due to the earlier availability of all-*trans* retinal because of its faster release from photoactivated visual pigment. In the salamander retina, blue-sensitive cones and green rods contain the same visual pigment, SWS2. The kinetics of retinol formation, however, are faster in the cone than in the rod (Ala-Laurila et al., 2006), underlining the importance of the higher retinol dehydrogenase activity in the former.

5.2 Retinoids and lipofuscin formation

In both rod and cone photoreceptors, photoactivation of the visual pigment occurs through the photoisomerization of the 11-*cis* retinal to the all-*trans* form. In both types of photoreceptors, all-*trans* retinal released from the photoactivated pigment is reduced to all-*trans* retinol within the OS (Figure 10). The reduction requires NADPH (Futterman et al., 1970) and, at the same time, achieves both the removal of retinal and initiation of the recycling of all-*trans* retinal back to the 11-*cis* form. Retinal is a reactive aldehyde and a photosensitizer (Delmelle, 1978; Krasnovsky and Kagan, 1979; Matsumoto et al., 1975; Rozanowska and Sarna, 2005), and its toxicity may play a role in several diseases of the retina.

All-*trans* retinal can form a Schiff base with amine groups, including the one on phosphatidylethanolamine, a major lipid component of OS membranes (Fliesler and Anderson, 1983). A second molecule of all-*trans* retinal can then react with the Schiff base, forming *bis*-retinoids, such as A2PE (Ben-Shabat et al., 2002b; Liu et al., 2000). A2PE is a precursor of A2E, the best characterized among the *bis*-retinoids. The release and removal of all-*trans* retinal that occur as a part of the light detection process are thought to be associated with the formation of lipofuscin (Katz and Redmond, 2001; Katz and Robinson-Jr, 2002; Sparrow et al., 2010), the fluorescent pigment that accumulates with age in the RPE lysosomal compartment. Lipofuscin and A2E can accumulate in the RPE of both rod-dominant and cone-dominant retinas, as shown by recent studies on the *Nrl*^{-/-} mouse (Figure 11) (Boyer et al., 2012b).

There are several lines of evidence in support of the relation between retinal and lipofuscin. First, lipofuscin levels are greatly decreased in mice that lack RPE65 (Katz and Redmond, 2001), indicating that lipofuscin formation requires the generation of 11-*cis* retinal. Second, *bis*-retinoids constitute the major extractable component of lipofuscin (Kim et al., 2007;

Sparrow et al., 2012). Third, lipofuscin accumulation requires the phagocytosis of the photoreceptor OS by the RPE, and in the absence of phagocytosis, retinoid-derived fluorescent material accumulates in the photoreceptors (Katz et al., 1987). Finally, all-*trans* retinal can form A2PE in rod OS membranes. This includes the formation of A2PE from all-*trans* retinal added exogenously to membranes (Liu et al., 2000), as well as from retinal released endogenously from rhodopsin following bleaching (Ben-Shabat et al., 2002b). Taken together, these pieces of evidence have led to the proposal that *bis*-retinoid precursors of lipofuscin, such as A2PE, form in the photoreceptor OS from all-*trans* retinal that is escaping reduction to retinol (Katz and Redmond, 2001; Katz and Robinson-Jr, 2002; Sparrow et al., 2010). Subsequently, *bis*-retinoids enter the RPE through the daily phagocytosis of photoreceptor outer segments (Young, 1967), ending up in the lysosomal compartment where they accumulate in the form of lipofuscin. Consistent with this notion, mice that lack retinol dehydrogenases, the enzymes that oxidize all-*trans* retinol to retinal show increased A2E accumulation in the RPE (Chrispell et al., 2009; Maeda et al., 2005).

5.3 A2E formation from 11-*cis* retinal

While it is clear that retinoids are involved in lipofuscin, recent evidence shows 11-*cis* retinal itself may be a major source of lipofuscin and A2E (Boyer et al., 2012a). These results are based on the finding that mice raised in the dark have the same levels of lipofuscin and lipofuscin precursors as animals raised in cyclic light. Furthermore, direct addition of 11-*cis* retinal to rod photoreceptors results in the generation of lipofuscin precursors in the absence of light. This surprising result challenges the existing notion that A2E and lipofuscin arise from all-*trans* retinal generated from the bleaching of the visual pigments. These data indicate that 11-*cis* retinal may have a major role in events resulting in retinal degeneration.

5.4 Distribution of A2E in the RPE

The accumulation of lipofuscin is one of the most striking features of the aging human eye (Delori et al., 1995; Feeney, 1978; Wing et al., 1978). Through interaction with blue light, lipofuscin was proposed to contribute to the development of age-related macular degeneration (Birnbach et al., 1994; Holz et al., 2001; Roberts et al., 2002). The exact composition of lipofuscin is not known (Ng et al., 2008; Sparrow et al., 2010); however, *bis*-retinoids are a major component of lipofuscin. More than 20 *bis*-retinoids have been identified in the last two decades (Sparrow et al., 2010), but the best-studied *bis*-retinoid is still A2E (Eldred and Lasky, 1993; Parish et al., 1998; Sakai et al., 1995). There are numerous studies suggesting a toxic role for A2E to the RPE, particularly in its oxidized form (Kim et al., 2008; Wang et al., 2006). A detoxifying role for A2E itself has been suggested as it removes the highly toxic free all-*trans* retinal (Maeda et al., 2011; Maeda et al., 2009a; Roberts et al., 2002). The inhibition of A2E formation through the limitation of normal retinoid processing has become a primary target of ocular pharmaceuticals.

No immunological techniques are suitable for determining the spatial localization of A2E, and therefore the spatial relationship of A2E and lipofuscin has been hard to establish. The Bernstein laboratory utilized 8 mm tissue punches on human RPE and surprisingly found no correlation with the lipofuscin distribution reported by others (Bhosale et al., 2009; Wing et al., 1978). We have recently developed a method for the localization of *bis*-retinoid utilizing a mass spectrometry-based technique, MALDI imaging (Grey et al., 2011). In this method, mass spectra are collected over the surface, allowing the relative levels of individual molecular ions (such as A2E) to be measured and visualized across the entire tissue. This information is directly comparable to fluorescence measurements; therefore, the spatial distributions of individual molecular ions (such as A2E, its oxides, precursors, and degradation products) can be determined and correlated with the measured fluorescence.

The distributions of A2E and its oxides were determined in three murine models, *Abca4*^{-/-} mice (with a rapid accumulation of A2E (Mata et al., 2000)), *Sv129* mice (the base strain for the *Abca4*^{-/-}), and *C57BL/6* mice (Grey et al., 2011). In the murine RPE, we found a marked agreement between the MALDI images of A2E and the fluorescence of lipofuscin in the RPE. Age and the higher accumulation of A2E in the *Abca4*^{-/-} model increased the correlation of lipofuscin and A2E (Figure 12, A&B). The normalized profiles of relative lipofuscin fluorescence and A2E quantity along a cross-section of an eye (Figure 12, C) reinforce these observations. *In vitro* studies had demonstrated up to nine oxygens could be detected on A2E exposed to oxidizing conditions (Ben-Shabat et al., 2002b); however, the tissue profiling studies only detected A2E with an addition of a maximum of 3 oxygens. The distribution of oxidized forms of A2E, which are proposed to be more harmful than A2E itself (Ben-Shabat et al., 2002a), followed closely that of A2E but at much reduced levels. Although the quantity of A2E changed with age and strain, the fraction of oxidized A2E to unoxidized A2E was constant (0.17 ± 0.03 oxygen per A2E).

Post mortem human eyes from fetal age to 98 years have also been analyzed in a similar manner (Ablonczy et al., 2012b). Contrary to the murine models, the distribution of A2E in the human eyes did not correlate with lipofuscin fluorescence (Figure 12, D-F), regardless of age. Our measurements of lipofuscin fluorescence reinforced prior data (central distribution of lipofuscin which increases with age, see (Wing et al., 1978)). However, A2E was most abundant in the far periphery (50-100-fold more than in the center), independent of age. These findings are in agreement with the data obtained from the tissue punch study (Bhosale et al., 2009), and demonstrate that lipofuscin fluorescence is not an adequate monitor of A2E in the human eye.

6. Therapeutic use of retinoids and retinoid-like compounds

Diseases resulting in visual impairment arising from the disrupting retinoid metabolism have been recognized for some years and excellent reviews cover this area (Travis et al., 2007). Night blindness resulting from vitamin A deficiency was identified as the result of the rod photoreceptors lacking chromophore, (Dowling, 1964) and indeed many of these studies led to the initial findings in retinoid cycling. There has been recent interest in the use of retinoids and small molecules mimicking retinoids as therapeutic agents acting as inhibitors of the visual cycle as a means of preventing the accumulation of the lipofuscin that occurs in Stargardt's disease and macular degeneration (Golczak et al., 2008; Maiti et al., 2006; Palczewski, 2010). Primary amines, which can sequester all-*trans* retinal, have been shown to exert a protective role against light-induced damage in mouse models of retina degeneration (Maeda et al., 2011). Furthermore, successful use of small molecules and retinoid derivatives to ameliorate the cone degeneration reported in animal models of LCA2 have been reported (Maeda et al., 2009b; Rohrer et al., 2005). The small molecules have the advantage in not being light sensitive and also in not forming photoreactive pigments with the opsins (Fan et al., 2011). Methods of sustained delivery of the retinoids have also been developed for the potential use of these hydrophobic agents (Tang et al., 2010). The use of these retinoid-like compounds as therapeutic agents is an active area of investigation.

7. Conclusions and Future Directions

Although our understanding of the retinoid visual cycle has progressed significantly throughout the last decade, several recent observations show there are still many questions to be addressed. RPE65 has been shown to be present in cones; however, the role of RPE65 within this cell type remains unknown. How does its presence fit in with what is known about the cone visual cycle? As RPE65 in cones does not act as an isomerase, is its role that of a retinoid binding protein? Cone opsins have been shown to mislocalize in the absence of

the native chromophore, but the mechanism responsible for this has yet to be deduced. What is the process by which cone opsins are trafficked, and why does the absence of chromophore inhibit this process? Recent studies suggest IRBP has a role in cone pigment regeneration, but the details are still unclear. The assumption that A2E and lipofuscin precursors arise from all-*trans* retinal generated by light has been challenged as 11-*cis* retinal is found to generate the same precursors independent of light. Finally, why does the posterior pole of the human eye exhibit strong fluorescence without the presence of significant levels of A2E? Currently, much interest lies within limiting retinoid metabolism to prevent the accumulation of *bis*-retinoids. The lack of correlation between A2E and lipofuscin in the human eye clearly shows that further study is needed. New analytical imaging techniques, experiments focused on the novel cone visual cycle and transduction mechanisms, animal models which more closely model the human retina, and experimental investigation of the human retina are needed to address these poorly understood areas to further understand the retinal degenerative diseases affecting human health and well-being.

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Figure 1. Chemical structures of retinoids involved in vision or used in characterizing opsin structure and function

(A) Synthesis of 11-*cis* retinal from β -carotene and recycling of all-*trans* retinal. **(B)** Structures of retinoids. 1 = 11-*cis* retinal; 2 = β -carotene; 3 = all-*trans* retinal; 4 = all-*trans* retinol; 5 = all-*trans* retinyl β -ester (R represents fatty acid); 6 = 11-*cis* retinol; 7 = 9-demethyl 11-*cis* retinal; 8 = 13-demethyl 11-*cis* retinal; 9 = β -ionone; 10 = A2E.

Figure 2. Mouse retina and photoreceptor cell morphology

(A) The retina is located in the posterior portion of the eyecup (left) and is organized into multiple distinct layers (right). (B) Schematic of rod and cone photoreceptor cells. RPE = retinal pigment epithelium; OS = outer segment; IS = inner segment; ONL = outer nuclear layer; INL = inner nuclear layer; IPL = inner plexiform layer; GCL = ganglion cell layer.

Figure 3. Sequence identity among the human rod and cone opsins

Opsin secondary structure is represented in two-dimensions based on alignment with the known structure of bovine rhodopsin. Residues that are identical between the human rod and human red, green, and blue cone opsins are denoted as black circles with the conserved amino acid residues identified.

Figure 4. Classic visual cycle

Retinoids are metabolized and shuttled between the RPE (purple) and both rod and cone OS (depicted generically in green). RAL = retinal; ROL = retinol; RDH = retinol dehydrogenase; LRAT = lecithin:retinol acyltransferase.

Figure 5. Cone visual cycle

Retinoids are metabolized and shuttled between the Müller glia (orange) and cones (blue). Currently, the isomerase of the Müller glia remains unknown (depicted as *). ARAT = Acyl CoA:retinol acyltransferase.

Figure 6. RPE65 in mouse cones

(A) Cross section of *C57BL/6* mouse eyecup shows RPE65 staining in both the RPE and the cone OS. (RPE65 = green; SWS1 opsin = red; DAPI = blue) (B) RPE65 is localized within rosettes in the ONL of *Nrl*^{-/-} mouse retina. (RPE65 = green; PI = propidium iodide = red) (C) RPE65 co-localizes with SWS1-opsin, a marker for cone OS, within the rosette of the *Nrl*^{-/-} mouse retina as shown by yellow. (RPE65 = green; SWS1 opsin = red).

Figure 7. RPE65 in human cone photoreceptors

Frozen section from temporal peripheral area of a human retina immunostained for RPE65 shows the protein is localized within the cone OS of the human retina. Images shown (A) composite of transmitted light and RPE65 fluorescence, (B) composite of RPE65 and propidium iodide fluorescence and (C) RPE65 fluorescence alone Trans = transmitted light; RPE65 = green; PI = propidium iodide = red.

Figure 8. RPE65 in lizard cone photoreceptors

(A-B) Cross-section of lizard retina stained with Hematoxylin/Eosin (H&E) or Periodic Acid Schiff (PAS) shows cones with large IS and tapered OS, as previously described (Makaretz and Levine, 1980). (A'-B') Higher magnification show the paraboloid structure (*). (C) Diagram of lizard cone shows various compartments. (D) RPE65 staining is present within the paraboloid of IS using DAB-color development. (E) Rabbit IgG serves as control. (F-G) Immunofluorescence shows RPE65 present in both RPE and cone IS. (RPE65 = green; DAPI = blue).

Figure 9. Kinetics of all-*trans* retinol formation in the outer segments of single isolated photoreceptors from *C57BL/6* and *Nr1h3*^{-/-} mouse retinas
 Retinas obtained from (A) *C57BL/6* or (B) *Nr1h3*^{-/-} mice. The insets show dark-adapted isolated photoreceptors: left panel = the bright field; middle = fluorescence images before bleaching; and the right panel = after bleaching for (A) 30 min and (B) 33 sec. Note the appearance of retinol fluorescence. Scale bars = 5 μ m. Data re-plotted from (Chen et al., 2009).

Figure 10. Reactions that generate and eliminate all-*trans* retinal in the photoreceptor outer segment

all-*trans* Retinal generated on decay of the photo-bleached pigment (see Figs. 4 and 5) can be reduced to all-*trans* retinol or react with an amine such as phosphatidylethanolamine to generate precursors to A2E.

Figure 11. Lipofuscin and A2E in the RPE from a rod-dominant and a cone-dominant retina (A) *C57BL/6* mouse, (B) *Nrf1*^{-/-} mouse, (C) Fluorescence emission spectra of lipofuscin granules, (D) Total lipofuscin fluorescence levels in the RPE of 12-month-old mice, (E) Total A2E levels in the RPE of 12-month-old mice. *Nrf1*^{-/-} mice were used as a cone-dominant model. True color RPE fluorescence micrographs (490 nm excitation). Data re-plotted from (Boyer et al., 2012b).

Figure 12. Lipofuscin fluorescence and A2E MALDI images in murine and human eyes
 The spatial distribution of lipofuscin fluorescence (A) and A2E (B) in the right eye of a 6-month-old *Abca4*^{-/-} mouse. C, cross-sectional profiles along the arrows indicated in the previous panels show a good correlation between lipofuscin and A2E. The fluorescence image was collaged from individual fields taken with a fluorescent microscope ($\lambda_{\text{exc}} = 488$ nm). The MALDI image of A2E was collected at 150 μm resolution. Bar = 1 mm. The spatial distributions of lipofuscin fluorescence (D) and A2E (E) in a 94-year right human eye. F, cross-sectional profiles along the arrows indicated in the previous panels show that lipofuscin (which is central) does not correlate with A2E (which is peripheral). The fluorescence image was acquired in a Xenogen IVIS 200 bioluminescence imaging system ($\lambda_{\text{exc}} = 450\text{--}490$ nm, $\lambda_{\text{em}} = 575\text{--}650$ nm) at a final resolution of 500 μm . The MALDI image of A2E was collected at 300 μm resolution. Bar = 1 cm. The intensity is coded by the indicated false color scale. The images are oriented: dorsal – top; ventral – bottom; nasal – left; temporal – right.

Table 1
Analogue pigments with salamander opsins

Salamander opsins were expressed in COS-1 cells and pigments generated and purified as described by Das et al. (Das et al., 2004).

Retinal	Red rod (RH1)	Green rod/blue cone (SWS2)	Red cone (M/LWS)	UV cone (SWS1)
11- <i>cis</i> retinal [†]	500 nm	432 nm	560 nm	356 nm
11- <i>cis</i> 9-demethyl retinal [‡]	468 nm (stable meta I)	412 nm	509 nm (stable meta II)	357 nm
11- <i>cis</i> 13-demethyl retinal [‡]	500 nm	432 nm	No pigment	ND

[†]Data from Das et al. (2004);

[‡]unpublished data from Kono and Crouch although a spectrum of 11-*cis* 13-demethyl retinal in bovine RH1 (Nelson et al., 1970) and an action spectrum of salamander rods regenerated with the same analogue have been shown to have the same absorption maximum (Corson et al., 2000).
 ND = not determined.

Table 2
Effects of ligands on human opsin activity

Activity is defined as the effect on the respective opsin's ability to activate transducin in a ligand-dependent manner as previously described (Kono and Crouch, 2011).

Compound	Structure	<i>red rod opsin (RH1)</i>	<i>activity blue cone opsin (SWS1)</i>	<i>red/green cone opsin (M/LSW)</i>
1,3 dimethylcyclohexane		inverse agonist [§]	inverse agonist [§]	inverse agonist [‡]
β-ionone		agonist [§]	agonist [§]	inverse agonist [‡]
9- <i>cis</i> C17 aldehyde		agonist [§]	ND	Inverse agonist [‡]
11- <i>cis</i> retinol		agonist [‡]	inverse agonist [‡]	inverse agonist [‡]
11- <i>cis</i> retinal		inverse agonist [‡]	inverse agonist [‡]	inverse agonist [‡]

[§]Unpublished results;
[‡]Kono and Crouch, 2011;
[‡]Ala-Laurila et al., 2009; ND= not determined.