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Constitutively Active Rhodopsin and Retinal Disease

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Abstract

Rhodopsin is the light receptor in rod photoreceptor cells of the retina that initiates scotopic vision. In the dark, rhodopsin is bound to the chromophore 11-*cis* retinal, which locks the receptor in an inactive state. The maintenance of an inactive rhodopsin in the dark is critical for rod photoreceptor cells to remain highly sensitive. Perturbations by mutation or absence of 11-*cis* retinal can cause rhodopsin to become constitutively active, which leads to the desensitization of photoreceptor cells and, in some instances, retinal degeneration. Constitutive activity can arise in rhodopsin by various mechanisms and can cause a variety of inherited retinal diseases including Leber congenital amaurosis, congenital night blindness, and retinitis pigmentosa. In this review, the molecular and structural properties of different constitutively active forms of rhodopsin are overviewed and the possibility that constitutive activity can arise from different active-state conformations is discussed.

Keywords

Biased agonism; functional selectivity; G protein-coupled receptor; night blindness; phototransduction; protein conformation; receptor activation; retinal degeneration; signal transduction

INTRODUCTION

Rhodopsin is a member of the G protein-coupled receptor (GPCR) family of membrane proteins. Bovine rhodopsin was the first GPCR to have its primary, secondary, and tertiary structures determined (Hargrave et al., 1983; Nathans & Hogness, 1983; Ovchinnikov Yu, 1982; Palczewski et al., 2000; Schertler, Villa, & Henderson, 1993). These studies revealed a structure comprised of 7 transmembrane alpha helices (TM1-TM7) connected by extracellular (EC1-EC3) and cytoplasmic (CP1-CP3) loops and an amphipathic alpha helix (H8) that sits parallel to the membrane surface (Fig. 1). The human gene for rhodopsin was isolated and sequenced in the mid 1980's (Nathans & Hogness, 1984). The rhodopsin gene is a hot spot for inherited mutations causing retinal disease (Mendes, van der Spuy, Chapple, & Cheetham, 2005; Nathans, Merbs, Sung, Weitz, & Wang, 1992; Stojanovic & Hwa, 2002).

Rhodopsin is the light receptor that initiates scotopic vision in rod photoreceptor cells of the retina upon photon capture. The receptor is embedded at a high concentration in disc membranes of rod outer segments (**ROS**) (Fig. 2A). Intense efforts to understand the structure and function of this light receptor have been ongoing for quite some time, especially after the initial discovery that a single point mutation in the rhodopsin gene causes retinitis pigmentosa (**RP**) (Dryja et al., 1990), a retinal degenerative disease. Even with these efforts, the mechanistic description of rhodopsin activity is incomplete. Since the initial discovery, more than 100 point mutations have been discovered in the rhodopsin gene that cause retinal disease (Garriga & Manyosa, 2002; Mendes et al., 2005; Nathans et al., 1992; Stojanovic & Hwa, 2002).

Under normal function, rhodopsin is covalently bound to 11-*cis* retinal and is inactive in the dark (Fig. 2B). Rhodopsin must be activated by light to initiate vision. Constitutive activity in rhodopsin (i.e., receptor activation in the absence of light stimulation) can arise because of mutation or absence of bound 11-*cis* retinal and can cause a range of inherited retinal diseases including Leber congenital amaurosis (**LCA**), congenital night blindness (**CNB**), and RP (Rao, Cohen, & Oprian, 1994; Robinson, Cohen, Zhukovsky, & Oprian, 1992; Sieving et al., 1995; Woodruff et al., 2003). The phenotypes promoted by the different constitutively active forms of rhodopsin that cause these diseases are variable. The reason for this variability is unclear and, therefore, the molecular and structural basis of these diseases must be better understood. In this review, the structural and molecular properties of different constitutively active forms of rhodopsin known to cause disease will be overviewed (Table 1). A discussion is also included about how variable phenotypes can arise from different constitutively active forms of rhodopsin.

RHODOPSIN ACTIVITY

Physiology of Rhodopsin Activity

Photoactivation of rhodopsin results in the recruitment and activation of the heterotrimeric G protein transducin (Fig. 2B), which triggers a set of biochemical reactions called phototransduction that culminate in the closure of ion channels leading to the hyperpolarization of the photoreceptor cell and a reduction in intracellular Ca^{2+} concentrations (reviewed in (Arshavsky, Lamb, & Pugh, 2002; Burns & Arshavsky, 2005; Burns & Baylor, 2001; Ridge, Abdulaev, Sousa, & Palczewski, 2003; Yau & Hardie, 2009)). Rhodopsin is comprised of the apoprotein opsin covalently bound to the chromophore 11-*cis* retinal via a protonated Schiff base linkage at Lys296 in TM7. When bound to 11-*cis* retinal, rhodopsin exhibits maximal absorbance of light (λ_{max}) at about 500 nm (Wald & Brown, 1953). Photon capture by rhodopsin results in the isomerization of 11-*cis* retinal to all-*trans* retinal, which triggers a series of structural changes in the receptor (Ye et al., 2010). The result of these changes is a sequence of spectrally distinct intermediate states that eventually culminate in the formation of the active metarhodopsin II (**MII**) state (reviewed in (Ernst et al., 2014; Kandori, Shichida, & Yoshizawa, 2001; Okada, Ernst, Palczewski, & Hofmann, 2001; Ritter, Elgeti, & Bartl, 2008; Shichida & Imai, 1998; Wald, 1968)). Crystal structures for many of the photointermediates of rhodopsin are now available, which provide insights about the sequence of structural changes accompanying

rhodopsin activation (Choe et al., 2011; Nakamichi & Okada, 2006a, 2006b; Ruprecht, Mielke, Vogel, Villa, & Schertler, 2004; Salom et al., 2006).

The MII state activates transducin by promoting the exchange of GDP for GTP (Fig. 2B), thereby initiating phototransduction (Emeis, Kuhn, Reichert, & Hofmann, 1982; Kibelbek, Mitchell, Beach, & Litman, 1991). The decay of the MII state of rhodopsin is accompanied by the release of all-*trans* retinal from the chromophore-binding pocket, which leaves the receptor in the apoprotein opsin form. A set of enzymatic reactions called the retinoid or visual cycle regenerates 11-*cis* retinal from all-*trans* retinal (reviewed in (Kiser, Golczak, Maeda, & Palczewski, 2011; Saari, 2012; Tang, Kono, Koutalos, Ablonczy, & Crouch, 2013; Travis, Golczak, Moise, & Palczewski, 2007)). Opsin must reconstitute with 11-*cis* retinal to form rhodopsin and once again be ready to capture a photon to initiate phototransduction.

Several events occur upon photoactivation of rhodopsin in addition to events required to hyperpolarize photoreceptor cells. Signaling must be terminated, which is achieved, in part, by a competing set of events that deactivate rhodopsin (Fig. 2B). These events include mono-, di- and tri-phosphorylation of the receptor by rhodopsin kinase and binding of arrestin to the cytoplasmic surface of the receptor (Bennett & Sitaramayya, 1988; Kennedy et al., 2001; McDowell, Nawrocki, & Hargrave, 1993; Mendez et al., 2000; Ohguro, Johnson, Ericsson, Walsh, & Palczewski, 1994; Papac, Oatis, Crouch, & Knapp, 1993; Thompson & Findlay, 1984). Phosphorylation of light-activated rhodopsin at multiple residues is required for arrestin binding (Vishnivetskiy et al., 2007). Photoactivation of rhodopsin triggers translocation of transducin and arrestin between the ROS and rod inner segments (**RIS**)/perinuclear region of photoreceptor cells (Fig. 2A) (Elias, Sezate, Cao, & McGinnis, 2004; Mendez, Lem, Simon, & Chen, 2003; Slepak & Hurley, 2008; Sokolov et al., 2002; Zhang et al., 2003), which acts as a light adaptation mechanism for these cells (Calvert, Strissel, Schiesser, Pugh, & Arshavsky, 2006).

Rod photoreceptor cells are exquisitely sensitive and can generate a response upon activation of a single rhodopsin molecule by a single photon (Baylor, Lamb, & Yau, 1979; Hecht, Schlaer, & Pirenne, 1942). Rhodopsin contributes to the sensitivity of photoreceptor cells and facilitates a single photon response by maintaining an inactive state in the dark and by promoting a highly efficient isomerization of 11-*cis* retinal to all-*trans* retinal, which occurs with a quantum yield of 0.67 (Dartnall, 1968). This efficient isomerization is a direct result of the protein environment rhodopsin provides for the chromophore (Becker & Freedman, 1985). The single photon response is also possible, in part, because of the large signal amplification occurring in subsequent stages of phototransduction (Baylor, 1996; Stryer, 1991).

Activation of even a small number of rhodopsin molecules by low levels of background light can desensitize photoreceptor cells (Baylor, Matthews, & Nunn, 1984). Thus, it is critical for rhodopsin to remain inactive in its dark state for maximal sensitivity. Despite the engineering of rhodopsin to allow maximal sensitivity of photoreceptor cells, spontaneous activation of rhodopsin is observed on rare occasions in complete darkness, which results in a photoreceptor cell response equivalent to that promoted by a single photon (Yau,

Matthews, & Baylor, 1979). This spontaneous activity results in rod dark noise and sets the sensitivity threshold for the detection of light (Aho, Donner, Hyden, Larsen, & Reuter, 1988). Molecular switches have been engineered into the structure of rhodopsin to lock the receptor in an inactive state and minimize spontaneous activation that can reduce the sensitivity of photoreceptor cells.

Molecular Switches that Lock Rhodopsin in an Inactive State

When bound to 11-*cis* retinal, several molecular switches in the rhodopsin structure are locked in place to keep the receptor in an inactive state (Figs. 1A and 3) (reviewed in (Ahuja & Smith, 2009; Hofmann et al., 2009; Nygaard, Frimurer, Holst, Rosenkilde, & Schwartz, 2009; Trzaskowski et al., 2012)). These switches are observed in bovine rhodopsin crystal structures and involve both interactions between amino acid residue side chains and amino acid residue side chains with water molecules (Angel, Chance, & Palczewski, 2009; Okada et al., 2002; Pardo, Deupi, Dolker, Lopez-Rodriguez, & Campillo, 2007). There are several molecular switches in the vicinity of the chromophore that help maintain the inactive state of the receptor. A hydrogen bond network formed by Glu122 and Trp126 in TM3 and His211 in TM5 surrounds the β -ionone ring of 11-*cis* retinal. This hydrogen bond network forms a constraint between TM3 and TM5. The β -ionone ring of 11-*cis* retinal is in direct contact with Trp265, which along with Pro267 and Ala269 form a molecular switch that includes residues from the conserved CWxP motif in TM6. This CWxP motif molecular switch is proposed to function as a rotamer toggle switch (Crocker et al., 2006; L. Shi et al., 2002).

Also in the vicinity of the chromophore is a critical ionic lock formed by ionic interactions between the protonated Schiff base at Lys 296 and Glu113 in TM3 (Fig. 3B) (Sakmar, Franke, & Khorana, 1989; Zhukovsky & Oprian, 1989). This ionic lock forms a constraint between TM7 and TM3. Upon attaining the metarhodopsin I (**MI**) state, an inactive precursor to the MII state, Glu181 in EC2 becomes the predominant counterion to the protonated Schiff base (Ludeke et al., 2005; Martinez-Mayorga, Pitman, Grossfield, Feller, & Brown, 2006; Yan et al., 2003), thereby releasing the TM3-TM7 constraint. Once the receptor attains the MII state, the Schiff base is deprotonated and the charge of Glu113 is neutralized by uptake of a proton (Arnis & Hofmann, 1993; Jager, Fahmy, Sakmar, & Siebert, 1994; Matthews, Hubbard, Brown, & Wald, 1963). Both Glu113 and Glu181 are part of a hydrogen bond network near the vicinity of the protonated Schiff base that also includes residues from EC2 and water molecules (Li, Edwards, Burghammer, Villa, & Schertler, 2004; Okada et al., 2002).

A second ionic lock involves the D(E)RY motif, a highly conserved motif among GPCRs (Mirzadegan, Benko, Filipek, & Palczewski, 2003). This ionic lock forms a constraint between TM3 and TM6 and is comprised of ionic interactions between Glu134 and Arg135 in TM 3 and a hydrogen bond network between Arg135 in TM3 and Glu247 and Thr251 in TM6 (Choe et al., 2011; Palczewski et al., 2000). Activation of the receptor results in the disruption of these molecular interactions and uptake of a proton by Glu134 (Arnis, Fahmy, Hofmann, & Sakmar, 1994; Fahmy, Sakmar, & Siebert, 2000). Release of constraints in the D(E)RY motif molecular switch can be decoupled from the release of constraints in the

protonated Schiff base molecular switch under certain conditions (Mahalingam, Martinez-Mayorga, Brown, & Vogel, 2008).

Another conserved motif among GPCRs that plays a role in locking the receptor in an inactive state is the NPxxY motif (Fritze et al., 2003; Mirzadegan et al., 2003). Residues in the molecular switch involving the NPxxY motif form constraints between TM7 and H8 or TM7, TM1, and TM2. The TM7-H8 constraint is mediated by the aromatic side chains of Tyr306 on TM7 and Phe313 on H8. The TM1-TM2-TM7 constraint is mediated by a hydrogen bond network formed by Asn55 on TM1, Asp83 on TM2, and Asn302, Ala299, and Ser298 (Ala298 in the human sequence) on TM7.

The D(E)RY and NPxxY motifs are found in the cytoplasmic region of rhodopsin (Fig. 3A). The molecular switch harboring the D(E)RY motif is decoupled, in terms of molecular interactions, from the chromophore-binding pocket. This decoupling is due to a hydrophobic barrier formed by Leu76 and Leu79 in TM2, Leu128 and Leu131 in TM3, and Met253 and Met257 in TM6, which separates this cytoplasmic molecular switch from the other molecular switches that are coupled to the chromophore-binding pocket (Li et al., 2004; Standfuss et al., 2011). Isomerization of 11-*cis* retinal releases constraints present in molecular switches coupled to the chromophore-binding pocket and rearranges the hydrogen bond network in a manner that couples the D(E)RY motif to the chromophore-binding pocket via residues in the NPxxY motif molecular switch (Choe et al., 2011; Standfuss et al., 2011). The result is an extended hydrogen bond network that spans from the chromophore-binding pocket to transducin bound on the cytoplasmic surface of rhodopsin. The major conformational changes in rhodopsin arising from the release of molecular switch constraints include an outward tilting and rotation of the cytoplasmic portion of TM6 and the elongation of TM5 (Fig. 1B) (Choe et al., 2011).

CONSTITUTIVE ACTIVITY IN RHODOPSIN THAT CAUSES DISEASE

Leber Congenital Amaurosis and Vitamin A Deficiency

LCA and vitamin A deficiency eliminate or reduce the pool of 11-*cis* retinal in the retina, thereby resulting in the presence of the apoprotein opsin rather than rhodopsin in ROS membranes. LCA is a heterogeneous group of inherited diseases that results in early vision loss (reviewed in (den Hollander, Roepman, Koenekoop, & Cremers, 2008)). LCA is named after Theodor Leber, who made the first description of the disease (Leber, 1869). Among genes with mutations causing LCA include *Lrat* and *Rpe65* (Gu et al., 1997; Marlhens et al., 1997; Thompson et al., 2001), which code for critical retinoid cycle enzymes lecithin-retinol acyltransferase (**LRAT**) and retinal pigment epithelium-specific 65 kDa protein (**RPE65**), respectively. LCA caused by defects in these genes is inherited in an autosomal recessive manner. Defects in LRAT and RPE65 appear to cause LCA by a common mechanism (Fan, Rohrer, Frederick, Baehr, & Crouch, 2008). In the absence of either enzyme, 11-*cis* retinal cannot be regenerated, which results in the presence of only the apoprotein opsin in ROS membranes and non-functional rod photoreceptor cells accompanied by a slowly progressing retinal degeneration (Batten et al., 2004; Redmond et al., 1998).

Vitamin A deficiency is a cause of night blindness due to diet (Hecht & Mandelbaum, 1938, 1940; Wald, Jeghers, & Arminio, 1938; Wald & Steven, 1939). Since vitamin A is a precursor to 11-*cis* retinal (Wald, 1968), deficiency of vitamin A in the diet can reduce the levels of 11-*cis* retinal available to form rhodopsin. Decreased levels of vitamin A in the diet result in increased levels of opsin in the retina, which causes decreased sensitivity of rod photoreceptor cells and eventual night blindness and retinal degeneration (Dowling & Wald, 1958, 1960). The retinal degeneration caused by vitamin A deficiency progresses much more rapidly than that promoted by a defect in RPE65 (Hu et al., 2011).

The increased levels of chromophore-free opsin generated in both vitamin A deficiency and LCA caused by defects in LRAT or RPE65 can be detrimental to photoreceptor cells. Opsin exhibits constitutive activity that is sufficient to initiate signaling in photoreceptor cells (Cornwall & Fain, 1994; Fan, Woodruff, Cilluffo, Crouch, & Fain, 2005). Since spontaneous activation of rhodopsin decreases the sensitivity of photoreceptor cells (Aho et al., 1988; Baylor, Matthews et al., 1984), constitutively active opsin will desensitize photoreceptor cells. Also, the constitutive activity of opsin can cause retinal degeneration (Woodruff et al., 2003). Thus, the desensitization and death of photoreceptor cells observed in conditions that eliminate or decrease the levels of 11-*cis* retinal in the retina can be a direct consequence of constitutive activity in the apoprotein opsin.

Opsin – Active Apoprotein—The efficiency of opsin in initiating phototransduction is only 10^{-6} – 10^{-5} times that of light-activated rhodopsin (Fan et al., 2005; Melia, Cowan, Angleson, & Wensel, 1997). Thus, the constitutive activity of opsin is very low and is often undetectable in *in vitro* assays at neutral pH that monitor the activation of transducin by opsin (e.g. (Rao et al., 1994)). The low level of constitutive activity in opsin, however, is sufficient to promote a response in photoreceptor cells (Cornwall & Fain, 1994; Fan et al., 2005). Moreover, the constitutive activity of opsin in photoreceptor cells triggers some of the signal termination mechanisms displayed by light activation of rhodopsin, with some differences.

Constitutive activity in opsin results in monophosphorylation of up to 20% of the receptor in photoreceptor cells by rhodopsin kinase (Fan et al., 2010). This pattern of phosphorylation contrasts with phosphorylation promoted by light activation of rhodopsin, which results in the phosphorylation of multiple residues in the receptor (Kennedy et al., 2001; McDowell et al., 1993; Mendez et al., 2000; Ohguro et al., 1994; Papac et al., 1993; Thompson & Findlay, 1984). Monophosphorylation of opsin likely is not sufficient to promote binding with arrestin (Vishnivetskiy et al., 2007), however, the constitutive activity of opsin does trigger the translocation of arrestin into the ROS (Mendez et al., 2003). In contrast to light-activated rhodopsin, constitutively active opsin does not trigger the translocation of transducin from the ROS to the RIS/perinuclear region (Mendez et al., 2003).

In the dark, rhodopsin is locked into an inactive state because of the presence of 11-*cis* retinal in the chromophore-binding pocket. Since opsin is free of chromophore, the structure is less constrained and can form multiple conformational substates in ROS membranes (Kawamura et al., 2013). It is unclear whether or not the constitutive activity in opsin originates from an active state conformation that is similar to that of the MII state generated

by light-activation of rhodopsin. Under acidic conditions or in crystals formed by detergent-solubilized receptor, opsin can achieve a conformation similar to that of the active MII state (Park, Scheerer, Hofmann, Choe, & Ernst, 2008; Scheerer et al., 2008; Vogel & Siebert, 2001). Detergent-solubilized opsin in crystals, however, may achieve the MII state because of a bound detergent molecule occupying the chromophore-binding pocket (Park et al., 2013). Moreover, under physiological conditions at neutral pH and in a lipid bilayer, opsin does not form a MII-like active state (Tsukamoto & Farrens, 2013; Vogel & Siebert, 2001). Thus, it is ambiguous as to whether a low photoreceptor response occurs because opsin forms an active state different from the MII state with lower activity or is a result of a minor population of opsin molecules achieving a MII-like active state.

Congenital Night Blindness

CNB is a vision disorder affecting scotopic vision, mediated by rod photoreceptor cells, without impairing photopic vision, mediated by cone photoreceptor cells (Dryja, 2000; Lem & Fain, 2004). CNB can be caused by inherited defects in several different genes and the inheritance patterns can differ depending on the causative gene. Mutation in the rhodopsin gene was the first to be identified as a cause of CNB (Dryja, 2000). Four different point mutations in rhodopsin have been identified that cause autosomal dominant CNB (Table 1): G90D (Sieving et al., 1995), T94I (al-Jandal et al., 1999), A292E (Dryja, Berson, Rao, & Oprian, 1993), and A295V (Zeitz et al., 2008). Patients that have these mutations in rhodopsin share common clinical features. Night blindness in these patients occurs with an early onset and the condition is generally non-progressive. Significant retinal degeneration is not observed in patients with these mutations.

The rhodopsin mutants causing CNB are properly folded and can bind 11-*cis* retinal. Each of the identified mutations has been shown to cause constitutive activity in the mutant receptor, which is thought to underlie the pathogenesis of the disease. Two of the mutations occur in TM2 (G90D and T94I) and the other two mutations occur in TM7 (A292E and A295V) (Figs. 1 and 3). Despite being present in different transmembrane helices, each of the affected amino acid residues is found near the chromophore-binding pocket in close proximity to the Schiff base linkage between the side chain of Lys296 and 11-*cis* retinal (Fig. 3).

G90D – Active Dark State—The G90D rhodopsin mutant is the most extensively studied of the rhodopsin mutants causing CNB. The properties of this mutant share several similarities with those of the other mutants causing CNB. The G90D mutation in rhodopsin leads to complete night blindness in patients from early childhood and is inherited in an autosomal dominant manner (Sieving et al., 1995). Night blindness results from desensitization of rod photoreceptor cells and is not accompanied by significant retinal degeneration, as is observed in RP. Patients experience a loss of sensitivity of rod photoreceptor cells that is analogous to desensitization occurring due to a low level of background light (Baylor, Nunn, & Schnapf, 1984). This desensitization of rod photoreceptor cells is a result of constitutive activity promoted by the G90D mutation in rhodopsin (Rao et al., 1994; Sieving et al., 1995).

The G90D mutation does not affect the proper folding and transport of rhodopsin to ROS (Naash et al., 2004; Sieving et al., 2001). The mutant apoprotein can bind 11-*cis* retinal (Kawamura, Colozo, Ge, Muller, & Park, 2012; Sieving et al., 2001), albeit more slowly compared to the wild-type apoprotein (Gross, Xie, & Oprian, 2003; Toledo et al., 2011). The primary structural impact of replacing a Gly residue with the charged Asp residue appears to be a perturbation in the chromophore-binding pocket (Singhal et al., 2013). An altered chromophore-binding pocket is suggested by a blue-shifted λ_{max} displayed by the mutant and a solvent accessible chromophore-binding pocket in the dark state of G90D rhodopsin (Kaushal & Khorana, 1994; Kawamura et al., 2012; Rao et al., 1994; Zvyaga, Fahmy, Siebert, & Sakmar, 1996).

The spectral properties of 11-*cis* retinal are sensitive to the surrounding protein environment (Sakmar et al., 1989; Zhukovsky & Oprian, 1989). The blue-shifted λ_{max} promoted by the G90D mutation is typically attributed to the replacement of Glu113 by Asp90 as the counterion for the protonated Schiff base at Lys296 (Jager et al., 1997; Rao et al., 1994). The replacement of Glu113 by Asp90 as the counterion disrupts constraints normally imposed by the protonated Schiff base molecular switch, thereby promoting the activation of the receptor (Singhal et al., 2013). The functional effect of disrupting this molecular switch can readily be observed in *in vitro* studies where the opsin form of the G90D mutant can activate higher levels of transducin than wild-type opsin (Rao et al., 1994; Toledo et al., 2011). This difference in transducin activation may not be relevant *in vivo* where the binding of arrestin may negate the higher levels of activity of the mutant opsin (Dizhoor et al., 2008).

Solvents are normally excluded from the chromophore-binding pocket of rhodopsin in the dark state but gain access upon light-activation of the wild-type receptor (Leioatts et al., 2014; Wald & Brown, 1953). Thus, the solvent accessibility of the chromophore-binding pocket in the dark state of the G90D mutant suggests that an active state is attained even when the mutant is bound to 11-*cis* retinal. Several observations from *in vitro* studies support the notion that the chromophore-bound dark state of the G90D mutant can be constitutively active. The dark state of G90D rhodopsin from heterologous expression systems exhibits some of the structural hallmarks of the active MII state, such as neutralization of Glu113 and movement of the cytoplasmic half of TM6 (Fahmy, Zvyaga, Sakmar, & Siebert, 1996; Kim et al., 2004; Zvyaga et al., 1996). Dark-state G90D rhodopsin embedded in native ROS membranes from transgenic mice also display characteristics expected for an active state (Kawamura et al., 2012). The constitutive activity in the dark-state mutant does not appear to be a result of thermal isomerization of bound 11-*cis* retinal (Dizhoor et al., 2008), but instead, likely related to the replacement of Glu113 by the mutant Asp residue as the counterion for the protonated Schiff base at Lys296 (Singhal et al., 2013).

Currently, there are divergent views on whether the constitutive activity originating from the chromophore-free opsin or dark-state rhodopsin bound to chromophore underlies the pathogenesis of CNB. The origin of constitutive activity has significant implications on the type of therapeutics possible to combat the disease (Jin, Cornwall, & Oprian, 2003). Electrophysiology studies on a *Xenopus laevis* model expressing low levels of the G90D mutant point to a scenario where the constitutive activity of the apoprotein opsin causes

CNB (Jin et al., 2003). This *Xenopus laevis* model exhibits desensitized rod photoreceptor cells that can be resensitized by the addition of exogenous 11-*cis* retinal. These results are consistent with the notion that the constitutive activity of the apoprotein opsin form of the mutant desensitizes photoreceptor cells and that the binding of exogenously added 11-*cis* retinal to the opsin mutant can lock the receptor into an inactive state thereby reversing the detrimental effects. These results, however, are inconsistent with observations in patients with CNB caused by the G90D rhodopsin mutation where reversal of desensitization in rod photoreceptor cells does not occur even after 12 h of dark adaption, a timeframe in which regeneration of rhodopsin by 11-*cis* retinal would be complete.

Observations in the *Xenopus laevis* model also contrast with those made in a transgenic mouse model expressing G90D rhodopsin (Dizhoor et al., 2008; Sieving et al., 2001). These mice display effects that more closely resemble those in patients harboring the G90D mutation in rhodopsin. The mutant rhodopsin desensitizes rod photoreceptor cells in the dark and the desensitization cannot be reversed by supplementing cells with exogenous 11-*cis* retinal (Dizhoor et al., 2008). These results suggest that G90D rhodopsin is already bound to 11-*cis* retinal and that it is the constitutive activity of the dark state that underlies the desensitization of photoreceptor cells. While it appears that the constitutive activity of the chromophore-bound dark state of G90D rhodopsin is sufficient to desensitize rod photoreceptor cells, a possible role for the chromophore-free opsin form of the mutant in CNB cannot be ruled out.

T94I, A292E, A295V – Active Dark State—The other rhodopsin mutants causing CNB have not been studied as extensively as the G90D mutant. Similarities in phenotype promoted by the different mutants may indicate that common mechanisms underlie the pathogenesis of the disease. The chromophore-free opsin form of all mutants exhibit increased activity, as assessed by transducin activation, compared to that of wild-type opsin under *in vitro* conditions (Dryja et al., 1993; Gross, Rao, & Oprian, 2003; Rao et al., 1994; Zeitz et al., 2008). The level of constitutive activity exhibited by the opsin mutants is different and occurs in the following order: A292E > G90D $\approx$ A295V > T94I (Gross, Rao et al., 2003; Zeitz et al., 2008). The level of constitutive activity of some mutants is correlated to the level of phosphorylation by rhodopsin kinase (Rim & Oprian, 1995). It must be noted again that the increased constitutive activity observed for mutant opsins *in vitro* may not be relevant *in vivo* where arrestin binding can counteract the increased activity of chromophore-free opsin to maintain similar levels of activity as wild-type opsin (Dizhoor et al., 2008). The increased level of constitutive activity of mutant opsins compared to that of wild-type opsin does indicate, however, that the mutations can promote an active state of the receptor.

Similar to the G90D mutation, the T94I, A292E, and A295V mutations may cause constitutive activity by releasing the constraint formed by the ionic interaction between Glu113 and protonated Schiff base at Lys 296 (Singhal et al., 2013). The T94I and A295V mutants, like the G90D mutant, exhibit a blue-shifted λ_{max} (Gross, Rao et al., 2003; Ramon, del Valle, & Garriga, 2003; Zeitz et al., 2008), which is indicative of altered electrostatics of the protonated Schiff base at Lys296 resulting from a disrupted ionic interaction between Glu113 and Lys296 (Jager et al., 1997). Since the T94I and A295V mutations result in

hydrophobic mutated residues, the Glu113-Lys296 constraint may be disrupted in an indirect manner and cause changes to the electrostatic environment of the protonated Schiff base or other regions of contact with the chromophore. Surprisingly, the A292E mutant exhibits a $\lambda_{\max}$ that is similar to that of the wild-type receptor (Dryja et al., 1993; Gross, Rao et al., 2003). The substitution in the A292E mutant results in a charged Glu292 residue that is predicted to replace Glu113 as the counterion for the protonated Schiff base at Lys296 in a similar manner as Asp90 in the G90D mutant (Kim et al., 2004). The absence of change in the $\lambda_{\max}$ may indicate that the replacement of Glu113 with Glu292 as the counterion does not significantly alter the electrostatic environment of the protonated Schiff base at Lys296.

The T94I and A292E mutants, like the G90D mutant, exhibit effects in the dark state that are characteristic of the light-activated wild-type receptor such as conformational changes and solvent accessibility of the chromophore-binding pocket (Kim et al., 2004; Ramon et al., 2003). Thus, constitutive activity in the dark state of all mutants may underlie the pathology in CNB. The mutants discussed also introduce changes that may be unrelated to the pathogenesis of the disease, such as changes in the MII decay rate and stability of the protein molecule (Table 1).

Retinitis Pigmentosa

By far, the largest share of mutations detected in the rhodopsin gene cause RP, the most common inherited retinal degenerative disease (Berson, 1993; Hartong, Berson, & Dryja, 2006; Shintani, Shechtman, & Gurwood, 2009). Mutations in the rhodopsin gene account for about 15% of all retinal degenerative diseases and are by far the largest cause of autosomal dominant RP (Dalke & Graw, 2005; Hartong et al., 2006). The receptor defects caused by different mutations in rhodopsin are variable and can be broadly classified as those causing receptor misfolding, mistrafficking, and constitutive activity (Malanson & Lem, 2009; Mendes et al., 2005). Regardless of the receptor defect promoted by mutation, the end result is the death of photoreceptor cells. Rhodopsin mutants that are constitutively active and cause RP differ from those that cause CNB since they result in photoreceptor cell death. The mechanism by which constitutive activity arises in these mutants and causes photoreceptor cell death can differ depending on the specific mutation introduced. At least 3 different mechanisms by which constitutive activity can arise in rhodopsin because of mutation and cause retinal degeneration are discussed.

S186W and D190N – Thermal Activation—Thermal activation of rhodopsin occurs in rare instances and sets the threshold for the sensitivity to light (Aho et al., 1988). In these cases, thermal energy rather than the energy from light drives the isomerization of 11-*cis* retinal to activate rhodopsin (Gozem, Schapiro, Ferre, & Olivucci, 2012; Luo, Yue, Ala-Laurila, & Yau, 2011). The S186W and D190N mutations in rhodopsin cause autosomal dominant RP, (Matias-Florentino, Ayala-Ramirez, Graue-Wiechers, & Zenteno, 2009; Ruther et al., 1995; Tsui, Chou, Palmer, Lin, & Tsang, 2008). Both these mutants can exhibit activity in the absence of light because of increased rates of thermal activation of the receptor (Liu et al., 2013). Patients with the S186W mutation have a more severe phenotype and earlier onset compared to patients with the D190N mutation.

Ser186 and Asp190 are found on EC2 and are in close proximity to the chromophore-binding pocket (Figs. 1 and 3). Both S186W and D190N mutants can fold properly, bind 11-*cis* retinal, and exhibit spectral properties indistinguishable from wild-type rhodopsin (Janz & Farrens, 2003; Liu et al., 2013). Patients and knockin mice that express the D190N mutant have desensitized rod photoreceptor cells (Sancho-Pelluz et al., 2012), an expected outcome for cells expressing a constitutively active mutant. In contrast to constitutively active mutants causing CNB, the D190N mutant does not exhibit solvent accessibility in its chromophore-binding pocket in the dark state (Janz & Farrens, 2003), which indicates that the mutation itself does not promote an active state via changes to protein structure like in CNB-causing mutants.

The thermal stability of the dark state of rhodopsin is often investigated by monitoring the decay of absorbance at 500 nm, the $\lambda_{\max}$ of the dark-state receptor, at elevated temperatures. This thermal decay of absorbance at 500 nm derives from two sources: thermal isomerization of bound 11-*cis* retinal and hydrolysis of the Schiff base linking the chromophore to Lys296 (Liu, Liu, Fu, Zhu, & Yan, 2011). Both the S186W and D190N mutants display increased rates of thermal isomerization of 11-*cis* retinal compared to that displayed by wild-type rhodopsin (Janz & Farrens, 2003; Liu et al., 2013). The thermal isomerization rate for the S186W mutant is higher than that for the D190N mutant, which may be the reason for the more severe phenotype observed in patients with the S186W mutation (Liu et al., 2013).

Thermal fluctuations of the protein structure forming the chromophore-binding pocket can contribute to the thermal isomerization of 11-*cis* retinal in rhodopsin (Lorenz-Fonfria, Furutani, Ota, Ido, & Kandori, 2010). The S186W and D190N mutations may reduce constraints in the chromophore-binding pocket by disrupting the hydrogen bond network involving residues in EC2 and Glu113 (Li et al., 2004; Okada et al., 2002). The disruption of this hydrogen bond network may increase the level of thermal fluctuations in the chromophore-binding pocket to effectively lower the energetic barrier for isomerization of 11-*cis* retinal to activate the receptor.

G90V – Active Dark State and Thermal Activation

Mutations at Gly90 in TM2 present a unique situation where substitution with different amino acid residues results in different diseases. As discussed earlier, a substitution of Gly90 with the charged Asp residue results in a constitutively active receptor causing CNB, which is not accompanied by retinal degeneration. In contrast, a substitution of Gly90 with the hydrophobic Val residue also results in a constitutively active receptor but causes autosomal dominant RP (Neidhardt, Barthelmes, Farahmand, Fleischhauer, & Berger, 2006), which results in retinal degeneration.

Several common effects are observed regardless of the amino acid substitution at Gly90 (Table 1) (Toledo et al., 2011). The G90V mutant like the G90D mutant exhibits a blue-shifted $\lambda_{\max}$, reconstitutes with 11-*cis* retinal slowly, and exhibits solvent accessibility in the chromophore-binding pocket of the dark state (Toledo et al., 2011). Moreover, the apoprotein opsin form of the mutant exhibits constitutive activity at similar levels to that of G90D opsin. These similarities with the G90D mutant suggest that the dark state of G90V

rhodopsin also achieves an active state, perhaps by disrupting the ionic interaction between Glu113 and the protonated Schiff base at Lys 296 in an indirect manner.

With the similarities exhibited by G90V and G90D rhodopsin, why then do the two mutants cause different diseases? The only significant difference between G90V and G90D rhodopsin is observed in the thermal stability of the dark state of each mutant as assessed by monitoring the decay of the λ_{max} . G90V undergoes rapid thermal bleaching at a temperature where G90D experiences minimal thermal bleaching (Toledo et al., 2011). Thus, it appears that under physiological conditions, G90V may experience increased levels of thermal activation in the dark whereas G90D experiences very little. Constitutive activity in G90V rhodopsin may arise from both an active dark state and thermal activation, ultimately causing retinal degeneration.

K296E – Active Apoprotein and Stable Arrestin Interactions

Mutations at Lys296 prevent the covalent linkage between 11-*cis* retinal and the receptor. Thus, these mutants exist as the apoprotein opsin. Two mutations at this amino acid position have been detected in patients with autosomal dominant RP, K296E and K296M (Keen et al., 1991; Sullivan, Scott, Falls, Richards, & Sieving, 1993; Vaithinathan, Berson, & Dryja, 1994). Patients with the K296E mutation in rhodopsin exhibit a severe retinal degeneration with rapid onset (Keen et al., 1991). The *in vitro* properties of the K296M mutant are similar to those of the K296E mutant (Rim & Oprian, 1995; Yang, Snider, & Oprian, 1997). Thus, both of these mutants may cause disease by similar mechanisms. The K296E mutant has been studied more extensively and, therefore, the discussion here will be centered on this mutant.

Since the K296E mutant is unable to bind 11-*cis* retinal, constitutive activity is expected since the mutant is in the opsin form. As expected, the K296E mutant activates transducin in the absence of light (Chen, Shi, Concepcion, Xie, & Oprian, 2006; Li, Franson, Gordon, Berson, & Dryja, 1995; Moaven et al., 2013; Robinson et al., 1992; Yang et al., 1997). The constitutive activity in K296E opsin and the mechanism by which it causes retinal degeneration, however, are different from that of wild-type opsin. The constitutive activity in K296E opsin is higher than that in wild-type opsin (Robinson et al., 1992). The higher level of activity in K296E opsin may lead to the observed constitutive phosphorylation of the receptor at multiple sites, which in turn promotes a tight association between the mutant and arrestin (Chen et al., 2006; Li et al., 1995; Rim & Oprian, 1995).

The constitutive activity in K296E does not directly cause retinal degeneration as it does for wild-type opsin. Rather, it is the effects related to the tight association between the K296E mutant and arrestin that underlies retinal degeneration. In photoreceptor cells, the tight association of K296E with arrestin quenches signaling and, therefore, photoreceptor cells are not desensitized and degeneration occurs in a transducin-independent manner (Chen et al., 2006; Li et al., 1995; Moaven et al., 2013). Interestingly, in the absence of arrestin, K296E opsin behaves similarly as wild-type opsin by causing retinal degeneration in a transducin-dependent manner (Chen et al., 2006). A consequence of a stable K296E mutant-arrestin complex in photoreceptor cells is the recruitment of the endocytic adapter protein AP-2, which results in effects leading to photoreceptor cell death (Moaven et al., 2013).

HOW CONSTITUTIVE ACTIVITY CAN CAUSE DIFFERENT PHENOTYPES

Different Levels of Activity as an Underlying Cause of Different Phenotypes

All classes of constitutively active mutants discussed are able to activate the phototransduction cascade in the absence of light and thereby desensitize rod photoreceptor cells (Chen et al., 2006; Sancho-Pelluz et al., 2012; Sieving et al., 1995). While this light-independent activity underlies the observed pathology in a variety of retinal diseases, different constitutively active mutants can promote different physiological outcomes. Some constitutively active mutants can cause night blindness with minimal retinal degeneration while others cause retinal degeneration with varying severity that eventually leads to complete blindness. While apparently disparate, the different phenotypes have been proposed to lie on the same spectrum only differing in severity that is dependent on the level of constitutive activity promoted by the mutation (Lem & Fain, 2004; Malik et al., 2013). Thus, mutations leading to the relatively mild phenotype of CNB would promote less receptor activity compared to those mutations leading to the more severe phenotypes observed in RP. The level of receptor activity as an explanation for variable phenotypes may be too simplistic, however, and the possibility that the receptor adopts different active-state conformations must be additionally considered.

Do All Constitutively Active Mutants Adopt the Same Active-State Conformation?

In the classical view of GPCR signaling, the receptor exists in an equilibrium between two states (Leff, 1995): an inactive state (**R**) and an active state (**R***) (Fig. 4A). In the dark, rhodopsin is present exclusively in the **R** state. Light activation of rhodopsin shifts the equilibrium towards the **R*** state, which is equivalent to the MII state. Within this framework, constitutive activity occurs when the equilibrium is shifted in a manner that causes an appreciable population of **R*** to be present under basal conditions in the absence of light (Samama, Cotecchia, Costa, & Lefkowitz, 1993; Spalding, Burstein, Wells, & Brann, 1997). The level of activity in a constitutively active mutant will then be solely determined by the number of receptors adopting the **R*** state. Thus, constitutively active mutants of rhodopsin with higher activity will shift the equilibrium towards the **R*** state to a greater extent than mutants with lower levels of activity. In this linear view, the number of receptors adopting the active **R*** state will also dictate the level of downstream events such as transducin activation, phosphorylation by rhodopsin kinase, and arrestin binding (Fig. 2B). Any variations observed in phenotype must then be a direct consequence of the number of constitutively active mutants adopting the **R*** state under basal conditions.

The classical view is restrictive in that the active states of light-activated rhodopsin and the different constitutively active mutants must be equivalent. Recent evidence suggests that a single GPCR can adopt multiple active states and that different active states can differentially interact with downstream signaling proteins (Figs. 4B and 4C), thereby promoting distinct cellular responses (reviewed in (Galandrin, Oligny-Longpre, & Bouvier, 2007; Kenakin, 2007; Kobilka & Deupi, 2007; Park, 2012; Perez & Karnik, 2005; Rajagopal, Rajagopal, & Lefkowitz, 2010; Seifert, 2013; Urban et al., 2007)). The conformations of different active states of a GPCR are beginning to be characterized structurally (Kim et al., 2013; J. J. Liu, Horst, Katritch, Stevens, & Wuthrich, 2012; Wacker

et al., 2013). It is unknown whether the distinct active states arise sequentially or non-sequentially from an inactive receptor (Figs. 4B and 4C). Rhodopsin, similarly to other GPCRs, also has the ability to adopt multiple active states (reviewed in (Park, 2012)), some of which may become favored because of mutation. At least two distinct active states have been detected for rhodopsin that form in a sequential manner (Knierim, Hofmann, Ernst, & Hubbell, 2007; Mahalingam et al., 2008).

A central question then becomes whether mutations causing constitutive activity in rhodopsin promote the same or different active-state conformation as that attained upon light activation of the wild-type receptor, the MII state. Electrophysiology studies on transgenic mice expressing G90D rhodopsin suggest that this constitutively active mutant adopts a different active-state conformation compared to the MII state of light-activated rhodopsin (Dizhoor et al., 2008). Several *in vitro* studies are consistent with this notion that the G90D mutant forms a distinct active-state conformation. The G90D mutant effectively activates transducin and is phosphorylated by rhodopsin kinase similarly as wild-type rhodopsin, however, the interaction between the opsin form of the mutant and arrestin is severely impaired (Rim & Oprian, 1995; Vishnivetskiy et al., 2013). Thus, the constitutively active G90D mutant appears to adopt a conformation with impaired ability to bind arrestin. Differences are observed in the electron paramagnetic resonance (EPR) spectra of spin-labeled G90D mutant and light-activated rhodopsin (Kim et al., 2004), which supports the notion that the conformation of the constitutively active state of the G90D mutant is different from the light-activated state of rhodopsin. Interestingly, the EPR spectra of spin-labeled A292E mutant, which also causes CNB, is similar to that of the G90D mutant (Kim et al., 2004), which may indicate that constitutively active mutants causing CNB attain a common active-state conformation.

Under normal conditions, activation of rhodopsin is coupled to transducin activation, phosphorylation by rhodopsin kinase, and arrestin binding (Fig. 2B). Since the constitutively active state of the G90D mutant adopts a conformation that activates transducin but has an impaired ability to bind arrestin, downstream events can be uncoupled under certain circumstances. Mutations at Arg135 result in a conformation that has the opposite effect as the G90D mutation on downstream events. The opsin form of the R135L mutant, which causes autosomal dominant RP (Sung et al., 1991), adopts a conformation that is phosphorylated by rhodopsin kinase and can bind arrestin but cannot activate transducin (Shi et al., 1998). Thus, rhodopsin can achieve multiple conformational states that can differentially interact with downstream signaling partners and raises the possibility that different classes of constitutively active mutants attain distinct active-state conformations with potentially different cellular effects.

The possibility that different constitutively active mutants achieve different active-state conformations is suggested by observations on E134Q and M257Y mutants of rhodopsin, which are experimentally determined constitutively active mutants (Cohen, Yang, Robinson, & Oprian, 1993; Han, Smith, & Sakmar, 1998). In contrast to the disease-causing mutations discussed so far, these mutations do not occur near the chromophore-binding pocket. These mutations likely promote constitutive activity by affecting constraints in the D(E)RY and NPxxY motif molecular switches (Deupi, Edwards et al., 2012), but independently of the

protonated Schiff base molecular switch in the chromophore-binding pocket. EPR studies of spin-labeled receptors reveal that both the E134Q and M257Y mutants have different active-state conformations compared to both light-activated rhodopsin and constitutively active mutants that cause CNB (Kim et al., 2004; Kim, Altenbach, Thurmond, Khorana, & Hubbell, 1997). Thus, there appears to be multiple active-state conformations that constitutively active mutants can achieve that are different from the conformation of the MII state generated from light-activation of rhodopsin.

SUMMARY AND CONCLUDING REMARKS

Constitutive activity in rhodopsin can arise due to a variety of reasons and cause disease (Table 1). It is interesting to note that all known mutations to date that cause inherited retinal disease because of constitutive activity occur at residues near the chromophore-binding pocket (Fig. 3). In the absence of bound 11-*cis* retinal, the apoprotein opsin itself can adopt an active state due to diminished constraints. The apoprotein can form because of the absence of available 11-*cis* retinal, as occurs in LCA and vitamin A deficiency, or because of mutation at Lys296 (K296E and K296M), as occurs in RP. The apoprotein formed because of mutation is not equivalent to that formed by the wild-type receptor in LCA or vitamin A deficiency. The mutant apoprotein exhibits higher activity and is hyperphosphorylated leading to stable interactions with arrestin, which underlies the pathology. In contrast, conditions causing the formation of the wild-type apoprotein result in much lower levels of activity and it is the activity itself that underlies the pathology.

Rhodopsin bound to 11-*cis* retinal can exhibit constitutive activity by at least two mechanisms. Rhodopsin is engineered to prevent the thermal isomerization of 11-*cis* retinal. Mutation can effectively reduce the energetic barrier to isomerization, thereby making thermal isomerization of 11-*cis* retinal a more frequent event in the dark, as is observed in the RP mutants S186W and D190N. Mutations can also cause constitutive activity in chromophore-bound rhodopsin by disrupting the ionic interaction between the protonated Schiff base at Lys296 and Glu113 in the dark state (Fig. 3B), as occurs in the CNB mutants G90D, T94I, A292E, and A295V. Some mutants, such as the RP mutant G90V, can cause constitutive activity by both of these mechanisms.

The level of activity promoted by mutation likely plays some role in the variable phenotypes observed for different mutants. It is not yet clear whether all constitutively active mutants achieve the same active-state conformation or whether some achieve different active-state conformations that exhibit different levels of activity or promote different downstream events. As discussed, several observations suggest that at least some constitutively active mutants achieve a different active-state conformation compared to that achieved by light-activated rhodopsin. Multiple molecular switches are engineered into the structure of rhodopsin that keep the receptor in an inactive state in the dark and promote the activation of the receptor upon isomerization of 11-*cis* retinal (Fig. 3). While these molecular switches work in concert under normal conditions, they can be uncoupled under certain circumstances (Kim et al., 1997; Mahalingam et al., 2008). This uncoupling may occur in some instances because of mutation and result in distinct active states that display different activity levels

and downstream effects (Deupi, Standfuss, & Schertler, 2012), thereby resulting in different phenotypes.

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Abbreviations

CNB	congenital night blindness
CP	cytoplasmic loop
EC	extracellular loop
EPR	electron paramagnetic resonance
GPCR	G protein-coupled receptor
H8	amphipathic alpha helix 8
$\lambda_{\max}$	maximal absorbance of light
LCA	Leber congenital amaurosis
LRAT	lecithin-retinol acyltransferase
MI	metarhodopsin I
MII	metarhodopsin II
RIS	rod inner segment(s)
ROS	rod outer segment(s)
R	inactive state
R*	active state
RP	retinitis pigmentosa
RPE65	retinal pigment epithelium-specific 65 kDa protein
TM	transmembrane alpha helix

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Figure 1. Structure of rhodopsin

A, The secondary structure of human rhodopsin is shown with residues causing constitutive activity and retinal disease when mutated highlighted in black, except for Lys296. Residues forming molecular switches are colored as follows: green, protonated Schiff base switch; yellow, CWxP motif switch; cyan, TM3-TM5 hydrogen bond network switch; blue, NPxxY motif switch; red, D(E)RY motif switch. Residues forming the CWxP, NPxxY, and D(E)RY motifs are highlighted in bold. **B**, Crystal structures of the inactive state of bovine rhodopsin (colored, PDB: 1U19) and the MII state of bovine rhodopsin (gray, PDB: 3PXO) were aligned with PyMOL. Residues causing constitutive activity and retinal disease when mutated are depicted as black spheres. 11-*cis* retinal is depicted as pink spheres. Helices in the inactive state structure are colored as follows: blue, TM1; cyan, TM2; green, TM3; lime green, TM4; yellow, TM5; orange, TM6; red, TM7; purple, H8.

Figure 2. Rod photoreceptor cells and phototransduction

A, Cartoon depiction of a rod photoreceptor cell. The cartoon of the cell on the left shows the structure of a rod photoreceptor cell with disc membranes in the ROS and mitochondria, Golgi apparatus, endoplasmic reticulum, and nucleus in the RIS/perinuclear region. Rhodopsin is embedded in disc membranes of the outer segment. The cartoons of the cell in the middle and on the right illustrate the levels of transducin (green) and arrestin (blue) in the ROS and RIS/perinuclear region in the dark and in the light. **B**, Life cycle of rhodopsin. Rhodopsin is covalently bound to 11-*cis* retinal in the dark. Light isomerizes 11-*cis* retinal to all-*trans* retinal, which promotes the activation of rhodopsin and formation of the MII state. MII binds and activates the heterotrimeric G protein transducin (green) to initiate phototransduction. MII is inactivated via phosphorylation by rhodopsin kinase and the binding of arrestin (blue). The MII state decays to opsin upon release of all-*trans* retinal from the chromophore-binding pocket. Opsin must reconstitute with 11-*cis* retinal to regenerate rhodopsin.

Figure 3. Molecular switches in rhodopsin

A, The inactive-state structure of bovine rhodopsin (PDB: 1U19) is shown with residues forming molecular switches that lock rhodopsin into an inactive state highlighted as colored spheres (green, protonated Schiff base switch; yellow, CWxP motif switch; cyan, TM3-TM5 hydrogen bond network switch; blue, NPxxY motif switch; red, D(E)RY motif switch). Residues that cause constitutive activity and retinal disease when mutated are shown as black spheres, except for Lys296. 11-*cis* retinal is shown as pink spheres. **B**, The region surrounding the chromophore 11-*cis* retinal (pink sticks) is shown to highlight residues causing constitutive activity and retinal disease when mutated (black sticks, except for Lys296) and residues forming the protonated Schiff base molecular switch (green sticks).

Figure 4. Models of receptor activation

A, The classic two-state model describes the equilibrium between an inactive receptor (R) and an active receptor (R*). **B**, The non-sequential multi-state model describes the equilibrium between an inactive receptor (R) and different active states with distinct conformations (R*, R**, and R***) that arise non-sequentially from the inactive receptor. **C**, The sequential multi-state model describes the equilibrium between an inactive receptor (R) and different active states with distinct conformations (R* and R**) that arise sequentially from the inactive receptor. The number of different active states rhodopsin or other GPCRs can form is unknown and, therefore, the number of different active states may be greater or fewer than those depicted in the multi-state models.

Table 1

Properties of constitutively active forms of rhodopsin that cause retinal disease

Constitutively active form	Properties of the constitutively active receptor
<u>Leber congenital amaurosis and vitamin A deficiency</u>	
Opsin	<ul style="list-style-type: none"> Activity that is 10^{-6} – 10^{-5} times that promoted by light-activated rhodopsin (Fan et al., 2005; Melia et al., 1997). Monophosphorylated by rhodopsin kinase (Fan et al., 2010). Triggers translocation of arrestin but not transducin (Mendez et al., 2003).
<u>Congenital night blindness</u>	
G90D	<ul style="list-style-type: none"> Blue-shifted $\lambda_{\max}$ (480–485 nm) (S. Jager et al., 1997; Kaushal & Khorana, 1994; Kawamura et al., 2012; Rao et al., 1994; Zvyaga et al., 1996). Slower 11-<i>cis</i> retinal binding kinetics (Gross, Xie et al., 2003; Toledo et al., 2011). Chromophore-binding pocket exhibits solvent accessibility in the dark state (Kawamura et al., 2012; Toledo et al., 2011; Zvyaga et al., 1996). Dark state and opsin exhibit structural features of an active state (Fahmy et al., 1996; Kawamura et al., 2012; J. M. Kim et al., 2004; Singhal et al., 2013; Zvyaga et al., 1996)^I. Increased transducin activation by opsin (Rao et al., 1994; Toledo et al., 2011). Slower rate of MII formation but faster rate of MII decay. Forms additional intermediate upon photobleaching (Toledo et al., 2011; Zvyaga et al., 1996). Similar MII decay observed under certain conditions (Gross, Rao et al., 2003). Decreased thermal stability of the dark state and increased thermal stability of opsin (Singhal et al., 2013)^I. Increased phosphorylation of opsin (Rim & Oprian, 1995). Decreased arrestin binding (Rim & Oprian, 1995; Singhal et al., 2013; Vishnivetskiy et al., 2013)^I. Mutated residue replaces Glu113 as the counterion for the protonated Schiff base at Lys296 (Singhal et al., 2013)^I. No transducin translocation (Nash & Naash, 2006).
T94I	<ul style="list-style-type: none"> Blue-shifted $\lambda_{\max}$ (478 nm) (Ramon et al., 2003) Similar 11-<i>cis</i> retinal binding kinetics (Gross, Xie et al., 2003). Chromophore-binding pocket exhibits solvent accessibility in the dark state (Ramon et al., 2003). Increased transducin activation by opsin (Gross, Rao et al., 2003). Slower rate of MII decay (Gross, Rao et al., 2003; Ramon et al., 2003). Decreased thermal stability of the dark state (Ramon et al., 2003). Mutated residue predicted to form hydrophobic interactions with Lys296 to disrupt the protonated Schiff base molecular switch (Singhal et al., 2013).
A292E	<ul style="list-style-type: none"> Unchanged $\lambda_{\max}$ (500 nm) (Dryja et al., 1993; Gross, Rao et al., 2003). Decreased stability of opsin (Gross, Xie et al., 2003). Dark state exhibits structural features of an active state (J. M. Kim et al., 2004). Increased transducin activation by opsin (Dryja et al., 1993). Increased phosphorylation of opsin (Rim & Oprian, 1995). Slower rate of MII formation but faster rate of MII decay. Forms additional intermediate upon photobleaching (Gross, Rao et al., 2003).

Constitutively active form	Properties of the constitutively active receptor
A295V	<ul style="list-style-type: none"> • Mutated residue predicted to replace Glu113 as the counterion for the protonated Schiff base at Lys296 (Singhal et al., 2013). • Blue-shifted $\lambda_{\max}$ (482 nm) (Zeititz et al., 2008). • Increased transducin activation by opsin (Zeititz et al., 2008). • Faster rate of MII decay (Zeititz et al., 2008). • Mutated residue not predicted to interact with Lys296 but may interact with Trp265 to disrupt the protonated Schiff base molecular switch (Singhal et al., 2013).
<u>Retinitis pigmentosa</u>	
G90V	<ul style="list-style-type: none"> • Blue-shifted $\lambda_{\max}$ (489 nm) (Toledo et al., 2011). • Slower 11-<i>cis</i> retinal binding kinetics (Toledo et al., 2011). • Chromophore-binding pocket exhibits solvent accessibility in the dark state (Toledo et al., 2011). • Increased transducin activation by opsin (Toledo et al., 2011). • Faster rate of MII decay and forms additional intermediate upon photobleaching (Toledo et al., 2011). • Decreased thermal stability of the dark state. Less thermally stable compared to G90D mutant (Toledo et al., 2011).
S186W	<ul style="list-style-type: none"> • Decreased thermal stability of the dark state: increased rate of thermal isomerization of 11-<i>cis</i> retinal and hydrolysis of the Schiff base linkage (M. Y. Liu et al., 2013).
D190N	<ul style="list-style-type: none"> • Decreased thermal stability of the dark state: increased rate of thermal isomerization of 11-<i>cis</i> retinal and hydrolysis of the Schiff base linkage (Janz & Farrens, 2003; Janz, Fay, & Farrens, 2003; M. Y. Liu et al., 2013).
K296E	<ul style="list-style-type: none"> • Cannot bind 11-<i>cis</i> retinal and is constitutively active <i>in vitro</i> (Robinson et al., 1992; Yang et al., 1997). • Constitutively phosphorylated and tightly bound to arrestin, which prevents constitutive activity <i>in vivo</i> (Chen et al., 2006; T. Li et al., 1995; Rim & Oprian, 1995). • Arrestin present in ROS in the dark (Chen et al., 2006; T. Li et al., 1995). • Some of the mutant is mislocalized to RIS (Chen et al., 2006; Moaven et al., 2013). • Mutant-arrestin complex can recruit endocytic proteins (Moaven et al., 2013).
K296M	<ul style="list-style-type: none"> • Cannot bind 11-<i>cis</i> retinal and is constitutively active <i>in vitro</i> (Rim & Oprian, 1995; Yang et al., 1997). • Constitutively phosphorylated and bound to arrestin (Rim & Oprian, 1995).

¹ Studies reported in (Singhal et al., 2013; Vishnivetskiy et al., 2013) were conducted on a rhodopsin background containing the N2C and D282C mutations, which stabilize the receptor molecule.