

FERNANDA BALEN

**EM BUSCA DE NOVOS MÉTODOS DE TRATAMENTO
PARA A RETINOSE PIGMENTAR CAUSADA POR
MUTAÇÕES NA RODOPSINA**

Tese apresentada ao Programa de Pós-Graduação em Biologia Celular e Tecidual do Instituto de Ciências Biomédicas da Universidade de São Paulo, para obtenção do Título de Doutor em Ciências.

Área de concentração: Biologia Celular e Tecidual

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ABSTRACT

Balen F. Finding new approaches to treat retinitis pigmentosa caused by mutations in the photoreceptor rhodopsin [Ph.D. Thesis (Cell and Developmental Biology)]. São Paulo: Instituto de Ciências Biomédicas, Universidade de São Paulo; 2012.

Retinitis Pigmentosa (RP) is an inherited disease that progressively leads to blindness. To date, more than 150 mutations associated with RP are known in rhodopsin. *In vitro* studies have shown that most of these mutations cause misfolding of rhodopsin. It has been hypothesized that molecular instability of the rhodopsin structure is responsible for disease severity in patients, but there is still no effective therapy to treat RP. Administration of vitamin A or retinoid derivatives is being used to rescue correctly folded rhodopsin and to slow down the degeneration, however, this treatment alone cannot cure RP. The focus of this thesis was to test the hypothesis that molecules other than retinal can help to rescue folded rhodopsin and/or reduce photoreceptor cell death. For that, the binding of other ligands to rhodopsin, was investigated *in vitro*, by studying the effects of the ligands on WT rhodopsin as well engineered rhodopsin mutants, and *in vivo* by making use of different rat models of RP. The major findings of this thesis are: I) The RP mutations, Asn-15-Ser (N15S) and Pro-23-His (P23H) were studied and characterized *in vitro*. Expression of these mutants in the presence of 9-*cis* and 11-*cis* retinal reveals that they differ in characteristics and severity, despite their global classification into the same class. N15S is slightly defect in structure, stability and cellular localization. P23H, on the other hand, is severely impaired at the molecular and cellular levels. II) Binding of small molecules, namely metal ions (Zn^{2+} , Cu^{2+} , Fe^{2+} , Ni^{2+} , Mg^{2+} and Mn^{2+}), the anthocyanin cyanidin-3-glucoside (C3G) and the chlorophyll-derivative chlorin e6 (Ce6), was tested. It was shown that these ligands directly interact with rhodopsin *in vitro*. Biophysical evidence indicated differential effects of these ligands on rhodopsin function, structure and dynamics. Ce6 was shown to be the best candidate to confer stability to the rhodopsin protein *in vitro*. III) Assessment of the effects of Ce6 on the stability of rhodopsin was tested *in vivo*: (a) First, normal rats, Sprague Dawley (SD), were subjected to light-damage. Treatment with Ce6 appears to have only a minor effect on prevention of retinal damage. (b) Secondly, the effects of Ce6 on RP progression *in vivo* were evaluated in the P23H and Ser334ter (S334ter) rat models. Histological and functional analysis indicated

that Ce6 seems to exert a positive functional effect by slowing the rate of P23H photoreceptor degeneration *in vivo*. In contrast, Ce6 increased the photoreceptor degeneration of the S334ter rat *in vivo*. Collectively, the studies presented in this thesis enhance the knowledge related to several RP mutations, namely P23H, N15S and S334ter, which were found to cause misfolding of the photoreceptor rhodopsin *in vitro* and/or mediate the RP disease *in vivo*. It also conveys a new possibility for treatment of the RP disease with the identification of molecules (retinals, divalent metal ions, porphyrins and anthocyanins) that could aid in the stability and folding and modulate photoreceptor structure and function, thus paving the way to selectively target this receptor and aid directly in vision-rescue strategies.

Key-words: Retinitis Pigmentosa. Rhodopsin. Photoreceptors.

RESUMO

Balen F. Em busca de novos métodos de tratamento para a retinose pigmentar causada por mutações na rodopsina [Tese (Doutorado em Biologia Celular e Tecidual)]. São Paulo: Instituto de Ciências Biomédicas, Universidade de São Paulo; 2012.

Retinose Pigmentar (RP) é uma doença hereditária que progressivamente conduz à cegueira. Atualmente, são conhecidas mais de 150 mutações da rodopsina. Estudos *in vitro* mostraram que a maioria destas mutações altera a conformação da rodopsina sugerindo que a instabilidade molecular da estrutura da rodopsina é responsável pela gravidade da RP em pacientes. Todavia, uma terapia efetiva para tratar a RP ainda não foi estabelecida. Administração de vitamina A ou derivados de retinóides são usados como tratamento para mediar a correta formação da rodopsina ou reduzir a degeneração. Contudo, o uso exclusivo desse tratamento não é suficiente para curar a RP. O objetivo desta tese foi testar a hipótese de que outras moléculas, além dos retinóides, poderiam influenciar na conformação correta da rodopsina e/ou reduzir a morte dos fotorreceptores. Com este objetivo, foi investigada *in vitro* e *in vivo* a ligação de outros compostos moleculares à rodopsina. Os efeitos destes compostos foram estudados *in vitro* na rodopsina selvagem e mutantes, e *in vivo*, utilizando diferentes modelos de ratos. Os principais resultados desta tese são: I) As mutações da RP Asn-15-Ser (N15S) e Pro-23-His (P23H), foram estudadas e caracterizadas *in vitro*. A expressão destas mutações na presença de retinóides 9-*cis* ou 11-*cis* revelou que ambas apresentam diferenças em características e severidade, apesar de serem globalmente classificados dentro da mesma classe. O N15S apresentou estrutura, estabilidade e localização celular levemente anormal. O P23H mostrou-se altamente anormal, tanto em nível molecular quanto celular. II) A ligação de pequenas moléculas à rodopsina foi investigada, utilizando-se os íons metálicos Zn^{2+} , Cu^{2+} , Fe^{2+} , Ni^{2+} , Mg^{2+} e Mn^{2+} , a antocianina cianidina-3-O-glicosídeo (C3G) e o derivado da clorofila clorina e6 (Ce6). Verificou-se que estes ligantes interagem diretamente com a rodopsina *in vitro*. Evidências biofísicas indicaram efeitos diferenciados destes ligantes na função, estrutura e dinâmica da rodopsina. Entre todos, Ce6 conferiu maior estabilidade à rodopsina *in vitro*. III) A avaliação dos efeitos do Ce6 na estabilização da rodopsina foi testada *in vivo*: (a) Primeiramente, ratos Sprague Dawley (SD) normais foram submetidos à degeneração por luz. O tratamento com Ce6 apresentou um efeito muito pequeno na prevenção da degeneração retiniana induzida por

fototoxicidade. (b) Em seguida, o efeito do Ce6 na progressão da RP foi avaliado nos ratos modelos P23H e Ser334ter (S334ter). Análises histológicas e funcionais indicaram que o Ce6 parece exercer um efeito positivo diminuindo a taxa de degeneração dos fotorreceptores dos ratos P23H. Por outro lado, o Ce6 aumentou a degeneração dos fotorreceptores do rato S334ter. Coletivamente, os estudos apresentados aumentam o conhecimento relacionado às diversas mutações da RP, P23H, N15S e S334ter, as quais causam a má-formação da rodopsina *in vitro* e/ou medeiam a RP *in vivo*. Este estudo também conduz a novas possibilidades de tratamento da RP por meio da identificação de moléculas (retinóides, íons metálicos divalentes, porfirinas e antocianinas) que podem ajudar na estabilização e formação da rodopsina, assim como na modulação da estrutura e função do fotorreceptor, objetivando a seleção específica do receptor e auxiliando diretamente em estratégias para o resgate da visão comprometida pela RP.

Palavras-chave: Retinose Pigmentar. Rodopsina. Fotorreceptores.

1 CHAPTER 1

1.1 INTRODUCTION

Vision is an important sensory model system for vertebrates since it most directly mediates the interaction with the exterior world. Not surprisingly, the eye has evolved to be an organ of extreme perfection and complexity. This was elegantly pointed out by Charles Darwin, who said:

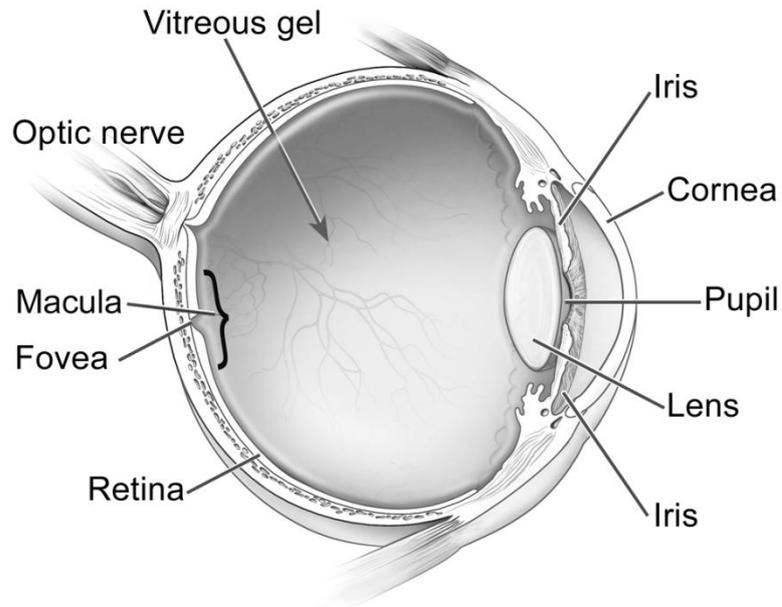
To suppose that the eye, with all its inimitable contrivances for adjusting the focus to different distances, for admitting different amounts of light, and for the correction of spherical and chromatic aberration, could have been formed by natural selection seems, I freely confess, absurd in the highest possible degree. Yet reason tells me, that if numerous gradations from a perfect and complex eye to one very imperfect and simple, each grade being useful to its possessor, can be shown to exist; if further, the eye does vary ever so slightly, and the variations be inherited, which is certainly the case; and if any variation or modification in the organ be ever useful to an animal under changing conditions of life, then the difficulty of believing that a perfect and complex eye could be formed by natural selection, though insuperable by our imagination, can hardly be considered real. How a nerve comes to be sensitive to light, hardly concerns more than how life itself first originated; but I may remark that several facts make me suspect that any sensitive nerve may be rendered sensitive to light, and likewise to those coarser vibrations of the air which produce sounds (Darwin, 2006). Charles Darwin 1809-1882.

The “perfection” of the eye can be well illustrated by the capability of adaptation that the eye presents in front of specific needs of the organisms. Vision in humans has evolved to accommodate both, daylight and night vision. Nocturnal animals have their visual systems optimized for night activity. Deep sea animal vision is adapted to the limited radiation that penetrates their habitat.

1.1.1 The vertebrate eye

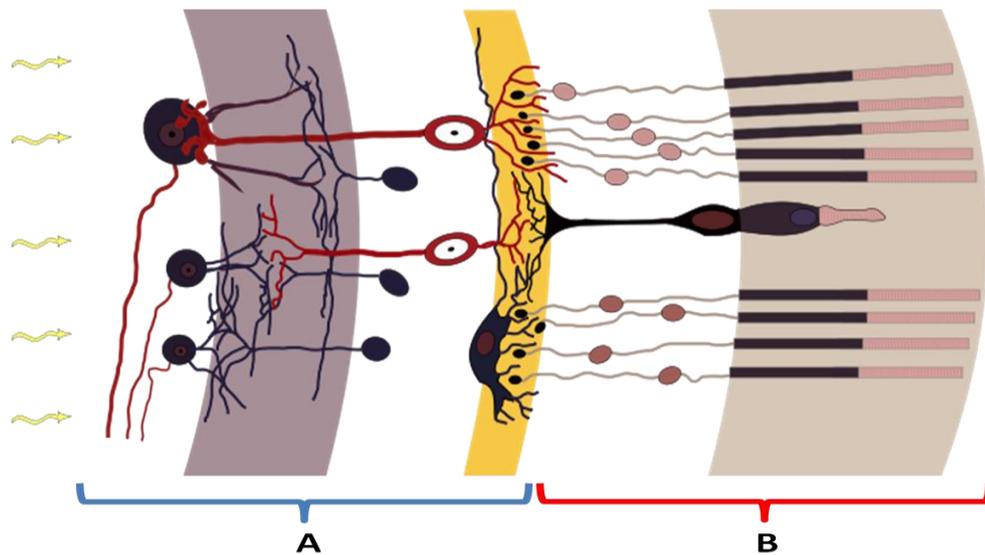
The retina is a very complex and layered structure that lines the back of the eye (Fig.1.1 and 1.2). It has a highly ordered anatomical organization, with a few basic classes of cells located in the outer nuclear layer (rods and cones), inner nuclear layer (bipolar, horizontal, and amacrine cells) and ganglion cell layer (ganglion cells).

Figure 1.1 - Structure of the eye.



SOURCE: Eye diagram is a courtesy of the National Eye Institute, National Institutes of Health. With permission.

Figure 1.2 - Axial organization of the retina.



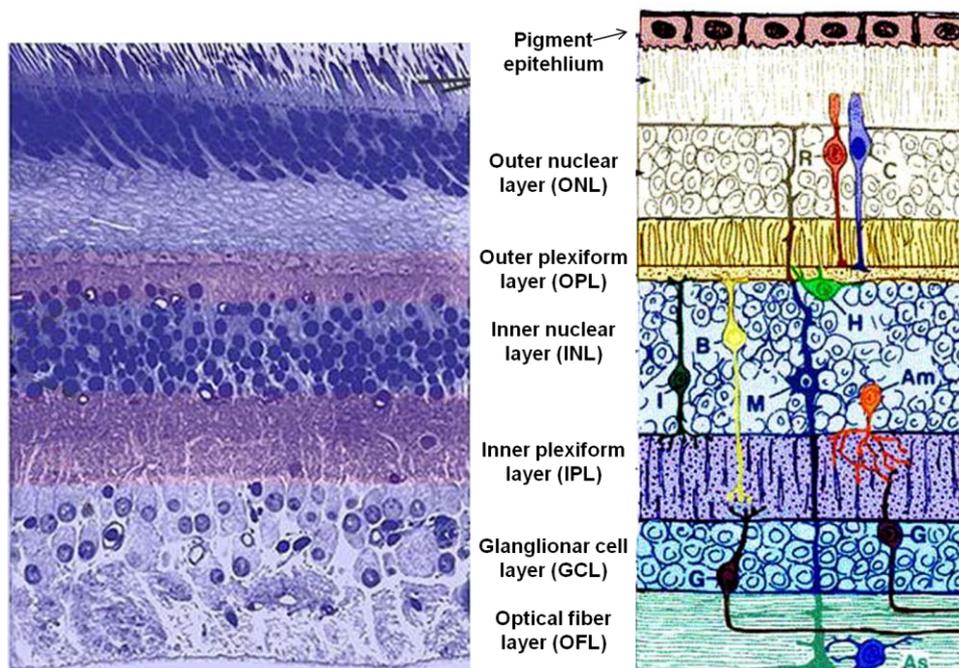
SOURCE: Drawing is adapted from (Cajal, 1900). With permission.

Figure 1.3 shows the three layers of neuronal cell bodies (outer nuclear, inner nuclear and ganglion cell layers) interconnected by synapses in the two plexiform layers (outer and inner).

Light must pass through the entire retinal thickness to reach the outer segments of photoreceptors (rods and cones), where the phototransduction occurs. To avoid image distortion and loss, Müller glial cells, whose cell bodies are located in the inner nuclear layer, appear to act as living fiber optics (Franze et al., 2007).

Photoreceptor axons contact the dendrites of bipolar cells and horizontal cells in the outer plexiform layer (OPL). In turn, the bipolar cells transmit the signal to ganglion cell dendrites and amacrine cells in the inner plexiform layer. Last, the ganglion cells send their axons through the optic fiber layer to the optic disk to make up the optic nerve (Molavi, 1997). Photoreceptors, bipolar, and ganglion cells release glutamate to mediate the information from retina to the brain.

Figure 1.3 - Light micrograph of a vertical section through the human retina and a scheme identifying the cells type.



SOURCE: Figure adapted from (Kolb, 2011). With permission.

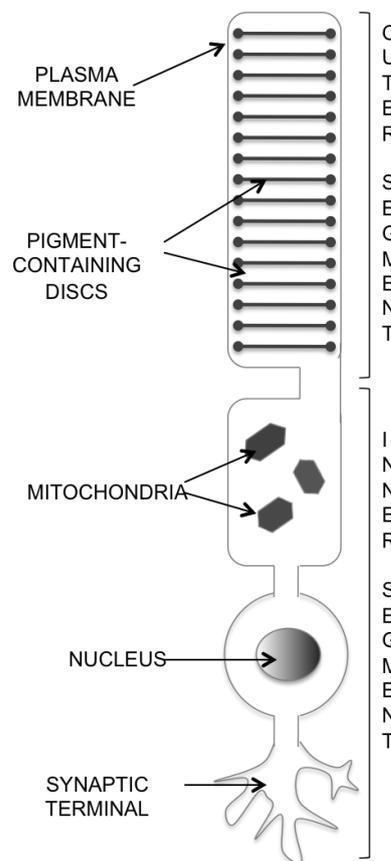
The photoreceptor cells, rods and cones, are responsible for different parts of visual perception. Rods, the most abundant photoreceptor cells, are responsible for black and white vision under dim light conditions. Rod mediated vision is very sensitive to light, but do not enable color vision. Cone mediated vision are responsible for day light vision. They present high resolution and are sensitive to color and details.

Besides rods and cones, photosensitive ganglion cells containing melanopsin were also described in mammals (Foster et al., 1991; Lucas et al., 2001; Provencio et al., 2000). They

are involved in various reflexive responses of the brain and body to the presence of light, for example the regulation of circadian rhythms, the pupillary reflex and other non-visual responses to light.

Anatomically, vertebrate rods and cones photoreceptor cells contain an outer segment, which carries the photosensitive pigments; an inner segment, a nucleus and a synaptic terminal (Figure 1.4). The rod outer segment (ROS) is packed by a large number of discs, where the visual photoreceptor molecule rhodopsin is located. Rhodopsin consists of the apoprotein opsin and the, Vitamin A derivative, chromophore *11-cis* retinal (Wald, 1968).

Figure 1.4 - Schematic structure of the rod photoreceptor.



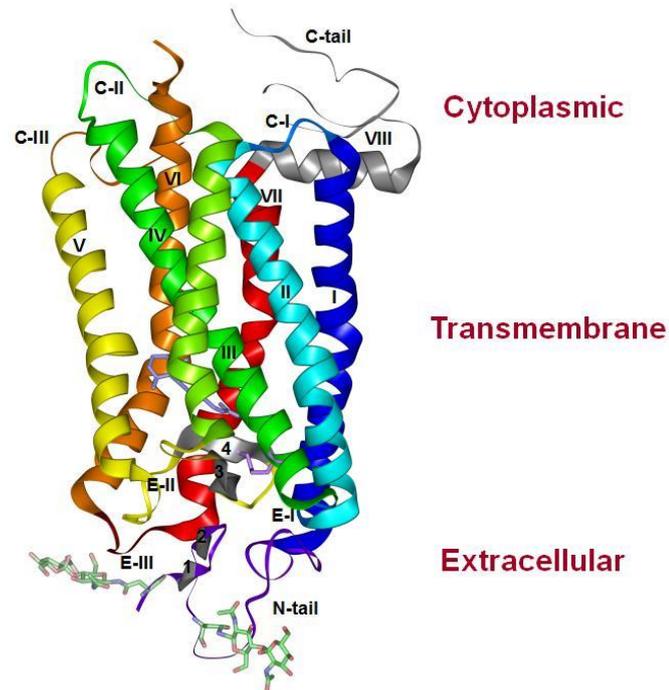
SOURCE: Balen, 2012

1.1.2 Rhodopsin

The photoreceptor molecule rhodopsin is a membrane protein, and a prototypical member of the large G protein-coupled receptor (GPCR) family, the largest family of cell surface receptors. Rhodopsin (Figure 1.5) is made up of a cytoplasmic domain (CP), a

seven-transmembrane helical domain (TM) and an extracellular domain (EC). Rhodopsin contains a covalently linked ligand, *11-cis* retinal, a Vitamin A derivative, that stabilizes the folded receptor (Figure 1.5). Visual signal transduction is initiated when a photon induces isomerization of *11-cis* retinal to *all-trans* retinal. This event triggers the rearrangement of the TM domain, resulting in the light-activated, Metarhodopsin II (Meta II) state. The Meta II state of rhodopsin is the active functionally state of the protein and initiates the visual signal transduction cascade. As a first step, Meta II binds to the G protein, transducin (Gt). Gt then activates phosphodiesterase (PDE), which hydrolyzes cyclic GMP (cGMP). This event ultimately leads to the hyperpolarization of the cell through the closure of ion channels. When the cell is hyperpolarized, the electrical potential inside the cell is more negative than in darkness. In contrast, in the dark state, cGMP keeps the channels in the photoreceptor membrane open, and Na^+ - Ca^{2+} influx depolarizes the membrane. The signal can be shut down through two mechanisms: (1) through Meta II decay, a process of dissociation of protein and ligand to opsin and free *all-trans* retinal, or (2) through phosphorylation of Meta II by rhodopsin kinase, followed by binding of arrestin to the phosphorylated C-terminal end of rhodopsin (Chabre et al., 1988). The ligand free opsin formed when Meta II decays can readily uptake *11-cis*-retinal, thus regenerating rhodopsin.

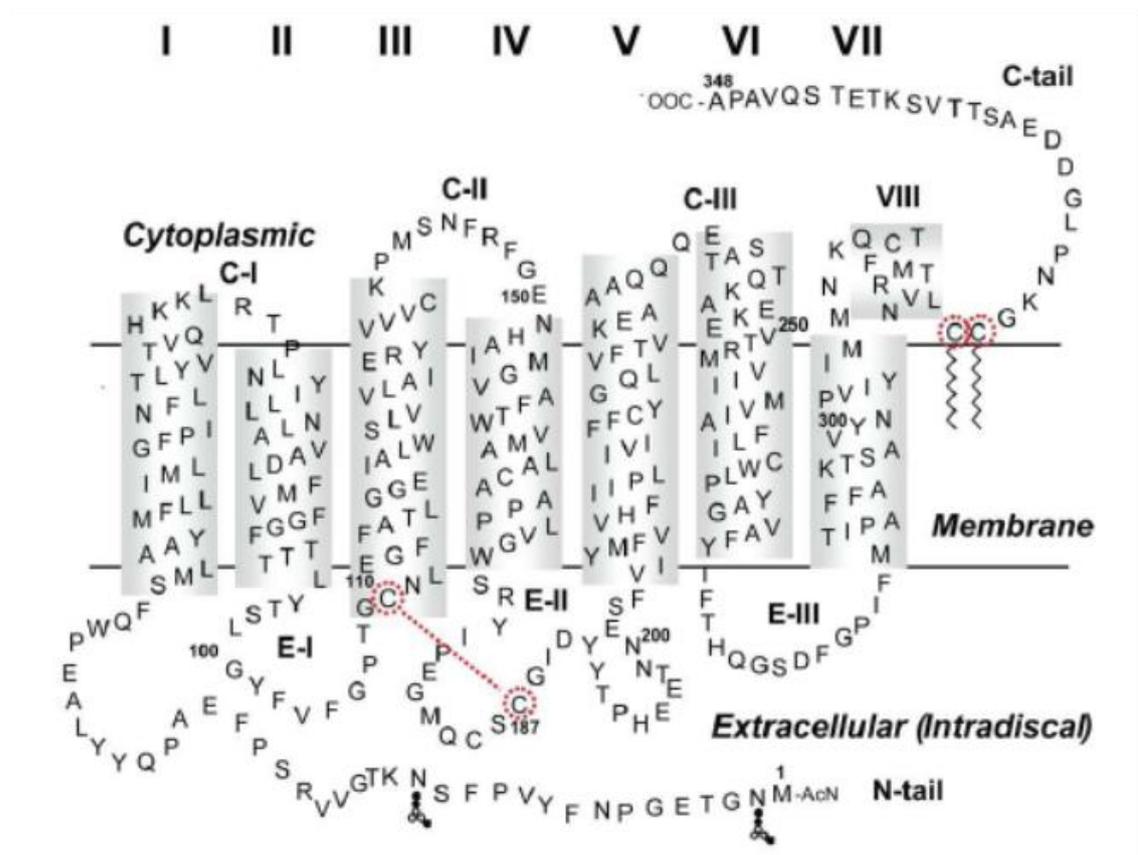
Figure 1.5 - Tertiary structure of rhodopsin.



SOURCE: Balen, 2012

The primary structure of rhodopsin (bovine) contains 348 amino acids. This sequence was determined using amino acid sequencing (Hargrave et al., 1983; Ovchinnikov Yu et al., 1982). Figure 1.6 shows the secondary structure model of rhodopsin showing the seven TM helices, the EC domain and the C-terminus domain, which faces the CP, along with the disulfide bond (Cys110 and Cys187) and palmitoylation sites (Cys322 and Cys323). The structure is divided in about 50% TM, 25% CP and 25% EC domains. Rhodopsin was the first protein of the GPCR family to be crystallized (Palczewski et al., 2000). Rhodopsin is the best studied and the prototypic member of the GPCR family.

Figure 1.6 - Secondary structure of rhodopsin.



SOURCE: Balen, 2012

Since the correct folding of the rhodopsin protein is extremely important for its functionality, many studies were conducted to understand the folding and unfolding mechanisms of rhodopsin. It has been demonstrated that there is a minimum amount of helix content necessary to maintain the binding of *11-cis* retinal to the protein (Ridge et al., 1995). Unfolding studies using single molecule force spectroscopic approaches, point at specific areas of rigidity in the structure of rhodopsin (Sapra et al., 2006, 2008; Tastan et al., 2007). Furthermore, *in silico* simulations of thermal unfolding suggested formation of a folding core between the EC and TM domains (Rader et al., 2004; Tastan et al., 2007). The structure of rhodopsin is also prone to misfolding when amino acid replacements are made (Anukanth and Khorana, 1994; Garriga et al., 1996; Kaushal and Khorana, 1994; Liu et al., 1996). Misfolding here is characterized by the inability of the protein to bind *11-cis* retinal, necessary for the correct function of the protein in the visual system.

1.1.3 Allosteric ligands

In addition to retinal isomers, rhodopsin has been shown to bind a number of other ligands. They all bind in locations other than the retinal binding pocket and are thus by definition allosteric ligands. These ligands are reviewed below.

Zinc

Zinc, after iron, is the second most abundant trace metal in the human body (Aggett and Comerford, 1995). It is present in high concentrations in the eye, especially in the retina (Grahn et al., 2001). Numerous normal retinal functions are dependent on Zn^{2+} , including retinal development, synaptic transmission, light response and Vitamin A metabolism (Grahn et al., 2001). Zn^{2+} deficiency leads to night blindness, changes in dark-adaptation, ultra-structural changes in the retina and the retinal pigment epithelium, and age-related macular degeneration (Ugarte and Osborne, 2001). Furthermore, patients with the retinal degeneration disease retinitis pigmentosa (see below) exhibit decreased serum Zn^{2+} levels (Karcioglu et al., 1984). However, especially for age-related macular degeneration, opposing roles have been proposed for Zn^{2+} . While supplementation with Zn^{2+} is the most widely used intervention strategy for patients with this disease, its presence in Drusen, deposits of accumulated extracellular material containing misfolded proteins which especially occur in patients with age-related macular degeneration, has been linked to increased aggregation of proteins (Lengyel and Peto, 2008). There are probably multiple proteins responsible for these physiological effects (Grahn et al., 2001), but one particularly well established target is the photoreceptor rhodopsin, to which Zn^{2+} binds directly and specifically with milli- to micromolar affinities depending on the membrane environment studied (Shuster et al., 1992). The crystal structure of rhodopsin revealed four putative Zn^{2+} binding sites (Okada et al., 2002; Palczewski et al., 2000), shown in Figure 1.7 as purple spheres. The extracellular and transmembrane Zn^{2+} binding sites have been proposed to be the physiologically relevant ones (Park et al., 2007; Stojanovic et al., 2004), but X-ray absorption spectroscopy (Toledo et al., 2008), NMR spectroscopy (Patel et al., 2005) and proteolytic digestion (Shuster et al., 1992) have suggested the primary binding sites to be located in the EC domain.

Figure 1.7 - Cartoon representation of dark state rhodopsin highlighting the presence of Zn²⁺ bound.



The four Zn²⁺ ions bound are represented as spheres and colored in magenta. Images were generated using VMD visualization software (Humphrey et al., 1996).

SOURCE: The structure shown is PDB identification 1L9H (Okada et al., 2002; Palczewski et al., 2000).

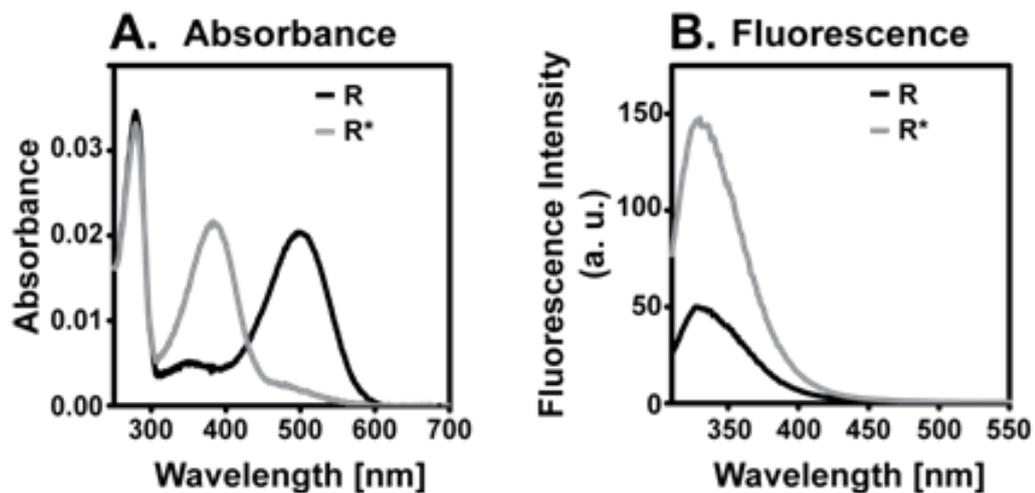
Anthocyanins

Anthocyanins are plant pigments belonging to the large group of phenolic compounds, the flavonoids. They are abundant in a diet rich in fruits and vegetables. Anthocyanin consumption confers a broad spectrum of health benefits, especially in vision, where they have been implicated to have protecting, enhancing and restoring abilities. They also have anti-oxidant, anti-cancer, anti-diabetic, anti-inflammatory, anti-aging and cardioprotective properties (Bagchi et al., 2004; Kaushal and Khorana, 1994; Lila, 2004; Zafra-Stone et al., 2007). Recently, anthocyanins from blackcurrants were shown to enhance the regeneration of rhodopsin, directly linking anthocyanin action to vision-related functional effects (Matsumoto et al., 2003). In the dark, *11-cis* retinal bound via Schiff base linkage to Lys296 in the TM domain of rhodopsin, gives rise to a chromophore with absorbance maximum at 500nm (Figure 1.8A). Illumination results in formation of the activated Meta II state of the protein with characteristic absorbance maximum at 380nm (Figure 1.8A), in which the *all-trans* retinal isomer is bound. Slowly – in the order of minutes – the Schiff base is hydrolyzed and *all-trans* retinal leaves the protein, forming opsin. This Meta II decay can be

followed by tryptophan fluorescence (Figure 1.8B). The assay consists of illumination of the sample, and measuring the rate of fluorescence increase, as the tryptophans are exposed, corresponding to the rate of retinal release.

Opsin is then able to bind free *11-cis* retinal and the 500nm chromophore is restored. This process is referred to as regeneration. The faster rates of rhodopsin regeneration in the presence of anthocyanins are potentially of functional importance because under dim-light conditions it is critical to ensure that rhodopsin is quickly available after activation to participate in a renewed visual signal transduction cascade.

Figure 1.8 - Application of different spectroscopic methods to the analysis of rhodopsin.

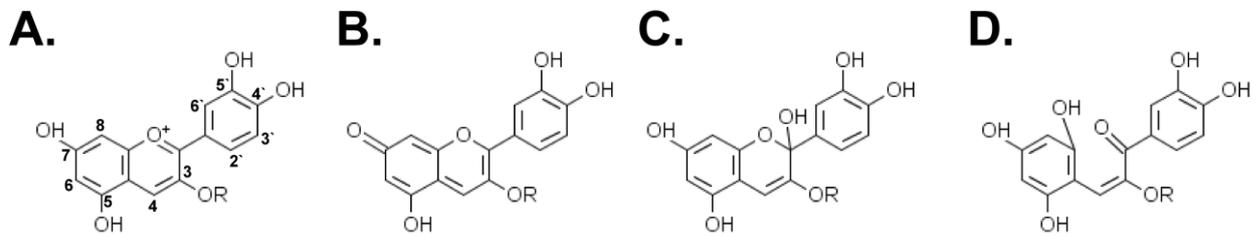


(A) Absorbance spectrum. For absorbance spectrum, rhodopsin in the dark (R; black line) and after illumination (R*; grey line) are shown. The rhodopsin concentration is $0.5\mu\text{M}$. (B) Fluorescence emission spectrum (excitation wavelength 295nm) in the dark (R; black line) and 60min after illumination (R*; grey line). The rhodopsin concentration is $0.5\mu\text{M}$.

SOURCE: Figure adapted from (Tirupula et al., 2009). With permission.

Anthocyanin cyanidin-3-O-glucoside (C3G) binds directly to rhodopsin in the dark and upon light activation, probably in its cytoplasmic domain (Yanamala, 2009). The apparent affinity of C3G for dark-adapted rhodopsin is $50\text{-}100\mu\text{M}$ based on ^1H NMR data recorded at pH 6, but the affinities for light-activated and opsin states is likely higher and remain to be determined. Evidence was provided that the mode of interaction not only varies with activation-state but also with pH, as C3G exists in multiple chemical forms, namely the flavylium ion, the quinoidal base, the carbinol pseudobase and the chalcone, which are shown in Figure 1.9 (Veitch and Grayer, 2008).

Figure 1.9 - Chemical structures of pH dependent equilibrium species of the cyanidin-3-glucoside.



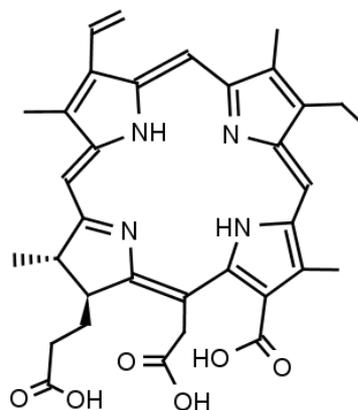
(A) Flavylium cation. (B) Quinoidal base example. (C) Carbinol pseudobase. (D) Chalcone. 'R' in the chemical structures of C3G represents the glucoside sidechain.

SOURCE: Figure adapted from (Yanamala et al., 2009). With permission.

Chlorin e6

Porphyrins are an omnipotent class of naturally occurring compounds with many important biological representatives such as hemes and chlorophylls. The chlorophyll derivative chlorine e6 (Ce6) (Figure 1.10) is believed to enhance sensitivity of rhodopsin to red light in deep-sea fish rhodopsin (Douglas et al., 1999; Isayama et al., 2006).

Figure 1.10 - Chemical structure of Ce6.



SOURCE: Balen, 2012

In vivo studies with salamander and mouse models have confirmed that Ce6 can effectively enhance vision in these animals (Washington et al., 2007). A decrease in the 500nm peak of a UV-Vis spectrum of salamander rhodopsin was observed by illuminating the sample at 668nm, a wavelength at which Ce6 absorbs while rhodopsin has less response (Isayama et al., 2006). Additionally, mice administered with Ce6, showed a two-fold increase in electroretinogram b-wave amplitudes (the mass response to the eye) as a response to red and blue light (Washington et al., 2007). An energy transfer between Ce6 and *11-cis* retinal

has been proposed (Isayama et al., 2006). *In vitro* studies demonstrated that Ce6 physically binds to rhodopsin and modulates its structure and function (Yanamala, 2009).

1.1.4 Retinitis pigmentosa

Mutations in rhodopsin are associated with the retinal degeneration disease retinitis pigmentosa (RP). RP reflects a large group of genetically heterogeneous disorders that affect the photoreceptors and retinal pigment epithelium (RPE) diffusely across the entire fundus but begin with initial geographic involvement in either the periphery or the macula (Sieving, 2010). It is one of the major causes of blindness in the world, affecting 1 in 4000-5000 people (Kannabiran, 2008). The disease is marked by early rod photoreceptor dysfunction and progressive degeneration of rods, which impairs vision in dim light and causes loss of peripheral vision, that is “tunnel vision” (Sieving, 2010), and later cones (Lam, 2005). This sequence of events explains why patients initially present night blindness, and only later in life presents diurnal visual impairment (Hamel, 2006). Typical RP is then described as rod-cone dystrophy, where rods are more affected than cones. However, some of the allied forms primarily cause cone photoreceptor loss and initially manifest with a reduction in central visual acuity (Sieving, 2010).

The pathologic mechanism underlying the observation that at the latest stages in the disease retinal degeneration is observed is still not clear. Family-related retinal degeneration with intraneural retinal pigmentation was described as early as 1855 (Sieving, 2010), and although “retinitis” implies an inflammatory or infectious cause, histopathology studies shows no evidence of macrophage invasion or other inflammatory response in the photoreceptor layer or elsewhere in the retina (Sieving, 2010). Studies attributed a genetic basis to the majority cases of RP and recently concluded that apoptosis is the last stage contributing to photoreceptor cell death (Chang et al., 1993; Portera-Cailliau et al., 1994; Reme et al., 1998). Belmonte et al. (2006) showed that Rac1, a member of the GTPase family with very low molecular weight, may participate in the cell death process when the degeneration is mediated by light or inherited. Moreover, after degeneration, there was an increase in the activity of Rac1, and furthermore Rac1 expressing photoreceptors were shown to be TUNEL-positive. TUNEL is a common method for detecting DNA fragmentation, by labeling the terminal end of nucleic acids, which results from apoptotic signaling cascades.

No evidence of racial or ethnic predisposition has been observed. However, men may be affected slightly more than women because of X-linked conditions (Sieving, 2010). The age-at-onset of symptoms and diagnosis is variable and can range from early childhood to mid-age adults. The diagnostic criteria is assessed through 4 parameters (Hamel, 2006); 1) Functional signs: which are characterized by night blindness as the first symptom, followed by photophobia; visual acuity is preserved in early and mid ages. 2) Visual field: marked by patchy losses of peripheral vision evolving to ring-shaped scotoma, tunnel vision is the last step. 3) Fundus: presence of pigmentary deposits similar to bone spicules in the peripheral retina; thinning of the retinal vessels; waxy pallor of the optic nerve; various degrees of retinal atrophy. 4) Electroretinogram: decrease of the amplitudes of a- and b- waves; scotopic system, rods, predominates over photopic system, cones.

Methods for clinical diagnosis are based on the presence of night blindness and peripheral visual defects, lesions in fundus, ERG changes, and progressive deterioration of all of these signs (Hamel, 2006). The ERG is the best method to detect the disease, since patients can be asymptomatic, for example presenting only night blindness and no other clinical signs.

1.1.5 Types of retinitis pigmentosa

RP is extremely heterogeneous, both clinically and genetically. Cases where there are no family histories evident are known as isolate, sporadic or simplex. By inheritance, RP can be classified in autosomal dominant (AD) (30%), autosomal recessive (AR) (20%) and X-linked recessive (XL) (15%) or sporadic/simplex traits (30%), and be part of the group of Leber congenital amaurosis (5%). These are among the more common modes of inheritance in RP and the relative prevalence of each form varies between populations (Kannabiran, 2008; Musarella and Macdonald, 2011). There are also rare forms of RP that are called X-linked dominant, mitochondrial and digenic (presents mutations in two different genes) (Musarella and Macdonald, 2011). Clinically, they are known as non-syndromic RP. Mutations in 16 different genes were found to cause autosomal dominant retinitis pigmentosa (ADRP) (Kannabiran, 2008). Autosomal dominant forms are usually the mildest forms, some cases starting after the age of 50, although severe disease can also appear (Hamel, 2006). The most frequent cause of ADRP is a mutation found in the rhodopsin gene, affecting 20-25% of the patients, especially in North America and Europe. In North America, a single point mutation, Pro23His, accounts for 12% of the cases, although not significantly in Europe. Mutations on

the RDS gene is the second most predominant set of mutations in RP, 3.5 to 9% were estimated followed by the RP1, which was estimated to be 4-8% of the cases. Other mutations appear to be less frequent and are implicated in less than 5% of the cases (Kannabiran, 2008).

Autosomal recessive retinitis pigmentosa (ARRP) is a group of disorders that includes juvenile and early-onset forms of RP. This group overlaps with another neurodegenerative disease called Leber's congenital amaurosis (LCA). 21 genes were identified to cause ARRP. However, the frequency of mutations from all of these genes accounts for approximately a third of ARRP patients, with individual genes contributing to less than 5% of disease (Kannabiran, 2008). There are two genes that were found to cause X-linked RP, RP2 and RP3 (known as RP GTPase regulator (*RPGR*)). *RP2* account for 10-20% of recessive XLRP and mutations in *RPGR* account for 50-80% of recessive XLRP. Another six loci were also mapped but the genes were not identified yet (Kannabiran, 2008). X-linked forms also start early and are frequently associated with myopia. Transmission of the disease is recessive in most cases; however there are some families in which dominant inheritance with affected females is found (Hamel, 2006).

Many syndromes are associated with different types of pigmentary retinopathies, and are known as syndromic RP. The most frequent are the Usher and Bardet Biedl syndromes (BBS). Usher syndrome is the most frequent and is typically associated with neurosensory deafness. BBS is marked by obesity (already in childhood), mental retardation or mild psychomotor delay, post axial polydactyly, hypogenitalism and renal problems and RP associated, frequently as cone-rod type of dystrophy (Hamel, 2006). Other less frequent syndromes encompass those that cause renal abnormalities such as Senior Loken syndrome (SLS) and Alport syndrome, dysmorphic syndromes such as Cohen, Jeune and Cockayne syndromes, metabolic syndromes such as Methylmalonic aciduria with homocystinuria, Abetalipoproteinemia (Bassen Korntzweig disease), Bietti's disease, Cystinosis, Mucopolysaccharidoses, Zellweger (cerebro-hepato-renal) syndrome, Hyperoxaluria type I with retinal atrophy in spots, Neonatal adrenoleukodystrophy with leopard spots fundus, Infantile Refsum disease, Adult Refsum disease, Peroxisomal disorders other than Refsum disease, Peroxisomal disorders other than Refsum disease and neurological disease, neuronal ceroid lipofuscinosis, Joubert syndrome, autosomal dominant cerebellar ataxia type II (SCA7), Myotonic dystrophy and Hallervorden-Spatz syndrome (Hamel, 2006).

1.1.6 Current therapies

Although innumerable studies have been performed, there is no effective therapy to treat RP. Meanwhile, many studies are focusing on alternative therapies to prevent, treat or slow down the disease.

In order to slow down the degeneration process, light protection and treatment with vitamins is recommended. Animal studies suggested that light exposure was damaging for the transgenic photoreceptors (Wang et al., 1997), and it is recommended to patients with pigmentary retinopathies to avoid direct light exposure, by either wearing dark glasses or yellow-orange spectacles, which minimizes photophobia. The use of eye shades and lateral protection is also recommended to protect against light coming from the sides (Hamel, 2006). Treatment with vitamins A and E are believed to protect the photoreceptors by trophic and antioxidant effects. A clinical trial of nutritional supplements for adults diagnosed with the common forms of RP was conducted (Kupfer, 1993). The course of retinal degeneration was monitored by electroretinogram recordings. On average, patients taking 15,000 IU of vitamin A daily showed slower, and those taking 400 IU of vitamin E showed faster retinal degeneration. The molecular mechanism of the Vitamin A effect is the rescue of correctly folded rhodopsin by retinal. However, 15,000 IU only slowed and could not prevent retinal degeneration, and a daily vitamin A intake exceeding 25,000 IU over the long-term is toxic. Later, similar studies were conducted and showed complementary or adverse results (Berson et al., 1993a, 1993b, 2004, 2010).

Different therapies have therefore been explored (Kannabiran, 2008). Gene therapy methods have been geared towards correction of the specific gene mutated, by means of gene replacement for recessive gene mutations or suppression and replacement of mutant mRNAs for dominant gene mutations. Encapsulated cells releasing neurotrophic factors have been implemented with the aim of promoting survival and growth of photoreceptors. Anti-apoptotic factors have been introduced by gene-delivery systems. Finally, stem cell transplantation has been considered. Each approach is briefly out-lined below.

Gene therapy is a promising therapy for many inherited human diseases. The technique requires efficient genotyping methods for identification of the implicated gene (Hamel, 2006). Ocular gene therapy (OGT) has been successfully tried in different animals and humans. First, identification of the genetic cause of the RP and genotyping of the patients was performed in order to proceed with the genetic modification of mutant ocular cells to

produce therapeutic effects. The strategy of the therapy differs accordingly with the type of disease or mutation target, and was already applied successfully in mouse (Bennicelli et al., 2008; Tan et al., 2009), rats (Drenser et al., 1998; Lewin et al., 1998; Weber et al., 2003), dogs (Acland et al., 2001; Veske et al., 1999; Weber et al., 2003), non-human primates (Weber et al., 2003), and humans (Bainbridge et al., 2008; Hauswirth et al., 2008; Maguire et al., 2008, 2009). Due to different outcomes from patients, more tests still need to be done in order to prove the efficacy for all patients.

Retinal implants using either epiretinal or subretinal implants is also a good therapy and have shown benefits in many tests with RP patients (Caspi et al., 2009; Humayun and de Juan, 1998; Yanai et al., 2007) and animals models (Chow and Chow, 1997; Zrenner et al., 1997). Clinical trials in human patients are already in Phase II, but still being improved.

Neurotrophic factors showed protective effects on photoreceptors degeneration, including neurotrophic factor (CNTF), Glial cell-line derived neurotrophic factor (GDNF), cardiotrophin-1, brain-derived neurotrophic factor (BDNF) and basic fibroblast growth factor (bFGF) (Hamel, 2006; Musarella and Macdonald, 2011) in animals (Bok et al., 2002; Frasson et al., 1999; Li et al., 2010; Liang et al., 2001; Tao et al., 2002) and are in the trial phase in humans (Sieving et al., 2006). Phase I and II are currently underway for treatment of atrophic macular degeneration and RP.

Retinal transplantation consists of the transplantantion of sheets of retinal cells, layers of photoreceptors or a complete retina (Hamel, 2006; Musarella and Macdonald, 2011). Alternatively, transplantation of photoreceptor precursors has also been tried (Musarella and Macdonald, 2011). Transplants in animal models and humans showed that transplanted material does survive but not all presented evidence of a therapeutic effect (Gal et al., 2000; MacLaren et al., 2006; Radtke et al., 2008).

Stem cell treatment is being used to regenerate degenerated retinas, based on its capability to give rise to specialized cells and also its capability of self-renewal (Lund et al., 2006). The donor cells and the host recipient are two major aspects of the procedure (Rivas and Vecino, 2009). Hippocampal, embryonic and bone marrow cells have been tested in models of retinal degenerative disorders and have showed high migratory and differentiation capacity. In contrast, the use of retinal stem/progenitor cells in suspension or as neurospheres exhibit poor migration, but were successful in expressing retina specific markers after transplantation. Although progress is being made, extrapolation to humans is yet to be demonstrated.

Neuroprotection using antiapoptotic factors or inhibitors of apoptosis is also being explored (Hamel, 2006; Kannabiran, 2008). Studies in mouse and rat models have shown protection of photoreceptor cells (Bennett et al., 1998; Leonard et al., 2007; Liu et al., 1999; Petrin et al., 2003).

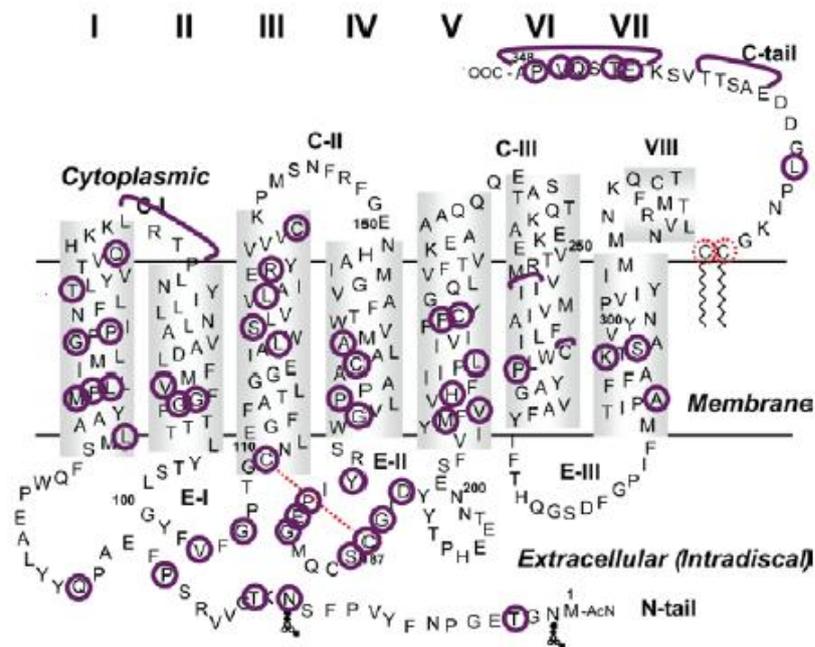
1.1.7 Rhodopsin as a target in retinitis pigmentosa

The causes for RP are heterogeneous, and mutations in many different genes can lead to RP. The first of these genes that has been identified as a genetic cause for RP was the rod photoreceptor rhodopsin. Now more than 150 mutations in rhodopsin have been identified in different RP patients (Cooper et al., 2007). From these, 92 are single point mutations in 64 amino acid positions (Figure 1.11). This is a remarkable number considering that rhodopsin is a protein with a total length of only 348 amino acids. 42 of these RP mutations have been studied *in vitro* and 31 of them (>70%) were found to cause misfolding of rhodopsin.

Many studies have focused on understanding the molecular mechanism underlying rhodopsin mutations leading to RP development. The majority of the mutations studied previously focused on the EC and TM domains of rhodopsin (Dryja et al., 1993, 1995). Most of these mutations mediate formation of an incorrect disulfide bond, leading to an incorrectly folded protein that cannot bind *11-cis* retinal (Kaushal and Khorana, 1994; Ridge et al., 1995). This finding suggested a structural coupling between the EC and TM regions, which is important for the correct folding of rhodopsin.

Since the discovery of misfolding of rhodopsin as one of the leading causes for RP (Kaushal and Khorana, 1994), much effort has concentrated on rescuing correctly folded rhodopsin by adding excess Vitamin A or retinoid derivatives. Recently, a clinical trial of nutritional supplements for adults with common forms of RP was conducted, and demonstrated that vitamin A treatment alone cannot cure RP (see above).

Figure 1.11 - Secondary structure model of rhodopsin showing the positions of the mutations that cause RP and congenital stationary night blindness.



SOURCE: Balen, 2012

1.1.8 Animal models of retinitis pigmentosa

Hereditary retinal degeneration affects human and other animals. The range of diseases that affect animals can be due to natural occurrence or obtained through genetic manipulation. These various mutations are of great importance as model system for the study of the disease evolution, pathogenesis and strategies for treatment. Specifically for RP, there are many animal models available and many of them mimic the disease that is found in humans.

The list of animal models that present mutations that occur naturally includes dogs, cats and chicken. Natural and induced mutations are studied in mouse, rats and pigs. These animal models were reviewed recently (Rivas and Vecino, 2009) and are summarized here.

Canine animal models of RP are considered the best models due to the similarity between dog and human eye disorders. Furthermore, since eye anatomy and size in dogs is similar to human, the same apparatus for physical studies can be used for dogs as that for humans. All canine diseases present rod-cone degenerations, where rod disease, dysfunction, and death precede the loss of cones. These diseases are analogous to human RP and are called Progressive Retinal Atrophy (PRA). The genes mutated include PDE6A and PDE6B encoding

the α and β subunits of the cGMP specific phosphodiesterase (autosomal recessive RP), RPE65 gene (leber congenital amaurosis), the RP GTPase regulator RPGR (X-linked RP) and rhodopsin RHO (autosomal dominant RP). A list of available canine models is provided below:

- erd - canine early retinal degeneration
- rcd-1 - rod-cone dysplasia type 1 - and rcd-2 - rod-cone dysplasia type 2
- RHO - rhodopsin, visual pigment of rod photoreceptor
- RPE65 – membrane-associated protein
- RPGR - mutations in the RP GTPase regulator
- RPGRIP1 – mutations in the RP GTPase regulator-interacting protein 1

Cats are good models too since they have a manageable eye size for examination and manipulation. Their visual neurophysiology is well characterized and their eyes are physiologically and anatomically similar to human. There are only two forms of feline PRA that are well studied:

- rdAc - autosomal recessive rod-cone degenerative disorder
- Rdy model - autosomal dominant early-onset retinal degenerative disease

Chicken eyes are also useful to study since they can be compared with human eyes in size, facilitating pathological examination. However, chicken eyes are very different from human eyes from a number of perspectives. In particular, the chicken eye is cone-dominated rather than rod-dominated. However, the conservation of gene order between chicken and humans is similar to that between human and mice genome, making this a potential model for the study of spontaneously occurring inherited blindness disease. There are two models of chicken that presents retinal degeneration:

- rd – retinal degeneration chicken strain
- rdd – retinal dysplasia and degeneration

Mice are the most studied animal models, and the rod-less mouse was the first animal model where retinal degeneration was described. Mouse and human have a genomic similarity of about 90%, and gene correlation can be precisely achieved. Advantages in the use of mouse

include the short life span (about 2 years), availability of protocols that are well established and the ease at which new single gene diseases can be mapped. Available mouse models are:

- Peripherin rds – retinal degeneration slow, rds-peripherin null mutation
- Rd – retinal degeneration, which includes rd1, rd4, rd8, rd10, rd12
- CEP290: rd16 – novel centrosomal protein
- I-255/256 – mutant opsin gene with 3-bp deletion of isoleucine at codon 255/256
- Knockout RPE65 (RPE65^{-/-}) - accumulates retinyl esters in the RPE and lacks 11-cis retinal
- P347S - proline-347 to serine mutation in rhodopsin
- Peripherin-rds 307 – mice heterozygous for the codon 307 mutation
- Q334ter - transgenic mice that express a truncated rhodopsin due to a mutation resulting in a stop codon at position 344
- Knockout rho (rho^{-/-}) – mutations in the rhodopsin gene (have no functional rhodopsin gene)
- VPP – mice express a mouse opsin gene containing three point mutations within a seven amino acid sequence near the N-terminus of the molecule
 - P23H - histidine for proline mutation
 - V20G – glycine for valine mutation
 - P27L – leucine for proline mutation

Rats exhibit retinal degeneration very similar to the retinal degeneration observed in humans. The benefits of using rat photoreceptors are that they have been well studied, therefore extensive knowledge of basic biological mechanisms is known. Morphologically, the rat eye is several times larger than the mouse, which simplifies surgical manipulations and electroretinographic evaluation; they also breed as rapidly as mice, generating large litters in a short gestational time. The following lines are available:

- RCS – Royal College of Surgeons. Retinal pigment epithelium (RPE) is unable to phagocytose shed photoreceptor outer segments.
- P23H – histidine at proline 23 position in rhodopsin. Rat carries a mutant mouse opsin gene in addition to the endogenous native opsin genes

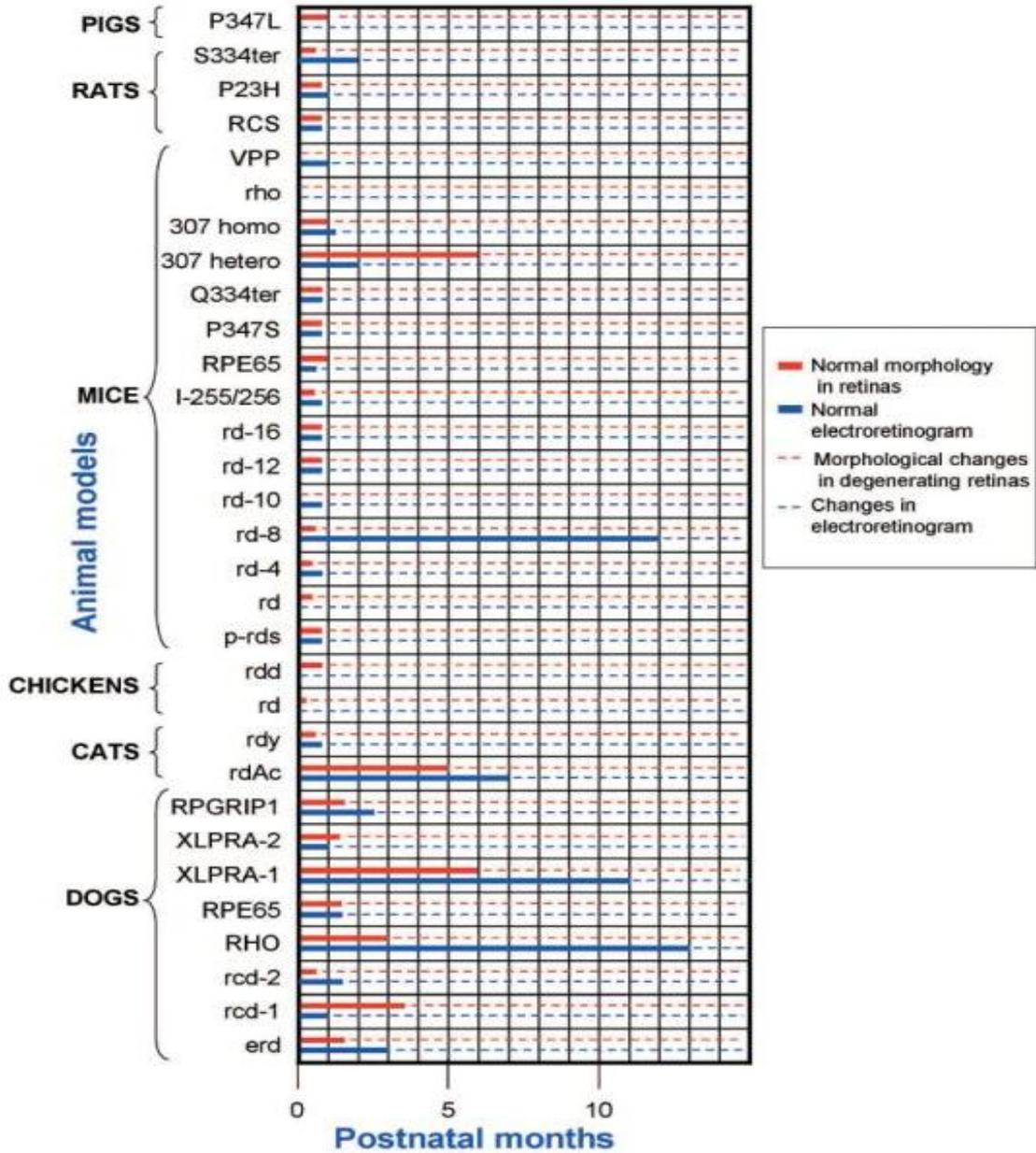
- S334ter – the opsin transgene contains a termination codon at residue 334, resulting in the expression of a rhodopsin protein lacking the last 15 C-terminal amino acids

Pigs are genetically engineered animal models. Pig eyes are similar in size to the human eyes. They have a similar number and distribution of rod and cone cells as humans, which makes them an excellent model for RP. Among other large mammals, the porcine retina is even more similar to the human retina in size, and tools applied for diagnostics can be used without adaptations.

- P347L - lysine for proline at position 347 in rhodopsin
- P347S - serine for proline at position 347 in rhodopsin

The figure below represents the time of initiation of retinal degeneration in each animal model (Figure 1.12). Knowledge of the different degeneration times is important in order to decide which model is more relevant to specific studies such as conducted as part of this thesis.

Figure 1.12 - Schematic representation of the initiation of the retinal degeneration in each animal model.



Continuous lines: normal retina (non-degenerating); discontinuous lines: degeneration of the retina; Red: morphological states; Blue: electroretinographic state; each square represents a postnatal age of the animal (months).

SOURCE: Figure is taken from (Rivas and Vecino, 2009). With permission.

1.2 OPEN QUESTIONS AND THESIS OBJECTIVES

1.2.1 Open questions

Previous studies by Khorana and coworkers have addressed the identification of the amino acids stabilizing rhodopsin and have established that there is tight coupling between the extracellular, transmembrane and cytoplasmic domains in rhodopsin (Khorana, 2000). Disruption of this coupling causes irreversible misfolding. Recent simulations of misfolding of the rhodopsin tertiary structure have provided a mechanistic understanding of these findings (Rader et al., 2004). A major core of residues contributing to rhodopsin stability was identified at the interface between the extracellular and transmembrane domains. A small additional region at the conserved DERY motif in the cytoplasmic domain was also implicated in playing a role in rhodopsin stability. More than 90% of the residues in the large core cause misfolding upon mutations (Rader et al., 2004). Furthermore, recent findings with RP patients and molecular studies have correlated these molecular studies with RP patients studies (Iannaccone et al., 2006). This indicates that it may be possible to develop new avenues for RP treatments based on stabilization of rhodopsin structure. Such new avenues are needed because there is, so far, still no cure or treatment for RP.

Here we explore novel approaches for the stabilization of opsin/rhodopsin through small molecules. Rhodopsin has been shown to be a receptor for anthocyanins (Lila, 2004) and its spectral range can be extended by interacting with porphyrin compounds (Washington et al., 2004). Members of the flavonoid group of phytochemicals, anthocyanins are mostly found in teas, honey, wines, fruits, vegetables, nuts, olive oils, cocoa and cereal. Anthocyanins pigments and the flavonoids associated are known to protect against many kinds of diseases. The enhancement of night-time vision by anthocyanins is known (Lila, 2004). Porphyrins are an omnipotent class of naturally occurring compounds with many important biological representatives such as hemes and chlorophylls. Previous evidence from our laboratory suggests that porphyrin and anthocyanin compounds both stabilize rhodopsin and/or opsin (Yanamala, 2009).

In addition, studies have shown that providing patients with more retinal, via administration of Vitamin A, can only partially slow down RP. So far, many alternative approaches have been proposed, but none have an entirely positive effect on reversing the

disease. The hypothesis that molecules other than retinal can alone or in conjunction with retinal rescue folded rhodopsin may lead to new treatments of RP.

1.2.2 Objectives and Approaches

This thesis aims to investigate the hypothesis that molecules other than retinal can rescue folded rhodopsin and/or reduce photoreceptor cell death, which could potentially lead to new treatments for RP. To address the open questions, the following specific aims are proposed:

Specific aim 1: In vitro molecular characterization of WT and different rhodopsin mutants

A study comparing two RP mutations N15S and P23H with WT rhodopsin was performed. Rhodopsin and mutants were expressed in COS-1 and HEK-293 cells followed by purification after reconstitution with *9-cis* or *11-cis* retinal, during or after expression in the cells. This provided a quantitative understanding of the role of retinal in the rescue of folded rhodopsin. Using thermal denaturation, Total Reflectance Fourier Transform Infrared (ATR/FT-IR) and fluorescence spectroscopy, SDS-PAGE mobility, glycosylation studies, and subcellular localization using confocal microscopy, a quantitative comparison of molecular properties of the mutants as compared to the WT was assessed.

Specific aim 2: Effects of ligand binding on rhodopsin structure and stability

First, stability studies using the protein rhodopsin from native source was carried out. Next, the effect of different compounds such as metal ions, porphyrins and anthocyanins were tested in order to assess their effect on the stability of rhodopsin. UV-visible, fluorescence and CD spectroscopy were used in order to compare of molecular properties of the WT rhodopsin alone and in presence of the different ligands tested.

Specific aim 3: *In vivo* drug treatment of light-damaged and mutant rats with hereditary RP disease

The aim was to test whether Ce6 is able to prevent apoptosis in Sprague Dawley rats submitted to light-induced degeneration, and in P23H and S334ter rat models of RP. Morphological and functional (ERG) analyses were performed.

7 CHAPTER 7: INTEGRATION OF *IN VITRO*, *IN VIVO* RAT MODEL AND PATIENT STUDIES IN RETINITIS PIGMENTOSA AND FUTURE STUDIES

7.1 SUMMARY

In parallel to our molecular and *in vivo* model studies, our collaborator Alexander Iannaccone at the Retinal Degeneration & Ophthalmic Genetics Service & Lions Visual Function Diagnostic Laboratory, Hamilton Eye Institute, Department of Ophthalmology, and University of Tennessee Health Science Center, USA, conducted patient studies of P23H and N15S mutants. This provides us with the opportunity to compare *in vitro*, *in vivo* and patient data directly. Here, I therefore present a summary of the major findings of my work integrated with the patient studies. To this end, the manifestations, variability in severity and disease progression rates obtained from patients pertaining to families carrying either the N15S or P23H mutation is compared with the *in vitro* biochemical properties of the corresponding mutant rhodopsin. The results are integrated into a comprehensive model for the role of rhodopsin as a target for small molecules in RP degeneration and rescue. Finally, the prospects for novel approaches to prevent and treat RP in animals and patients are discussed.

7.2 INTRODUCTION

Based on clinical studies, ADRP is divided into two classes – class A and B (Cideciyan et al., 1998). Class A is characterized by severe rod function abnormalities based on ERG and perimetry measurement, which results in consistent early night blindness while there is measurable cone-mediated activity even later in life. On the other hand, Class B shows measurable rod function (partial ERG preservation) as well as preserved cone function and is characterized by variable onset of night blindness (some asymptomatic). Class B is further divided into subclasses B1 and B2. B1 presents regional variation of retinal damage, while B2 indicates a diffuse disease condition with no regional predilection. Therefore, in class B mutants, cone loss is spatially and temporally related to rod loss and the rate of cone loss is slower than that of rods.

Bridging the gap between the classifications of mutants based on disease phenotype in patients and the classifications based on *in vitro* biochemical properties of *RHO* mutations, requires a detailed comparison between the clinical manifestations of the mutations and the molecular properties of the proteins. Many rhodopsin mutations have been studied *in vitro* and are classified according to its *in vitro* behavior (Kaushal and Khorana, 1994; Sung et al., 1991, 1993). *In vitro* classification of ADRP mutants has been carried out based on their molecular phenotype, with three classes (Kaushal and Khorana, 1994): Class I, II and III. Class I resembles WT rhodopsin where the mutants fold correctly, bind the *11-cis* retinal chromophore normally, but are inefficient in the inactivation of transducin, the cognate G protein complex. Class II mutants do not traffic out of the endoplasmic reticulum and do not bind *11-cis* retinal. Class III mutants are expressed at low levels, poorly form rhodopsin chromophore and are also retained in the endoplasmic reticulum, presenting high mannose glycosylation.

Previously, several class B ADRP patients, carrying mutations in different domains of rhodopsin, namely in the extracellular domain, in the second intradiscal loop (P180A and G188R), and in the cytoplasmic domain, at the end of helix III (R135L and R135W) were studied (Iannaccone et al., 2006). The degrees of severity and progression rates in patients correlated with the computationally predicted and experimentally confirmed molecular properties of the respective rhodopsin protein (Iannaccone et al., 2006).

In this thesis, I investigated two mutants located at the same region of rhodopsin (N-terminus in the extracellular domain). They are both grouped in the same class, based on clinical phenotype and molecular properties. This provided an opportunity to test the hypothesis that clinical phenotype and molecular properties are related at a finer grained level. To this end, an in-depth comparison of the molecular data with the clinical characteristics studied by our collaborator is provided in this chapter.

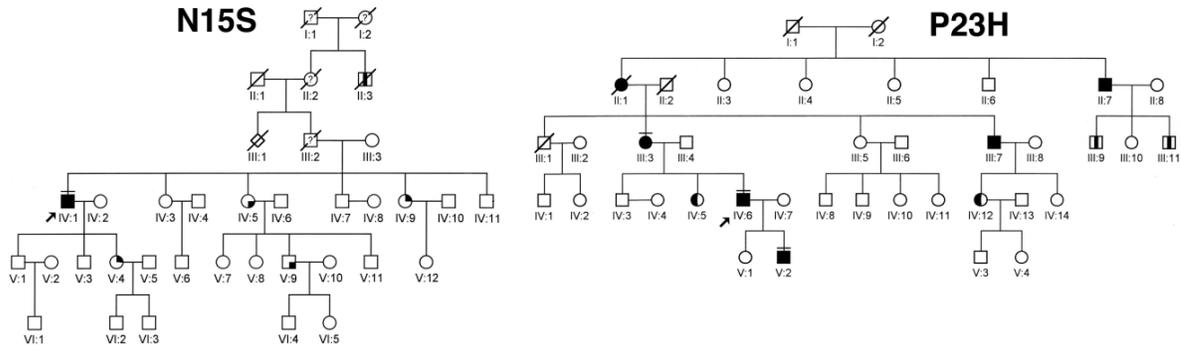
The *in vitro* data described in this thesis showed that the mutant rhodopsin proteins N15S and P23H are structurally impaired, but present different characteristics and severity of impairment. There is to date no rat or mouse model of N15S, so a comparison in an *in vivo* animal model is currently not possible. We are therefore fortunate to have access to *in vivo* patient data instead through our collaborator, Alex Iannaccone, University of Tennessee. His *in vivo* patient data, summarized in this chapter, below, confirms the phenotype observed *in vitro*. Even though both mutations belong to the same class, the investigated patients presented

remarkable differences. P23H affected patient show a greater disease severity than the N15S patient. This supports the conclusion that the instability of the rhodopsin protein structure is linked to disease phenotype and severity. Thus, it should be possible to lessen the disease burden of patients with strategies that stabilize the rhodopsin structure. In order to improve the conditions of RP affected patients, new strategies for treatment of RP are being explored, and are at different stages of trial. Here, the use of different ligands was tested, as a new approach for the stabilization of the structure of rhodopsin. We first demonstrated the success of this strategy *in vitro*. Since the results *in vivo* were promising, especially for Ce6 alone and in conjunction with Zn^{2+} , it was also tested if Ce6 would also help to stabilize the mutant protein *in vivo*. While we cannot test binding and stabilization effects on rhodopsin *in vivo*, we can use the end product – prevention of retinal degeneration as indirect evidence for a success in treatment. To this end, two studies were performed. First, a light-degeneration model, and secondly two inherited RP rat models. Ce6 did show some (albeit weak) evidence for a prevention of light-induced retinal degeneration, but did have a clearly positive effect in one of the models, P23H. Interestingly, a negative effect was observed in the other rat model, S334ter. These effects are likely attributable to the type of the mutation and its localization.

7.2.1 Summary of patient data carrying P23H and N15S mutations

Clinical studies were performed by Alexander Iannaccone are briefly summarized here. Two individuals of each family were investigated, each of them presenting one of the two RP mutations studied, P23H and N15S. The genealogies of the families are shown in Figure 7.1.

Figure 7.1 - Pedigrees of the two families investigated in this study.



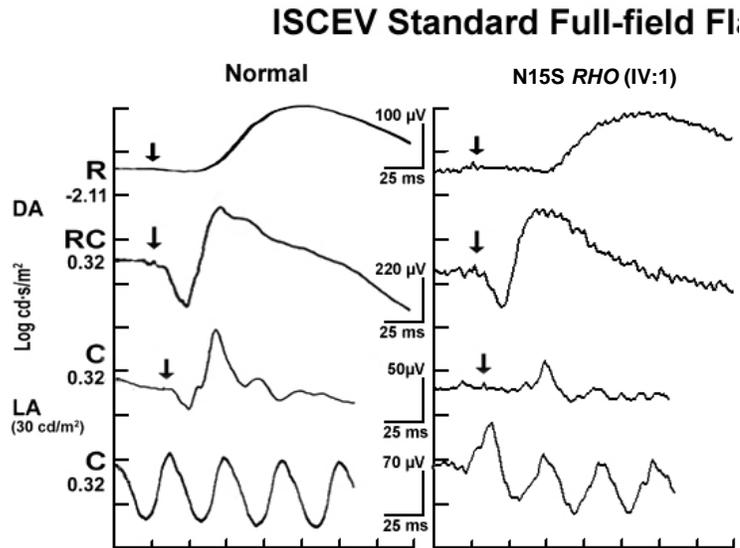
Proband from each family is shown by the arrow sign. Horizontal bar on top of the symbol correspond to subjects that were examined. Filled symbols identify affected individuals.

SOURCE: Not published. With permission.

Clinical studies included full-field flash ERG testing, Goldmann visual fields (GVFs), monochromatic automated perimetry (MAP) and dark adaptometry (DAPT). In all patients, the onset of night blindness was delayed (>30 years old) and visual acuity was $\geq 20/30$, but disease expression varied in severity across these *RHO* changes. Thus, both N-terminal mutations studied here are associated with a Class B phenotype.

The N15S mutation was associated with mild and sharply demarcated inferior retinal disease. Where in the inferior fields, both rod sensitivity loss (RSL) and cone sensitivity loss (CSL) were minimal at age 62, but rod dark adaptation was markedly and unevenly delayed across the entire visual field even after exposure to moderate amounts of ambient light. At age 66, Goldmann visual fields (GVFs) were virtually unchanged, CSL had slightly increased around fixation, and RSL had increased pericentrally. At age 62, rod, mixed, and cone ERGs of the N15S patient were reduced only about 80%, 60% and 75% of the lowest limits of normal, respectively, configuring a cone>rod pattern of retinal dysfunction. At age 66, rod and mixed ERGs had declined to about 50% of the normal lowest limits observed, whereas cone ERGs were unchanged. Formal DAPT testing at age 66 showed markedly delayed recovery kinetics of both rods and cones.

Figure 7.2 - Representative ISCEV-standard ERG response.



Full-field flash ERG testing of a N15S patient.

SOURCE: Not published. With permission.

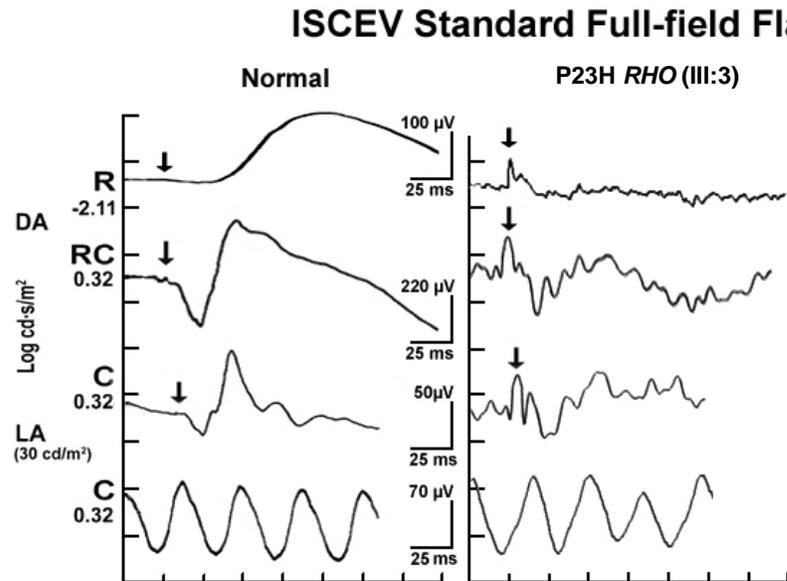
Similar to the N15S mutation, P23H also had regional (inferior and nasal) retinal disease predilection. This Class-B1 pattern was observed at age 12 (V:2) clinically and at the GVF as well as at the MAP level. However, unlike the N15S mutation, by age 41 (IV:6) the disease pattern transitioned to a widespread RSL>CSL (Class-B2 behaviour), followed by severe RSL and CSL with macular sparing only at age 74 (III:3). ON GVF testing, a peripheral island of nasal, inferior and temporal vision was also still present at age 74, but no regional/altitudinal pattern was evident. Also, in P23H patients, rod-driven ERGs were non-recordable to standard flashes since age 12 (V:2), but could be clearly elicited with brighter stimuli and b-wave splitting into separate cone- and a rod-driven peaks was also seen (loss of rod-driven sensitivity), as previously reported in class-B phenotypes (Iannaccone et al., 2006). This is in line with the partially preserved dark adaptation rod-mediated function on MAP. At this young age, mixed responses were fairly well preserved (~60%) and cone ERGs were normal. By age 41, mixed, transient cone, and flicker cone ERGs were reduced to about 10%, 45% and 75% of the lowest normal limits, respectively. At age 74, although markedly reduced in amplitude and markedly delayed, mixed and cone-driven ERGs (recorded with HK-loop electrodes) remained clearly recordable (~8%, 20% and 27% of the lowest normal limits, respectively). Overall, the P23H-associated phenotype was significantly more severe than the N15S phenotype.

Visual acuity is well preserved in both mutants, with most patients retaining $\geq 20/40$, although substantial inter-individual variability is observed, both between and within mutations. ERG measurements were also performed. Although instances of non-recordable ERGs have been reported in the literature, most patients retain measurable mixed ERG b-waves. Longitudinal measurements in the N15S proband (IV:1) with Class-B1 phenotype show a yearly rate of ERG b-wave loss from baseline of about 6.25%. This is very similar to that reported for of another Class-B1 mutant (P180A, 7%/yr) (Iannaccone et al., 2006). The ERG is much better preserved in N15S than in P23H.

It has been suggested that cone loss is spatially and temporally related to rod loss and the rate of cone loss is slower than that of rods in Class B mutants (Cideciyan et al., 1998). The *in vivo* data strongly support this hypothesis. Likewise, coexistence of Class-B1 and B2 features can occur in the same family in association with the P23H mutation, a transition that appears to be linked to disease stage. Despite evidence of regional disease and retention of rod function, the P23H mutation causes greater and earlier rod function loss than N15S, and an overall significantly greater disease severity.

The N15S phenotype is remarkable for the presence of a ring of pericentral disease, severely compromised kinetics of rhodopsin regeneration, and a cone > rod pattern of ERG dysfunction, but later in life rod disease progression continues to precede that of cones as postulated for Class-B phenotypes.

Figure 7.3 - Representative ISCEV-standard ERG response



Full-field flash ERG testing of a P23H patient.

SOURCE: Not published. With permission.

7.2.2 Comparison of P23H and N15S human patient data with *in vitro* studies

The clinical and functional characterizations of the two mutants are compared here to my molecular studies (subcellular localization using confocal microscopy, SDS-PAGE mobility, glycosylation studies, thermal stability and secondary structure content using spectroscopy and Attenuated Total Reflectance Fourier Transform Infrared (ATR/FT-IR), described in detail in Chapter 4). Being able to quantitatively compare the characteristics of the two mutants *in vitro* and *in vivo* allowed the study of the extent of severity in molecular and functional terms. The *in vivo* functional and clinical studies, expression profiles in cell culture and molecular/biophysical *in vitro* studies of the purified proteins all suggest that the phenotype of N15S is significant but is less than that of P23H. The correlation between the relative severities lends support to the hypothesis that the molecular properties of the mutant proteins are directly related to the clinical manifestations in patients. Despite the global classification into the same class, both from a clinical and a molecular perspective, a quantitative comparison reveals that the two mutations differ in severity of both, clinical manifestations, and molecular properties. Although not proving causality, this observation does provide strong support for the hypothesis that the molecular

properties of the mutant rhodopsin proteins are quantitatively linked to the phenotype observed in patients.

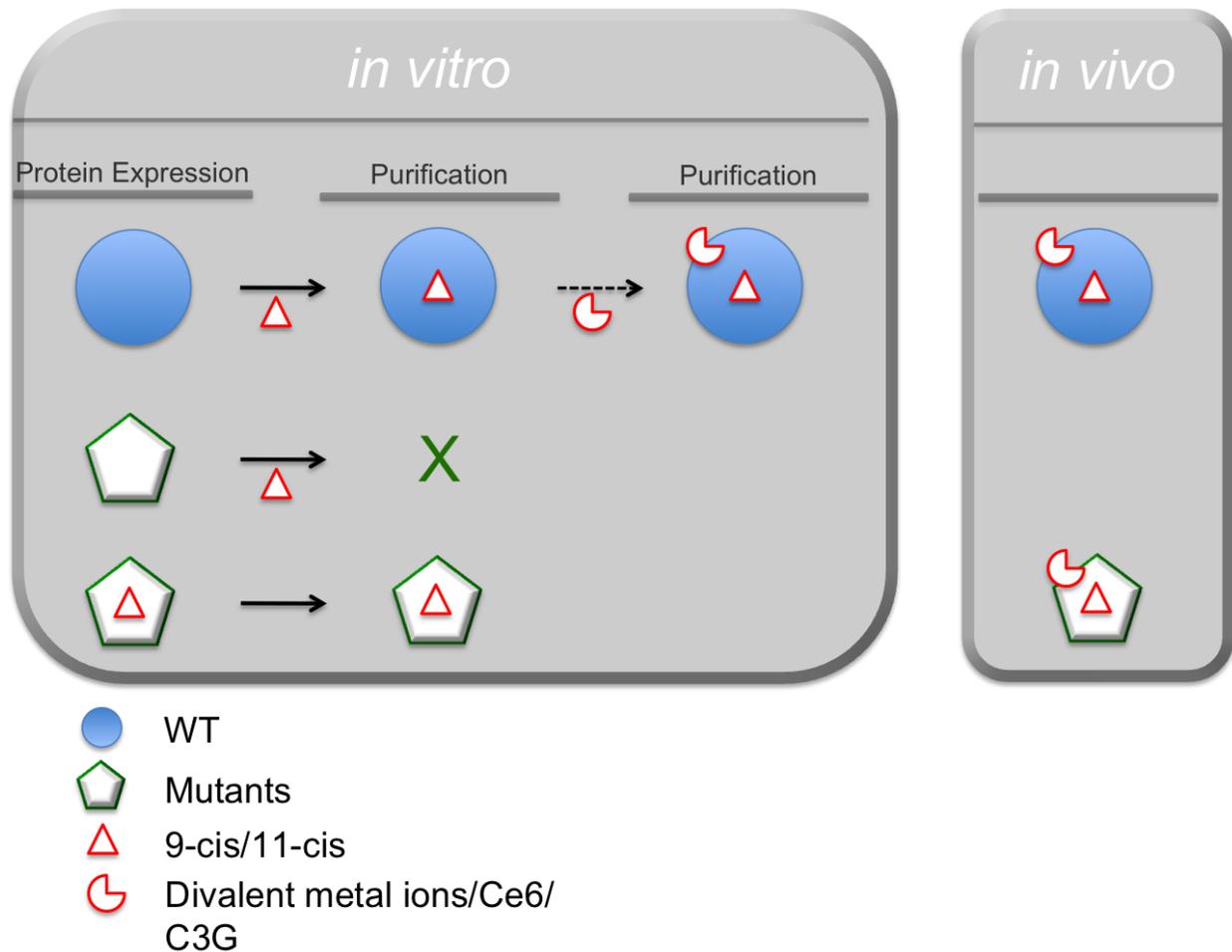
7.2.3 Comparison of Ce6 effects on RP progression *in vivo* versus *in vitro* model systems

Having established that stability of rhodopsin is related - possibly in a causal manner – to clinical phenotype of RP mutants P23H and N15S (Chapter 3) and also that stability can be enhanced by small molecules (Chapter 4), I then tested if the P23H phenotype can be rescued by the small molecule stability enhancer Ce6 in a rat model of P23H induced RP, described in Chapter 6. The results of ERG and retinal tissue analysis indicated that Ce6 exerts a positive functional effect by slowing the rate of photoreceptor degeneration of P23H rats *in vivo*. The studies indicated that Ce6 affects RP progression *in vivo* and the effects are likely linked to the binding of this molecule to rhodopsin as demonstrated *in vitro*.

7.3 FUTURE WORK - PROSPECTS FOR NOVEL APPROACHES TO PREVENT AND TREAT RP IN PATIENTS

This thesis investigated new approaches to treat RP. The emphasis was to find new treatments of those manifestations of disease caused by mutations in the photoreceptor rhodopsin. To this end the hypothesis was tested that compounds other than retinal can rescue folded rhodopsin and/or reduces photoreceptor cell death by means of exerting a direct effect on the structure and stability of rhodopsin. A conceptual view of the areas where rhodopsin can be targeted is shown schematically in Figure 7.4.

Figure 7.4 - Schematic representation of the *in vitro* and *in vivo* experimental steps taken in this work.



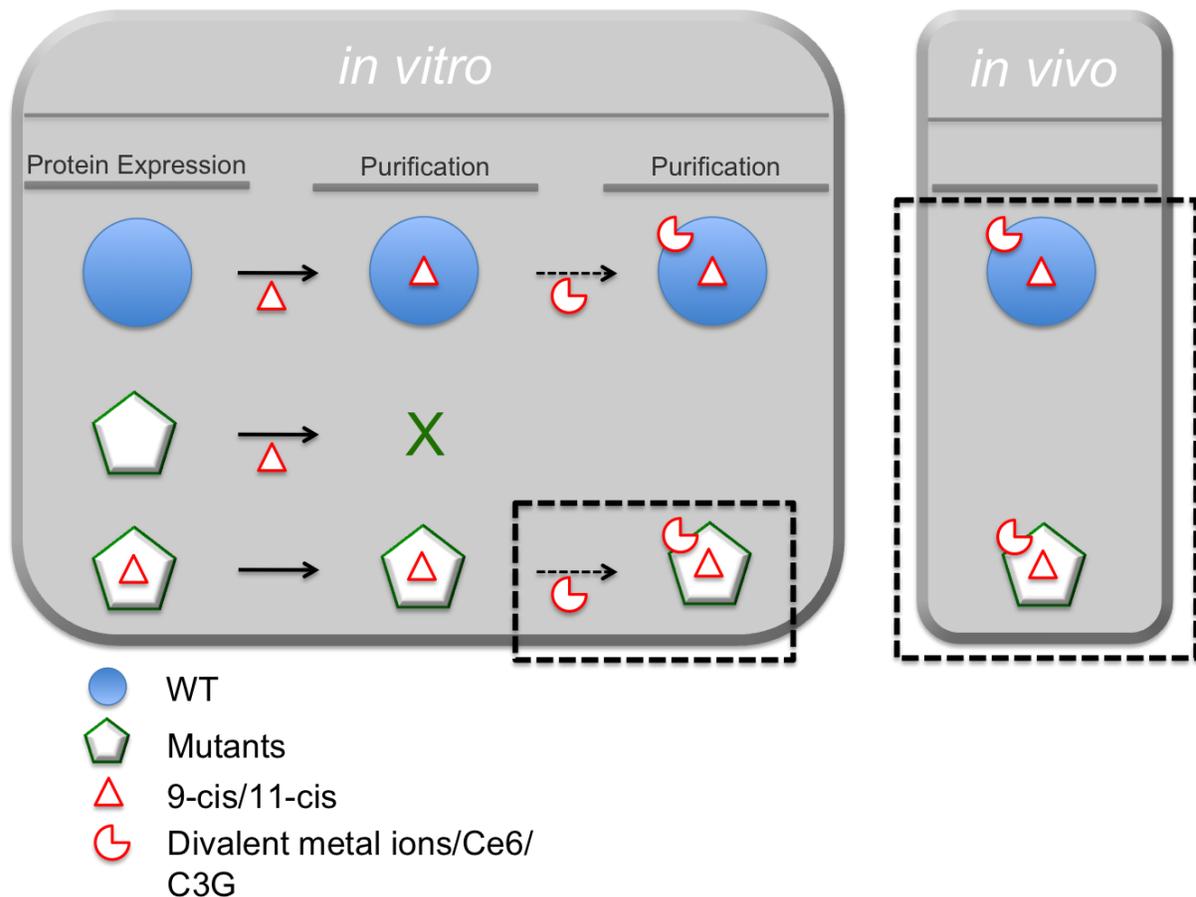
SOURCE: Balen, 2012

It was previously found and confirmed in this thesis for the particular mutants under study that the addition of 9-*cis* and 11-*cis* retinal during protein expression in cell culture improves the folding of rhodopsin mutants, increasing the amount of correctly folded rhodopsin. This *in vitro* result is in agreement with *in vivo* studies where vitamin A was administered to patients and partially slowed down the RP. However, retinals are able to only partially improve rhodopsin folding and large amounts of vitamin A are toxic. Therefore, in this thesis, other molecules were also tested *in vitro* and *in vivo*. Divalent metal ions (Zn^{2+} , Cu^{2+} , Fe^{2+} , Ni^{2+} , Mg^{2+} and Mn^{2+}), the anthocyanin compound C3G and the Chlorophyll derivative Ce6 were tested *in vitro* as a mean of stabilizing the structure of rhodopsin. All these molecules exerted an effect on opsin/rhodopsin structure at different extents and directions (stabilization and destabilization). Metal ions and Ce6

conferred a pronounced increase in the thermal stability of the secondary structure of rhodopsin. The Ce6 molecule was also tested in light-induced degeneration and hereditary rat models *in vivo* and has shown an effect on retinal protection. In contrast, C3G exerted a destabilizing effect on rhodopsin structure while enhancing the regeneration rates and were therefore only tested *in vitro*. This thesis work has shown that Ce6 but not C3G can be used to help in the rescue of folded rhodopsin and/or reducing photoreceptor cell death. This provides a novel avenue and a complementary one to current approaches to clinically study the disease and help patients that are affected with hereditary RP.

A number of areas for future exploitation of this finding and further consolidation of its premises are opened up by the work of this thesis. Shown in Figure 7.5 are my suggested areas of concentration for future work.

Figure 7.5 - Suggested concentration areas for future work.



SOURCE : Balen, 2012

In vivo experiments could be conducted to better evaluate the effect of Ce6 on the prevention of retinal degeneration caused by light-damage. Factors that could be exploited are the number of rats, which could be increased, dosage and frequency of treatment, as well age at which the treatment is performed that could be varied. The parameters should also be extended to RP affected rats.

In vitro studies need to fill the current gaps opened by the results of my findings. Since addition of 9-*cis* and 11-*cis* during the expression of the rhodopsin mutants, P23H and N15S, mediated the purification of high amounts of protein, further studies can be pursued where the addition of different molecules, such as the use of divalent metal ions alone and in conjunction with Ce6 can be investigated. To facilitate a direct comparison, the corresponding studies I have done in my thesis with WT rhodopsin need to be carried out with purified mutant proteins. Thus, all *in vivo* studies have to be matched with the corresponding *in vitro* studies. Given the promising results obtained in this thesis for WT rhodopsin *in vitro* for Ce6 alone and the combination of Ce6 and Zn²⁺, and the observation that Ce6 alone helps the P23H rats *in vivo*, strongly suggests, that the proposed approach will be successful.

Finally, to understand the negative results obtained with the *in vivo* rat model of RP, S334ter upon treatment of Ce6, *in vitro* analysis could be performed in order to comprehend the mechanism behind the effects of these molecules on the S334ter mutation. Specifically, we propose that Ce6 cannot bind to S334ter. This hypothesis needs to be tested experimentally. The stability of the mutant also needs to be investigated *in vitro* to see if stabilization in this case by binding of small molecules would be beneficial. Given that Ce6 is not a beneficial molecule for this particular rat model, other compounds, such as C3G and metal ions could be investigated.

7.4 IMPACT

Analysis of the interaction of various ligands with rhodopsin, results described in Chapter 3 of this thesis, demonstrated that there are different ligands to rhodopsin, other than *11*-retinal, that bind to rhodopsin. These are allosteric ligands and are believed to bind at the CP domain of rhodopsin (Yanamala, 2009). The interaction of these accessory ligands exerted different effects on rhodopsin. Ce6 and metal ions such as Zn^{2+} , Fe^{2+} , Cu^{2+} have shown to stabilize rhodopsin secondary and tertiary structure. C3G binding to rhodopsin resulted in the destabilization of retinal-protein interactions, which explains its effect on enhancement of the regeneration rates of rhodopsin. Yet, it was already known that Zn^{2+} deficiency causes retinal dysfunctions such as night blindness and neurodegeneration. Because Zn^{2+} binds directly to the photoreceptor rhodopsin and alters its stability, the stabilization of rhodopsin may be key to prevention and treatment of retinal dysfunctions. These findings can be of great potential importance for the treatment of retinal degeneration diseases, where mutations in the photoreceptor rhodopsin are known to be the major disease cause *in vivo*.

The stabilization obtained by Ce6 and Zn^{2+} *in vitro* and the effect of Ce6 *in vivo* can be potentially relevant to dysfunctions caused by nutrient deficiencies, but also for inherited diseases. *Retinitis Pigmentosa* (RP) is a disease that reflects a large group of genetically heterogeneous disorders marked by early rod photoreceptor dysfunction and progressive degeneration of rods and cones (Lam, 2005). The initial causes for RP are heterogeneous, and mutations in many different genes can lead to RP development (Berson, 1993c; Cooper et al., 1998). The first of these genes that has been identified as a genetic cause for RP was rhodopsin. Now more than 150 mutations in rhodopsin have been identified in different RP patients (Cooper et al., 1998). 42 of these RP mutations have been studied *in vitro* and 31 of them (>70%) were found to cause misfolding of rhodopsin (Rader et al., 2004). Since *in vitro* misfolding and cell-based retention in the endoplasmic reticulum have been proposed to be one of the molecular triggering events associated with retinal degeneration in RP in 1994 (Kaushal and Khorana, 1994) much effort has concentrated on rescuing correctly folded rhodopsin with excess Vitamin A or retinoid derivative. A clinical trial of nutritional supplements for adults with the common forms of RP (Kupfer, 1993) showed that vitamin A treatment alone cannot cure RP (Kupfer, 1993) but maybe additional means of stabilization of rhodopsin could open the way to a new and

more direct therapeutic tool, applicable to many different mutants of rhodopsin as long as their stabilization contributes to the mechanism of RP prevention/slow-down.

The studies presented here do not only have direct impact on vision. Rhodopsin is a prototypic member of the GPCR family, which accounts for 2 percent of the human genome and is the target for approximately 50 percent of current pharmaceutical compounds (Jacoby et al., 1997). Indeed, Zn^{2+} has been found to bind also to other members of the GPCR family and has been shown to have physiological roles in some of these receptors. For β 2-adrenergic, dopamine, and melanocortin receptors, Zn^{2+} is a positive allosteric modulator of agonist binding (Holst and Schwartz, 2003; Norregaard et al., 1998; Schetz et al., 1999; Schetz and Sibley, 1997). Other studies presented experimental evidence showing that Zn^{2+} also acts as a signaling molecule (Hershinkel et al., 2001). Inhibition of G protein activation was observed for rhodopsin (Sheikh et al., 1996), the β 2-adrenergic receptor and the parathyroid hormone receptor (Sheikh et al., 1999). These are only some GPCRs for which Zn^{2+} effects have been reported and it is likely that other GPCRs will be affected also. In this thesis, it has been shown that there can be potential benefits in combining Zn^{2+} with other allosteric modulators, such as Ce6, to modulate the stability of GPCRs as well as interactions of the GPCRs with their signaling ligands. It is of particular importance to note that Ce6 is believed to bind in the CP domain of rhodopsin (Yanamala, 2009), an area that is highly conserved in GPCRs in general. Thus, binding of allosteric CP ligands and stabilization of the respective receptors may be achieved not only in rhodopsin but also other GPCRs.

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