**FERNANDA BALEN**

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

Tese apresentada ao Programa de Pós-Graduacão 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

Orientadora: Prof[a]. Dr[a]. Dânia Hamassaki

Co-orientadora: Prof[a]. Dr[a]. Judith Klein-Seetharaman

Versão corrigida. Versão original eletrônica encontra-se disponível tanto na biblioteca do ICB quanto na biblioteca digital de teses e dissertações da USP (bdtd).

São Paulo
2012

## 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:

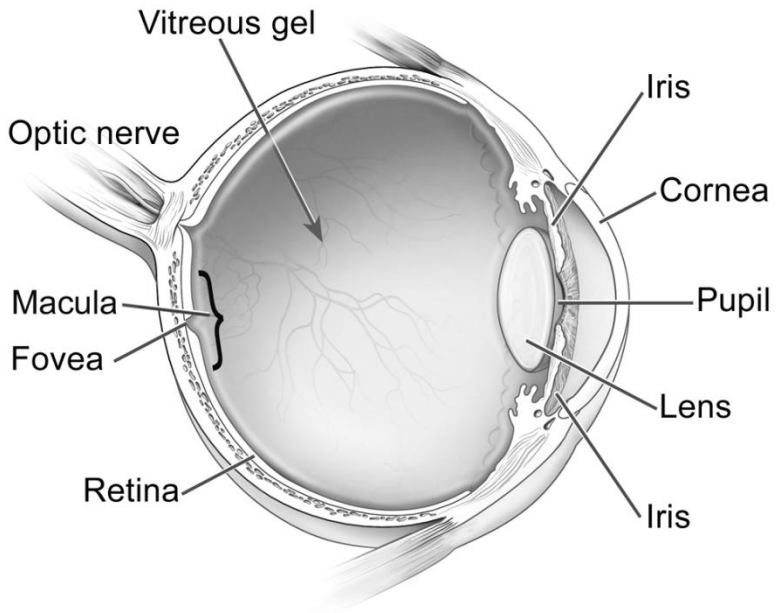
> To suppose that the eye, with all its inimitable contrivances for adjusting the focus to different distances, for admitting different amounts of light, and for the correction of spherical and chromatic aberration, could have been formed by natural selection seems, I freely confess, absurd in the highest 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

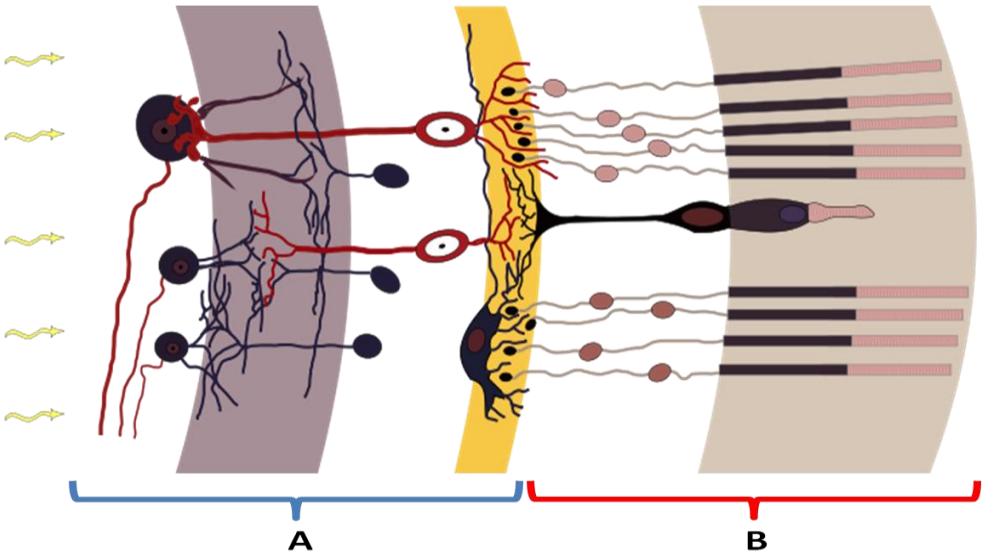
The retina is a very complex and layered structure that lines the back of the eye (Fig.1.1 and 1.2). It has a highly ordered anatomical organization, with a few basic classes of cells located in the outer nuclear layer (rods and cones), inner nuclear layer (bipolar, horizontal, and amacrine cells) and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



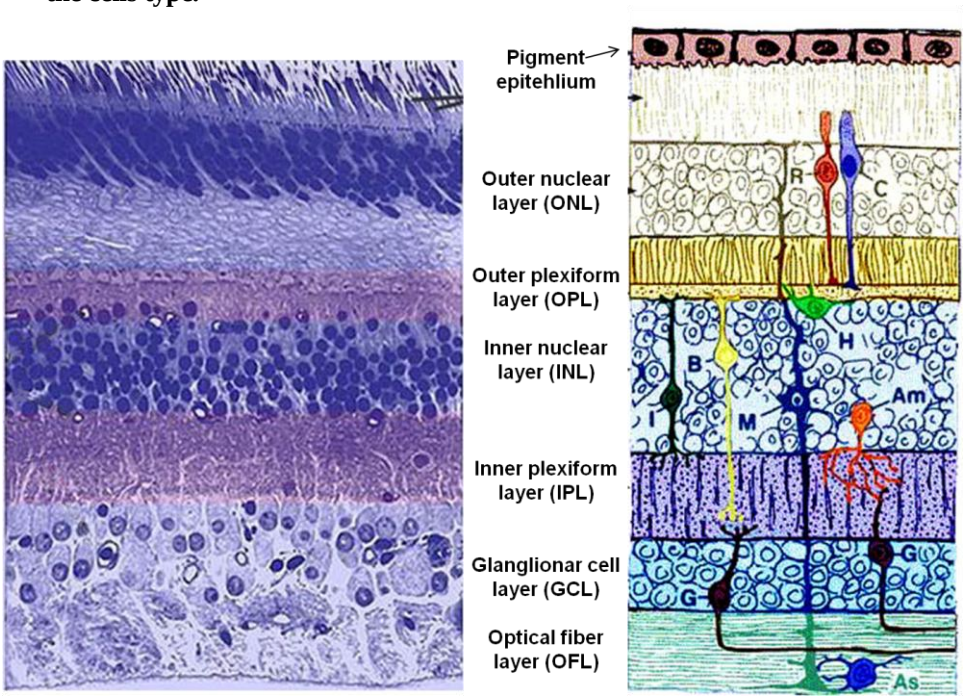

SOURCE: Drawing is adapted from (Cajal, 1900). With permission.

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.

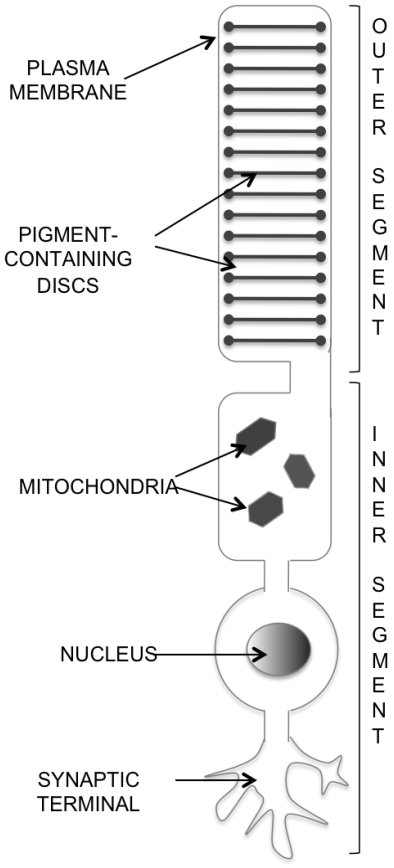
The photoreceptor cells, rods and cones, are responsible for different parts of visual perception. Rods, the most abundant photoreceptor cells, are responsible for black and white vision under dim light conditions. Rod mediated vision is very sensitive to light, but do not enable color vision. Cone mediated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