

# Retinoids for treatment of retinal diseases

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**Knowledge about retinal photoreceptor signal transduction and the visual cycle required for normal eyesight has increased exponentially over the past decade. Substantial progress in human genetics has facilitated the identification of candidate genes and complex networks underlying inherited retinal diseases. Natural mutations in animal models that mimic human diseases have been characterized and advanced genetic manipulation can now be used to generate small mammalian models of human retinal diseases. Pharmacological repair of defective visual processes in animal models not only validates their involvement in vision, but also provides great promise for the development of improved therapies for millions who are progressing towards blindness or are almost completely robbed of their eyesight.**

## Visual perception

The capacity of the brain to analyze and interpret information is limited ultimately by the sensory input it receives. For the human brain, visual perception is a major input pathway and an essential sense for understanding of the environment and social communication. The performance of this sense reflects an individual's genetic background, withstands many environmental insults and deteriorates with age [1–4]. Molecular investigations of the fundamental processes in vision initiation revealed that these can be divided into two stages: phototransduction, which propagates the light signal [5,6], and the visual (retinoid) cycle [7], which consists of metabolic pathways that regenerate the visual chromophore and thus sustain vision [8]. In reality, both pathways are fully integrated and complementary (Figure 1) [2,7,9]. Technical innovations and improved methodologies in proteomics and structural biology have led to substantial advances in our understanding of the molecular basis of vision [10,11]. Such advanced knowledge of the molecular basis of phototransduction and the visual cycle means that it is possible to manipulate these processes using highly specific retinoid-based therapies for disease states and visual deterioration due to aging and environmental insults [2,4,12]. The remarkable anatomy and physiology of the eye facilitate the use of unique and highly specific pharmacological approaches that are discussed in this review. Conceptually, these approaches can be extended to other biological systems that require delivery of therapeutics *in situ*.

## Phototransduction and the retinoid cycle

Sensitivity and adaptation to variable environmental light conditions are hallmarks of vision. Effective vision requires

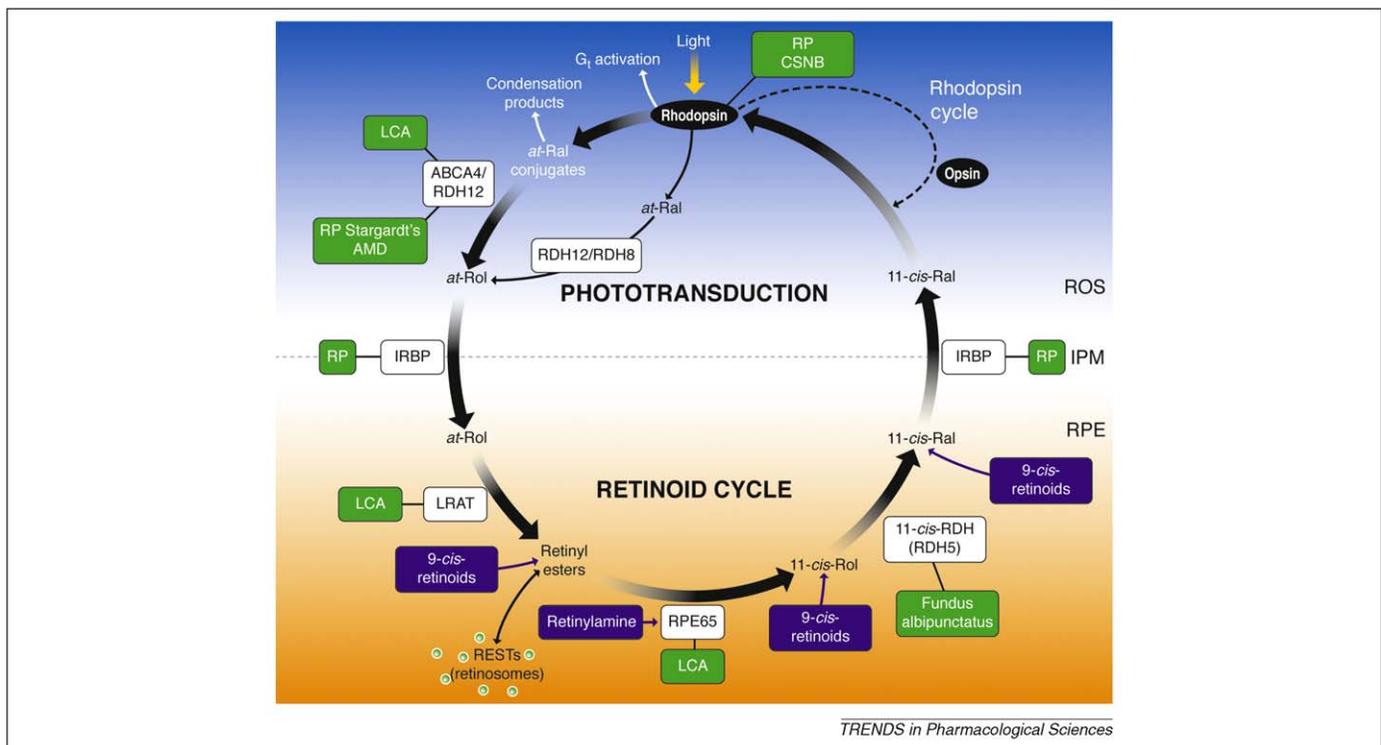
detection of light ranging from a single photon to a trillion photons per second and requires rapid restoration of the pre-illumination physiological state. This cyclic process is based on 11-*cis*-retinal, a light-sensitive chromophore derived from vitamin A that absorbs light in the visible range. When 11-*cis*-retinal binds to its cognate visual protein receptors (opsins), the resulting highly concentrated visual pigment is exquisitely sensitive to different wavelengths of light ranging from ~360 to 620 nm [13]. Production of 11-*cis*-retinal involves several enzymatic steps, collectively called the visual (retinoid) cycle, that is split between processes in photoreceptor cells and the adjacent retinal pigment epithelium (RPE).

Rhodopsin in rod photoreceptors and cone pigments in cone photoreceptors are the two classes of visual pigments that respond to light [13]. Their common chromophore, 11-*cis*-retinal, is covalently linked via a protonated Schiff base to a lysine side-chain amino group embedded within the opsin transmembrane domain, forming 11-*cis*-retinylidene. On photon absorption, the chromophore undergoes photoisomerization to all-*trans*-retinylidene, changing the visual pigments from an inactive to an active conformation [14]. In rods, this active form, known as Meta II, then recruits and binds intracellular G proteins, continuing the signaling cascade that culminates in visual perception (Figure 1) [15]. Various aspects of these phototransduction processes have been reviewed in depth elsewhere [5,6,16].

The retinoid (visual) cycle is a complex enzymatic pathway essential for regeneration of 11-*cis*-retinal. The maintenance of continuous vision and preservation of photoreceptor health require a continuous adequate supply of this aldehyde, so vertebrates have evolved the retinoid cycle to achieve this objective [2]. The pathway operates in sequential reactions in photoreceptor cells, in the RPE and back in photoreceptors, converting all-*trans*-retinal back to 11-*cis*-retinal via several chemical transformations. The classical vertebrate retinoid cycle contributes primarily to regeneration of rhodopsin in rod cells (Figure 1). The key enzyme in this pathway is retinoid isomerase or RPE65, which resides in the RPE. RPE65-dependent chromophore production might also be important for cone function [17]. However, the cone retinoid cycle seems to be supplemented by another metabolic pathway that is as yet genetically undefined [18–20].

The series of chemical reactions comprising the classical retinoid cycle is now well established (Figure 1). In rod cells, absorption of a photon of light by rhodopsin causes photoisomerization of 11-*cis*-retinylidene to all-*trans*-retinylidene, resulting in release of all-*trans*-retinal from

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**Figure 1.** Phototransduction and the visual (retinoid) cycle in vertebrates. Vision is triggered by light-dependent activation of rhodopsin or other visual pigments. In rod cells, this chromophore couples to the protein opsin, forming rhodopsin. Absorption of a photon of light by rhodopsin causes photoisomerization of 11-*cis*-retinal to all-*trans*-retinal. In turn, photoactivated rhodopsin generates activation of heterotrimeric G proteins, called transducin or G<sub>t</sub>, in photoreceptors. This G-protein-coupled receptor cascade is a classic cyclic nucleotide pathway that results in a decrease in cGMP levels (not depicted in the figure) and consequently hyperpolarization of the plasma membranes and ultimately reduction of glutamate secretion to secondary neurons. The visual cycle regenerates 11-*cis*-retinal from all-*trans*-retinal released from the chromophore-binding pocket of opsin. All-*trans*-retinal is reduced to all-*trans*-retinol in a reversible reaction catalyzed by RDH12 and RDH8, which are NADPH-dependent all-*trans*-retinol dehydrogenases. All-*trans*-retinol diffuses into the RPE, where it is esterified in a reaction catalyzed by lecithin:retinol acyl transferase to long-chain fatty acids. As a consequence of their propensity to aggregate, retinyl esters are stored in lipid-droplet-like structures called retinosomes. The all-*trans*-retinyl esters seem to be the substrate for RPE65, which converts them to 11-*cis*-retinol, which then is further oxidized back to 11-*cis*-retinal by RDH5, RDH11 and other NAD-dependent retinol dehydrogenases. 11-*cis*-Retinal formed in the RPE diffuses back into the ROS and COS, where it completes the cycle by recombining with opsins to form rhodopsin and cone pigments. Mutations in genes encoding proteins of phototransduction and the retinoid cycle are associated with various retinal diseases, some of which are indicated by the green boxes. Pharmacological intervention has been successful in animal models in a few instances, as indicated by the compounds in the blue boxes. IPM, interphotoreceptor matrix; IRBP, interphotoreceptor retinoid-binding protein; CSNB, congenital stationary night blindness; Ral, retinal; Rol, retinol; ABCA4, ATP-binding transporter 4.

the chromophore-binding pocket of opsin. Most of the all-*trans*-retinal that dissociates from opsin diffuses into the cytoplasm. A fraction that dissociates into the disc lumen also reaches the cytoplasm via transfer by ATP-binding cassette, transporter 4 (ABCA4) [21]. All-*trans*-retinal in the cytoplasm is reduced to all-*trans*-retinol in a reversible reaction catalyzed by an NADPH-dependent all-*trans*-retinol dehydrogenase (RDH). All-*trans*-retinol then diffuses into the RPE, where it is esterified in a reaction catalyzed by lecithin:retinol acyl transferase (LRAT). The acyl group is transferred from the sn1 position of ER phospholipids [22,23]. The most common lipids in the sn1 position are saturated long-chain fatty acids, with a palmitoyl group representing the predominant species. Thus, retinyl esters are dominated by retinyl palmitate, but also contain stearate (C18) and other minor species. These esters have a propensity to aggregate and in the RPE they form lipophilic droplets called retinosomes, which are discussed below. These all-*trans*-retinyl esters are presumably converted by the retinoid isomerase RPE65 to 11-*cis*-retinol, which is further oxidized back to 11-*cis*-retinal by RDH5, RDH11 and other RDHs. After diffusion from the RPE, 11-*cis*-retinal combines with opsin to form a light-sensitive pigment that is ready for another cycle of photoisomerization, signal transduction and regeneration.

### Retinoid metabolism and retinopathies

Retinoids are required for normal growth, vision, reproduction, and maturation and maintenance of the immune system [24]. Retinoids are also important regulators of metabolism in general [25]. All-*trans*-retinol is an essential micro-nutrient because it cannot be synthesized by animals and therefore must be absorbed as either retinol or retinyl esters from food of animal origin or generated from their precursor,  $\beta,\beta$ -carotene from plants. Dietary retinyl esters are hydrolyzed in the intestinal lumen, absorbed into intestinal enterocytes, re-esterified and incorporated into chylomicrons. They are then taken up by hepatocytes and either hydrolyzed and secreted after binding to the retinol-binding protein 4 (RBP4) complex or stored as lipid-droplet-like structures in cells called Ito or stellate cells. Thus, the liver is the largest storage depot for retinyl esters in the body [26,27].  $\beta,\beta$ -Carotene is symmetrically cleaved into two molecules of all-*trans*-retinal by members of the carotenoid oxygenase enzyme family [28–30]. Retinal is reduced to retinol, esterified and further processed in pathways that involve exogenous retinyl esters throughout the body [31]. Retinoid-metabolizing enzymes in the liver and peripheral tissues, including LRAT, transferases, hydrolases, diacylglycerol acyltransferase 1 (DGAT1) and acyl-coenzyme A transferase

(ACAT), are not highly specific, so they can process retinoid analogs as well.

Considering the fundamental role of retinoids in vision, it is not surprising that many forms of retinopathy are caused by defects in genes encoding proteins of the visual cycle (Figure 1) [2]. Storage of absorbed retinoids in the liver, their transport in the plasma and delivery to the RPE can all be impaired as a result of inactivating mutations in enzymes (such as LRAT), transport carrier proteins (such as RBP4) or receptors (such as STRA6). Progress in our understanding of these processes was possible because a large number of animal models of these defects were either generated or occur naturally [32]. In *Lrat*<sup>-/-</sup> and *Rpe65*<sup>-/-</sup> mice, lack of 11-*cis*-retinal leads to rapid degeneration of cone photoreceptors and progressive death of rods [33]. This phenomenon might involve the mechanism leading to the pathology seen in Leber congenital amaurosis (LCA) patients [17]. LCA has been attributed to continuous activation of visual phototransduction [34] due to basal activity of chromophore-free opsin [35–37], disordered vectorial transport of cone visual pigments lacking bound chromophore [38], instability of nonbound cone visual pigments or a combination of all these mechanisms.

More complete discussions of retinal diseases related to genetic alterations of phototransduction and the visual cycle can be found elsewhere [1,2,39,40]. Because components of the visual cycle and phototransduction are mostly non- or only partially redundant, genetic defects resulting in dysfunction of these proteins are manifest as retinopathies (for examples, see Figure 1). The severity of these genetic retinopathies is determined by the toxicity of the accumulated intermediates (e.g. condensation products of all-*trans*-retinal in Stargardt's disease) [41], the need of the product for cellular homeostasis (e.g. cGMP production in LCA caused by a mutation in guanylate cyclase) [42], the instability of the mutated protein structure [e.g. rhodopsin mutants in autosomal recessive retinitis pigmentosa (RP)] [43] and whether regulation of protein function is altered (e.g. Ca<sup>2+</sup> coordination in autosomal dominant cone-rod dystrophy mutants of guanylate cyclase-activating proteins) [44].

### All-*trans*-retinal, condensation products and degenerative retinal diseases

In many individuals the visual system degenerates with age. Prevention of vision loss requires a better understanding of the fundamental causes of age-dependent changes. Fortunately, our understanding of both retinoid metabolism outside the eye and production of 11-*cis*-retinal unique to the eye is accelerating [1,2,7]. Genetic mouse models are also now available to study these processes and their aberrations *in vivo* [45]. These advances allow the central question of what compromises photoreceptor cells and the underlying RPE to be addressed. Retinoids, despite being essential for vision, can also cause certain retinal pathologies when not tightly controlled.

Typically, retinoids are complexed with soluble proteins that protect them. These reactive compounds are bound by a number of retinoid-binding proteins and are rarely freely solubilized from membranes. Protection of retinoids also stems from their ability to cluster when esterified

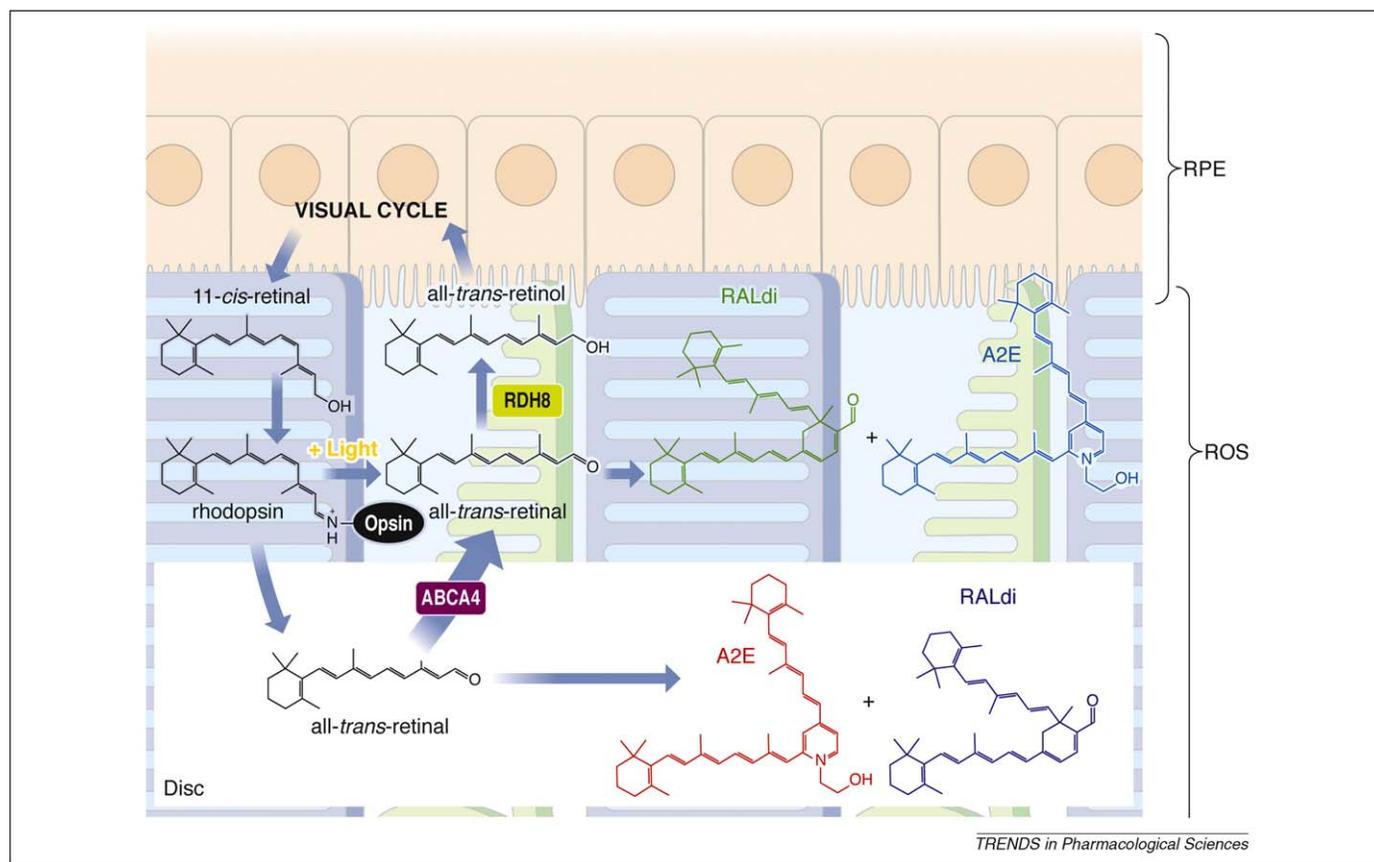
by long-chain fatty acids and to be stored in lipid-like droplets in the liver or as retinosomes in the eye [46–48].

To absorb light efficiently, visual pigments need to be very sensitive to light (11-*cis*-retinal requires a quantum efficiency of 0.65 [14]) and to be highly concentrated. Indeed, a large fraction of rhodopsin forms a paracrystalline structure in rod outer segments (ROS) [49–52] and cone pigments can form diffractable crystalline structures in cones [53,54]. ROS contain ~5 mM rhodopsin [55] that, if completely bleached, would yield an equal level of free all-*trans*-retinal. How can cells cope with such an aldehyde flux? Even less than 0.5% bleaching will produce toxic levels of all-*trans*-retinal if this retinoid is not properly cleared.

The efficiency of the mammalian visual system and the health of photoreceptors and RPE decrease significantly with age, suggesting that before cell death there are biochemical changes that slowly promote retinal damage. For example, an abnormally high flux of retinoids through the retinoid cycle can induce retinopathies in some mouse models [56–58]. This process in turn triggers host immune and other defense responses that culminate in retinal cell death. Even in the presence of an efficient and fully functional retinoid cycle, all-*trans*-retinal can condense, producing, among a myriad of other byproducts, di-retinoid pyridinium ethanamine (A2E) and all-*trans*-retinal dimer (RALdi) [59–61] (Figure 2). Initial condensation products are formed in the ROS and 10% of the ROS undergoes phagocytosis and accumulates in the RPE daily. Condensation products also accumulate with age [62] and these compounds can cause RPE cell toxicity under experimental conditions [63–65]. Patients affected by age-related macular degeneration (AMD), Stargardt's disease or other retinal diseases associated with accumulation of surrogate markers such as A2E all develop retinal degeneration [66].

Mutations in *ABCA4* cause Stargardt's macular degeneration [67], cone-rod dystrophy [68] and recessive RP [69,70]. Heterozygous mutations in *ABCA4* increase the risk of developing AMD as well [66]. A2E [71,72] and RALdi [61] are the major fluorophores of lipofuscins produced from all-*trans*-retinal [73] (Figure 2). As a consequence of aging, both A2E and RALdi can accumulate over a lifetime of light exposure [62], with toxic effects on RPE cells [74,75]. Patients affected by Stargardt's disease or AMD because of a disabled *ABCA4* gene and those affected by other retinal diseases associated with lipofuscin accumulation eventually develop retinal degeneration. *ABCA4* mutations are also linked to an increased risk of AMD [66]. However, no such degeneration was observed in *Abca4*<sup>-/-</sup> mice even though RPE atrophy was detected [21,41,76]. Thus, mice and humans do not always exhibit identical responses to fluorophore accumulation.

Recently, we showed that mice carrying double knockout of *Abca4* [21] and retinol dehydrogenase 8 (*Rdh8*), one of the main enzymes that reduce all-*trans*-retinal in ROS and cone outer segments (COS) [77], rapidly accumulate all-*trans*-retinal condensation products and exhibit accentuated RPE/photoreceptor dystrophy at an early age [58]. Retinas from these mice exhibited lipofuscin, drusen, basal laminar deposits, Bruch's membrane thickening and choroidal neovascularization. Importantly, the severity of their



**Figure 2.** Retinoid flow in the visual cycle and condensation of all-*trans*-retinal. After 11-*cis*-retinal binds to opsin, forming rhodopsin, the resulting visual chromophore, 11-*cis*-retinylidene, is photoisomerized to all-*trans*-retinylidene, the precursor of all-*trans*-retinal that is subsequently released. Most of the all-*trans*-retinal dissociates from opsin into the cytoplasm, where it is reduced to all-*trans*-retinol by RDHs, including RDH8. The fraction of all-*trans*-retinal that dissociates into the disc lumens is transported by ABCA4 back into the cytoplasm [21] before it is reduced. Thus, condensation products can be generated within both the disc lumens and the cytoplasm. Loss of ABCA4 and RDH8 exacerbates this condensation, reminiscent of an accelerated aging process. In humans, as a result of daily phagocytosis of part of the rod outer segments, lipofuscin fluorophores accumulate with age in the RPE, especially in RPE cells underlying the cone-rich macula [109,127]. Such accumulation has been considered to constitute one of the major risk factors for AMD, the predominant cause of legal blindness in developed countries [128]. Lipofuscin fluorophores are especially abundant in Stargardt disease, the most common juvenile form of macular degeneration [72]. A2E and RALdi, the major fluorophores of lipofuscin, are formed by condensation of phosphatidyl ethanolamine with two molecules of all-*trans*-retinal followed by oxidation and hydrolysis of the phosphate ester [129]. Various mechanisms have been proposed to explain the toxicity of A2E. These include its cationic detergent properties [130], physiological interference with RPE function [131,132] and radical reaction products induced by light-dependent oxidation [133].

visual dysfunction and retinopathy was exacerbated by light but attenuated by treatment with retinylamine, a visual cycle inhibitor that slows the flow of retinoids through the visual cycle, thus giving other oxidoreductase enzymes more time to detoxify retinaldehyde molecules. These findings provide direct evidence that aberrant production of toxic condensation byproducts of the visual cycle can lead to rapid and progressive retinal degeneration in mice and suggest a pharmacological method for amelioration of these conditions. The similarity of this retinopathy to human AMD means that these mice are invaluable for research aimed at ameliorating this devastating blinding disease.

Although the above studies strongly suggest retinoid toxicity, it still is unclear if high levels of retinal and/or its condensation products such as A2E actually cause these retinopathies or merely constitute non-specific manifestations of impaired retinoid metabolism. Recently, we reported that all-*trans*-retinal is probably responsible for photoreceptor degeneration in *Rdh8<sup>-/-</sup>Abca4<sup>-/-</sup>* mice [73]. Toxic effects of all-*trans*-retinal induce apoptosis through caspase activation and mitochondrial-associated cell death [73]. Therefore, excessive levels of this aldehyde must be

decreased to preserve retinal health. Although A2E formation could be a surrogate marker for aberrations in all-*trans*-retinal clearance, it might also represent a detoxification product and not the primary toxin previously supposed. Regardless of whether all-*trans*-retinal or A2E is responsible for retinal degeneration, prevention of all-*trans*-retinal accumulation will also stop production of A2E and other similar derivatives formed from this aldehyde.

### Retinoids are a diverse group of compounds with different biological activity

The fact that retinoids are often grouped together in the medical literature without distinction can be misleading. For example, retinol and retinoic acid have dramatic differences in biological activity and, even though retinol can be converted to retinoic acid, this transformation is highly regulated. Retinol has only limited biological activity *per se* [78] but it can be dehydrated to anhydroretinol, which might exert regulatory effects on the immune system [79] or it can be saturated in the 13–14 position to produce dihydroretinol precursors involved in metabolic processes [80,81]. In the retinoid cycle,

retinol is an excellent substrate for LRAT and is quickly converted into fatty acid esters. Their propensity to form oil droplets excludes fatty acid esters of retinol from the circulation. Retinol can be oxidized to retinal in a reaction catalyzed by a subset of short-chain alcohol dehydrogenases and medium-chain alcohol dehydrogenases. Because the redox potential of cells favors reducing conditions and the oxidation reaction is thermodynamically neutral, only a tiny fraction of retinol can be oxidized to fulfill the thermodynamic requirements of equilibrium. The all-*trans*-retinal formed probably does not exert direct biological activity, although there is speculation to the contrary [82]. Instead, this aldehyde is subsequently oxidized by aldehyde dehydrogenase to form retinoic acid in a highly regulated process. Retinoic acid is a potent mitogen involved, via nuclear transcription factors, in controlling the expression of a large number of genes. One of the most highly upregulated genes is *CYP26*, which encodes a P450 enzyme that oxidizes retinoic acid to inactive products. Thus, retinoic acid levels are kept relatively low and tightly controlled by its rates of biosynthesis and destruction.

Another issue is differences in biological processing exhibited by geometric isomers of retinoids. For example, 9-*cis*-retinal and 11-*cis*-retinal recombine with opsin to generate visual pigments, but all-*trans*-retinal and 13-*cis*-retinal, two isomers that are in thermodynamic equilibrium, do not. Indeed, all-*trans*-retinal can be converted to 11-*cis*-retinal through the retinoid cycle, but typically all-*trans*-forms are preferably stored as fatty acid esters in the RPE.

### Strategies for treating blinding retinal diseases caused by mutations in retinoid cycle genes

Conceptually, the simplest way to restore function is to replace defective genes by viral, nanoparticle or other gene therapy methods. RPE and photoreceptor cells take up and express recombinant constructs with great efficiency in many experimental settings. This strategy was used successfully in LCA animal models such as mice and dogs, and the positive effect of gene replacement therapy was sustained for several years after a single treatment of RPE65-null dogs [83,84]. Recently, several LCA patients were enrolled for gene therapy. Initial results revealed that this treatment at least partially restored vision in retinal areas of gene transfer in those individuals with blindness attributed to RPE65 mutations [85–87].

Retinoids undergo multiple transformations in the eye (Figures 1 and 2), and disabling mutations in genes encoding unique enzymes responsible for these transformations cause a deficiency in 11-*cis*-retinal production. Below we describe how such compromised transformations can be bypassed by supplying active chromophore(s).

Theoretically, decreasing production of the 11-*cis*-retinal chromophore using specific inhibitors of the visual cycle would be beneficial. Slowing the rapid turnover of retinoids should prevent excessive production of toxic all-*trans*-retinal and its condensation products. Excessive or greatly prolonged inhibition would be detrimental because, conceptually, this would decrease vision under extreme conditions, as observed in LCA. However, slowing the

visual cycle under strong illumination conditions would be beneficial.

### Retinosomes as a depot for chromophores or inhibitors of the visual cycle

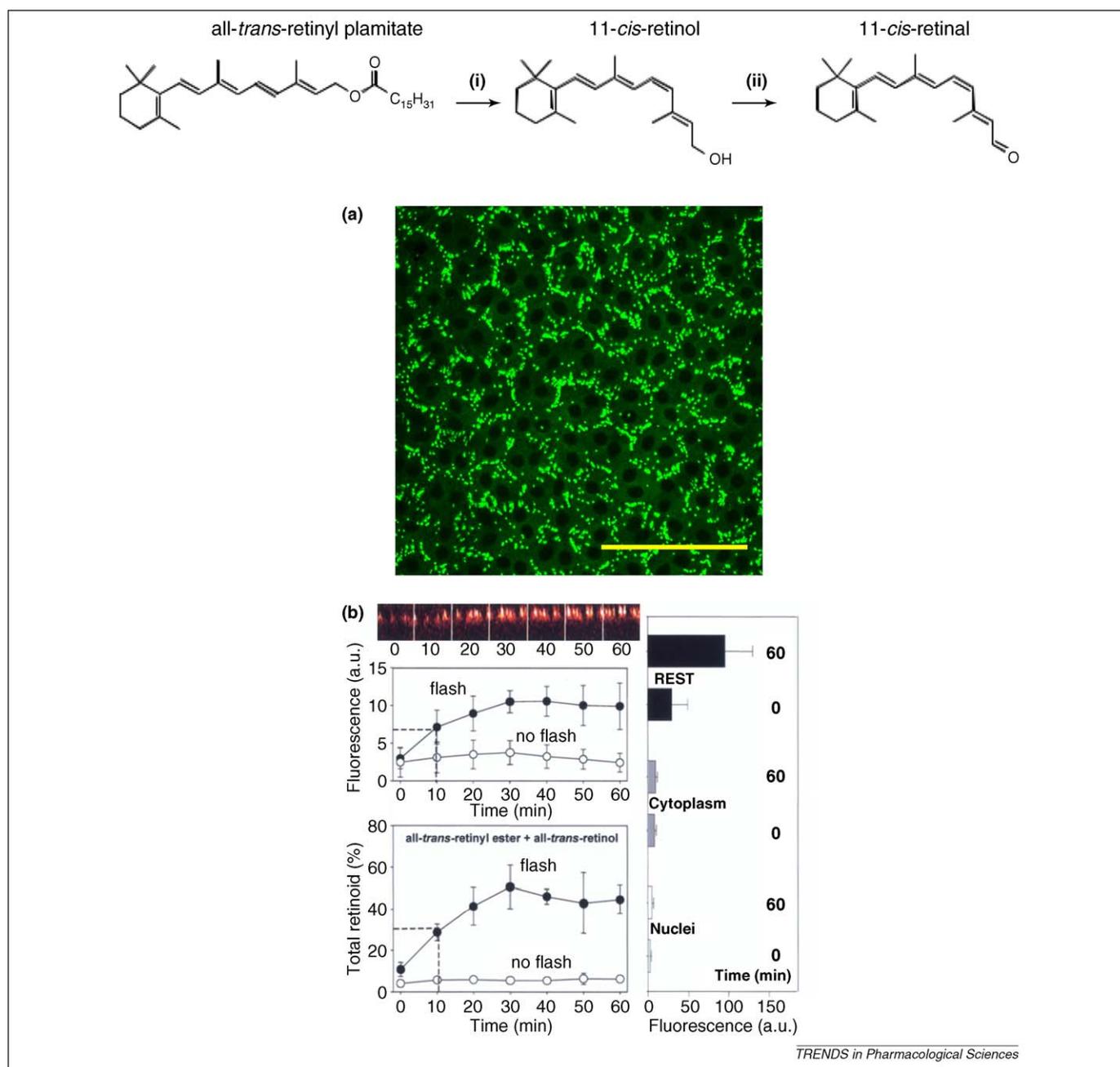
Retinosomes are storage particles that were discovered by a post-doctoral fellow in my laboratory, Dr Yoshikazu Imanishi. They bud off the ER, but their mechanism of formation has yet to be clarified. Retinosomes are composed of fatty acid retinyl esters, lipids and at least one other identified component, adipocyte differentiation-related protein [46,47,88,89]. As a result of the high UV sensitivity of retinoids, these structures can be imaged only under long-wavelength infrared light using two-photon microscopy (Figure 3A). It was then demonstrated that these particles expand under light and contract when light is removed, providing evidence that retinosomes participate in the regular visual cycle (Figure 3B). These storage particles become light-insensitive when the visual cycle is disabled by elimination of retinoid isomerase or LRAT activity. Retinosomes can also be used to store retinylamine because this compound can be amidated by LRAT [48]. Importantly, these structures can also be used to store artificial precursors of the chromophore in the form of 9-*cis*-retinyl esters.

### Pharmacological replacement of missing chromophore and its precursor

In the most severe cases, insufficient 11-*cis*-retinal production leads to congenital or progressive blindness in humans. LCA is an autosomal recessive, early-onset, severe retinal dystrophy that accounts for 5% of all such inherited disorders [90]. Pharmacological replacement of missing chromophore is applicable to diseases resulting from deficient chromophore biosynthesis. Examples include LCA arising from mutations in the *LRAT* and *RPE65* genes (Figure 1). Initial experiments aimed at bypassing the biochemical defect caused by absence of Rpe65 were performed by oral gavage of *Rpe65*<sup>-/-</sup> mice with 9-*cis*-retinal [91]. 9-*cis*-Retinal, which combines with opsin to form light-sensitive iso-rhodopsin [91], was initially selected because it is easier to synthesize and is more stable than 11-*cis*-retinal. Moreover, iso-rhodopsin has an absorbance maximum at 494 nm compared to 502 nm for rhodopsin, facilitating experimental identification of reconstituted iso-rhodopsin [92]. Further refinement and extensive testing identified 9-*cis*-retinyl acetate as a useful experimental compound [93] (Figure 4).

The use of *cis*-retinoids in the treatment of symptoms in LCA mouse models [91,94] seemed mechanistically sound and encouraging results were obtained in the treatment of aging mice [95]. Not only was it proved that *cis*-retinoids chaperone the mutant opsin to allow proper *in vivo* folding of P23H-opsin, but in experimental cell lines the rescued protein also formed pigment, acquired mature glycosylation and was transported to the cell surface [96]. Only photoactive *cis*-chromophores were beneficial; all-*trans*-retinal was ineffective.

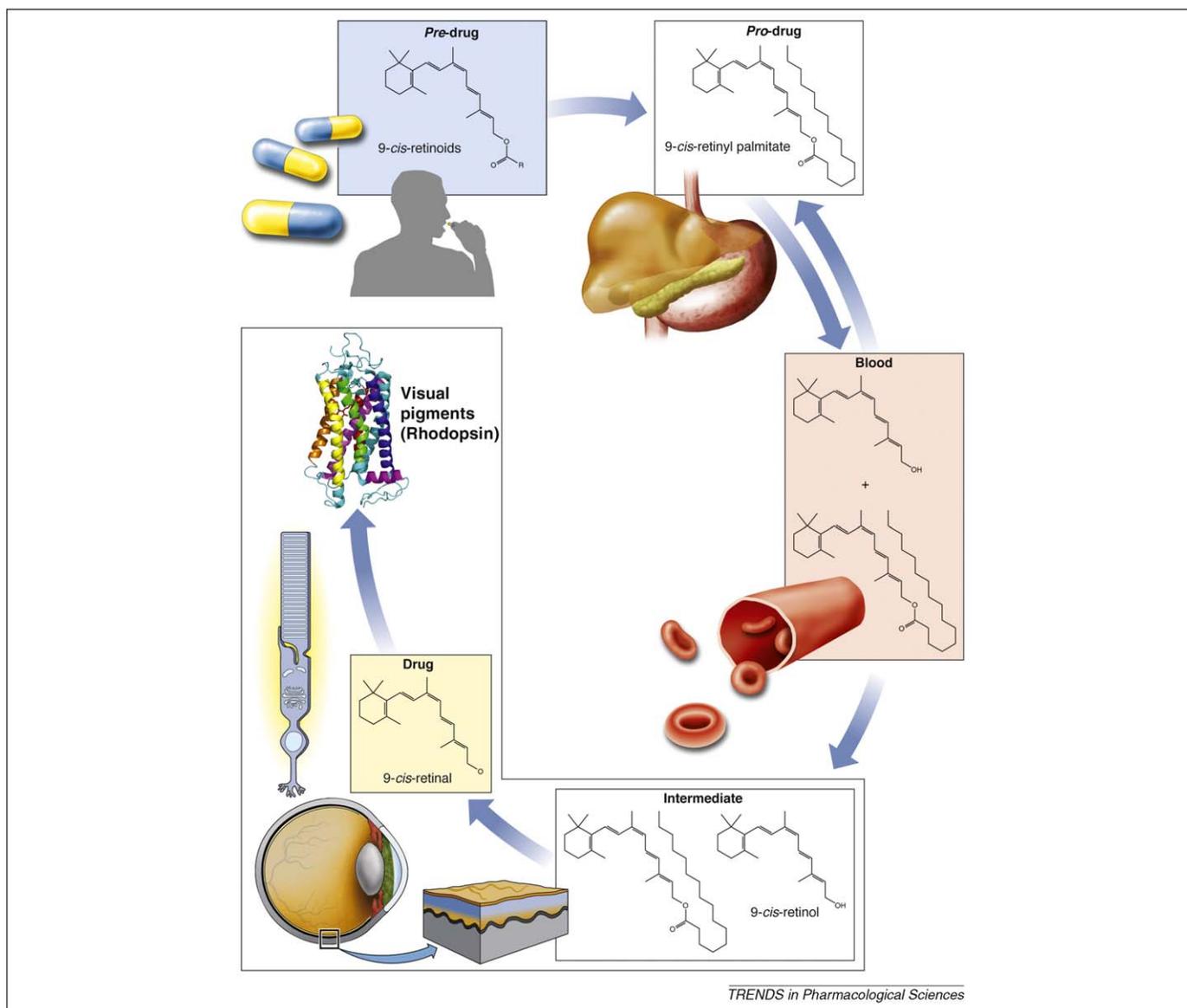
Dietary supplementation of *Rpe65*<sup>-/-</sup> mice with 9-*cis*-retinoids restored light sensitivity to levels found in wild-



**Figure 3.** Transformations of visual cycle retinoids in the RPE. All-trans-retinol diffuses from photoreceptor cells into the RPE, where it is esterified by LRAT to all-trans-retinyl esters. Hydrophobic retinyl esters then form retinosomes (RESTs). All-trans-retinyl esters are isomerized to 11-cis-retinol (reaction a) in a reaction that involves an RPE-abundant protein, termed RPE65. 11-cis-Retinol is then oxidized by 11-cis-RDH to 11-cis-retinal (reaction b). 11-cis-Retinol diffuses back into the rod and cone outer segments, where it completes the retinoid cycle by recombining with opsins to reform rhodopsin and cone pigments. **(a)** Retinosomes in RPE cells captured using two-photon microscopy (courtesy of Grazyna Palczewska, Polgenix Inc., Cleveland, OH). Fluorescence emission from the isolated intact mouse eye at 560–700 nm in green pseudocolor was observed after excitation by a 730-nm mode-locked Ti:Sapphire laser. Scale bar, 5  $\mu\text{m}$ . **(b)** Flash-dependent changes in fluorescence and all-trans-retinoid/all-trans-retinyl esters in the RPE cell layer of isolated mouse eyes. The top shows a row of images of optical sections of the retina perpendicular to the ocular tissue. RPE fluorescence (a.u., arbitrary unit) was quantified as a function of time. Numbers refer to minutes after the flash. The middle and bottom graphs show fluorescence quantified for retinoids and retinoid analyses by HPLC (all-trans-retinol and all-trans-retinyl esters; mean  $\pm$  SD,  $n = 3$ ), respectively. Dashed lines indicate the half-life for REST formation and the increase in all-trans-retinol and all-trans-retinyl esters. Light-dependent changes in the fluorescent signal in different subcellular compartments are shown on the right (copied from Ref. [46] with permission from Rockefeller University Press).

type animals, as assessed by both single-cell and ERG recordings. Similar recovery of visual function was reported following intraperitoneal injection of 11-cis-retinal into *Rpe65*<sup>-/-</sup> mice [97]. There are several advantages of 9-cis-retinoid over 11-cis-retinoid treatment. First, the 9-cis-compound is effective when taken orally. Second, because it is converted to prodrug forms that are stored in the liver, transported in the blood, selectively taken up by

the eye and stored in retinosomes (Figure 4a,b), storage particles that participate in the regular visual cycle [45–47,88,89] but can also be used to store artificial precursors of the chromophore from which the active compound is slowly released, a single high dose can produce a long-term therapeutic effect. The toxicity profile of 9-cis-retinoids has yet to be investigated. If these retinoids have a narrow therapeutic window, any toxic effects are likely to be



**Figure 4.** Delivery and action of 9-cis-retinoids. Retinyl esters are effective when taken orally. In the small intestine, 9-cis-retinyl esters are either hydrolyzed and esterified with fatty acyl coenzyme A, or *trans*-esterified with phospholipids before being transported in chylomicrons to the liver, where these intermediate products are found in lipid droplets. This cycle of hydrolysis and esterification can occur several times before storage in hepatic stellate cells. Fatty acid (mostly palmitate) esters and free 9-cis-retinol are then secreted into the systemic circulation, either bound to RBP or albumin or incorporated into chylomicrons. In the eye, these retinoids are probably hydrolyzed again as they pass from the choroid capillaries into the RPE by STRA6-dependent and -independent mechanisms. Fatty acid esters of 9-cis-retinol in the RPE are stored in specific lipid droplets called retinosomes. When required, these esters are hydrolyzed and oxidized to the drug 9-cis-retinal, which is then delivered to opsins in photoreceptors to form light-sensitive visual pigments.

long-lasting. A particular concern is the potential for long-term toxicity during pregnancy. Fortunately, such toxicity is unlikely to emanate in mammals from conversion to retinoic acid, a potent mitogen, because biosynthesis of the latter from retinol is tightly controlled. As in aging, rhodopsin regeneration after light exposure is more delayed in humans and mice with vitamin A deficiency because of either inadequate dietary intake or intestinal absorption [9]. Studies in mice have shown that age-related decreased retinal rod cell function cannot be explained by rod cell loss, abnormal retinal plasticity or any signs of retinal disease [98–100]. However, a dramatic age-associated slowing of rod-mediated dark adaptation after light exposure in humans was related to delayed regeneration of rhodopsin [98]. Deteriorating photoreceptor function documented in

mice at 10 and 14 versus 4 months of age was improved significantly by long-term monthly administration of the artificial chromophore 9-cis-retinyl acetate. These findings suggest one potential therapeutic approach for prevention of age-related retinal dysfunction [3].

In a highly discussed study, all-*trans*-retinol combined with vitamin E was tested as a remedy for RP patients lacking genotype characterization. The results of this study support a beneficial effect of 15,000 IU/day of vitamin A on the course of RP [101]. There was no indication that these patients were vitamin-deficient, so it seems inconceivable that the role of this treatment differed mechanistically from the known antioxidant role of vitamin A on the retina of this genetically heterogeneous diseased population. Surprisingly, vitamin A supplementation slows the

rate of photoreceptor degeneration caused by a threonine-17→methionine mutation in the opsin gene. The authors speculated that vitamin A supplementation could confer therapeutic benefit by stabilizing mutant opsins through increased availability of the chromophore [94], but chromophore production does not depend on a further increase in this precursor because it exists in significant excess in the mature retina.

An observation in *Abca4*<sup>-/-</sup> mice is also puzzling. Upon supplementation with vitamin A, these mice exhibited dramatically higher levels of retinyl esters in their liver and RPE and, more importantly, lipofuscin pigments such as A2E were significantly increased as well. Photoreceptor degeneration was also observed in 11-month-old albino mice. The author recommended that 'vitamin A supplementation should be avoided in patients with *ABCA4* mutations or other retinal or macular dystrophies associated with lipofuscin accumulation in the retinal pigment epithelium' [95]. However, A2E formation is related only to high levels of flux through the visual cycle, and high levels of the chromophore ester precursor have nothing in common with A2E formation, as evidenced by *Rpe65*<sup>-/-</sup> mice that completely lack A2E but still accumulate huge amounts of all-*trans*-retinyl esters [102].

The situation differs for Sorsby's fundus dystrophy, which is an autosomal dominant retinal degeneration caused by mutations in the tissue inhibitor of metalloproteinases-3 gene. During the course of this disease, a thickened membrane barrier between photoreceptor layers causes local vitamin A deprivation. Administration of vitamin A dramatically restored photoreceptor function [103]. Insufficient dietary vitamin A can also cause progressive deterioration of vision and ultimately blindness without genetic abnormalities [104], a major problem in underdeveloped countries. Dietary supplementation with vitamin A typically reverses this problem.

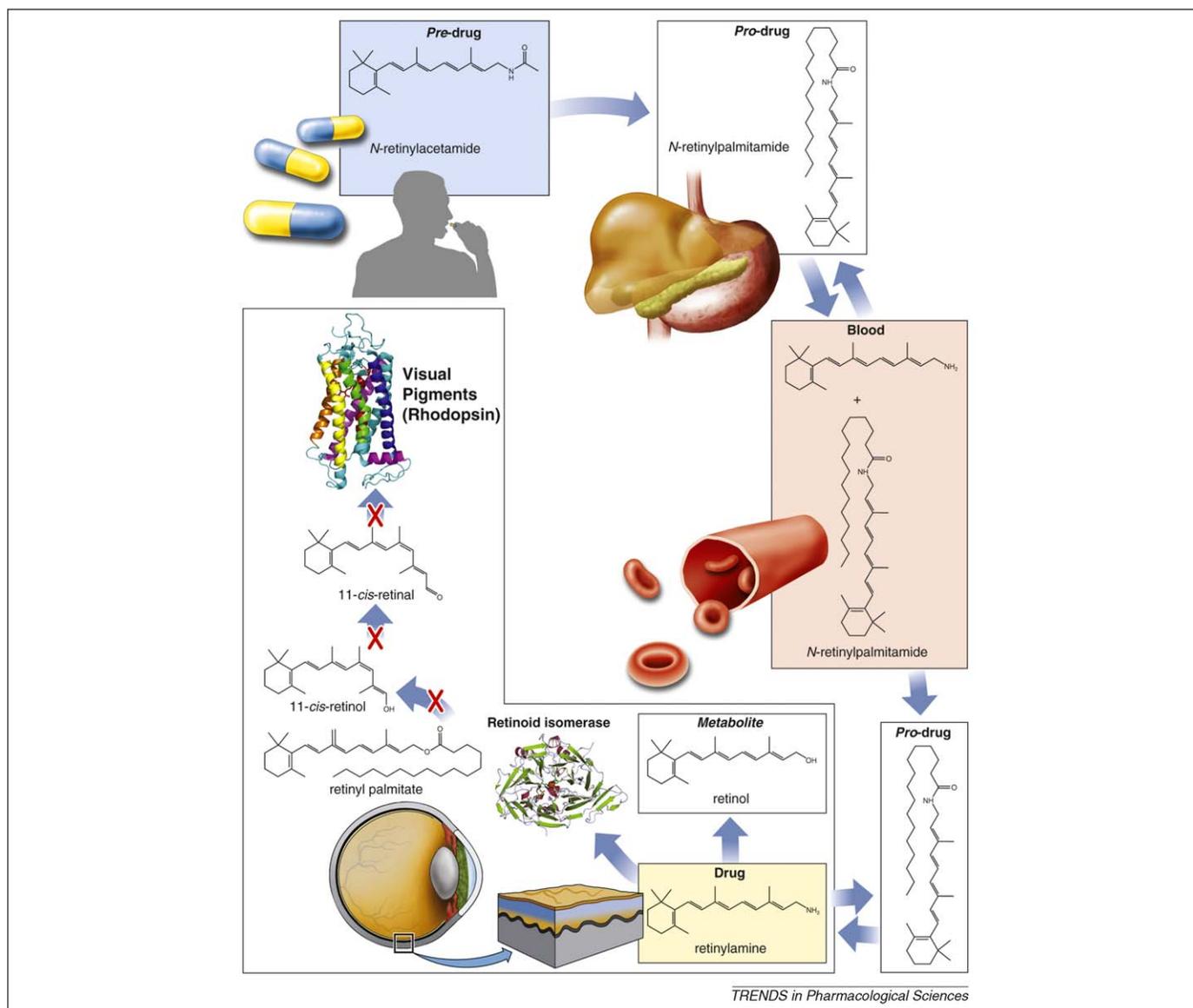
### Pharmacological inhibition of the retinoid cycle

Another treatment strategy is to slow the biosynthesis of chromophore by either inhibiting steps in the visual cycle or limiting availability of the all-*trans*-retinol precursor. This approach is applicable to diseases associated with accumulation of retinoid cycle intermediates. As noted above, impaired clearance of all-*trans*-retinal causes acute light-induced retinal toxicity [73] and induces the formation of fluorescent lipofuscin pigments such as A2E in RPE cells. Acute all-*trans*-retinal toxicity observed in light-induced photoreceptor damage might involve increased plasma membrane permeability and mitochondrial poisoning that leads to caspase activation and mitochondrial-associated cell death [73]. Accumulation of lipofuscin pigments is an important pathological feature of Stargardt's disease [105,106], but it is not limited to this inherited condition. Monitoring fundus autofluorescence is a non-invasive technique developed in the past decade that uses the fluorescent properties of lipofuscin to study the health and viability of the retina. Increased fundus autofluorescence by scanning laser ophthalmoscopy is commonly observed in patients with AMD [107,108]. Fluorescent material acquired in aged RPE has spectral properties similar to A2P identified in *Abca4*<sup>-/-</sup> mice [59,109]. Strong

fundus autofluorescence is also observed in patients with Best vitelliform macular dystrophy and in a subset of patients with cone-rod dystrophy [110]. Patients with dominant Stargardt's disease, caused by mutations in the *ELOVL4* gene, exhibit a dark choroid on fluorescein angiography, also owing to lipofuscin in RPE cells [111,112]. The major disadvantage of inhibiting the retinoid cycle is night blindness, with a resulting inability to drive. Also of concern is that prolonged inadequate production of chromophore will adversely affect the health of rod and, more severely, cone cells. The most potent and efficacious retinoid cycle inhibitor identified to date is retinylamine, a transition-state inhibitor of RPE65 [19,48,58,113–118]. The unusual fate of this retinoid, including its route of administration, hepatic storage, release into the circulation, uptake by the eye, storage and eventual release in retinosomes of the retina, is illustrated in Figure 5.

We systematically studied the effects of retinylamine and other potential inhibitors of visual function in mice. Prolonged and sustainable, but reversible, suppression of visual function was observed with retinylamine (Figure 5) as a result of its storage in a prodrug form, *N*-retinylamide [117]. The drug was directly compared with other inhibitors to assess their prevention of light-induced retinal damage. Retinylamine displayed higher efficacy, specificity and potency and lower transcriptional activation compared to *N*-(4-hydroxyphenyl)retinamide [119] and 13-*cis*-retinoic acid [120,121], whereas the other compounds tested were ineffective [114]. It should be noted that other investigators have proposed that *N*-(4-hydroxyphenyl)retinamide would cause immediate dose-dependent decreases in serum retinol and RBP in *Abca4*<sup>-/-</sup> mice [119]. This prediction is puzzling because excess retinoids are stored in the eye, which is highly resistant to vitamin A deprivation. In mice, it takes two generations, even after liver stores are depleted, for retinoids to be depleted from the eye. Methods to reduce circulating retinol would be inadequate to deplete retinoids in the eye because of the powerful protective mechanisms involved in retinoid transport (Figures 4 and 5) and retinoid retention within the RPE (Figure 5). Thus, it is possible that a small effect on retinoid metabolism in the eye could be accomplished by weak inhibition of RPE65 by *N*-(4-hydroxyphenyl)retinamide and of 11-*cis*-retinol dehydrogenase by 13-*cis*-retinoic acid [122]. However, a dramatic decrease in retinol delivery to peripheral tissues for a prolonged period could be detrimental to the health of the individual.

Is there a future for the use of inhibitors of the visual cycle for treatment of degenerative retinal diseases? We believe that these inhibitors could be effective therapies based on what we have learned about their effects in animal models of these diseases. For example, it was recognized that cone photoreceptor cells in *Rpe65*<sup>-/-</sup> mice degenerate more rapidly than rod photoreceptors [123], with similar observations for *Lrat*<sup>-/-</sup> mice [38,124]. In *Rpe65*<sup>-/-</sup>*rhodopsin*<sup>-/-</sup> mice, chromophore addition enhanced proper transport of cone opsins to outer segments while partially preserving cone structure and function in a compromised retina lacking rods because of rhodopsin elimination [38]. These findings are critical



**Figure 5.** Delivery and action of retinylamide. This amide ester can be taken orally. Once in the intestine, it is hydrolyzed and re-amidated with fatty acid coenzyme A and transported in chylomicrons to the liver, where it is stored as lipid droplets in hepatic stellate cells. This cycle of hydrolysis and re-amidation can occur several times. Fatty acid (mostly palmitate) amide and free retinylamine are then secreted into the systemic circulation, either bound to RBP or albumin, or incorporated into chylomicrons. The amides are probably hydrolyzed again as they pass from the choroid capillaries into the RPE in a STRA6-independent manner. There, retinylamine is stored in specific lipid droplets called retinosomes. When required, retinylamine is released and acts as a very potent transition-state inhibitor of RPE65, which catalyses the hydrolytic isomerization of retinyl esters. Suppression of this isomerization can last for weeks because of long-term storage. Retinylamine also conjugates with free retinal, preventing accumulation of other toxic retinal condensation products. Eventually, retinylamine is metabolized to retinol.

because of the importance of cones for human high-resolution spatial vision and color perception [125] and the need to evaluate cone status for any potential LCA therapy [126]. Children with *RPE65*-LCA exhibit cone photoreceptor loss in the first decade of life [118]. The central retinal RPE layer of the normal primate retina also exhibits higher retinoid isomerase activity than the more peripheral RPE, so we speculated that early cone photoreceptor loss in *RPE65*-LCA indicates that robust RPE65-based visual chromophore production is vital for cones [17]. Residual cone structure and function could be supported by a retinal-based alternative pathway for chromophore production [18]. Mice chronically treated with retinylamine showed a decrease in the number of cones that was ameliorated by administration of 9-*cis*-retinoids. Together these results suggest that a chronic lack of chromophore

leads to progressive loss of cones in mice and humans [118]. Thus, prolonged inhibition of the visual cycle, a currently tested approach for treatment of Stargardt's and AMD patients, poses a major unresolved problem. Moreover, night blindness, which accompanies this treatment, might severely limit patients, who can no longer drive at night, and suffer other serious inconveniences.

### Conclusions

Classical approaches can be combined with emerging technologies to address previously challenging therapeutic questions. By obtaining a proper molecular framework with which to view biological systems, new strategies can be evolved to develop better pharmacological agents to combat blinding diseases. Further progress in vision research and medicine will require a combi-

nation of multiple approaches and techniques to solve the complexities of retinal diseases. Only selected retinoids, when properly used, have the potential to combat retinal diseases. Compounds that can either inhibit the *trans-cis* isomerization step of the retinoid cycle or that recombine with opsin to form light-sensitive pigments could be stored in the liver and eye as prodrugs. This is highly unusual, but it would facilitate the use of novel and very powerful pharmacology. These properties rely on the ability of vertebrates to store inactive fatty acid acylated chromophore or retinoid inhibitors in cellular structures such as stellate cells in the liver and retinosomes in the eye.

### Conflict of interest statement

University of Washington, Acucela Inc., Retinagenix Inc. and QLT Inc. might commercialize some of the technology described in this work. KP is a consultant for QLT Inc. and Acucela Inc. and a co-founder of Retinagenix Inc.

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### References

- Thompson, D.A. and Gal, A. (2003) Vitamin A metabolism in the retinal pigment epithelium: genes, mutations, and diseases. *Prog. Retinal Eye Res.* 22, 683–693
- Travis, G.H. *et al.* (2007) Diseases caused by defects in the visual cycle: retinoids as potential therapeutic agents. *Annu. Rev. Pharmacol. Toxicol.* 47, 469–512
- Maeda, T. *et al.* (2009) Effects of long-term administration of 9-cis-retinyl acetate on visual function in mice. *Invest. Ophthalmol. Vis. Sci.* 50, 322–333
- Jackson, G.R. *et al.* (2006) Impact of aging and age-related maculopathy on inactivation of the  $\alpha$ -wave of the rod-mediated electroretinogram. *Vision Res.* 46, 1422–1431
- Yau, K.W. and Hardie, R.C. (2009) Phototransduction motifs and variations. *Cell* 139, 246–264
- Arshavsky, V.Y. *et al.* (2002) G proteins and phototransduction. *Annu. Rev. Physiol.* 64, 153–187
- McBee, J.K. *et al.* (2001) Confronting complexity: the interlink of phototransduction and retinoid metabolism in the vertebrate retina. *Prog. Retinal Eye Res.* 20, 469–529
- Rando, R.R. (1996) Polyenes and vision. *Chem. Biol.* 3, 255–262
- Lamb, T.D. and Pugh, E.N., Jr (2004) Dark adaptation and the retinoid cycle of vision. *Prog. Retinal Eye Res.* 23, 307–380
- Ridge, K.D. *et al.* (2003) Phototransduction: crystal clear. *Trends Biochem. Sci.* 28, 479–487
- Ridge, K.D. and Palczewski, K. (2007) Visual rhodopsin sees the light: structure and mechanism of G protein signaling. *J. Biol. Chem.* 282, 9297–9301
- Moise, A.R. *et al.* (2007) Delivery of retinoid-based therapies to target tissues. *Biochemistry* 46, 4449–4458
- Filipek, S. *et al.* (2003) G protein-coupled receptor rhodopsin: a prospectus. *Annu. Rev. Physiol.* 65, 851–879
- Palczewski, K. (2006) G protein-coupled receptor rhodopsin. *Ann. Rev. Biochem.* 75, 743–767
- von Lintig, J. *et al.* (2010) The biochemical and structural basis for *trans-to-cis* isomerization of retinoids in the chemistry of vision. *Trends Biochem. Sci.* DOI: 10.1016/j.tibs.2010.01.005
- Polans, A. *et al.* (1996) Turned on by  $\text{Ca}^{2+}$ ! The physiology and pathology of  $\text{Ca}^{2+}$ -binding proteins in the retina. *Trends Neurosci.* 19, 547–554
- Jacobson, S.G. *et al.* (2007) Human cone photoreceptor dependence on RPE65 isomerase. *Proc. Natl. Acad. Sci. U. S. A.* 104, 15123–15128
- Mata, N.L. *et al.* (2002) Isomerization and oxidation of vitamin A in cone-dominant retinas: a novel pathway for visual-pigment regeneration in daylight. *Neuron* 36, 69–80
- Schonthaler, H.B. *et al.* (2007) Evidence for RPE65-independent vision in the cone-dominated zebrafish retina. *Eur. J. Neurosci.* 26, 1940–1949
- Wang, J.S. *et al.* (2009) Intra-retinal visual cycle required for rapid and complete cone dark adaptation. *Nat. Neurosci.* 12, 295–302
- Molday, R.S. (2007) ATP-binding cassette transporter ABCA4: molecular properties and role in vision and macular degeneration. *J. Bioenerg. Biomembr.* 39, 507–517
- Saari, J.C. and Bredberg, D.L. (1989) Lecithin:retinol acyltransferase in retinal pigment epithelial microsomes. *J. Biol. Chem.* 264, 8636–8640
- Saari, J.C. *et al.* (1993) Retinol esterification in bovine retinal pigment epithelium: reversibility of lecithin:retinol acyltransferase. *Biochem. J.* 291, 697–700
- Chambon, P. (1996) A decade of molecular biology of retinoic acid receptors. *FASEB J.* 10, 940–954
- Ross, A.C. (2003) Retinoid production and catabolism: role of diet in regulating retinol esterification and retinoic acid oxidation. *J. Nutr.* 133, 291S–296S
- Paik, J. *et al.* (2004) Vitamin A: overlapping delivery pathways to tissues from the circulation. *J. Nutr.* 134, 276S–280S
- Blaner, W.S. *et al.* (2009) Hepatic stellate cell lipid droplets: a specialized lipid droplet for retinoid storage. *Biochim. Biophys. Acta* 1791, 467–473
- Voolstra, O. *et al.* (2009) NinaB is essential for *Drosophila* vision but induces retinal degeneration in opsin-deficient photoreceptors. *J. Biol. Chem.* 285, 2130–2139
- Fierce, Y. *et al.* (2008) *In vitro* and *in vivo* characterization of retinoid synthesis from beta-carotene. *Arch. Biochem. Biophys.* 472, 126–138
- Moise, A.R. *et al.* (2005) Related enzymes solve evolutionarily recurrent problems in the metabolism of carotenoids. *Trends Plant Sci.* 10, 178–186
- Yeum, K.J. and Russell, R.M. (2002) Carotenoid bioavailability and bioconversion. *Annu. Rev. Nutr.* 22, 483–504
- Baehr, W. and Frederick, J.M. (2009) Naturally occurring animal models with outer retina phenotypes. *Vision Res.* 49, 2636–2652
- Rohrer, B. *et al.* (2005) Cone opsin mislocalization in *Rpe65*<sup>-/-</sup> mice: a defect that can be corrected by 11-*cis* retinal. *Invest. Ophthalmol. Vis. Sci.* 46, 3876–3882
- Fan, J. *et al.* (2005) Opsin activation of transduction in the rods of dark-reared *Rpe65* knockout mice. *J. Physiol.* 568, 83–95
- Hofmann, K.P. *et al.* (1992) The role of arrestin and retinoids in the regeneration pathway of rhodopsin. *J. Biol. Chem.* 267, 15701–15706
- Palczewski, K. *et al.* (1994) Rod outer segment retinol dehydrogenase: substrate specificity and role in phototransduction. *Biochemistry* 33, 13741–13750
- Jager, S. *et al.* (1996) Opsin/all-*trans*-retinal complex activates transducin by different mechanisms than photolyzed rhodopsin. *Biochemistry* 35, 2901–2908
- Zhang, H. *et al.* (2008) Trafficking of membrane-associated proteins to cone photoreceptor outer segments requires the chromophore 11-*cis*-retinal. *J. Neurosci.* 28, 4008–4014
- Dryja, T.P. (1992) Doyne Lecture. Rhodopsin and autosomal dominant retinitis pigmentosa. *Eye* 6, 1–10
- Rattner, A. *et al.* (1999) Molecular genetics of human retinal disease. *Annu. Rev. Genet.* 33, 89–131
- Weng, J. *et al.* (1999) Insights into the function of Rim protein in photoreceptors and etiology of Stargardt's disease from the phenotype in *abc* knockout mice. *Cell* 98, 13–23
- Perrault, I. *et al.* (1996) Retinal-specific guanylate cyclase gene mutations in Leber's congenital amaurosis. *Nat. Genet.* 14, 461–464
- Dryja, T.P. *et al.* (1990) A point mutation of the rhodopsin gene in one form of retinitis pigmentosa. *Nature* 343, 364–366
- Sokal, I. *et al.* (1998) GCAP1 (Y99C) mutant is constitutively active in autosomal dominant cone dystrophy. *Mol. Cell* 2, 129–133
- Baehr, W. *et al.* (2003) The retinoid cycle and retina disease. *Vision Res.* 43, 2957–2958
- Imanishi, Y. *et al.* (2004) Noninvasive two-photon imaging reveals retinyl ester storage structures in the eye. *J. Cell Biol.* 164, 373–383

- 47 Imanishi, Y. *et al.* (2004) Retinosomes: new insights into intracellular managing of hydrophobic substances in lipid bodies. *J. Cell Biol.* 166, 447–453
- 48 Golczak, M. *et al.* (2005) Lecithin:retinol acyltransferase is responsible for amidation of retinylamine, a potent inhibitor of the retinoid cycle. *J. Biol. Chem.* 280, 42263–42273
- 49 Fotiadis, D. *et al.* (2004) The G protein-coupled receptor rhodopsin in the native membrane. *FEBS Lett.* 564, 281–288
- 50 Fotiadis, D. *et al.* (2003) Atomic-force microscopy: rhodopsin dimers in native disc membranes. *Nature* 421, 127–128
- 51 Liang, Y. *et al.* (2003) Organization of the G protein-coupled receptors rhodopsin and opsin in native membranes. *J. Biol. Chem.* 278, 21655–21662
- 52 Govardovskii, V.I. *et al.* (2009) Lateral diffusion of rhodopsin in photoreceptor membrane: a reappraisal. *Mol. Vis.* 15, 1717–1729
- 53 Corless, J.M. *et al.* (1982) Two-dimensional rhodopsin crystals from disk membranes of frog retinal rod outer segments. *Proc. Natl. Acad. Sci. U. S. A.* 79, 1116–1120
- 54 Corless, J.M. *et al.* (1994) Three-dimensional membrane crystals in amphibian cone outer segments. 1. Light-dependent crystal formation in frog retinas. *J. Struct. Biol.* 113, 64–86
- 55 Nickell, S. *et al.* (2007) Three-dimensional architecture of murine rod outer segments determined by cryoelectron tomography. *J. Cell Biol.* 177, 917–925
- 56 Maeda, A. *et al.* (2006) Retinol dehydrogenase (RDH12) protects photoreceptors from light-induced degeneration in mice. *J. Biol. Chem.* 281, 37697–37704
- 57 Wenzel, A. *et al.* (2001) The Rpe65 Leu450Met variation increases retinal resistance against light-induced degeneration by slowing rhodopsin regeneration. *J. Neurosci.* 21, 53–58
- 58 Maeda, A. *et al.* (2008) Retinopathy in mice induced by disrupted all-*trans*-retinal clearance. *J. Biol. Chem.* 283, 26684–26693
- 59 Mata, N.L. *et al.* (2000) Biosynthesis of a major lipofuscin fluorophore in mice and humans with ABCR-mediated retinal and macular degeneration. *Proc. Natl. Acad. Sci. U. S. A.* 97, 7154–7159
- 60 Kim, S.R. *et al.* (2007) Characterization of dihydro-A2PE: an intermediate in the A2E biosynthetic pathway. *Biochemistry* 46, 10122–10129
- 61 Fishkin, N.E. *et al.* (2005) Isolation and characterization of a retinal pigment epithelial cell fluorophore: an all-*trans*-retinal dimer conjugate. *Proc. Natl. Acad. Sci. U. S. A.* 102, 7091–7096
- 62 Yannuzzi, L.A. *et al.* (2004) Ophthalmic fundus imaging: today and beyond. *Am. J. Ophthalmol.* 137, 511–524
- 63 Finnemann, S.C. *et al.* (2002) The lipofuscin component A2E selectively inhibits phagolysosomal degradation of photoreceptor phospholipid by the retinal pigment epithelium. *Proc. Natl. Acad. Sci. U. S. A.* 99, 3842–3847
- 64 Kim, S.R. *et al.* (2006) Photooxidation of A2-PE, a photoreceptor outer segment fluorophore, and protection by lutein and zeaxanthin. *Exp. Eye Res.* 82, 828–839
- 65 Kim, S.R. *et al.* (2008) Mechanisms involved in A2E oxidation. *Exp. Eye Res.* 86, 975–982
- 66 Allikmets, R. (2000) Further evidence for an association of ABCR alleles with age-related macular degeneration. The International ABCR Screening Consortium. *Am. J. Hum. Genet.* 67, 487–491
- 67 Allikmets, R. *et al.* (1997) Mutation of the Stargardt disease gene (ABCR) in age-related macular degeneration. *Science* 277, 1805–1807
- 68 Cremers, F.P. *et al.* (1998) Autosomal recessive retinitis pigmentosa and cone-rod dystrophy caused by splice site mutations in the Stargardt's disease gene ABCR. *Hum. Mol. Genet.* 7, 355–362
- 69 Martinez-Mir, A. *et al.* (1998) Retinitis pigmentosa caused by a homozygous mutation in the Stargardt disease gene ABCR. *Nat. Genet.* 18, 11–12
- 70 Zhang, L. *et al.* (2005) Expression of functional G protein-coupled receptors in photoreceptors of transgenic *Xenopus laevis*. *Biochemistry* 44, 14509–14518
- 71 Parish, C.A. *et al.* (1998) Isolation and one-step preparation of A2E and iso-A2E, fluorophores from human retinal pigment epithelium. *Proc. Natl. Acad. Sci. U. S. A.* 95, 14609–14613
- 72 Delori, F.C. *et al.* (1995) *In vivo* measurement of lipofuscin in Stargardt's disease – fundus flavimaculatus. *Invest. Ophthalmol. Vis. Sci.* 36, 2327–2331
- 73 Maeda, A. *et al.* (2009) Involvement of all-*trans*-retinal in acute light-induced retinopathy of mice. *J. Biol. Chem.* 284, 15173–15183
- 74 Radu, R.A. *et al.* (2004) Light exposure stimulates formation of A2E oxiranes in a mouse model of Stargardt's macular degeneration. *Proc. Natl. Acad. Sci. U. S. A.* 101, 5928–5933
- 75 De, S. and Sakmar, T.P. (2002) Interaction of A2E with model membranes. Implications to the pathogenesis of age-related macular degeneration. *J. Gen. Physiol.* 120, 147–157
- 76 Allikmets, R. *et al.* (1997) A photoreceptor cell-specific ATP-binding transporter gene (ABCR) is mutated in recessive Stargardt macular dystrophy. *Nat. Genet.* 15, 236–246
- 77 Rattner, A. *et al.* (2000) Identification and characterization of all-*trans*-retinol dehydrogenase from photoreceptor outer segments, the visual cycle enzyme that reduces all-*trans*-retinal to all-*trans*-retinol. *J. Biol. Chem.* 275, 11034–11043
- 78 Chiu, H.J. *et al.* (2008) Vitamin A depletion causes oxidative stress, mitochondrial dysfunction, and PARP-1-dependent energy deprivation. *FASEB J.* 22, 3878–3887
- 79 O'Connell, M.J. *et al.* (1996) Retro-retinoids in regulated cell growth and death. *J. Exp. Med.* 184, 549–555
- 80 Moise, A.R. *et al.* (2005) Metabolism and transactivation activity of 13,14-dihydroretinoic acid. *J. Biol. Chem.* 280, 27815–27825
- 81 Moise, A.R. *et al.* (2008) Stereospecificity of retinol saturase: absolute configuration, synthesis, and biological evaluation of dihydroretinoids. *J. Am. Chem. Soc.* 130, 1154–1155
- 82 Ziouzenkova, O. *et al.* (2007) Retinaldehyde represses adipogenesis and diet-induced obesity. *Nat. Med.* 13, 695–702
- 83 Acland, G.M. *et al.* (2005) Long-term restoration of rod and cone vision by single dose rAAV-mediated gene transfer to the retina in a canine model of childhood blindness. *Mol. Ther.* 12, 1072–1082
- 84 Acland, G.M. *et al.* (2001) Gene therapy restores vision in a canine model of childhood blindness. *Nat. Genet.* 28, 92–95
- 85 Bainbridge, J.W. *et al.* (2008) Effect of gene therapy on visual function in Leber's congenital amaurosis. *N. Engl. J. Med.* 358, 2231–2239
- 86 Maguire, A.M. *et al.* (2008) Safety and efficacy of gene transfer for Leber's congenital amaurosis. *N. Engl. J. Med.* 358, 2240–2248
- 87 Cideciyan, A.V. *et al.* (2008) Human gene therapy for RPE65 isomerase deficiency activates the retinoid cycle of vision but with slow rod kinetics. *Proc. Natl. Acad. Sci. U. S. A.* 105, 15112–15117
- 88 Imanishi, Y. and Palczewski, K. Visualization of retinoid storage and trafficking by two-photon microscopy. *Methods Mol. Biol.* (in press)
- 89 Imanishi, Y. *et al.* (2008) Retinyl ester homeostasis in the adipose differentiation-related protein-deficient retina. *J. Biol. Chem.* 283, 25091–25102
- 90 Hanein, S. *et al.* (2004) Leber congenital amaurosis: comprehensive survey of the genetic heterogeneity, refinement of the clinical definition, and genotype–phenotype correlations as a strategy for molecular diagnosis. *Hum. Mutat.* 23, 306–317
- 91 Van Hooser, J.P. *et al.* (2002) Recovery of visual functions in a mouse model of Leber congenital amaurosis. *J. Biol. Chem.* 277, 19173–19182
- 92 Fan, J. *et al.* (2003) Isorhodopsin rather than rhodopsin mediates rod function in RPE65 knock-out mice. *Proc. Natl. Acad. Sci. U. S. A.* 100, 13662–13667
- 93 Batten, M.L. *et al.* (2005) Pharmacological and rAAV gene therapy rescue of visual functions in a blind mouse model of Leber congenital amaurosis. *PLoS Med.* 2, e333
- 94 Li, T. *et al.* (1998) Effect of vitamin A supplementation on rhodopsin mutants threonine-17→methionine and proline-347→serine in transgenic mice and in cell cultures. *Proc. Natl. Acad. Sci. U. S. A.* 95, 11933–11938
- 95 Radu, R.A. *et al.* (2008) Accelerated accumulation of lipofuscin pigments in the RPE of a mouse model for ABCA4-mediated retinal dystrophies following vitamin A supplementation. *Invest. Ophthalmol. Vis. Sci.* 49, 3821–3829
- 96 Noorwez, S.M. *et al.* (2003) Pharmacological chaperone-mediated *in vivo* folding and stabilization of the P23H-opsin mutant associated with autosomal dominant retinitis pigmentosa. *J. Biol. Chem.* 278, 14442–14450
- 97 Ablonczy, Z. *et al.* (2002) 11-*cis*-Retinal reduces constitutive opsin phosphorylation and improves quantum catch in retinoid-deficient mouse rod photoreceptors. *J. Biol. Chem.* 277, 40491–40498

- 98 Jackson, G.R. *et al.* (1999) Aging and dark adaptation. *Vision Res.* 39, 3975–3982
- 99 Gao, H. and Hollyfield, J.G. (1992) Aging of the human retina. Differential loss of neurons and retinal pigment epithelial cells. *Invest. Ophthalmol. Vis. Sci.* 33, 1–17
- 100 Jackson, G.R. *et al.* (1998) Aging and scotopic sensitivity. *Vision Res.* 38, 3655–3662
- 101 Berson, E.L. *et al.* (1993) A randomized trial of vitamin A and vitamin E supplementation for retinitis pigmentosa. *Arch. Ophthalmol.* 111, 761–772
- 102 Katz, M.L. and Redmond, T.M. (2001) Effect of Rpe65 knockout on accumulation of lipofuscin fluorophores in the retinal pigment epithelium. *Invest. Ophthalmol. Vis. Sci.* 42, 3023–3030
- 103 Jacobson, S.G. *et al.* (1995) Night blindness in Sorsby's fundus dystrophy reversed by vitamin A. *Nat. Genet.* 11, 27–32
- 104 Schoeff, L. (1983) Vitamin A. *Am. J. Med. Technol.* 49, 447–452
- 105 Birnback, C.D. *et al.* (1994) Histopathology and immunocytochemistry of the neurosensory retina in fundus flavimaculatus. *Ophthalmology* 101, 1211–1219
- 106 De Laey, J.J. and Verougstraete, C. (1995) Hyperlipofuscinosis and subretinal fibrosis in Stargardt's disease. *Retina* 15, 399–406
- 107 Lois, N. *et al.* (2002) Fundus autofluorescence in patients with age-related macular degeneration and high risk of visual loss. *Am. J. Ophthalmol.* 133, 341–349
- 108 Bindewald, A. *et al.* (2005) Classification of fundus autofluorescence patterns in early age-related macular disease. *Invest. Ophthalmol. Vis. Sci.* 46, 3309–3314
- 109 Delori, F.C. *et al.* (2001) Age-related accumulation and spatial distribution of lipofuscin in RPE of normal subjects. *Invest. Ophthalmol. Vis. Sci.* 42, 1855–1866
- 110 Wabbers, B. *et al.* (2006) Fundus autofluorescence in children and teenagers with hereditary retinal diseases. *Albrecht von Graefes Arch. Klin. Exp. Ophthalmol.* 244, 36–45
- 111 Zhang, K. *et al.* (2001) A 5-bp deletion in *ELOVL4* is associated with two related forms of autosomal dominant macular dystrophy. *Nat. Genet.* 27, 89–93
- 112 Karan, G. *et al.* (2005) Lipofuscin accumulation, abnormal electrophysiology, and photoreceptor degeneration in mutant *ELOVL4* transgenic mice: a model for macular degeneration. *Proc. Natl. Acad. Sci. U. S. A.* 102, 4164–4169
- 113 Golczak, M. *et al.* (2005) Positively charged retinoids are potent and selective inhibitors of the *trans-cis* isomerization in the retinoid (visual) cycle. *Proc. Natl. Acad. Sci. U. S. A.* 102, 8162–8167
- 114 Maeda, A. *et al.* (2006) Effects of potent inhibitors of the retinoid cycle on visual function and photoreceptor protection from light damage in mice. *Mol. Pharmacol.* 70, 1220–1229
- 115 Maeda, A. *et al.* (2006) Aberrant metabolites in mouse models of congenital blinding diseases: formation and storage of retinyl esters. *Biochemistry* 45, 4210–4219
- 116 Tu, D.C. *et al.* (2006) Inner retinal photoreception independent of the visual retinoid cycle. *Proc. Natl. Acad. Sci. U. S. A.* 103, 10426–10431
- 117 Golczak, M. *et al.* (2008) Metabolic basis of visual cycle inhibition by retinoid and nonretinoid compounds in the vertebrate retina. *J. Biol. Chem.* 283, 9543–9554
- 118 Maeda, T. *et al.* (2009) Loss of cone photoreceptors caused by chromophore depletion is partially prevented by the artificial chromophore pro-drug, 9-*cis*-retinyl acetate. *Hum. Mol. Genet.* 18, 2277–2287
- 119 Radu, R.A. *et al.* (2005) Reductions in serum vitamin A arrest accumulation of toxic retinal fluorophores: a potential therapy for treatment of lipofuscin-based retinal diseases. *Invest. Ophthalmol. Vis. Sci.* 46, 4393–4401
- 120 Radu, R.A. *et al.* (2004) Isotretinoin treatment inhibits lipofuscin accumulation in a mouse model of recessive Stargardt's macular degeneration. *Novartis Found. Symp.* 255, 51–63 (discussion 63–57, 177–178)
- 121 Radu, R.A. *et al.* (2003) Treatment with isotretinoin inhibits lipofuscin accumulation in a mouse model of recessive Stargardt's macular degeneration. *Proc. Natl. Acad. Sci. U. S. A.* 100, 4742–4747
- 122 Law, W.C. and Rando, R.R. (1989) The molecular basis of retinoic acid induced night blindness. *Biochem. Biophys. Res. Commun.* 161, 825–829
- 123 Znoiko, S.L. *et al.* (2005) Downregulation of cone-specific gene expression and degeneration of cone photoreceptors in the *rpe65<sup>-/-</sup>* mouse at early ages. *Invest. Ophthalmol. Vis. Sci.* 46, 1473–1479
- 124 Fan, J. *et al.* (2008) *Rpe65<sup>-/-</sup>* and *Lrat<sup>-/-</sup>* mice: comparable models of Leber congenital amaurosis. *Invest. Ophthalmol. Vis. Sci.* 49, 2384–2389
- 125 Rodieck, R.W. (1998) *The First Steps in Seeing*, Sinauer Associates
- 126 Jacobson, S.G. *et al.* (2005) Identifying photoreceptors in blind eyes caused by RPE65 mutations: prerequisite for human gene therapy success. *Proc. Natl. Acad. Sci. U. S. A.* 102, 6177–6182
- 127 Wing, G.L. *et al.* (1978) The topography and age relationship of lipofuscin concentration in the retinal pigment epithelium. *Invest. Ophthalmol. Vis. Sci.* 17, 601–607
- 128 Rattner, A. and Nathans, J. (2006) Macular degeneration: recent advances and therapeutic opportunities. *Nat. Rev. Neurosci.* 7, 860–872
- 129 Sparrow, J.R. *et al.* (1999) A2E, a lipofuscin fluorophore, in human retinal pigmented epithelial cells in culture. *Invest. Ophthalmol. Vis. Sci.* 40, 2988–2995
- 130 Eldred, G.E. (1993) Age pigment structure. *Nature* 364, 396
- 131 Yasukawa, T. *et al.* (2007) Glycooxidized particles mimic lipofuscin accumulation in aging eyes: a new age-related macular degeneration model in rabbits. *Albrecht von Graefes Arch. Klin. Exp. Ophthalmol.* 245, 1475–1485
- 132 Vives-Bauza, C. *et al.* (2008) The age lipid A2E and mitochondrial dysfunction synergistically impair phagocytosis by retinal pigment epithelial cells. *J. Biol. Chem.* 283, 24770–24780
- 133 Sparrow, J.R. *et al.* (2000) The lipofuscin fluorophore A2E mediates blue light-induced damage to retinal pigmented epithelial cells. *Invest. Ophthalmol. Vis. Sci.* 41, 1981–1989