Parallel visual cycles in the zebrafish retina

Fleisch, V C; Neuhauss, S C F
Parallel visual cycles in the zebrafish retina

Abstract

Vertebrate vision necessitates continuous recycling of the chromophore 11-cis retinal (RAL). The classical (or canonical) visual cycle employs a number of enzymes located in the photoreceptor outer segment and RPE (retinal pigment epithelium) of the retina to regenerate 11-cis RAL from all-trans RAL. Cone-dominant species are believed to utilize a second, intra-retinal, pathway for 11-cis RAL generation, involving retinal Müller glia cells. This review summarizes the efforts made in zebrafish to gain a better understanding of the role of these two visual cycles for rod and cone photoreceptor chromophore recycling.
Parallel visual cycles in the zebrafish retina

Valerie C. Fleisch¹ and Stephan C.F. Neuhauss²

¹ Department of Biological Sciences & Centre for Prions and Protein Folding Diseases
Biological Sciences, University of Alberta, Edmonton, Canada,

² University of Zurich, Institute of Molecular Life Sciences; Neuroscience Center Zurich and
Center for Integrative Human Physiology, Winterthurerstrasse 190, CH-8057 Zurich, Switzerland

Corresponding author:
Stephan C.F. Neuhauss
Telephone: +41 446356040
Telefax: +416356897
stephan.neuhauss@imls.uzh.ch

Abstract
Vertebrate vision necessitates continuous recycling of the chromophore 11-cis retinal (RAL). The classical (or canonical) visual cycle employs a number of enzymes located in the photoreceptor outer segment and RPE (retinal pigment epithelium) of the retina to regenerate 11-cis RAL from all-trans RAL. Cone-dominant species are believed to utilize a second, intra-retinal, pathway for 11-cis RAL generation, involving retinal Müller glia cells. This review summarizes the efforts made in zebrafish to gain a better understanding of the role of these two visual cycles for rod and cone photoreceptor chromophore recycling.
Article Outline

1. Introduction .................................................................................................................................. 3
  1.1. Vertebrate duplex retina .................................................................................................... 3
  1.2. Canonical retinoid recycling in vertebrate eyes ............................................................... 4
2. Alternative cone visual cycle in cone-dominant species .......................................................... 5
3. Model system zebrafish ............................................................................................................ 8
  3.1. Zebrafish as a model organism .................................................................................... 8
  3.2. Zebrafish visual system ............................................................................................... 9
4. Cone visual cycle in zebrafish ................................................................................................ 10
  4.1. Vision in canonical cycle-deficient zebrafish .............................................................. 10
  4.2. Müller cells are implicated in the intra-retinal visual cycle ........................................... 12
  4.3. Subfunctionalization of zebrafish Cralbp provides evidence for the existence of two pathways for visual pigment recycling in the zebrafish ........................................ 14
  4.4. Cone photoreceptors use both, the canonical and the intra-retinal visual cycle ...... 16
  4.5. A novel enzymatic coupling reaction for the formation of 11-cis RAL in cone photoreceptors in the carp (Cyprinus carpio) ................................................................. 18
  4.6. What may be the rationale behind having two vertebrate visual cycles ............... 19
5. Future directions ..................................................................................................................... 20
  5.1. Cone visual cycle in higher mammals ........................................................................... 20
  5.2. Transport of 11-cis ROL from Müller glia cells to cone photoreceptors ......... 22
  5.3. Implication for therapy of retinal disease (rod-cone, cone dystrophies) .......... 24
1. Introduction

1.1. Vertebrate duplex retina

Our visual perception of the world relies on the ability to detect differences in luminance, contrast and color with high temporal and spatial resolution. To achieve this remarkable feat, light perceiving eyes have evolved from quite simple organs, being capable to detect differences in luminance (for phototaxis) into highly complex image-forming vertebrate eyes (Lamb et al., 2007). Photoreceptors, the light-receptive cells in the neural retina have existed throughout evolution, however the “modern” vertebrate duplex retina, containing both rod and cone photoreceptors, has only been established by the time of the last common ancestor that humans share with jawless fish over 500 million years ago (Lamb et al., 2009). Our early ancestors exclusively relied on photopic (cone photoreceptor-mediated) vision. Scotopic vision, mediated through rod photoreceptors, has only evolved from cone photoreceptors as a specialization to low light-level environments later in vertebrate history. The majority of extant vertebrates are teleost species with most of them living in deep water under mainly scotopic conditions.

Rod and cone photoreceptors are morphologically distinct and show substantial differences in light sensitivity and overall kinetics (Kawamura and Tachibana, 2008; Tachibana et al., 2007). Whereas rod photoreceptors are extremely sensitive to light and are thus used under dim light (scotopic) conditions, cone photoreceptors allow for high-resolution color vision under daylight (photopic) conditions, during which rods become saturated. The opsins, mediating photon capture, also come in rod- (rod opsin) and cone (cone opsins)-specific variants.
For continuous vision, two main enzymatic pathways are required in the retina: phototransduction, the pathway converting light into a neuronal response, and the visual (or retinoid) cycle, regenerating bleached visual pigment. The vertebrate visual cycle (Wald, 1968) has been extensively studied, and components as well as enzymatic reactions including their kinetics are well-understood. It had been assumed that vertebrates utilize the same pathway for regenerating rod and cone visual pigment (11-cis RAL). Interestingly, rods and cones do partly utilize different subtypes of enzymes in phototransduction (for example different subunits of the transducin protein (Larhammar et al., 2009)). Considering this specialization and the apparent differences in cone versus rod light sensitivities and kinetics, it has been tempting to speculate that cone and rod photoreceptors might use cell type-specific enzymes/pathways for processes involving the retinoid cycle as well.

1.2. Canonical retinoid recycling in vertebrate eyes

The so-called canonical visual cycle (Wald, 1968) is a series of enzymatic reactions resulting in the regeneration of the visual pigment 11-cis RAL from all-trans RAL, which is the end-product of phototransduction. Thus, it is fundamental for the restoration of vision after light exposure. The vertebrate visual cycle involves two cellular compartments - photoreceptor outer segments and the RPE (retinal pigment epithelium) (Crouch et al., 1996; Lamb and Pugh, 2004; McBee et al., 2001a; McBee et al., 2001b; Rando, 2001; Saari, 2000) (Figure 1).

Retinoids are highly suited to capture photons, but their chemical properties also make them prone to degradation potentially leading to toxic products. This may well be the rationale to separate the regeneration of the visual pigment to another cell type to prevent photoreceptor toxicity. Even in cephalopods, where pigment regeneration takes place in the photoreceptors,
photon capture and pigment regeneration is intracellularly separated (Hara and Hara, 1976; Molina et al., 1992; Terakita et al., 1989).

In the photoreceptor outer segments absorption of a photon results in the cis to trans conversion of the Vitamin A derivative 11-cis retinal (RAL). The resulting all-trans RAL is first reduced to all-trans ROL by an NADPH-dependent dehydrogenase (Cideciyan et al., 2000; Lion et al., 1975; Rattner et al., 2000; Suzuki et al., 1993), and then transported from the photoreceptor to the second compartment involved in visual pigment recycling, the RPE. Upon arrival in RPE cells, all-trans ROL is esterified into retinylesters (REs) by the lecithin:retinol acyltransferase LRAT (Mondal et al., 2000; Ruiz et al., 1999; Saari and Bredberg, 1989; Saari et al., 1993), and then isomerized to 11-cis ROL by the isomerase RPE65 (or Isomerase I) (Gollapalli and Rando, 2003; Jin et al., 2005; Moiseyev et al., 2005; Redmond et al., 2005). Finally, 11-cis ROL is oxidized to 11-cis RAL by a retinol dehydrogenase (Cideciyan et al., 2000; Driessen et al., 1997; Gamble et al., 2000; Haeseleer et al., 1998; Lion et al., 1975; Simon et al., 1999; Suzuki et al., 1993). The recycled 11-cis RAL is transported back to photoreceptor outer segments where it re-assembles with the opsin to form functional visual pigment (Bok, 1985; Pepperberg and Clack, 1984) (Figure 1).

2. Alternative cone visual cycle in cone-dominant species

In recent years researchers have begun to challenge the concept of the existence of a single (the canonical) visual cycle mediating pigment regeneration in rod as well as in cone photoreceptors. In vivo data thus far had mainly stemmed from studies in nocturnal (and consequently rod-dominant) rodent animal models such as mouse and rat. These studies might have been biased,
since the vast majority of photoreceptors in the mouse retina are rods (more than 95%), and mouse vision primarily relies on rods.

Evidence supporting the existence of a second (cone-specific) retinoid cycle can be found in the older literature. Goldstein and co-workers had shown in the 60s and 70s that in the isolated frog neural retina (separated from the RPE) the cone but not the rod photoreceptor early receptor potential (ERP) recovered after bleaching and subsequent dark adaptation (Goldstein, 1967, 1968; Goldstein and Wolf, 1973; Hood and Hock, 1973). Moreover, it had been shown in the 1980s that in the retina of the cone-dominant chicken the major source of retinylesters are 11-cis REs localized inside the neural retina, whereas rod-dominant species, such as frogs and cows show a pre-dominance of all-trans REs in the RPE (Bridges and Alvarez, 1987; Rodriguez and Tsin, 1989). Bleached salamander rod outer segments are only capable of recovering their visual sensitivity upon administration of exogenous 11-cis RAL, but not ROL, whereas cones were able to utilize both retinoid sources (Jones et al., 1989).

Thus, the idea that rod and cone photoreceptors may utilize distinct pathways for regenerating their visual pigment has been around for a couple of decades. However, only fairly recent mostly biochemical studies conducted in cone-dominant species such as chicken and ground squirrel have confirmed this concept (Muniz et al., 2007).

Light exposure of the chicken retina leads to the accumulation of two pools of retinylesters – a pool of 11-cis REs in the neural retina itself, and a second pool of all-trans REs in the RPE. Importantly, light exposure was tightly associated with rising levels of 11-cis REs and lowering levels of 11-cis RAL in the retina and vice versa, reflecting the basic action of a visual cycle (Trevino et al., 2005; Villazana-Espinoza et al., 2006). Biochemical studies in chicken and ground squirrel have then provided ground-breaking new insights into how the suspected cone visual cycle might work. It was Mata and co-workers who demonstrated the existence of three
novel enzymatic activities localized to the retina of the cone-dominant species chicken and ground squirrel (Mata et al., 2002; Mata et al., 2005). These include a retinylester synthase which is thought to be the equivalent of LRAT in the canonical visual cycle, a retinol dehydrogenase able to oxidize 11-cis ROL, and last and most importantly, a novel isomerase, termed Isomerase II. This isomerase converts all-trans ROL into 11-cis ROL in the presence of CRBP and CRALBP. It is evidently different from RPE65, the isomerase of the canonical visual cycle, as its substrate is all-trans ROL as opposed to all-trans REs, and it is able to directly create 11-cis ROL without the formation of REs (Mata et al., 2002) (Figure 2).

However, actual in vivo data are scarce and data presented from studies performed in the mouse, the most widely used model system for retina research, have remained controversial. The mouse visual cycle has been mainly assessed in RPE65 knock-out mouse models. All studies have unequivocally shown that loss of the retinoid isomerase RPE65 leads to impaired rod function. Initial studies report that cones were unaffected by the absence of RPE65 protein (Redmond et al., 1998), whereas subsequent studies have argued that both rod and cone photoreceptors were affected in the knock-out. Thus, cone photoreceptors were thought to rely entirely on the canonical visual cycle for chromophore regeneration (Fan et al., 2003; Feathers et al., 2008; Seeliger et al., 2001; Wenzel et al., 2007). This hypothesis has been challenged by the recent findings of Wang and Kefalov, who provided clear evidence for an intra-retinal visual cycle in the rod-dominant mouse and salamander, which will be discussed in more detail below (Wang et al., 2009; Wang and Kefalov, 2009).

Additional in vivo studies, preferably in diurnal cone-dominant animal models, will likely contribute to a more in-depth understanding of the functional roles of the intra-retinal and the RPE-localized visual cycles in the vertebrate retina.
3. Model system zebrafish

3.1. Zebrafish as a model organism

The zebrafish has earned its status as one of the most widely used vertebrate animal models besides the mouse for a number of reasons. In contrast to larger vertebrate models, zebrafish are easy to maintain in large numbers, and a great amount of offspring can be bred weekly. *In vivo* studies in zebrafish embryos and larvae are facilitated by the transparency of the embryos in combination with their extraordinary rapid embryonic development.

Resources for genetic engineering in zebrafish are vast compared to other cone-dominant models, such as the chicken or ground squirrel. Gain- as well as loss of function studies can be conducted by using a variety of transgenic and knock-down as well as novel knock-out approaches. In addition, the zebrafish genome is fully sequenced and largely annotated.

One peculiarity of the zebrafish genome that it shares with all teleosts, is that frequently two orthologs of a given mammalian gene are found. Vertebrates have experienced 2 successive rounds of genome duplication (1R, 2R) during their evolution (Garcia-Fernandez and Holland, 1994; Lundin, 1993; Putnam et al., 2008). In addition to this tetraploidization, the teleost lineage underwent a 3rd round (R3) of genome duplication (Meyer and Van de Peer, 2005; Taylor et al., 2003). Whereas usually a large part of duplicated genes are being lost during the course of evolution, a considerable number of genes will generate either genes with novel functions (neofunctionalization) or genes with specific sub-functions (sub-functionalization) (Force et al., 1999). Through mutations in regulatory sequences, the expression domains of an ancestral gene may be split among the two paralogs arising through a duplication event. In such an event both
genes perform a kind of job sharing arrangement to fulfill the essential ancestral gene function and thereby evade mutational elimination by being under selective pressure (Force et al., 1999).

3.2. *Zebrafish visual system*

Zebrafish rely on a functional visual system already very early in development. As soon as embryos hatch from their chorions at around 3dpf (days post-fertilization), free-swimming larvae need to track food and avoid predators, necessitating a fully developed retina (Gahtan et al., 2005).

The overall structure and function of the retina is conserved to a high degree across vertebrate species. Accordingly, the zebrafish retina contains all the cell types found in any other vertebrate – retinal ganglion cells, bipolar cells and amacrine cells, photoreceptors and Müller glia cells. These cell types develop in an inside-out progression. After evagination of the optic lobes, ganglion cells are the first retinal cells to differentiate at around 32hpf (Burrill and Easter, 1995; Hu and Easter, 1999; Schmitt and Dowling, 1994, 1999). In succession first amacrine and horizontal cells develop around 50hpf, and the photoreceptor cells are the last cells to differentiate around 55hpf (Schmitt and Dowling, 1999). Functional synapses only develop after cell type differentiation, and thus signal transmission from photoreceptors to second-order neurons gets only fully functional around 5dpf (Biehlmaier et al., 2003). At 5dpf the larval zebrafish retina has developed synaptic connections between all retinal layers, and robust behavioral as well as electrophysiological responses (ERGs) can be measured (Baier, 2000; Branchek, 1984; Brockerhoff et al., 1995; Easter and Nicola, 1996, 1997; Neuhauss, 2003; Neuhauss et al., 1999).

Like humans, zebrafish have a duplex retina containing two types of photoreceptors – rods and cones. Zebrafish are a day-active diurnal species and hence predominantly use cone
photoreceptors for vision, just like humans. ERG recordings in the larval zebrafish have shown that rod-driven responses can only be measured at around 15dpf, and thus it is assumed that zebrafish rod photoreceptors do not significantly contribute to vision before this developmental stage (Bilotta et al., 2001; Branchek, 1984). As a result, the larval zebrafish retina can be considered functionally cone-dominant.

4. Cone visual cycle in zebrafish

4.1. Vision in canonical cycle-deficient zebrafish

The 11-cis ROL isomerase RPE65 is a key enzyme in the vertebrate visual cycle (Redmond et al., 2005; Redmond et al., 1998). It is highly expressed in the RPE of all vertebrates and is vital for regenerating the visual pigment 11-cis RAL. A variety of different mutations in RPE65 have been isolated in human patients presenting with mild-to-severe retinal dystrophies (Thompson et al., 2000). RPE65 and its effect on chromophore recycling has been studied extensively in the mouse. However due to conflicting data in different studies, the question if an intra-retinal visual cycle exists in rod-dominant species remained controversial until recently (Fan et al., 2003; Feathers et al., 2008; Redmond et al., 2005; Redmond et al., 1998; Seeliger et al., 2001; Wang et al., 2009; Wang and Kefalov, 2009; Wenzel et al., 2007).

In 2007, Schonthaler et al. were the first to tackle this controversy using the cone-dominant zebrafish (Schonthaler et al., 2007). Two orthologs of mammalian RPE65, which are likely to have arisen during the 3rd round of genome duplication in teleosts, were identified in the zebrafish genome. Only one paralog, RPE65a, is expressed in the zebrafish eye (in the RPE),
whereas *RPE65b* is localized to the ventricular zone, the upper and lower jaw and the pectoral fins during early development.

Schonthaler et al. took a dual approach to create a zebrafish model of *RPE65a* loss of function. First a morpholino-mediated knock-down approach was employed to block translation of Rpe65a protein. This technique reduced the protein level by at least 88%. Since the enzymatic activity of RPE65 is quite low, necessitating surprisingly high RPE65 protein levels in RPE cells, this reduction is expected to block any RPE65 dependent pathway. They additionally took advantage of Ret-NH₂ (retinylamine), a potent inhibitor of RPE65 (Golczak et al., 2005), and thus additionally blocking the canonical visual cycle by pharmacological means.

The impact of severely decreased *RPE65a* levels onto retinoid metabolism was assessed by HPLC analysis of retinoids in the larval zebrafish eye. Baseline levels of 11-*cis* RAL were decreased in *RPE65a* MO-injected larvae, as compared to wildtype siblings. This finding could be explained by immunostainings of rod photoreceptors, which showed deterioration of rod outer segments. Intense light treatment was used to bleach visual pigment in larvae, and pigment regeneration was assessed after a subsequent period of dark adaptation. Surprisingly, *RPE65a*-deficient larvae were able to regenerate the full amount of 11-*cis* RAL (Figure 3A).

To exclude that the regenerated 11-*cis* RAL did not stem from the remaining low levels of *RPE65a* due to only partial knock-down, Schonthaler et al. blocked the canonical visual cycle using the RPE65 inhibitor Ret-NH₂ (Golczak et al., 2005). Initial studies in cell culture revealed that Ret-NH₂ directly blocks RPE65 enzymatic activity, as cells incubated with the compound as well as all-\textit{trans} ROL were unable to synthesize 11-*cis* ROL in contrast to untreated cells. Bathing zebrafish larvae in a Ret-NH₂ solution and subsequent bleaching and re-dark adaptation resulted in the same level of 11-*cis* RAL reduction as had been measured in MO-injected larvae. Moreover, combining the MO and Ret-NH2 did not further exacerbate the phenotype (Figure 3A).
As the zebrafish visual system is fully functional by 5dpf, MO-treated larvae could be assessed for visually-mediated behavior. The OKR (optokinetic response) assays tracking eye movements of zebrafish larvae in response to a moving stimulus, and is a highly robust behavioral test for visual system function (Huang and Neuhauss, 2008; Neuhauss, 2003; Rinner et al., 2005b). Consistent with the previous result, \textit{RPE65a} deficiency did not result in decreased contrast sensitivity/visual resolution, as tested by the OKR.

Whereas the majority of researchers have found that in the RPE65 knock-out mouse, both rod and cone photoreceptors were severely impaired (Fan et al., 2003; Feathers et al., 2008; Seeliger et al., 2001; Wenzel et al., 2007), Redmond et al. had shown that although rod-driven ERG responses were abolished, cone function still remained intact. This finding led them to speculate that cones might use a different pathway for regenerating their pigment. Thus the question if an intra-retinal visual cycle existed in rod-dominant species remained unanswered until recently. Kefalov and colleagues provided direct \textit{in vitro} as well as \textit{in vivo} evidence for the existence of an intra-retinal visual cycle in the rod dominant salamander and mouse, as well as in primates including humans (Wang et al., 2009; Wang and Kefalov, 2009). As to the question why previous studies had concluded that the RPE is the only source of 11-\textit{cis} RAL in the mouse retina, Wang et al. speculate that 11-\textit{cis} ROL generated in Müller cells is only supplied to a tiny fraction of mouse photoreceptors. In the mouse retina only about 3\% of all photoreceptors are cones. Thus, increasing the mouse cone population by the \textit{nrl-/-} knock-out would not increase the chromophore recycling capacity. Moreover, RPE65 might be expressed in cone photoreceptors and a knock-out would thus directly impact the intra-retinal visual (Wang et al., 2009; Wenzel et al., 2007; Znoiko et al., 2005).

4.2. \textit{Müller glia cells are implicated in the intra-retinal visual cycle}
RPE65 is highly expressed in the RPE of all vertebrates, where the canonical visual cycle is located. The second, intra-retinal visual cycle is independent of the RPE, and thus some of the enzymatic steps for conversion of all-trans ROL to 11-cis RAL must be localized to a compartment different than the RPE. It had long been assumed that this cellular compartment might be the Müller glia cells of the retina. These specialized astrocytes perform an impressive number of different functions in the retina, including structural and metabolic support of cells, ion buffering, and neurotransmitter homeostasis (Bringmann et al., 2006), but also might have yet undiscovered additional roles. Das et al. have shown that cultured Müller cells can isomerize and esterify retinoids in cell culture (Das et al., 1992). It has been shown later that these cells possess an 11-cis ARAT activity, which catalyzes the synthesis of 11-cis REs from 11-cis ROL and whose activity is enhanced in the presence of CRALBP (Muniz et al., 2006). Very recently Wang et al. have provided direct *in vivo* evidence that Müller glia cells are essential for regeneration of visual pigment for cone photoreceptors in the salamander and the mouse (Wang et al., 2009; Wang and Kefalov, 2009). They showed that if physical contact between Müller cells and cone photoreceptors is disturbed (by physical separation using a suction electrode) or when the gliotoxin L-alpha-AAA is administered to the retina, cone chromophore cannot be regenerated (Wang et al., 2009; Wang and Kefalov, 2009).

Müller glia cells have also been shown to express components involved in the canonical visual cycle, such as RGR (retinal G-protein coupled receptor) (Chen et al., 1996; Shen et al., 1994), and the retinoid binding proteins CRBP (cellular retinol binding protein) and CRALBP (cellular retinalaldehyde binding protein) (Bunt-Milam and Saari, 1983; Eisenfeld et al., 1985; Fleisch et al., 2008).
4.3. Subfunctionalization of zebrafish Cralbp provides evidence for the existence of two pathways for visual pigment recycling in the zebrafish

In zebrafish Cone visual pigment regeneration seems to be independent of RPE65, the retinoid isomerase of the canonical visual cycle (Schonthaler et al., 2007). In addition, data from other cone-dominant species suggested the existence of a second, cone-specific pathway for 11-cis RAL regeneration.

Retinoid binding proteins such as CRALBP have a variety of functions in the vertebrate visual cycle. Mutations in human CRALBP lead to different types of photoreceptor dystrophies, such as Retinitis Pigmentosa and Bothnia dystrophy (Burstedt et al., 1999; Maw et al., 1997; Morimura et al., 1999), OMIM #180090. CRALBP function has been investigated in a mouse knock-out model (Saari et al., 2001). Lack of Cralbp protein in these animals led to delayed rhodopsin regeneration and dark adaptation after light exposure. Whereas levels of 11-cis RAL decreased, all-trans REs increased, suggesting that Cralbp plays a role in the isomerization step of the canonical visual cycle (Saari et al., 2001).

Intriguingly Cralbp is not only expressed in the RPE, where it is involved in the canonical cycle, but also in Müller glia cells (Saari and Crabb, 2005).

Whereas rodents (and humans) possess only one gene encoding CRALBP, zebrafish possess two variants of the mammalian CRALBP gene – cralbp a and cralbp b (Collery et al., 2008; Fleisch et al., 2008). Notably, these two paralogs are expressed in the two compartments believed to be involved in chromophore regeneration – cralbp a in the zebrafish RPE, and cralbp b in Müller glia cells, in both the larval and adult retina (Collery et al., 2008; Fleisch et al., 2008). This specific expression pattern suggests a sub-functionalization of the two cralbp genes which had arisen during teleost evolution and allowed us to separately assess the role of each paralog in retinoid recycling in the zebrafish (Fleisch et al., 2008). For this purpose morpholino (MO)
oligonucleotides directed against each paralog were designed and injected into larval zebrafish. HPLC analysis of retinoids was used to determine the implication of loss of \textit{cralbp a} and/or \textit{cralbp b} on retinoid recycling. Control and MO-injected larvae were dark adapted, bleached by high-intensity light and finally again dark adapted. In line with the findings published by Schonthaler et al., knock-down of the RPE-expressed Cralbp a, led to decreased levels of 11-\textit{cis} RAL (Figure 3B). Knocking-down Müller cell Cralbp b resulted in almost identically reduced (about 50\%) 11-\textit{cis} RAL levels in the zebrafish retina. Thus, both zebrafish CRALBP orthologs are clearly implicated in visual pigment regeneration in the zebrafish larva. To further prove this point, a combination of both morpholinos was administered. Measurements of 11-\textit{cis} RAL levels showed a synergistic effect, as loss of both Cralp proteins led to a reduction in retinal 11-\textit{cis} RAL to about 25\% of control levels. Residual 11-\textit{cis} RAL in the retina could be due to either residual Cralbp protein expression caused by incomplete morpholino penetrance and/or to incomplete bleaching of the visual pigment by the light treatment applied.

To exclude that photoreceptor degeneration (as opposed to defective chromophore recycling) caused the observed phenotype, we conducted standard histology as well as immunostainings using rod (1D1) and cone (zpr1)-specific antibodies and did not detect any signs of photoreceptor outer segment degeneration (Fleisch et al., 2008). Collery et al. additionally used an antibody against cone blue-opsin to test for cone integrity after Cralbp b knock-down. In concordance with our results, they did not detect any abnormalities in cone morphology or opsin expression (Collery et al., 2008; Fleisch et al., 2008). Our data in zebrafish mirror the data published in the Cralbp knock-out mouse model. Saari et al. showed that lack of Cralbp resulted in delayed dark adaptation as well as impaired 11-\textit{cis} RAL regeneration, which could be attributed to a defective visual cycle, as photoreceptors did not show any sign of degeneration (Saari et al., 2001).
4.4. **Cone photoreceptors use both, the canonical and the intra-retinal visual cycle**

Visually-mediated behavior (OKR assay) and ERG recordings were used to assess the effects of reduced 11-cis RAL levels on visual performance in *cralbp* MO-injected larvae (Fleisch et al., 2008). Studies have shown that rod photoreceptors do not contribute to larval vision until 15dpf (Bilotta et al., 2001; Branchek, 1984). Thus, the data obtained from these experiments exclusively reflect cone photoreceptor output.

ERG responses were measured in both single (*cralbp a* or *cralbp b*) MO- as well as double MO-injected larvae, and the ERG b-wave amplitude was used as a measure for outer retina function (Rinner et al., 2005a) (Figure 4A). Injection of either *cralp a* or *b* MO induced a highly significant reduction in the ERG b-wave amplitude, and co-injection further decreased the measured response to levels lower than 25% of controls (Figure 4B). Remarkably, Cralbp b deficiency led to a more pronounced effect on ERG b-wave amplitude than Cralbp a deficiency, suggesting that cone photoreceptors are more reliant on the intra-retinal (Müller cell) pathway for chromophore regeneration. One could speculate that this difference might be due to differences in remaining Cralbp protein levels. Due to the lack of a Cralbp antibody reliably recognizing zebrafish Cralbp in Western Blots on larval tissue, residual Cralbp a and b protein levels could not be measured. We have provided a partial answer by measuring *cralbp a* and *b* mRNA levels after MO injection. RT PCR experiments showed that *cralbp a* mRNA was reduced to 22% of control levels, whereas *cralbp b* mRNA was reduced to 29% of control levels (Fleisch et al., 2008). This suggests that the *cralbp a* and *b* knock-downs were of comparable efficiency. Thus, the more severe decline in cone function in the *cralbp b* knock-down indicates that the intra-retinal visual cycle is more relevant for zebrafish cone photoreceptors than the canonical visual cycle in the RPE.
In addition to recordings of retinal currents, the visually-mediated OKR (optokinetic response) assay was used to assess visual system performance in cralbp-deficient zebrafish larvae. Collery et al. reported that knock-down of both zebrafish Cralbp proteins resulted in a significant reduction in the number of saccades in the OKR (Collery et al., 2008). In contrast to that, we found that visual performance, measured as a contrast sensitivity function, was significantly impaired in zebrafish larvae injected with the Müller cell-specific cralbp b MO, but cralbp a MO-injected fish did not differ from wildtype controls (Fleisch et al., 2008) (Figure 5). Interestingly, Schonthaler et al. had similarly not observed an impaired OKR response in rpe65a MO-injected larvae, despite severely reduced 11-cis RAL levels as assessed by HPLC analysis (Schonthaler et al., 2007) (Figure 3B). These data support our hypothesis that the Müller cell-specific pathway mediated by cralbp b is more important for cone photoreceptor function.

In conclusion, our data and the data of Collery et al. suggest that two pathways for regenerating visual pigment exist in the zebrafish retina – one located to the RPE and one located to Müller glia cells. Cralbp seems to be indispensable for efficient 11-cis RAL production in both pathways. The cralbp b (Müller cell) dependent pathway is not reliant on rpe65a (Schonthaler et al., 2007), and thus most likely utilizes a different isomerase activity (similar to the Isomerase II catalytic activity identified in chicken/ground squirrel by Mata et al. (Mata et al., 2005)). Importantly, we have provided evidence that zebrafish cone photoreceptors utilize both sources of regenerated pigment. Knocking-down either cralbp paralog did not result in complete absence of 11-cis RAL, as cone photoreceptors are capable of utilizing the 2nd source of recycled visual pigment (from the RPE or Müller cells). It has been shown that cone photoreceptors possess a 11-cis ROL dehydrogenase activity, capable of oxidizing 11-cis ROL into 11-cis RAL (Jones et al.,
189; Mata et al., 2002), and are able to regenerate visual pigment from 11-cis ROL or 11-cis RAL (Jones et al., 1989).

Chromophore regeneration in mouse cone photoreceptors has been shown to be significantly delayed in the absence of Cralbp (Saari et al., 2001). This can be readily explained by the fact that the Cralbp protein is expressed in the RPE as well as Müller glia cells of the mouse retina (Saari and Crabb, 2005). Thus, loss of Cralbp function will affect both, the canonical as well as the intra-retinal visual cycle.

4.5. **A novel enzymatic coupling reaction for the formation of 11-cis RAL in cone photoreceptors in the carp (Cyprinus carpio)**

Mata et al. had identified three novel catalytic activities implicated in the intra-retinal visual cycle in the chicken and ground squirrel retina (Mata et al., 2002; Mata et al., 2005) - a retinylester synthase, a retinol dehydrogenase and a novel isomerase. The molecular identity of these enzymes has not been elucidated yet.

Cone but not rod photoreceptors seem capable of oxidizing 11-cis ROL to 11-cis RAL (Jones et al., 1989). Such an 11-cis retinol dehydrogenase (RDH) activity preferring NADP+ as a cofactor had then been isolated in chicken and ground squirrel microsomes, and suggested to be implicated in this cone-specific oxidation step (Mata et al., 2002; Mata et al., 2005).

Miyazono et al. investigated the metabolism of all-trans RAL and 11-cis RAL in purified carp (Cyprinus carpio) rod and cone photoreceptors. In contrast to the data from chicken and ground squirrel, the oxidation of 11-cis ROL into 11-cis RAL by RDHs was not effective. The authors speculated that the co-factor might be all-trans RAL rather than NADP+ (Miyazono et al., 2008). To test their hypothesis, they added all-trans RAL to the carp cone lysate. Addition of 11-cis
ROL plus all-trans RAL, but not NADP+, resulted in formation of 11-cis RAL as well as all-trans ROL. Miyazono et al. concluded that this ALOL coupling reaction, which they detected exclusively in carp cone but not rod photoreceptors, might be the enzymatic activity underlying 11-cis ROL oxidation in cone photoreceptors. Heat treatment of cone lysates abolished the ALOL reaction, suggesting that it is mediated by an enzyme (complex).

The identity of this enzyme and the molecular mechanisms underlying the ALOL coupling reaction are currently still unknown. Moreover it remains unclear why different retinol dehydrogenase enzymatic activities have been identified in the carp versus the chicken and ground squirrel retina.

4.6. What may be the rationale behind having two vertebrate visual cycles?

The discussed recent data indicate that the canonical visual cycle can regenerate both rod and cone pigments. This begs the question why two separate visual cycles have emerged during the course of evolution. One reason may be found in the high demand of regenerating capacity of the more numerous rods under photopic conditions.

This competition is aggravated by the rods being driven into saturation under photopic conditions with a maximal demand for pigment regeneration. As rod and cone photoreceptors compete for 11-cis RAL, the high demand for visual pigment by rod photoreceptors under photopic conditions might therefore restrict the availability of chromophore to cone photoreceptors in their working range (Mata et al., 2002; Muniz et al., 2007). Samardzija et al. created an RPE65 knock-in mouse model carrying the R91W mutation to investigate the consequences of chromophore insufficiency on cone function (Samardzija et al., 2009). Their data revealed that under conditions of limited 11-cis RAL supply, rod and cone opsins compete for the remaining visual pigment. In their
mouse model, rod photoreceptors out-competed cone photoreceptors, which ultimately led to cone degeneration.

On the other hand, one could speculate that the intra-retinal visual cycle might be evolutionary older than the canonical visual cycle localized to the RPE. Rod photoreceptors have evolved from cone photoreceptors during vertebrate evolution, and thus a chromophore recycling pathway supplying 11-cis RAL to cone photoreceptors must have existed before the emergence of rod photoreceptors. Most recent data from zebrafish, salamander, mouse and primates have shown that cones most likely are able to utilize both visual cycles for pigment regeneration (Fleisch et al., 2008; Wang et al., 2009; Wang and Kefalov, 2009). Therefore it is likely that the Müller cell-based pathway has evolved at a later step in evolution, at the time when rod photoreceptors started to populate the vertebrate retina. This led to reduced availability of chromophore for cone photoreceptors, as rods are saturated under photopic conditions, but still consume 11-cis RAL. Consequently, cone photoreceptors needed another additional source of chromophore supply.

5. Future directions

5.1. Cone visual cycle in primates

Until very recently it was claimed by the majority of researchers working with rodent models that rod-dominant species entirely rely on the canonical visual cycle for chromophore recycling (Fan et al., 2003; Feathers et al., 2008; Seeliger et al., 2001; Wenzel et al., 2007). The data arguing in favor of a second, cone-specific visual cycle, acquired in the cone-dominant models chicken, ground squirrel and zebrafish had been acknowledged, but it had been speculated that this discrepancy might be due to species differences. Primates, including humans, highly rely on cone
photoreceptors for daylight vision. However rod photoreceptors outnumber cones, which are concentrated in the fovea of the retina, by far and in consequence primates and humans could be considered rod-dominant (with the exception of the foveal region). Hence the existence of an intra-retinal visual cycle in humans has been questioned.

The studies of Wang et al. are an important contribution towards settling the question if all vertebrates have two pathways for 11-cis RAL recycling (Wang et al., 2009; Wang and Kefalov, 2009). In their first study they investigated if the rod-dominant salamander and mouse retina promotes cone visual pigment regeneration (Wang et al., 2009). Microspectrophotometry as well as electrophysiological recordings (single-cell and ERG) revealed that visual pigment is regenerated in the salamander retina independent of the RPE (and thus the canonical visual cycle). Similarly, ERG recordings in the isolated mouse retina indicated substantial pigment regeneration.

In a subsequent study they expanded their study to include primates, including humans (Wang and Kefalov, 2009). Eyecups were isolated from the RPE, and recovery of ERG responses measured after bleaching of photoreceptors. The primate, including the human retina showed robust dark adaptation in cones but not rods. This cone response recovery could be blocked by the gliotoxin L-alpha-AAA and could subsequently be reversed by addition of exogenous 11-cis ROL.

In summary, these data convincingly demonstrate that rod- as well as cone-dominant species possess an intra-retinal visual cycle, which acts to regenerate 11-cis RAL for cone photoreceptors, independent of the RPE. Thus, this Müller glia cell-dependent pathway is evolutionally conserved among vertebrates.
5.2. Transport of 11-cis ROL from Müller glia cells to cone photoreceptors

Both, the canonical as well as the proposed intra-retinal visual cycle, are each localized to two different retinal compartments. Retinoids are largely water insoluble and potentially toxic to the cell, and thus need to be in complex with a chaperone protein for transportation between different cellular compartments. IRBP (interphotoreceptor retinoid-binding protein) is localized to the sub-retinal space (Loew and Gonzalez-Fernandez, 2002) and is known to be able to bind retinoids (Shaw and Noy, 2001). It therefore has been hypothesized to play a role in the transport of 11-cis RAL from the RPE to rod outer segments (Bunt-Milam and Saari, 1983; Fong et al., 1984; Lamb and Pugh, 2006; Pepperberg et al., 1993). Surprisingly, no evidence of an impairment of the canonical visual cycle has been found in irbp-/- mice (Palczewski et al., 1999; Ripps, 2001).

This obvious discrepancy has been revisited by two recent studies (Jin et al., 2009; Parker et al., 2009). Both studies suggest that the lack of a detectable visual cycle phenotype in those mice could have been due to the genetic background of the mouse strain used in the original studies (which had been uncontrolled for an rpe65 mutation). They document a clear reduction in cone function in the novel irbp-/- model, however differ greatly in a number of experimental results, and present two quite contrary hypothesis of IRBP function. Whereas Jin et al. demonstrate rod and cone photoreceptor degeneration, and postulate that the severe cone outer segment deterioration is due to opsin mistrafficking caused by 11-cis RAL deficiency, Parker et al. show normal cone opsin localization and no photoreceptor degeneration. Recovery of 11-cis RAL after photobleach was delayed in irbp-/- mice, and accumulation of 11-cis RAL in the RPE and all-trans ROL in the retina was observed (Jin et al., 2009). These data suggest that IRBP has a role in retinoid trafficking in the RPE. Parker et al. specifically examined the impact of IRBP loss on cone photoreceptor function. ERG recordings in irbp-/- mice revealed decreased cone responses,
which could be rescued by the administration of exogenous 11-

Cis ROL (Parker et al., 2009). Consequently, IRBP is likely to be implicated supplying regenerated chromophore to mouse photoreceptors.

The two datasets of Jin and Parker et al. conflict in one major point – whereas Jin et al. argue that degeneration of photoreceptor outer segments underlies reduced cone function, Parker et al. state that it is caused by a deficiency in retinoid recycling.

The function of zebrafish Irbp has not been addressed yet. It is noteworthy that zebrafish possess two IRBP orthologs, – irbp, and irbp-like that are arranged head-to-tail in the zebrafish genome (Nickerson et al., 2006). It has been shown that irbp is expressed in the zebrafish RPE as well as in cone and rod photoreceptors (Stenkamp et al., 1998). Whereas Nickerson et al. confirmed that Irbp is expressed in the RPE and photoreceptors, Irbp-like was found to be expressed in the inner nuclear layer of the zebrafish retina (Nickerson et al., 2006). This specific expression pattern makes it tempting to speculate that zebrafish Irbp might be involved in transporting 11-

Cis RAL from the RPE to rod photoreceptors, whereas Irbp-like is localized perfectly to transport 11-

Cis ROL from Müller glia cells to cone photoreceptors.

Müller cell processes do not reach photoreceptor outer segments, but do surround the inner segment (Sarantis and Mobbs, 1992). Thus, in the intra-retinal visual cycle 11-

Cis ROL must be transported from Müller glia cells to the cell bodies of cone photoreceptors. It has been shown that chromophore can diffuse from the inner to the outer segment in cone but not rod photoreceptors (Jin et al., 1994). Thus, if IRBP provides a retinoid transport mechanism between the Müller glia cell to the cone photoreceptor cell body, 11-

Cis RAL could then readily diffuse to the outer segment to recombine with opsin to form functional pigment.
5.3. **Implication for therapy of retinal disease (rod-cone, cone dystrophies)**

Human patients suffering from photoreceptor dystrophies exhibit mutations in a great number of genes involved in the canonical visual cycle (RPE65, RDHs, LRAT, CRALBP; for a review see (Travis et al., 2007)) Hence, it is very likely that mutations in the genes encoding for the yet unidentified enzymatic components of the Muller glia cell-dependent pathway, are implicated in human retinal disease. Identification of these genes should be of prior importance to the retina research community. Moreover, the molecular mechanisms of the intra-retinal visual cycle need to be understood in order to develop appropriate therapeutics.

It has been shown that cone photoreceptors are affected by defective canonical as well as intra-retinal chromophore regeneration. It is still not clear to which extent cone photoreceptors utilize each respective pathway. Thus mutations in genes that affects specifically one or the other pathway, might affect cone vision differently.
6. References


disruption of the gene encoding interphotoreceptor retinoid-binding protein or arrestin. Biochemistry 38, 12012-12019.


entrap 11-cis-retinal leading to loss of cone function and cell death. Hum Mol Genet 18, 1266-1275.


Figure 1. Canonical visual cycle in vertebrates
**Figure 1. Canonical visual cycle in vertebrates**

Rod photoreceptors exclusively use the RPE for recycling their visual pigment through a series of enzymatic reactions. For a detailed description of the pathway refer to (Lamb and Pugh, 2004). Enzymatic activities are depicted by numbers: 1, all-*trans* ROL dehydrogenase; 2, retinylester synthase; 3, retinylester hydrolase; 4, isomerase; 5, 11-*cis* ROL dehydrogenase. Transport of retinoids between the rod outer segment and the RPE (indicated by dashed arrows) is thought to involve the retinoid-binding protein IRBP (Bunt-Milam and Saari, 1983; Fong et al., 1984; Jin et al., 2009; Lamb and Pugh, 2006; Parker et al., 2009; Pepperberg et al., 1993).
Figure 2
Figure 2. Vertebrate cone photoreceptors utilize two retinoid cycles to recycle their visual pigment

Cone photoreceptors use two parallel visual cycles: the canonical cycle localized to the RPE (depicted in green arrows), as well as an intra-retinal cycle involving Müller glia cells (depicted in red arrows). IRBP is hypothesized to play a role in transporting retinoids between the photoreceptors and the RPE, as well as Müller glia cells (Bunt-Milam and Saari, 1983; Fong et al., 1984; Jin et al., 2009; Lamb and Pugh, 2006; Parker et al., 2009; Pepperberg et al., 1993). For a comprehensive review of the intra-retinal visual cycle please refer to (Muniz et al., 2007).

Enzymatic activities are depicted by numbers: 1, all-trans ROL dehydrogenase; 2, retinylester synthase; 3; retinylester hydrolase; 4, isomerase; 5, 11-cis ROL dehydrogenase. Novel enzymatic activities unique to the intra-retinal visual cycle (in red arrows) include an isomerase (Mata et al., 2005), a RE hydrolase (Bustamante et al., 1995), a retinylester synthase (Mata et al., 2002; Muniz et al., 2006), and a retinol dehydrogenase (Mata et al., 2002; Miyazono et al., 2008).
Figure 3. HPLC analysis of ocular retinoids in visual cycle-impaired zebrafish larvae

(A) Injection of *rpe65* MO as well as administration of the Rpe65 inhibitor retinylamine (Ret-NH2) lead to equally reduced levels of 11-*cis* RAL after bleaching and subsequent dark adaptation, without showing a synergistic effect. (B) Knock-down of both zebrafish *cralbp a* and *b* severely impact chromophore regeneration; the combined knock-down exacerbates the effect.

For experimental details see (Schonthaler et al., 2007) and (Fleisch et al., 2008)
Figure 4
Figure 4. Electrophysiological assessment of visual system function in visual cycle-impaired zebrafish larvae

(A) ERG recordings of zebrafish larvae with reduced *cralbp* *a* and *b* levels illustrate impaired vision as a consequence of reduced chromophore levels; interestingly Cralbp b (Müller cell-specific paralog) seems to be more important for visual pigment recycling. (B) A comparison of the averaged saturated b-wave amplitudes of *cralbp* *a*, *b* and double MO-injected larvae highlights these observations: lack of either *cralbp* paralog leads to significantly reduced visual function; the effect of *cralbp* *b* knock-down is more severe than the effect of *cralbp* *a* knock-down. For experimental details see (Schonthaler et al., 2007) and (Fleisch et al., 2008)
Figure 5. Behaviorial assessment of visual system function in visual cycle-impaired zebrafish larvae

cralbp a (RPE-specific paralog) as well as rpe65 (expressed in the zebrafish RPE) knock-down have no effect on visual system performance, measured by the OKR, whereas cralbp b (Müller cell-specific paralog) knock-down results in decreased contrast sensitivity.

For experimental details see (Schonthaler et al., 2007) and (Fleisch et al., 2008)