

## **Regulation of retinalaldehydes isomerization in the chicken inner retina.**

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### **Background:**

#### **Classical and Non-canonical Visual Cycles**

It is known that vitamin A is a ubiquitous chromophore utilized in all photopigments described to mediate photic responses. After light exposure, the vitamin A must be recycled to regenerate the photopigment and restore light sensitivity. It is well established that the visual cycle of classical photoreceptor cells (PRCs), rods and cones, occurs in the retinal pigment epithelium (RPE) (Muniz et al., 2009). The transport depends on facilitating factors, cellular retinal binding protein (CRALBP) and cellular retinol binding protein (CRBP) present in the RPE, and interphotoreceptor retinoid binding protein (IRBP). The visual cycle utilizes RPE65 that uses all-trans retinyl esters synthesized by lecithin retinol acyl transferase (LRAT) as the substrate. However, recent reports have demonstrated that an alternative visual cycle could be acting in cones regardless of the RPE presence (Trevino et al., 2005). Muller glia cells have the ability to isomerize and esterify retinoids, two key functions supporting the existence of a cone visual cycle (Muniz et al., 2006). The putative cycle would use an as yet unidentified retinyl ester synthase, a novel retinol dehydrogenase different from that present in the classical visual cycle, and an isomerase different to RPE65. In addition, Muller cells show the expression of CRALBP and CRBP, similar to RPE cells (revised in Muniz et al., 2007).

The Retinal G protein coupled receptor (RGR) is a 32-kDa protein expressed in internal membranes of the RPE and Müller glial cells of mammals (Jiang et al 1993, Pandey et al 1994). Dark-adapted RGR contains predominantly all-*trans*-retinal (RAL), coupled via a Schiff base to a Lys residue, similar to rhodopsin (Hao et al 1999). RGR has absorption maxima ( $\lambda$  max) at 370 and 469 nm from the protonated and unprotonated forms of this Schiff base respectively (Shen et al 1994). Exposing RGR to light induces photoisomerization of the all-*trans*-RAL to 11-*cis*-RAL (Hao et al 1999). RGR appears to play an important role: Mutations in the *RGR* gene cause the severe inherited blinding disease, retinitis pigmentosa (Morimura et al 1999), suggesting that RGR is required for normal functioning of the retina.

Recent results suggest that the RGR-opsin regulates the activities of all-*trans*-Retinyl Dehydrogenase and LRAT in a light dependent fashion. The mechanism of this regulation is not clear. It may involve direct modulation of the catalytic activities or changes in the stability of a protein complex through a second messenger system (Radu et al 2008). Also RGR-opsin may interact with other enzymes of the visual cycle.

We have previously demonstrated the presence of intrinsically photosensitive retinal ganglion cells (ipRGCs) in the retina of wild type (WT) chickens as well as light responses mediated by the inner retina of blind birds, GUCY1\* (Contin et al., 2006, 2010; Valdez et al., 2009). Moreover, our group confirmed the presence of photopigments, photoisomerases and retinal chromophores (11-*cis* RAL and all-*trans* RAL) in the inner retina and RGCs that could be responsible for the photosensitivity observed (Guido et al, ARVO 2010).

### **Working Hypothesis:**

In recent studies, we characterized the expression of RGR in the inner retina. We confirm the expression of RGR in the Müller glial cells by specific glia cells markers. This expression is mainly located in the ganglion cells layer (Diaz et al, ARVO 2011). We hypothesized that RGR-opsin could regulate a novel visual cycle in the inner retina by interacting with certain enzymes present in Müller cells.

**Some relevant questions that we would like to answer are:**

- 1- What is the function of RGR-opsin in the inner retina?
- 2- Could RGR regulate the retinyl ester metabolism in the Müller Glial Cells?

**Specific aims:**

Our main goal is to determine if RGR-opsin regulates somehow a novel visual cycle in the inner retina to replenish the chromophore of intrinsically photosensitive RGCs.

To elucidate this, we propose the following specific objectives:

- 1- Determine the activity of the different enzymes involved in the visual cycle taking place in the inner retina under different light conditions.
- 2- Determine the activity of the enzymes mentioned above in the different retinal cell layers of lyophilized retinas.
- 3- Determine RGR expression in Müller cells cultures.

**Results:**

We assessed the retinylaldehydes present in the different retinal layers from animals exposed to light or dark. Cobb Hardig chickens (WT) were grown in constant darkness (DD) and then were killed following the *Guide to the Care and Use of Experimental Animals* published by the Canadian Council on Animal Care and approved by the local animal care committee (School of Chemistry, National University of Córdoba, Exp. 15-99-39796).

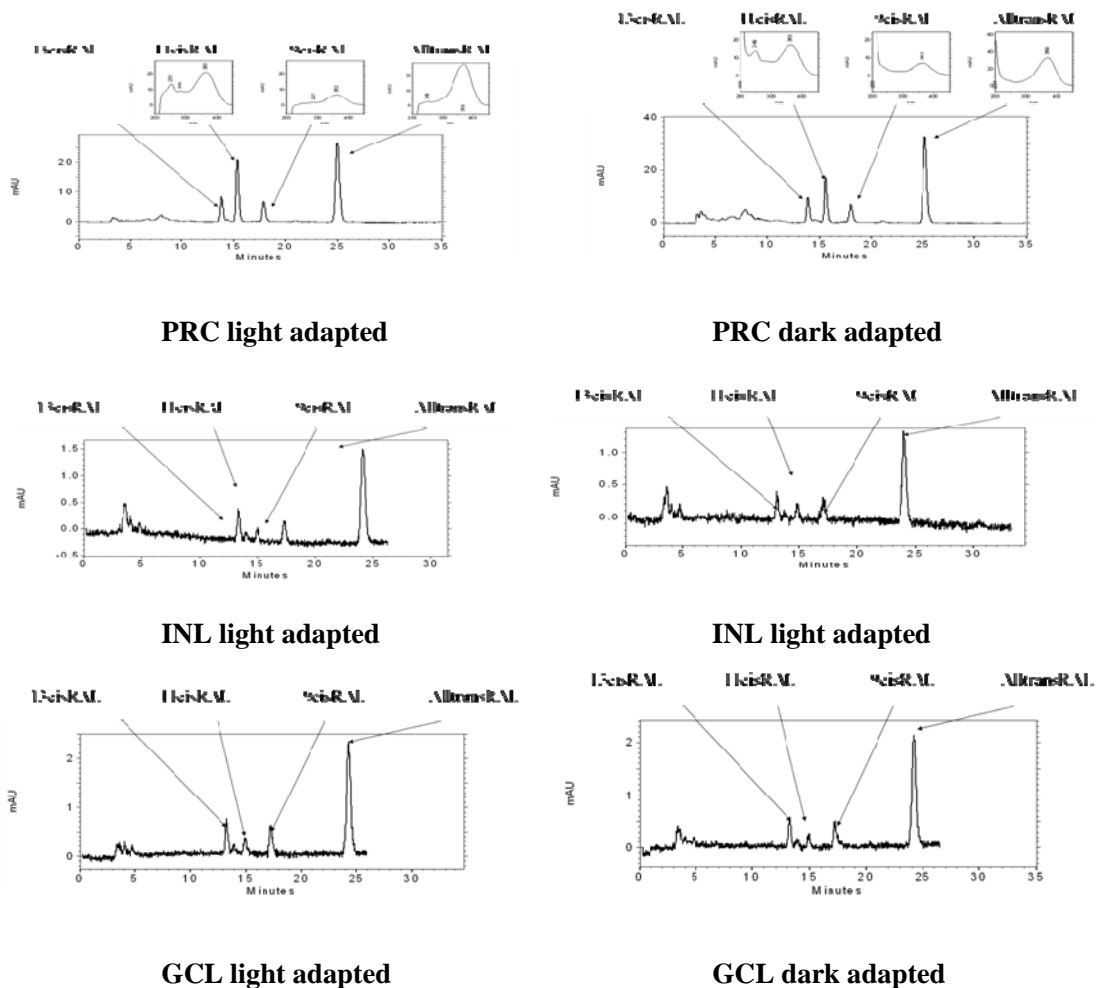
Highly enriched preparations in PRCs, inner nuclear layer cells (INL) and RGCs were obtained as previously described by Guido *et al* (1999). Briefly, after lyophilization, each retina was glued to adhesive tape by the RGC layer (white color), and this layer was separated from the rest of the retina by attaching another piece of tape to the pigment epithelium surface, pressing, and removing the second piece of adhesive tape with a cell layer attached. Each time this procedure is repeated, a different retinal layer is obtained attached to the tape. The pigment epithelium (dark gray color) was removed on the first piece of tape, whereas the PRC layer (orange color) was obtained attached to the second and third tapes, and cells from the inner nuclear layer (INL) were removed on the fifth and sixth. Remaining cells from the PRC layer and INL obtained on the fourth and seventh tapes, respectively, were discarded. This procedure was repeated at least eight times with each retina until a preparation of RGC was obtained on the basal tape.

To measure 11-*cis* and all-*trans* retinals, retinoids from the different retinal layers were extracted by the formaldehyde method before being analyzed on isocratic HPLC (eluting at 1% dioxane/hexane 2 ml min<sup>-1</sup>; 4.6×250 cm, 5 µm silica column) according to the method described by Mata et al. Retinals were monitored at 365 nm. Retinoids were identified by comparison of their retention times to authentic standards, and their online photodiode array UV spectra. Quantification was performed by comparing peak areas of retinoid with those from calibration curves obtained from authentic standards (Waters Millennium Software).

We found two different pattern of isomerization into the retina. One is the classical isomerization in the photoreceptor layer. In the dark condition, PRCs have a large amount of 11-cis-retinal. (Table 1 and Fig. 2). When we turned the light on, we found an increase of all-Trans retinal. These results are similar to observations described in the classical retinoid cycle.

On the contrary, RGCs have a different pattern of isomerization. In the dark condition, RGCs show a minimum amount of 11-cis-retinal. When the light is turned on, the amount of all-Trans retinal decreases but the 11-cis retinal increases approximately twice (Figure 2). These observations seem to be totally opposite to those obtained for the classical visual cycle described in PRCs.

Furthermore, this finding supports the hypothesis about a differential photoisomerization process taking place in the inner retina and catalyzed by RGR or other photoisomerase.

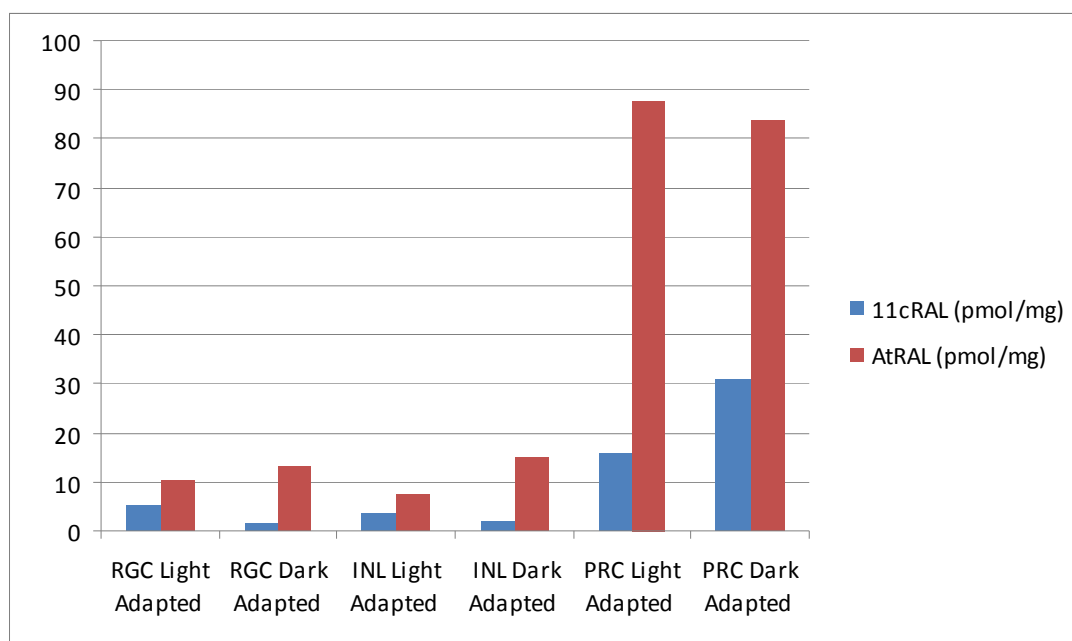


**Figure 1:** Straight Phase HPLC analysis of aldehydes from retina's layers. PRC= Photoreceptor Cells; INL= Inner Nuclear Layer; GCL= Ganglion Cell Layer.

## HPLC Quantification

	11cRAL (pmol/mg)	AtRAL (pmol/mg)
RGC Light Adapted	5,25	10,28
RGC Dark Adapted	1,53	13,27
INL Light Adapted	3,71	7,43
INL Dark Adapted	2,13	14,89
PRC Light Adapted	16,05	87,89
PRC Dark Adapted	31,05	83,88

**Table 1:** Aldehydes quantification in inner retina in dark and light condition. Abrv. RGC= Retinal Ganglion Cells; INL= Inner Nuclear Layer; PRC= Photoreceptor Cells; 11cRAL= 11-cis-Retinal Aldehyde; AtRAL= All trans Retinal Aldehyde.



**Figure 2:** Aldehydes isomerization in retinal layers; amount of 11-cis-Retinal Aldehyde or All trans Retinal Aldehyde in two different light conditions.

### Isomerase activity

Moreover, we assessed the isomerase activity in the different retinal layers. For this, retinal layer preparations were pre-incubated with palmitoyl CoA in 100 mM Tris pH 8, 2 mM CaCl<sub>2</sub>, 2 mM MgCl<sub>2</sub> (reaction buffer) for 5 minutes at room temperature. The substrate, 11-cis retinol or all-trans retinol, was delivered in 2 µl of ethanol to the reaction buffer containing either cellular retinaldehyde binding protein (CRALBP), bovine serum albumin (BSA), or reaction buffer alone,

and pre-incubated for 5 minutes at room temperature. The protein samples and retinol samples were then combined and incubated at 37° C for 30 minutes. The final reaction mixture was 100 μM palmitoyl CoA, 30 μM CRALBP or 1% BSA, 10 μM retinol and protein in increasing amounts (between 10 and 50 μg). Total reaction volume was 1ml. The reaction was stopped with 1ml ice cold ethanol. Retinoids were then extracted with hexane and analyzed by HPLC. Results are shown in the Table 2.

Sample	ARAT activity	Isomerase activity
	(ATRP pmol/mg protein)	(11cisRP pmol/mg protein)
<b>PRC Dark adapted</b>	96	24
<b>INL Dark adapted</b>	32	24
<b>RGC Dark adapted</b>	50	-

**Table 2:** Isomerase activity in different retinal layers. ARAT = Acyl-Coa retinil acetyl transferase; ATRP=all trans retinyl palmitate; 11cisRP= 11 cis retinyl palmitate.

### Conclusion:

During my stay at Dr. Tsin's Laboratory, I have obtained some preliminary promising results related to the chromophore regeneration occurring in the inner retina of chicken, specifically in RGCs. I have successfully learned the complete methodology for obtaining and analyzing the presence of retinoids in living tissues, using and processing the material from the samples collected in our lab of Argentina, and helping the colleagues at the UTSA laboratory. After this successful experience, we have consolidated collaboration with this foreign group, to continue investigating the possible ways that chromophore regeneration could be occurring in the inner retina. It was a successful experience for me, at both scientific and personal level. I met people working in the same area, and I could interact and share experiences with them.



Doctor Tsin's Laboratory and I in the lunch. In this picture I am placed in the left corner wearing a black t-shirt (white arrow) while Dr. A. Tsin is in the back of the table, in the right corner (yellow arrow).