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The Visual Cycle in the Inner Retina of Chicken and the Involvement of Retinal G-Protein-Coupled Receptor (RGR)

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Abstract The vertebrate retina contains typical photoreceptor (PR) cones and rods responsible for day/night vision, respectively, and intrinsically photosensitive retinal ganglion cells (ipRGCs) involved in the regulation of non-image-forming tasks. Rhodopsin/cone opsin photopigments in visual PRs or melanopsin (Opn4) in ipRGCs utilizes retinaldehyde as a chromophore. The retinoid regeneration process denominated as "visual cycle" involves the retinal pigment epithelium (RPE) or Müller glial cells. Opn4, on the contrary, has been characterized as a bi/tristable photopigment, in which a photon of one wavelength isomerizes 11-*cis* to all-*trans* retinal (Ral), with a second photon re-isomerizing it back. However, it is unknown how the chromophore is further metabolized in the inner retina. Nor is it yet clear whether an alternative secondary cycle occurs involving players such as the retinal G-protein-coupled receptor (RGR), a putative photoisomerase of unidentified inner retinal activity. Here, we investigated the role of RGR in retinoid photoisomerization in Opn4x (*Xenopus* ortholog) (+) RGC primary cultures free of RPE and other cells from chicken embryonic retinas. Opn4x (+) RGCs display significant photic responses by calcium fluorescent imaging and photoisomerize exogenous all-*trans* to 11-*cis* Ral and other retinoids. RGR was found to be expressed in developing retina and in primary cultures; when its expression was knocked down, the levels of 11-*cis*, all-*trans* Ral, and all-*trans* retinol in cultures exposed to light were significantly higher and those in all-*trans* retinyl esters lower than in dark controls. The results support a novel role for RGR in ipRGCs to modulate retinaldehyde levels in light, keeping the balance of inner retinal retinoid pools.

Keywords Retinoids · Inner retina · Melanopsin · Retinal ganglion cells · Retinal G-protein-coupled receptor · Photoisomerization · Visual cycle

Introduction

The vertebrate retina comprises at least three different types of photoreceptor (PR) cells: (1) the visual PR cone and rod cells responsible for day and night vision, respectively, and (2) the intrinsically photosensitive retinal ganglion cells (ipRGCs) [1, 2] which express the non-visual photopigment melanopsin (Opn4) [3] and are involved in the regulation of a number of non-image-forming tasks and subconscious activities (the photic entrainment of circadian rhythms, pupillary light reflexes, melatonin suppression in the pineal gland, and sleep, among others) [4–9]. Interestingly, all known visual and non-visual photopigments utilize retinaldehyde as a chromophore, isomerized from 11-*cis* retinal to all-*trans* retinal upon light exposure and released from the photoreceptor cells to supportive surrounding cells for full regeneration to make up the active form of the photopigment. For this, the vertebrate retina has developed a specific process of retinoid recycling named "visual cycle", which involves the retinal pigment epithelium (RPE) mainly for rods [10] and Müller glial cells for cones [11, 12]. In contrast, Opn4 has been characterized as a bi/tristable photopigment, in which a photon of one particular wavelength isomerizes 11-*cis* to all-*trans* retinal (Ral) while a second photon of another wavelength re-isomerizes it back

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without releasing it from the opsin [13, 14]; however, little is known about chromophore regeneration and metabolism in the inner retina. In heterologous cellular systems for Opn4 expression supplied with the exogenous addition of retinoids, light responses are dependent on the administration of selected retinoids, mainly 11-*cis* and 9-*cis* retinal, which elicit the most significant photic responses; the addition of all-*trans* retinal triggers weaker responses to those obtained with 11-*cis* retinal, these responses increasing with longer exposures to bright illumination and at longer wavelengths, or illumination with white light or arrestin co-expression [15–17]. Moreover, full ipRGC activity in response to light pulses of 1-min duration with light of 480 nm is recovered after only a brief dark adaptation [18]. In addition, recovery of activity in the dark requires a process in which all-*trans* retinal is converted back to 11-*cis* retinal, as reported using recombinant Amphioxus Opn4 [19]. However, since the restoration of activity in the dark is not fully consistent with a purely bi/tristable photopigment, other explanations may apply, such as a second alternative cycle of support to regenerate the chromophore, as seen in *Drosophila* [20, 21]. If this is the case, other players, such as the retinal G-protein-coupled receptor (RGR), a putative photoisomerase of unknown function in the inner retina of vertebrates, may be involved in this process. RGR is an opsin expressed in the inner membranes of the RPE and in the end-feet of Müller glial cells [22]. It was first described as a putative photoisomerase located in the RPE and the retina [23], sharing some sequence homology with the bovine rhodopsin (23 %) and the squid retinochrome (26 %) [24]. RGR exhibits two clear peaks of maximal absorption: one at 370 nm, near UV, and the other at 469 nm, in the blue region [23]. RGR uses all-*trans* retinal as a chromophore, and upon light stimulation, part of the *trans* isomer is photoisomerized into 11-*cis* retinal. This observation, together with some shared homology with the retinochrome, sustains the hypothesis that RGR may act as a photoisomerase [25]. However, RGR exhibits low photosensitivity compared with rhodopsin (~30 %), and its chromophore is not released after isomerization, making it an inefficient photoisomerase. Though RGR function was not yet fully clarified, the retinas in knockout (KO) mice for RGR looked morphologically normal, the only visible effect being a failure to regenerate rhodopsin, likely due to the accumulation of retinyl esters causing retinal degeneration with photoreceptor cell loss [26]. Based on evidence reported, it can be inferred that RGR does not act as a photoisomerase, but rather regulates the recycling of retinal. RGR was shown to co-purify with other proteins, such as cRDH [27]. In another series of experiments involving KO for RGR or for RPE65, it was found that retinas lacking RPE65 were unable to generate 11-*cis* retinal even though they expressed RGR [28, 29]. Though RGR may not be a typical photoisomerase, it nevertheless plays an important role in the retina, as reported by Chen et al. [26]. In fact, years later, it was shown that RGR regulates the traffic of retinyl esters within the RPE cells by means of the modulation of enzyme activities, as a potential negative regulator, in the formation of retinyl esters on all-*trans* retinyl ester hydrolase (REH) and lecithin retinyl acyl transferase (LRAT) in light [30]. RGR interaction with *cis*-RDH (RDH5) to isomerize all-*trans* retinal to 11-*cis* retinal under light conditions [28] may be a strong indication that there is an alternative route for the regeneration of 11-*cis* retinoids in the visual cycle. Here, we investigated the possible role of RGR in retinoid photoisomerization in ipRGCs of birds.

Materials and Methods

Animal Handling

For studies involving immunochemistry, we used 10-day-old chickens (*Gallus gallus domesticus*). Chickens were anesthetized with 2.5 mL/kg Equitesin (426 mg chloral hydrate, 96 mg pentobarbital, 212 mg $MgSO_4$, 3.5 mL propylenglycol, and 1 mL ethanol; final volume, 10 mL) and killed by decapitation.

All experiments were performed in accordance with the Use of Animals in Ophthalmic and Vision Research of ARVO, approved by the local animal care committee (School of Chemistry, Universidad Nacional de Córdoba; Exp. 15-99-39796).

Retinal Fixation and Sectioning

After enucleation, the eyes were hemisected equatorially and the gel vitreous was removed from the posterior eyecup. Samples were fixed for 30 min at 20 °C in 4 % paraformaldehyde plus 3 % sucrose in 0.1 M phosphate buffer, pH 7.4. Fixed samples were washed three times in phosphate-buffered saline (PBS; 0.05 M phosphate buffer, 195 mM NaCl, pH 7.4), cryo-protected in PBS plus 30 % sucrose, soaked in embedding medium (O.C.T. compound, Tissue-Tek) for 10 min, and freeze-mounted onto aluminum sectioning blocks. Transverse sections nominally 14 mm thick were cut consistently from the posterior pole of the eye, near the dorsal portion of the pecten, and thaw-mounted on Super-Frost glass slides (Fisher Scientific). Sections from control and treated eyes from the same individual were placed consecutively on each slide to ensure equal exposure to reagents. Sections were air-dried and stored at −20 °C until use.

Immunohistochemistry

Retinal sections were fixed for 30 min in 4 % paraformaldehyde in PBS, washed in PBS, and incubated with the specific antibody, as described [31]. The cells were then rinsed in PBS

and incubated with Alexa Fluor 488 goat anti-rabbit and Alexa Fluor 546 goat anti-mouse IgG at dilution of 1:1,000 for 1 h at room temperature.

Primary Cultures of Embryonic RGCs

RGCs were purified from embryonic day 8 (E8) neural chicken retinas dissected in ice-cold Ca^{+2}–Mg^{+2}-free Tyrode's buffer containing 25 mM glucose, as previously reported [32]. Briefly, cells were treated with papain (P3125, Sigma Aldrich) for 25 min at 37 °C and Deoxyribonuclease I (18047-019, Invitrogen) and rinsed with soybean trypsin inhibitor and Dulbecco's modified Eagle's medium (DMEM). After dissociation, the cell suspension from five retinas was poured into Petri dishes pretreated with 2.5 μg/mL protein A followed by incubation at 37 °C for 30 min with an anti-chicken Opn4x polyclonal antibody (Bio-Synthesis Inc., Lewisville, TX). After being washed exhaustively, identical aliquots of the remaining bound RGCs were harvested in DMEM containing B27 (dilution, 1:50, *v*/*v*; Life Technologies, Invitrogen, GIBCO, Carlsbad, CA), forskolin from *Coleus forskohlii* (4.25 μg/mL in DMSO; Sigma Aldrich, St. Louis, MO), and recombinant human BDNF (50 μg/mL; R&D Systems, Minneapolis, MN) and seeded in Petri dishes previously treated with 10 μg/mL polylysine and 5 μg /mL laminin. Primary cell cultures were incubated at 37 °C under constant 5 % CO_2–air flow in a humid atmosphere for 3 days and further characterized with specific retinal cell-type markers by immunochemistry.

Immunocytochemistry

Cultured cells were fixed for 30 min in 4 % paraformaldehyde in PBS and coverslips were washed in PBS, treated with blocking buffer (PBS supplemented with 0.1 % BSA, 0.1 % Tween 20, and 0.1 % $NaNO_3$), and incubated with the respective antibodies, as described [33, 34]. They were then rinsed in PBS and incubated with goat anti-rabbit IgG Alexa Fluor 488 or goat anti-mouse IgG Alexa Fluor 546 (monoclonal antibodies, 1:1,000) for 1 h at room temperature. In some experiments, the samples were incubated with Hoechst stain (0.001 mg/mL; 94403 Sigma). Coverslips were finally washed thoroughly and visualized by confocal microscopy (FV1000; Olympus, Tokyo, Japan).

Antibodies

The antibodies used were monoclonal anti-α-tubulin, clone DM1A (dilution, 1:1,000; T9026 Sigma Aldrich); monoclonal anti-RPE65 (dilution, 1:100; MAB5428 Millipore, Chemicom), polyclonal anti-chicken Opn4x (dilution, 1:1,000; Bio-Synthesis Inc.), polyclonal anti-chicken RGR (dilution, 1:500; kindly gifted by Dr. Andrew Tsin, UTSA, USA), and polyclonal anti-RGR (dilution, 1:100; LS-A1043-50, LifeSpan BioSciences Inc.)

RNA Isolation and RT-PCR

Total RNA from RGC cultures was extracted following the method of Chomczynski and Sacchi using the TRIzol™ kit for RNA isolation (Invitrogen). RNA integrity was checked in 1 % agarose gel and quantified by UV spectrophotometry (Epoch Microplate Spectrophotometer, Biotek). Finally, 2 μg of total RNA was treated with DNAse (Promega) to eliminate contaminating genomic DNA. cDNA was synthesized with M-MLV (Promega) using oligo(dT).

The oligonucleotide sequences used for RT-PCR from the *Gallus gallus* sequences were as follows:

Brn3:
Forward: CCATCCTGCACGAGCCCAAGTA
Reverse: GCCCCGTAGCAAGGTCTCATCAA
Gq:
Forward: TCAAAACATCTTCACTGCCATG
Reverse: TCCACGTCGCTGAGATAGTATT
RGR:
Forward: TTGAAGACGCTGGTCATTTGC
Reverse: CTGCCATATTCCTCCTCTGTAGTT
Opn4x:
Forward: TGCTTTGTCAACAGCTTGCACAGA
Reverse: CAGCAATAATCTGTATGGTGCCTTC
GAPDH:
Forward: AGGCGAGATGGTGAAAGTCG
Reverse: TCTGCCCATTTGATGTTGCT

Polymerase Chain Reaction

PCR reactions were carried out according to Contin et al. [35], with an initial denaturation step of 1 min at 94 °C, 25 cycles of 60 s at 94 °C, 50 s at 60–65 °C, and 90 s at 72 °C, and a final 5-min elongation step at 72 °C. Amplification products were separated by 2 % agarose gel electrophoresis and visualized by ethidium bromide staining.

RGR Knockdown

A synthetic morpholino antisense oligonucleotide sequence against the chicken RGR mRNA (NM_001031216)—RGR Morpholine (5′-GATGTGAAGTGACCATTCCTGTGAT; Gene Tools LLC, Philomath, OR)—was used to knock down RGR expression. We transfected oligos into primary cultures at different oligo-to-polyethylenimine (PEI) ratios (20 kDa; 764965 Sigma Aldrich). The greatest reductions in RGR mRNA level were obtained by mixing 5 μg of oligo (200 nM final concentration), 5 μL of PEI 20 kDa, and

DMEM to a final volume of 150 μL. This mixture was added to the media of the dishes. The cells were incubated with the transfection media at 37 °C for 4 h. Following transfection, the medium was replaced with specific growing medium for 24 h. This medium was then in turn replaced with DMEM containing 1 % BSA and 5 μM all-*trans* RAL, and the incubation continued for an additional 3 h. The all-*trans* RAL-containing medium was replaced with minimum DMEM and selected plates were incubated at 37 °C in the dark for 1 h, whereas other cells were exposed to fluorescent light (1,000–1,200 lx) for 1 h. Next, the medium was rinsed and the cultures were harvested by scraping and resuspended in PBS 1× plus protease inhibitor (p33360, Invitrogen).

Retinoid Analysis

Primary cultures were homogenized in phosphate buffer containing 200 mM hydroxylamine. One milliliter of ethanol was added and retinoids were extracted twice with 3 mL of hexane. The samples were centrifuged at 3,000×*g* for 5 min. The organic phases were collected, dried under a stream of argon gas, and redissolved in 200 μL of hexane. The hexane solutions were analyzed by normal-phase high-performance liquid chromatography (HPLC) using 0.5 % dioxane for retinaldehyde-oxime, all-*trans* retinol, and 0.1 % all-*trans* retinyl palmitate in hexane at a flow rate of 1.5 mL/min on a silica column (Agilent-Zorbax-Rx Sil 5 μm, 150 × 4.6 mm; retinaldehyde-oxime and all-*trans* ROL/Zorbax-Sil 5 μm, 250 × 4.6 mm; all-*trans* retinyl palmitate) in an Agilent model 1260 liquid chromatograph equipped with a photodiode array detector (Agilent Technologies, Wilmington, DE). The identity of each retinoid (including the syn- and anti-oximes of each retinaldehyde) was confirmed by online spectral analysis and co-elution with authentic retinoid standards. We obtained all-*trans* ROL, all-*trans* RAL, and all-*trans* retinyl palmitate from Sigma and 11-*cis* retinal from Santa Cruz (sc-208843). Retinaldehyde-oxime standards were prepared by reacting 11-*cis* RAL or all-*trans* RAL with hydroxylamine, as described [36]. Quantitation of the retinoids was carried out by comparing the sample peak areas to the calibration curves established for each retinoid standard using the published molar extinction coefficients [37].

Calcium Imaging By Fluo-4 AM Fluorescence Microscopy

The cells were grown in an eight-well Lab-Tek recording chamber (Nunc™, Rochester, NY) in a colorless DMEM (GIBCO) containing 0.1 % of Pluronic acid F-127 and 5 μM Fluo-4 AM (Invitrogen-Molecular Probes) Ca^{2+} indicator dye for 60 min at 37 °C. The fluorescence imaging technique was performed as described, with modifications by using Ca^{2+}-sensitive indicator Fluo-4 AM excited at 515 nm (25.8-μW laser intensity) with a laser coupled to a confocal microscope (Olympus FluoView-300). The emitted fluorescence was captured every 2 s using a PlanApo N ×60 Uplan SApo oil-immersion objective (NA, 1.42; Olympus). The 12-bit 4 × 4 binned fluorescence images for each photo were used to quantify the fluorescence levels in the cells using the Fluoview 10.1 software; the mean fluorescence intensity in each cell was background-corrected by subtracting the mean fluorescence of an area with no cells. The mean intensity over a particular area of cells in a selected field was measured in each captured image series. Changes in the fluorescence levels were quantified as the ratio between each relative intensity level measured after a light stimulus of 1,000 lx (F) and the mean of intensities of serial pictures before stimulation (F_0). Values of F/F_0 are not linearly related to changes in $[Ca^{+2}]_i$, but are intended to provide a qualitative indication of variations in $[Ca^{+2}]_i$. No significant vehicle effects or changes in focus were detected. Responses were considered significant when the ratio at the peak differed from the baseline levels by at least 20 %.

Statistics

Sigma plot XI was used to perform the statistical analysis. Statistical analyses involved a two-way analysis of variance with SNK post hoc tests or Student's t tests, where appropriate (significance at $p \leq 0.05$).

Results

Intrinsic Photosensitivity in Opn4x (+) RGC Cultures: Calcium Responses and Retinaldehyde Isomerization

We first examined the intrinsic photosensitivity of RGC primary cultures purified from embryonic neural chicken retinas at day 8 (E8), which were further immunopurified with an anti-chicken Opn4x antibody, as described in "Materials and Methods", and kept in cultures for several days (Fig. 1) in the presence or not of exogenous all-*trans* retinal. RGCs clearly expressing the photopigment Opn4x in their somas and processes (Fig. 1a) were used to determine their response to light by assessing changes in intracellular Ca^{+2} levels using the fluorescent indicator Fluo-4 AM and confocal microscopy of individual cultured RGCs. It was also investigated whether the cultured cells were able to photoisomerize all-*trans* retinaldehyde. The results shown in Fig. 1b indicate that most cells in each culture displayed a significant variation in relative somatic Ca^{+2} levels after light stimulation, with differential responses, as illustrated for each individual cell (lighter colors). Interestingly, some cells showed sustained depolarization lasting longer than 1.5 min after a few seconds of light stimulation; the darker blue color represents the average for positive responses. Other cells in the cultures did not respond to the photic stimuli; their relative Ca^{+2} levels are indicated in light red and the average is shown in

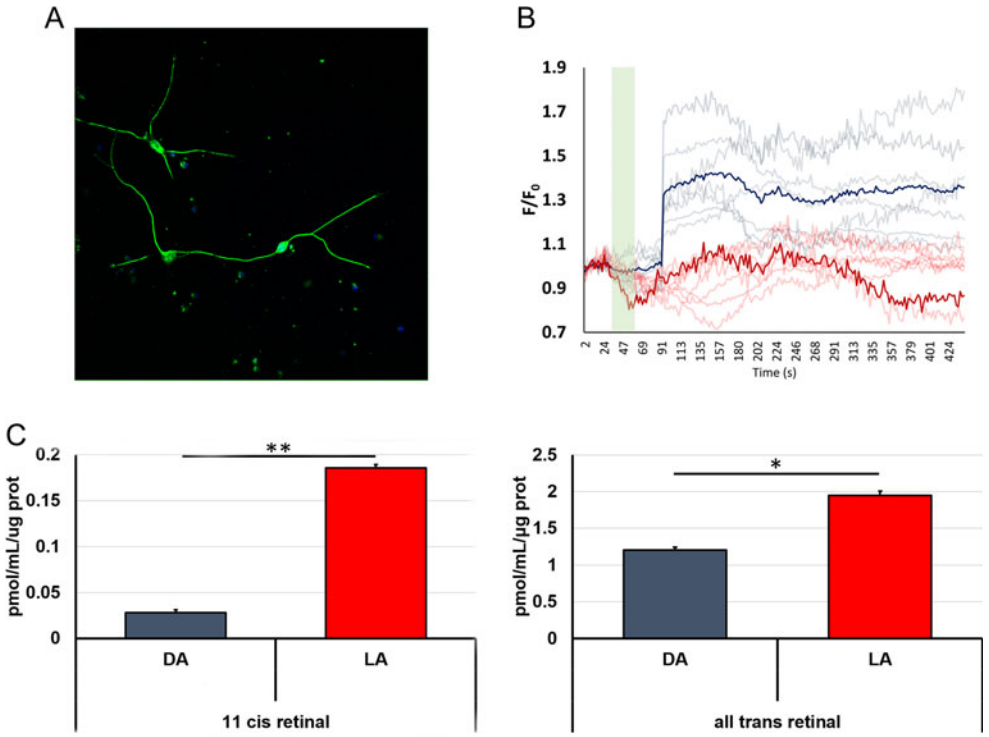


Fig. 1 Characterization of primary cultures of RGCs from chicken embryonic retinas. **a** Cultures express melanopsin X (*green*, Opn4) in cell somas and processes, displaying a typical morphology of neuronal cells. **b** Cells in the culture exhibit a significant response to light by increasing intracellular Ca^{2+} levels, measured by Fluo-4 fluorescent microscopy. The *dark blue line* represents the average response of photosensitive cells, while the *dark red line* indicates the mean response of non-photosensitive cells. **c** Photoisomerization of exogenous retinal in primary cultures quantified by HPLC. *Left panel* Levels of 11-*cis* retinal present in dark-adapted (*DA*, *blue bar*) or light-adapted (*LA*, *red bar*) cultures. The statistical analysis (*t* test) revealed a significant light/dark difference (**$p < 0.01$). *Right panel* Levels of all-*trans* retinal present in dark-adapted (*DA*, *blue bar*) or light-adapted (*LA*, *red bar*) cultures. The statistical analysis revealed a significant effect of light as compared with the dark controls (*$p < 0.05$). Results are the mean ± SEM ($n = 3$–5/group)

darker red. Furthermore, cells in the culture fed with exogenous all-*trans* retinal in the dark were able to photoisomerize this to 11-*cis* retinal after 60 min of photic stimulation with white light of 1,000 lx (Fig. 1c). It is to be noted that as endogenous retinal levels were barely detectable in the cultures and not sufficient to evoke robust light responses, we added exogenous all-*trans* retinal in all the experiments. The cells were allowed to take up exogenous all-*trans* retinal (5 μM) for 3 h, after which it was removed from the medium and the cells were exposed to light for 1 h. Although there is some basal isomerization of all-*trans* to 11-*cis* retinal in the dark, light exposure produced a significant eightfold increase in 11-*cis* retinal levels as well as a onefold increase in all-*trans* retinal levels. The statistical analysis revealed a significant effect of light for both retinal isomers (*$p < 0.05$ and **$p < 0.01$; Fig. 1c).

RGR Expression and Function in Opn4x (+) RGC Cultures

In addition to the expression of the non-visual photopigment Opn4x in the RGCs of embryonic chicken retinas, we began by investigating the presence of the putative photoisomerase RGR in samples from embryonic and postnatal retinas, as shown in Fig. 2. In the developing retina at E8, RGR(+) fluorescence immunostaining (in green) appears in the forming ganglion cell layer (GCL) at the inner part of the nascent retina and on the other side of the retina, close to the edge that will become the RPE (Fig. 2a). At postnatal days, RGR immunofluorescence is visualized in the GCL, in the different sub-laminas of the inner plexiform layer (IPL), in the outer plexiform layer (OPL), in the outer segments of photoreceptor cells (PRC), and at the RPE (Fig. 2b). Moreover, a band of 32 kDa was seen by Western blot in highly enriched preparations of PRC + RPE, INL, and GCL in the whole postnatal chicken retina of wild-type animals or GUCY1* birds suffering retinal degeneration. Also, positive immunoreactivity was observed for the typical band of RGR in samples from the primary cultures of RGC at E8; other bands detected by the specific primary antibody were found, likely corresponding to shorter or longer RGR isoforms [38]. As expected, no immunoreactivity was detected in the samples from NIH3T3 fibroblasts. In addition, the positive immunolabeling for the visual cycle protein RPE65 was only visualized in the preparation of PRC + RPE, clearly showing the specificity of immunoreactivity and the purity of the retinal preparations (Fig. 2c).

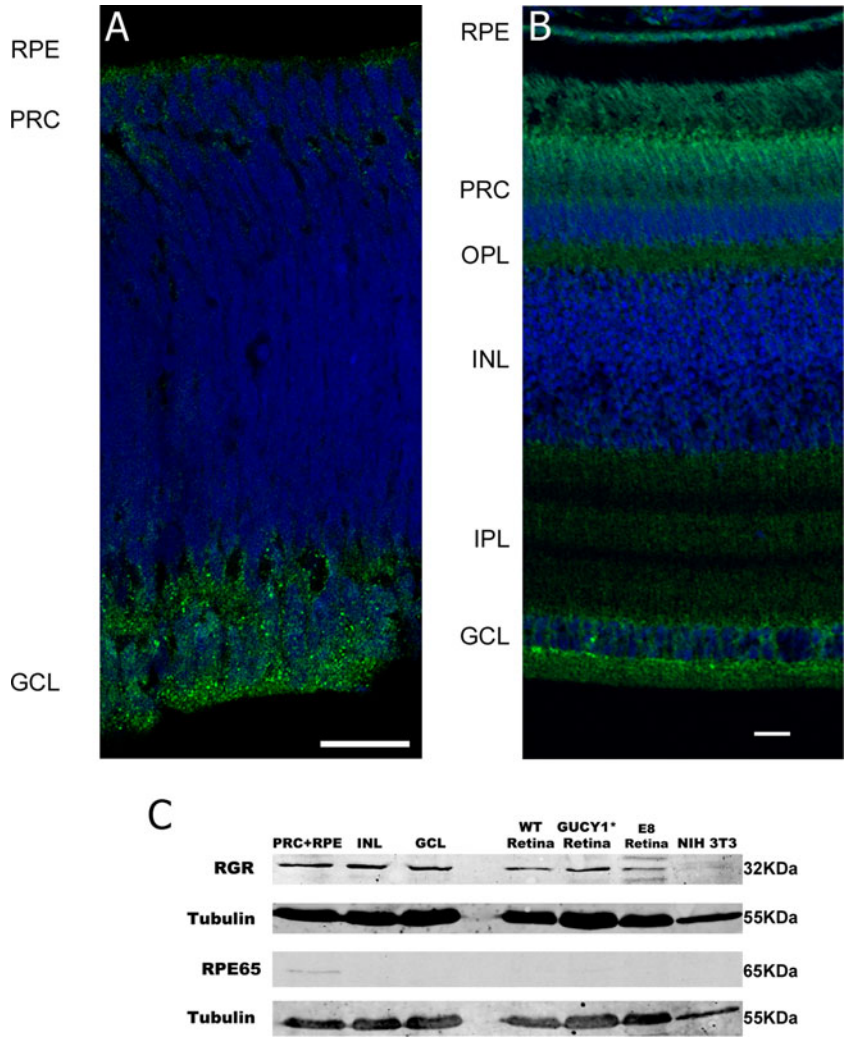


Fig. 2 Expression of RGR in the chicken retina. **a**, **b** Immunofluorescence of RGR in embryonic retina at day 8 (*E8*) (**a**) or at postnatal day 7 (**b**). Nuclear staining with DAPI is visualized in *blue*. RGR immunoreactivity shown in *green* denotes its localization in the ganglion cell layer (*GCL*) of the developing retina, whereas it is widespread in the postnatal retina with strong immunolabeling in the end-feet of Müller cells surrounding the GC nuclei. *Scale bar*, 50 μm. **c** Western blot for RGR with a specific antibody that detected the corresponding band of 32 kDa in retinal samples from highly enriched preparations of photoreceptor cells and retinal pigment epithelium (*PRC + RPE*), inner nuclear layer (*INL*), and ganglion cell layer (*GCL*). Also, RGR was detected in homogenates of whole retinas of WT or GUCY1 chickens, but was undetected in NIH3T3 cells. Tubulin was used as a loading control and RPE65 as a control of RPE contamination. RPE65 was only detected in the PRC + RPE sample. *OPL* outer plexiform layer, *IPL* inner plexiform layer

To further investigate the presence of RGR in the primary cultures, we assessed the positive immunoreactivity present in the Opn4x (+) neurons visualized in Fig. 3a. As shown in Fig. 3b, a typical neuronal cell in the culture stained for α-tubulin (in green) showed positive RGR immunolabeling (red) in its cell soma and processes. While there is a significant co-localization of RGR with tubulin in the axon and dendrites (yellow label), the co-localization in the somatic compartment is weak. In the first description of RGR [22], it was shown to be present as an intracellular opsin. Using electron microscopy, the RGR was located in the endoplasmic reticulum (ER), more specifically in the smooth ER. Figure 3b shows the first report of RGR in neuronal cells. In neurons, the smooth ER—more abundant in the axons—is a wide net stretching from the perinuclear area to the synaptic contacts. The high co-localization with tubulin can be explained by the expression of RGR in the smooth ER; the lack of such co-localization in the soma is due to the low staining of tubulin in the somatic area. Figure 3c shows the expression of RGR in cultured Müller cells (a positive control), in which there is a strong perinuclear labeling and some vesicular staining, as previously reported by electron microscopy [22]. Moreover, primary RGC cultures were further characterized with the specification markers shown in Fig. 3d and compared with the total postnatal retina (Ret), in which positive expression was seen for the *Brn3*, *G protein q*, *RGR*, and *Opn4x* transcripts.

In a second step, we proceeded to knock down RGR expression, as shown in Fig. 3e, by using a specific morpholino (MO or ASO) antisense oligonucleotide complementary to the RGR mRNA sequence. We then assessed the retinoid levels in primary RGC cultures that were fed with exogenous all-*trans* retinal for 3 h and then washed and light- (LA) or dark-adapted (DA) in the presence or not of the RGR MO antisense. After all-*trans* retinal administration, the levels of 11-*cis* retinal were higher than the basal content in the dark and significantly elevated upon light exposure in the control cultures (Fig. 4a). Also, the levels of all-*trans* retinal were higher in the light condition than the controls in the dark, as were the levels of all-*trans* retinyl palmitate from light-adapted cultures compared to the dark controls

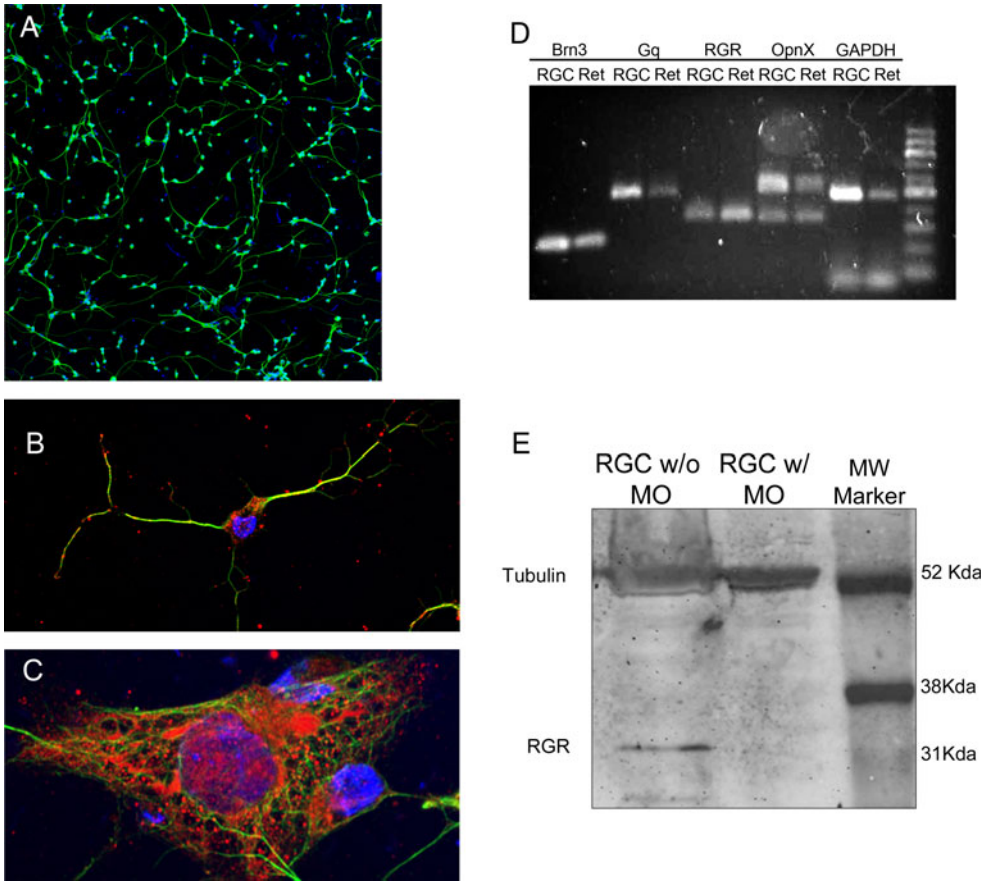


Fig. 3 Expression of RGR in embryonic RGC cultures. **a** Morphological characterization of cultured cells by α-tubulin (+) immunofluorescence (*green*). **b** Localization of RGR immunoreactivity in RGC primary cultures. RGR (*red*) was localized in inner cellular membranes with a strong labeling surrounding the nucleus; the cytoskeleton of the cell (α-tubulin) is visualized in *green*. **c** A positive control for RGR immunolabeling shows a typical Müller cell grown in culture for 15 days displaying a strong labeling of inner membranes and smooth endoplasmatic reticulum-like compartments. **d** Detection of mRNAs for RGR, a RGC specification marker (BRN3), and components of the rhabdomeric-like photocascade (Gq protein and melanopsin X, *Opn4X*) by RT-PCR in embryonic RGC primary cultures. Total retina (*Ret*) was used as a positive control and GAPDH as a housekeeping gene. **e** Knockdown of RGR protein in RGC cultures after treatment with (*w/*) or without (*w/o*) a specific RGR morpholino (*MO*) antisense oligonucleotide. The decrease in RGR expression was confirmed by Western blot. The lane of the culture treated with the MO (*RGC w/ MO*) clearly shows a significant decrease in RGR protein as compared with the band in the sample without treatment (*RGC w/o MO*)

(Fig. 4d). However, the levels of all-*trans* retinol in the dark occurred after the exogenous addition of all-*trans* retinal and were significantly reduced by light (Fig. 4c). When RGR expression was substantially knocked down, the retinoid levels were drastically affected, as shown in the ASO groups: the levels of 11-*cis* retinal, all-*trans* retinal, and all-*trans* retinol were significantly elevated in the light-adapted samples after RGR ASO treatment compared with those from cultures in the light control condition, whereas the levels of all-*trans* retinyl palmitate remained elevated in the light condition and RGR ASO treatment, but did not significantly differ from those kept in the control light condition. In the dark-adapted cultures, the RGR knockdown caused some differential effects on the retinoid levels: the levels of 11-*cis* retinal and all-*trans* retinol were unaffected compared with the dark controls, whereas all-*trans* retinal levels were significantly reduced in the ASO-treated cultures compared with the dark controls; the levels of all-*trans* retinyl palmitate on the other hand were increased in the dark after ASO treatment.

Discussion

Our findings constitute the first report showing that primary cultures of RGCs expressing the photopigment Opn4x are able to take up and isomerize exogenous all-*trans* retinal to 11-*cis* retinal and to other retinoids, such as the alcohol all-*trans* retinol and the retinyl ester all-*trans* retinyl palmitate, strongly indicating the presence and activity of both a retinal dehydrogenase and a retinol acyl transferase in the inner retina. Moreover, Opn4 (+) cultures exposed to light showed a significant increase in the levels of 11-*cis* retinal and all-*trans* retinaldehyde as compared with the dark controls, clearly suggesting the bi/tristability of this photopigment. Light also

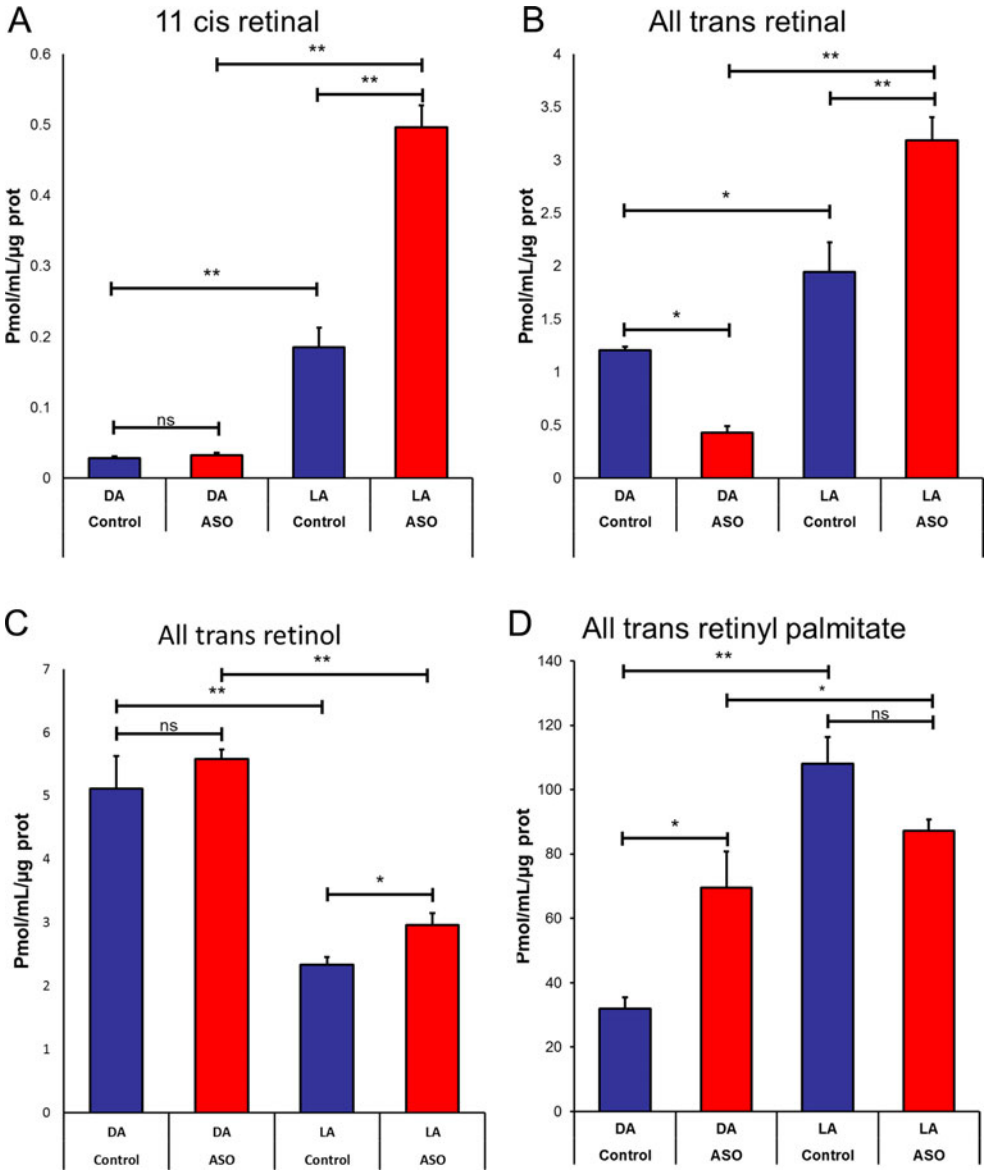
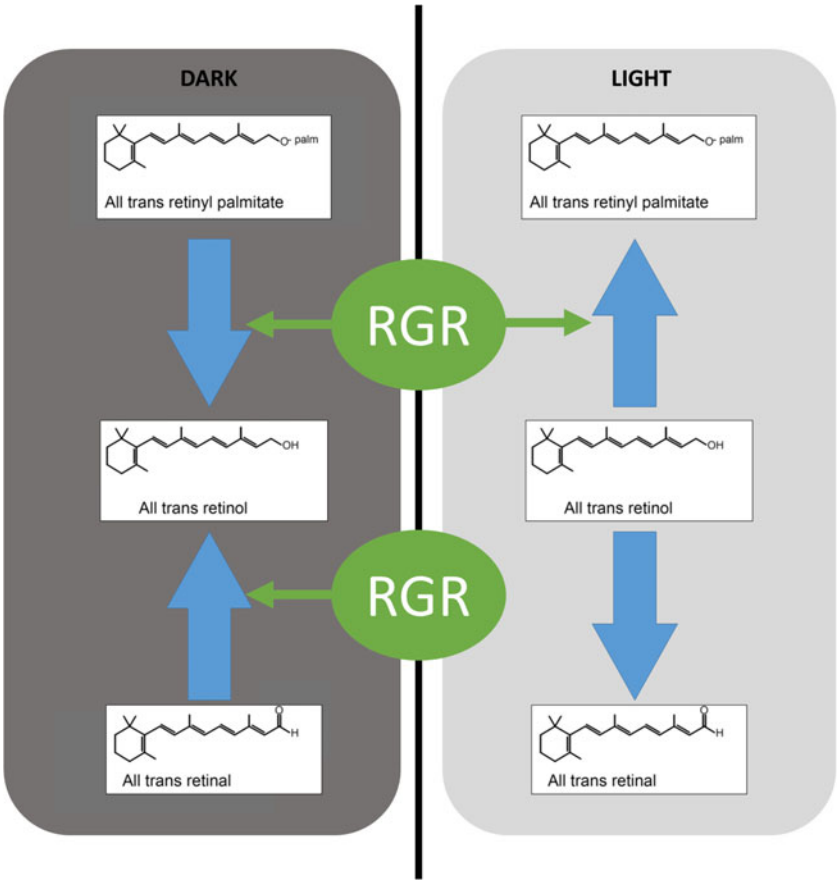


Fig. 4 Retinoid levels in cultured embryonic RGCs. Retinoid levels in dark- (*DA*) and light (*LA*)-adapted cultures of embryonic RGCs after transfection with a RGR antisense morpholino oligonucleotide (*red bar*, *ASO*) or untreated (*blue bars*, *Control*). **a** Levels of 11-*cis* retinal in cells cultured in the dark or exposed to cold white light for 2 h (*blue bars*, *Control*). Results are the mean picomoles per milliliter per microgram of protein ± SEM ($n = 3$). The statistical analysis denotes a significant difference between the dark and light values (**$p < 0.01$). In cells treated with ASO, there is a significant difference between the dark and light values ($p < 0.05$). **b** The levels of all-*trans* retinal show a significant difference between the light/dark controls (**$p < 0.01$). This difference was further increased in the presence of RGR ASO ($p < 0.01$). **c** The levels of all-*trans* retinol differ significantly between the light/dark untreated controls, with lower levels in the light condition than in the dark condition ($p < 0.01$). In the presence of RGR ASO, this condition was partially reverted. **d** The levels of all-*trans* retinyl palmitate show a significant difference between the light/dark control. Retinyl palmitate was higher in the light than in the dark condition ($p < 0.01$). In cultures treated with RGR ASO, no difference was detected in the light condition, whereas in the dark condition the levels of retinyl palmitate were higher than in the untreated controls

differentially affects the levels of the other retinoids: it substantially decreases all-*trans* retinol content and increases the levels of all-*trans* retinyl palmitate. These observations are in line with the idea that light regulates the pool of retinoids in the RGCs, increasing the pool of retinyl esters as well as the two isomers of retinal (see Scheme 1).

RGR was shown to be present in chicken developing retina at the very early embryonic days, around E8, located particularly in the GCL and primary cultures of RGCs at E8 expressing Opn4x and displaying net photosensitivity. It is noteworthy that RGR expression persists in the GCL at postnatal days. Although it was proposed to act as a photoisomerase, further



Scheme 1 Turnover of retinoids in the avian inner retina in the light and dark. *Right panel* Under physiological conditions and in the light, the levels of retinols are substantially decreased. RGR may enhance the levels of all-*trans* retinyl palmitate likely by positively modulating the enzymatic activity of retinyl acyltransferase. Thus, RGR may regulate the pool of retinyl esters in the inner retina, as previously reported in the RPE. *Left panel* Under physiological conditions and in the dark, in contrast to the light, the production of all-*trans* retinol is favored and RGR may act negatively on the hydrolysis of retinyl esters, leading to a diminution in all-*trans* retinal and all-*trans* retinyl palmitate levels, as shown in Fig. 4. RGR may also regulate the dehydrogenase activity responsible for the production of retinols. Overall, RGR may play a novel role in the GCL as a modulator of the retinoid pools (retinals, retinols, and retinyl esters) in order to maintain their balance for further use after light stimulation and for opsin (Opn4) formation

investigation into its role in the outer retina shows that it likely functions as a potential negative regulator of REH and LRAT in light [30]. The role of RGR in the inner retina and particularly in ipRGCs has not yet been examined. In the current study, we found that the levels of 11-*cis*, all-*trans* retinals, and all-*trans* retinol were substantially higher than in the light controls in RGC cultures in which RGR expression was significantly knocked down, whereas the content of all-*trans* retinyl palmitate slightly decreased after RGR knockdown, without differing significantly from the light control. Overall, these observations strongly suggest that under physiological conditions and in light, RGR modulates the enzyme activities that maintain the balance in the pool of all-*trans* retinals and retinyl esters (see Scheme 1) and, in particular, acts positively on the retinyl ester synthase; when RGR expression is knocked down, on the other hand, the balance in the pool of retinoids is shifted to increase the retinal and retinol content (see Scheme 1). This is likely due to the decrease in the synthesis of retinyl palmitate and accumulation of its precursor, all-*trans* retinol. It is thus possible that the increase in the levels of all-*trans* retinal is to provide higher availability of retinol as a substrate for the retinol dehydrogenase.

In addition, under physiological conditions and in the dark, RGR may positively contribute to increase the pool of retinols in ipRGCs as a consequence of a diminution in all-*trans* retinal and all-*trans* retinyl palmitate (see Scheme 1) as compared with the levels in the light. In contrast, when RGR expression was knocked down, there was a significant decrease in the level of all-*trans* retinal and an increase in all-*trans* retinyl palmitate content, with no significant changes in all-*trans* retinol levels. These observations suggest that RGR, in some way, affects dehydrogenase activity and negatively regulates the hydrolysis of retinyl palmitate, as previously reported in RPE cells [30].

It is known that in the outer retina, 11-*cis* retinal can be regenerated by two different visual cycles involving the RPE or Müller cells [12]. After 11-*cis* retinal photoisomerization into all-*trans* retinal in the outer segments of PRCs, it is then

reduced to alcohol by multiple membrane-bound retinal RDHs (RDH5, RDH8, or RDH12) in the PRCs [39]. The all-*trans* retinol is then diffused into the RPE and delivered to the intradiscal lumen, where it is esterified by a LRAT to form all-*trans* retinyl esters. As the cycle progresses, 11-*cis* retinal is regenerated and eventually rebound to the photopigment to reconstitute a functional rhodopsin [40, 41]. A second visual cycle of retinal regeneration occurs between cone photoreceptors and Müller glial cells, the conversion being very similar to the classical visual cycle involving the RPE, in which the substrate for the main enzyme is a retinyl ester as opposed to a retinyl alcohol in the cone visual cycle. A retinolisomerase named dihydroceramide desaturase-1, identified as a putative isomerase II driving the alternative retinoid isomerization (all-*trans* retinol to 11-*cis* retinol), was recently described [42] and shown to be also present in the GCL. The 11-*cis* retinol formed can be stored as 11-*cis* retinyl ester by action of the 11-*cis* ARAT. To this end, two new 11-*cis*-specific retinyl ester synthases were described in the retina. Multifunctional *O*-acyltransferase [43] and diacylglycerol *O*-acyltransferase-1 [44] were found to be present in retinal Müller cells and also in the GCL.

Based on our observations and taking into account some similarities between ipRGCs and rhabdomeric photoreceptors of invertebrates in terms of expression of specification markers, the biochemical nature of the phototransduction cascade, and opsin homology, we hypothesize that a novel secondary visual cycle may take place in Opn4-expressing ipRGCs to further support the regeneration of 11-*cis* retinal and the pool of retinoids for later supply via a supplementary alternative process, as described in *Drosophila* [20, 21]. In this alternative cycle, RGR may play a novel role as a modulator of the retinoid pools (retinals, retinols, and retinyl esters) in light, maintaining a balance for further use after light stimulation (Scheme 1).

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