Early Onset and Differential Temporospatial Expression of Melanopsin Isoforms in the Developing Chicken Retina

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PURPOSE. Retinal ganglion cells (RGCs) expressing the photopigment melanopsin (*Opn4*) display intrinsic photosensitivity. In this study, the presence of nonvisual phototransduction cascade components in the developing chicken retina and primary RGCs cultures was investigated, focusing on the two *Opn4* genes: the *Xenopus* (*Opn4x*) and the mammalian (*Opn4m*) orthologs.

METHODS. Retinas were dissected at different embryonic (E) and postnatal (P) days, and primary RGC cultures were obtained at E8 and kept for 1 hour to 5 days. Samples were processed for RT-PCR and immunochemistry.

RESULTS. Embryonic retinas expressed the master eye gene *Pax6*, the prospective RGC specification gene *Brn3*, and components of the nonvisual phototransduction cascade, such as *Opn4m* and the G protein q (*Gq*) mRNAs at very early stages (E4–E5). By contrast, expression of photoreceptor cell markers (*CRX*, red-opsin, rhodopsin, and α-transducin) was observed from E7 to E12. Opn4m protein was visualized in the whole retina as early as E4 and remained elevated from E6 to the postnatal days, whereas Opn4x was weakly detected at E8 and highly expressed after E11. RGC cultures expressed *Gq* mRNA, as well as both Opn4 mRNAs and proteins. Opn4m was restricted exclusively to the GC layer at all ages, whereas Opn4x was limited to the forming GC layer and optic nerve at E8, but by E15, its expression was mostly in Prox1(+) horizontal cells.

CONCLUSIONS. The early expression onset of nonvisual phototransduction molecules could confer premature photosensitivity to RGCs, while the appearance of Opn4x expression in horizontal cells suggests the identification of a novel type of photosensitive cell in birds. (*Invest Ophthalmol Vis Sci.* 2011; 52:5111–5120) DOI:10.1167/iovs.11-75301

In vertebrates, a third group of retinal photoreceptors has recently been identified as intrinsically photosensitive retinal ganglion cells (ipRGCs).[1,2] These cells are responsible for conveying photic information to the brain concerning ambient illumination conditions and regulate distinct non–image-forming (NIF) tasks (e.g., synchronization of circadian clocks and pupillary light reflexes).[3–5] Likely evolved from a common ancestor with rhabdomeric photoreceptors of invertebrates, these ipRGCs express the photopigment melanopsin (Opn4),[1,2,6] an opsin closely related to the invertebrate Gq-coupled visual pigment.[2,3,7–11] Moreover, based on data from specification markers, horizontal and amacrine cells in the inner retina could be considered sister cells of ipRGCs.[12,13]

The biochemical events of phototransduction operating in the ipRGCs involve the activation of phospholipase C (PLC)[8,10,14] and of the phosphoinositide (PIP) cycle[15] in a manner similar to that of the invertebrate photocascade.[8–10,14,15]

Vertebrates have evolved two separate *Opn4* genes: *Opn4x* (also named *Opn4-1*[16] or *Opn4a*[17]) and *Opn4m* (also named *Opn4-2*[16] or *Opn4b*[17]) which are orthologs of the nonmammalian and mammalian vertebrate genes, respectively.[10,11,16–20] In the chicken retina, different laboratories have reported the expression of *Opn4* genes in cells of the outer nuclear (ONL), inner nuclear (INL), and ganglion cell (GCL) layers.[10,11,16–20] These two genes encode at least five different isoforms, two of which show blue-light sensitivity.[16] Moreover, detectable expression of both melanopsin transcripts (*Opn4x* and *Opn4m*) has been observed in retinas of GUCY1* chickens, an avian model of blindness lacking functional rod and cone photoreceptors that shows persistent light responses in diverse NIF tasks.[5] GUCY1* birds are clinically blind since hatching, even though their retinas are morphologically normal during the first weeks and only become degenerated after 2 to 3 months.[5]

The goal of the current work was to investigate the onset of expression of the nonvisual phototransduction machinery during development in the embryonic chicken retina and in isolated RGCs. To this end, we first examined the expression of Opn4x and Opn4m at the mRNA and protein levels in whole developing retina at different embryonic stages, as well as in immunopurified RGC cultures from embryonic day (E)8 retinas. Second, we investigated the cellular localization of Opn4 proteins in the retina during development as well as in the mature retinas of wild-type (WT) and blind birds (GUCY1*).

MATERIALS AND METHODS

Materials

Aqueous mounting medium (FluorSave) was from Calbiochem (San Diego, CA). The secondary antibodies used for WB were goat anti-rabbit 800CW and goat anti-rat 800CW (IRDye, dilution 1:25,000) from Li-COR (Lincoln, NB). Protease inhibitor and other biochemical reagents were from Sigma-Aldrich; B-27 supplement 50× was from Invitrogen-Gibco (Grand Island, NY). α-Tubulin (α-Tub) was detected by the mouse monoclonal DM1A antibody (1:1000 for WB; Sigma-Aldrich). The primary antibody against chicken Opn4x was raised in rabbit using the specific Opn4x peptide 1: RQKRDLLPDSYSCSEE.[21] The antibody against the chicken Opn4m was raised in rat and generated with the specific Opn4m peptide: CKHGNRELQKQYHR[20] (Bio-Synthesis Inc., Lewisville, TX).

Chicken Thy-1, Antisera Preparation, and Purification

Preparation of anti-chicken Thy-1 sera was performed by Bio-Synthesis, Inc. They synthesized the NH2-KNITVIKDKLEKC-OH peptide sequence conjugated with KLH and then immunized two rabbits. After 6 weeks they bled the rabbits and tested the serum by ELISA. A total of 100 to 150 mL of crude serum with five boosts and four bleeds were purified by affinity column purification.

Primary Cultures of Embryonic RGCs

RGCs from E8 neural retinas dissected in ice-cold Ca_2-Mg_2–free Tyrode's buffer containing 25 mM glucose were immunopurified with the anti Thy-1 antibody as described.[10,15,22] The cell cultures were highly enriched in RGCs (>93% according to different RGC markers)[10,15,22] and incubated from 1 hour to 5 days at 37°C under constant 5% CO_2-air flow in a humid atmosphere.

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

Preparation of Optic Nerve and Outer Plexiform Layer Samples

After decapitation, both eyes were dissected from the head, cutting at the level of the optic nerve (ON) head. The skull was then opened carefully to expose the ON still attached to the brain, and approximately 2 to 3 mm from each side (until the optic chiasma) was dissected and either fixed for antibody labeling or homogenized in PBS for Western blot analysis (see respective sections). Eyes were opened at the iris, and the lens and vitreous humor were removed. The eye cups were rinsed twice in 4 mL of cold 0.25 M sucrose, immediately frozen in liquid N_2, and lyophilized overnight at −40°C. Highly enriched preparations in the outer plexiform layer (OPL) were obtained as previously described.[23] Briefly, each lyophilized retina was sandwiched between adhesive tape attached to the RGC and retinal pigment epithelium (RPE) surfaces, After the upper piece of adhesive tape with RPE attached was removed, another piece of tape was applied to the remaining retina, and a different layer was removed, bound to the uppermost tape. This process was repeated successively, such that the RPE (dark gray color) was attached to the first piece of tape, the photoreceptor cell (PRC) layer (orange color) to the second and third tapes, and the OPL to the fourth and fifth tapes. OPL samples were then homogenized in PBS for Western blot analysis (see below).

Immunohisto(cyto)chemistry

Embryonic chick eyes were dissected from the head in cool PBS; the cornea, lens and pectens discarded; and the posterior eye cup fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) overnight at 4°C; ON was fixed in the same way, and cultured cells were fixed for 30 minutes (4% paraformaldehyde in PBS). Eye cups and ON were cryoprotected in sucrose and sectioned by cryostat. Sections and fixed coverslips were washed in PBS, treated with blocking buffer (PBS
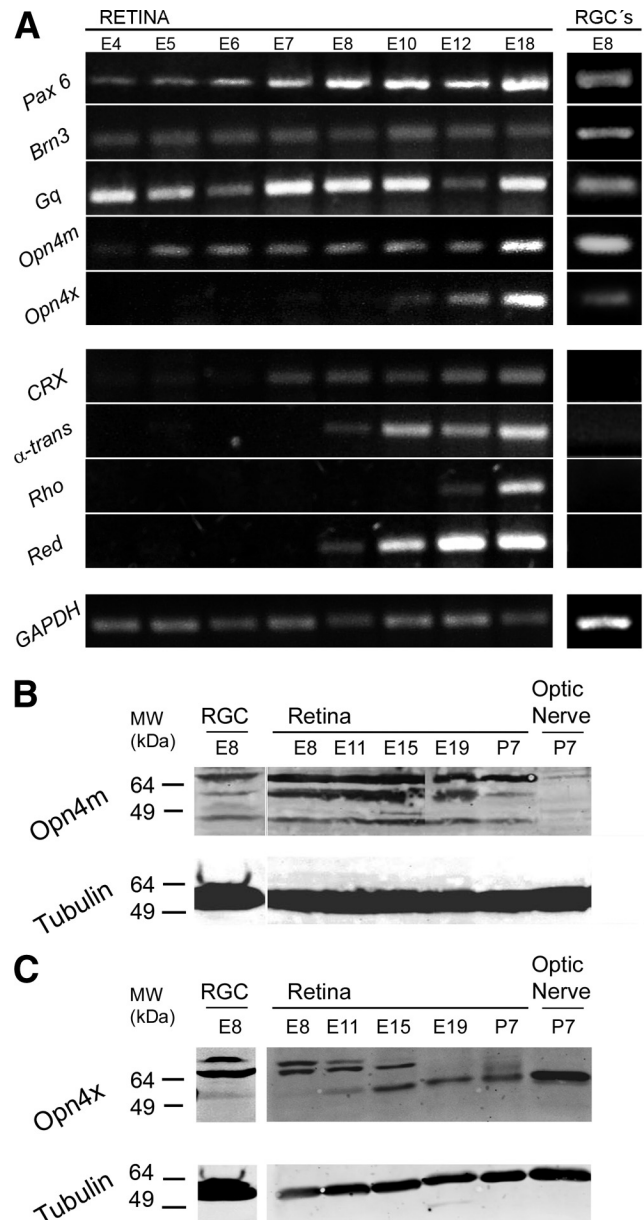

Figure 1. Temporal profiles of gene expression in the developing chicken retina. (**A**) Analysis of mRNA expression in the whole chicken retina from E4 to E18 and in primary cultures of E8 chicken RGCs (RGC-E8). Chicken embryonic retinas were dissected from E4 to E18, and RGCs were immunopurified from E8 retina and cultured for 1 hour. mRNA expression was assessed by RT-PCR from RGC-E8 and retinal samples collected from E4 to E18. Retinal cell specification markers: *Pax-6, Brn3,* and *CRX.* G proteins: α-transducin (α-trans) and q (*Gq*). Photopigments: melanopsin (*Opn4*) isoforms (mammalian orthologue *Opn4m* and *Xenopus* orthologue *Opn4x*), rhodopsin (*Rho*), and red cone opsin (*Red*). *GADPH,* housekeeping gene. *Left:* gene expression in developing whole retina; *right:* gene expression in RGC-E8 cultures. Results shown are representative of those in four independent experiments. Expression of Opn4m (**B**) and Opn4x (**C**) proteins was detected in the whole retina from E8 to E19 and P7, in the ON at P7, and in primary RGC cultures at E8 (RGC). Protein assays were performed by WB of 50 μg protein for each sample and specific primary antibodies against Opn4m, Opn4x, and α-Tubulin (Tubulin). For Opn4m, immunoblots showed three bands of ~48, 62, and 65 to 70 kDa, whereas for Opn4x, there were three bands of ~55 to 60, 65, and 70 kDa. Results shown are representative of three blots from independent experiments.

supplemented with 0.1% BSA, 0.1% Tween 20, and 0.1% NaN$_3$) and incubated with the different antibodies: anti-chicken Opn4x (1:1000 at 4°C for 24 hours) and anti-chicken Opn4m (1:200 at 4°C for 24 hours), together with anti-rhodopsin (Rho4d2, 1:1000),[24] ON bipolar cell marker (115a10; 1:100)[25], anti-neurofilament monoclonal antibody (8A1; 1:1000; Santa Cruz Biotechnology, Santa Cruz CA) or anti-cone α-transducin (GaT2, 1:500; Santa Cruz Biotechnology). They were then rinsed in PBS and incubated with goat anti-rabbit IgG Alexa Fluor 488 (Opn4x) and goat anti-mouse IgG Alexa Fluor 546 (monoclonal antibodies), or goat anti-rat IgG Alexa Fluor 488 (Opn4m; 1:1000) for 1 hour at room temperature (RT). In some experiments, samples were incubated with propidium iodide (0.05 mg/mL) or DAPI (300 nM). For the double-immunolabeling experiments with Opn4x and Prox1 (Abcam Ltd., Cambridge, MA) rabbit polyclonal antibodies, the sections were first incubated in Opn4x (1:2000) overnight, washed and incubated in goat anti-rabbit IgG-Alexa Fluor 488 for 1 hour at RT, washed again, refixed in 3% paraformaldehyde+4% sucrose in PBS for 1 hour, washed and permeabilized with 0.1% Triton X-100, and reblocked in saturating buffer supplemented with 2% horse serum, 3% BSA, and 0.05% Triton X-100 for 2 hours at RT. The sections were then incubated overnight at 4°C in Prox1 (1:1000) in the same saturation buffer, washed, and treated with secondary goat anti-rabbit IgG-Alexa Fluor 546 for 1 hour at RT. Slides and coverslips were finally washed thoroughly and viewed by confocal microscopy (FV1000; Olympus, Tokyo, Japan).

Western Blot Analysis

Homogenates of whole chicken retinas from embryonic or postnatal days, ON samples, OPL preparations, and primary RGC cultures resuspended in PBS buffer containing protease inhibitors were processed for WB by using published procedures.[26] Homogenates were resuspended in sample buffer and separated by SDS-gel electrophoresis on 10% polyacrylamide gels (50 μg total protein/lane), transferred onto nitrocellulose membranes, blocked for 1 hour at RT with 5% skimmed milk in PBS, and incubated overnight at 4°C with specific antibodies in the incubation buffer (2.5% skimmed milk and 0.1% Tween 20 in PBS). The membranes were washed three times for 15 minutes each in washing buffer (0.1% Tween 20 in PBS) and incubated with the corresponding secondary antibody in incubation buffer during 1 hour at RT followed by three washes with washing buffer for 15 minutes each. For Li-COR IRDye antibodies, the membranes were scanned (Odyssey IR Imager; LI-Cor Biosciences). After incubation with Opn4 antibodies, the membranes were stripped with NaOH 0.5 M and incubated in blocking buffer containing α-tubulin antibody.

RNA Isolation and cDNA Synthesis

Total RNA from RGC cultures was extracted according to the method of Chomczynski and Sacchi,[27] using a kit for RNA isolation (TRIzol;
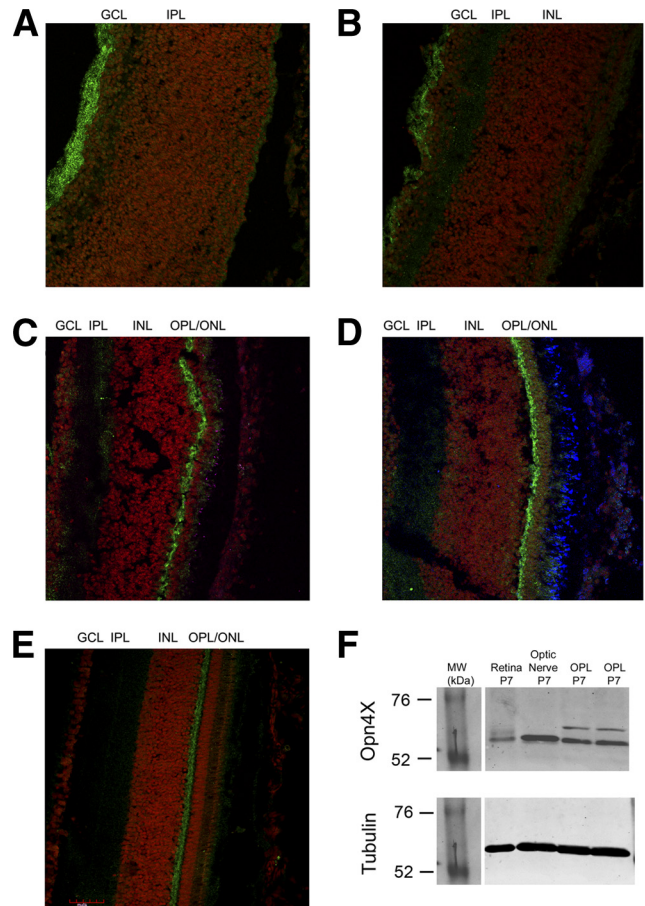

FIGURE 3. Immunochemistry for Opn4x-like protein in the chicken retina during development. **(A–E)** Retinal sections were immunolabeled for Opn4x (*green*), rhodopsin (*blue*), and propidium iodide (*red* nuclear staining) and visualized by confocal microscopy with specific primary antibodies at 40× magnification. Positive immunoreactivity was visualized for Opn4x in the axonal fibers of the forming GCL at E8 to E11 **(A, B)** and in the OPL at E15 to E19 and P7 **(D–F)**. Positive immunoreactivity for rhodopsin seen at E19 **(D)** demarks the outer segments of the rod PRCs. Scale bar, 40 μm. **(F)** Expression of Opn4x protein in the whole retina, the ON, and OPL at P7. Protein assays were performed by WB with 50 μg of protein for each sample and specific primary antibodies against Opn4x and α-tubulin (tubulin). For Opn4x, immunoblots showed bands of ~55 to 60 and 65 kDa. Results shown are representative of three blots from independent experiments.
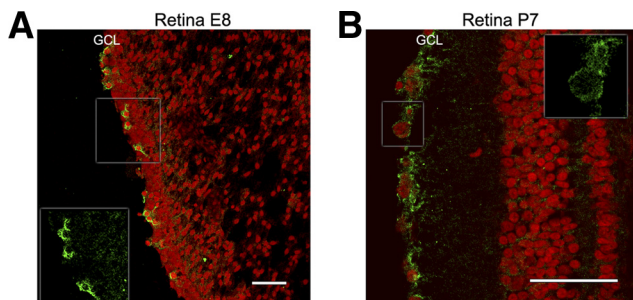
Invitrogen). RNA integrity was checked in 1.5% agarose gel and quantified by UV spectrophotometry (Gene Quant spectrophotometer; GE Healthcare, Madrid, Spain). Finally, 1 to 2 μg of total RNA was treated with DNase (Promega, Madrid, Spain) to eliminate contaminating genomic DNA. cDNA was synthesized with M-MLV (Promega), using oligo(dT).

Oligonucleotides were designed on computer (Vector NTI Advance 10; Invitrogen) to generate 100- to 400-bp products. The oligonucleotide sequences used for RT-PCR from the *Gallus gallus* sequences were as follows:

GAPDH forward: 5′AGG CGA GAT GGT GAA AGT CG 3′, reverse: 5′ TCT GCC CAT TTG ATG TTG CT 3′; Pax6 forward: 5′ GGG AGT AGA GGC ACG CAG ATG T 3′, reverse: 5′ GGA GTC GCT ACT CTC GGC TTA CTA 3′; Brn3 forward: 5′ CCA TCC TGC ACG AGC CCA AGT A 3′, reverse: 5′ GCC CCG TAG CAA GGT CTC ATC AA 3′; CRX forward: 5′ GCA CAG CCC AGA GCA TGA TGT CC 3′, reverse: 5′ CTC CAG GAT GTC CAA CTG CGC C 3′; Opn4m forward: 5′ TCT CGC CGT AGA ACA TCC 3′, reverse: 5′ GAA GTG TTT CAG AGC AAG GTA GGA 3′; Opn4x forward: 5′ TGC TTT GTC AAC AGC TTG CAC AGA G 3′,

FIGURE 2. Immunohistochemistry for Opn4m-like protein in the chicken retina at E8 and P7. Retinal sections were immunolabeled for Opn4m (*green*) and propidium iodide (*red*) and visualized by confocal microscopy with a specific primary antibody at 20× and 40× magnification. *Insets*: positive immunoreactivity was visualized in cell somas for Opn4m in both the forming and mature GCL, as seen at E8 and P7, respectively. Scale bar, 20 μm.

reverse: 5′ CAG CAA TAA TCT GTA TGG TGC GCT TC 3′; Gq forward: 5′ TCA AAA CAT CTT CAC TGC CAT G 3′, reverse: 5′ TCC ACG TCG CTG AGA TAG TAT T 3′; α-transducin forward: 5′ AAG GAC CTC AAC TTC AGG ATG T 3′, reverse: 5′ CAG TCC TTG AGG TTC TCC TTG 3′; red opsin forward: 5′CAC GAA GAG GAG GAC ACC AC 3′, reverse: 5′ CGA GAT CTG GTT GAT GAC GCT 3′; and rhodopsin forward: 5′AGG GCC AAG ACT TCT ACG TGC 3′, reverse: 5′ATG ATC CAG GAG AAC GCG ACC3′.

Statistics

Statistical analyses involved one- or two-way analysis of variance (ANOVA) with Duncan post hoc tests or Student's *t*-tests, when appropriate (significance at $P < 0.05$).

RESULTS

Onset of Nonvisual Gene Expression in the Embryonic Retina and RGCs

The presence of specific markers for prospective RGCs, *Pax6* and *Brn3*, was detected as early as E4, whereas the PRC-specific marker *CRX* became detectable by E7 (Fig. 1A, left). When we examined the components of the nonvisual phototransduction cascades, G protein q (*Gq*) mRNA together with trace *Opn4m* transcripts were observed at E4, whereas *Opn4m* expression became clearly detectable by E5 and remained robust throughout the different stages examined through E18. By contrast, *Opn4x* appeared several days later in the whole embryonic retina, exhibiting trace levels by E7 to E8 and robust levels by E10 to E12. The onset of expression of components of the visual photocascade was substantially delayed, as observed for G protein α-transducin (α-trans) and red cone opsin

by E8 to E10 and rhodopsin by E12. When the presence of RNAs was investigated in immunopurified RGC primary cultures, and compared with RNAs at E18 from the whole retina, we observed detectable mRNA for *Opn4m*, *Opn4x*, and *Gq* in the primary cultures, but not for transcripts of red cone opsin, rhodopsin, or α-trans (Fig. 1A, right).

Onset of Nonvisual Opsins in the Developing Retina and RGCs

We examined both Opn4x and Opn4m in embryonic and posthatching retina and RGC cultures. Western blot analysis revealed positive immunoreactivity for Opn4m at all embryonic stages examined. There were three main bands of ~48, 62, and 67 kDa. The 48- and 67-kDa bands were prominent at all times examined, whereas the 62-kDa band decreased markedly at P7. Binding was also found in RGC cultures, showing the same three bands seen in whole retina, but with more intense staining at 48 and 67 kDa. There was no clear binding in ON preparations from P7 chicks.

Immunoreactivity associated with Opn4x was observed in the whole retina from E8 onward, exhibiting three bands of ~56, 65, and 70 kDa (Fig. 1C). The two Opn4x-immunoreactive bands with higher MW were strongly stained at E8 to E11 and decreased by E15; the 56-kDa band appeared at E11, increased by E15, and remained elevated at later stages, with an additional band appearing at ~62 kDa around the time of hatching. The 56-kDa band was strongly stained in ON samples at P7. Positive binding was also observed in RGC cultures, in which only the two bands of higher MW were clearly observed.
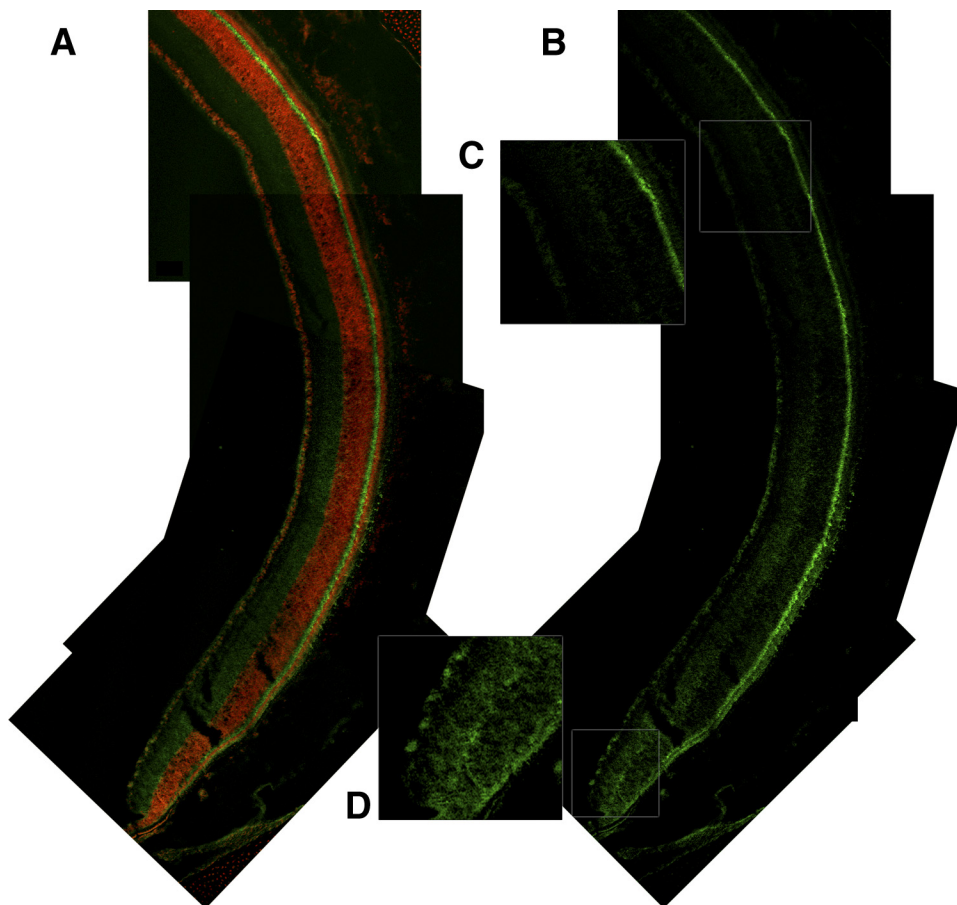
FIGURE 4. Opn4x immunoreactivity in mature chicken retina. Serial retinal sections from marginal to central zones of a retina at P7 were stained for propidium iodide (*red*) and Opn4x (*green*) (**A**) or Opn4x only (**B**, **C**) with the specific primary antibody and visualized by confocal microscopy at 40× (**A**, **B**) or higher magnification (**C**, **D**). Opn4x immunoreactivity was present throughout the retina, including the OPL and GCL. Discrete areas of central and peripheral retina are highlighted in the *insets* in (**C**) and (**D**), respectively. Scale bar, 40 μm.

Expression of Opn4 Proteins in the Developing Chicken Retina and RGC Cultures

We investigated the presence and localization of both Opn4 proteins in sections of developing retina and RGC cultures. Opn4m immunostaining was mostly restricted to cell soma membranes within the GCL in all ages examined (Fig. 2). In contrast, Opn4x was mainly located in axonal fibers of the forming GCL at E8 to E11 (Figs. 3A, 3B), with strong immunoreactivity appearing in the OPL by E15 and later (Figs. 3C, 3E). Immunoblotting for Opn4x in P7 samples clearly showed the 56-kDa band in whole retinal and ON samples, whereas an additional band of ~65 kDa was observed in enriched OPL preparations (Fig. 3F). Moreover, in serial sections of a mature WT chicken retina from the marginal to the central zone, Opn4x was clearly visualized throughout the OPL as well as in clusters of cells in the GCL, mainly in the periphery (Fig. 4). In addition, immunostaining of Opn4m and Opn4x proteins was clearly visualized in RGC cultures (Supplementary Figs. S1, S2, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-75301/-/DCSupplemental) visible as clusters of Opn4(+) cells (Supplementary Fig. S1). Quantification of numbers of immunostained cells indicated that RGC cultures contained 10.7% ± 3.0% Opn4x(+) cells, and 23.2% ± 2.2% Opn4m(+) cells. Supplementary Figures S2G–I (http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-75301/-/DCSupplemental) show intense Opn4x labeling in neurites of an individual RGC kept in culture for 5 days.

In addition, compared with Opn4x immunoreactivity in normal chickens (Fig. 5A), retinal sections of retinal degeneration GUCY1* chickens showed markedly stronger immunostaining in the different layers (Fig. 5B) and was especially intense in the GCL with bundles of strongly labeled processes. Discrete staining of axonal elements was observed in sections of normal ON (data not shown) and more strongly in GUCY1* ON (Fig. 5C; Supplementary Fig. S3, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-75301/-/DCSupplemental).

Cell Layer Characterization of Opn4x Expression in the Developing Chicken Retina

To identify cells expressing Opn4x at E15 and later in the OPL, we examined the co-localization of this photopigment with different markers for cells in the INL (horizontal cells, HCs; bipolar cells, BCs) and ONL (rod and cone and PRCs) (Figs. 6–8). No co-localization was observed by confocal microscopy between Opn4x immunoreactivity and PRC staining with anti-rhodopsin (restricted to outer segments) and anti-cone transducin (Fig. 6) or with a marker for "ON" type BCs (115a10) within the INL (Fig. 7). By contrast, positive co-localization was visualized in the OPL and adjacent INL between Opn4x and Prox1, an HC nuclear marker in both WT (Figs. 8A–C) and GUCY1* chickens (Fig. 8D). Opn4x immunofluorescence clearly labeled the cell soma and neurites of typical HCs (Figs. 8B, 8C). At longer incubation times, immunofluorescence could be clearly visualized in distinct inner plexiform layer (IPL) sublaminas, which did not correspond with "ON" BC axonal arborization (Fig. 7). We also observed that Opn4x immunoreactivity in the HC juxtaposed bipolar terminals in the OPL.

DISCUSSION

Opn4 was initially discovered in *Xenopus* and later found in the brain, iris, and retinal cells from most vertebrates tested.[2,6,19,28–32] In mammals Opn4 is only expressed in a small RGC subset[2,6,29,32,33] and was identified as the photopigment conferring intrinsic photosensitivity to these cells.[1,3,9,14]
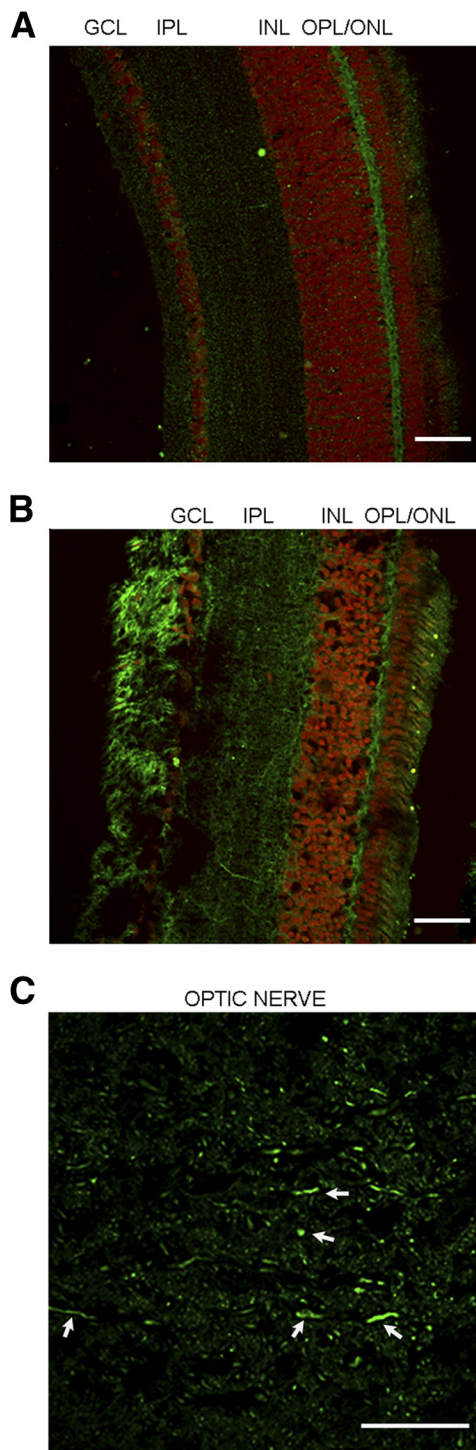

FIGURE 5. Opn4x immunoreactivity in sections of a wild-type retina (**A**), GUCY1* chicken retina (**B**), and ON (**C**). Sections were immunolabeled for Opn4x (*green*) and propidium iodide (*red*) and visualized by confocal microscopy at 40× magnification. Positive Opn4x immunoreactivity was visualized mainly in the OPL of wild-type chicks and intensely in different layers of the GUCY1* retina and ON. (**C**, *white arrows*) Intense Opn4x immunolabeling in axonal fibers. Scale bar, 40 μm.

Mammals uniquely express Opn4m, whereas nonmammalian vertebrates express both genes.[11] In mammals, melanopsin-expressing RGCs project to brain areas involved in diverse visual and NIF tasks regulated by light.[34–36] The function of
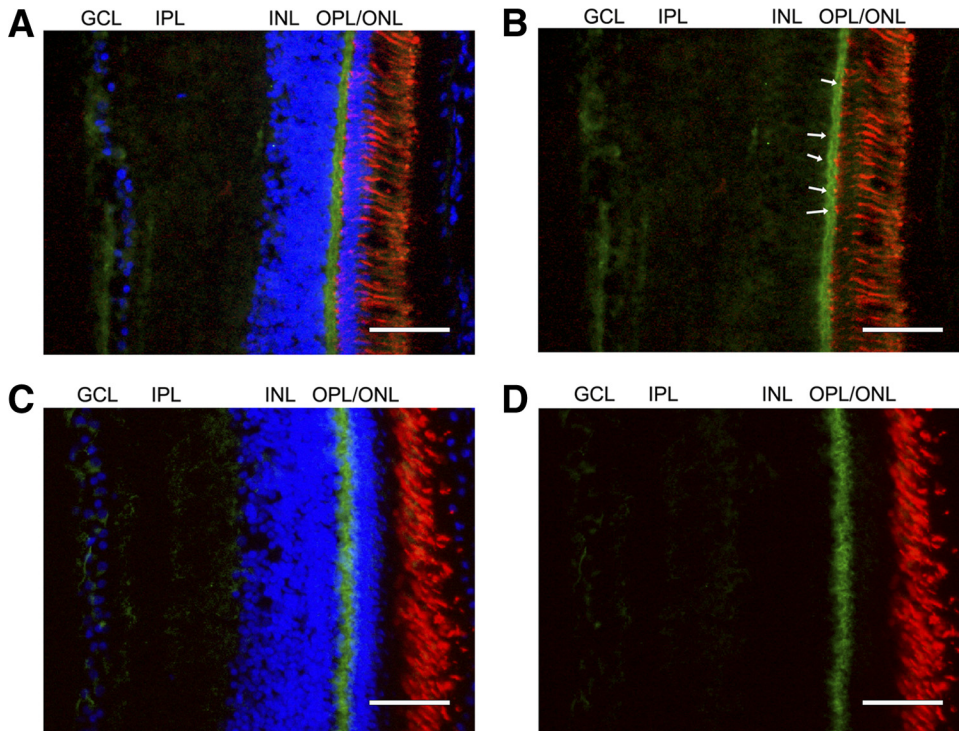
FIGURE 6. Characterization of immunoreactivity for Opn4x and INL and ONL components with specific markers of the chicken retina at P7. Consecutive retinal sections were immunolabeled for Opn4x (*green*; A–D) and nuclear staining with DAPI (*blue*; **A**, **C**), together with GTa1-α cone transducin (*red*; **A**, **B**) and rhodopsin (rho4d2, *red*; **C**, **D**), and visualized by confocal microscopy with specific primary antibodies at 20× magnification. Merging of immunofluorescence signals is shown in (**B**) and (**D**) with no DAPI staining; no co-localization was found between Opn4x and the two photoreceptor antibody markers as marked by *white arrows* in **B**. Scale bar, 50 μm.

Opn4x is not known but is thought to be similar. Opn4x is lacking in different eutherian mammals,[11,35] likely coinciding with a nocturnal period of mammalian evolution[37] and a general reduction in photosensitivity. More extensive photodetection systems have been retained in birds and other vertebrates during evolution, involving the pineal organ and encephalic
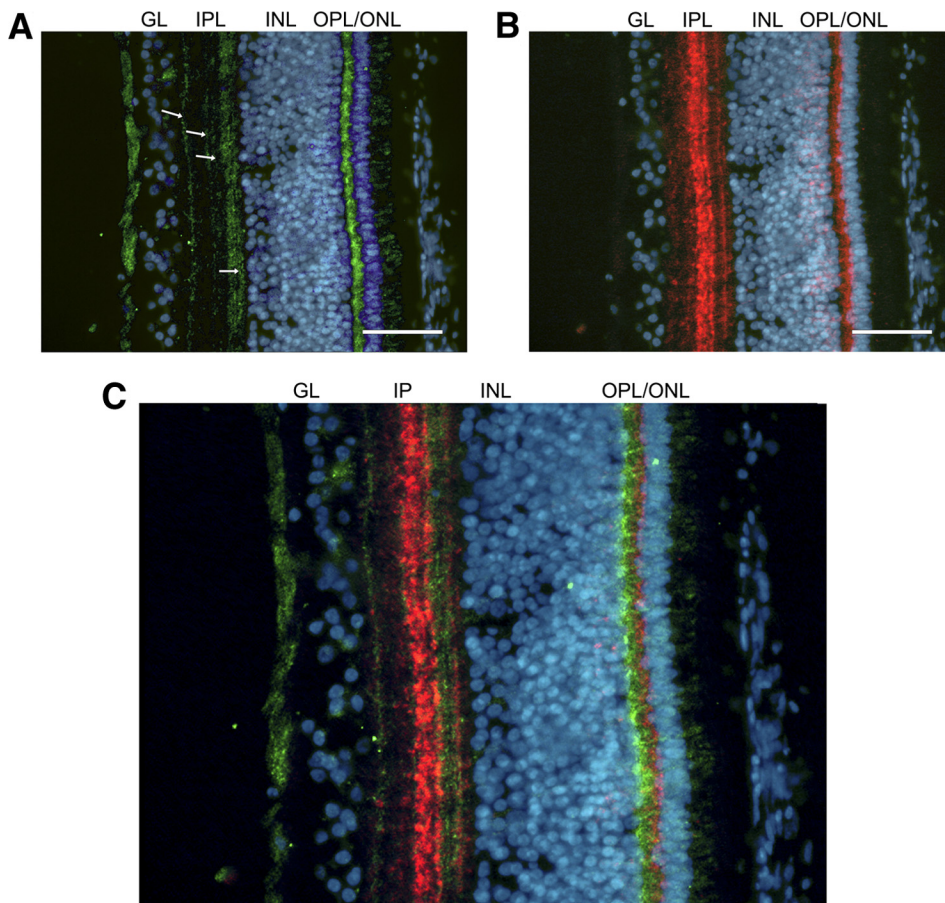

FIGURE 7. Characterization of immunoreactivity for Opn4x and "ON" bipolar cells in the chicken retina at P7. Retinal sections were immunolabeled for Opn4x (*green*; **A**, **C**) and an "ON" bipolar cell marker (115A10) (*red*; **B**, **C**) and nuclear staining by DAPI (*blue*; **A–C**). Marked Opn4x staining can be seen in HCs and axons in the nerve fiber layer, as well as in discrete sublamina within the IPL (*arrows*, **A**). "ON" bipolar cell axons also ramified in the IPL, but merged images of the double-stained sections show no overlap between the two plexi (**C**). Furthermore, the close nonoverlapping apposition of Opn4x in HC dendrites in the lower OPL and 115A10 staining of "ON" bipolar cell dendrites in the upper OPL can be clearly seen (**C**). Faint labeling of photoreceptor outer segments is due to autofluorescence. Scale bar, 50 μm.
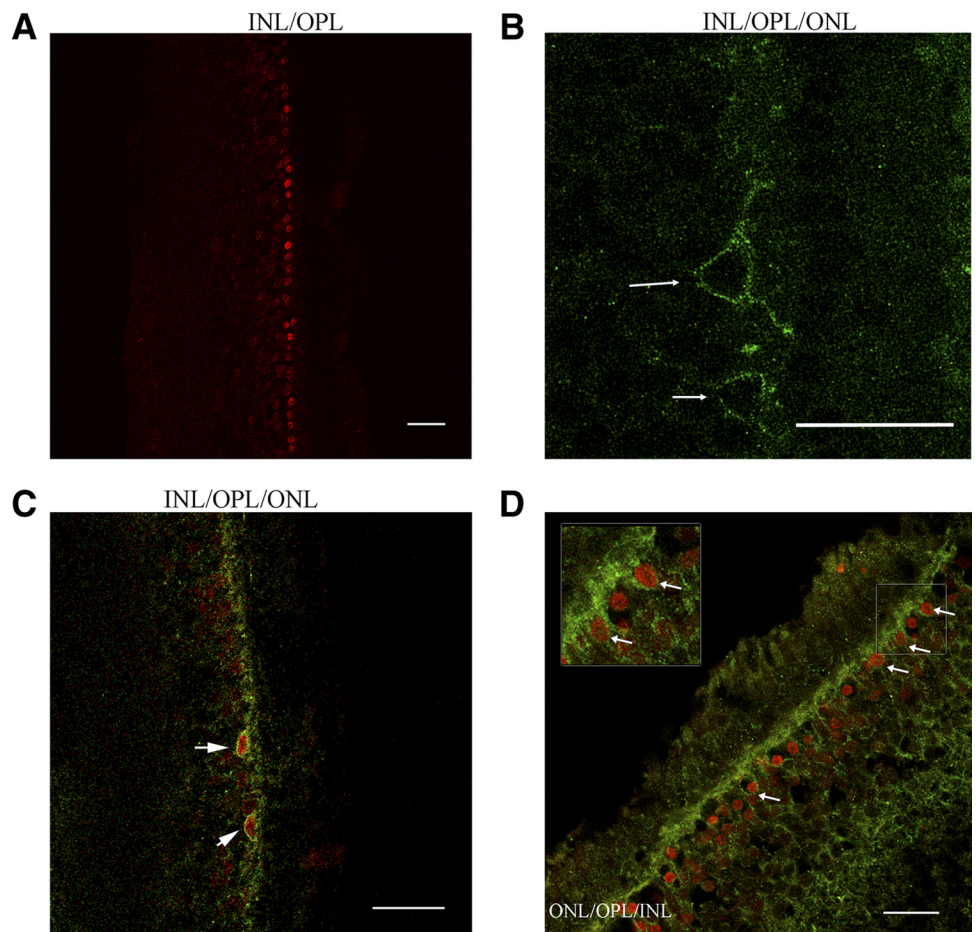
FIGURE 8. Co-localization of Opn4x immunoreactivity with Prox1, an HC marker in the retina of WT and GUCY1* chickens. Immunohistochemistry for Prox1 (*red*; **A**, **C**, **D**) and Opn4x (*green*; **B-D**) in the chicken retina at P7. Retinal sections were immunolabeled for Prox1 (**A**) or Opn4x (**B**), or sequentially stained for both antibodies and merged (WT, **C**; GUCY1*, **D**). *Arrows*: (**B**) A typical axonless candelabrum HC stained for Opn4x;in; (**C**) and (**D**) and *inset* in (**D**) Opn4x and Prox1 co-localization. Scale bar, 20 μm.

photoreceptors; within the retina, this would include noncanonical PRCs.[38] These nonclassic photoreceptors can be active in measuring day length and implicated in diverse light-regulated processes (i.e., seasonal reproduction and migration).

Initial expression of both Opn4 orthologues is confined to the emerging RGCs (Fig. 1). The expression of each orthologue correlates with a distinct phase of RGC development: onset of *Opn4m* and *Gq* mRNA coincides with RGC birth around E4,[12,39] and early *Opn4m* appearance has been reported in mammals.[40–44] The onset of Opn4x expression is markedly later, commencing around E8 and coinciding with the time at which retinotectal projections are forming and arriving at laminas of the optic tectum by E6 to E8.[45–50] Strong Opn4x expression is observed in axonal fibers of RGCs and persists in the ON throughout maturation. The spatial distribution of Opn4m protein differed greatly from that of Opn4x at later times in retinal development. Whereas Opn4m remained expressed exclusively in an RGC subpopulation at all ages examined, in addition to RGC localization, Opn4x was switched on in HCs by E15. Previous in situ hybridization studies showed quite widespread distribution for both orthologues, with detectable mRNA in all cellular layers.[11,20] Antibody staining patterns in the present study were more restricted, possibly because protein levels in many cells are below detection limits. Transgenic mice expressing reporter genes under Opn4 promoter control revealed far more positive cells than detected by IHC.[51] Indeed the retinal pattern we see for Opn4m protein distribution is identical with that of mammals, restricted to the RGC layer at all ages examined. On the other hand, previous in situ hybridization studies more closely resembled the anti-Opn4x antibody data.[17] *Opn4x* mRNA was shown to be rhythmically expressed in the mature chicken retina,[18,19] and was particularly expressed in the outer INL.[19] Using the same antibody, Opn4x staining was previously detected in RGCs and general neurons in the INL, including HC, although no cell-type identification was performed.[21,52] We were unable to detect any immunostaining in rod or cone photoreceptors, or bipolar cells ("ON" subtype). It should be stressed that Opn4x continued to be expressed by an RGC subpopulation, as witnessed from the immunostaining of discrete axons in P7 ON. The target region(s) innervated by these Opn4x-positive RGCs is currently unknown.

Immunoblotting for both Opn4 proteins revealed the presence of several main bands (~48–67 kDa for Opn4m, and 56–70 kDa for Opn4x). The in silico analysis for both Opn4 proteins from their mRNA sequences identify long (L), short (S), and super short (SS) isoforms of 58.5, 47.7, and 35.9 kDa, respectively, for Opn4m, and a long (L) and a short (S) isoform of 60.3 and 47.6 kDa, respectively, for Opn4x.[16] The persistence of the three Opn4m-immunoreactive bands at all embryonic ages and in cultured RGCs, and their relative absence from ON, indicates the these forms are all confined to RGC and dendrites. They may correspond to the three alternatively spliced Opn4–2 isoforms cloned.[16] Alternatively, the uppermost band may result from posttranslational modifications such as glycosylation.[26] Opn4x immunoblots showed age-related changes in band profiles, with a transition from an early pattern (E8-E11) dominated by high molecular mass bands toward one with a mainly low molecular mass isoform (E15 onward) enriched in RGC axons. This low-mass isoform may correspond to the short Opn4x isoform Opn41S previously identified[16] and suggests that it serves specific roles in retinal photosensitivity. The robust expression in HC is seen as a
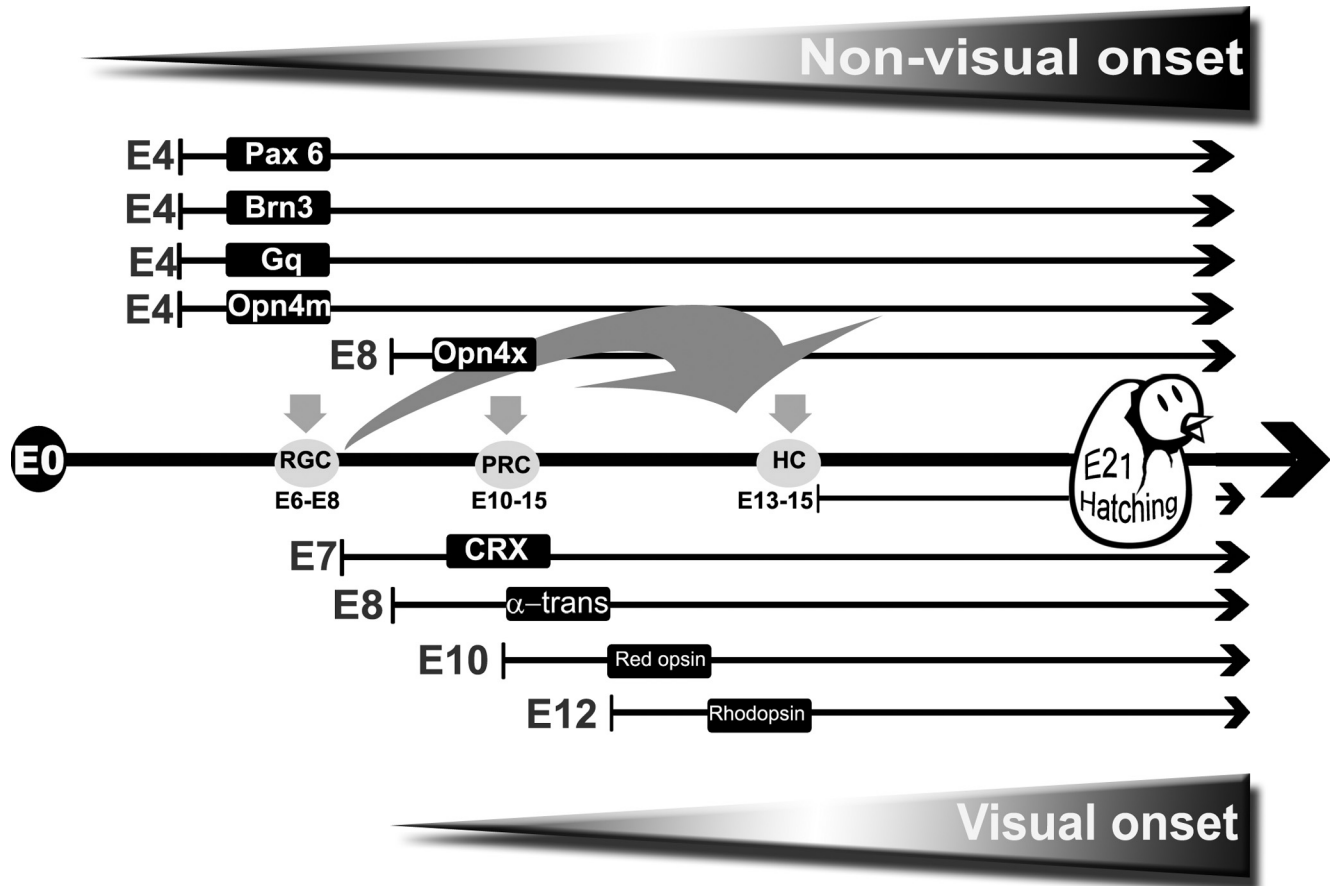
SCHEME 1. Time line for the onset of expression of opsins and photocascade elements in the visual and nonvisual systems in the developing chicken retina. Embryonic retinas expressed specification genes for prospective RGCs, such as *Pax6* and *Brn3*, and components of the nonvisual phototransduction cascade like *Opn4m* and *Gq* mRNAs at very early stages (E4–E5). On the contrary, the expression of PRC markers (*CRX*, red-opsin, rhodopsin, and α-transducin) was observed from E7 to E12. Opn4m protein was visualized in the whole retina as early as E4 and remained elevated from E6 to postnatal days, mainly localized in the GCL, whereas Opn4x was first detected at E8 in axonal fibers of the GCL. By E15 and onward, it was highly expressed in the OPL, mostly in HCs, but in a few RGCs. The ON RGCs were mostly mature by E8, the PRCs after E10. HCs differentiated and migrated to the INL by E13 to E15.

higher mass form, possibly corresponding to Opn41L,[16] and again indicates a selective function in inner retinal processing.

After cell fate determination, HC precursors migrate, differentiate, and acquire the appropriate morphology for their function in visual perception.[53] In the chicken retina, three different cell types (H1, H2, and H3) were characterized by a distinct morphology[54]: H1 is an axon-bearing HC, while H2 "stellate" and H3 "candelabrum-shaped" are axonless HCs. Concomitantly with HC birth and migration between E10 and E15[55,56] Opn4x (+) immunoreactivity appeared in the cell somas of Prox1 (+) HC and displayed prominent labeling of the lower OPL. Morphologically, some of these Opn4x cells are candelabrum axonless HCs, which mainly connect to cone pedicles.[53,55] Close imbrications of cone pedicles and Opn4x-immunopositive HC processes were seen in our IHC studies. Remarkably, cone HCs in fish are directly light sensitive and express Opn4m.[13] In the chicken, Opn4x could play a role in receptive field construction (cones, horizontal cells, bipolar cells, and RGCs). It is intriguing to speculate how Opn4x may modify HC responses to light, since they are normally hyperpolarized in response to rod and cone input after light onset, whereas activation of Opn4x would blunt such activity through an opposing depolarization. Such influences would be further influenced by two additional factors: first, circadian fluctuations in Opn4x levels,[19] with the highest expression in outer INL occurring at ZT4; second, direct recording of Opn4x-

dependent light-induced currents indicates less reproducible and lower amplitude responses than Opn4m.[11] Finally, shared Opn4x expression by HC and RGCs in birds suggests that they are ancestrally related cells.[12]

Opn4 has been phylogenetically related to invertebrate rhodopsin, which couples to invertebrate Gq protein,[57–60] and full-length *Amphioxus* melanopsin activates Gq with an efficiency similar to that of Gq-coupled visual pigments.[61] On the contrary, expression of components of the visual phototransduction cascade was noticeably delayed: α-transducin and red cone opsin were first detected at E8 to E10, and rhodopsin at E12, after the appearance of the PRC marker CRX around E6 and E7. This agrees with the sequence of timing for the onset of PRC markers and visual photopigments reported previously[62–64]; red and green cone opsins were detected first at E14 and rhodopsin around E15. The developmental onset of both visual and nonvisual photocascade elements is summarized in Scheme 1. Primary cultures of E8 RGCs also expressed noncanonical photocascade elements such as the *Gq* and the two *Opn4* genes, but not Gα-transducin or red cone opsin, both markers of PRCs (Fig. 1; Supplementary Figs. S1, S2, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-75301/-/DCSupplemental). Strikingly, RGC cultures are intrinsically photosensitive and respond to light by triggering an invertebrate-like phototransduction cascade.[10,15] In addition, GUCY1* chickens which lack functional classic photoreceptors respond to light that mediates photic entrainment of

feeding rhythms and pupil reflexes and express both *Opn4x* and *Opn4m* transcripts in their retinas.[5] Opn4x immunoreactivity was highly expressed in GUCY1* retina, particularly in the GCL, INL, and OPL and with intense staining in RGC axons (Fig. 5). Rodent models of photoreceptor degeneration show either suppression[65,66] or no effect[67] on downstream Opn4m expression. In the present case, Opn4x over-expression may be a compensatory mechanism for the absence of classic rod and cone input.

CONCLUSIONS

Taking all this information together with findings in previous work,[10,15,64] developing chicken retina exhibits early expression of components for the nonvisual phototransduction cascade. This early photosensitivity mediated by *Opn4* in ipRGCs may act as an immature form of vision, since these cells may also project to image-forming visual areas,[2,51,68,69] supporting the idea that "second sight comes first."[17,70,71] The appearance of Opn4x expression in HC by E15 strongly suggests the existence of a novel type of photosensitive retinal cell in birds, implicating novel connections between outer and inner retina and a higher degree of complexity in mechanisms of light detection, processing, and transmission to the brain.

Acknowledgments

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