Horizontal cells expressing melanopsin x are novel photoreceptors in the avian inner retina

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In the vertebrate retina, three types of photoreceptors—visual photoreceptor cones and rods and the intrinsically photosensitive retinal ganglion cells (ipRGCs)—converged through evolution to detect light and regulate image- and nonimage-forming activities such as photic entrainment of circadian rhythms, pupillary light reflexes, etc. ipRGCs express the nonvisual photopigment melanopsin (OPN4), encoded by two genes: the *Xenopus* (*Opn4x*) and mammalian (*Opn4m*) orthologs. In the chicken retina, both OPN4 proteins are found in ipRGCs, and Opn4x is also present in retinal horizontal cells (HCs), which connect with visual photoreceptors. Here we investigate the intrinsic photosensitivity and functioning of HCs from primary cultures of embryonic retinas at day 15 by using calcium fluorescent fluo4 imaging, pharmacological inhibitory treatments, and *Opn4x* knockdown. Results show that HCs are avian photoreceptors with a retinal-based OPN4X photopigment conferring intrinsic photosensitivity. Light responses in HCs appear to be driven through an ancient type of phototransduction cascade similar to that in rhabdomeric photoreceptors involving a G-protein q, the activation of phospholipase C, calcium mobilization, and the release of the inhibitory neurotransmitter GABA. Based on their intrinsic photosensitivity, HCs may have a key dual function in the retina of vertebrates, potentially regulating nonvisual tasks together with their sister cells, ipRGCs, and with visual photoreceptors, modulating lateral interactions and retinal processing.

retina | light responses | horizontal cell | phototransduction | melanopsin

In the vertebrate retina, photoreceptors can be classified according to their function as canonical or noncanonical. The first group comprises specialized ciliary retinal neurons, cones, and rods in the outer retina, which participate in the image-forming processes associated with day/night vision. The second group is composed of intrinsically photosensitive retinal ganglion cells (ipRGCs) in the inner retina, which preferentially participate in the processing of photic inputs related to nonimage-forming tasks (photic synchronization of circadian rhythms, pupillary light reflexes, inhibition of pineal melatonin, etc.) (1–7). The photopigment melanopsin (OPN4) (1) confers photosensitivity to ipRGCs as clearly shown through later experiments with OPN4 knockout mice or OPN4 heterologous expression in nonretinal cells for loss or gain of function, respectively (8–11). Through evolution, OPN4 appears to have been encoded by at least two genes in vertebrates: *Opn4x* and *Opn4m*, the *Xenopus* and mammalian ortholog genes, respectively (12). Interestingly, the first-appearing vertebrates, nonmammals including fish, amphibians, and birds, possess both *Opn4* genes whereas mammals only have *Opn4m*. Evidence suggests that during the course of evolution, mammals lost some visual opsins and *Opn4x* as they entered the nocturnal niche. In the chicken retina, different laboratories have reported the expression of *Opn4* genes in outer nuclear, inner nuclear, and ganglion cell layer (GCL) cells (12–21). At the protein level, OPN4M was shown to be restricted to ipRGCs, whereas OPN4X was found in the GCL at embryo day 8 (E8) but mostly in PROX1 (+) horizontal cells (HCs) by E15 (21); the homeobox gene PROX1 is a universal HC marker that is expressed from E8 forward (22, 23). These and other experiments raised a crucial question: Can Opn4x (+) HCs intrinsically respond to light? HCs are retinal interneurons lying adjacent to the outer plexiform layer and implicated in visual perception and the regulation of signaling between cones, rods, and bipolar cells, enhancing image contrast, color discrimination, and light adaptation. HCs are involved mainly in the lateral interactions of the outer retina (24, 25). Once determined, retinal HC precursors migrate, differentiate, and express a set of specific markers; the final inner nuclear positioning occurs around E15 (23, 26), the same time at which a very strong expression of OPN4X was observed (21). Molecular comparative biology indicates that rods and cones have evolved from a common ciliary photoreceptor precursor, whereas RGCs, amacrine cells, and HCs may have evolved from a common precursor with rhabdomeric photoreceptors (27). Two studies provide strong evidence of photoresponses in HCs in teleosts (28, 29), shown to express vertebrate ancient (VA) opsin and OPN4 and to intrinsically respond to light well beyond rod and cone responses (28–31). In this work we investigated the intrinsic photosensitivity of HCs from embryonic avian retina, the role of the nonvisual photopigment Opn4x in these photic responses, and the neurochemical features of the events triggered by the light stimuli.

Results

Characterization of HC Primary Cultures Expressing OPN4x. HCs isolated from the 2.5% phase of a BSA gradient obtained from disaggregated embryonic chicken retinas at E15 displaying positive immunoreactivity for the typical HC marker PROX1 (≥85%) after flow cytometry (Fig. S1) were cultured for 2–3 d under constant illumination conditions and immunostained for OPN4x. Fig. 1 shows OPN4x (+) immunoreactivity in most cells of the

Significance

In the vertebrate retina, three types of photoreceptors—visual photoreceptor cones and rods and intrinsically photosensitive retinal ganglion cells (ipRGCs)—converged through evolution to detect light and regulate image- and nonimage-forming activities. ipRGCs express the photopigment melanopsin (OPN4), encoded by two genes: the *Xenopus* (*Opn4x*) and mammalian (*Opn4m*) orthologs. In the chicken retina, both OPN4 proteins are found in ipRGCs. Opn4x is also present in retinal horizontal cells (HCs) connecting with visual photoreceptors. We show that HCs displaying intrinsic photosensitivity constitute photoreceptors requiring Opn4x expression and retinaldehyde, acting through an invertebrate-like phototransduction cascade and GABA release. These mechanisms may enable HCs to regulate nonvisual tasks with ipRGCs and lateral interactions with visual photoreceptors.

Author contributions: L.P.M., N.M.D., and M.E.G. designed research; L.P.M. and N.M.D. performed research; M.E.G. contributed new reagents/analytic tools; L.P.M., N.M.D., and M.E.G. analyzed data; and M.E.G. wrote the paper.

The authors declare no conflict of interest.

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2.5% phase. These cells also expressed PROX1 after subsequent immunopurification by an anti-OPN4x antibody (Fig. S2 *A* and *B*). Cultured cells also expressed mRNAs for another HC marker, *Islet-1*, and for *Gq*, the member of the G-protein family involved in the rhabdomeric-like photocascade (Fig. S2*C*). By contrast, only trace inmmunoreactivity associated with RETP1 (≤6%) and NF-200 (≤5%), markers of photoreceptor cells and RGCs, respectively, was observed in the cultures (Fig. S2), and only when cultures were maintained for 5 d or longer were a few Muller glial cells seen using the specific marker glutamine synthase.

Retinal cells from the 3% and 4% phases of the gradient also expressed OPN4x and upon magnification showed much longer neurites than those in HC cultures, likely denoting OPN4x (+) RGCs (Fig. 1).

Endogenous Levels of Retinaldehyde Isomers and Light Responses in HCs. To function as a photopigment, OPN4 requires the presence of sufficient retinaldehyde isomers as the active chromophore (6–11). Results shown in Fig. 2 *C* and *D* indicate that HC cultures contained detectable basal levels of all-trans retinal. Nevertheless, to evoke significant light responses, 0.6 μM of exogenous all-trans retinal were added to the cultures in most experiments. Light response was then assessed by Ca^{2+} fluorescent imaging with Fluo4 in cells from disaggregated embryonic retinas at E15 kept in culture for several days (Fig. S3). When photic responses were evaluated in HC cultures, pulses of white light (1,000 lx) of different durations ranging from 5 to 50 s were able to promote significant increases in relative Ca^{2+} levels compared with basal fluorescence seen in the dark ($P < 0.001$ by ANOVA) (Figs. 2 and 3). Responses were further increased with the addition of exogenous all-trans retinal (Fig. 2*B*). After light stimulation of different durations, relative levels of Ca^{2+} increased 50% from 5 s of exposure to 20–50 s (Fig. 3), and the time to peak revealed that the highest response in each experiment did not attain saturation along the photic stimuli. Moreover, when HCs were exposed to prolonged illumination stimuli of longer duration (5–10 min), sustained responses were observed in intracellular Ca^{2+} levels, peaking after 1 min of light onset ($\Delta F = 1.34 \pm 0.06$), decreasing by 50% after 3 min, and returning to basal levels minutes later.

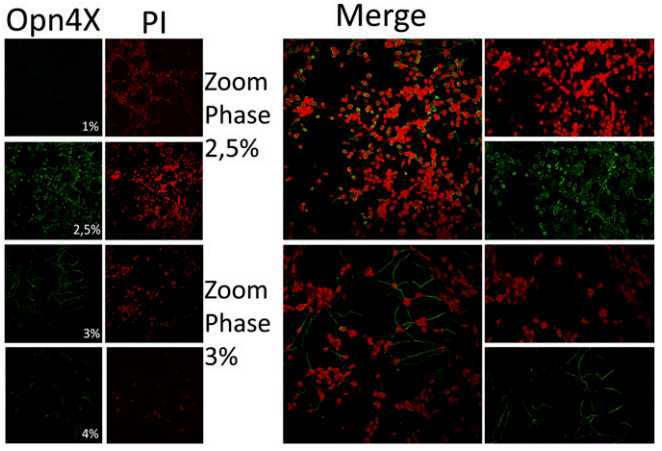


Fig. 1. Characterization of primary cultures of embryonic retinal cells obtained from the different phases of a discontinuous BSA gradient at day 15. (*Left*) Cell cultures from the different phases (2.5%, 3%, 4%) show positive immunoreactivity for Opn4x (green) in cell somas and processes; propidium iodide (PI, red) staining denotes cellular nuclei. (*Right*) Magnification (200×) of microphotographs from cell cultures from the 2.5–3% BSA gradient phases showing immunofluorescence colocalization (merge) between PI and Opn4x in cell somas and processes. OPN4 (+) immunoreactivity demarks the typical morphology of neuronal cells with shorter or longer processes as indicated in the selected square further magnified on the right.

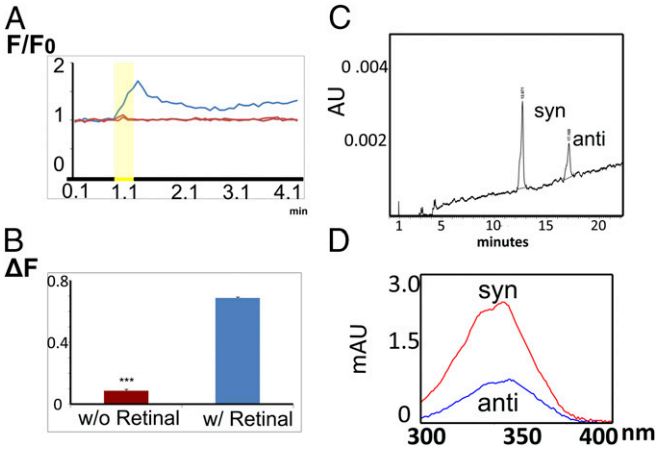


Fig. 2. Light-induced increase in Ca^{2+} levels measured by Fluo-4 AM fluorescent microscopy measured in HC cultures with (w/) or without (w/o) the addition of exogenous all-trans retinal (0.6 μM). (*A*) Intracellular increase in Ca^{2+} levels after light exposure for 10 s (yellow mark) without (w/o) exogenous all-trans retinal in individual cells (red) compared with levels with (w/) exogenous all-trans retinal (blue line) representing the average of photic responses in individual cells. (*B*) Graphical representation of relative fluorescent Ca^{2+} levels (ΔF) in HC cultures with (w/) or without (w/o) all-trans retinal (0.6 μM) administration in the culture medium. (*C* and *D*) Endogenous levels of all-trans retinal in primary cultures of HCs by HPLC determination (*C*) in both all-trans retinal stereoisomers, *syn* (peak at 357 nm) and *anti* (peak at 361 nm), by UV spectrum (*D*). Data are mean ± SEM ($n = 3$). Calcium fluorescent imaging was assessed as stated in *Materials and Methods*.

The decline in light responses was probably due to fluorescent indicator saturation after continuous light exposure. When two brief pulses of bright light were given to the cultures, separated by an interval of 5 min in the dark to allow for cell recovery, Ca^{2+} levels peaked immediately after the photic stimulation for both pulses (Fig. 3*E*). In addition, when light pulses of different wavelengths were tested in the Opn4x (+) HC cultures, using LEDs of similar intensities at 395–400 nm, 460 nm, and 625 nm for 5 s, the highest responses were visualized with blue light (Fig. S3*D*).

OPN4X-Mediated Intrinsic Photosensitivity in HCs. We next investigated the role of OPN4X in conferring HC intrinsic photosensitivity by knocking down its expression and assessing the effect of light-evoked responses on fluorescent Ca^{2+} levels. Fig. S4 shows that the specific shRNA for *Opn4x* significantly decreased OPN4X expression (Fig. S4 *A*, *B*, *E*, and *F*). Light increases in intracellular somatic Ca^{2+} levels in control cultures treated with a scrambled sequence RNA (ssRNA) (Fig. 4 *A* and *B*) were completely attenuated in those cells positively transfected with Opn4x shRNA (Fig. 4 *A* and *B*; Fig. S4 *C* and *D*). Fig. 4*B* indicates an almost 90% decrease compared with the control and a significant treatment effect ($P < 0.001$). In another series of experiments, when a chemically synthesized opsinamide was used to inhibit OPN4 activity as previously shown (32), no photic increases in fluorescent Ca^{2+} levels were found in HC cultures compared with cells treated with vehicle only (Fig. S5).

Light-Triggered Events in HCs. To investigate the molecular events triggered by light downstream of OPN4X activation, we treated Opn4x-immunopurifed HC cultures with different Ca^{2+} chelators, retinal bleachers, or Gq protein and phospholipase C (PLC) inhibitors to examine their effect on fluorescent Ca^{2+} levels after light stimulation. The elevation in somatic Ca^{2+} levels by light observed in cultures treated with vehicle (Fig. 5 *F* and *G*) were completely abolished by treatment with U73122, a PLC inhibitor (Fig. 5 *A* and *G*), or with hydroxylamine, a retinal bleacher that strongly affects photopigment activity (Fig. 5 *B* and *G*). Suramin

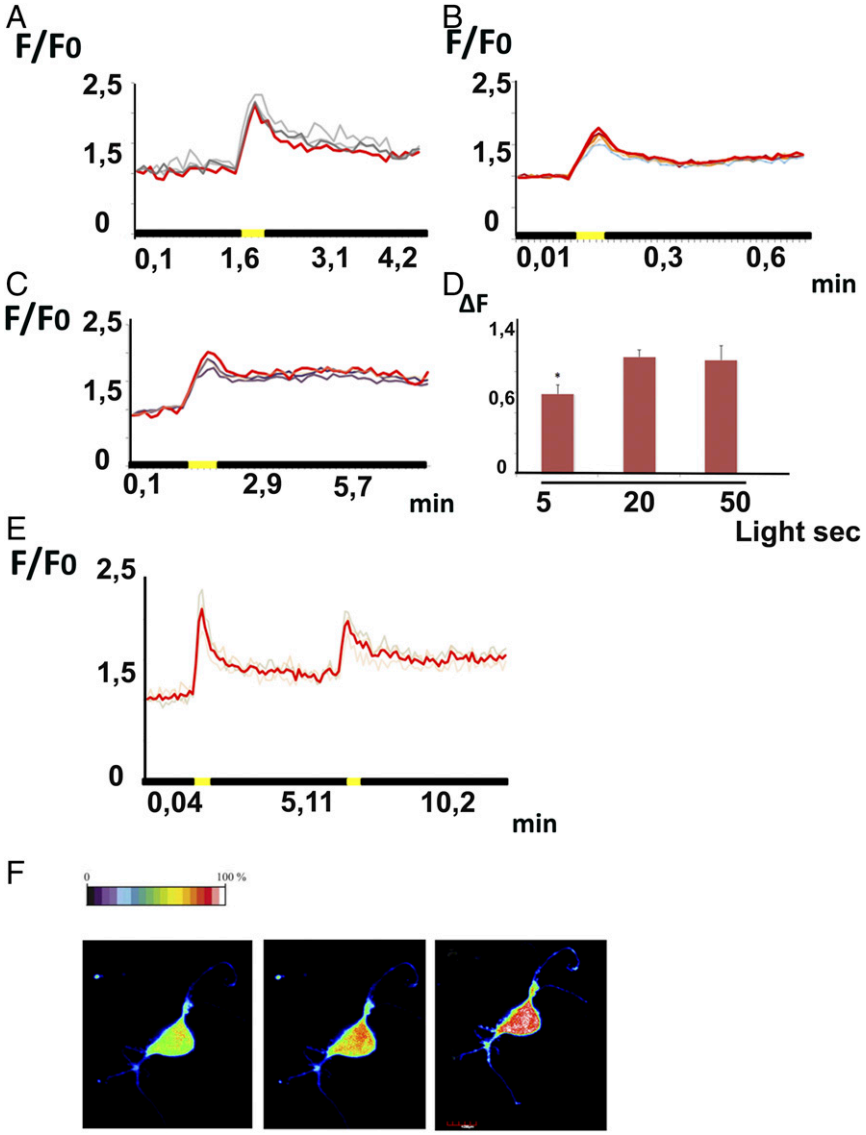


Fig. 3. Light-induced changes in intracellular Ca^{2+} levels measured by Fluo-4 AM fluorescent microscopy in HC cultures. (*A–C* and *F*) Individual cells in culture exhibit a significant response to light by increasing intracellular Ca^{2+} levels as seen in lighter color recordings. The red line represents the average response of all photosensitive cells assessed. Cells were exposed to a single brief white light pulse of 1,000 lx for 5 (*A*), 20 (*B*), or 50 s (*C*) or to two brief white light pulses of 1,000 lx for 20 s (*E*). Light duration is marked with yellow in the axis. Plots shown in *A–C* and *E* represent the F/F_o ratio for the changes in fluorescence levels 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_o). (*D*) Graphical representation of relative fluorescent Ca^{2+} levels (ΔF) calculated as indicated in *Materials and Methods* after 5 s, 20 s, and 50 s of light stimulation compared with dark controls. Data are mean ± SEM ($n = 25$ from three independent experiments); $^*P < 0.05$ by Student *t* test. (*F*) A typical HC (magnification: 600×) showing several processes grown after 3 d in culture loaded with Fluo-4 AM and displaying a significant increase in intracellular calcium fluorescence levels after light exposure, visualized in a pseudocolor scale with the highest response in red. Ca^{2+} levels were measured by Fluo-4 AM fluorescent microscopy as described in *Materials and Methods*.

analogs were shown to act as direct antagonists of heterotrimeric G proteins (33) and reported to impair light responses in ipRGCs (11). We examined the effect of 100 μM suramin on HCs applied for 15–20 min before light stimulation and found that light responses were substantially abolished after treatment (Fig. 5 *C* and *G*). On the contrary, when specific inhibitors of the visual phototransduction cascade were used, such as the pertussis toxin (500 nM) that disrupts signaling through the Gi/o protein α-transducin or zaprinast (0.1 M), a specific phosphodiesterase 6 (PDE6) inhibitor (6, 17), light responses were not affected in the cultures (Fig. S5 *D* and *E*).

In an attempt to elucidate the calcium sources responsible for the light increases observed in somatic Ca^{2+} levels and because it is known that 2-aminoethoxydiphenyl borate (2-APB) is a membrane-permeable blocker of the inositol 1,4,5-trisphosphate (IP_3)-induced Ca^{2+} release from internal stores (34), we investigated the effect of 2-APB on light responses in HCs; the treatment with 100 μM of 2-APB before light exposure significantly inhibited light-mediated increases in intracellular Ca^{2+} levels (Fig. 5 *D* and *G*). Furthermore, photic responses were substantially blocked when HC cultures were treated with EGTA (1 mM), a known extracellular Ca^{2+} chelating agent, before light stimulation (Fig. 5 *E* and *G*).

HCs can regulate photoreceptor functioning upon depolarization, and GABA release may be implicated in lateral inhibition (35–37). To investigate its potential utilization as a neurotransmitter and its release in these cells under the different

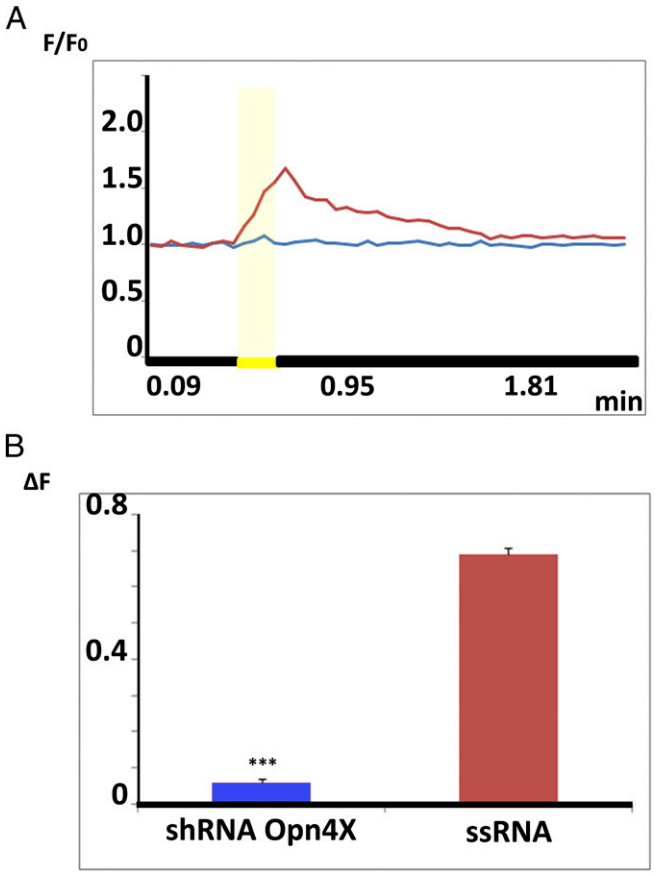


Fig. 4. Calcium fluorescent imaging of HC cultures treated with the Opn4x shRNA. (*A*) Significant light-induced changes in intracellular Ca^{2+} levels found in HC cultures treated with the control ssRNA (red line) were drastically affected in cell cultures treated with the specific Opn4x shRNA (blue line). (*B*) Graphical representation of relative fluorescent Ca^{2+} levels (ΔF) after a brief white light pulse of 1,000 lx for 5 s in Opn4x shRNA-treated cell cultures compared with ssRNA-treated cells (control). Data are mean ± SEM (n = 15 from three independent experiments); ***$P < 0.001$ by Student t test.

light conditions examined, HCs were loaded with ^{3}H-GABA in the dark, the culture medium was washed out, and the cells were then exposed to bright light for 30 min or kept in the dark. HCs in culture take up radiolabeled GABA from the extracellular medium under control conditions (Fig. 6), and light stimulation significantly increased its release to the extracellular medium compared with dark controls ($P < 0.001$).

Discussion

Within the retina, HCs project laterally and integrate inputs from visual photoreceptor rods and cones, providing them with negative and positive feedback to generate an antagonistic, and to a lesser degree agonistic, center-surround receptive field (Scheme 1, *Scenarios 1* and *3*) (25). Each HC receives chemical synaptic inputs from many rods and cones and, in return, generates a feedback signal that alters HC neurotransmitter release. Through this neural network comprising HCs, rods, cones, and bipolar cells, light in the retina generates a representation of spatial contrast and visual detection of edges, color discrimination, and light adaptation (35, 36). HC feedback is critical for establishing the antagonistic receptive field-surround of the subsequent neuron in the visual path (the bipolar cell). HCs are thus responsible for lateral interaction and inhibition (24, 34, 35). In the dark, HCs are depolarized as a consequence of glutamate release by visual photoreceptors, which in turn causes the release of GABA by HCs onto cone synapses promoting a neurochemical inhibition, proton release, or ephaptic signaling (35). Conversely, when the light is on, cones and rods become hyperpolarized, stopping the release of glutamate and thus preventing the depolarization and subsequent HC response. In addition to their typical role in the retinal circuits, in nonmammalian vertebrates HCs may conserve vestiges of ancient photoreceptor cells (Scheme 1, *scenario 2*) as clearly demonstrated for ipRGCs (2, 6, 17, 18). In teleosts, it has been reported that retinal HCs expressing VA opsin or OPN4 can intrinsically respond to light (28, 29). OPN4 is found in brain, iris, and retinal cells of vertebrates (1, 3, 5, 6, 12, 17–19) and highly conserved through evolution (38). In mammals OPN4 is confined to ipRGCs; however, the distribution of multiple Opn4s (Opn4m and Opn4x) in diverse retinal cell types of nonmammalian vertebrates suggests a biological function of higher complexity (see review in ref. 6). In the chicken retina, HCs express *Opn4* mRNA (14, 19) and Opn4x protein (21). OPN4 is an ortholog of the Gq-coupled invertebrate opsins (6), and the photocascade operating in the OPN4 (+) ipRGCs clearly involves the participation of PLC, Ca^{2+} mobilization,

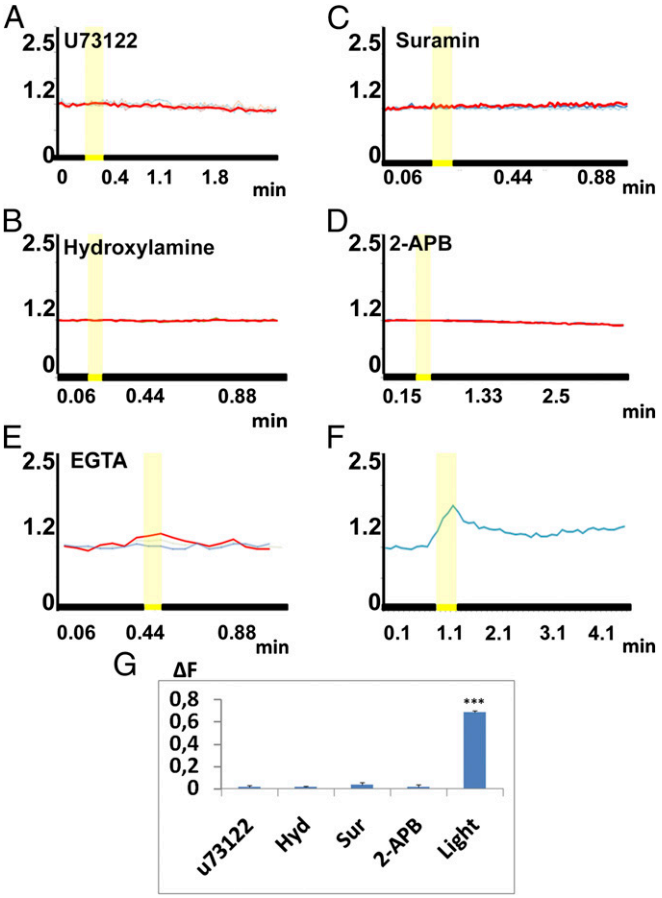


Fig. 5. Effect of retinal bleachers and nonvisual phototransduction cascade inhibitors on intracellular Ca^{2+} after light exposure in HC cultures. Significant light-induced changes (F/F_o) in intracellular Ca^{2+} levels visualized in HC cultures treated with the vehicle (control) (*F*) were drastically affected in cell cultures treated with the PLC inhibitor U73122 (5 μM) (*A*); the retinal bleacher hydroxylamine (Hyd, 30 mM) (*B*); the G-protein q inhibitor suramin (Sur, 100 μM) (*C*); the blocker of the IP_3-induced Ca^{2+} release 2-APB (100 μM) (*D*); or a chelating agent, 1 mM EGTA (*E*). (*G*) Graphical representation of relative fluorescent Ca^{2+} levels (ΔF) after a brief white light pulse of 1,000 lx for 5 s in cell cultures treated with the effectors compared with vehicle-treated controls. Data are mean ± SEM (n = 20 from three independent experiments); ***$P < 0.001$ by Student t test.

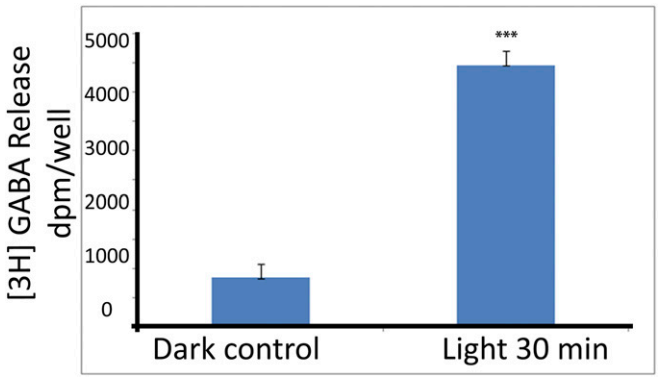


Fig. 6. ^{3}H-GABA release in HC cultures after 30 min of light exposure. HCs were incubated for 30 min at 37 °C with ^{3}H-GABA (0.5–1 mCi, specific activity 87.1 Ci/mmol) in the dark, the culture medium was washed out, and cells were exposed to bright light for 30 min or kept in dark. Graphical representation showing ^{3}H-GABA release in primary HC culture after 30 min of light (1,000 lx) and dark controls. Data are mean ± SEM (n = 40 from three independent experiments); ***P < 0.001 by Student t test.

and TRP channel activation (18, 39–41). The embryological origin of these cells within the retina and other molecular features strongly suggest that ipRGCs and HCs are likely to derive from a common ancient photoreceptor cell. The present paper provides evidence that Opn4x-expressing HCs kept in culture in the presence of retinaldehyde intrinsically respond to light with significant and sustained increases in intracellular Ca^{2+} from internal IP_3 stores but also in part from the extracellular medium. The cascade depends on G-protein q and PLC activation and ultimately leads to the release of intracellular GABA. Light-sensitive HCs can provide a plus-local regulation of visual photoreceptor function, further modifying neurotransmitter release and thus light intensity perception, because under this illumination condition HCs may increase negative feedback with a much stronger lateral inhibition of neighbor photoreceptors. One possible function of OPN4 is depolarization of the melanopsin-containing HCs themselves, which may inhibit the hyperpolarization responses of HCs triggered by light-dependent hyperpolarization of photoreceptor cells and thus regulate the negative feedback to visual photoreceptors. Accordingly, the OPN4-mediated depolarization of HCs by light may contribute to membrane potential regulation in the photoreceptor cells, modulating visual function. For its part, negative feedback reduces the dynamic range of the cone synapse by diminishing the maximal release rate of cones. By boosting neurotransmitter release from cones, positive feedback may recover the dynamic range lost in the negative feedback. Jackman and colleagues (25) propose that positive and negative feedbacks spread out differentially in the retina. Their results suggest that positive feedback acts locally; because there is no evidence of factors preventing positive feedback from causing runaway, this crucial role could be taken on by HCs expressing melanopsin. HCs cooperate in image-forming pathways with feedback signaling to cones and feed forward signaling to bipolar cells (42, 43). Here we provide evidence that HCs in culture release ^{3}H-GABA upon 30 min of light exposure. Several mechanisms for HC feedback to photoreceptors have been proposed. HCs contain GAD, the synthesizing GABA enzyme (44, 45), and exhibit a direct GABAergic synaptic connection to ON and OFF bipolar cells (46) and to visual photoreceptors (47), showing that vesicular GABA release from HCs is required for feedback inhibition of photoreceptors.

Despite basic similarities, cell number, layer length, photopigment localization, and physiological responses differ significantly in the inner retina of birds compared with that of primates; in fact, the avian retina is especially rich in HCs and amacrine cells and presents higher complexity of interconnections and intraretinal visual processing. The complex processing occurring in higher forebrain areas in primates is achieved at a lower level in birds, within the inner retina perhaps indicating one of the crucial differences between primate and avian visual systems.

Our findings demonstrate that HCs are retinal photoreceptors expressing a retinaldehyde-based Opn4x photopigment that renders cells intrinsically photosensitive. In nonmammalian vertebrates these retinal interneurons may have a dual function, somehow regulating nonvisual tasks such as those in which ipRGCs are involved or lateral interactions with visual photoreceptors such as typical HCs (Scheme 1). Our findings leave many open questions as to the way in which this intrinsic photosensitivity affects in retinal circuits or why these sensitive interneurons were evolutionarily preserved in some species. The findings provide evidence of intrinsically photosensitive retinal HCs in birds that significantly increases the complexity of retinal neural circuits for light perception and visual processing.

Materials and Methods

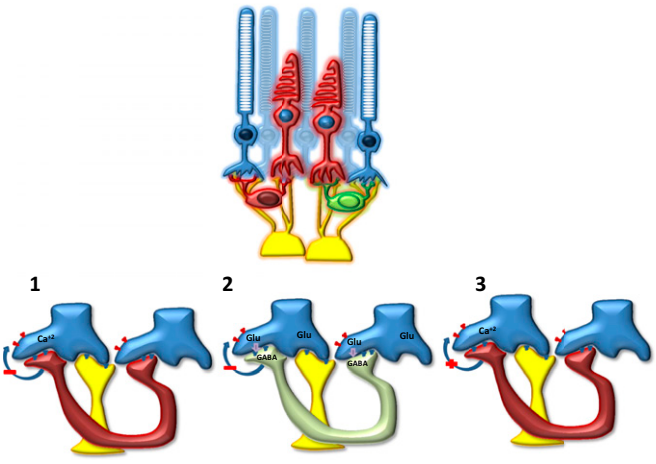
For complete information on materials and methods, see *SI Materials and Methods*.

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

Primary Cultures of Embryonic Horizontal Cells. HCs were purified from chicken neural retinas at E15 by a BSA discontinuous gradient of concentrations ranging from 1 to 4 % (wt/vol) as previously reported (48). Highly enriched HC cultures were characterized by specific HC markers as previously shown. See *SI Materials and Methods* for further information.

RNA Isolation and RT-PCR. Total RNA from HC primary cultures was extracted using the TRIzol kit for RNA isolation (Invitrogen) (7, 48). See *SI Materials and Methods* for further information.

An initial denaturation step of 1 min at 94 °C, 25 cycles of 60 s at 94 °C, 50 s at 60°–65 °C, 90 s at 72 °C, and a final 5-min elongation step at 72 °C.



Scheme 1. Schematic representation of three different scenarios within the retina involving the synapses between visual photoreceptors and HCs after light stimulation. (*Scenario 1*) Upon light stimulation, classic HCs project laterally and integrate inputs from visual photoreceptor cones and rods, providing critical negative feedback for establishing an antagonistic receptive field. (*Scenario 2*) Intrinsically photosensitive HCs depolarize upon light stimulation, releasing GABA on the ephaptic synapse with visual photoreceptors, causing further hyperpolarization and a decrease in glutamate release. (*Scenario 3*) HCs may also transmit a positive feedback signal to cone terminals, elevating intracellular Ca^{2+} and accelerating neurotransmitter release.

Amplification products (Table S1) were separated by 2% agarose gel electrophoresis and visualized by ethidium bromide staining.

Opn4x Knockdown. The shRNA sequence to suppress Opn4x (GAAGCTAACTGGAGTGATG) and a scrambled DNA target sequence (ACTCCATACTCGACACCTA) were created using the siRNA Wizard InvivoGen. cDNAs encoding shRNAs were inserted in a discistronic vector pSuper.neo+GFP (pSuperRNAi System-OligoEngine) under the control of the H1 RNAIII polymerase promoter. The transfection marker GFP was under the control of the PGK promoter. The resulting plasmids were referred to as Opn4x shRNA and ssRNA. The plasmids were mixed with Lipofectamine 2000 and added to the retinal cultures 12 h after plating.

Calcium Imaging by Fluo-4 AM Fluorescence Microscopy. Cells were grown in an eight-well Lab-Tek recording chamber (NuncTM) 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 (7) 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). Changes in 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 (Fo). ΔF was calculated as the difference between the maximal (peak) and minimal values of relative fluorescence in light responses for each assessment. See *SI Materials and Methods* for further information.

Retinoid Analysis. Primary cultures were homogenized in phosphate buffer containing 200 mM hydroxylamine and processed for retinoid analysis by HPLC as reported (7). See *SI Materials and Methods* for further information.

^{3}H-GABA Release. HCs were incubated for 30 min at 37 °C with ^{3}H-GABA (0.5–1 μCi) in 500 μL of buffer containing 140 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Hepes, and 10 mM glucose, adjusted to pH 7.4 with Tris base. The primary cultures were washed several times in fresh buffer to remove the excess ^{3}H-GABA and exposed for 30 min to a 1,000-lx light stimulus. Supernatant was recovered, and radioactivity in the medium was determined in a scintillation counter. Fractional release was calculated as the ratio of radioactivity released/total radioactivity taken up by the culture.

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