Neuropsin (OPN5)-mediated photoentrainment of local circadian oscillators in mammalian retina and cornea

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The molecular circadian clocks in the mammalian retina are locally synchronized by environmental light cycles independent of the suprachiasmatic nuclei (SCN) in the brain. Unexpectedly, this entrainment does not require rods, cones, or melanopsin (OPN4), possibly suggesting the involvement of another retinal photopigment. Here, we show that the ex vivo mouse retinal rhythm is most sensitive to short-wavelength light but that this photoentrainment requires neither the short-wavelength–sensitive cone pigment [S-pigment or cone opsin (OPN1SW)] nor encephalopsin (OPN3). However, retinas lacking neuropsin (OPN5) fail to photoentrain, even though other visual functions appear largely normal. Initial evidence suggests that OPN5 is expressed in select retinal ganglion cells. Remarkably, the mouse corneal circadian rhythm is also photoentrainable ex vivo, and this photoentrainment likewise requires OPN5. Our findings reveal a light-sensing function for mammalian OPN5, until now an orphan opsin.

OPN5 | photoentrainment | circadian rhythm | retina | cornea

Most mammalian tissues contain autonomous circadian clocks that are synchronized by the suprachiasmatic nuclei (SCN) in the brain (1). The SCN clock itself is entrained by external light cycles through retinal rods, cones, and melanopsin (OPN4)-expressing, intrinsically photosensitive retinal ganglion cells (ipRGCs) (2, 3). The retina also manifests a local circadian clock, which regulates many important functions, such as photoreceptor disk shedding, photoreceptor gap-junction coupling, and neurotransmitter release (4–6). Surprisingly, local retinal photoentrainment does not require the SCN, and it also does not require rods, cones, or ipRGCs (7, 8). Thus, the *rd1/rd1;Opn4*−/− mouse retina, which has lost essentially all rods and cones due to degeneration and also has an ablated *Opn4* gene (3), remains synchronized to light/dark cycles both in vivo and ex vivo (7).

To determine the photopigment(s) responsible for local circadian entrainment in the retina, we took a candidate gene approach. Because some cone nuclei may persist in degenerate *rd1/rd1* retinas (9), and murine short-wavelength–sensitive cone opsin (OPN1SW) has been reported to be present in the ganglion cell layer (10), we tested the necessity of this pigment for local retinal circadian photoentrainment. We also tested for the involvement of two orphan pigments, encephalopsin (OPN3) (11) and neuropsin (OPN5) (12), both of which are expressed in mammalian retina and, when expressed heterologously, form light-sensitive pigments that activate G proteins (13–17). The function of OPN3 in mammals is unknown despite its widespread expression in neural tissues (18). OPN5 appears to be a deep-brain photopigment in the hypothalamus of birds and is thought to contribute to seasonal reproduction (19–22); it has been immunolocalized to the mammalian inner retina (13, 16) (SI Text); however, to date, no retinal function for this mammalian pigment has been identified. We did not examine two other pigments, retinal G protein-coupled receptor (RGR)

opsin (23) and peropsin (RRH) (24). RGR opsin participates in retinoid turnover (25, 26), whereas RRH is expressed principally in the retinal pigment epithelium (24), a cell layer absent in the photoentrainable ex vivo retina preparation (7).

Results

Wavelength Dependence of ex Vivo Retinal Circadian Photoentrainment. We first examined the wavelength dependence of retinal circadian photoentrainment with *Per2::Luciferase* mouse retinas (27). The luciferase luminescence from these retinas comes predominantly from the inner retina (28, 29), although autonomous rhythms have also been found in the outer retina (28). Pairs of retinas from these mice were exposed ex vivo to 9-h/15-h light/dark cycles for 4 d, with the two retinas in each pair subjected to opposite-phase light/dark entrainment (7) (SI Materials and Methods). Immediately after cyclic light exposure, the luciferase luminescence in each retina was measured for 4 d in continuous darkness to determine the circadian phase. In previous work with white light (7), paired retinas under these conditions showed opposite phases, with peak luminescence occurring at roughly 4 h after the respective light-to-dark transition. To determine wavelength dependence, we replaced white light with monochromatic light-emitting–diode light at 370 nm, 417 nm, 475 nm, 530 nm, or 628 nm; peak photon flux at each of the last

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four wavelengths was identical (9×10^{13} photons cm$^{-2}\cdot$s^{-1}) but was 10-fold less (9×10^{12} photons cm$^{-2}\cdot$s^{-1}) at 370 nm to avoid tissue toxicity at higher UV intensities. After 4 d of entrainment, the paired retinas exposed to 370-nm light/dark cycles had stable, nearly opposite entrainment phases, as did retinas exposed to 417-nm light/dark cycles (Fig. 1*A*). In contrast, the paired retinas exposed to 475-nm light/dark cycles showed only a moderate, ~6-h difference in their phases. Paired retinas exposed to 530-nm or 628-nm light/dark cycles did not photoentrain, with each having phases identical to retinas kept in continuous darkness (Fig. 1*A*, rightmost vertical bar). Thus, circadian photoentrainment in ex vivo retinas is most sensitive to UV-A and violet light.

To confirm this spectral sensitivity, we tested retinas for acute phase shifts under different wavelengths. *Per2::Luciferase* retinas were cultured in continuous darkness. At different phases during their free-running circadian rhythms, we administered equal quanta of a 3-h light pulse at 417 nm or 475 nm and measured the resulting phase delay or phase advance in the rhythm to generate a phase-response curve. At all phases, 417-nm light triggered phase shifts that were consistently larger than those phase shifts triggered by equal quanta of 475-nm light (Fig. 1*B*), in agreement with the photoentrainment experiment.

OPN1SW Pigment Is Not Required for ex Vivo Retinal Photoentrainment.
Because photoentrainment was most sensitive to UV-A and violet light, OPN1SW [wavelength of maximal absorption (λ_{max}) = 360 nm] could be responsible. We therefore tested retinas from mice lacking OPN1SW (*Opn1sw*$^{-/-}$;*Per2::Luciferase*) (30). Retinas from these animals photoentrained to white light just as WT (i.e.,

Per2::Luciferase) did (Fig. 1*C*). Furthermore, in response to an acute violet light pulse (3 h, 417 nm) of various intensities administered at circadian time (CT) 9 or CT 20, *Opn1sw*$^{-/-}$;*Per2::Luciferase* retinas phase-shifted with sensitivity identical to the sensitivity of WT retinas (Fig. 1*D*). The quantitatively similar photosensitivities exhibited by WT and *Opn1sw*$^{-/-}$ retinas to violet light demonstrate that OPN1SW is not required for retinal photoentrainment ex vivo.

OPN5, but Not OPN3, Is Required for ex Vivo Retinal Photoentrainment.
Mouse OPN5 has a λ_{max} in the UV-A region (380 nm) when heterologously expressed (13, 16). For OPN3, heterologously expressed fish and insect homologs have a λ_{max} of 460 nm and 500 nm, respectively (14), but the λ_{max} of mammalian OPN3 is still unknown. We examined both OPN3 and OPN5 with the gene KO approach. We generated *Opn3*$^{-/-}$ and *Opn5*$^{-/-}$ ("*Opn5*$^{-/-}$ line 1") mice by homologous recombination (*SI Materials and Methods* and Fig. S1 *A* and *B*). We bred these lines into the *Per2::Luciferase* line to perform ex vivo photoentrainment experiments (retinas cultured for 4 d under 9-h/15-h white light/dark cycles). We found that the *Opn3*$^{-/-}$;*Per2::Luciferase* retinas photoentrained normally (despite a substantially weaker circadian luminescence amplitude), but *Opn5*$^{-/-}$;*Per2::Luciferase* retinas completely failed to entrain, maintaining phases identical to WT controls kept in continuous darkness (Fig. 2). We also generated an *Opn5*$^{flox/flox}$ mouse line (*SI Materials and Methods* and Fig. S1*C*), which allowed us to ablate OPN5 in all tissues ("*Opn5*$^{-/-}$ line 2") after crossing it with a *ROSA-FLP* line and an *EIIa-Cre* line. Retinas from transheterozygotes of the two *Opn5*$^{-/-}$ lines, in a *Per2::Luciferase* background, similarly
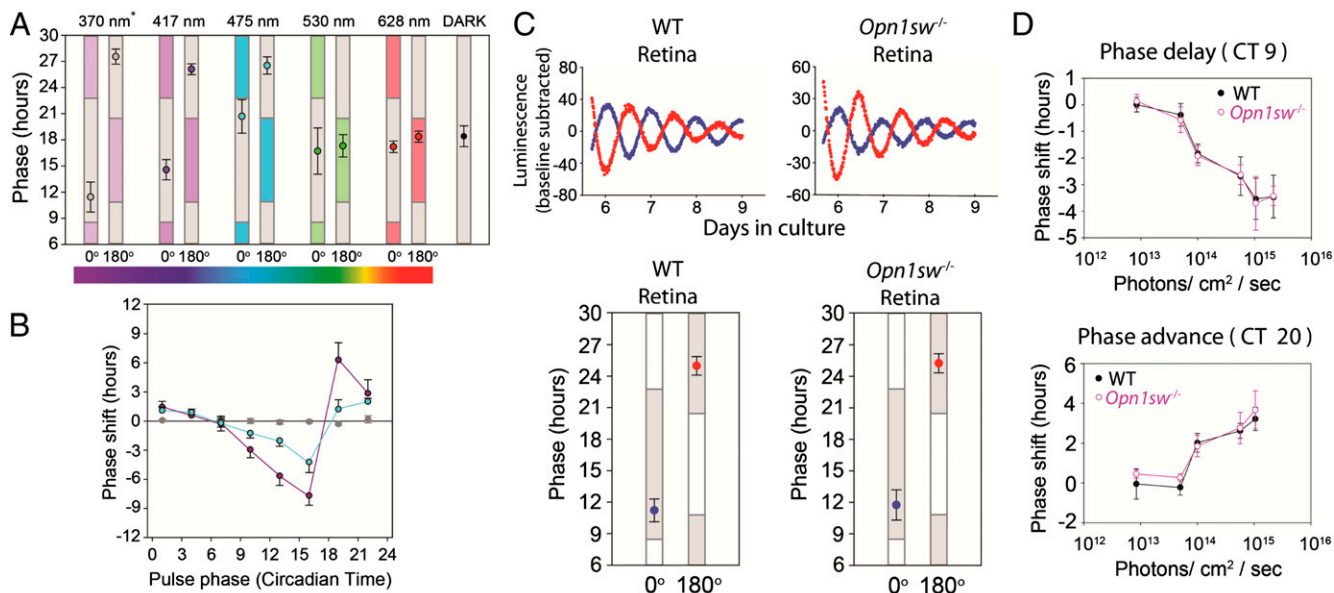

Fig. 1. Sensitivity of retinal circadian photoentrainment to short-wavelength light and lack of effect of the *Opn1sw*$^{-/-}$ genotype. (*A*) Wavelength dependence of ex vivo photoentrainment. Pairs of retinas from *Per2::Luciferase* mice were cultured in light/dark cycles (vertical colored bars) or in continuous darkness (full gray bar) for 4 d. Points represent mean phase (peak of bioluminescence) on the day following light/dark exposure ± 1 SEM for 370-nm and 417-nm light (n = 6 retinal pairs each; $P < 0.001$ for 0°/180° comparison, 0°/dark control comparison, and 180°/dark control comparison, one-way ANOVA, Tukey post hoc), 475-nm light (n = 7 pairs; P = 0.038 for 0°/180° comparison, P = 0.007 for 180°/dark comparison, and P > 0.5 for 0°/dark comparison), and 530-nm and 628-nm light (n = 5 pairs each; all comparisons not significant in post hoc analyses). *The 370-nm light was at 9×10^{12} photons cm$^{-2}\cdot$s^{-1}, but all other wavelengths were at 9×10^{13} photons cm$^{-2}\cdot$s^{-1}. (*B*) Phase-response relation of *Per2::Luciferase* retinal rhythms after exposure to a 3-h pulse (1.5×10^{15} photons cm$^{-2}\cdot$s^{-1}) of 417-nm light (purple) or 475-nm light (blue), together with dark-handling controls (gray). "Pulse phase" denotes the phase of the retinas at the time of the pulse. Points represent mean ± SEM and are connected by straight lines for a given wavelength ($n \geq 5$ for each point). (*C*, *Upper*) Dark-recorded bioluminescence traces (with background luminescence subtracted) from a pair of *WT;Per2::Luciferase* or *Opn1sw*$^{-/-}$;*Per2::Luciferase* retinas after 4 d of culturing at the 0° (blue) or 180° (red) position of the light/dark photoentrainment apparatus (white, 5 W·m^{-2}). (*C*, *Lower*) Time of peak bioluminescence of retinas as in *A*. Bars indicate periods of darkness (gray) and light exposure (white) in entrainment cycles. WT, n = 6 retinal pairs; *Opn1sw*$^{-/-}$, n = 7 retinal pairs. (*D*) Intensity-response relation of *WT;Per2::Luciferase* (black) and *Opn1sw*$^{-/-}$;*Per2::Luciferase* (red) retinal rhythms after exposure to a 3-h pulse of 417-nm light of various intensities at CT 9 and CT 20. Points represent mean ± SEM and are connected by straight lines for a given genotype ($n \geq 5$ for each point).
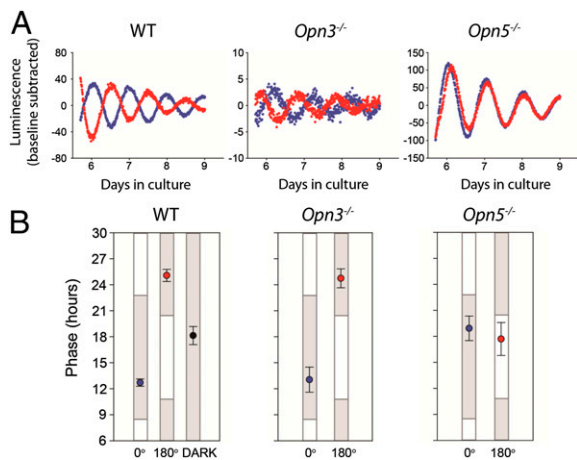
Fig. 2. Disruption of photoentrainment in $Opn5^{-/-}$, but not $Opn3^{-/-}$, retinas. (*A*) Dark-recorded bioluminescence traces (with background luminescence subtracted) from a pair of *WT;Per2::Luciferase*, *Opn3*$^{-/-}$*;Per2::Luciferase* or *Opn5*$^{-/-}$*;Per2::Luciferase* retinas after 4 d of culturing at the 0° (blue) or 180° (red) position of the light/dark photoentrainment apparatus. The WT data are from the same experiment as shown in Fig. 1*C*. (*B*) Time of peak bioluminescence of retinas (points, mean phase ± SEM) photoentrained at 0° (blue) and 180° (red) positions. Controls (DARK) are retinas maintained in continuous darkness. Bars indicate periods of darkness (gray) and light exposure (white; 5 W·m^{-2}) in entrainment cycles. WT, $n = 7$ retinal pairs; $Opn3^{-/-}$, $n = 6$ retinal pairs; $Opn5^{-/-}$ line 1 (main text), $n = 7$ retinal pairs.

failed to entrain to light/dark cycles. However, retinas from heterozygous $Opn5^{+/-}$;*Per2::Luciferase* littermates of the two $Opn5^{-/-}$ lines entrained normally (Fig. S2). Thus, both $Opn5^{-/-}$ lines appeared to behave identically. All experiments below (except for X-Gal labeling) were done only with $Opn5^{-/-}$ line 1.

To determine if the nonphotoentrainable phenotype arose secondarily from possibly widespread retinal dysfunction, we performed basic histological, electrophysiological, and visual function tests (*SI Materials and Methods*) on $Opn5^{-/-}$;*Per2::Luciferase* animals. Their retinas showed normal histology, with normal patterns of expression of rod/cone opsins and OPN4 (Fig. S3). $Opn5^{-/-}$ and WT mice showed similar electroretinogram responses to identical light flashes in both dark- and light-adapted states (Fig. 3*A*) (when assayed between 6 and 12 h after lights-on in a 12-h/12-h light/dark cycle; we did not perform measurements at other CTs). $Opn5^{-/-}$; *Per2::Luciferase* mice also had a normal optokinetic tracking reflex to a rotating grating of different spatial frequencies, unlike *rd1/rd1; Opn4*$^{-/-}$ mice, which showed a near-complete lack of visual tracking (Fig. 3*B*). Finally, the circadian rhythm of wheel-running activity of $Opn5^{-/-}$ mice showed entrainment to 12-h/12-h light/dark cycles, and reentrained to a phase advance in the external light/dark cycles, although more slowly than WT mice (Fig. 3*C*). When kept in constant darkness, $Opn5^{-/-}$ and WT mice had virtually identical free-running periods ($Opn5^{-/-}$: 23.89 ± 0.04 h, $n = 7$; WT: 23.91 ± 0.06 h, $n = 6$; $P = 0.762$, Student's t test). Thus, overall, no substantial retinal or circadian dysfunction was observed.

OPN5-Dependent ex Vivo Corneal Photoentrainment. The mammalian cornea has a strong circadian rhythm ex vivo (27). We also detected $Opn5$ transcripts by RT-PCR in both fresh and cultured WT corneas (Fig. S4). Therefore, we tested whether the corneal circadian luciferase rhythm is light-entrainable ex vivo and, indeed, found this to be the case (Fig. 4); the corneal luciferase rhythm was nearly opposite in phase to the luciferase rhythm of the retina (i.e., peaking at subjective dawn rather than subjective dusk). Furthermore, OPN5 is essential for this corneal photoentrainment because this function was abolished ex vivo (Fig. 4) in two different strains of OPN5-KO mice ($Opn5^{-/-}$;*Per2::Luciferase*

line 1 and transheterozygous $Opn5^{-/-}$;*Per2::Luciferase* mice obtained from crossing $Opn5^{-/-}$ lines 1 and 2; Fig. S2). By contrast, corneal photoentrainment persisted in mice lacking OPN1SW ($Opn1sw^{-/-}$; *Per2::Luciferase*) or OPN3 ($Opn3^{-/-}$;*Per2::Luciferase*) (Fig. 4). For comparison, we tested the pituitary gland (another strongly rhythmic tissue) for photoentrainment ex vivo. The circadian rhythm of cultured pituitary glands was not sensitive to light, and we did not detect $Opn5$ transcripts in this tissue (Fig. S5).

Identity of Cells Expressing OPN5. In the absence of any validated specific antibody against OPN5 (*SI Text*), we used $Opn5$-promoter activity to perform initial localization of $Opn5$ expression in the retina. The $Opn5^{-/-}$ line 1 mice carry a knock-in *tau-lacZ* fusion gene in the $Opn5$ gene locus (Fig. S1*B*), allowing us, in principle, to reveal $Opn5$-expressing cells by X-Gal staining. Puncta of X-Gal precipitate were found in about 4,800 cells in the retina, evenly spaced in the ganglion cell layer (Fig. 5*A*, *Right*). Colocalization experiments comparing X-Gal labeling and immunohistochemistry with a ganglion-cell marker, retinal binding protein with multiple splicing (RBPMS) (31, 32) (Fig. 5*B*), suggested that the X-Gal–positive neurons constitute a small subset of ganglion cells rather than displaced amacrine cells. The punctate X-Gal signal in $Opn5^{-/-}$ retinas was typically confined to the periphery of the labeled somata, unlike, for example, the diffuse X-Gal signal observed in the somata, proximal dendrites, and axons of ipRGCs in $Opn4^{-/-}$ retina (33). Such punctate X-Gal labeling has been reported (34), particularly in situations of low expression of β-gal coded for by *lacZ* (34), with confinement to subcellular organelles such as the endoplasmic reticulum (35); in our case, this pattern could be caused by weak $Opn5$-promoter activity. The $Opn5^{-/-}$ line 2 mice likewise carry a knock-in *lacZ* gene in the $Opn5$ gene locus (Fig. S1*C*). It gave a similar puncte-labeling pattern with X-Gal (Fig. 5*C*), totaling ~5,200 cells per retina, broadly similar to line 1. To reveal the $Opn5$-expressing neurons better, we tried staining $Opn5^{-/-}$ line 1 retinas with a fluorescent substrate of β-gal [5-chloromethylfluorescein di-β-D-galactopyranoside
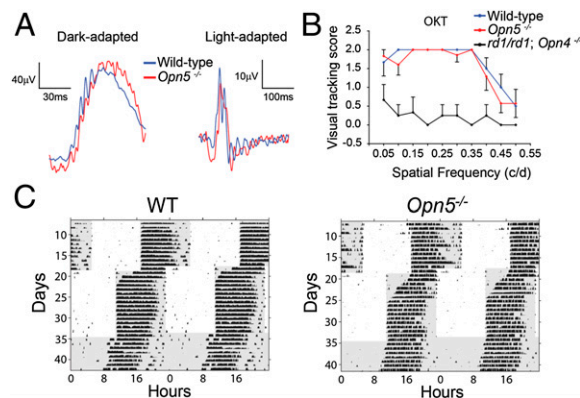

Fig. 3. Normal visual functions in $Opn5^{-/-}$ mice. (*A*) Dark- and light-adapted electroretinograms (ERGs) on anesthetized WT (blue) and $Opn5^{-/-}$ (red) mice. For dark-adapted ERGs, a flash intensity of 3 cd·m^{-2} was administered over a dark background. Light-adapted ERGs consisted of 900-cd·m^{-2} white light over a background of 340-cd·m^{-2} white light. Each trace is the average of ~150 trials. (*B*) Optokinetic tracking responses of WT (blue), $Opn5^{-/-}$ (red), and *rd1/ rd1;Opn4*$^{-/-}$ (black) mice to rotating gratings with different spatial frequencies (0.05–0.5 cycles per degree). Scores were given based on the head movements of the mice, with 0 = no visual tracking, 1 = ambiguous tracking, and 2 = obvious visual tracking. WT, $n = 6$; $Opn5^{-/-}$, $n = 7$; rd1/rd1;Opn4$^{-/-}$, $n = 4$. (*C*) Representative actograms of individual WT and $Opn5^{-/-}$ mice. Actograms are double-plotted so that each horizontal trace represents two consecutive days, with the second day replotted as the first day on the trace beneath it. Periods of darkness are indicated by shaded areas. Black marks represent the number of wheel revolutions in 5-min bins. All $Opn5^{-/-}$ animals were from line 1.
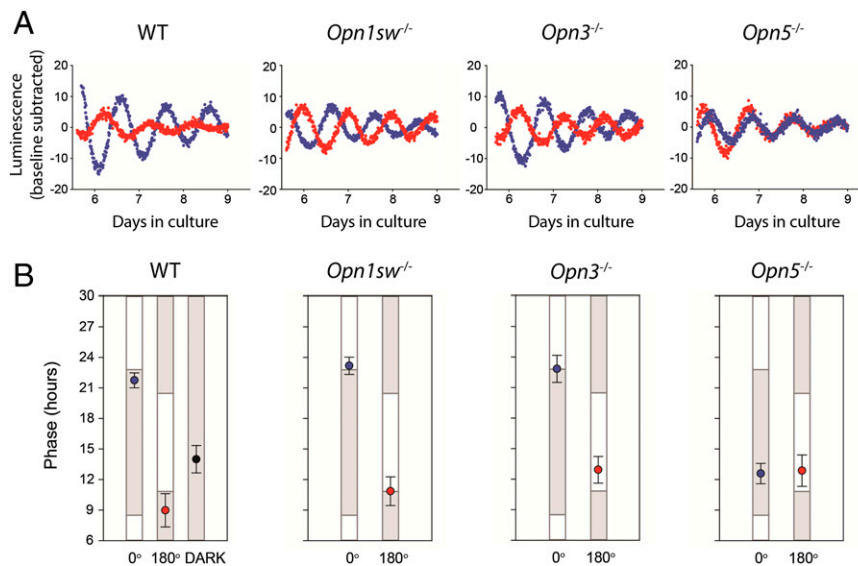
Fig. 4. Loss of photoentrainment in *Opn5⁻/⁻* cornea. (*A*) Dark-recorded bioluminescence traces from a pair of WT, *Opn1sw⁻/⁻*, *Opn3⁻/⁻*, or *Opn5⁻/⁻* line 1 corneas (all in a *Per2::Luciferase* background) after 4 d of culturing at the 0° (blue) or 180° (red) position of the light/dark photoentrainment apparatus. (*B*) Time of peak bioluminescence of corneas (points, mean phase ± SEM) photoentrained at 0° (blue) and 180° (red) positions. Controls (DARK) are corneas maintained in continuous darkness. Bars indicate periods of darkness (gray) and light exposure (white, 5 W·m⁻²) in entrainment cycles. WT, n = 7 corneal pairs; *Opn1sw⁻/⁻*, n = 6 corneal pairs; *Opn3⁻/⁻*, n = 5 corneal pairs; *Opn5⁻/⁻* line 1, n = 6 corneal pairs.

(CMFDG)] and then injecting Alexa Fluor 555/488 hydrazides into a small number of the labeled neurons. Unfortunately, the CMFDG labeling appeared to be cytotoxic, causing membrane leakage and rapid dissipation of the injected dyes (*SI Materials and Methods*). Only four of 20 injected cells successfully retained enough dye to show discernable cell bodies and primary dendrites, with three cells showing a well-defined axon heading toward the optic disk, confirming them as retinal ganglion cells (Fig. S6). We also tried several commercial antibodies against the β-gal coded for by *lacZ* but failed to detect any labeling in the retina. Finally, we could not observe any X-Gal labeling or anti–β-gal immunolabeling in the cornea, possibly also due to its very low *Opn5*-promoter activity [a situation not unlike the absence of X-Gal labeling in non–M1-subtype ipRGCs in an *Opn4-tau-lacZ* knock-in mouse line, which also have low *Opn4*-promoter activity (2, 33, 36–38)].

To confirm the necessity of retinal ganglion cells for local retinal circadian photoentrainment, we performed ex vivo photoentrainment experiments on *Math5⁻/⁻* retinas, in which >80% of ganglion cells are absent developmentally (39). We found that the intrinsic rhythmicity of these retinas was intact and of normal amplitude, but their photoentrainment had largely disappeared (Fig. 5*D*). *Opn5* transcript expression was also greatly reduced in these retinas (Fig. 5*E*). Taking all results together, we tentatively conclude that OPN5 is expressed primarily in a small subset of *Math5*-dependent retinal ganglion cells.

Discussion

We show in this work that OPN5 is required for the photoentrainment of the murine retinal and corneal circadian clocks ex vivo. The concordance between the wavelength dependence of the retinal entrainment and the absorption spectrum of OPN5 (13, 16) lends further support to the notion that OPN5 functions as a photopigment for these clocks. As such, we have identified a function for mammalian OPN5, previously an orphan opsin. It remains to be confirmed whether OPN5 is also required for photoentrainment of the retinal rhythm in vivo. Mouse cornea and lens, unlike their human counterparts, transmit UV-A (40), a property exploited by the abundant blue-cone pigment, OPN1SW,

in the mouse retina, and presumably also by OPN5. Even in humans, there is likely enough transmitted blue light in full sunlight for OPN5 to signal, because OPN5's sensitivity at the human blue cone pigment's λ_{max} of 430 nm is only one log-unit lower than at its own λ_{max} of 380 nm [based on heterologous expression (13, 16)]. The *cis*-retinal required by OPN5 as a chromophore (13, 16) could potentially be accessed in the inner retina in the same way as by ipRGCs, possibly from the Müller glial cells (41). For corneal OPN5, interphotoreceptor retinoid-binding protein in the aqueous and vitreous humors (42) could potentially transport *cis*-retinal via diffusion from the retina. Because OPN5 appears to be bistable (13, 16), similar to bistable/tristable OPN4 (43–45), this property may explain its sustained photosensitivity under extended culture conditions ex vivo.

It is remarkable that the four mouse retinal pigments (rhodopsin, S/M-cone opsins, and OPN4) already known, which cooperatively photoentrain the SCN, nonetheless appear not to entrain the mouse retinal circadian clock. Instead, the latter function is left to OPN5. Conversely, so far, OPN5 does not appear to have an essential role in SCN photoentrainment (2, 3), although a more subtle role by this pigment still cannot be ruled out, as implicated by the slower reentrainment of *Opn5⁻/⁻* mice to a phase advance in the experiment illustrated in Fig. 3*C*. The division of labor between OPN5 and the other retinal pigments may suggest a selective advantage in maintaining segregation of the light-signaling pathway for local retinal photoentrainment and the light-signaling pathway for SCN photoentrainment, for reasons presently unknown. Given that OPN5 appears to be expressed in a subset of ganglion cells (albeit of unknown subtype) and that OPN5 and S-cone opsin (at least in the mouse) both have a λ_{max} in the UV-A spectral window, it is not immediately clear how OPN5-expressing cells manage to avoid commingling photosignals from their intrinsic OPN5 pathway and those photosignals from the synaptically conveyed rhodopsin-cone pigments/OPN4 pathway. One solution to this problem would be that the OPN5-expressing cells, via unique circuitry, are functionally isolated from the mainstream light signaling in the retinal network. Another possibility would be that the two light pathways use distinct signal codes, such as electrical activities with different
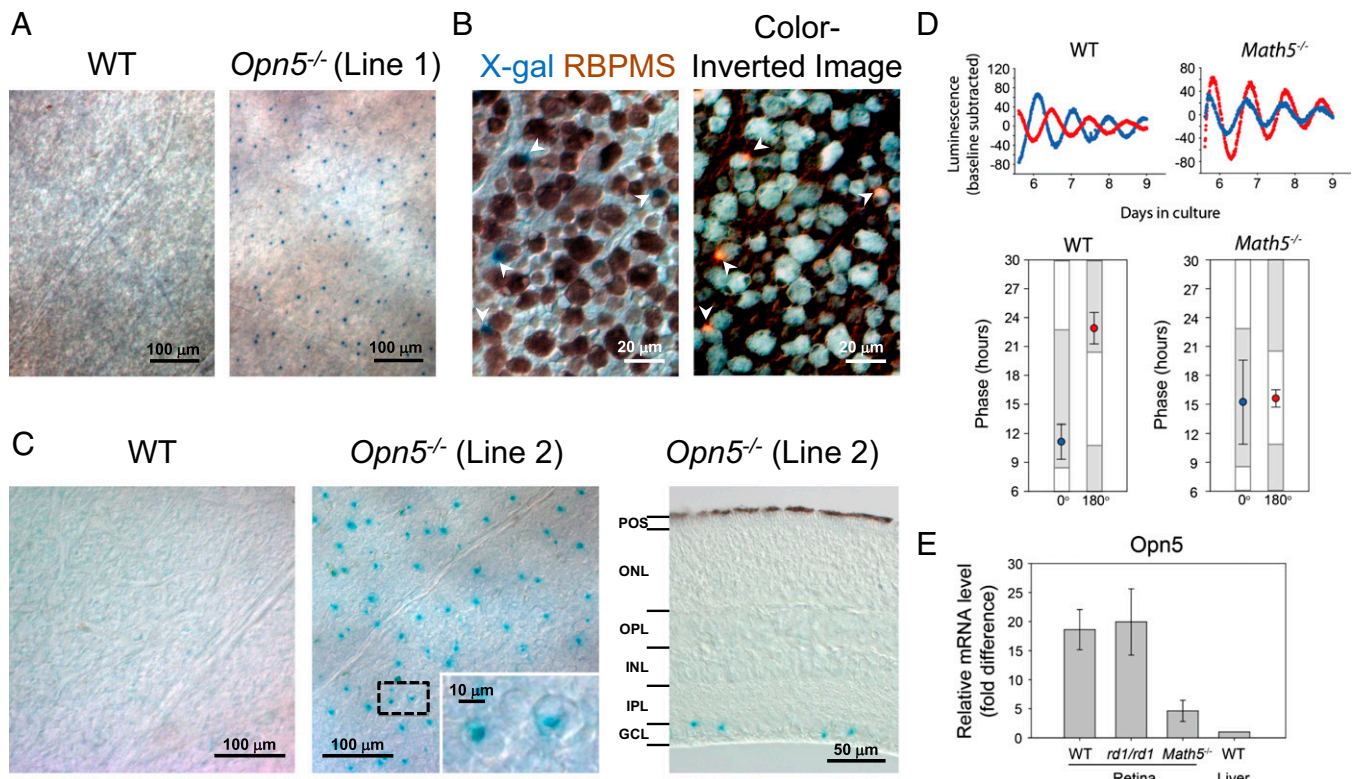
Fig. 5. Apparent *Opn5* expression in retinal ganglion cells. (*A*) X-Gal staining in flat-mount adult WT and *Opn5⁻/⁻* line 1 retinas. Blue puncta in *Opn5⁻/⁻* retinas are X-Gal precipitate. (*B, Left*) Colocalization of X-Gal (blue puncta) and immunosignal from an antibody against retinal binding protein with multiple splicing (RBPMS; brown), a retinal ganglion cell marker, in a small subset of cells in a flat-mount *Opn5⁻/⁻* retina. White arrowheads show examples of colocalization. (*B, Right*) Color-inverted and contrast-adjusted version of the image on the left for distinguishing the X-Gal labeling and immunosignal more easily. (*C, Left* and *Middle*) X-Gal staining of postnatal day 8 (P8) WT and *Opn5⁻/⁻* line 2 retinas at about one-third of the distance from the optic nerve head to the retinal periphery. (*Inset*) Two positive cells enlarged. (*C, Right*) Ten-micrometer cryosection of an X-Gal–stained P10 *Opn5⁻/⁻* line 2 retina, showing positive cells situated in ganglion cell layer (GCL). X-Gal staining was not detected in the inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), outer nuclear layer (ONL), or photoreceptor outer segments (POS). (*D*) Lack of photoentrainment in ex vivo *Math5⁻/⁻* retinas. (*Top*) Dark-recorded bioluminescence traces from a pair of *Per2::Luciferase* retinas (*Left*) or *Math5⁻/⁻;Per2::Luciferase* (*Right*) retinas after 4 d of culturing at the 0° (blue) or 180° (red) position of the light/dark photoentrainment apparatus. (*Bottom*) Time of peak bioluminescence of *Per2::Luciferase* or *Math5⁻/⁻;Per2::Luciferase* retinas (points, mean phase ± SEM) photoentrained at 0° (blue) and 180° (red) positions (*n* = 4 each). (*E*) Relative levels of *Opn5* transcript as assayed by quantitative RT-PCR on total retinal or liver RNA. *Opn5* transcript levels from retinas of *Per2::Luciferase*, *rd1/rd1;Per2::Luciferase*, or *Math5⁻/⁻;Per2::Luciferase* mice were normalized to the *Gapdh* levels of each respective sample and then compared with the normalized *Opn5* transcript level in *Per2::Luciferase* liver using the delta-delta threshold cycle ($2^{-\Delta\Delta Ct}$) method (*SI Materials and Methods*).

temporal properties that can be distinguished from each other by their respective decoders. Alternatively, the nature of the light signal downstream of OPN5 for local retinal photoentrainment may be entirely different from the conventional electrical signals (i.e., action potentials) sent to the SCN for photoentraining these nuclei. Thus, for example, OPN5-triggered signal transduction could lead to an electrically silent biochemical reaction rather than a change in membrane potential. If so, this characteristic may also imply that even though OPN5 is present in retinal ganglion cells, its signaling will not necessarily be transmitted to the brain. In contrast, ipRGCs make use of intrinsic OPN4-generated signals and also synaptically driven rod/cone signals (both electrical in nature), either synergistically or individually, for phase-shifting the SCN clock in the brain (2, 3).

The identity of the rhythmic cells in the retina remains unclear. In our experiments, *Math5⁻/⁻* retinas showed a luciferase rhythm of largely unattenuated amplitude despite the loss of most *Opn5* transcripts and the loss of photoentrainment (Fig. 5*C*). The simplest interpretation of these findings is that the majority of the rhythmic cells in the retina do not express OPN5, although their exact locations are still unclear (28, 29). If OPN5 cells are indeed distinct from the rhythmic cells, the important question arises of how light signals from OPN5 are transmitted to these rhythmic cells, especially if the OPN5-generated signal is

nonelectrical downstream. Separately, it appears that the ex vivo retinal rhythm has a lower amplitude in the *Opn3⁻/⁻* genotype than in WT (Fig. 2*A*), raising the possibility that although OPN3 is not necessary for local photoentrainment of most inner retinal cells, it nonetheless may have a role in influencing the intrinsic retinal rhythmic activity, perhaps by synchronizing the rhythmic cells with a mechanism that is still unknown. For example, we cannot rule out the possibility that OPN3 is required for the photoentrainment of rhythmic outer retinal neurons, which contribute a minority of the overall *Per2::Luciferase* signal in the retina (28, 29).

The photoentrainment of the cornea ex vivo provides the first evidence, to our knowledge, for photosensory function in the mammalian cornea. Although insects, reptiles, amphibians, and birds have long been known to use extraretinal photoreceptors, this corneal photosensitivity via OPN5 is a rare example of opsin-dependent, extraretinal photoreception in mammals [with another being the OPN4-mediated intrinsic photosensitivity of some mammalian irises (46)]. The fact that photoentrainment of the cornea likewise requires OPN5 instead of the other known retinal pigments may suggest an overall concerted ocular (i.e., not just retinal) photoentrainment that is segregated from SCN photoentrainment. We currently know nothing about the OPN5-expressing cell type or the nature of OPN5 signaling in the cornea, although the latter

may, in principle, be similar to OPN5 signaling in the retina. The identity of the corneal rhythmic cells is likewise unknown.

OPN4 was initially identified by virtue of its role in entrainment of the SCN clock, but it is now known to be involved in a broad range of functions, including pupillary light reflex, SCN photoentrainment, visual irradiance coding, retinal vascular development, and negative phototaxis during mammalian development (reviewed in refs. 36–38). By the same token, OPN5 may have other functions besides circadian photoentrainment of the retina and cornea.

Materials and Methods

Opn3$^{-/-}$ and *Opn5*$^{-/-}$ mouse lines were generated by homologous recombination. Photoentrainment assays were carried out by culturing pairs of tissues in specified medium under a 9-h/15-h light/dark cycle at antiphase for 4 d and then recording the rhythms of luciferase bioluminescence in darkness. Light-induced phase shifts were determined from cultured retinas by measuring the bioluminescence rhythms before and after exposure of the retinas to a 3-h light pulse at selected phases of their rhythms. X-Gal labeling, immunohistochemistry, and quantitative RT-PCR were done with standard protocols. Detailed methods are described in *SI Materials and Methods*.

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