Wounding Induces Facultative Opn5-Dependent Circadian Photoreception in the Murine Cornea

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Purpose. Autonomous molecular circadian clocks are present in the majority of mammalian tissues. These clocks are synchronized to phases appropriate for their physiologic role by internal systemic cues, external environmental cues, or both. The circadian clocks of the in vivo mouse cornea synchronize to the phase of the brain's master clock primarily through systemic cues, but ex vivo corneal clocks entrain to environmental light cycles. We evaluated the underlying mechanisms of this difference.

Methods. Molecular circadian clocks of mouse corneas were evaluated in vivo and ex vivo for response to environmental light. The presence of opsins and effect of genetic deletion of opsins were evaluated for influence on circadian photoresponses. Opn5-expressing cells were identified using Opn5Cre;Ai14 mice and RT-PCR, and they were characterized using immunocytochemistry.

Results. Molecular circadian clocks of the cornea remain in phase with behavioral circadian locomotor rhythms in vivo but are photoentrainable in tissue culture. After full-thickness incision or epithelial debridement, expression of the opsin photopigment Opn5 is induced in the cornea in a subset of preexisting epithelial cells adjacent to the wound site. This induction coincides with conferral of direct, short-wavelength light sensitivity to the circadian clocks throughout the cornea.

Conclusions. Corneal circadian rhythms become photosensitive after wounding. Opn5 gene function (but not Opn3 or Opn4 function) is necessary for induced photosensitivity. These results demonstrate that opsin-dependent direct light sensitivity can be facultatively induced in the murine cornea.

Keywords: opn5, cornea, neuropsin, circadian rhythm, corneal wound

Most tissues in mammals exhibit autonomous molecular circadian clocks that adopt specific phases within the entrained animal, typically by responding to timing cues from the brain's master clock, the suprachiasmatic nuclei (SCN).1 The phase of the SCN is synchronized to the 24-hour solar cycle through light information relayed by Opn1-expressing retinal ganglion cells.2 Loss of the SCN causes behavioral sleep-wake and hormone rhythms to become arrhythmic.3,4 While peripheral clocks may remain oscillatory in SCN-lesioned animals, the phases of these clocks scatter within the same organism.5 The full mechanisms by which the SCN maintains synchrony of specific peripheral clocks is an area of active inquiry, but it appears that most individual tissue clocks synchronize their rhythms to the SCN using some combination of systemic circulating factors (particularly glucocorticoids6), neural input (particularly through the autonomic nervous system7), or oscillating physiologic parameters (such as body temperature8,9).

Two tissues—retina and skin—appear to be exceptions to this mechanism. Circadian clocks within the retina and exposed skin of mice are directly photoentrained (synchronized by photons) to environmental light cycles and can do so regardless of the locomotor activity phase of the animal10–12. Both of these phenomena require the function of the opsin protein OPN5 (neuropsin).11,12 Interestingly, the retina uses a combination of classical (rhodopsin) and novel (Opn5) opsins for local clock entrainment.11,13 In mammals, OPN5 is one of the “noncanonical” opsin photoreceptors, along with OPN3 and OPN4, which mediate the effects of light outside of classical visual pathways.2 OPN5 is maximally sensitive to short-wavelength light (380 nm, λmax)14,15 and in mice contributes to circadian photoentrainment of individual tissues.13 photoentrainment of locomotor rhythms,16 and development of retinal vasculature.17 In birds, OPN5 functions as a photoreceptor in both the retina and hypothalamic areas.18–20
The murine cornea expresses robust circadian rhythms of clock gene expression in the majority of cells throughout the epithelium and endothelium both in vivo and ex vivo. Synchronization of corneal phase to the light-dark cycle appears to be influenced by both melatonin and glucocorticoids, suggesting circadian systemic cues set the phase of this tissue from the master clock. However, murine cornea has also been shown to express OPN4 and OPN5. OPN4 expression in the cornea appears to occur in neural tissue and contribute to light-induced photosensitivity (allogynia). Murine cornea has also been shown to be directly phototransmissible ex vivo via an Opn5-dependent mechanism. The current study was designed to determine whether murine cornea primarily uses systemic humoral or local photic mechanisms for its entrainment and synchronization to the light-dark cycle. We also wished to assess the contributions of the noncanonical opsin in corneal photoreception.

**METHODS**

**Animals**

All animal experiments were carried out according to Institutional Animal Care and Use Committee guidelines at University of Washington, Seattle, WA, and adhere to the ARVO statement for the Use of Animals in Ophthalmic and Vision Research. Opn5Cre;Ai14 was created as described in Nguyen et al. Offspring were analyzed in this study. Opn3Cre;Ai14 mice were described in Panda et al. and Pde6brd1/rd1 mice were described in Panda et al. for the use of melatonin in the cornea and melanopsin function and behaviorally free-run rather than entrain to light-dark cycles. By maintaining these animals in light-dark 12:12 (LD 12:12) cycles, desynchrony between the fellow corneas using Lumicycle Analysis software will occur in all animals. By assessing the phase of the circadian clock in specific tissues via measurement of clock-gene expression, it can be determined whether a given tissue remains synchronized to the behavioral (SCN) clock or directly to the light-dark cycle.

**Animal Behavior and Behavioral Desynchrony**

OPN4+/−; Pde6b−/− mice lack functional rods, cones, and melanopsin function and behaviorally free-run rather than entrain to light-dark cycles. By maintaining these animals in light-dark 12:12 (LD 12:12) cycles, desynchrony between the external light-dark cycle and the SCN-driven phase of behavior will occur in all animals. By assessing the phase of the circadian clock in specific tissues via measurement of clock-gene expression, it can be determined whether a given tissue remains synchronized to the behavioral (SCN) clock or directly to the light-dark cycle. OPN4+/−; Pde6b−/− mice were singly housed in cages that contained running wheels, and wheel revolutions were monitored by a microswitch connected to a computer. Behavioral traces were collected and analyzed using ClockLab software (Actimetrics, Evanston, IL, USA). Behavioral onsets were monitored in free-running mice using a fit-line to the average onsets over at least 7 days. After at least 21 days in a 12-hour light/12-hour dark cycle, behavior was observed for days at which mice were beginning behavioral onset coincident with lights-on or (in a separate group of mice) lights-off. On these days, mice were euthanized with CO2 asphyxiation and their eyes were quickly removed and transferred to ice-cold HBSS (Gibco, USA). In dim white light, corneas were removed using two scalpels and cultured on cell culture inserts (PICMOR; Millipore, USA). They were incubated in sealed petri dishes in DMEM (Cellgro; USA) containing B-27 serum-free supplement (Life Technologies, USA) 0.1 mM D-Luciferin potassium salt (Biosynth; USA), 352.5 μg/mL sodium bicarbonate, 10 mM HEPES, and 25 U/mL penicillin, 25 μg streptomycin (Life Technologies). Dishes were cultured at 36°C in a Lumicycle photomultiplier tube luminometer machine (Actimetrics, USA) contained within an air-jacketed incubator. Because the phase of cornea cultures is influenced by the time of dissection, all experiments here used sealed cultures throughout the experiment, and the phase of the fellow cultured cornea was used as a phase control for treatment groups. For light pulses, one cornea culture was transferred in an insulated shuttle box to another incubator equipped with an LED array for 90 minutes and exposed to light at the indicated wavelength/intensity. For each light pulsed cornea, the fellow cornea was transferred to the LED incubator at the same time in a separate shuttle box covered with aluminum foil. Phase shifts were calculated as the phase difference between the fellow corneas using Lumicycle Analysis software (Actimetrics). For ex vivo photoentrainment, cornea pairs (right and left eye) from individual mice were maintained as sealed organotypic tissue cultures and exposed to oppositely phased LD 9:15 light-dark cycles (2 × 1014 photons cm−2 s−1, 415 nm) for 4 days after dissection (this light cycle has previously been shown to be optimal for demonstrating ex vivo entrainment of retina, cornea, and skin). The dishes were then transferred to constant darkness, and the phases of the Per2luciferase luminescence rhythms were measured using Lumicycle Analysis software (Actimetrics).

Relaxing incisions were made for most experiments so that the cornea would lie flat on the cell culture insert. Images were made of tdTomato fluorescence from Opn5Cre;Ai14 corneas using a fluorescence microscope. For time course experiments, an individual Opn5Cre;Ai14 cornea was maintained at 36°C on the microscope stage using a stage top incubator (Bioscience Tools, USA). For algerbrush experiments, an algerbrush with a 0.5-mm burr (Strong Vision Technology, Jackson, MI, USA) was used to deepen epithelialize a region of the cornea of a euthanized mouse.
prior to the eye being removed. For these corneas, small relaxation incisions were made away from the deep epithelialization region.

**Immunofluorescence**

Whole corneas were fixed in 4% Paraformaldehyde (PFA)/PBS at room temperature (RT) for 15 minutes followed by permeabilization with Triton X-100 1% for 30 minutes. After this, corneas were incubated for 1 hour in blocking buffer (2.5% normal goat serum, 2.5% normal donkey serum, 2% gelatin, 1% BSA, 0.2% Triton X-100). Corneas were incubated in different primary antibodies (Table 2) overnight at 4°C. After incubation, samples were rinsed in PBS, incubated with secondary antibody goat anti-rabbit–Alexa 633 (Invitrogen, USA) for 1 hour at RT, and rinsed in PBS, incubated with secondary antibody goat anti-rabbit–Alexa 633 (Invitrogen, USA) for 1 hour at RT, and mounted with ProLong Gold Antifade Mountant with 4′,6-diamidino-2-phenylindole (DAPI) (Invitrogen).

For EdU incorporation, corneas were incubated as described above. EdU was added to the media for a relaxation incisions were made away from the deep epithelialization region.

![Image](https://via.placeholder.com/150)

### Table 1. Primers used in RT-PCR

| Transcript (Amplon Size) | Forward | Reverse |
|--------------------------|---------|---------|
| Opn5 (218 bp)            | 5′-AGCTTTTGGAGGCGACAG-3′ | 5′-CGACACACGAGAAGACTTC-3′ |
| Opn4 (180 bp)            | 5′-TCTGGATCCCGACGATC-3′ | 5′-TGAACTGTGCTGTTGCT-3′ |
| Opn3 (227 bp)            | 5′-CTGGCTGAATCGACCTTC-3′ | 5′-GTATGCTAAGATGACCTGTT-3′ |
| Hprt1 (142 bp)           | 5′-TCAGTCAAGGAGGACATAAA-3′ | 5′-GGGTCTACTGCTTAAACAG-3′ |
| Gapdh (75 bp)            | 5′-CAAGGAGTAAGAAGCCCTGAC-3′ | 5′-CAGATGGGATAGGCGCTC-3′ |
| β-actin (188 bp)         | 5′-AGGTGACAGCATGTCTCCTG-3′ | 5′-GCTGCCCTCAACACCTTAC-3′ |
| Per2 (216 bp)            | 5′-CCAACAGACGACGACAGC-3′ | 5′-TCTCAGCTAAACACAGATC-3′ |
| Per1 (91 bp)             | 5′-CCACGCTTATACCGAGAAG-3′ | 5′-ATGGTCAAGGAAGAAGCCT-3′ |
| Bmal1 (200 bp)           | 5′-GACATTCATCTCAACATCAGCG-3′ | 5′-GATCCCTGATCCTCCTTTG-3′ |
| Dbp (118 bp)             | 5′-CGAAGACGTCATGATGCAG-3′ | 5′-GTTCCCCAAATGCTAAGA-3′ |

### Table 2. Antibodies used in Immunofluorescence

| Antibodies | Source | Concentration | Catalog number |
|------------|--------|---------------|----------------|
| Cytokeratin 12 (KRT12) | Proteintech | 1:200 | 24789-1-AP |
| Cytokeratin 14 | Proteintech | 1:100 | 10143-1-AP |
| Pax6 | Proteintech | 1:200 | 12532-1-AP |
| Cytokeratin 15 | Proteintech | 1:200 | 10137-1-AP |
| Anti-mouse TCR γ/δ antibody (GL3) | Biolegend | 1:500 | 118101 |
| CD11c monoclonal antibody (N418) | invitrogen | 1:200 | 14-0114-81 |
| CD11b monoclonal antibody (M1/70) | invitrogen | 1:200 | 14-0112-82 |
| APC anti-mouse CD45 (30-F11) | Biolegend | 1:200 | 105111 |
| Keratocan anti-mouse | gift | 1:200 | 52 |

of clock gene transcript abundance in corneas of behaviorally desynchronized mice (see Methods). We have previously used this experimental design to demonstrate direct phototainment of murine retina11 and skin12 clocks to light-dark cues, while other tissues such as pituitary and liver remain tied to behavioral phase. *Opn4−/−; Pde6b−/−/rd1/rd1* mice were exposed to 12-hour light/12-hour dark cycles, and the onset of their running wheel activity was used as a measure of the phase of their internal, SCN-driven phase (Fig. 1A). After at least 3 weeks, we monitored their activity for times at which one cohort of mice began activity at the dark-to-light transition and another began activity at the light-to-dark transition (as has been described previously12). On the day at which the behavioral onsets of the two groups of mice were at opposite phases relative to the light cycle, tissues were collected at four time points across the day. We analyzed the transcript abundance of the clock genes *Per2, Per1, Bmal1, and Dbp* relative to *β-actin* using RT-PCR from the corneas of each cohort. Unlike previous results from retina and skin,12,31 analyses of the phases in the rhythmic expression of clock genes in the cornea using linear harmonic regression sine waves (fit using CircWave 1.433) revealed an alignment with behavioral phase, not the light-dark cycle (Fig. 1B). This indicates that the circadian clocks of the healthy cornea are not directly synchronized by light in vivo.

However, these data are not consistent with our earlier observation that the *Per2Luciferase* rhythms of wild-type corneas can be photoentrained ex vivo,11 and it is possible that the mutation of *Opn4* or *Pde6b* could impair the cornea’s photosensitivity. We next tested ex vivo photoentrainment for 4 days (see Methods) in corneas of wild-type; *Per2Luciferase* or *Opn4−/−; Pde6b−/−/rd1/rd1*; *Per2Luciferase* mice (Fig. 1C). Upon transfer to constant darkness, the phases of the *Per2Luciferase* luminescence rhythms of cornea pairs from the same animals displayed opposite phases reflecting the phase of the previous light-dark cycle (Fig. 1C), confirming ex vivo photoentrainment of corneal tissue and demon-
FIGURE 1. Cornea circadian clocks photoentrain ex vivo but not in vivo. (A) Wheel-running activity of Opn4−/−; Pde6bΔ1/Δ1; Per2Luciferase mice in light (white)/dark (gray) cycles. Tissue was collected when the behavioral onset coincided with either lights-off (blue frame) or lights-on (red frame). (B) RT-PCR of the indicated transcripts using the ΔΔCt method relative to β-actin transcript. Open circles represent values from individual corneas from either in-phase/lights-off (blue) or out-of-phase/lights-on (red) mice. n = 5 for each time point, for
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Curves represent fit sine waves using CircWav software. The solid points with error bars represent the center of gravity ± SEM and the P value of the fit rhythm compared to the horizontal average of the data. (C) Per2Luciferase bioluminescence rhythms after 4 days of oppositely phased light-dark cycles. Red and blue traces represent cultures of individual corneas from the same mouse from either a wild-type; Per2Luciferase (left); Pde6b+/rd1, Per2Luciferase (right).

FIGURE 2. Photic responses of circadian clocks in cultured corneas emerge over time in culture. (A) Per2Luciferase rhythms from corneas receiving a 90-minute 415-nm light pulse (blue) or a dark pulse (gray) at the time indicated by a red arrow. (B) Phase delays of light-treated corneas compared to dark-handled controls of the same animals. Bars represent mean ± SEM. P < 0.05 in ANOVA with Tukey post hoc analysis where noted. n = 8 for each group. (C) Phase response curve of cultured corneas receiving a 90-minute 2 × 10¹⁴ photons cm⁻² s⁻¹ pulse of 415-nm light at the indicated circadian phase. n = 38.

stratifying that Opn4 and Pde6b are not required for ex vivo photoentrainment of mouse corneas.

Opn5-Dependent Photic Responses of the Circadian System in Cornea Emerge Ex Vivo

To further characterize the light response of cultured corneas, we administered 90-minute pulses of 2 × 10¹⁴ photons cm⁻² s⁻¹ 415-nm light to corneas during early subjective night and measured resulting circadian phase changes. After 1 day of culture, there was no shift in the Per2Luciferase luminescence rhythms of a light-treated cornea compared to a dark-treated control from the fellow eye (Fig. 2A). However, after approximately 3 days of culture, large phase delays were observed in light-pulsed corneas (Fig. 2B). When similar light pulses were administered at phases across the circadian cycle on the fourth day of culture, phase delays and advances were observed at specific times of day characteristic of a “type 1” phase response curve (PRC) (Fig. 2C).

In previous work, we observed that the photoentrainment of circadian clocks in the cornea was dependent on the expression of Opn5.¹¹ To test whether the spectral sensitivity of the photic phase shifting in corneas fit with this observation, we exposed corneas to 2 × 10¹⁴ photons cm⁻² s⁻¹ monochromatic light after 4 days in culture at a phase predicted to elicit a large phase delay (based on the PRC of Fig. 2C). Light of 400 nm and 415 nm produced large phase shifts, whereas 530-nm light did not shift the phase of the cornea clocks (Fig. 3A). This is consistent with the function of a short wavelength-sensitive photoreceptor. To test the involvement of noncanonical opsin expression, we administered 415-nm light pulses to corneas from Opn3–/–, Opn4–/–, Opn5–/–, and Opn4–/–; Opn5–/– mice on the fourth day in culture. Wild-type, Opn3–/–, and Opn4–/– corneas responded with similar phase shifts, which were significantly different from dark-handled controls (Fig. 3B, P < 0.05 ANOVA, Tukey post hoc). The circadian phase of the Opn5–/– and Opn4–/–; Opn5–/– corneas did not respond to light pulses, confirming the necessity of Opn5 in the corneal light response (Fig. 3B).
Induction of Opn5 Gene Expression in Cultured Cornea

From these data, it appeared that direct, Opn5-dependent shortwave light sensitivity of the cornea developed following explantation. One possibility for this would be that Opn5 expression is upregulated following dissection. We assessed the level of opsin transcript relative to β-actin in corneas maintained in organotypic culture. A large increase of Opn5 transcript expression was observed after 2 days in culture (Fig. 4A). A smaller but significant increase in Opn4 transcript was also detected relative to the fresh cornea (day 0). Transcript levels were variable but not significantly increased compared to nonwounded areas (Supplementary Fig. S1A).

The wounded area showed an increase of 14.4 ± 4.3 ± 0.8 cells/mm² in the same area of nonwounded cornea (paired t-test, P = 0.017, n = 5; Supplementary Fig. S1B).

We sought to determine if the type 1 cells were nascent cells rapidly dividing along wound sites or if these were preexisting cells being triggered to express the opsin. Immediately after dissection, corneas from Opn5Cre; Ai14 mice were incubated with EdU (Click-iT EdU; Thermo Fisher), which is incorporated into cells undergoing the DNA synthesis phase of cell division. Proliferating cells incorporating the modified uridine were then imaged by detection of a fluorescent tag (Alexa Fluor, 647 nm). Many recently divided cells were readily identified within 12 hours of culture, and by 24 hours, the corneal epithelium was populated by 1.2 × 10⁴ ± 641 cells/mm² labeled with EdU (Fig. 5A). When we compared the tdTomato expression from the Ai14 transgene with the EdU-labeled cells, there was little coexpression. Only 10.1% ± 3.1% (n = 6) of the tdTomato-positive type 1 cells also contained EdU by 24 hours (Figs. 5B, 5C). Thus, it is likely that the majority of the cells that induced Opn5 were cells already resident in the cornea at the time of dissection or injury. Similarly, type 1 cells induced by an epithelial debridement did not incorporate EdU (Supplementary Fig. S2C).

To further validate this finding, we exposed cultured corneas to ARA-C to inhibit synthesis of DNA and block cell division. A total of 50 μM Ara-C ex vivo was sufficient to block proliferating cells at 24 hours as measured by EdU (Fig. 5D). The same treatment had no discernible impact on the induction of type 1 Opn5Cre; Ai14 expressing cells (Fig. 5E). ARA-C treatment also had no effect on the period or amplitude of the circadian rhythm as measured by Per2 luciferase activity in cultured corneas, indicating that the treatment was not toxic (Supplementary Fig. S2).

Identity of Opn5-Expressing Corneal Cells

The type 1 corneal cells with induced Opn5Cre; Ai14 are found throughout the epithelium, from basal to suprabasal...
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**Figure 4.** Opn5 induction after culture of cornea. (A) Relative abundance of the indicated transcript compared to β-actin using the ΔΔCt quantitative PCR method in fresh or cultured mouse corneas. Bars represent mean ± SEM, n = 4. (B) White-light contrast image of cornea in C at time 0. **Yellow dashed line** shows sites of relaxation incisions. (C) Fluorescence images of an Opn5Cre; Ai14 cornea taken at the indicated time postdissection. **Red arrows** in 21-hour image highlight relaxation incisions. (D) Fluorescence images of Opn5Cre; Ai14 (red) expression and DAPI-stained nuclei (blue) of a type 0 cell. **Scale bar:** 50 μm. **Right panel** shows Z projection of image at left. e, epithelium; s, stroma. (E) Fluorescence images of Opn5Cre; Ai14 (red) expression and DAPI-stained nuclei (blue) of type 1 cells in en face (left) or diagonal-cut (right) corneas. **Scale bar:** 50 μm.

The cells were colabeled with antibodies for K14, K12,36,37 and PAX6,38,39 confirming their identity as epithelial cells (Figs. 6A–C). Furthermore, we did not observe any

layers, and show typical morphology of epithelial cells (Fig. 4E).
colabeling of the Opn5<sup>Cre</sup>; Ai14 cells with K15, distinguishing these cells from limbal epithelial cells (Fig. 6D).<sup>30</sup> Finally, because of their induction after wounding, morphology, and lack of proliferative markers, we also considered that they bear a resemblance to corneal γδ T cells.<sup>31,42</sup> However, we did not detect colabeling with TCR<sub>γδ</sub> GL3 or CD45 and tdTomato (Figs. 6E, 6F). Thus, the damage-induced Opn5<sup>+</sup> expressing type 1 cells appear to be central corneal K14<sup>+</sup> epithelial cells.

The identity of the larger type 0 cells is still underdetermined. Because of the morphology, location in the upper stroma/basal epithelium, and lack of proliferative markers, we considered the type 0 cells may be resident dendritic cells or macrophages.<sup>43,44</sup> However, these cells were not colabeled with CD11c (dendritic cells) or CD11b (macrophages) (Supplementary Fig. S3A). Rather, we observed colabeling with a keratocan antibody, suggesting that these cells are a subset of keratocytes.<sup>32</sup> It should be noted that the Opn5<sup>Cre</sup>; Ai14 reporter system is a lineage-marking loxP-based reporter. This means that cells expressing tdTomato at the start of an experiment may be expressing Opn5 actively or may be lineage-derived from Opn5-expressing cells. While the type 1 epithelial cells were induced during each experiment (indicating active Opn5-expression), the type 0 cells were present throughout the experiments. Also, because the type 0 cells are present at times when the cornea circadian clocks are not light responsive (Fig. 2), it is unlikely that they are responsible for the phenomena.

**DISCUSSION**

We have demonstrated that in vivo, circadian phase entrainment of corneal rhythmicity is driven by systemic cues linked to the phase of SCN and behavior rather than the direct light-dark cycle. This result corroborates earlier findings from corneas of Opn4:TeNT mice.<sup>45</sup> However, once the cornea has been explanted, it becomes phototentrainable and exhibits phase shifts to light. This induced photosensitivity is maximally sensitive to short visible wavelengths and requires Opn5 (but not Opn4 or Opn3). Photosensitivity corresponds to induction of Opn5 expression, which appears to localize in a preexisting, small subset of corneal epithelial cells concentrated in areas of injury. Taken together, these results demonstrate that corneal injury results in induction of local photosensitivity in the corneal epithelium via an Opn5-dependent mechanism that is sufficient to permit direct circadian phototentrainment.

Opsin-mediated extraretinal photoreception has long been known in fish,<sup>46,47</sup> amphibians,<sup>48,49</sup> reptiles,<sup>50,51</sup> and birds.<sup>19,52</sup> In brains of birds, pinopsin is expressed in the pineal gland and regulates melatonin synthesis,<sup>53</sup> and Opn5 is expressed in the hypothalamus and regulates breeding based on seasonal changes in day length.<sup>19,20</sup> Similarly, teleost fish express opsins in many tissues, and these tissues are photosensitive to prolonged light exposure.<sup>54</sup> Until recently, it was believed that in mammals, only the retina demonstrated opsin-mediated photosensitivity. The catalog of vertebrate tissues that have been found to express opsins has expanded dramatically in recent years.<sup>55</sup> Mice have been shown to express opsins in their skin,<sup>15,56</sup> as well as in their birds.<sup>19,52</sup> In brains of birds, pinopsin is expressed in the pineal gland and regulates melatonin synthesis,<sup>53</sup> and Opn5 is expressed in the hypothalamus and regulates breeding based on seasonal changes in day length.<sup>19,20</sup> Similarly, teleost fish express opsins in many tissues, and these tissues are photosensitive to prolonged light exposure.<sup>54</sup>

Recent evidence suggests that the anterior segment of the eye, including the cornea, contains photoreceptors.<sup>62,63</sup>

**FIGURE 5.** Cells with induced Opn5 are resident cells. (A) Fluorescence image of EdU incorporation in a whole-mounted cornea after 24 hours in culture. Scale bar: 200 μm. (B) EdU-incorporated cells (green) and Opn5<sup>Cre</sup>; Ai14 tdTomato cells (red) after 24 hours in culture. Scale bar: 200 μm. (C) Same as B but with higher magnification. Scale bar: 50 μm. Arrows point to two colabeled cells. (D) EdU-incorporated cells in vehicle (left) and two examples of 50 μM ARA-C-treated corneas as whole mounts after 24 hours in culture. (E) Brightfield illumination (left) and fluorescence image (center) of an Opn5<sup>Cre</sup>; Ai14 cornea immediately after dissection into 50 μM ARA-C. The same cornea showing tdTomato-positive cells after 24 hours.
For example, the branch of the trigeminal nerve that innervates the cornea expresses Opn4. One potential role for this extra-retinal opsin is the photo-allodynia associated with corneal stress or migraine headache models, which may result from photic signals directly through these OPN4 cells in the trigeminal nerve. The current work now demonstrates a second opsin (Opn5) is expressed in cornea; however, Opn5 appears to be expressed inducibly in corneal epithelial cells rather than in neurons, and it appears to be necessary and sufficient for induction of circadian photosensitivity. To our knowledge, the current study is the first to demonstrate inducible circadian photosensitivity in any tissue and the first to show rapid induction of opsin expression following injury.

The circadian clock and the time of wounding have an impact on the rate of healing in both skin and cornea. Light has been linked to enhanced dermal wound healing (or “photobiomodulation”); however, the necessary wavelengths, optimal procedures, and mechanisms of action remain unclear. In the cornea, the rate of mitosis in healing corneas is reduced in darkness compared to a light-dark cycle. One possible mechanism for altered healing in light-dark cycles could be enhanced synchronization of local circadian clocks allowing for a coordinated healing response. Opn5 is induced along wound sites, which is consistent with a model of local clock synchronization influencing healing. Alternatively, it is possible that circadian entrainment is a by-product of other Opn5-mediated signals that are involved in wound healing. Future studies will assess the role of Opn5 and light in corneal wound healing directly and whether any role is mediated by circadian clock-dependent mechanisms.

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