Melatonin Entrains PER2::LUC Bioluminescence Circadian Rhythm in the Mouse Cornea

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Purpose. Previous studies have reported the presence of a circadian rhythm in PERIOD2::LUCIFERASE (PER2::LUC) bioluminescence in mouse photoreceptors, retina, RPE, and cornea. Melatonin (MLT) modulates many physiological functions in the eye and it is believed to be one of the key circadian signals within the eye. The aim of the present study was to investigate the regulation of the PER2::LUC circadian rhythm in mouse cornea and to determine the role played by MLT.

Methods. Corneas were obtained from PER2::LUC mice and cultured to measure bioluminescence rhythmicity in isolated tissue using a Lumicycle or CCD camera. To determine the time-dependent resetting of the corneal circadian clocks in response to MLT or IIK7 (a melatonin type 2 receptor, MT_2, agonist) was added to the cultured corneas at different times of the day. We also defined the location of the MT_2 receptor within different corneal layers using immunohistochemistry.

Results. A long-lasting bioluminescence rhythm was recorded from cultured PER2::LUC cornea and PER2::LUC signal was localized to the corneal epithelium and endothelium. MLT administration in the early night delayed the cornea rhythm, whereas administration of MLT at late night to early morning advanced the cornea rhythm. Treatment with IIK7 mimicked the MLT phase-shifting effect. Consistent with these results, MT_2 immunoreactivity was localized to the corneal epithelium and endothelium.

Conclusions. Our work demonstrates that MLT entrains the PER2::LUC bioluminescence rhythm in the cornea. Our data indicate that the cornea may represent a model to study the molecular mechanisms by which MLT affects the circadian clock.

Keywords: melatonin, cornea, circadian rhythm, entrainment

Circadian rhythms are an ubiquitous feature of living systems. Accumulating evidence suggests that dysfunction of circadian rhythms due to genetic mutations or environmental factors contributes to the development of many diseases.[1–4] The retinal circadian clock was the first extra-SCN (suprachiasmatic nucleus) circadian oscillator to be discovered in mammals[5,6] and several studies have now demonstrated that many of the physiological, cellular, and molecular rhythms that are present in the retina are under the control of circadian clocks.[4] Experimental evidence also indicates that other ocular structures may contain circadian clocks that drive circadian rhythms in the eye. Diurnal fluctuations have been reported in IOP,[7,8] photoreceptor disk shedding and phagocytosis,[9–13] axial chamber length, choroidal volume, corneal curvature, and cornea thickness.[14–22] The changes in axial chamber length, corneal curvature, and thickness are believed to contribute to variations of astigmatism and refractive errors observed at different times of the day.[23–25] Further investigations also have shown that a diurnal rhythm in the light- dark cycle is important for normal corneal growth and development,[26] and that renewal of the corneal epithelium shows a daily rhythm.[27]

The PERIOD2::LUCIFERASE (PER2::LUC) knockin mice in which the firefly *luciferase* gene was fused into the *Period 2 (Per2)* gene, have been used to study circadian rhythms in many different tissues and/or organs,[28] including in the eye. These rhythms are thought to be driven by circadian oscillators endogenous to a variety of structures throughout the eye. For example, endogenous circadian clocks have been identified in mouse photoreceptors,[29] retina,[29–33] RPE,[32] and the cornea.[28,34] Circadian clocks in the photoreceptors are directly entrained by changes in light/dark. However, because photoreceptors, and a handful of specialized ganglion cells, are the only cells in the eye capable of detecting light/dark changes, there must be signals emanating from photoreceptors that propagate time-of-day information throughout the eye.

Among the many neurotransmitters present in the retina, melatonin (MLT) is believed to be a key signal that regulates circadian rhythms.[35] Melatonin is synthesized by the photoreceptors and by the pineal gland with high levels at night and lower levels during the day.[36,37] Retinal MLT is involved in the modulation of many important ocular functions by acting on melatonin receptors (MT_1 and MT_2) that are present in these structures.[38,39] For example, MLT affects the amplitude of the scotopic electroretinogram[39–41] and protects RPE cells,[42] photoreceptors,[41,43] and ganglion cells.[41,44]

The aim of the present study was to further define the cellular location of corneal PER2::LUC bioluminescence rhythm and to test whether MLT can synchronize the PER2::LUC bioluminescence rhythm.

Materials and Methods

Animals

The PER2::LUC mice[28] (C57/Bl6) at approximately 12 weeks of age were used in this study ($n = 196$). All the procedures were approved by the Institutional Animal Care and Use Committee at Morehouse School of Medicine. Mice were raised at Morehouse School of Medicine in 12 hours light and 12 hours dark with lights on (zeitgeber time [ZT] 0) at 06:00 and lights off (ZT 12) at 18:00 hours. Water and food were available ad libitum. The light was supplied with fluorescent tubes and the light intensity ranged from 200 to 400 lux at cage level.

Tissue Culture Preparation and Measurement of Bioluminescence

Mice were anesthetized with CO_2 and killed by cervical dislocation. The eyes were removed and the cornea was carefully separated under a dissecting microscope. The cornea was placed on the culture membrane (epithelial layer up) (Millicell-CM, PICM030-50; Millipore, Billerica, MA, USA) in a 35-mm Petri dish with 1.2 mL of 199 medium (Cambrex, Walkersville, MD, USA) containing 0.1 mM D-Luciferin K salt (Molecular Imaging Products, Bend, OR, USA). Dishes were sealed and kept at 37°C. The cultures were prepared under fluorescent tubes between ZT (lights on at ZT0) 8 to 10. The bioluminescence emitting from the cornea was measured with photomultiplier tubes (Lumicycle; Actimetrics, Wilmette, IL, USA). To identify the sources of bioluminescence within the different layers, the cornea was sectioned with a razor blade and placed on a culture membrane in a Petri dish containing 199 medium. The bioluminescence images of a cornea section was obtained by a Zeiss AxioObserver Z1 microscope (Zeiss, Oberkochen, Germany) with an ×10 Fluar objective lens (Zeiss), Marzhauzer scanning stage with LUDL Mac 5000 controller, and a Stanford Photonics (Palo Alto, CA, USA) XR Mega 10Z cooled intensified charge-couple device (CCD) camera in a custom-built light-tight chamber maintained at 37°C (see Evans et al.[45] for details). Bioluminescence images were collected every 15 minutes for 3 days. Continuous images of cornea PER2::LUC bioluminescence were converted and analyzed for a quantification of bioluminescence counts by Image-J software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA).

Immunohistochemistry

Mice were killed and corneas were dissected at ZT 9 to ZT 10. Tissues were fixed overnight in 4% paraformaldehyde PBS solution and cryoprotected in a 30% sucrose solution for 48 hours. Tissue was then frozen and embedded in Tissue Tek OCT compound (Sakura, Finetek, Torrance, CA, USA). Then, 10 μm frozen sagittal cornea sections were made and mounted on a positive charged slide glass (VWR Vista Vision Histo Bond; VWR Inc., Radnor, PA, USA). Afterward, the sections were washed in PBS (3 × 10 minutes) and permeabilized with 0.3% Triton X-100 in a blocking buffer containing 1% BSA for 60 minutes at room temperature (RT). Sections were incubated overnight at 4°C with a 1:2000 dilution of 10 mg/mL biotin-conjugated goat anti-firefly luciferase antibody (Abcam, Hartford, CT, USA). To determine the cellular localization of MT_2 receptors in the cornea, sections were incubated for 3 hours with a 1:500 dilution of 0.8 mg/mL rabbit anti-MT_2 antibody or with the control peptide antigen (Alamone Labs, Jerusalem, Israel). Sections were then incubated with the biotinylated goat anti-rabbit IgG secondary antibody (1:200; Vector Laboratories, Inc., Burlingame, CA, USA). After washing sections with washing solution (PBS with 0.3% Triton X-100), sections were incubated with horseradish peroxidase conjugated with streptavidin (Vectastain Elite ABC kit; Vector Laboratories, Inc.) for 45 minutes at RT. Peroxidase activity was revealed with 3′,3′-diaminobenzidine (DAB) tetrahydrochlorate using a DAB kit (Vector Laboratories, Inc.). Sections were then washed with distilled water and mounted with a mounting medium (VectaMount AQ Mounting Medium; Vector Laboratories, Inc.).

Drug Treatments

Melatonin (Sigma-Aldrich Corp., St. Louis, MO, USA) was first dissolved in ethanol (8 mg/mL). It was then diluted to 100 μM in PBS (100 nM final concentration). The IIK7 (*N*-butanoyl-2-[2-methoxy-6*H*-isoindolo(2,1-a)indol-11-yl]ethanamine; Sigma) was dissolved in DMSO 30 mg/mL then diluted with PBS to 1 mM (1 μM final concentration) or 1 μM (1 nM final concentration). These concentrations were selected on the basis on the compound affinity for each of the murine MLT receptors.[39] Controls were prepared with the same procedure, but without the active compound. After 3 to 4 days of bioluminescence recording, the culture dishes containing cornea were gently removed from the Lumicycle and 1.2 μL of drug solutions or vehicles were added to the culture dishes. They were then re-sealed, returned to the Lumicycle and placed in the same positions that they were occupying before the treatment. The culture dishes were kept in the Lumicycle until the end of the experiment without a drug washout.

Analysis of Phase-Shifts

Bioluminescence recordings emitted from cornea cultures were detrended by a 24-hour moving average subtraction method and smoothed by a 2-hour moving average. The circadian peak phase was determined as the highest point of the curve picked by Origin (Origin Laboratories, Northampton, MA, USA) software. The amount of phase-shift (in hours) was calculated by comparing the regression lines fitted to the circadian peaks before and after the treatment.[32] Phase-shifts in individual cornea were averaged in a 4-hour bin at circadian time (CT) 0 to 4, CT 4 to 8, CT 8 to 12, CT 12 to 16, CT 16 to 20, and CT 20 to 24 and normalized with respective control groups. Two-way ANOVA with post hoc Tukey test was performed to compare the difference between experimental groups and time points.

Results

Circadian Rhythms of Per2::LUC Bioluminescence in Cultured Cornea

Figure 1A shows a representative measurement of cornea bioluminescence obtained from a PER2::LUC mouse. Similar to results reported in a previous study,[28] we could readily detect a robust circadian rhythm in PER2::LUC bioluminescence that could persist for more than 80 days in culture with weekly medium exchanges (Fig. 1B). The average circadian peak phase of PER2::LUC bioluminescence in cornea was ZT 13.13 ± 0.09 hours, just after dark onset, and the average circadian period was 22.83 ± 0.04 hours (mean ± SEM).

Localization of the PER2::LUC Bioluminescence Rhythms in Cornea

The mouse cornea contains five different layers (epithelium, anterior limiting lamina, stroma, posterior limiting lamina, and

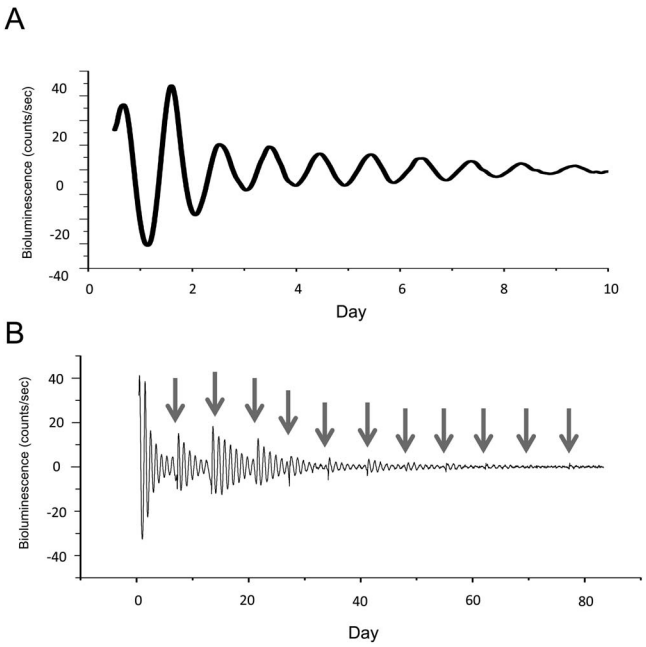

Figure 1. The PER2::LUC bioluminescence rhythms in mouse cornea. Bioluminescence obtained from a cultured PER2::LUC mouse cornea was measured with a Lumicycle for 10 days (**A**). Rhythmic PER2::LUC bioluminescence was observed for more than 80 days when cultures received weekly medium exchange (**B**). *Arrows* indicate the time of medium exchanges.

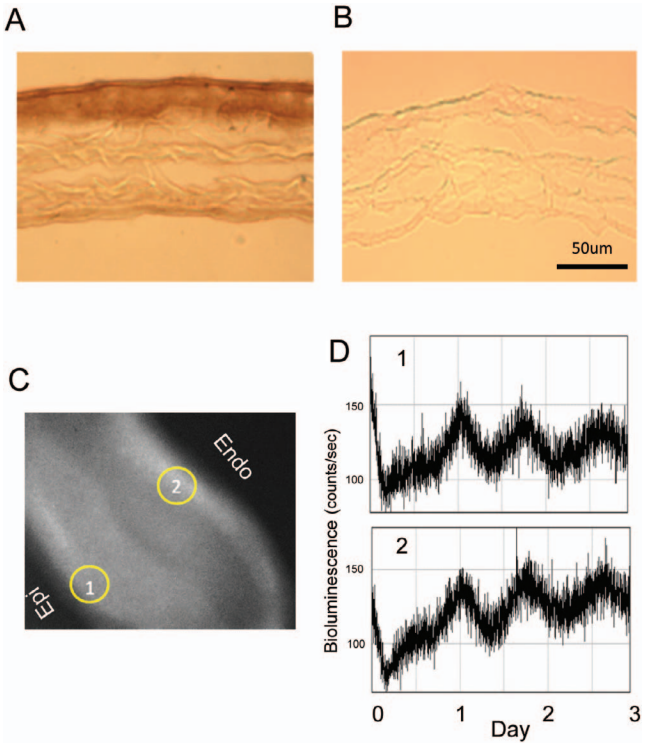

Figure 2. The localization of PER2::LUC to the cornea layers. Cornea obtained from PER2::LUC mice was immunostained with anti-firefly luciferase antibody (**A**). Specific staining was only observed in epithelium and endothelium layers of cornea (**A**). Control (i.e., tissue incubated without the primary antibody) did not show any immunoreactivity (**B**). Images of PER2::LUC bioluminescence obtained with the CCD camera (**C**). Circadian bioluminescence rhythms in epithelium (**D1**) and endothelium (**D2**) were recorded for 3 consecutive days.

endothelium). Using anti-luciferase antibody, we identified strong specific immunoreactivity in corneal epithelium and endothelium (Fig. 2A). No immunoreactivity was detected in controls (Fig. 2B). To validate the result obtained with immunohistochemistry, we investigated the cellular location of the PER2::LUC bioluminescence rhythm in cornea using a CCD camera imaging system. As shown in Figure 2C, PER2::LUC bioluminescence was mostly detected in the epithelium and endothelium layers. The phase and the period of the circadian rhythms in the epithelium and endothelium were similar (Fig. 2D).

Effect of Melatonin on the PER2::LUC Bioluminescence Rhythms

We then generated a phase response curve (PRC) to MLT. On the third day of culture, corneas were treated with 100 nM MLT or a vehicle at several different phases of the circadian cycle and then the bioluminescence was continuously measured for an additional 5 days (Figs. 3A, 3B). The individual phase-shift for each cornea rhythm was plotted to produce the PRC (Fig. 3C). A two-way ANOVA analysis revealed a significant interaction effect between groups and treatment time points ($P < 0.001$, Fig. 3D). Melatonin significantly phase-delayed cornea rhythms by 3.02 hours when applied at CT 12 to 16 ($P < 0.05$, Tukey test) and significantly phase-advanced cornea rhythms by 4.97 hours when applied at CT 20 to 24 ($P < 0.05$, Tukey test) and 2.88 hours at CT 0 to 4 ($P < 0.05$, Tukey test).

Effect of Melatonin Receptor Agonist on PER2::LUC Bioluminescence Rhythms

In Figure 4A, we show the PRC of the PER2::LUC bioluminescence rhythm to IIK7, a MT_2 receptor agonist, at a concentration of 1 nM (i.e., at a concentration that activates only MT_2 receptors[39]). At this concentration, II7K significantly phase-delayed cornea rhythms by 2.46 hours at CT 12 to 16 ($P < 0.05$, two-way ANOVA followed by Tukey test; Fig. 4C) and

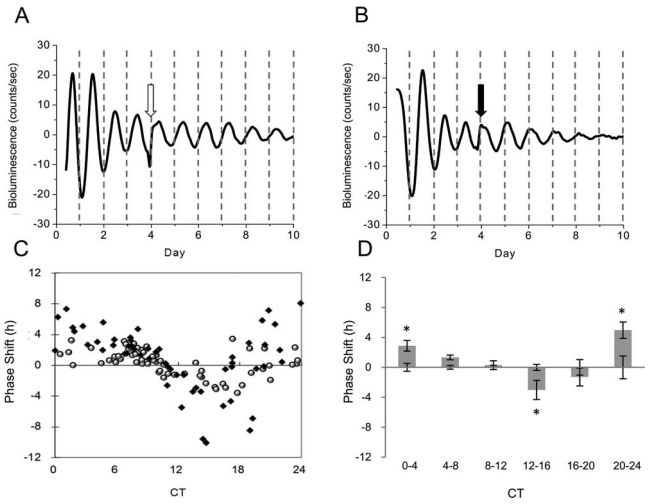

Figure 3. Phase response curve of PER2::LUC bioluminescence rhythm to MLT administration. Representative examples of PER2::LUC bioluminescence rhythms in response to vehicle (**A**) and MLT (**B**). The *arrows* indicate when the Vehicle (Veh) or MLT was added to the culture dishes respectively. The value of the phase-shift for each individual cornea rhythm was plotted to create a PRC (**C**). *White circles* indicate cultures treated with Veh and *black squares* indicate culture treated with MLT. Data were divided into six bins at 4-hour intervals for statistical analysis (**D**) (Tukey test, $^*P < 0.05$). $n = 5$ to 8 for each bin.

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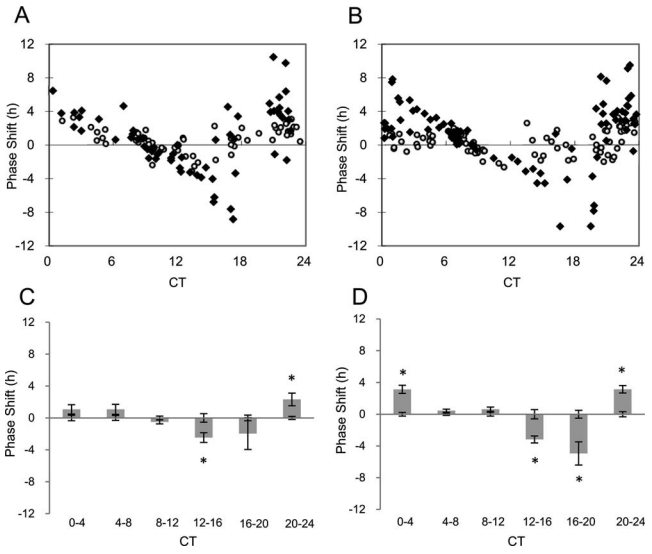

FIGURE 4. Phase response curve of PER2::LUC bioluminescence rhythm to IIK7 administration: 1 nM of IIK7 induced a significant phase-shift at CT 12 to 16 and CT 20 to 24 (A); 1 μM of IIK7 also induced significant phase-shift at CT 0 to 4 and CT 12 to 24 (**B**). *White circles* indicate cultures treated with Veh and *black squares* indicate culture treated with IIK7. Data were divided to six bins at 4-hour intervals for statistical analysis ([**C**] for 1 nM and [**D**] for 1 μM) (Tukey test, $^*P < 0.05$). $n = 7$ to 17 for each bin.

significantly phase-advanced cornea rhythms by 2.32 hours at CT 20 to 24 ($P < 0.05$, two-way ANOVA followed by Tukey test). We then increased the dosage of IIK7 to 1 μM where both MT_1 and MT_2 can be activated[39] (Fig. 4B). As seen with the lower concentration, administration of 1 μM of IIK7 also phase-delayed cornea rhythms by 3.18 hours at CT 12 to 16 ($P < 0.05$, two-way ANOVA followed by Tukey test, interaction effect $P < 0.001$, Fig. 4D), 4.17 hours at CT 16 to 20 ($P < 0.05$, Tukey test) and phase-advanced cornea rhythms by 3.14 hours at CT 20 to 24 ($P < 0.05$, Tukey test), 3.13 hours at CT 0 to 4 ($P < 0.05$, Tukey test). Consistently with the data obtained with IIK7 administration, MT_2 immunoreactivity was observed in the corneal epithelium and endothelium (Fig. 5).

Discussion

In this study, we investigated the PER2::LUC bioluminescence circadian rhythm in the cornea and we localized PER2::LUC expression to the epithelial and endothelial layers of the mouse cornea. We also demonstrated that MLT can phase-shift this circadian rhythm, possibly by acting on MT_2 receptors. Consistent with what has been previously shown,[28] we observed a robust and persistent (up to 80 days) PER2::LUC bioluminescence rhythm with a phase and period similar to what has been reported previously in the same animal model.[28] The mouse cornea is composed of epithelium, anterior limiting lamina, stroma, posterior limiting lamina, and endothelium layers.[46] Although previous studies have reported bioluminescence circadian rhythms in this tissue,[28,34] the localization of the PER2::LUC bioluminescence was still unknown. Our study indicates that the corneal epithelium and the endothelium contain a circadian clock driving the circadian rhythm in this tissue (Fig. 2) and MLT can entrain these circadian rhythms (Fig. 3).

As previously mentioned, accumulating experimental evidence indicates that circadian rhythms control important physiological functions of corneal epithelium and endothelium. The renewal of the corneal epithelium has been shown to exhibit circadian regulation,[27] and the mitotic rate and proliferation of corneal epithelial cells has been shown to have circadian variation.[47,48] Furthermore, it has been reported that mitotic rhythm can be phase-shifted by MLT.[49] These circadian variations affect the process of corneal epithelial wound healing[50] and influence the action of drugs on the corneal epithelium.[51,52] Similarly, it has been reported that IIK7 enhanced wound healing in rabbit epithelium, and that the positive effect of MLT administration on wound healing was blocked by DH97, a selective MT_2 antagonist, thus suggesting MT_2 signaling as important for corneal wound healing in rabbit.[53] Although a previous study has suggested that corneal mitotic activity is not driven by MLT, because such a rhythm persists after the photoreceptors were destroyed,[54] it is worthwhile to mention that MLT of pineal origin could still have affected and driven corneal mitotic rhythm.

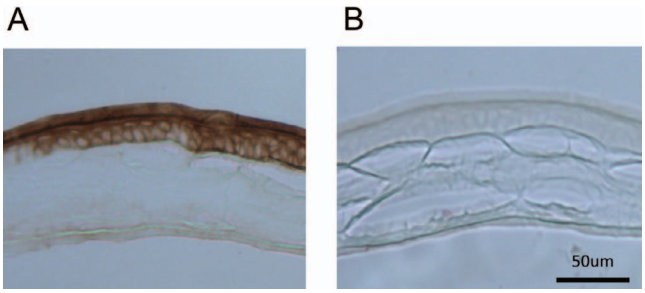

FIGURE 5. Immunoreactivity of MT_2 receptor in the cornea. Immunoreactivity of MT_2 was localized to the corneal epithelium and endothelium (**A**). Control (i.e., tissue incubated with the control peptide antigen) did not show any immunoreactivity (**B**).

Our data indicate that activation of the MT_2 receptor can induce a phase-shift of corneal PER2::LUC bioluminescence rhythms (Fig. 4A). Although a higher concentration of IIK7 seems to increase the amplitude of the phase-shifts (Figs. 4C, 4D), a careful inspection of the individual data points (Figs. 4A, 4B) does not support this conclusion. Hence, we believe that the corneal PER2::LUC bioluminescence rhythm is phase-shifted predominantly by activation of MT_2 receptors. The observation that MLT or IIK7 can still produce a phase-shift (advance) in the early subjective morning (Figs. 3, 4) when MLT levels are low indicates that the action of MLT on the regulation of circadian rhythms may vary among different tissues.

Melatonin receptor immunoreactivity has been localized to the corneal epithelium and endothelium in many species.[53,55–59] Our results indicate that, in the mouse, MT_2 receptors are expressed in the corneal epithelium and endothelium (Fig. 5) and MLT acts directly on the cells expressing PER2::LUC bioluminescence.

The action of MLT and its associated receptors on the regulation of circadian rhythms is well established (see Dubocovich et al.,[60] Tosini et al.[61]). Indeed MLT, via MT_2, receptors can phase-shift electrical activity in the SCN[62,63] and locomotor activity.[63,64] Our new data further support this hypothesis. We have recently reported that disruption of MLT signaling affects clock gene expression in photoreceptors, and may affect the expression of these genes in the inner retina and/or ganglion cell layer.[65] These data together with our new results indicate that the action of MLT on the circadian clock is cell specific.

Previous studies have shown that in the retina, MLT is synthetized by photoreceptors[36] and it is believed that retinal MLT is rapidly metabolized in the retina.[66] Although we believe that the MLT synthetized by the photoreceptors is responsible

for the entrainment of the corneal circadian rhythms, we cannot exclude that MLT of pineal origin (i.e., circulating MLT) may also reach the corneal tissue and entrain the corneal circadian clock. Indeed, several studies have shown that administration of exogenous MLT via different routes (intraperitoneal injection or via drinking water) can reach the eye and can alter retinal physiology.[39,41,67]

In conclusion, our study demonstrates that the cornea is a robust model to study circadian rhythms and that the epithelium and endothelium layers of the cornea contain circadian clocks. These circadian rhythms may be entrained by MLT via MT_2 receptor signaling. Our study also indicates that corneal epithelial and endothelial cells may represent a new tool to study the molecular mechanisms by which MLT can entrain the circadian clock.

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