Circadian and light-driven regulation of rod dark adaptation

Yunlu Xue1,2,4, Susan Q. Shen1,3,4, Joseph C. Corbo1,3 & Vladimir J. Kefalov1,2

Continuous visual perception and the dark adaptation of vertebrate photoreceptors after bright light exposure require recycling of their visual chromophore through a series of reactions in the retinal pigmented epithelium (RPE visual cycle). Light-driven chromophore consumption by photoreceptors is greater in daytime vs. nighttime, suggesting that correspondingly higher activity of the visual cycle may be required. However, as rod photoreceptors are saturated in bright light, the continuous turnover of their chromophore by the visual cycle throughout the day would not contribute to vision. Whether the recycling of chromophore that drives rod dark adaptation is regulated by the circadian clock and light exposure is unknown. Here, we demonstrate that mouse rod dark adaptation is slower during the day or after light pre-exposure. This surprising daytime suppression of the RPE visual cycle was accompanied by light-driven reduction in expression of Rpe65, a key enzyme of the RPE visual cycle. Notably, only rods in melatonin-proficient mice were affected by this daily visual cycle modulation. Our results demonstrate that the circadian clock and light exposure regulate the recycling of chromophore in the RPE visual cycle. This daily melatonin-driven modulation of rod dark adaptation could potentially protect the retina from light-induced damage during the day.

The retina provides vertebrate animals with information about the world around them and the overall light intensity. Detailed visual information is generated by rod and cone photoreceptors, which are responsible for dim- and bright-light vision, respectively. The function of the retina is modulated by daily changes in ambient light conditions and by an intrinsic circadian clock1. These mechanisms regulate many retinal functions, including melatonin synthesis2, the electrical coupling between photoreceptors3,4, and synaptic transmission5, to fine-tune visual processing in the retina6. The susceptibility to light-induced retinal damage is also higher in subjective (circadian) night than in subjective day7. Although the mechanisms by which the circadian clock regulates this process is not understood, it is likely to be related to the light-sensing visual pigments in photoreceptors.

Light detection is initiated in the retina when a photon is absorbed by the visual pigment in photoreceptors. This causes the conversion of the visual chromophore 11-cis retinal to its all-trans form, activating the visual pigment and triggering the phototransduction cascade that ultimately results in the electric response of the cell8. Resetting of the photoactivated (bleached) visual pigment to its ground state requires removal of the spent all-trans chromophore from photoreceptors and its recycling back to its 11-cis form in the RPE cells (RPE visual cycle; for both rods and cones) or in the retinal Müller cells (retina visual cycle; for cones only)9,10. Notably, even though rods are saturated during the day, their visual pigment still continuously cycles through bleaching and regeneration. As a result, in rod-dominant species like mouse and human, rods consume the bulk of the chromophore recycled by the RPE visual cycle11, while chromophore recycled by the retina visual cycle allows cones to rapidly regenerate their visual pigment12,13. The accumulation of retinoid byproducts with age or as a result of a dysfunctional visual cycle can cause retinal degeneration and blindness14.

1Washington University School of Medicine, St. Louis, Missouri 63110, USA. 2Department of Ophthalmology & Visual Sciences, Washington University School of Medicine, St. Louis, Missouri 63110, USA. 3Department of Pathology & Immunology, Washington University School of Medicine, St. Louis, Missouri 63110, USA. 4Graduate Program in Division of Biological & Biomedical Sciences, Washington University School of Medicine, St. Louis, Missouri 63110, USA. Correspondence and requests for materials should be addressed to V.J.K. (email: kefalov@wustl.edu)
Chromophore consumption varies greatly during the day-night cycle. During the day, the visual pigments in rods and cones are photobleached at a high rate, whereas a minimal amount of chromophore is used and recycled at night. This day/night difference in chromophore consumption prompted us to ask: is pigment regeneration under the regulation of the circadian clock, in accordance with chromophore demand? Does light modulate the efficiency of chromophore recycling? One of the key processes modulated by both the circadian clock and light exposure is melatonin synthesis, which is suppressed during the circadian daytime and by light. Thus, we addressed these questions by electrophysiological recordings and molecular analysis of retinas of melatonin-proficient (C3H/f+/- and CBA/CaJ) and melatonin-deficient (C57BL/6J and 129S2/Sv) mouse strains.

**Results**

**Rod dark adaptation in melatonin-proficient mice is regulated by the circadian clock.** The goal of our study was to determine if pigment regeneration is regulated by the circadian clock or by light. Each of these two retinal signals strongly regulates the expression of melatonin at night, which in turn affects many processes in the retina. Thus, we investigated rod dark adaptation in melatonin-proficient (C3H/f+/- and CBA/CaJ) and melatonin-deficient (C57BL/6J and 129S2/Sv) mouse strains. We began with the melatonin-proficient C3H/f+/- strain of mice, first testing their in vivo electroretinogram (ERG) responses. We observed robust dark-adapted (scotopic) responses with a normal waveform (Fig. 1A). Measurements of their maximal a-wave amplitudes (r_max) at subjective night (solid squares, n = 15) and subjective day (open squares, n = 16) (*p < 0.05). Here and in all subsequent figures, DA refers to the initial dark-adapted value of the a-wave maximal response or sensitivity.

![Figure 1. Effect of the circadian clock on rod dark adaptation in melatonin-proficient mice.](image)

(A) Representative dark-adapted scotopic in vivo ERG responses to various light intensities from melatonin-proficient C3H/f+/- mice. (B) Representative dark-adapted scotopic in vivo ERG responses from melatonin-proficient CBA/CaJ mice revealing b-wave deficit. (C) Normalized in vivo ERG scotopic a-wave maximal response (a-wave r_max/DA) recovery in C3H/f+/- mice following 90% pigment bleach at t = 0 at subjective night (solid squares, n = 15) and subjective day (open squares, n = 16) (*p < 0.05). (D) Normalized in vivo ERG scotopic a-wave sensitivity (a-wave Sf/a-wave Sf DA) recovery in C3H/f+/- mice following 90% pigment bleach at t = 0 at subjective night (solid squares, n = 15) and subjective day (open squares, n = 16) (*p < 0.05). Here and in all subsequent figures, DA refers to the initial dark-adapted value of the a-wave maximal response or sensitivity.
In order to determine whether pigment regeneration is regulated by the circadian clock, we next examined the kinetics of rod dark adaptation in C3H/f+ mice in subjective night and subjective day. The dark adaptation experiments were performed with mice that were dark adapted for 30 hours (for the CT 18 time point) or 18 hours (for the CT 6 time point). As mouse rod pigment regeneration and dark adaptation are typically complete within one hour, such conditions allowed for full dark-adaptation prior to the experiment for both time points. This notion was also supported by the comparable scotopic a-wave sensitivities at CT 18 and CT 6. A bright 30 seconds light exposure, estimated to bleach >90% of the visual pigment, instantly reduced the a-wave amplitude to near threshold levels (Fig. 1C), while the a-wave sensitivity declined by ~1000-fold, as the flash intensity required to produce a measurable response had to be increased by 3 log units (Fig. 1D). Consecutive measurements of these parameters in darkness over the next two hours revealed the gradual dark adaptation of the rods as their pigment regenerated. Notably, the recovery of both the amplitude and sensitivity of the a-wave over the first ~30 min of dark adaptation was significantly (p < 0.05, two-tailed Student's t-test) slower for the subjective day group than for the animals in subjective night (Fig. 1C,D). As a result, the initial recovery of rod a-wave response in subjective day was delayed by ~8 minutes compared to that in subjective night (Fig. 1C). Similarly, the rod sensitivity 30 min after the bleach in subjective day was 2-fold lower than that in subjective night (Fig. 1D). Eventually, the recovery levels became comparable, so that the tails of the dark adaptation (final 25% of rmax and final 5-fold of Sf recovery) were similar in the two groups. These results demonstrate that the time course of rod dark adaptation in C3H/f+ mice is delayed during the subjective day compared to subjective night. Thus, mouse rod dark adaptation is modulated by the circadian clock.

We sought to establish the regulation of rod dark adaptation in another melatonin-proficient strain, CBA/CaJ. However, the ERG recordings from these mice revealed a prominent b-wave amplitude loss and extended a- and b-wave implicit times (Fig. 1B), reminiscent of the phenotype caused by mutation in Gpr179, a G-protein coupled receptor in ON-bipolar cells. Thus, this strain of CBA/CaJ mice proved unsuitable for our physiological analysis. The abnormal ERG responses from these mice also call for caution when using this strain for molecular analysis of the retinal circadian clock.

### Rod dark adaptation in melatonin-proficient mice is regulated by light history

In addition to the circadian clock, exposure to light may also directly affect animal physiology, particularly in the light-sensitive retina. Thus, we next sought to determine if rod dark adaptation is regulated by light history. To accomplish this, we compared the dark adaptation of C3H/f+ mouse rods in vivo at 12 o’clock (noon) but dark adapted for 30 hours (subjective day, CT 6), or pre-exposed to light in the morning and then dark adapted for 1 hour before the experiment (objective day, zeitgeber time ZT 6). The 1 hour of darkness was sufficient to fully dark-adapt the rods in unanesthetized mice and restore their in vivo ERG a-wave sensitivity and maximal response amplitude (Table 1, compare values for subjective day, dark-adapted for 18 hours, and objective day, dark-adapted for 1 hour). Comparison of rod dark adaptation in subjective and objective day demonstrated that both a-wave maximal response (Fig. 2A) and a-wave sensitivity (Fig. 2B) recovered significantly more slowly during the objective day. Thus, our results revealed that rod dark adaptation in melatonin-proficient mice is suppressed by pre-exposure to light.

|                  | Subjective night | Subjective day | Objective day |
|------------------|------------------|----------------|---------------|
| C3H/f+          | (n = 15)         | (n = 16)       | (n = 7)       |
| a-wave r max    | 312 ± 21         | 291 ± 31 (NS)  | 338 ± 29 (NS) |
| a-wave S f      | 223 ± 30         | 267 ± 39 (NS)  | 296 ± 37 (NS) |
| C57BL/6J        | (n = 9)          | (n = 10)       | (n = 10)      |
| a-wave r max    | 598 ± 24         | 532 ± 18 (*)   | 700 ± 22 (**) |
| a-wave S f      | 751 ± 39         | 589 ± 15 (**)  | 856 ± 55 (**) |
| 129S2/Sv        | (n = 6)          | (n = 8)        | (n = 7)       |
| a-wave r max    | 277 ± 29         | 269 ± 36 (NS)  | 301 ± 28 (NS) |
| a-wave S f      | 249 ± 46         | 208 ± 37 (NS)  | 255 ± 27 (NS) |

Table 1. Dark-adapted scotopic in vivo ERG parameters of C3H/f+, C57BL/6J and 129S2/Sv mice at subjective night, subjective day and objective day. r DA max is the maximal amplitude of a-wave. S f DA is the a-wave sensitivity. NS: p > 0.05, *p < 0.05, **p < 0.01, subjective day: tested compared to subjective night group, objective day: tested compared to the subjective day group.
Notably, the effect of light on the rod visual cycle was substantially more prominent than that induced by the circadian clock (Fig. 1).

Given the electrophysiological findings that light exposure suppresses rod dark adaptation, we hypothesized that there was an underlying molecular downregulation of the visual cycle in objective day. Accordingly, we conducted RNA-seq and differential expression analysis of the eyes of subjective day and objective day groups. A total of 1,460 genes were found to be significantly differentially expressed (FDR = 0.05) (Supplemental Table S1), with most of them (1,298 or 89%) expressed at lower levels in objective day than subjective day. Among the genes with altered expression, we identified two known visual cycle genes, Rpe65 and Rdh12 (Fig. 2C). RPE65 converts all-trans retinal esters (atRE) into 11-cis retinol (11cROL) in the RPE 20, while RDH12 converts all-trans retinal (atRAL) to all-trans retinol (atROL) in photoreceptors21. Interestingly, Rpe65 levels were 30% lower in objective day than subjective day, whereas Rdh12 levels were 29% higher in objective day than subjective day. The downregulation of Rpe65 would delay the recycling of chromophore in the RPE and the overall visual cycle 22, whereas Rdh12 upregulation would accelerate the reduction of toxic all-trans retinal and its clearance from the...
To verify the RNA-seq results, we examined \( Rpe65 \) transcript levels by quantitative RT-PCR (qRT-PCR) in an independent set of biological replicates. In good agreement with RNA-seq, we found that \( Rpe65 \) levels were 27% lower in objective day than subjective day by qRT-PCR (Fig. 2D). Overall, these molecular studies suggest that the light-driven slowing of rod dark adaptation may be mediated by \( Rpe65 \)-dependent suppression of the RPE visual cycle.

Rod dark adaptation in melatonin-deficient C57BL/6J mice is not affected by the circadian clock or light history. We next investigated whether the changes in dark adaptation we observed in C3H/HeJ mouse rods were mediated by melatonin. To address this question, we examined the function of melatonin-deficient C57BL/6J mouse rods using in vivo ERG recordings at subjective day (CT 6), subjective night (CT 18), and objective day (ZT 6). First, we measured the dark-adapted scotopic intensity-response curves at subjective day, subjective night, and objective day. The results revealed that the ERG a-wave amplitudes were slightly increased in subjective night compared to subjective day (Fig. 3A–C). A further increase in a-wave amplitudes was observed in objective day (Fig. 3D). Then we performed the dark adaptation test as above to probe the operation of the C57BL/6J RPE visual cycle at subjective day, subjective night, and objective day. In contrast to the melatonin-proficient C3H strain, we found that the kinetics of C57BL/6J rod dark adaptation at CT 6, CT 18, and ZT 6 were identical as measured by both a-wave amplitude (Fig. 3D) and sensitivity (Fig. 3E).
We obtained similar results from another melatonin-deficient mouse strain, 129S2/Sv. As in C3H mice, the scotopic a-wave maximal amplitude and sensitivity of 129S2/Sv mice were comparable among subjective night, subjective day and objective day (Table 1). However, unlike the case of C3H mice and similar to the C57BL/6J mice, the circadian time or light exposure of 129S2/Sv mice prior to the experiment failed to modulate the dark adaptation of their rods (Fig. 4A,B). Consistent with this observation, we found no significant difference in the transcript levels of \textit{Rpe65} in objective day vs subjective day of 129/Sv mice as examined by qRT-PCR (Fig. 4C). Thus, rod dark adaptation in melatonin-deficient C57BL/6J and 129S2/Sv mice was not affected by the circadian clock or light exposure history.

**Discussion**

**Melatonin-mediated regulation of mouse rod dark adaptation.** The circadian clock and light regulate many processes in the retina\textsuperscript{25}. However, despite the large difference in the rates of visual pigment photoreactivation at night and during the day, it was not previously known whether the recycling of chromophore and the regeneration of visual pigment are also subject to such regulation. Our results clearly demonstrate that both the circadian clock and light exposure slow down the dark adaptation of rods in melatonin-proficient mice during the day. Thus, rod pigment regeneration in these mice is modulated by the combined effects of the circadian clock and light, such that rod dark adaptation is substantially slowed in the daytime, when rods are largely saturated.

Although the exact mechanism of this regulation is still unclear, our finding that \textit{Rpe65} expression is suppressed by light, together with its previously described diurnal regulation in the melatonin-proficient CBA/CaJ strain\textsuperscript{1}, suggest that it likely involves modulation of the efficiency of chromophore recycling by...
the RPE visual cycle. As this is the only mechanism for regeneration of the rod visual pigment and the rate-limiting step for rod dark adaptation\(^4^{,11}\), slowing the RPE visual cycle would cause a corresponding delay in the regeneration of rod pigment and in the dark adaptation of rods.

Melatonin is produced not only by the pineal gland but also locally in the retina by photoreceptor cells at night\(^26\), where it regulates many aspects of mammalian retinal physiology (see ref. 16 for review). The rhythmicity of melatonin biosynthesis also drives diurnal retinal dopamine synthesis, which peaks during the day\(^35\), further amplifying the robustness of the retina-intrinsic circadian clock. Melatonin receptors have been identified in various ocular cell types, including photoreceptors, RPE, and Müller cells\(^2,27^{,28}\). However, many commonly used inbred strains of laboratory mice, including C57BL/6J, 129S2/Sv and BALB/c, have lost their ability to produce melatonin due to mutations in key melatonin biosynthesis enzymes\(^29^{,30}\). In contrast, C3H mice still retain their ability to synthesize melatonin. Thus, our observation that rod dark adaptation is subject to regulation by the circadian clock and light in C3H, but not in C57BL/6J or 129S2/Sv mice, suggests a role for melatonin in this process. Thus, the simplest explanation for our results is that the efficiency of the RPE visual cycle is modulated by the daily oscillation of melatonin.

The daily modulation of the RPE visual cycle and rod-mediated vision. The high sensitivity of rods enables them to detect low light levels and mediate dim light vision. However, the high amplification that produces this exquisite rod sensitivity also results in the saturation of the rods at moderately bright light conditions\(^31\). Despite this fact, the rod visual pigment continues to undergo bleaching and regeneration throughout the day. As this process involves multiple enzymatic reactions both in the rods and in the RPE cells, it imposes a significant metabolic load on the visual system. Therefore, the downregulation of the RPE visual cycle during the day by both the circadian clock and light would conserve energy without significantly compromising rod-mediated vision. The corresponding acceleration of all-trans retinal reduction in the rods, as suggested by the observed upregulation of Rdh12, would minimize the toxic effects of this compound\(^32\) and prevent the formation and accumulation of related toxic byproducts\(^33\). At the same time, as the cones rely predominantly on the alternative retina visual cycle for the bulk of their light damage at night\(^37^{,38}\), our finding that the RPE visual cycle is faster at night provides a mechanistic explanation for this observation. Therefore, a rhythmic melatonin-driven diurnal suppression of the RPE visual cycle may protect the retina from degeneration by lowering the susceptibility of photoreceptors to light damage during the day. Indeed, lack of melatonin-dependent RPE visual cycle regulation could be involved in the enhanced age-dependent retinal degeneration in mice lacking the melatonin receptors MT1 and MT2\(^6,39\). Conversely, enhancing the diurnal suppression of the RPE visual cycle by oral intake of melatonin could be one of the factors that reduce the risk of human age-related macular degeneration (AMD)\(^40\).

**Methods**

**Animals.** The maintenance and treatment of the mice was in compliance with the protocols approved by the Washington University Animal Studies Committee. Melatonin-deficient C57BL/6 and melanin-proficient CBA/CaJ and C3H/f\(^+/+\) mice were purchased from Jackson Laboratory (Bar Harbor, ME). The C3H/f\(^+/+\) mice are also known as C3A.BLIA-Pde6b\(^+/+\), originally generated by Willem J. de Grip (Erasmus Universiteit, Netherlands)\(^5^{,41}\). Melatonin-deficient 129S2/Sv mice were purchased from Charles River Laboratories (Wilmington, MA). Unlike the original C3H strain, the C3H/f\(^+/+\) mice used in our study were free of the PDE6b mutation that causes retinal degeneration, and had normal retinal morphology and light-driven responses\(^5\). All animals used in this study were free of the rd8 mutation\(^42\). The animals were raised in a 12hr:12hr light-dark cycle and entrained in a 420 cd m\(^{-2}\) light environment for a week before the experiments. Age-matched animals were grouped into three categories: subjective night, subjective day, and objective day. Subjective night groups were dark-adapted in a light-proof cabinet for 30 hours and tested at 18 circadian time (CT; midnight). Subjective day groups were dark-adapted for 18 hours and tested at CT 6 (noon). Objective day groups were dark-adapted for 1 hour and tested at 6 Zeitgeber time (ZT, noon).

**In vivo electroretinography (ERG).** The method for studying mouse rod dark adaptation in vivo using LKC® ERG system had been described in detail previously\(^15\). Briefly, dark-adapted animals were anesthetized with ketamine/xylazine cocktail (100/20 mg/kg) by intraperitoneal injection. The pupils of the anesthetized animals were dilated with a drop of 1% atropine sulfate solution and the animals were transferred to a 37°C heating pad with a feedback anal thermal probe. The reference electrode was inserted subcutaneously beneath the scalp and 2.5% Gonak hypromellose ophthalmic demulcent solution...
was applied to the cornea. A contact lens electrode was positioned on the cornea of each eye to detect electrical signals from retina. Excessive Gonak solution was removed from the eyes with tissue paper and the animals were allowed to stabilize in darkness for 15 minutes before beginning the recordings. Test flashes from a 530 nm LED, ranging from $2.5 \times 10^{-5}$ cds m$^{-2}$ to the 25 cds m$^{-2}$ limit, were used to elicit photoresponses from each eye, and white Xenon flashes were used to produce saturated photoresponses. Sufficient time was allowed between individual test flashes to allow full recovery of the retina and avoid gradual response run-down due to light adaptation. For dark adaptation testing, a bright green (505 nm) LED light was applied to both eyes for 30 seconds to photobleach an estimated 90% of the visual pigment. The recovery of the ERG responses was monitored at fixed post-bleach time points within 2 hours after the bleach. The maximal response amplitude, $r_{\text{max}}$, was recorded at the brightest light intensity, and $S_f$ was estimated as the ratio of dim flash response amplitude and the corresponding flash intensity in the linear range of the intensity-response curve, about 20% to 30% of the maximum. The post-bleach maximal amplitude ($r_{\text{max}}$) and sensitivity ($S_f$) were normalized to their dark adapted pre-bleach level, $r_{\text{DA}}^{\text{pre}}$ and $S_{f}^{\text{pre}}$, respectively.

**RNA-seq.** RNA-seq was performed in two biological replicates per condition (objective day vs. subjective day), each consisting of four eyes. Eyes were harvested and rapidly dissected in the dark. The anterior portion of the eye including the lens was removed, and the remaining tissue (posterior sclera, choroid, RPE, and retina) was rinsed in cold sterile HBSS with calcium and magnesium (Gibco) and stored in TRIzol (Invitrogen) at $-80 \degree C$. For extraction, the tissue was homogenized with a pestle and then passed through a needle. Total RNA was extracted and purified using the RNeasy Mini Kit (Qiagen) with on-column DNaseI digestion (Qiagen). Integrity of total RNA was verified on a Agilent 2100 Bioanalyzer. Poly-A selection and synthesis of the cDNA library for sequencing was conducted as described 43. The four samples were indexed and sequenced on a single lane of a HiSeq 2500 sequencer (1 × 50 bp). To analyze the sequencing data, raw reads were demultiplexed and aligned to *Mus musculus* Ensembl release 72 with Tophat v2.0.948, and Bowtie2 v2.1.0.49 HTSeq47 was used to estimate gene abundance, and differential expression analysis was conducted with EdgeR48. See Supplementary Table S1 and Gene Expression Omnibus (GEO) accession number GSE68470 for raw and processed RNA-seq data.

**Quantitative RT-PCR (qRT-PCR).** Quantitative RT-PCR was performed in five biological replicates per condition (objective day vs. subjective day), each consisting of two eyes at 5–6 weeks old. Eyes were harvested and dissected and total RNA was prepared as described above for RNA-seq. Complementary DNA (cDNA) was prepared as previously described24, and qRT-PCR was conducted with SYBR-Green primers49 and Rpe65 primers50 were previously published. Three technical replicate PCR reactions were performed for each biological replicate. The $\Delta$Ct values from biological replicates were averaged and the SEM across biological replicates was calculated. The $\Delta$Ct values were used to calculate statistical significance with a two-tailed Student’s t-test.

**Statistics.** Unless noted otherwise, all data is presented as mean ± SEM. Two tailed unequal Student’s t-test was used to examine the significance of difference between two sample groups. Statistical significance was reported when $p < 0.05$.

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Y.X., S.Q.S., J.C.C. and V.J.K. designed the experiments, Y.X. and S.Q.S. carried out the experimental work, Y.X., S.Q.S., J.C.C. and V.J.K. wrote the manuscript. All authors reviewed the manuscript.

Additional Information
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