Effect of Circadian Clock Gene Mutations on Nonvisual Photoreception in the Mouse

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PURPOSE. Mice lacking rods and cones retain pupillary light reflexes that are mediated by intrinsically photosensitive retinal ganglion cells (ipRGCs). Melanopsin is necessary and sufficient for this nonvisual photoreception. The mammalian inner retina also expresses the potential blue light photopigments cryptochromes 1 and 2. Previous studies have shown that outer retinal degenerate mice lacking cryptochromes have lower nonvisual photic sensitivity than retinal degenerate mice, suggesting a role for cryptochrome in inner retinal photoreception.

METHODS. Nonvisual photoreception (pupillary light responses, circadian entrainment, and in vitro sensitivity of intrinsically photosensitive retinal ganglion cells) were studied in wild-type, *rd/rd*, and circadian clock-mutant mice with and without *rd/rd* mutation.

RESULTS. Loss of cryptochrome in retinal degenerate mice reduces the sensitivity of the pupillary light response at all wavelengths but does not alter the form of the action spectrum, suggesting that cryptochrome does not function as a photopigment in the inner retina. The authors compounded the *rd/rd* retinal degeneration mutation with mutations in other essential circadian clock genes, *mPeriod* and *Bmal1*. Both *mPeriod1*^{-/-}; *mPeriod2*^{-/-}; *rd/rd* and *Bmal1*^{-/-}; *rd/rd* mice showed significantly lower pupillary light sensitivity than *rd/rd* mice alone. A moderate amplitude (0.5 log) circadian rhythm of pupillary light responsiveness was observed in *rd/rd* mice. Multi-electrode array recordings of ipRGC responses of *mCryptochrome1*^{-/-}; *mCryptochrome2*^{-/-} and *mPeriod1*^{-/-}; *mPeriod2*^{-/-} mice showed minimal sensitivity decrement compared with wild-type animals. *mCryptochrome1*^{-/-}; *mCryptochrome2*^{-/-}; *rd/rd*, *mPeriod1*^{-/-}; *mPeriod2*^{-/-}; *rd/rd* and *Bmal1*^{-/-}; *rd/rd* mice all showed comparable weak behavioral synchronization to a 12-hour light/12-hour dark cycle.

CONCLUSIONS. The effect of cryptochrome loss on nonvisual photoreception is due to loss of the circadian clock nonspe-

cifically. The circadian clock modulates the sensitivity of nonvisual photoreception. (*Invest Ophthalmol Vis Sci.* 2012;53:454–460) DOI:10.1167/iovs.11-8717

Mice lacking all classical visual photoreceptors (rods and cones) continue to evince a number of light-mediated behaviors and physiology, including entrainment of circadian rhythms,^{1–3} pupillary light responses,^{4,5} and photic suppression of pineal melatonin.⁶ These effects are mediated by a population of intrinsically photosensitive retinal ganglion cells (ipRGCs)⁷ that project specifically to nonvisual centers such as the suprachiasmatic nuclei of the hypothalamus and the olivary pretectum.⁸

ipRGCs express the opsin family member melanopsin,^{9,10} an invertebrate-like opsin that forms a functional photopigment when expressed in heterologous cell culture^{11–14} or in non-ipRGC ganglion cells.¹⁵ Retinal degenerate mice lacking melanopsin lose all nonvisual photoreception,^{16,17} and ipRGCs lacking melanopsin do not show intrinsic light responses.^{17–19} Thus melanopsin appears both necessary and sufficient for ipRGC photosensitivity.

The murine inner retina also expresses cryptochrome family members *mCry1* and *mCry2*.²⁰ Cryptochromes are a conserved family of photopigments that mediate blue-light growth in plants^{21,22} and circadian rhythm entrainment in insects^{23,24} (see Ref. 25 for review). We have previously shown that, compared with retinal degenerate mice, retinal degenerate mice lacking cryptochromes have reduced behavioral synchronization to light-dark cycles, reduced light-mediated *c-fos* induction in the suprachiasmatic nuclei, and reduced pupillary light responses,^{26–28} suggesting a role for cryptochromes in inner retinal photoreception.

In mammals, cryptochromes are essential components of the time-delayed transcription-translation feedback loop that underlies circadian pacemaking; mice lacking both *mCry1* and *mCry2* lose all free-running circadian rhythms.^{29–31} This raises the question whether the observed additivity of loss of cryptochrome and outer retinal degeneration on nonvisual responses reflects a role for cryptochrome as an auxiliary photoreceptive protein in the inner retina or whether such additivity is a nonspecific result of loss of circadian rhythmicity in the whole animal. To distinguish these possibilities, we have further analyzed the physiology of cryptochrome mutant mice and mice lacking circadian rhythms from mutations in the *Period* and *Bmal1* families of circadian clock genes.

MATERIALS AND METHODS

Mice

C3H/HeJ mice (*rd/rd*; Jackson Laboratories, Bar Harbor, ME) were crossed with circadian clock mutants *mCry1*^{-/-}; *mCry2*^{-/-}, *mPer1*^{-/-}; *mPer2*^{-/-}, and *Bmal1*^{-/-} and were backcrossed to create *mCry1*^{-/-}; *mCry2*^{-/-}; *rd/rd*, *mPer1*^{-/-}; *mPer2*^{-/-}; *rd/rd*, and *Bmal1*^{-/-}; *rd/rd*

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mice. Genotypes were verified by PCR analysis of distal tail snips, as previously described (*mCry1*^{-/-30}; *mCry2*^{-/-31}; *mPer1*^{-/-} and *mPer2*^{-/-32}; *Bmal1*^{-/-33}). C57Bl/6 mice were used as wild-type controls. Mice were housed individually in running wheel cages enclosed in light-tight cabinets. Wheel running activity was monitored in 6- to 8-month-old mice and analyzed using a personal computer platform (Actimetrics, Evanston, IL). All experiments were conducted under an approved animal studies protocol and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Histology

Eyes enucleated from euthanized 6- to 8-month-old mice were immediately fixed in 10% formalin at room temperature overnight. Fixed globes were embedded in glycol methacrylate, cut into 3- μ m sections, and stained with toluidine blue. Inner and outer retinal cell counts were determined by counting nuclei at a location one 40 $\times$ field away from the optic nerve. ANOVA was performed on counts averaged from at least six different sections.

Melanopsin Staining

Enucleated globes of adult mice, age 1 to 8 months, were fixed in 4% paraformaldehyde and flat-mounted. Retinas were incubated overnight in 1:5000 of N15 anti-melanopsin antibody,¹⁰ followed by rhodamine-conjugated secondary antibody. Retinas were scored by a masked observer for a total number of brightly stained cells per retina using an epifluorescence microscope (Olympus, Tokyo, Japan).

Pupillary Light Recordings

Six- to 8-month-old mice were dark-adapted overnight and then exposed to various fluences of narrow bandpass-filtered 470-nm light for 60 seconds. Nonanesthetized mice were video recorded in a completely dark room using infrared illumination as described.²⁸ Irradiance measurements were determined with a calibrated radiometer (Macam, Scotland). Irradiance-response relations and action spectra were generated as previously described.³⁴ Briefly, for each combination of wavelength and genotype, irradiance-response relations were constructed and fit with a Michaelis-Menten equation. The log IR₅₀ (log irradiance that results in half-maximal pupillary response) for each irradiance-response curve was calculated and used to construct action spectra.

Multielectrode Array Recordings

mCry1^{-/-}; *mCry2*^{-/-} and *mPer1*^{-/-}; *mPer2*^{-/-} mice were euthanized on postnatal day (P) 8 by CO₂ narcosis followed by cervical dislocation. The dissected retina was placed on an array of 60 recording electrodes (Multi Channel Systems, Reutlingen, Germany) and was perfused with a bicarbonate-buffered physiologic solution as described.¹⁸ Both recording environment and perfusion fluid were maintained at 30.8°C. To suppress spontaneous activity during recording, the retina was kept under pharmacologic blockade using both glutamatergic and cholinergic inhibitors as described.¹⁸ Raw electrical signals in response to light stimuli provided by a xenon source (Sutter Instruments, Novato, CA) were amplified, filtered, and digitized through an A/D card (National Instruments, Austin, TX) and analyzed offline using custom software.³⁵

RESULTS

Retinal Degenerate Mice Lacking Cryptochromes Show Equivalently Reduced Light Sensitivity to All Wavelengths

If cryptochrome were functioning as a photopigment or an auxiliary photopigment in the inner retina, it would be expected to contribute to the action spectrum for inner retinal photoresponses. The action spectrum for pupillary light responses in *rdta*; *cl* mice,⁵ and for circadian phase shifting in

these animals¹⁷ is well fit with an opsin template with peak sensitivity of approximately 480 nm. Identical action spectra have been reported for ipRGC responses *in vitro*.^{7,18} To determine whether cryptochrome contributes to the shape of this action spectrum, irradiance response relationships were measured for seven wavelengths of light in *rd/rd* and *rd/rd*; *mCry1*^{-/-}; *mCry2*^{-/-} mice (Supplementary Fig. S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-8717/-/DC_Supplemental). Retinal degenerate mice lacking cryptochrome showed approximately 10-fold reduced sensitivity at all wavelengths compared with *rd/rd* mice alone. However, the shape of the resultant action spectrum was identical for retinal degenerate mice with and without cryptochromes (Fig. 1). This suggests either that cryptochrome does not substantially participate in the photoreceptive event in inner retinal photoreception (at least that mediating the pupillary light response) or that the action spectrum of cryptochrome is indistinguishable from that of melanopsin. The latter possibility appears unlikely given the flavin-based spectrum associated with cryptochrome, which is not fit by an opsin template.³⁶

Pupillary Light Responses of Circadian Clock Gene Mutant Mice with and without Outer Retinal Degeneration

To determine whether the reduced photic sensitivity of retinal degenerate mice lacking cryptochrome is specific to cryptochrome or generic to genes causing loss of free running circadian rhythmicity, we intercrossed *rd/rd* mice with *mPeriod1*^{-/-}; *mPeriod2*^{-/-} (*mPer1*^{-/-}; *mPer2*^{-/-}) and *Bmal1*^{-/-} mice and then backcrossed these mice to generate *Bmal1*^{-/-}; *rd/rd* and *mPer1*^{-/-}; *mPer2*^{-/-}; *rd/rd* animals. We measured pupillary light responses of *mPer1*^{-/-}; *mPer2*^{-/-}, *mPer1*^{-/-}; *mPer2*^{-/-}; *rd/rd*, *Bmal1*^{-/-}, and *Bmal1*^{-/-}; *rd/rd* mice to 470 nm blue light. Neither *mPer1*^{-/-}; *mPer2*^{-/-} nor *Bmal1*^{-/-} mice showed decrement in PLR sensitivity relative to wild-type animals (data not shown). Consistent with previous reports,²⁶ *rd/rd* mice showed approximately 1 log reduced sensitivity compared with wild-type animals. However, both *mPer1*^{-/-}; *mPer2*^{-/-}; *rd/rd* (Fig. 2A) and *Bmal1*^{-/-}; *rd/rd* (Fig. 2B) showed significantly reduced PLR compared with *rd/rd* mice. The loss of PLR sensitivity was slightly less than that seen between *rd/rd* and *mCry1*^{-/-}; *mCry2*^{-/-}; *rd/rd* mice (which was closer to 1 log²⁸) but was nonetheless significant. Therefore, reduced pu-

FIGURE 1. Action spectrum of pupillary light response of *rd/rd* and *rd/rd*; *mCry1*^{-/-}; *mCry2*^{-/-} (derived from data in Supplementary Fig. S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-8717/-/DC_Supplemental).

FIGURE 2. Irradiance-response relationship of pupillary light responsiveness in (A) *mPer1*^{-/-};*mPer2*^{-/-} and (B) *Bmal1*^{-/-} mice with and without compounded *rd/rd* mutation. *n* = 4 for all points. Error bars, SEM.

pupillary light responsiveness is a general finding in retinal degenerate mice with mutations rendering the circadian clock nonfunctional and not specific to a loss of cryptochrome function.

Circadian Clock Gene Mutations Do Not Alter Retinal Degeneration, ipRGC Number, or ipRGC Photosensitivity

To determine whether the loss of function in *rd/rd* mice with clock gene mutations is due to a deficiency at the level of the retina, the anatomic and functional integrity of the retina were analyzed. Histologic analysis of eyes from these mice demonstrated that all circadian clock mutant mice had grossly normal appearing inner and outer retinal architecture (Fig. 3). Cell counts in outer nuclear layer (rod and cone) columns and in the retinal ganglion cell layer revealed minimal difference in numbers of nuclei (Supplementary Table S1, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-8717/-/DCSupplemental>). All *rd/rd*-containing strains showed complete outer retinal degeneration with loss of all outer segments and preservation of less than a monolayer of the outer nuclear layer. No significant differences in numbers of retinal ganglion cells could be appreciated between retinal degenerate strains.

To determine whether clock gene mutations affect melanopsin expression, we stained retinas from wild-type,

Bmal1^{-/-}, *mPer1*^{-/-};*mPer2*^{-/-}, and *mCry1*^{-/-};*mCry2*^{-/-} mice with anti-melanopsin antiserum. As assessed by retinal flat mount, the morphology of melanopsin-containing ipRGCs was not changed by the presence of clock mutations (Supplementary Fig. S2, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-8717/-/DCSupplemental>). Quantitation of melanopsin-containing cells per retina showed no decrement from wild-type in any of the clock-mutant backgrounds. Indeed, though C57Bl/6 and *Bmal1*^{-/-} retinas had comparable numbers of ipRGC per retina (301 ± 24 and 304 ± 27 , respectively, *n* = 8 wild-type, *n* = 5 *Bmal1*^{-/-}), higher numbers of ipRGC per retina were observed in *mPer1*^{-/-};*mPer2*^{-/-} (419 ± 56 , *n* = 9) and *mCry1*^{-/-};*mCry2*^{-/-} (511 ± 84 , *n* = 6) retinas.

If the clock genes are necessary for ipRGC function, it is possible the response of the cells would be impaired in mutant cells. To assess ipRGC sensitivity, we measured irradiance response functions to 480 nm blue light of individual P8 ipRGCs ex vivo using multielectrode array recording.¹⁸ The sensitivity responses of multiple ipRGCs can be measured simultaneously from a single retina. P8 retinas are used because excessive spontaneous activity of non-ipRGCs in adult animals obscures ipRGC responses. Previous work has demonstrated that P8 ipRGC responses are melanopsin dependent and show an action spectrum identical with that for PLR and circadian phase shifting.¹⁸ Three types of ipRGC cells are physiologically distinguishable by multielectrode array recording in P8 retina: fast onset, relatively sensitive, fast off type (type 1, ~70% of cells); slow onset, insensitive, slow off type (type 2, ~15% of cells), and very fast onset, very sensitive, slow off type (type 3, ~15% of cells). All three types were present in statistically equivalent numbers in wild-type, *mCry1*^{-/-};*mCry2*^{-/-}, and *mPer1*^{-/-};*mPer2*^{-/-} retinas. Cell firing waveforms were qualitatively similar for all three genotypes (Supplementary Fig. S3, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-8717/-/DCSupplemental>). Irradiance response relationships to 480 nm light revealed no change in ipRGC sensitivity for type 1, 2, or 3 cells (Supplementary Fig. S3, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-8717/-/DCSupplemental>).

Pupillary Light Responses of *rd/rd* Mice Show Diurnal Variation

The finding that all three clock mutant backgrounds reduced the PLR sensitivity of *rd/rd* mice suggests a circadian modulation of PLR. To determine whether PLR of *rd/rd* mice is regulated by the circadian clock, we tested responses to multiple irradiances of 470 nm light at four times of day in the same cohort of mice, corresponding to zeitgeber times (ZT) 2, 8, 14, and 20 (Fig. 4). A significant circadian rhythm of PLR was observed, with sensitivity maximal during daytime at ZT2 and ZT8, and trough levels at ZT 14 and 20. The overall magnitude of sensitivity was approximately 0.5 log (Fig. 4).

Diurnal and Free Running Behavior of Circadian Clock Gene Mutant Mice with Outer Retinal Degeneration

To determine whether retinal degeneration exacerbated the behavioral phenotypes of mice with circadian mutations, we observed the wheel running behavior of mice lacking *mCry* genes, *mPer* genes, or *Bmal1* with and without *rd/rd*. Representative actograms from mice kept in a 12-hour light/12-hour dark cycle with a phase shift of the light-dark cycle and the dark-dark cycle are shown in Figure 5. Wild-type and *rd/rd* mice typically demonstrated entrainment with nocturnal phase preference in 12-hour light/12-hour dark, gradual re-entrainment to an altered phase of 12-hour light/12-hour dark, and free-running rhythmicity in dark-dark with a period of approximately 23.6 hours (Figs. 5A, 5B). In contrast, cryptochrome

FIGURE 3. Representative thin section retinal histology of (A) wild-type, (B) *mCry1*^{-/-};*mCry2*^{-/-}, (C) *mPer1*^{-/-};*mPer2*^{-/-}, (D) *Bmal1*^{-/-}, (E) *rd/rd*; *mCry1*^{-/-};*mCry2*^{-/-}, (F) *rd/rd*; *mPer1*^{-/-};*mPer2*^{-/-}, and (G) *rd/rd*; *Bmal1*^{-/-}. RPE, retinal pigment epithelium; OS, outer segment; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer.

mutant mice demonstrated an immediate shifting of phase with a new light cycle, as previously reported²⁷ (Fig. 5C). In contrast to the strong masking behavior seen in cryptochrome mutant mice, *Period* and *Bmal1* mutant mice both demonstrated relatively weak rhythmicity in light-dark conditions (Figs. 5E, 5G). When kept in constant darkness, *mCry1*^{-/-}; *mCry2*^{-/-}; *mPer1*^{-/-};*mPer2*^{-/-}, and *Bmal1*^{-/-} mice are behaviorally arrhythmic.^{37,38} In general *Bmal1*^{-/-} exhibited the least overall activity and the weakest rhythmicity, with one third of the animals showing no rhythmic behavior in any lighting condition. *Period* mutant mice had an intermediate phenotype exhibiting moderate overall levels of activity and some visible rhythmicity in every animal. In all cases, however, the three clock mutant phenotypes were indistinguishably arrhythmic in dark-dark.

The addition of outer retinal degeneration to clock mutation induced more arrhythmicity in light-dark conditions in some animals. With the addition of *rd/rd*, the robustness of masking in some cryptochrome mutants decreased as activity became less consolidated to dark hours. A subset of *mPer1*^{-/-}; *mPer2*^{-/-};*rd/rd* and *Bmal1*^{-/-};*rd/rd* became arrhythmic in all lighting conditions, whereas some mice retained some weak rhythmicity. With the addition of retinal degeneration, some *Bmal1*^{-/-} mice exhibited some rhythmicity in light-dark only but with a strange phase relationship; 2 of 6 mice had weak

rhythmicity in 12-hour light/12-hour dark, with activity onset at 1 hour after lights off, and two other mice exhibited a diurnal preference but with activity onset delayed to the middle of the light phase. A subset of *Bmal1*^{-/-};*rd/rd* mice exhibited arrhythmicity in all lighting conditions.

DISCUSSION

Cryptochromes belong to the photolyase family of blue-light photoreceptors and are found in organisms ranging from cyanobacteria to humans.³⁹ Cryptochromes were initially identified in *Arabidopsis* as a mutation causing loss of blue light-regulated hypocotyl growth.⁴⁰ Cloning of this locus revealed the founding members of the family, aCry1 and aCry2.²² Like photolyase, these proteins contain binding regions for potential chromophores MTHF and FAD. Biochemical studies have demonstrated that *Arabidopsis* cryptochrome functions as a light-dependent autokinase, confirming the photoreceptive function of this branch of the protein family.^{41,42} In *Drosophila* cryptochrome was identified in a screen for circadian rhythm mutations and was subsequently found not to affect the central circadian oscillator of the fly but to render it insensitive to light pulses.^{23,24} Although both *glass* mutant and *cryptochrome* mutant flies have reduced sensitivity to circadian

FIGURE 4. Diurnal rhythm of pupillary light response in *rd/rd* mice. (A) Irradiance response of pupillary light response at four times of day (ZT 2, 8, 14, 20). $n = 8$ per time point. (B) IR_{50} (irradiance required for 50% pupillary constriction) for individual *rd/rd* mice tested at four times in a 12-hour light/12-hour dark cycle. Error bars, SEM.

FIGURE 5. Representative diurnal and circadian wheel running behavior of circadian clock mutant mice with and without compounded *rd/rd* mutation. (A) Wild-type. (B) *mCry1*^{-/-}; *mCry2*^{-/-}. (C) *mPer1*^{-/-}; *mPer2*^{-/-}. (D) *Bmal1*^{-/-}. (E) *rd/rd*; *mCry1*^{-/-}; *mCry2*^{-/-}. (F) *rd/rd*; *mPer1*^{-/-}; *mPer2*^{-/-}. (G) *rd/rd*; *Bmal1*^{-/-}. Data are double plotted. Gray areas represent periods of light.

phase shifting to light, double mutants are oblivious to external lighting conditions.⁴³ Light sensitivity can be restored to flies by the expression of cryptochrome only in the circadian clock pacemaking cells in the lateral brain nuclei, suggesting cryptochrome function is cell autonomous.⁴⁴ In flies, cryptochrome is thought to function in mediating light-dependent degradation of the clock gene *timeless*. In Schneider 2 cell lines, transfected cryptochrome undergoes a light-dependent degradation, suggesting it is a functional photopigment in flies.^{36,45}

Several lines of argument have suggested that cryptochrome may function as a photopigment involved in circadian entrainment and other nonvisual functions in mice. First, cryptochrome family members 1 and 2 are expressed in the inner retina (and in many other locations).^{20,46} Second, vitamin A depletion studies have suggested that near-total depletion of the outer retina does not abrogate circadian entrainment or pupillary light responsiveness⁴⁷; however, loss of cryptochrome expression in a vitamin A-depleted animal reduces nonvisual photoreception.⁴⁸ Third, compared with retinal degenerate mice, retinal degenerate mice lacking cryptochromes show reduced *c-fos* activation in the suprachiasmatic nuclei after light pulse, have weaker behavioral masking responses to light, and show reduced pupillary light responses (although such animals do retain at least some photic sensitivity in all assays).^{26,28}

These findings must be reconciled with data demonstrating that the opsin family member melanopsin serves as a photopigment for the ipRGC. Unlike cryptochrome loss (which attenuates but does not eliminate nonvisual photoreception), in the setting of outer retinal degeneration or dysfunction, melanopsin loss completely abolishes nonvisual photoreception; mice with outer retinal degeneration or lacking outer retinal function that also lack melanopsin show no circadian entrainment or pupillary light responses.⁴⁹ Similarly, ipRGC function is eliminated in melanopsin mutant mice.⁵⁰ The action spectra for nonvisual photoreception matches the action spectrum of ipRGC function.⁷ Melanopsin also appears to form a functional photopigment when expressed in *Xenopus* oocytes or mammalian cell lines; in at least some of these lines, the melanopsin action spectrum matches that of ipRGC function.^{11,14} Thus, melanopsin appears both necessary and sufficient for nonvisual photoreception.

Cryptochromes have an additional strong phenotype in mice. Although *Drosophila* lacking cryptochrome still show a

functional circadian pacemaker,^{23,24} mice lacking cryptochromes show no free-running circadian rhythms.^{29,30} Cryptochromes bind with Period proteins and form the negative limb of a transcription-translation feedback loop, inhibiting transcription of their own genes mediated by BMAL and CLOCK proteins. Indeed, the argument has been made that cryptochrome function has changed during evolution from primarily a photoreceptive clock component to a “blind” core clock component. In this model, the effects of cryptochrome mutations on photoreception in *rd/rd* animals would be indirect; by affecting the circadian clock and those aspects of physiology under its control, photosensitivity might be reduced. Alternatively, cryptochrome may function in a pleiotropic manner, perhaps functioning as an accessory photopigment to modulate melanopsin-dependent photoreception.

The data in the present studies strongly support the first model that reduced nonvisual photoreception in cryptochrome mutant mice is a nonspecific consequence of loss of the circadian clock. Were cryptochrome functioning an accessory pigment, one would expect it to contribute to the action spectrum for nonvisual photoreception. In its absence, one might expect to see a shift in sensitivity. Both absorption spectra and action spectra suggest that the action spectrum of cryptochrome should dissociate from that of melanopsin at shorter wavelengths. However, we find that retinal degenerate mice lacking cryptochrome have an identical action spectrum shape (matching that of melanopsin); cryptochrome-mutant mice with retinal degeneration are simply 10-fold less sensitive to light at all wavelengths than animals with retinal degeneration alone.

A second strong prediction of this model is that loss of circadian clock function from other mutations should also affect nonvisual photoreception. We find this to be the case. Behaviorally, retinal degenerate mice lacking *Cryptochrome* function, *Period* function, or *Bmal1* all behaved similarly; indeed, it would be difficult to distinguish behaviorally among these three genotypes (although all are readily distinguished from wild-type and *rd/rd* mice). In each case, loss of the circadian clock gene alone diminished diurnal rhythmicity (residual rhythmicity in these mice is attributable to “masking” or the direct effect of light and darkness on locomotor behavior) and loss of outer retina compounded this arrhythmicity. Interestingly, as has been previously noted, *Bmal1*^{-/-} animals show very weak masking in light-dark activity.³³ This is mod-

erately compounded in mice with outer retinal degeneration. Similarly, the pupillary light response sensitivity of *mPer1*^{-/-}; *mPer2*^{-/-}; *rd/rd* mice and *Bmal1*^{-/-}; *rd/rd* mice is reduced compared with that of *rd/rd* mice alone (similar to what has been previously reported for *cryptochrome* mutant animals²⁸). This demonstrates that the loss of photosensitivity for both behavioral and pupillary light responses seen when *cryptochrome* mutations are compounded with outer retinal degeneration is not specific for *cryptochrome* but is seen in all mutations that eliminate the molecular circadian clock.

Although these results do demonstrate further interactions between the circadian clock and light-mediated behavior and physiology, they do not support the hypothesis that *cryptochrome* functions as a photopigment in the inner retina of the mouse. Rather, a functioning molecular circadian clock is a requirement for normal nonvisual photoreception, including behavioral entrainment, masking, and pupillary light responses. The finding that the photic sensitivity of ipRGC in vitro is not reduced in either animals lacking *cryptochrome* or *period* function further suggests that the reduced light sensitivity in *rd/rd* animals lacking these genes (compared with *rd/rd* alone) is mediated downstream of the ipRGC itself (although with the caveat that, for technical reasons, studied mice were early postnatal and we cannot exclude that clock gene function might influence adult ipRGC sensitivity). Measurement of pupillary light response in *rd/rd* animals at different times of day suggests a substantial circadian rhythm to this response (with increased sensitivity seen during the daytime hours). A parsimonious explanation for the loss of sensitivity seen in pupillary light responses of clockless mice with outer retinal degeneration is that loss of the clock nonspecifically "defaults" the sensitivity of the pupil response to a low level, comparable to nighttime sensitivity or less. In conclusion, the reduced PLR sensitivity and the confounded behavioral rhythmicity in circadian clock mutant *rd/rd* animals demonstrates that the circadian system modulates the response to nonvisual retinal output, and this likely occurs downstream of the retina.

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