Nonvisual light responses in the *Rpe65* knockout mouse: Rod loss restores sensitivity to the melanopsin system

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Intrinsically photosensitive retinal ganglion cells (ipRGCs) expressing the photopigment melanopsin (OPN4), together with rods and cones, provide light information driving nonvisual light responses. We examined nonvisual photoreception in mice lacking RPE65, a protein that is required for regeneration of visual chromophore in rods and cones. Although *Rpe65* knockouts retain a small degree of rod function, we show here that circadian phase shifting responses in *Rpe65*$^{-/-}$ mice are attenuated far beyond what has been reported for rodless/coneless mice. Furthermore, the number of melanopsin-immunoreactive perikarya and the extent of dendritic arborizations were decreased in *Rpe65* knockout mice compared with controls. To assess the nature of the photoreceptive defect in *Rpe65* null mice, we eliminated either rods or melanopsin from *Rpe65*$^{-/-}$ retinas by generating (*i*) *Rpe65*$^{-/-}$ mice carrying a transgene (*rdta*) that results in selective elimination of rods and (*ii*) double knockout *Rpe65*$^{-/-}$*;Opn4*$^{-/-}$ mice. Surprisingly, rod loss in *Rpe65* knockout mice resulted in restoration of circadian photosensitivity. Normal photoentrainment was lost in *Rpe65*$^{-/-}$*; Opn4*$^{-/-}$ mice, and, instead, a diurnal phenotype was observed. Our findings demonstrate that RPE65 is not required for ipRGC function but reveal the existence of a mechanism whereby rods may influence the function of ipRGCs.

circadian | entrainment | chromophore | isomerohydrolase

Entrainment of circadian rhythms, pupillary constriction, masking of locomotor activity, and suppression of pineal melatonin are examples of ''non-image-forming,'' light-dependent phenomena, so called because they persist in the absence of the photoreceptors that subserve vision, the retinal rods and cones (1). In rodless/coneless mice, these light responses depend on melanopsin (*Opn4*), an inner retinal opsin that is localized to a small subset of intrinsically photosensitive retinal ganglion cells (ipRGCs) (2–6). Melanopsin-containing ipRGCs send projections to brain nuclei that are involved in circadian entrainment and the pupillary light reflex (4, 7, 8). In melanopsin knockout mice, ipRGCs lose their photosensitivity; however, nonvisual photoresponses, although attenuated, are not lost (9, 10). Although these data indicate redundancy among rods, cones, and ipRGCs, the specific role(s) of each of these three photoreceptors and their possible interactions in nonvisual photoreception are not understood.

Heterologously expressed melanopsin forms a functional photopigment that signals through an invertebrate-like phosphoinositide phototransduction cascade (11–13). A retinaldehyde chromophore is necessary to achieve light responses, and although recent studies suggest that melanopsin may be a bistable photopigment that is able to reisomerize chromophore within the opsin molecule (12–14), we do not yet have a full understanding of chromophore regeneration in ipRGCs. In vertebrate rods, the light-sensitive chromophore 11-*cis*-retinaldehyde is bound to opsin apoprotein and is isomerized by light to *all-trans*-retinaldehyde. The *all-trans* product is released by the opsin and, in a series of enzymatic reactions taking place in both photoreceptor outer segments and the adjacent RPE (retinal pigment epithelium), is reisomerized into the 11-*cis* form. This process, the retinoid or visual cycle, is critical for sustained photoreception (for review, see ref. 15). Whether melanopsin shares components of the visual cycle is not known.

To determine whether melanopsin uses a pathway for chromophore regeneration that is similar to that of rods, we examined circadian photoentrainment in mice with targeted disruption of *Rpe65* (16), a gene encoding the recently identified retinoid isomerohydrolase (17–19). *Rpe65*$^{-/-}$ mice lose all cone function and almost completely lack rod function (20, 21). At high light intensities, a small electroretinographic signal can be detected from insensitive rods functioning through a 9-*cis*-retinaldehyde chromophore (16, 20, 22). Because circadian responses to light in rodless/coneless mice are unattenuated (23), we hypothesized that if melanopsin-containing ipRGCs recycled chromophore by using RPE65, then *Rpe65*$^{-/-}$ mice would show loss of circadian photosensitivity. Reduced sensitivity of the pupillary light reflex in *Rpe65*$^{-/-}$ mice lacking rod and cone function suggests that melanopsin is sensitive to RPE65 loss (21, 24).

Here, we report dramatically decreased circadian photosensitivity accompanied by inner retinal remodeling in *Rpe65*$^{-/-}$ mice. We characterize the photoreceptor input to the nonvisual photic system of *Rpe65*$^{-/-}$ mice by generating mice lacking both RPE65 and rods (*Rpe65*$^{-/-}$*;rdta*) or RPE65 and melanopsin (*Rpe65*$^{-/-}$;*Opn4*$^{-/-}$). We demonstrate that ablation of rods rescues the circadian and morphological defects resulting from RPE65 loss, and we propose mechanisms by which visual photoreceptors might alter ipRGC function in the *Rpe65*$^{-/-}$ retina.

Results

Locomotor Activity Rhythms. To determine whether loss of RPE65 affects circadian photoreception, we examined circadian locomotor activity rhythms in *Rpe65*$^{-/-}$, *Rpe65*$^{+/-}$, and *Rpe65*$^{+/+}$ mice under entrained and free-running conditions (Fig. 1 *A–C*). All three genotypes entrained to a 12-h light/12-h dark (LD) cycle; however, the phase angle of activity onset in relation to the LD cycle was altered for *Rpe65*$^{-/-}$ mice compared with the two control groups (Fig. 1*D*). Whereas control mice began their

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Abbreviations: ipRGC, intrinsically photosensitive retinal ganglion cell; LD, 12-h light/12-h dark; DD, constant darkness; CT, circadian time; IPL, inner plexiform layer; TBS, Tris-buffered saline.

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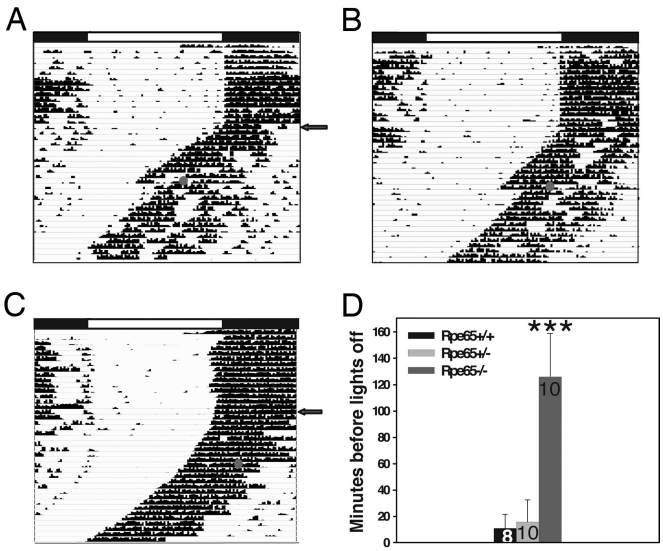

Fig. 1. Locomotor activity rhythms in *Rpe65* mice. (*A–C*) Representative running wheel records of WT (*Rpe65*$^{+/+}$) (*A*), *Rpe65* heterozygote (*Rpe65*$^{+/-}$) (*B*), and homozygous *Rpe65* knockout (*Rpe65*$^{-/-}$) (*C*) mice. Each horizontal line represents 24 h, with successive days plotted beneath each other. Activity was first recorded in a light/dark cycle (lights on at 0500 h and off at 1700 h) as indicated by open and filled bars at the top of each record. Animals were then transferred to DD (arrows) and given a 15-min light pulse (515 nm and 0.1 μW) at CT 16 (4 h after activity onset; filled circle). (*D*) Mean number of minutes (±SEM) by which activity onset preceded dark onset (ZT12). ***, $P < 0.001$ compared with controls, one-way ANOVA with post hoc Tukey's honestly significant difference test.

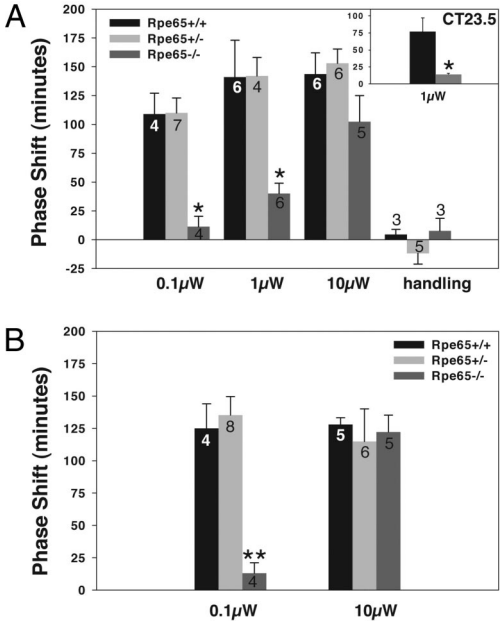

Fig. 2. Phase shifting to light is reduced in *Rpe65*$^{-/-}$ mice. (*A*) Magnitude of phase delays produced by 15 min of 515-nm light at three different irradiances. Handling controls were removed from their cages at CT 16 and placed in the light-pulse apparatus exactly as with experimental animals except that no light pulse was given. (*Inset*) Mean phase shift to a 15-min light pulse applied at CT 23.5, which shifts locomotor activity in the opposite direction (advance shift). (*B*) Phase shifts to 15 min of 480-nm light at CT 16. Error bars indicate SEM. *, $P < 0.05$ compared with controls; **, $P < 0.01$ compared with controls, one-way ANOVA with post hoc Tukey's honestly significant difference test. μW values are per cm^2.

nightly bout of activity just before lights off (*Rpe65*$^{+/+}$, 10.1 ± 8.6 min before lights off; *Rpe65*$^{+/-}$, 15.6 ± 16.0 min before lights off), activity onset in *Rpe65*$^{-/-}$ mice occurred significantly earlier (125 ± 32 min before lights off; $P = 0.0003$, ANOVA, post hoc Tukey's test). However, on the first day of constant darkness (DD), the time of activity onset did not differ among genotypes (*Rpe65*$^{+/+}$, 16.1 ± 0.1 h, $n = 6$; *Rpe65*$^{+/-}$, 15.5 ± 0.4 h, $n = 10$; *Rpe65*$^{-/-}$, 15.4 ± 0.2 h, $n = 9$; $P = 0.38$, ANOVA), suggesting that the knockout animals were not entrained with an advanced phase angle but were exhibiting light-dependent positive masking of locomotor activity. Alpha, the length of the activity phase, did not differ among genotypes (*Rpe65*$^{+/+}$, 7.96 ± 0.18 h; *Rpe65*$^{+/-}$, 8.16 ± 0.38 h; *Rpe65*$^{-/-}$, 8.50 ± 0.18 h; $P = 0.77$, ANOVA). Free-running period length in DD did not differ among *Rpe65*$^{-/-}$ mice and controls (*Rpe65*$^{+/+}$, 23.61 ± 0.07 h, $n = 6$; *Rpe65*$^{+/-}$, 23.56 ± 0.06 h, $n = 18$; *Rpe65*$^{-/-}$, 23.53 ± 0.08 h, $n = 9$; $P = 0.84$, ANOVA), suggesting that core circadian clock function in the knockouts was normal.

Phase Shifting Sensitivity. To test for possible defects in circadian photosensitivity of *Rpe65* knockout mice, we measured phase shifting responses to 15-min pulses of monochromatic light administered at circadian time (CT) 16, 4 h after activity onset in DD (Figs. 1 and 2). Light given at this time of the circadian day induces maximal delay shifts in mice (25). At a wavelength and irradiance that is subsaturating for both C57 WT mice and those lacking rods and cones [515 nm and 0.1 μW/cm^2 (23, 26)], *Rpe65*$^{+/+}$ and *Rpe65*$^{+/-}$ mice showed robust phase delays (109 ± 18 and 110 ± 13 min), whereas no phase shifts distinguishable from handling controls could be detected in *Rpe65*$^{-/-}$ mice (Fig. 2*A*; 11 ± 9 min). Similar results were found for a light pulse given at CT 23.5, a time when light produces phase advances instead of delays (Fig. 2*A Inset*; *Rpe65*$^{+/+}$, 76 ± 20 min, $n = 5$; *Rpe65*$^{-/-}$, 13 + 2 min, $n = 6$; $P < 0.03$, ANOVA, post hoc Tukey's test) and for a 480-nm light pulse of the same irradiance (Fig. 2*B*; *Rpe65*$^{+/+}$, 156 ± 11 min; *Rpe65*$^{+/-}$, 135 ± 14 min; *Rpe65*$^{-/-}$, 13 ± 8 min; $P = 0.001$, ANOVA, post hoc Tukey's test). However, as irradiance was increased to saturating levels (1–10 μW/cm^2), the magnitude of phase shifts in *Rpe65*$^{-/-}$ mice also increased, indicating that the circadian system of *Rpe65* knockout mice is capable of responding to high-intensity light as well as entraining to repeated long-duration light signals (Fig. 2). In response to 10 μW/cm^2 light, mean phase shift for knockout animals reached control levels (Fig. 2; at 515 nm, *Rpe65*$^{+/+}$, 143 ± 5 min; *Rpe65*$^{-/-}$, 102 ± 22 min; $P = 0.28$, ANOVA; at 480 nm, *Rpe65*$^{+/+}$, 128 ± 5 min; *Rpe65*$^{-/-}$, 122 ± 13 min; $P = 0.85$, ANOVA).

Melanopsin Immunocytochemistry. We identified melanopsin-immunoreactive retinal ganglion cells in flat-mounted retinas. Numbers of melanopsin-positive RGCs were decreased in *Rpe65*$^{-/-}$ mice compared with WT controls (Fig. 3*A*; $P < 0.0005$, ANOVA, post hoc Tukey's test). Furthermore, when patterns of dendritic arborization were examined in retinal cross sections, dendritic reorganization was evident in *Rpe65*$^{-/-}$ mice. In WT mice ($n = 6$), dendrites of melanopsin ganglion cells were evident in two substrata of the inner plexiform layer (IPL), the physiologic "ON" and "OFF" layers near the inner and outer regions of the IPL, respectively. Although melanopsin-immunostained dendrites were visible in the outer (OFF) plexus, staining of the inner (ON) plexus was greatly reduced in all *Rpe65*$^{-/-}$ mice ($n = 6$) compared with controls (Fig. 3*B*).

Circadian Photosensitivity and Melanopsin Cell Number Is Restored in *Rpe65*$^{-/-}$;*rdta* Mice. To determine the source of the much reduced photic input to the circadian system of *Rpe65*$^{-/-}$ mice, we eliminated rod photoreceptors in *Rpe65*$^{-/-}$ mice by crossing them with transgenic *rdta* mice, in which attenuated diphtheria

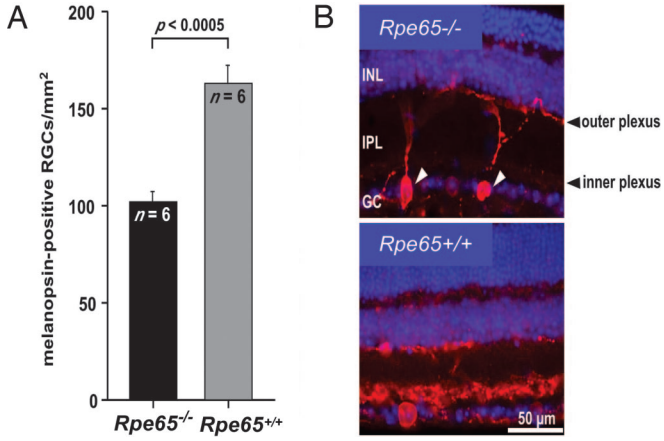

Fig. 3. *Rpe65*$^{-/-}$ mice have fewer melanopsin-positive ipRGCs than WT controls. (*A*) Number of immunofluorescent retinal ganglion cells per mm^2 estimated from flat-mounted retinas. Black bar, *Rpe65*$^{-/-}$; gray bar, *Rpe65*$^{+/+}$. (*B*) Vertical retinal sections showing melanopsin immunofluorescence in red. Cell nuclei are stained blue with DAPI. *Upper*, *Rpe65*$^{-/-}$; *Lower*, *Rpe65*$^{+/+}$. INL, inner nuclear layer; GC, ganglion cell layer.

toxin is expressed under the control of the rhodopsin promoter (27). Our crosses yielded littermates of four different genotypes: *Rpe65*$^{-/-}$, *Rpe65*$^{+/-}$, *Rpe65*$^{+/-}$;*rdta*, and *Rpe65*$^{-/-}$;*rdta*; these genotypes were used in phase shifting experiments identical to those described above. We reasoned that if rods were the source of light input for entrainment and phase shifting in the *Rpe65*$^{-/-}$ mice, then their ablation should result in complete loss of circadian photoresponses. To our surprise, the reverse occurred. *Rpe65*$^{-/-}$;*rdta* mice showed phase shifts in response to a 0.1 μW/cm^2 light pulse as large as those of control *Rpe65*$^{+/-}$ and *Rpe65*$^{+/-}$;*rdta* animals, whereas, as expected, the phase shifts of *Rpe65*$^{-/-}$ littermates were significantly reduced (Fig. 4 *A–C*; $P = 0.01$, ANOVA, post hoc Fisher's least significant difference test).

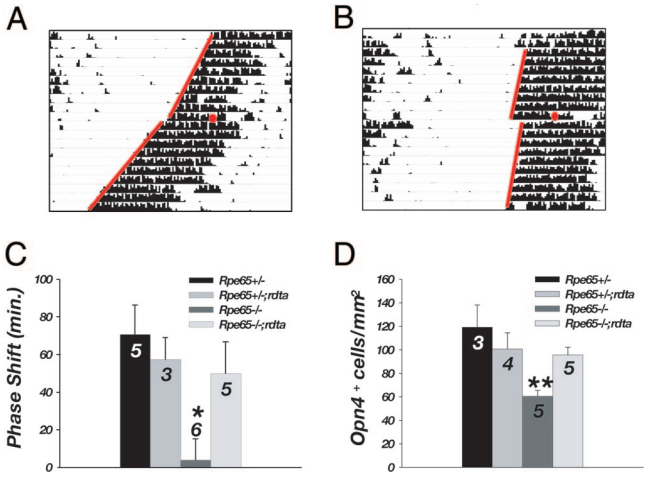

Fig. 4. Circadian photosensitivity and melanopsin cell number are restored in *Rpe65*$^{-/-}$ mice that lack rods. (*A–C*) Phase shifting responses to 15 min of 0.1-μW, 515-nm light at CT 16 in littermate *Rpe65;rdta* mice. (*A* and *B*) Wheel-running records of an *Rpe65*$^{-/-}$ (*A*) and *Rpe65*$^{-/-}$;*rdta* (*B*) mouse in DD. Filled circles indicate time of light pulse. Best-fit lines are drawn through activity onsets on the days before and after the pulse. (*C*) Histogram showing mean phase shifts ± SEM. *, $P < 0.05$ compared with the three control groups, ANOVA, post hoc Fisher's least significant difference test. (*D*) Mean number of melanopsin immunoreactive cells ± SEM estimated from flat mounts of *Rpe65;rdta* retinas. **, $P < 0.01$ compared with all controls, ANOVA, post hoc Tukey's honestly significant difference test.

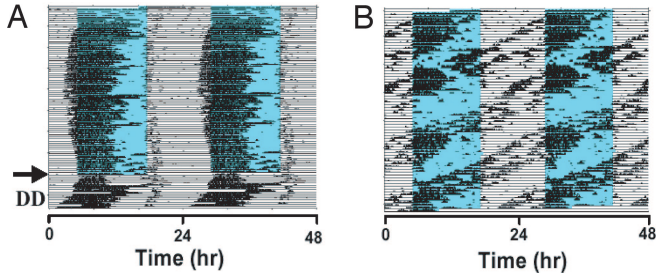

Fig. 5. Circadian phenotypes of *Rpe65*$^{-/-}$;*Opn4*$^{-/-}$ mice. Representative wheel-running records of a diurnally entrained (*A*) and a free-running (*B*) mouse. Arrow in *A* indicates day of release into DD. Records are double-plotted so that each horizontal trace represents 48 h; subsequent day's records are shown to the right and beneath the day before. The light/dark cycle (lights on at 0500 h and off at 1700 h) is indicated by shading in each actogram (blue shaded areas indicate times when lights are on).

The number of melanopsin-immunoreactive cells was also restored in *Rpe65*$^{-/-}$;*rdta* mice and did not differ from controls, whereas significantly fewer cells were once again observed in retinas of *Rpe65*$^{-/-}$ mice (Fig. 4*D*; $P < 0.05$, ANOVA, post hoc Tukey's test). Thus, rod loss resulted in restoration of both phase shifting sensitivity and melanopsin cell number.

Diurnal Phenotype of *Rpe65*$^{-/-}$;*Opn4*$^{-/-}$ Mice. To test whether the much reduced nonvisual photoresponses in *Rpe65*$^{-/-}$ mice could be attributed to input from the melanopsin ipRGCs, we examined circadian entrainment in *Rpe65*$^{-/-}$;*Opn4*$^{-/-}$ mice. In a LD cycle, mice lacking both RPE65 and melanopsin could not achieve the nocturnally entrained activity rhythms that are typical of normal mice. In LD at an intensity of white fluorescent light of 40 μW/cm^2, 30% of animals (7 of 22) restricted their activity to coincide with the light phase; the remaining 70% free-ran through the light/dark cycle while displaying positive masking (a light-dependent increase in activity; Fig. 5). When the intensity of the light phase of the LD cycle was increased to 150 μW/cm^2, most of the mice (12 of 14) adopted a diurnal phenotype. Activity onset for mice displaying the diurnal phenotype did not coincide exactly with the time of lights on but occurred slightly earlier. Furthermore, upon release into DD, activity onset for the free runs of all diurnal mice began from the time of lights on (Fig. 5*A*). The anticipation of lights on, coupled with a free run from diurnal phase, demonstrates that the activity rhythms of these mice were diurnally entrained and not simply masked by the light.

Triple Mutants (*Rpe65*$^{-/-}$;*Opn4*$^{-/-}$;*rdta*). Because a small number of cones persist in adult *rdta* retinas (27), we generated triple mutant *Rpe65*$^{-/-}$;*Opn4*$^{-/-}$;*rdta* mice to control for the possibility that cones were responsible for the nonvisual light responses observed in *Rpe65*$^{-/-}$;*rdta* mice. If, as reported by Seeliger *et al.* (20), cone function is eliminated in *Rpe65*$^{-/-}$ mice, nonvisual light responses in *Rpe65*$^{-/-}$;*rdta* mice should be the result of melanopsin input alone, and triple mutant mice without RPE65, melanopsin, and rods should show no circadian responses to light. We placed 2-month-old *Rpe65*$^{-/-}$;*Opn4*$^{-/-}$;*rdta* mice into a LD cycle followed by constant light of two different intensities (40 μW/cm^2, $n = 4$; 150 μW/cm^2, $n = 5$). In both light conditions, all animals free-ran through the light/dark cycle and showed no masking or other light responses upon transfer to constant light (Fig. 6). These results provide an independent confirmation of the absence of cone function in *Rpe65*$^{-/-}$ mice and demonstrate that *Rpe65*$^{-/-}$;*rdta* photosensitivity is not a result of input from cones.

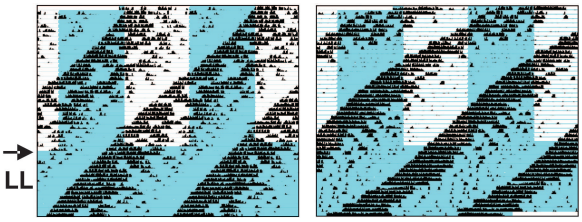

Fig. 6. Circadian locomotor activity of *Rpe65*$^{-/-}$;*Opn4*$^{-/-}$;*rdta* mice. Double-plotted locomotor activity records from representative animals in a light/dark cycle (lights on at 0500 h and off at 1700 h), followed by either dim (10 μW/cm^2, *Left*) or bright (100 μW/cm^2, *Right*) constant light. Blue shading indicates times when lights are on.

Discussion

In this study, we have shown that circadian photosensitivity in *Rpe65*$^{-/-}$ mice is decreased to a far greater degree than in other mouse models with more complete rod and cone loss or dysfunction (23, 26, 28–30). These results are consistent with previous reports of slowed reentrainment to a reversed LD cycle (31) and decreased sensitivity of transient and steady-state pupillary light reflexes in *Rpe65*$^{-/-}$ mice (24, 32). In the absence of rod and cone input, melanopsin-containing ipRGCs are required for the maintenance of nonvisual photic responses (5, 6). It follows that loss of nonvisual photosensitivity in the *Rpe65*$^{-/-}$ mice indicates loss of ipRGC sensitivity. Decreased ipRGC sensitivity in adult *Rpe65*$^{-/-}$ mice has also been demonstrated recently by Fu *et al.* (24), who showed a 20- to 40-fold decrease in magnitude of the light responses of individual ipRGCs.

Despite a pronounced decrease in ipRGC sensitivity in *Rpe65*$^{-/-}$ mice, the striking restoration of phase shifting responses that occurs when rod cells are removed by using the *rdta* construct demonstrates that ipRGCs are capable of functioning without RPE65 (at least well enough to maintain normal photoentrainment) and therefore that melanopsin can use a chromophore regeneration pathway that is different from that of rods. A likely possibility, suggested by evidence from recent studies (12–14), is that melanopsin can function as a bistable opsin that is capable of *in situ* photoisomerization of *all-trans*- to 11-*cis*-retinaldehyde in a manner similar to that of invertebrate opsins. Isomerization of 11-*cis*-retinal in the *Rpe65*$^{-/-}$ mouse has been reported in response to a bright flash of light (33). That response persisted in the absence of retinal G protein-coupled receptor (RGR), a known photoisomerase (33). Light activation of a bistable melanopsin might explain this result and the increased magnitude of phase shifts that we observed in *Rpe65*$^{-/-}$ mice as light intensity was increased (Fig. 2). Another possibility is that ipRGCs might regenerate chromophore by sharing retinoid processing enzymes with adjacent Müller cells. RGR has been localized to Müller cells (34) along with RDH10, an *all-trans* retinol dehydrogenase that generates *all-trans*-retinal, the substrate of RGR (35). A unique retinoid cycle has also been reported in cones, involving shuttling of retinoids between cone photoreceptors and retinal Müller cells (36). Because Müller cells span the entire retina, ipRGCs could have access to retinoid processing enzymes in these cells. Finally, melanopsin might use an alternate chromophore such as 9-*cis*-retinaldehyde. 9-*cis*-Retinal and its corresponding photopigment, isorhodopsin, accumulate in retinas of *Rpe65*$^{-/-}$ mice after prolonged dark adaptation and have been proposed as the source of the small light-evoked electroretinographic response recorded from these animals (22). 9-*cis*-Retinal could be produced from the overabundant supply of *all-trans*-retinal in the *Rpe65*$^{-/-}$ retina (37), but it has never been reported in WT retinas and was undetectable in *Rpe65*$^{-/-}$ mice maintained in cyclic lighting (22). However, if 9-*cis*-retinal were contained exclusively in ipRGCs, it would likely be undetectable by current chromatographic methods because of the very small number of these cells in the retina.

Our finding that rod loss restores sensitivity to the nonvisual system of *Rpe65*$^{-/-}$ mice suggests that rods were somehow inhibiting ipRGC function. If ipRGCs can function without RPE65-based chromophore recycling, then their decreased sensitivity in *Rpe65*$^{-/-}$ mice must be an indirect result of RPE65 loss. The most parsimonious explanation for this result is that chromophore-starved rods produce an indirect inhibition by depleting retinaldehyde from ipRGCs. Such a mechanism is consistent with our *Rpe65*$^{-/-}$;*rdta* result, because removal of rods would eliminate them as a chromophore sink and allow ipRGCs to retain their chromophore and function. It is also in agreement with data showing that 9-*cis*- or *all-trans*-retinaldehyde increases pupil sensitivity in *Rpe65*$^{-/-}$;*gnat1*$^{-/-}$;*cnga3*$^{-/-}$ mice (24).

Alternatively, a decrease in ipRGC sensitivity could be created by an inhibitory drive from rods onto ipRGCs. Lupi *et al.* (29) reported that mice carrying the *rdta* mutation exhibit an increased magnitude of circadian phase shifts compared with WT controls. In addition, *rdta* was accompanied by tonic activation of retinal ganglion cells, as measured by *c-fos* expression. Thus, even without the reduction of ipRGC sensitivity created by chromophore depletion in *Rpe65*$^{-/-}$ mice, it appears that rod loss resulting from the *rdta* transgene releases ipRGCs from inhibition. Another study reported that retinal degeneration slow (*rds*) mice, which carry a mutation in the *peripherin* gene that disrupts outer segment formation (38, 39), have a higher threshold for entrainment to light/dark cycles than *rd*/*rd* mice (40). Furthermore, suppression of activity in response to light (i.e., negative masking), a phenomenon believed to be mediated largely by ipRGCs (41), was decreased in *rds* mice compared with *rd*/*rd* mice. Unlike *rd*/*rd* mice, which carry a mutation in the rod-specific phosphodiesterase β-subunit that disrupts phototransduction (42), the degenerating rods of *rds* mice remain functional. The researcher therefore proposed that the presence of these rods, through an unknown mechanism, reduced sensitivity of the *rds* circadian system. Rod inhibition of ipRGCs in *rds* mice, which, like *Rpe65*$^{-/-}$ mice, also retain some rod function, could explain these results.

Although our data suggest that rods may inhibit ipRGC function, rod loss due to the *rd*/*rd* mutation does not alter circadian photosensitivity, and in the primate retina, ipRGCs are strongly activated by rods (43). A key difference between *rd*/*rd* and *rdta* retinas is the timing of photoreceptor loss, which begins earlier and proceeds more rapidly in *rdta* mice than in *rd*/*rd* mice. It has therefore been suggested that early rod loss may cause reorganization of the developing retina in such a way that circadian responsivity is increased (29). It is possible that abnormal rod signaling in *Rpe65*$^{-/-}$ mice may alter retinal development in an opposite way, reducing ipRGC sensitivity.

In support of this hypothesis, we found a small but significant decrease in the number of melanopsin-immunoreactive cells, accompanied by changes in the stratification of melanopsin-containing dendrites in the IPL of *Rpe65*$^{-/-}$ mice. Inner retinal remodeling has been described in mice with photoreceptor degeneration and includes retraction of deafferented bipolar cell terminals after photoreceptor cell loss, horizontal cell neurite sprouting, and, at advanced ages, retinal ganglion cell loss (44). Photoreceptor degeneration in *Rpe65*$^{-/-}$ mice is mild in the early weeks of life (the mice retain 8–9 rows of photoreceptor nuclei up to 14 weeks of age, vs. 10–11 rows in WT), and ganglion cell loss would not be expected at the age at which histology was performed in our study ($\approx$12 weeks). However, alterations in visual input have been shown to block the postnatal maturation of ganglion cell dendrites into appropriate ON or OFF sublaminae of the IPL (45). Dendrites of melanopsin-containing gan-

glion cells are postsynaptic to both bipolar and amacrine cells and thus may receive rod and/or cone input (46). Furthermore, in the mouse retina, ipRGCs receive rod input through amacrine cells in the off sublamina of the IPL (46). Rods in *Rpe65* knockout mice may behave as if under continuous background illumination because of activation of transducin by unliganded opsin (47). This abnormal input from rods in the $Rpe65^{-/-}$ retina may give rise to a developmental situation that results in selective stratification of ipRGC dendrites in the off layer of the IPL. One caveat to this interpretation is that loss of chromophore may affect melanopsin expression, and it is unclear whether the loss of immunostaining that we observed is due to loss of melanopsin expression in ipRGCs or loss of cell bodies. *In situ* hybridization for the melanopsin message, or immunostaining for pituitary adenylate cyclase-activating polypeptide, which is coexpressed in melanopsin-containing ipRGCs (48), could clarify this issue.

When melanopsin was eliminated from retinas of $Rpe65^{-/-}$ mice by generating $Rpe65^{-/-}$;$Opn4^{-/-}$ mice, photoentrainment was grossly abnormal, demonstrating that the residual ability of $Rpe65^{-/-}$ mice to entrain to light/dark cycles and to phase shift to bright light is largely the result of ipRGC input. Moreover, because cone function is completely lost in $Rpe65^{-/-}$ mice, the remarkable diurnal phenotype observed in $Rpe65^{-/-}$;$Opn4^{-/-}$ mice must stem from the desensitized rod system that remains after the loss of RPE65 function (20). If so, one might expect scotopic light levels below the sensitivity range of both cones and melanopsin to produce a similar effect in WT mice. Indeed, diurnal entrainment has been described in $C3H^{+/+}$ mice maintained at an illuminance of 0.1 lux (40).

In a recent review of temporal niche switching in nocturnal rodents, Mrosovsky and Hattar (49) report cases of diurnal behavior in mice lacking either melanopsin ($Opn4^{-/-}$) or both melanopsin and rod function ($Opn4^{-/-}$;$Gnat1^{-/-}$). Because $Opn4^{-/-}$ mice show deficits in negative masking (41) and mice with rod and cone degeneration lack positive masking (i.e., stimulation of activity by light) (28, 30), a diurnal phenotype could arise when negative masking is decreased by loss of melanopsin, leaving rod/cone-driven positive masking. How the circadian clock is then entrained to the diurnal phase remains to be explained.

It is clear from the data presented here that interactions exist between the different classes of photoreceptors that mediate nonvisual photoresponses. The different entrainment phenotypes we observed in $Rpe65^{-/-}$, $Rpe65^{-/-}$;*rdta*, and $Rpe65^{-/-}$;$Opn4^{-/-}$ animals suggest that nonvisual function may differ dramatically depending on the specific type and rate of photoreceptor loss and may have important clinical implications. Mutations in RPE65 result in several types of incurable human retinal degenerative diseases, including Leber's congenital amaurosis, autosomal recessive retinitis pigmentosa, and rod–cone dystrophy (50). Disturbed sleep patterns have been reported in patients with retinitis pigmentosa (51), and circadian rhythm and sleep disorders exist in blind patients (52). Our results suggest that patients with retinal degenerative disease could experience entrainment phenotypes that vary depending on the extent and type of photoreceptor loss. A more detailed understanding of the retinal pathways by which nonvisual light signals are processed may lead to therapies that are capable of ameliorating the consequences of nonvisual pathologies of the retina.

Materials and Methods

Animal Models. Littermate $Rpe65^{+/+}$, $Rpe65^{+/-}$, and $Rpe65^{-/-}$ mice were used in this study. Original breeding pairs were generously donated by Michael Redmond (National Institutes of Health, Bethesda). $Rpe65^{-/-}$;*rdta* mice were generated by cross-breeding $Rpe65^{-/-}$ mice with transgenic *rdta* mice (27) and intercrossing the resulting $Rpe65^{+/-}$;*rdta* mice to obtain $Rpe65^{-/-}$;*rdta*, $Rpe65^{+/-}$, $Rpe65^{-/-}$, and $Rpe65^{+/-}$;*rdta* littermates. $Rpe65^{-/-}$;$Opn4^{-/-}$ mice were generated by cross-breeding $Opn4^{-/-}$ animals, kindly provided by Samer Hattar (Johns Hopkins University, Baltimore) and King-Wai Yau (Johns Hopkins University School of Medicine, Baltimore), with $Rpe65^{-/-}$ mice. *Rpe65*, *rdta*, and *Opn4* genotypes were determined by PCR as described in refs. 4, 16, and 27. Animals used in these experiments were raised in our vivarium under a LD cycle with light intensities between 1 and 10 $\mu W/cm^2$. Experiments and procedures were carried out in accordance with Association for Assessment of Laboratory Animal Care policies and approved by the University of Virginia Animal Care and Use Committee.

Locomotor Activity Recording. Two-month-old male and female mice were placed in individual running wheel cages in light-tight boxes at 21°C and 50% relative humidity with ad lib food and water. Locomotor activity was measured as running wheel revolutions recorded in 1-min bins and analyzed with CLOCKLAB software (Actimetrics, Wilmette, IL). At the beginning of each experiment, activity was recorded for 7–14 days in a LD cycle with lights on at 0500 h and off at 1700 h. Mean light intensity from fluorescent bulbs (F40CW/RS/EW; Philips, Eindhoven, The Netherlands) was 40 $\mu W/cm^2$ at the level of the mouse in its cage. Procedures in the dark were carried out under infrared light.

Phase Shift Experiments. For phase shift experiments, animals in LD were released into DD. On the 10th to 12th day of DD, when mice were 80–82 days of age, they were removed from their cages under infrared illumination at CT 16 or 23 (4 or 11 h after activity onset) and given a 15-min monochromatic light pulse (515 or 480 nm; half bandwidth of 10 nm) at an irradiance of 0.1, 1.0, or 10 $\mu W/cm^2$ (26) as measured with a radiometer (UDT 350; United Detector Technology, Santa Monica, CA). CLOCKLAB software was used to fit lines through 10 days of activity onsets before and after the light pulse, and phase shifts were determined by measuring the difference in minutes between the two lines on the day after the light pulse. When pre- and postpulse free-running period (τ) differed by >30 min, data were excluded from analysis. Handling controls were treated identically, except that no light pulse was given. CLOCKLAB software was used to calculate free-running period over the first 20 days in DD from animals that received no light pulses.

Melanopsin Immunostaining and Cell Counts. The melanopsin-specific antiserum UF006, without affinity purification, was used for immunolabeling of mouse retinas (9).

Animals were killed, the eyes were quickly excised, and the corneas were removed. Eye cups were fixed at 4°C for 24 h in freshly prepared 4% paraformaldehyde (Electron Microscopy Sciences, Fort Washington, PA) in PBS. Lenses were removed, and retinas to be flat-mounted were dissected from eye cups, spread on filter paper, and processed in 1.5-ml microfuge tubes. Eye cups destined for sectioning were cryoprotected at 4°C for 24 h in 30% sucrose in PBS, embedded in OCT medium (Sakura Finetek, Torrance, CA), frozen, sectioned (16–20 μm), and thaw-mounted on gelatin-coated slides.

Flat mounts or sections were washed three times (10 min at 4°C) in Tris-buffered saline (TBS) (Quality Biological, Gaithersburg, MD) and blocked for 30 min at 4°C in 1.5% normal goat serum (Vector Laboratories) in TBS. After three washes in TBS (10 min at 4°C), the tissue was incubated for 24 h at 4°C in a 1:2,500 dilution of the antiserum in a TBS buffer containing 1% BSA, 0.25% carrageenan lambda, and 0.3% Triton X-100. The tissue was then washed three times in TBS (10 min at 22°C) and incubated for 1 h at 22°C in Cy3-conjugated anti-rabbit IgG antibody (Jackson ImmunoResearch) diluted 1:500 in TBS in-

cubating buffer. After three final washes in TBS (10 min at 22°C), flat mounts were removed from filter paper and transferred to glass slides. Both flat mounts and sections were mounted in DAPI-containing Vectashield (Vector Laboratories), coverslipped, and sealed with clear fingernail polish.

Images were captured on a Zeiss epifluorescence microscope equipped with a SPOT charge-coupled device camera. Image files were pseudocolored and enhanced for brightness and contrast by using PHOTOSHOP 6.0 (Adobe Systems, San Jose, CA). In each retinal quadrant, 3 pictures corresponding to an area of 0.61 mm^2 were taken sequentially, from the periphery to the center (optic nerve), for a total of 12 per retina. The cell count in each of the 12 frames was converted to cell number per mm^2 and averaged to give a mean cell count per mm^2 for each animal. Statistical comparisons of cell counts were made with GRAPHPAD INSTAT (GraphPad, San Diego).

Statistical Analysis. One-way ANOVA followed by a post hoc Tukey's honestly significant difference test or Fisher's least significant difference test was used to determine differences between groups. Null hypotheses were rejected at the 0.05 level. Values are expressed as mean ± SEM.

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