Retinal pathways influence temporal niche

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In mammals, light input from the retina entrains central circadian oscillators located in the suprachiasmatic nuclei (SCN). The phase of circadian activity rhythms with respect to the external light:dark cycle is reversed in diurnal and nocturnal species, although the phase of SCN rhythms relative to the light cycle remains unchanged. Neural mechanisms downstream from the SCN are therefore believed to determine diurnality or nocturnality. Here, we report a switch from nocturnal to diurnal entrainment of circadian activity rhythms in double-knockout mice lacking the inner-retinal photopigment melanopsin (OPN4) and RPE65, a key protein used in retinal chromophore recycling. These mice retained only a small amount of rod function. The change in entrainment phase of *Rpe65*$^{-/-}$*;Opn4*$^{-/-}$ mice was accompanied by a reversal of the rhythm of clock gene expression in the SCN and a reversal in acute masking effects of both light and darkness on activity, suggesting that the nocturnal to diurnal switch is due to a change in the neural response to light upstream from the SCN. A switch from nocturnal to diurnal activity rhythms was also found in wild-type mice transferred from standard intensity light:dark cycles to light:dark cycles in which the intensity of the light phase was reduced to scotopic levels. These results reveal a novel mechanism by which changes in retinal input can mediate acute temporal-niche switching.

circadian | diurnality | melanopsin | retina | Rpe65

In mammals, the external light:dark cycle is the predominant environmental cue that synchronizes the central circadian clock in the suprachiasmatic nuclei (SCN) of the hypothalamus to the 24-h solar day. The temporal niche (nocturnal, diurnal, crepuscular, etc.) occupied by an animal reflects the phase relationship between activity and the synchronized clock. Several fundamental features of the relationship of the SCN to light, such as the association between the timing of resetting light pulses and phase changes in the clock (i.e., phase-response curves), are very similar among mammalian species regardless of their temporal niche (1, 2). Daily rhythms of SCN electrical activity, metabolism, and expression of genes that comprise the core molecular clock mechanism are also similarly timed with respect to the external light:dark cycle in both nocturnal and diurnal mammals [for review, see Smale *et al*. (3)]. These similarities have led to the widely accepted idea that temporal niche is not controlled through pathways that communicate light information to the clock or by the response of the clock to light, but rather by neural mechanisms downstream from the SCN.

However, temporal-niche switching has recently been reported in mouse models of photoreceptor dysfunction, suggesting that input pathways may play a more important role in determining temporal niche than was previously believed (4–7). Retinal rods, cones, and intrinsically photosensitive retinal ganglion cells containing the photopigment melanopsin (OPN4) all provide light information for mammalian photo-entrainment (8, 9). We have previously reported a robust temporal-niche-switching phenotype in *Rpe65*$^{-/-}$*;Opn4*$^{-/-}$ mice with targeted deletion of both melanopsin and RPE65, the retinoid isomerohydrolase essential for recycling the 11-*cis*-retinaldehyde chromophore of rods and cones (7). *Rpe65*$^{-/-}$*;Opn4*$^{-/-}$ mice show either diurnal or free-running phenotypes, with the majority of animals (≈80%) displaying a diurnal activity pattern in 12-h-light:12-h-dark (LD12:12) cycles of 150 μW/cm^2 (7).

The sensitivity of the rod system in *Rpe65*$^{-/-}$ mice is decreased by more than three log units (10–12) and cone function is believed to be completely lost (10, 13, 14). In the absence of melanopsin, the remaining light input to the circadian system of *Rpe65*$^{-/-}$*;Opn4*$^{-/-}$ mice is most likely mediated by highly insensitive rods. Here, we characterized circadian and masking behaviors of *Rpe65*$^{-/-}$*;Opn4*$^{-/-}$ mice and tested a possible nonphotic mechanism for temporal-niche switching in these animals. Using *Rpe65*$^{-/-}$*;Opn4*$^{-/-}$ mice carrying the *mPer2*^{luciferase} knockin reporter (15), we demonstrate a reversal in the phase relationship between SCN clock gene expression in the SCN and the light cycle. We further show that in wild-type mice, dim light:dark conditions that should mimic the rod-only retinal phenotype of *Rpe65*$^{-/-}$*;Opn4*$^{-/-}$ mice lead to diurnal entrainment. Our data suggest that the temporal-niche switching in these two models results from channeling light through rod pathways and raises the possibility that intensity-dependent alterations in retinal light signaling may be a widespread mechanism for acutely regulating temporal niche.

Results

Reentrainment, Short Photoperiod Response, and Free-Running Period. When released into constant darkness (DD), *Rpe65*$^{-/-}$*;Opn4*$^{-/-}$ mice with diurnal activity patterns free-ran from the time of lights-on, indicating that their circadian clock was diurnally entrained (7). Clock entrainment in *Rpe65*$^{-/-}$*;Opn4*$^{-/-}$ mice was also seen when animals with diurnal activity patterns were subjected to a 4-h delay of the light:dark cycle (Fig. 1*A*). A slow reentrainment (43.8 ± 7 days, $n = 6$) of the circadian activity rhythm to the shifted light:dark cycle was apparent as activity onset moved from the beginning of the original light phase to the shifted light phase.

Because differences in free-running period length (τ) may affect the ability of *Rpe65*$^{-/-}$*;Opn4*$^{-/-}$ mice to entrain to light:dark cycles (16), we compared τ in DD in *Rpe65*$^{-/-}$*;Opn4*$^{-/-}$ and *Rpe65*$^{+/-}$*;Opn4*$^{+/-}$ mice that were entrained in a light:dark cycle and in *Rpe65*$^{-/-}$*;Opn4*$^{-/-}$ mice that free-ran throughout the light:dark cycle (Fig. 1*B*). τ over days 1–21 in DD did not differ between nocturnal double-heterozygous controls and diurnally entrained double knockouts (23.53 ± 0.10 h, $n = 6$ vs. 23.47 ± 0.11 h, $n = 8$), suggesting that clock function was normal in the double-knockout mice. However, τ in *Rpe65*$^{-/-}$*;Opn4*$^{-/-}$ mice that free-ran through the light:dark cycle was significantly

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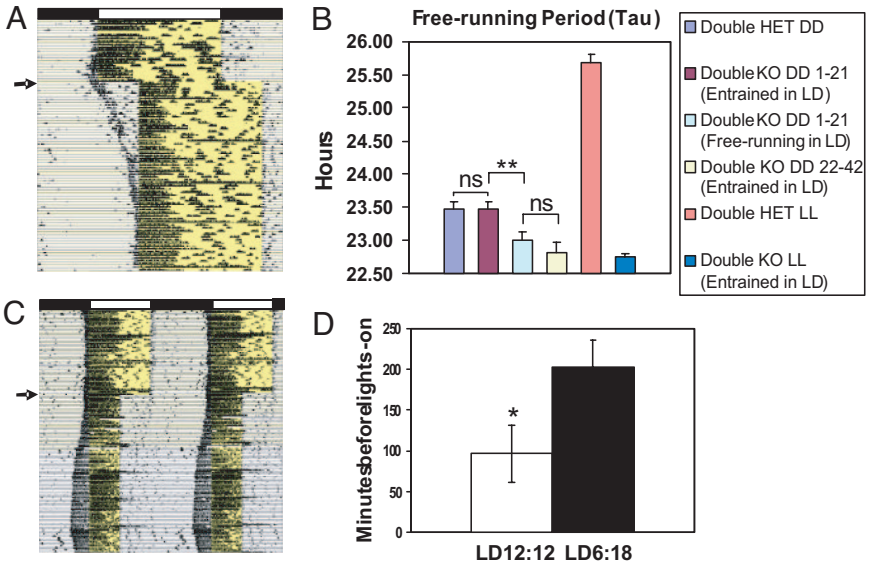

Fig. 1. Entrainment and free-running periods of *Rpe65*$^{-/-}$;*Opn4*$^{-/-}$ mice. (*A*) Wheel-running record of a representative *Rpe65*$^{-/-}$;*Opn4*$^{-/-}$ mouse housed for 19 days in a LD12:12 cycle before receiving a 4-h phase delay of the light:dark cycle (the arrow indicates the day of delay). Each horizontal line represents 24 h, with subsequent daily records plotted beneath each other. The original LD12:12 cycle is indicated by black (dark) and white (light) bars at the top of the record, and the light phase is highlighted in yellow within the activity record. Note the slow reentrainment and the immediate positive masking response. (*B*) Analysis of free-running periods in DD or LL for *Rpe65*$^{-/-}$;*Opn4*$^{-/-}$ (Double KO) and *Rpe65*$^{+/-}$;*Opn4*$^{+/-}$ (Double HET) mice. The free-running period was calculated over days 1–21 or days 22–42 in DD from animals previously entrained (entrained in LD) or free-running (free-running in LD) for 4 weeks in a LD12:12 cycle. The free-running period in LL was calculated over days 1–21 in LL after transfer from DD ns, not significantly different. **, $P < 0.01$. ANOVA, Tukey's post hoc comparison. (*C*) Running-wheel activity record of a representative *Rpe65*$^{-/-}$;*Opn4*$^{-/-}$ mouse maintained in a LD12:12 cycle for 40 days before being exposed to a short day photoperiod of 8-h-light:16-h-dark for 100 days. The arrow indicates the day of light cycle change. The record is double plotted so that each horizontal line represents 48 h, and successive days are shown to the right and beneath the previous day. The light:dark cycle is the same as that in *A*. (*D*) Histograms showing mean number of minutes (±SEM) by which activity onset preceded lights-on in *Rpe65*$^{-/-}$;*Opn4*$^{-/-}$ mice ($n = 7$) in LD12:12 (open bar) or LD6:18 (filled bar). *, $P < 0.05$, Student's *t* test. Activity onsets were calculated over the entire 40-day period in LD12:12 and the 100-day period in LD6:18.

shorter than that of the entrained groups (23.00 ± 0.13 h, $n = 10$ vs. 23.47 ± 0.11 h, $n = 8$; $P < 0.005$ ANOVA, Tukey's post hoc comparison). Because after-effects of entrainment may be responsible for the longer τ observed in the entrained groups, we also measured τ from diurnally entrained *Rpe65*$^{-/-}$;*Opn4*$^{-/-}$ mice maintained in DD for >3 weeks. τ of mice in 22–42 h DD (22.82 ± 0.16 h, $n = 4$) did not differ from that observed in *Rpe65*$^{-/-}$;*Opn4*$^{-/-}$ mice that free-ran in light:dark cycles (LD). The similarity in the free-running period of these two groups suggests that τ of double knockouts that free-ran throughout the light:dark cycle is not intrinsically different from that of diurnally entrained double-knockout mice once the after-effects of entrainment wore off. Upon transfer from DD to constant light (LL), *Rpe65*$^{-/-}$;*Opn4*$^{-/-}$ mice ($n = 4$) showed an absence of any free-running period change, whereas robust period lengthening was observed in nocturnal double-heterozygous controls ($n = 4$) (Fig. 1*B*). The amplitude of rhythmicity in LL, measured over a period of 30 days, was also greater for double knockouts than for double heterozygotes (fast Fourier transform relative power: 0.06 ± 0.01, $n = 6$ vs. 0.02 ± 0.009, $n = 5$; $P < 0.05$).

Because of the lack of RPE65, the retinoid isomerohydrolase critical for chromophore regeneration, the remaining functional photoreceptors in the *Rpe65*$^{-/-}$;*Opn4*$^{-/-}$ retina may bleach rapidly in the presence of light, producing the neural equivalent of one pulse of light every day instead of a full 12-h photophase. In mice, a single pulse of light given once a day will entrain circadian activity rhythms such that the beginning of daily activity coincides roughly with the light pulse (16). To test the possibility that this type of abnormal photoreceptor response to light may produce the observed diurnal phenotype, we transferred *Rpe65*$^{-/-}$;*Opn4*$^{-/-}$ mice from a LD12:12 cycle into a short-day photoperiod of 6-h-light:18-h-dark (LD6:18) (Fig. 1*C*). Under these conditions, photoperiodic changes in circadian activity rhythms were observed. The phase of activity onset became significantly earlier with lights-on compared to LD12:12 (96.4 ± 34 min before lights-on in LD12:12 vs. 203.2 ± 32 min in LD6:18; $P < 0.05$, *t* test, $n = 7$) (Fig. 1 *C* and *D*). Because the time of lights-on remained the same, these results indicate that *Rpe65*$^{-/-}$;*Opn4*$^{-/-}$ mice detect the duration, and not simply the onset, of the light phase. They further suggest that these mice are able to regenerate chromophore through an RPE65- and melanopsin-independent mechanism.

Masking to Light and Dark Pulses. In addition to its effect in entraining the circadian oscillator, light can also have direct stimulatory or suppressive effects on activity. These have been termed "masking" effects because although they do not entrain the clock, they can obscure (or mask) its true phase. In nocturnal species such as the mouse, light normally inhibits activity, whereas darkness stimulates it. To determine whether masking responses might be reversed in *Rpe65*$^{-/-}$;*Opn4*$^{-/-}$ mice, we tested masking to 3-h pulses of both light (150 μW/cm^2 white fluorescent light) and darkness in diurnally entrained *Rpe65*$^{-/-}$;*Opn4*$^{-/-}$ mice and nocturnal double-heterozygous controls. A light pulse given during the early night stimulated wheel running in *Rpe65*$^{-/-}$;*Opn4*$^{-/-}$ mice while suppressing it in controls (Fig. 2 *A–C*). Dark pulses, on the other hand, strongly suppressed activity in double knockouts (Fig. 2 *D* and *F*) but did not significantly increase the activity of controls (Fig. 2 *E* and *F*). These data show that masking responses to both light and dark are reversed in *Rpe65*$^{-/-}$;*Opn4*$^{-/-}$ mice. Moreover, the fact that circadian entrainment and masking, two separate nonvisual light responses, are reversed in *Rpe65*$^{-/-}$;*Opn4*$^{-/-}$ mice suggests a general reversal of the way in which the light signal is interpreted.

Activity-Dependent Entrainment. The apparent reversal of masking responses to light in *Rpe65*$^{-/-}$;*Opn4*$^{-/-}$ mice raises the question

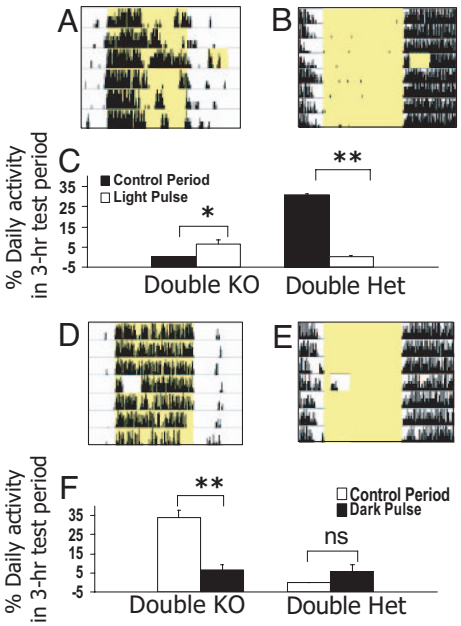

Fig. 2. Masking responses to 3-h light or dark pulses. (*A*, *B*, *D*, and *E*) Representative 24-h running-wheel records of a double-knockout *Rpe65*$^{-/-}$;*Opn4*$^{-/-}$ (*A* and *D*) and a double-heterozygous *Rpe65*$^{+/-}$;*Opn4*$^{+/-}$ (*B* and *E*) mouse administered either a 3-h light pulse in the early night or a 3-h dark pulse in the early part of a LD12:12 cycle. Subsequent days are plotted one beneath the other on the *y* axis. Yellow shading indicates time of lights-on. (*C* and *F*) Histograms showing mean percentage of daily wheel revolutions (±SEM) during the 3-h light or dark pulse and during the same 3-h control period on the day before the pulse. *, $P < 0.05$; **, $P < 0.001$, Student's *t* test.

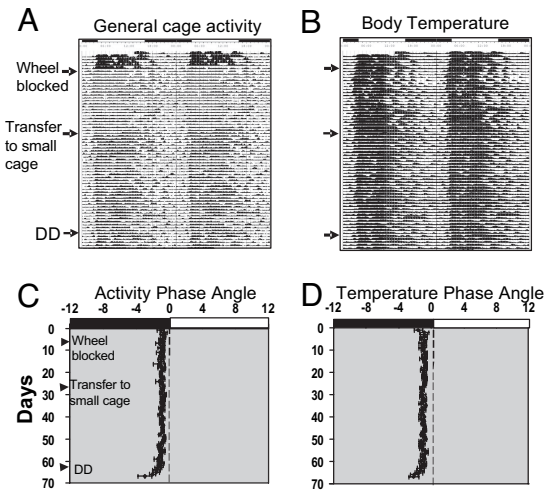

Fig. 3. Wheel blocking does not affect the entrainment phase of *Rpe65*$^{-/-}$;*Opn4*$^{-/-}$ mice. (*A* and *B*) Double-plotted records showing daily cage activity (*A*) and body temperature rhythms (*B*) from a representative *Rpe65*$^{-/-}$;*Opn4*$^{-/-}$ mouse implanted with a transponder. The arrows indicate the day of wheel blocking, transfer to a small cage without a wheel, and release into DD. (*C* and *D*) Mean phase angles (±SEM) of daily cage activity (*C*) and body temperature elevation (*D*) ($n = 5$). Phase angle is defined as the difference in hours between the daily onset of activity or body temperature increase and the light:dark cycle (where the time of lights on is 0 h). The light:dark cycle (lights on from 04:00–16:00) is shown by black (dark) and white (light) bars at the top of each record.

of whether activity that is directly stimulated by light can feed back onto the circadian clock to entrain it to a diurnal phase. Such activity-dependent entrainment has previously been demonstrated in rodents in response to vigorous wheel-running activity (17–19). We tested for activity-induced entrainment in diurnally entrained *Rpe65*$^{-/-}$;*Opn4*$^{-/-}$ mice by blocking access to running wheels while simultaneously recording circadian rhythms of body temperature and overall cage activity by using intraabdominal transponders (Fig. 3). In LD12:12 with wheels freely available, *Rpe65*$^{-/-}$;*Opn4*$^{-/-}$ mice exhibited diurnal rhythms of both activity and body temperature. When wheels were blocked, an expected decrease in amplitude of the activity rhythm was observed; however, the phase of entrainment was unchanged and remained unchanged even after the animals were transferred to smaller cages without a wheel. Upon release into DD, the mice began to free-run from their diurnal phase. These data indicate that the diurnal entrainment of *Rpe65*$^{-/-}$;*Opn4*$^{-/-}$ mice did not depend on the stimulation provided by wheel-running activity.

Clock Phase in SCN and Peripheral Tissues. In addition to the SCN, many peripheral tissues contain self-sustained circadian oscillators whose rhythms maintain specific phase relationships with the SCN. These phase relationships are lost after SCN lesion, indicating that the SCN coordinates the phases of peripheral oscillators (15). To assess the level at which the nocturnal-to-diurnal switch had occurred in *Rpe65*$^{-/-}$;*Opn4*$^{-/-}$ mice, we measured bioluminescence rhythms from cultured SCN and peripheral tissues of diurnally entrained *Rpe65*$^{-/-}$;*Opn4*$^{-/-}$ and nocturnal control mice carrying the real-time clock gene reporter *mPer2*^{luc} (15). Prior to culture, both experimental and control groups were stably entrained to LD12:12 for a minimum of 3 weeks. In static culture, explanted SCN, pineal gland, cornea, and lung exhibited robust circadian rhythms of bioluminescence over several days. A clear phase difference was apparent between all cultured tissues of diurnally entrained *Rpe65*$^{-/-}$;*Opn4*$^{-/-}$;*mPer2*^{luc} mice and those of the nocturnal control animals (Fig. 4 *A–D*). We examined the phase of PER2::LUC-expression rhythms in the SCN and in the periphery by measuring the peak of the bioluminescence rhythm on the second day of culture (Fig. 4*E*). Peak PER2::LUC expression in the SCN of control mice occurred near the time of the light-to-dark transition, in agreement with data from the original characterization of this reporter by Yoo *et al*. (15). In contrast, SCN peak phase in the *Rpe65*$^{-/-}$;*Opn4*$^{-/-}$;*mPer2*^{luc} mice was 9.5 h out of phase compared with wild-type and double-heterozygous controls. These phase differences are consistent with the difference in the phase angle of locomotor activity observed between *Rpe65*$^{-/-}$;*Opn4*$^{-/-}$ mice and control animals. As previously reported by Yoo *et al*., we found that peak expression of PER2 in peripheral tissues was phase-delayed relative to the SCN. However, the magnitude of the phase differences was significantly greater in *Rpe65*$^{-/-}$;*Opn4*$^{-/-}$ mice compared with control animals ($P < 0.005$; ANOVA, Tukey's post hoc comparison). These differences may reflect a more decentralized regulation of circadian oscillators in the periphery. Peak phases of tissues from *Rpe65*$^{+/-}$;*Opn4*$^{-/-}$;*mPer2*^{luc} and *Rpe65*$^{-/-}$;*Opn4*$^{+/-}$;*mPer2*^{luc} mice that also entrain nocturnally in LD cycles were not different from those of the nocturnal control animals shown in Fig. 4 (data not shown).

Diurnal Entrainment of Wild-Type Mice in Dim LD Cycles. Diurnal entrainment occurs in *Rpe65*$^{-/-}$;*Opn4*$^{-/-}$ mice in which rods are believed to provide the only photoreceptor input to the circadian system. We therefore hypothesized that reducing light levels below the thresholds of cones and melanopsin would produce a diurnal phenotype in wild-type mice. To test this hypothesis, we placed 12 C57BL/6J wild-type mice into running wheels in a standard LD12:12 cycle of 150 μW/cm^2 for 2 weeks and then decreased the intensity of the light during the 12-h light phase to 0.0001 μW/cm^2. This intensity is approximately three orders of

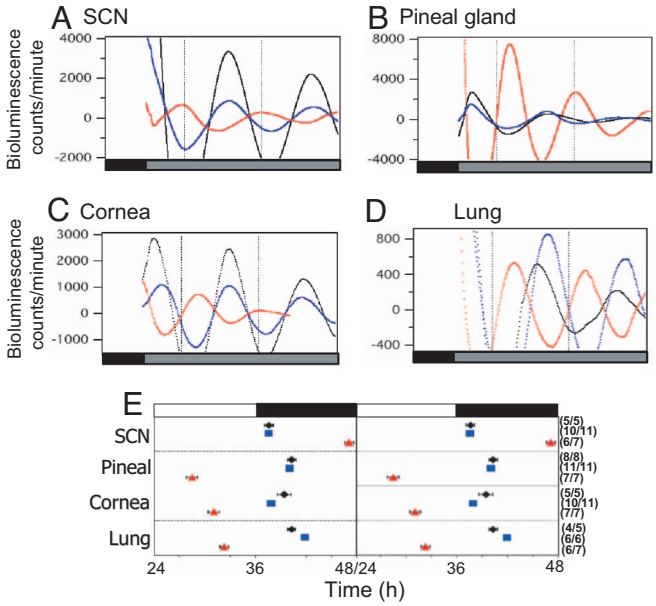

Fig. 4. Real-time monitoring of mPER2::LUCIFERASE expression. (*A–D*) Representative records of detrended mPER2::LUC bioluminescence rhythms from different tissues of one diurnally entrained *Rpe65*$^{-/-}$;*Opn4*$^{-/-}$; *mPer2*^{luc} mouse (red) and two nocturnal controls, a *mPer2*^{luc} knockin mouse (black) and one *Rpe65*$^{+/-}$;*Opn4*$^{+/-}$;*mPer2*^{luc} mouse (blue). (*E*) Peak phases of mPER2::LUC bioluminescence rhythms from SCN and pineal gland oscillators of *mPer2*^{luc} knockin (black diamonds), *Rpe65*$^{+/-}$;*Opn4*$^{+/-}$;*mPer2*^{luc} (blue squares), and *Rpe65*$^{-/-}$;*Opn4*$^{-/-}$;*mPer2*^{luc} (red triangles) mice. Data are double plotted for ease of interpretation. The mean time of peak (±SEM) is plotted against the time of the light:dark cycle, which is indicated by black (dark) and white (light) bars above the graph, on the day of the tissue explant. The phase of the rhythm peak was determined during the 24–48 h interval after the start of each culture. The number of rhythmic cultures out of the number of total cultures tested is indicated in parentheses to the right of each data point.

magnitude below the threshold estimated for cone pathways (20 and Maureen McCall, personal communication) and the threshold of the intrinsic melanopsin light response (21). We found that after entraining nocturnally to the standard-intensity LD cycle, 4 of 12 wild-type mice reentrained diurnally to the dim LD cycle. Diurnal entrainment occurred after 6–8 weeks of what appeared to be a free-run through the LD cycle (Fig. 5 *A* and *B*). When released into DD, the activity rhythms of these mice free-ran from the diurnal phase, indicating that the circadian clock was indeed diurnally entrained. Although 8 of 12 mice did not entrain diurnally, their activity was nonetheless strongly affected by the dim light:dark cycle. Two of these mice free-ran while exhibiting relative coordination to the light phase [supporting information (SI) Fig. S1 *A* and *B*]. In the remaining six mice, changes in the phase angle of activity and increases in activity at the light:dark transitions were observed (Fig. S1 *C* and *D*).

Discussion

We have previously reported that ≈80% of *Rpe65*$^{-/-}$;*Opn4*$^{-/-}$ mice show diurnal entrainment in a light:dark cycle of 150 μW/cm^2,whereas 20% free-ran (7). When the light intensity was reduced to 40 μW/cm^2, only 30% of mice entrained with a diurnal phase, whereas 70% free-ran (7), suggesting that the light intensity used in our experiments is close to the threshold which these animals are able to perceive. Here, we show that diurnally entrained *Rpe65*$^{-/-}$;*Opn4*$^{-/-}$ mice exhibit a reversal of both masking responses to light and the timing of SCN mPER2 expression with respect to the external LD cycle and that ≈30% of wild-type mice housed in dim LD cycles also exhibit diurnal entrainment. Collectively, these data suggest that the diurnal entrainment of both *Rpe65*$^{-/-}$;*Opn4*$^{-/-}$ mice and wild-type mice in dim LD is the result of channeling light through rod pathways and indicate that retinal pathways themselves can influence an organism's temporal niche.

In wild-type mice, two types of light-dependent masking have been described: suppression of activity by high intensity light (negative masking), mediated primarily by the melanopsin system (22), and stimulation of activity by lower intensity light (positive masking), driven by rods and/or cones (6, 23). In *Rpe65*$^{-/-}$ mice, the rod system remains functional even under photopic light conditions, although sensitivity is greatly reduced (10). The high levels of activity observed during the light phase in *Rpe65*$^{-/-}$;*Opn4*$^{-/-}$ mice might result from the loss of melanopsin-driven negative masking while rod-mediated positive masking is promoted (7). Here, we showed that *Rpe65*$^{-/-}$;*Opn4*$^{-/-}$ mice exhibited positive masking (i.e., enhancement of activity) by light but also a remarkable suppression of activity by darkness. Two recent studies have reported that masking responses to light do not require an intact SCN or a functional circadian clock (24, 25). One of these studies (24) was carried out in hamsters; however, both suggest that circadian entrainment and masking may be mediated by different brain areas. If so, then the reversal of both of these nonvisual responses in *Rpe65*$^{-/-}$;*Opn4*$^{-/-}$ mice suggests that a general reversal in the response to the light signal at the level of the retina has occurred.

Entrainment of the free-running circadian clock by wheel-running activity has been demonstrated in wild-type mice and in anophthalmic mice (17–19). In these animals, intervals of wheel availability that recur every 24 h entrain the circadian system in such a way that onset of daily activity precedes the wheel-running

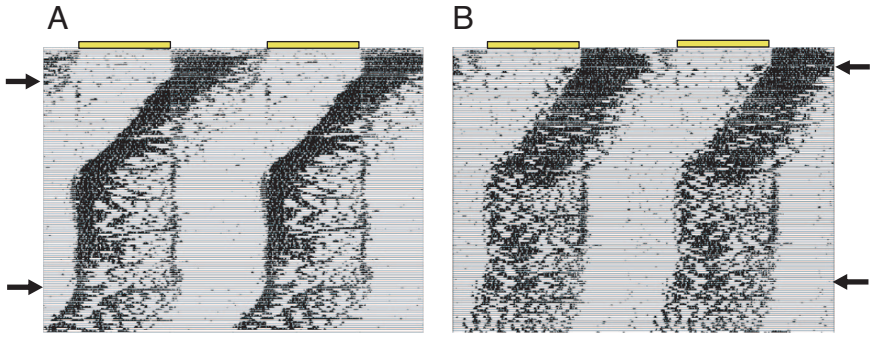

Fig. 5. Double-plotted wheel-running records of two wild-type C57BL/6J mice that entrained diurnally to the dim-light:dark cycle. Yellow bars at the top of each record indicate the time of lights-on. For the first 2 weeks of each record, animals were housed in a standard-intensity (150 μW/cm^2) LD12:12 cycle. On the days indicated by the upper arrows, the light intensity during the light phase was dimmed to 0.0001 μW/cm^2 by covering the fluorescent bulbs with a neutral density filter. On the day indicated by the lower arrow, mice were released into DD.

bout by several hours. In mice entrained by periods of wheel availability, activity occurred on average 9 h after the onset of general activity. This phase relationship and the ability to entrain also varied as a function of each animal's individual free-running period (17, 18). However, in diurnally entrained *Rpe65*$^{-/-}$;*Opn4*$^{-/-}$ mice, the onset of general activity, wheel running, and body temperature rhythms coincided. Moreover, free-running periods in long-term DD did not differ between *Rpe65*$^{-/-}$;*Opn4*$^{-/-}$ mice that entrained to LD cycles and those that failed to entrain, and upon wheel blocking, we observed no change in the phase or period of the activity rhythms of diurnally entrained mice. These results indicate that wheel-running activity is not required to entrain the circadian clock of *Rpe65*$^{-/-}$;*Opn4*$^{-/-}$ mice to a diurnal phase and argue against a nonphotic mechanism of entrainment.

The question of whether there are differences in the phase of SCN clock gene expression between diurnal and nocturnal mammals that might lead to diurnal or nocturnal activity patterns has been investigated in studies examining clock gene phase in several different day-active species (26–31). In all of these species, rhythms of *Period* gene expression in the SCN peaked at approximately the same time of day as in nocturnal rodents, suggesting that the phase of clock gene expression in the SCN relative to the light:dark cycle is conserved across mammalian groups regardless of whether they are diurnal or nocturnal. We found that the PER2::LUC rhythm in the SCN of diurnally entrained *Rpe65*$^{-/-}$;*Opn4*$^{-/-}$ mice peaked near the time of lights-on instead of near lights-off, as has been previously reported in nocturnal mice (15). This reversal in the phase relationship between the LD cycle and the PER2 peak with respect to what has been described for both nocturnal and diurnal animals suggests that the nocturnal-to-diurnal switch in *Rpe65*$^{-/-}$;*Opn4*$^{-/-}$ mice is the result of changes upstream of the SCN.

A reversal of the phase relationship between *Period* gene expression in the SCN and the LD cycle has also been found in a subset of mole rats, *Spalax ehrenbergi*, a subterranean rodent with atrophied eyes and massive reduction in visual sensitivity (30). Individuals in this subset display nocturnal activity patterns in contrast to the diurnal pattern typical of this species (30). The authors of that study suggested that the upstream switch is a unique characteristic of the *S. ehrenbergi* clock, reflecting the animal's subterranean ecotope. Our data suggest that such a switching mechanism may be more widespread. In support of this idea, Bachleitner *et al*. (32) recently reported a shift of morning and evening activity peaks in *Drosophila melanogaster* exposed to dim-light:bright-light cycles. The shift in activity was accompanied by a shift in the phase of clock gene expression in a subset of pacemaker neurons.

In addition to the diurnal entrainment that we report here in *Rpe65*$^{-/-}$;*Opn4*$^{-/-}$ and wild-type mice, there are several documented examples of complete reversals of temporal niche in nocturnal rodents (6, 33–35) and in a nocturnal primate (*Eulemur albifrons*) exposed to dim LD (36). Increased activity during the light phase also occurs in mice with severe vitamin-A depletion (4, 5) and in mice lacking cryptochromes (37). Because rods, cones, and melanopsin require a vitamin-A chromophore, vitamin-A depletion may reduce both outer retinal photoreceptor and melanopsin function to produce a phenotype similar to that of *Rpe65*$^{-/-}$;*Opn4*$^{-/-}$ mice. It is unclear, but very interesting, how the loss of cryptochromes, which are key components of the molecular clock mechanism in mammals (38–40), might produce a diurnal phenotype. Mice lacking *Bmal1*, another component of the molecular clock, also show an increase in the percentage of activity that occurs during the light phase in light:dark cycles (41).

The finding that manipulation of light intensity can produce temporal-niche switching in wild-type animals suggests that a common mechanism produces phase reversals in *Rpe65*$^{-/-}$;*Opn4*$^{-/-}$ mice and in wild-type mice during dim LD cycles. It further demonstrates that normal photoreceptor input channels can be manipulated in such a way as to affect entrainment phase. It is conceivable that species-specific changes in phase resulting from changes in light intensity may be a widespread mechanism for regulating temporal niche.

Materials and Methods

Animals. All procedures used in this study were approved by the University of Virginia Animal Care and Use Committee and are in compliance with guidelines established by the Association for Assessment of Laboratory Animal Care. Mixed strain (C57BL6 × 129s) *Rpe65*$^{-/-}$;*Opn4*$^{-/-}$ mice from our colony and double-heterozygous littermate controls were used in these experiments. For real-time reporting of PERIOD2::LUCIFERASE, *Rpe65*$^{-/-}$;*Opn4*$^{-/-}$ mice were crossed with *mPer2*^{luc} knockin mice kindly provided by Joseph Takahashi (Northwestern University, Evanston, IL). The resulting *Rpe65*$^{+/-}$;*Opn4*$^{+/-}$;*mPer2*^{luc} mice were crossed with *Rpe65*$^{-/-}$;*Opn4*$^{-/-}$ mice to obtain experimental *Rpe65*$^{-/-}$;*Opn4*$^{-/-}$;*mPer2*^{luc} mice and double-heterozygous littermate controls. PCR genotyping was performed according to published protocols (15, 42, 43). All mice used in these experiments were 2–4 months of age.

Running-Wheel Activity Recording. Mice were housed in individual running-wheel cages in light-tight boxes with temperature and relative humidity set at 21°C and 50%, respectively. Food and water were available ad libitum. Locomotor activity was measured as the number of wheel revolutions recorded in 1-min bins and analyzed with Clocklab software (Actimetrics). Mean intensity from fluorescent bulbs (Philips F40CW/RS/EW) was 150 μW/cm^2 measured at the level of the mice in their cages. To reduce light intensity, fluorescent bulbs were wrapped with black plastic sheeting. Transmittance over the visible spectrum was measured with a USB4000 Spectrometer (Ocean Optics) and remained unchanged after the addition of these filters. Procedures in dim light and in the dark were carried out with the aid of infrared viewers.

Masking to Light and Dark Pulses. Three-hour pulses of either light (white fluorescent, 150 μW/cm^2) or dark were administered to mice that had been maintained in LD12:12 for at least 2 weeks, by either turning lights on for 3 h from 1–4 h after the time of lights-off or by turning lights off for 3 h from 1–4 h after the time of lights-on. Masking responses were determined by calculating the percentage of daily activity (i.e., the number of wheel revolutions during the pulse divided by the total number of revolutions for that day times 100) during the 3-h pulse and during the same 3-h control period on the previous day.

Wheel Blocking. Five *Rpe65*$^{-/-}$;*Opn4*$^{-/-}$ mice that had been diurnally entrained for at least 3 weeks were implanted intraabdominally with a battery-free transponder (model G2 E-mitter, Mini Mitter) for telemetric recording of body temperature and gross motor activity. Immediately after recovery from surgery, animals were returned to their home running-wheel cages (47 × 26.5 × 20.5 cm). During this time, animals had free access to the running wheel. Body temperature and gross motor activity were collected in 10 min bins by using a Vital View telemetry system (Mini Mitter). After 5 days, running wheels were blocked and mice were monitored for ≈3 additional weeks. The mice were then placed into small cages (29 × 18 × 12.5 cm) without a running wheel and monitored for 5 more weeks. After this time period, lights were turned off and body temperature and activity were monitored for 5 days in DD to assess the phase of circadian entrainment. Data analysis was performed by using Clocklab and ActiView (Mini Mitter) software. Phase angles for general cage activity and body temperature were determined by measuring the number of minutes between activity onset or body-temperature rise and the time of lights-on.

PER2::LUC Recordings. The running-wheel activity of *Rpe65*$^{-/-}$;*Opn4*$^{-/-}$;*mPer2*^{luc} mice, control *Rpe65*$^{+/-}$;*Opn4*$^{+/-}$;*mPer2*^{luc} mice, and *mPer2*^{luc} mice in LD12:12 was recorded for 3–4 weeks. Animals were anesthetized with CO_2 and decapitated approximately 1 h before lights-off, a time of day chosen for previous experiments of this type to minimize disruption to tissues caused by a light-to-dark transition in the middle of the day (44). For pineal dissection, the pineal on the skull cap was removed and placed in cold Hanks' balanced salt solution (HBSS). The pineal gland was identified and separated from the meninges under a dissecting microscope. For SCN dissection, brains were rapidly removed and placed in cold HBSS. Coronal sections (300 μm) were cut on a vibratome, and the SCN-containing region was dissected from each slice. Whole cornea and hand-sliced pieces of lung ≈1 mm square were dissected in

cold HBSS. Tissues were cultured in sealed 35-mm Petri dishes containing 1.2 ml of culture medium and maintained in a light-tight incubator at 37°C. Culture medium consisted of DMEM (D2902, Sigma) with 10 mM Hepes, 2% B27 supplement, 352.5 μg/ml $NaHCO_3$, 3.5 mg/ml D-glucose, 25 μg/ml penicillin, 25 units/ml streptomycin, and 0.1 mM beetle luciferin, the substrate of the luciferase enzyme. The SCN and pineal were cultured on a Millicell culture insert, and other tissues were cultured free-floating. Bioluminescence was measured with a photomultiplier tube (Hamamatsu) or a Lumicycle photomultiplier tube detector assembly (Actimetrics), beginning immediately after tissue placement into an incubator and continuing for 3–5 days. Photon counts were collected in either 1- or 10-min bins, and detrended by subtracting the 24-h running average from the normalized data and then smoothing the data to 3-h running means. Peak phase was calculated as the highest point of the smoothed data in the 24–48-h interval after the initiation of the culture. Mean peak phases of tissues were calculated for each experimental group, and statistical differences were determined with ANOVA followed by a post hoc Tukey's test.

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