

## CONES ARE REQUIRED FOR NORMAL TEMPORAL RESPONSES TO LIGHT OF PHASE SHIFTS AND CLOCK GENE EXPRESSION

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In mammals, non-visual responses to light involve intrinsically photosensitive melanosin-expressing retinal ganglion cells (ipRGCs) that receive synaptic inputs from rod and cone photoreceptors. Several studies have shown that cones also play a role in light entrainment, photic responses of the suprachiasmatic nucleus (SCN), pupil constriction, and sleep induction. These studies suggest that cones are mainly involved in the initial response to light, whereas melanosin provides a sustained input for non-visual responses during continued light exposure. Based on this idea, we explored the effects of the absence of middle-wavelength (MW)-cones on the temporal responses of circadian behavior and clock gene expression in light. In mice lacking MW-cones, our results show a reduction in behavioral phase shifts in response to light stimulations of short duration at 480 and 530 nm, but no alteration for short-wavelength (360-nm) light exposures. Similarly, induction of the period gene *mPer1* and *mPer2* mRNAs in the SCN are attenuated in response to light exposures of mid to long wavelengths. Modeling of the photoresponses shows that mice lacking MW-cones have an overall reduction in sensitivity that increases with longer wavelengths. The differences in photic responsiveness are consistent with the idea that cones provide a strong initial phasic input to the circadian system at light-onset and may confer a priming effect on ipRGC responses to sub-threshold light exposures. In summary, the contribution of MW-cones is essential for the normal expression of phase shifts and clock gene induction by light in mammals. (Author correspondence: ouria.benyahya@inserm.fr)

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## INTRODUCTION

In mammals, behavior, metabolism, and physiological functions are regulated on a circadian basis by an endogenous clock located in the suprachiasmatic nucleus (SCN) of the hypothalamus. To maintain synchrony with the day/night cycle, the central clock is reset (entrained) daily by environmental light. In mammals, this photic information is transmitted from the retina to target neurons in the SCN via intrinsically light-sensitive, melanopsin-expressing ganglion cells (ipRGCs) that also receive inputs from rod and cone photoreceptors (Berson et al., 2002; Hattar et al., 2002; Belenky et al., 2003; Dacey et al., 2005; Viney et al., 2007).

In mutant and transgenic mice lacking specific photopigments, altered non-visual responses to light are observed following retinal degeneration of rods and/or cones, genetic knockout of the melanopsin photopigment gene (*Opn4*), or transgenic invalidation of rod/cone phototransduction pathways. Depending on the retinal alteration and composition of the light stimulus, the functional absence of one or more of these photopigments generally leads to deficits in light-induced phase shifts, entrainment, SCN neuronal responses, pupillary light reflex (PLR), and masking (Ebihara & Tsuji, 1980; Lucas et al., 2003; Mrosovsky, 2003; Mrosovsky & Hattar, 2003, 2005; Panda et al., 2002, 2003; Dkhissi-Benyahya et al., 2007; Mure et al., 2007; Drouyer et al., 2007, 2008; Thompson et al., 2008).

The results of these and other studies suggest that rods and cones contribute to the response at light-onset, whereas melanopsin provides a sustained response to continuous light exposure. The phasic input component from outer retinal photoreceptors and sustained input from ipRGCs are observed in electrophysiological retinal recordings of ipRGCs (Berson et al., 2002; Dacey et al., 2005; Schmidt et al., 2008), in SCN responses to light (Drouyer et al., 2007; Mure et al., 2007), and in pupil responses (Lucas et al., 2001; Hattar et al., 2003; Gamlin et al., 2007; Mure et al., 2009). Recently, it has also been suggested that in mice, cones are required for the initial light-dependent induction of sleep (Altimus et al., 2008). In a mouse model specifically lacking middle-wavelength (MW)-cones (maximum absorbance = 508 nm), we have previously shown an abnormal phase angle of entrainment and specific deficits in phase-shifting responses to mid- and long-wavelength light pulses of different irradiances (Dkhissi-Benyahya et al., 2007). Data from this study also suggested that the absence of MW-cones altered the responses to short-duration light exposure at 480 nm, but we did not examine temporal effects at other wavelengths.

In the present investigation, we extended this study by further exploring the consequences of the absence of MW-cones on phase shifts for light exposures of different durations at wavelengths from 360 to 530 nm. In addition, we examined the photic induction of the period genes *mPer1*

and *mPer2* to determine whether clock gene expression in the SCN is differentially affected by the duration and wavelength of the light pulse. We hypothesized that the absence of cone photoreceptors would result in deficits in the response, in particular, for short-duration light exposures.

## METHODS

### Animals

The *TRβ*<sup>-/-</sup> null mice used in this study have been described previously (Gauthier et al., 1999; Dkhissi-Benyahya et al., 2007) and are characterized by the absence of MW-expressing cones. Inactivation of the *TRβ* gene results in impairment of auditory function, but no alteration in development, metabolism, or neurological functions has been described in these animals (Forrest et al., 1996). Heterozygous mice were derived in an inbred 129SV background. Control homozygous animals were from the same genetic background and were obtained by inter-crossing heterozygous animals. All experiments were done with male mice between 6 and 8 wks of age at the start of the experiment. Animals were housed in Plexiglas cages under a 12 h/12 h light-dark (12L/12D) cycle, with food and water ad libitum. All treatment of animals was in strict conformity with current international regulations on animal care, housing, breeding, and experimentation and those of the journal for ethical biological rhythm research (Portaluppi et al., 2008).

### Phase-Shifting Assay

For monitoring locomotor activity, mice were housed individually in cages equipped with passive infrared motion captors placed over the cages, and continuous data were collected in 1-min bins using a computerized data acquisition system (Circadian Activity Monitoring System, INSERM, France). Activity records were analyzed with the Clocklab software package (Actimetrics, Evanston, IL).

Singly housed male mice (n = 8 for each genotype) were first entrained in a 12L/12D cycle for 20 days. Subsequently, animals were maintained in constant darkness (DD) at least for 10 days. Phase shifts were studied using monochromatic light pulses of an equivalent irradiance of  $2.8 \times 10^{14}$  photons/cm<sup>2</sup>/s for durations of 1, 5, and 15 min. The data reported herein for light pulses of 480 nm were presented in a previous study (Figure 5, Dkhissi-Benyahya et al., 2007). We then completed this experiment by analyzing phase shifts at two additional wavelengths (360 and 530 nm, half-bandwidth, 10 nm) using the same exposure durations (1, 5, and 15 min) and the same irradiance level ( $2.8 \times 10^{14}$  photons/cm<sup>2</sup>/s). The ultraviolet (UV) light source was a high-pressure mercury vapor

lamp, equipped with an additional Kodak UV glass filter to isolate the 365-nm band. Kodak neutral density filters were used to adjust the irradiance level. The order of presentation of duration and wavelength (and dark control) was randomized. The light stimulus was applied at CT16 (4 h after activity-onset). Dark controls were handled in the same way but did not receive a light pulse. The stimulator (light source and chamber) has been described previously (Dkhissi-Benyahya et al., 2000). After the light pulse, animals were returned to their home cages, and activity was monitored in DD for an additional 15–20 days before the next light pulse in order to calculate the amplitude of the light-induced phase shift. The magnitude of a light-induced phase shift was determined from the difference between the regression lines of the activity-onsets before and after the light stimulation using the built-in Clocklab functions and extrapolated to the day following the light pulse. The transient responses during the 3–4 days immediately after the pulse were discounted.

### **In Situ Hybridization: Light Induction of *mPer1* and *mPer2* in the SCN**

In order to compare the sensitivity of the behavioral response of the circadian system in wild-type and MW-coneless mice to clock gene induction by light, *mPer* expression was assayed in the SCN following pulses of monochromatic light. Animals ( $n = 12$  for each genotype) were kept for 20 days in a 12L/12D cycle and then transferred to DD for at least 10 days before light stimulation. Animals were exposed to a single monochromatic light pulse of an equivalent irradiance of  $2.8 \times 10^{14}$  photons/cm<sup>2</sup>/s using either 480- or 530-nm wavelength at one of the three durations (1, 5, and 15 min) that were applied in the study of behavioral phase shift. One hour after the beginning of the light pulse, mice were sacrificed by cervical dislocation under dim red light, and the brains were removed and immediately immersed in ice-cold 4% paraformaldehyde (PFA) and fixed at 4°C overnight. Brains were then dehydrated with ethanol (30%, 50%, and 70%, 2 h each, and 100% for 4 h at 4°C) and transferred to xylene at room temperature. Xylene was changed once before being replaced by 50% xylene/50% paraffin. Then, tissue was placed in paraffin, which was exchanged three times at 63°C before pouring it in embedding blocks. Coronal sections (7  $\mu$ m) of the mouse brain were made and fixed in 4% PFA, rinsed twice with phosphate-buffered saline (PBS; 5 min), followed by proteinase K (20  $\mu$ g/mL in 50 mM Tris-Hcl [pH 7.6] including 5 mM EDTA) for 5 min at 37°C. Sections were then rinsed once with PBS (5 min), 4% PFA for 20 min, then acetylated twice in 0.1 M TEA, and washed again with PBS and dehydrated in a graded ethanol series. In situ hybridization was performed overnight with either denatured antisense or sense riboprobe for *mPer1* and *mPer2*, as previously described (Albrecht

et al., 1997; Sun et al., 1997), hybridized with  $^{35}\text{S}$ -uridine triphosphate (New England Nuclear, Charlotte, NC, USA) in a humid chamber at  $55^{\circ}\text{C}$ . After hybridization, sections were then rinsed with  $5\times$  SSC ( $0.75\text{M}$  NaCl,  $0.075\text{M}$  sodium citrate), treated with  $10\ \mu\text{g}/\text{mL}$  ribonuclease A at  $37^{\circ}\text{C}$  for 30 min, rinsed with stringency washes of SSC ( $2\times$  to  $0.1\times$  SSC), dehydrated in a graded ethanol series, dried, and exposed to Hyperfilm for 1–4 days. For each animal, all sections from the rostral to the caudal SCN were divided into two series to assess the light induction of *mPer1* and *mPer2* in the same animal. Tissue was visualized by fluorescence of Hoechst dye-stained nuclei (not shown). Silver grains were visualized by dark-field illumination. Images were captured using a Spot II camera and software system.

Quantification was performed by densitometry analysis of hybridization signals of *mPer1* and *mPer2* using computer-assisted image analysis (Biocom, Les Ulis, France). Optical density of label was measured bilaterally from digitized images of the SCN. This method, previously described (Dkhissi-Benyahya et al., 2000; Rieux et al., 2002), is based on quantification of the total optical density of the labeling, defined as the integral sum of the surface area of all pixels in the SCN multiplied by their corresponding optical density value. The optical density of specific signal was calculated by subtracting the background density value determined from an adjacent area of the hypothalamus that did not contain staining. The background for each animal was systematically measured, and a threshold value was determined for detection of label significantly above the background level. Measures were made bilaterally, and every section of the SCN of each series was analyzed and averaged for each animal. The identity of the individual animals was unknown to the observer during the analysis.

### Statistical Analyses

Significant differences between the response among genotypes and light-duration conditions were determined using a two-way analysis of variance (ANOVA), followed, when significant ( $p < 0.05$ ), by post hoc Student Newman-Keuls to evaluate the differences in clock gene mRNA expression and phase-shift amplitude between genotypes for each duration. Values are shown as mean  $\pm$  SEM.

## RESULTS

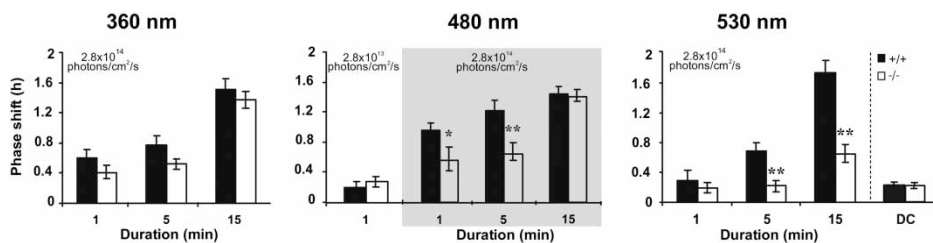
### Temporal Responses to Light-Induced Phase Shifts Are Altered in MW-Coneless Mice

To examine the effects of the absence of MW-cones on light-induced phase shifts, we exposed animals to a pulse of monochromatic light at

wavelength of 360 or 530 nm for the three light-pulse durations (Figure 1). In Figure 1, we include data from the 480-nm phase shifts reported previously (Dkhissi-Benyahya et al., 2007; enclosed in shaded square). In both genotypes, the shortest-duration light exposure used (1 min) at 360 nm induced a significant phase shift in comparison with the dark-control condition ( $0.22 \pm 0.02$  h for wild-type mice;  $0.21 \pm 0.02$  h for MW-coneless mice). With longer durations of UV light exposure (from 1 to 15 min), the magnitude of the phase shift increased proportionately in the two groups of mice. No significant differences in the magnitude of the phase shift of locomotor activity were observed between MW-coneless and wild-type mice for the three light durations tested at this wavelength (Figure 1).

At 480 nm and for the irradiance level of  $2.8 \times 10^{14}$  photons/cm<sup>2</sup>/s, both genotypes showed significant phase shifts compared to dark controls starting with the short 1-min light pulse, and there was a significant increase in the response with increase of the exposure duration from 1 to 15 min (Figure 1). However, MW-coneless mice showed significantly attenuated phase shifts compared to wild-type mice for the exposure durations of 1 and 5 min ( $p < 0.05$ ; Figure 1). For the 15-min light exposure, equivalent phase shifts were induced in the two groups of mice.

At 530 nm, the 1-min-duration pulse was insufficient to induce a significant phase shift compared to dark controls in either wild-type and MW-coneless mice (Figure 1), indicating that for this wavelength the irradiance used ( $2.8 \times 10^{14}$  photons/cm<sup>2</sup>/s) was below the threshold to elicit



**FIGURE 1** Attenuated phase-shifting response to light in MW-coneless mice. Mean phase shifts ( $\pm$  SEM) in wild-type (+/+) and MW-coneless (-/-) mice ( $n = 8$  for each genotype) for the three light durations tested (1, 5, and 15 min) at 360, 480, 530 nm at an equal irradiance of  $2.8 \times 10^{14}$  photons/cm<sup>2</sup>/s. An additional 1 log unit lower irradiance level was tested at 480 nm. Data in the shaded part of the histogram at 480 nm are from Dkhissi-Benyahya et al. (2007). A significant difference between genotypes is observed for light pulses of short duration (1 and 5 min) at 480 nm and at 530 nm for durations of 5 and 15 min. No differences between genotype were observed for the three durations at 360 nm. Dark controls (DC), handled in the same way but that did not receive a light pulse, show no significant difference between genotypes ( $0.22 \pm 0.02$  h for wild-type mice;  $0.21 \pm 0.02$  h for MW-coneless mice). Asterisks indicate a statistically significant difference between the two genotypes (ANOVA:  $p < 0.05$ ; post hoc Newman-Keuls tests comparing genotypes at each duration: \* $p < 0.05$ ; \*\* $p < 0.01$ ).

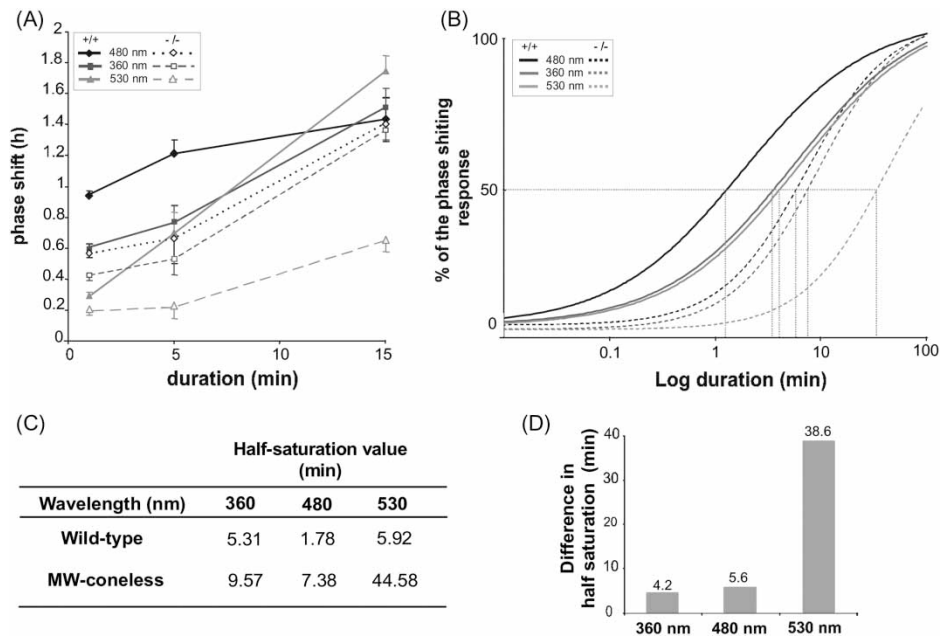
a response. A 5-min light pulse produced a significant phase shift in the wild-type mice but was still insufficient to induce a phase shift in the MW-coneless mice. At the longest duration (15 min), light pulses of 530 nm induced significant phase shifts in both groups, but the phase shift of MW-coneless mice was significantly attenuated compared to that of the wild-type mice (Figure 1).

We speculated that the difference in the phase shift observed for the 1-min pulse at 480 nm but not at 530 nm could be due to the higher sensitivity of the circadian system in the mid-wavelength region of the spectrum (Lucas et al., 2003; Dkhissi-Benyahya et al., 2007). Thus, we additionally exposed the mice to a short 1-min pulse at 480 nm but used a 1 log unit lower irradiance ( $2.8 \times 10^{13}$  photons/cm<sup>2</sup>/s; Figure 1). In this case, however, neither genotype showed a significant phase shift ( $0.20 \pm 0.08$  h for wild-type mice and  $0.28 \pm 0.07$  h for MW-coneless mice), indicating the photon dose was below threshold (as was the case for the 1-min light pulse duration of higher irradiance exposure at 530 nm).

We then used these data to model the response functions for stimulus durations at the different wavelengths. Figure 2a shows a composite graph of the phase-shift responses for both genotypes for the three light durations and three wavelengths tested. These data were then fit using a four-parameter logistic function to obtain dose-response curves (Figure 2b). As we did not assume that the response would be univariant (Dkhissi-Benyahya et al., 2007; Thompson et al., 2008), the best fit was obtained by calculating these functions separately for each genotype (see further details in figure legend). Half-saturation (duration necessary to induce 50% of the maximum response) values for each dose-response curve were obtained (Figure 2c), and the differences in the time required to attain the half-saturation response between the two genotypes were plotted (Figure 2d). At short and mid wavelengths, the difference between genotypes in the time required for half-saturation was short (360 nm = 4.2 min; 480 nm = 5.6 min), but for the longer wavelength (530 nm) it was extremely long (38.6 min), indicating a relative decrease in photic sensitivity that increases at longer wavelengths in mice lacking MW-cones.

### Photic Induction of *mPer* Genes in the SCN of Wild-Type and MW-Coneless Mice

In order to determine if light induction of clock genes are also altered by the absence of MW-cones, we exposed mice to equal irradiance ( $2.8 \times 10^{14}$  photons/cm<sup>2</sup>/s) light pulses of 480 and 530 nm using the three light-exposure durations (1, 5, and 15 min; Figure 3a, 3b). Since with the 360 nm light pulses no differences in phase-shift responses were observed between genotypes (Figure 1), we decided to focus on the



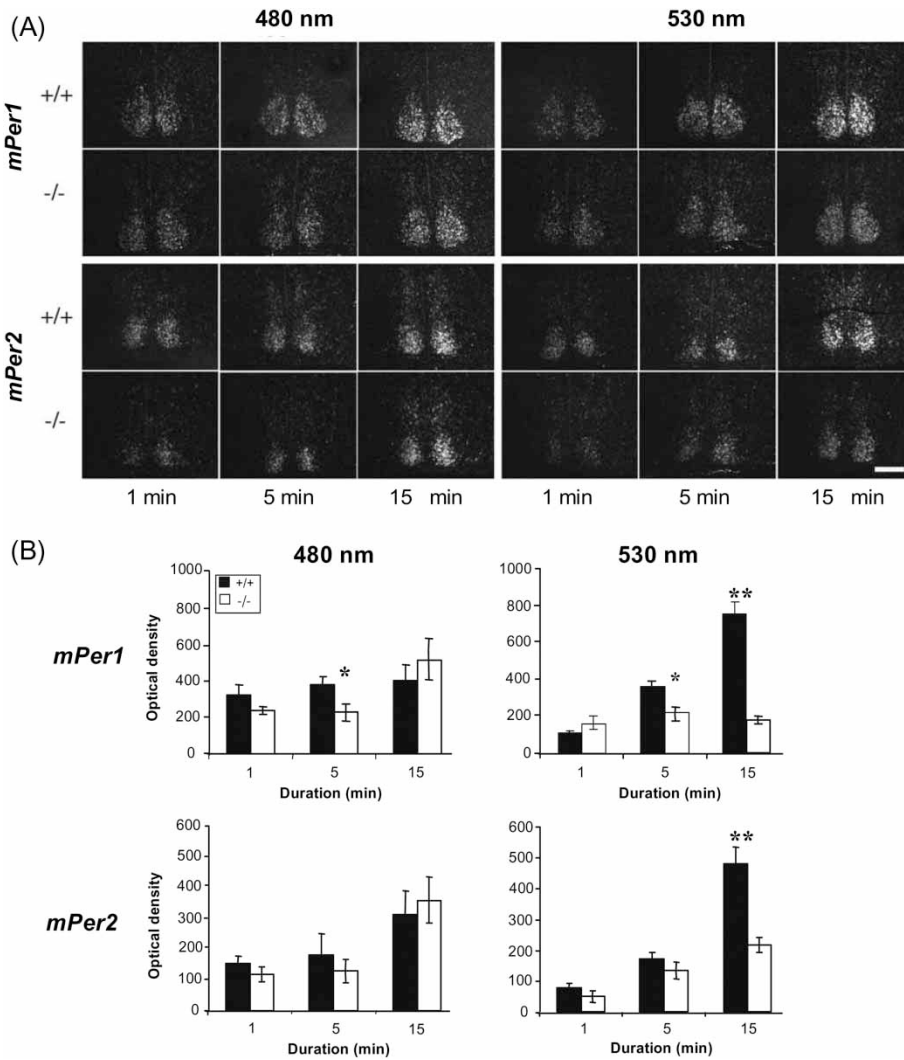
**FIGURE 2** Reduced sensitivity to light in MW-coneless mice. (a) Plots of mean phase shifts ( $\pm$  SEM, data from Figure 1) for different light durations (1, 5, 15 min) and wavelengths (360, 480, 530 nm) in wild-type (+/+) and MW-coneless mice (-/-). (b) Dose-response curves calculated using a four-parameter logistic function. For all curves, the minimum was set to the mean value of the dark controls (0.22 h) and the maximum to the mean values of phase shifts for a 15-min pulse at the three wavelengths (1.56 h). This maximum corresponds to the mean saturation value of phase shifts at the three wavelengths and for the irradiance level of  $2.8 \times 10^{14}$  photons/cm<sup>2</sup>/s. The slope was determined separately for each genotype, using the averaged data for 360 and 480 nm and is slightly different in wild-type (0.72) and MW-coneless (1.08) mice.  $R^2$  values varied from 0.82 to 0.97. (c) Half-saturation values for wild-type and for MW-coneless mice at the three wavelengths. (d) Histogram illustrate the differences between the two genotypes in half saturation values for each wavelength.

photic induction of *mPer* genes for wavelengths that showed alteration of the behavioral response in the MW-coneless mice.

Similar to observations for the behavioral phase shifts, *mPer1* and *mPer2* mRNAs were significantly induced, compared to the background level, by the 480-nm light pulse for the three durations in both genotypes (Figure 3a). Although *mPer1* gene expression did not increase with increased light duration ( $p = 0.45$ ), *mPer2* mRNA expression increased significantly from 1 to 15 min ( $p < 0.05$  in wild-type mice and  $p < 0.005$  in MW-coneless mice; Figure 3b). At 480 nm, a significant difference between genotypes was observed only for *mPer1* following the 5-min pulse ( $p < 0.05$ ).

The 530-nm light pulse of 1-min duration did not induce *mPer1* and *mPer2* above background levels in both groups. Increasing the duration of the light pulse from 1 to 15 min produced significant photic induction





**FIGURE 3** Induction of *mPer1* and *mPer2* in the SCN of wild-type (+ / +) and MW-coneless (- / -) mice by monochromatic light (480 nm or 530 nm) of different pulse durations (1, 5, and 15 min) at a constant irradiance level of  $2.8 \times 10^{14}$  photons/cm<sup>2</sup>/s (n = 12 for each genotype). (a) Representative photographs of *mPer1* and *mPer2* mRNA in situ hybridization in the SCN 60 min following the initial exposure to light delivered at CT16. (b) Quantification of *mPer1* and *mPer2* mRNA levels from in situ hybridization data from wild-type (+ / +) and MW-coneless (- / -) mice. Results are expressed as mean  $\pm$  SEM. Asterisks indicate a statistically significant difference (ANOVA:  $p < 0.05$ ; post hoc Newman-Keuls tests comparing genotypes at each duration: \* $p < 0.05$ ; \*\* $p < 0.001$ ).

of both *mPer1* and *mPer2* in wild-type mice ( $p < 0.001$  for both genes), whereas in MW-coneless mice only *mPer2* expression significantly increased. The MW-coneless mice showed a significant reduction in *mPer1* expression for the 5-min light exposure and for both *mPer1* and *mPer2* for the 15-min light exposure (15 min,  $p < 0.0001$ ).

## DISCUSSION

Several studies have provided convincing evidence that cones play a role in circadian entrainment and in SCN neuronal responses to light (Aggelopoulos & Meissl, 2000; Hattar et al., 2003; Lucas et al., 2003; Dacey et al., 2005; Dkhissi-Benyahya et al., 2007; Drouyer et al., 2007), pupil constriction (Lucas et al., 2003), masking (Thompson et al., 2008), and sleep induction (Altimus et al., 2008). Our previous study (Dkhissi-Benyahya et al., 2007) had shown that the specific absence of MW-cones alters phase-shifting responses for different irradiances. The present results extend these findings by demonstrating that in the absence of MW-cones, light-induced phase shifts and *mPer1* and *mPer2* mRNA induction in the SCN are attenuated in an exposure-duration- and wavelength-dependent manner. These alterations in sensitivity are not likely to be due to an absence of the nuclear thyroid hormone receptors per se, since the differences in responsiveness are wavelength dependent, and the MW-coneless mice display normal responses in the UV region of the spectrum.

### Phase-Shifting Responses to Monochromatic Light Are Altered in MW-Coneless Mouse

Although both wild-type and MW-coneless mice show increased responses to longer-duration light exposures, the absence of MW-cones results in attenuated behavioral responses and a general decrease in sensitivity that is wavelength dependent. At 480 nm, the deficits in MW-coneless mice are observed for the 1- and 5-min durations, whereas at 530 nm deficits are observed for the 5- and 15-min durations. For the 530-nm stimulations and at the irradiance level used ( $2.8 \times 10^{14}$  photons/cm<sup>2</sup>/s), the 1-min light pulse was below threshold for both genotypes, since the phase shifts are not significantly different from that of dark controls. Above this threshold level, a deficit in the phase shift for the MW-coneless mice is first observed with the 5-min duration. Since the peak of MW-cone sensitivity is at 508 nm (longer than the peak sensitivity of melanopsin at 480 nm), the difference in the responses between the wild-type and MW-coneless mice with the 5- and 15-min exposures is also related to the proportionately greater contribution of MW-cones at this wavelength (Dkhissi-Benyahya et al., 2007). One might thus speculate that the responses to a 1-min pulse but at a higher irradiance would have induced significantly different phase shifts in the two genotypes. On the contrary, at 480 nm, the 15-min-duration light pulse produced equivalent saturation level responses in both genotypes (Dkhissi-Benyahya et al., 2007), whereas at 530 nm only the wild-type mice attained a saturating response. Since the half-saturation value at 530 nm is ~44 min (Figure 2c), it could

be expected that MW-coneless mice would require a very long duration to attain a saturating response.

These results are consistent with the view that melanopsin ipRGCs provides a sustained signal throughout the duration of the light stimulus and for long temporal integration, whereas cones contribute during the initial part of the response due to their phasic response at light-onset (Berson et al., 2002; Dacey et al., 2005; Tu et al., 2005; Dkhissi-Benyahya et al., 2007; Drouyer et al., 2007; Mure et al., 2007; Wong et al., 2007; Schmidt et al., 2008). In these electrophysiological studies, a high-amplitude, short-duration phasic input originating from cones (and eventually rods) occurs within the first 500 ms following light-onset. In contrast, the sustained melanopsin response appears after a delay ranging from seconds to minutes depending on light intensity (Berson et al., 2002; Dacey et al., 2005; Tu et al., 2005; Dkhissi-Benyahya et al., 2007; Drouyer et al., 2007; Mure et al., 2007; Wong et al., 2007; Schmidt et al., 2008). In line with this hypothesis, we have previously shown that bright-light exposures aimed at desensitizing rods and cones will abolish the initial phasic SCN responses while minimally affecting the sustained melanopsin components (Drouyer et al., 2007), whereas melanopsin-invalidated mice conserve robust phasic responses but lack the sustained components (Mure et al., 2007). For downstream responses involving sensory and/or motor integration, phasic and sustained components may be expressed on a more extended time scale. This is observed, for example, in pupillary constriction (Gamlin et al., 2007; Mure et al., 2009) or in masking (Mrosovsky & Hattar, 2003), where in the *Opn4*<sup>-/-</sup> mouse cones and rods are unable to maintain the response under continuous light in the absence of sustained input from melanopsin ipRGCs.

In our experiments, the initial phasic influence of MW-cones is particularly obvious for the 1-min-duration light pulse at 480 nm where, based on the difference in response amplitude between the two genotypes, cones appear to account for nearly 50% of the response (Figure 1). Furthermore, a 5-fold increase in duration of light exposure from 1 to 5 min only produces a 25% (non-significant) increase.

The absence of MW-cones does not affect phase-shifting responses at 360 nm, although relative sensitivity is slightly reduced, since the calculated dose-response curve is shifted to longer durations (Figure 2b). This result is coherent with the greatly reduced relative sensitivity of MW-cones at this wavelength ( $\sim 2$  log units) and implies that short-wavelength cones and, perhaps, melanopsin account for behavioral phase-shift responses in the UV region of the spectrum. Since the relative sensitivity of melanopsin is also reduced in the UV region ( $\sim 1.5$  log units), the robust phase-shift response is due either to a relatively large contribution of the short-wavelength (SW)-cones (Thompson et al., 2008) or their possible priming effect on the intrinsic response of ipRGCs for which this

short-wavelength light is of low- or sub-threshold irradiance. Validation of this priming effect could represent a form of spectral “interaction” that widens the wavelength sensitivity output of ipRGCs in both the UV region (via UV-cones) and to longer wavelengths (via MW-cones). A priming effect between outer retinal photoreceptors and melanopsin, first suggested by Dacey et al. (2005) and Wong et al. (2007), has recently been experimentally demonstrated by Schmidt et al. (2008), who showed that synaptic input from MW-cones confers to ipRGCs a higher overall light sensitivity and a shift in spectral sensitivity from 480 nm towards longer wavelengths.

### Light Induction of *mPer1* and *mPer2* in the SCN Is Altered in MW-Coneless Mouse

Direct photic input to the SCN leads to the induction of clock genes, including *Per1* and *Per2* (Hamada et al., 2001; Yan & Silver, 2002). However, specific wavelength and light-duration-dependent effects on *mPer* gene expression have not been examined previously. We show here that the absence of MW-cones affects the photic induction of *mPer1* and *mPer2* in the SCN of MW-coneless mice in a pattern that is consistent with the observed effects of 480- and 530-nm light pulses on behavioral phase shifts. Following 530-nm light stimulations, significant differences in *mPer1* expression between the genotypes were observed for both the 5- and 15-min exposures but not for the 1-min exposure (below threshold), in agreement with the behavioral results. At 480 nm, a significant difference in *mPer1* expression was only observed for the 5-min light exposure, whereas an expected difference for the 1-min exposure was not observed. At 530 nm, the 15-min light pulse induced equivalent amounts of *mPer1* expression and phase shift; *mPer2* induction by light is less affected by the absence of MW-cones, and a significantly reduced expression is only seen at the longest duration used (15 min).

Our study differs from the only previous one that examined the consequences of photoreceptor loss on photic induction of clock genes in the SCN, which reported a similar induction of mPER1 protein in wild-type and *rd/rd* mice (Alvarez-Lopez et al., 2006). This discrepancy could be related to the experimental design (30-min stimulation of white light versus 1, 5, and 15 monochromatic light pulses), the photoreceptor mutation (*rd/rd* or MW-coneless mice), and/or gene versus protein assay.

In conclusion, our results confirm the view that circadian photoreception is based on multiple photoreceptors functioning in concert within a system where each photoreceptor provides specific contributions based on its response domain and the photic environment in terms of the spectrum, irradiance, and duration of light. Although cones are not necessary for circadian entrainment and gene expression, their presence is required

for normal responses and for optimization of the system's capacity to respond to a range of light intensities and durations. For example, phasic responses from cones to light-onset may account for the greater efficiency of multiple short-duration light pulses to shift the human circadian timing system compared to a single long-duration exposure of higher total irradiance (Gronfier et al., 2007). By providing a phasic input to the mid-wavelength region of the spectrum, MW-cones increase the overall sensitivity of the system, shift the response to longer wavelengths, and widen the spectral bandwidth. The same is most likely valid for the SW-cone contribution in the short-wavelength region of the spectrum.

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