CIRCADIAN RHYTHMS

Spectral Responses of the Human Circadian System Depend on the Irradiance and Duration of Exposure to Light

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In humans, modulation of circadian rhythms by light is thought to be mediated primarily by melanopsin-containing retinal ganglion cells, not rods or cones. Melanopsin cells are intrinsically blue light–sensitive but also receive input from visual photoreceptors. We therefore tested in humans whether cone photoreceptors contribute to the regulation of circadian and neuroendocrine light responses. Dose-response curves for melatonin suppression and circadian phase resetting were constructed in subjects exposed to blue (460 nm) or green (555 nm) light near the onset of nocturnal melatonin secretion. At the beginning of the intervention, 555-nm light was equally effective as 460-nm light at suppressing melatonin, suggesting a significant contribution from the three-cone visual system (λ_{max} = 555 nm). During the light exposure, however, the spectral sensitivity to 555-nm light decayed exponentially relative to 460-nm light. For phase-resetting responses, the effects of exposure to low-irradiance 555-nm light were too large relative to 460-nm light to be explained solely by the activation of melanopsin. Our findings suggest that cone photoreceptors contribute substantially to nonvisual responses at the beginning of a light exposure and at low irradiances, whereas melanopsin appears to be the primary circadian photopigment in response to long-duration light exposure and at high irradiances. These results suggest that light therapy for sleep disorders and other indications might be optimized by stimulating both photoreceptor systems.

INTRODUCTION

In mammals, daily rhythms of sleepiness and alertness, physiology and metabolism, and gene expression are driven endogenously by neurons in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus. A small subset of retinal ganglion cells (RGCs) projects directly to the SCN and synchronizes the circadian timing system, ensuring that daily changes in behavior are timed appropriately with the solar cycle. Light-induced activation of SCN neurons also acutely suppresses pineal gland synthesis of the hormone melatonin, which is only released during the biological night. These nonvisual light responses persist in humans with impaired or absent vision, suggesting that rod and cone photoreceptors are not required (*1*–*4*). In mice deficient in rod and cone function, nonvisual light responses are mediated exclusively by intrinsically photosensitive RGCs (ipRGCs) that express the blue light–sensitive photopigment melanopsin (λ_{max} = ~480 nm) (*5*–*9*). In humans, circadian phase resetting, melatonin suppression, and objective measures of alertness are most sensitive to short-wavelength light, suggesting a primary role for melanopsin in regulating human nonvisual light responses (*10*–*14*). Consistent with these findings, we recently reported that circadian, neuroendocrine, and neurobehavioral light responses to bright light were short wavelength–sensitive in a pair of blind individuals without rod and cone function (*4*). Hence, in the absence of visual photoreceptor signaling, melanopsin cells in the inner retina are sufficient to drive nonvisual light responses (*6*, *7*, *15*–*17*).

In intact retinae, however, ipRGCs receive indirect synaptic input from rod and cone photoreceptors (*18*–*20*). Moreover, melanopsin-null mice show intact phase resetting, melatonin suppression, and pupillary light responses; these responses are abolished only after rod and cone signaling pathways are also eliminated (*6*, *7*, *21*, *22*). These findings suggest that melanopsin and visual photoreceptors are complementary in regulating non–image-forming responses. Nonetheless, in humans, it is still widely assumed that cone photoreceptors play a marginal role, if any, in driving circadian and neuroendocrine light responses. Given that cone photoreceptors are more sensitive to light intensity and have more rapid, transient, response dynamics compared to the intrinsic melanopsin-driven RGC response (*8*, *20*), we hypothesized that it should be possible to determine the relative importance of the three-cone visual system by manipulating the irradiance and spectral content of light exposures. To test this hypothesis, we compared the relative effectiveness of retinal exposure to 460-nm versus 555-nm light—appearing blue and green to the normal human eye, respectively—at eliciting melatonin suppression and circadian phase-shift responses.

RESULTS

Short-wavelength shift in sensitivity for melatonin suppression in continuous light

We measured melatonin suppression and phase shifting in young healthy subjects (ages 18 to 30 years) exposed to 6.5 hours of continuous narrow-bandwidth, short-wavelength (460 nm; n = 24) or longer-wavelength (555 nm; n = 24) light during the night (Fig. 1A).

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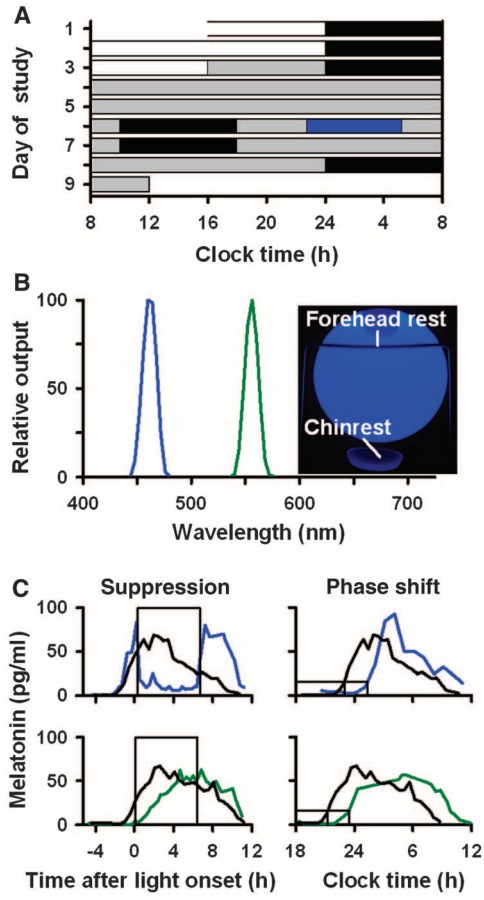

Fig. 1. Protocol for assessing melatonin suppression and phase-shift responses. (**A**) Subjects participated in a 9-day inpatient protocol. White bars indicate exposure to ambient room light (<190 lux), and gray bars indicate exposure to dim ambient light (<3 lux). Black bars show scheduled sleep episodes in darkness (<0.002 lux), and the blue bar indicates the 6.5-hour light intervention. (**B**) On the evening of day 6, each subject was exposed to 6.5 hours of 460- or 555-nm light. The blue and green traces show the relative spectral content for a pair of representative light exposures to 460- and 555-nm light, respectively. The inset shows a frontal view of the modified Ganzfeld dome used to administer the light exposure. (**C**) Melatonin suppression and phase-shift responses are shown for two representative subjects exposed to 460-nm light (top traces) or 555-nm light (bottom traces) at 12.85 log photons cm^{-2} s^{-1}. In each plot, black traces show melatonin on the day before the light exposure. In the left column, colored traces show melatonin suppression during the 6.5-hour light exposure, with open boxes marking the timing of the light intervention. In the right column, colored traces show the melatonin rhythm on the day after the light exposure, and drop lines indicate the timing of the dim-light melatonin onset (clock time at which melatonin level exceeds 25% of the peak-to-trough fitted amplitude).

The 460-nm light was selected on the basis of the initially reported ~460-nm peak of spectral sensitivity for melatonin suppression in humans (*10*, *14*), whereas the 555-nm light stimulus was selected to activate the three-cone photopic visual system maximally. Fixed-irradiance light exposures were given to each individual near the onset of nocturnal melatonin secretion using a modified Ganzfeld dome (Fig. 1B), with irradiance values spanning a 3–log unit range (half-peak bandwidth = 10 to 14 nm).

In most subjects, exposure to 460-nm light elicited a relatively constant degree of melatonin suppression during the light exposure, whereas exposure to 555-nm light elicited an initially strong suppression of melatonin, which gradually recovered to baseline values even in the continued presence of light (Fig. 1C) (*12*). To determine the relative spectral sensitivity of melatonin suppression during the 6.5-hour light intervention, we compared the log ED_{50} (median effective dose; irradiance required to elicit a half-maximal response) for the dose response to 460-nm versus 555-nm light exposures in quarterly intervals (Fig. 2, A and B). During the first quarter, there was no difference in spectral sensitivity (Q1: $F_{3,45} = 0.59$, $P = 0.62$), whereas by the second quarter there was a relative decrease in sensitivity to 555-nm light compared to 460-nm light. In the third and fourth quarters of exposure, the log ED_{50} for the dose response to 555-nm light was significantly higher than in response to 460-nm light (Q3: $F_{3,45} = 6.67$, $P < 0.001$; Q4: $F_{3,45} = 9.21$, $P < 0.001$) (Fig. 2B), indicating that short-wavelength light was much more effective than longer-wavelength light at suppresing melatonin in the latter half of the 6.5-hour light exposure. To examine the kinetics of melatonin suppression sensitivity in greater detail, we constructed serial dose-response curves for exposure to 460- and 555-nm light in 30-min intervals across the 6.5-hour light intervention (Fig. 2C). Relative to 460-nm light, the sensitivity of melatonin suppression to 555-nm light decayed exponentially ($R^2 = 0.99$), with a half-life of 37.85 min (Fig. 2D). At the start of the light exposure, the log relative sensitivity of melatonin suppression to 555-nm versus 460-nm light was essentially identical (−0.048 log unit). By the fourth hour of exposure to continuous light, however, melatonin suppression sensitivity to 555-nm light was 0.88 log unit lower compared to 460-nm light, which matches the predicted difference in log relative sensitivity at these wavelengths for a vitamin A1–based photopigment with peak sensitivity to 481-nm light (*23*).

Robust circadian phase shifting in response to longer-wavelength light

After exposure to 460-nm light, phase shifts of the melatonin rhythm exhibited a nonlinear dose response saturating at −3.19 hours (R^2 = 0.73; Fig. 3A). By comparison, the dose response for phase resetting to 555-nm light did not appear to have the same shape (Fig. 3, A and B) and the slope was significantly different from the curve to 460-nm light ($F_{1,48} = 10.17$, $P < 0.01$). Given that the dose-response curves converged at lower irradiances (Fig. 3B), the log ED_{50} for phase shifting to 555-nm light tended to be higher than the response to 460-nm light, but the difference in log ED_{50} values did not reach statistical significance (0.53 log unit; $F_{1,48} = 2.94$, $P = 0.093$). To test whether the dose response to 555-nm light could be explained solely by a single-photoreceptor model, we fit a univariant curve to the data with the same slope as the dose response to 460-nm light (*10*, *14*). The resulting curve fit was poor ($R^2 = 0.12$; Fig. 3C), demonstrating that phase-shift responses to 555-nm light are better described by a dose-response curve in which the slope is not constrained ($R^2 = 0.53$; Fig. 3B) or, perhaps, by a more complex model that incorporates combined photoreceptor drive from visual photoreceptors and melanopsin. In addition, phase-resetting responses at the 12 lowest irradiances tested (<13 log photons cm^{-2} s^{-1}) were an hour greater than predicted by comparison to a forced univariant curve fit (for λ_{max} = 480 nm; −1.00 hour ±

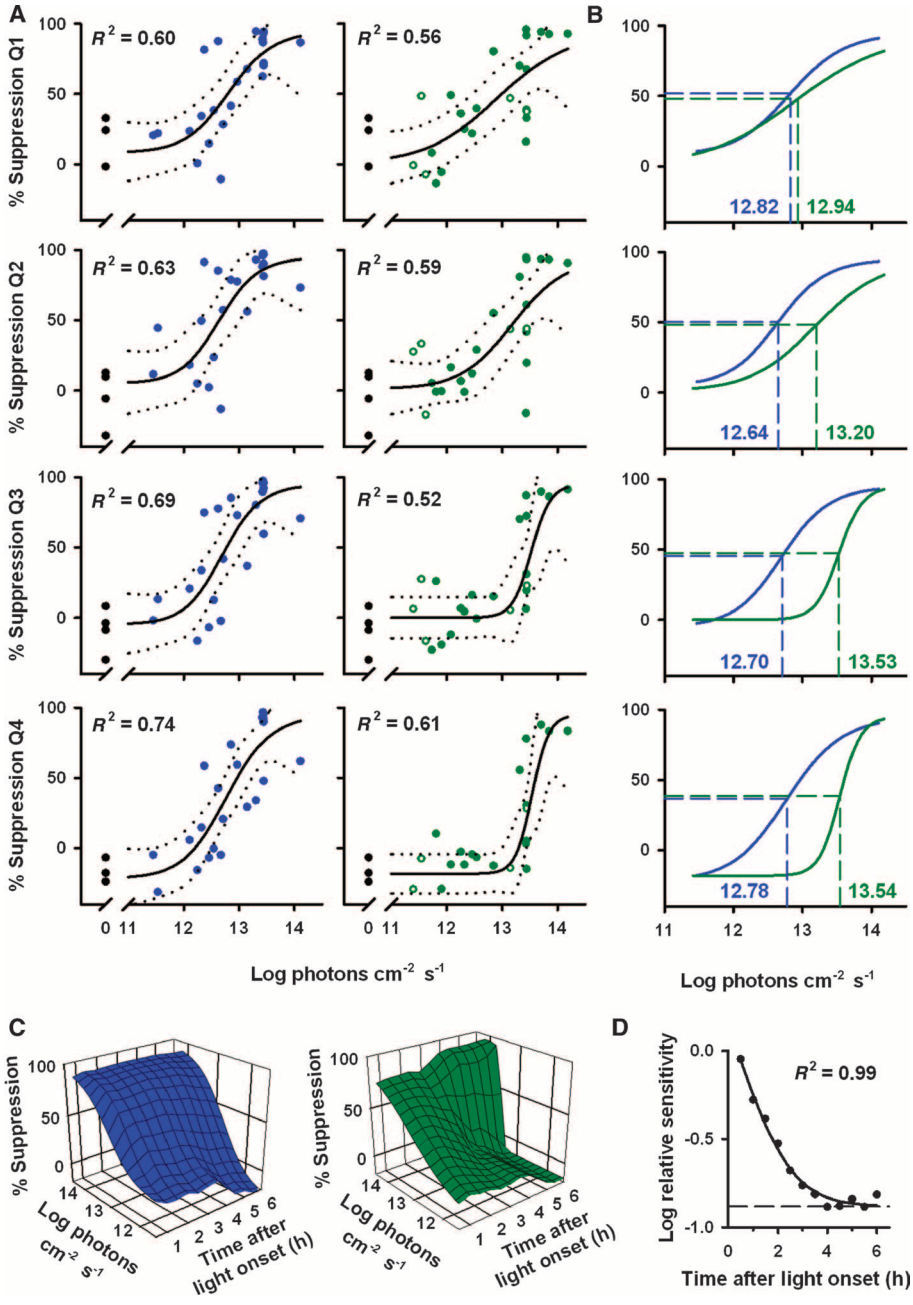

Fig. 2. Melatonin suppression sensitivity to 460-nm versus 555-nm light exposure. (**A**) Dose-response curves for melatonin suppression are shown in response to 460-nm light (left, blue circles) and 555-nm light (right, green circles) in quarterly intervals (Q1 to Q4, top to bottom) across the 6.5-hour light exposure. Closed and open circles show suppression of plasma or salivary melatonin, respectively, in individual subjects. Black traces show the best-fit dose-response curve with 95% confidence intervals. Black filled circles at 0-log irradiance show melatonin suppression in response to darkness. (**B**) The dose-response curves are overlaid, demonstrating a short-wavelength shift in spectral sensitivity during the light exposure. Horizontal dashed lines indicate the half-maximal melatonin suppression response, and vertical dashed lines show the corresponding log ED_{50} values, which are labeled in each plot. (**C**) The dose response for melatonin suppression to 460-nm light (left) remained relatively constant during the light exposure, whereas the dose response to 555-nm light (right) exhibited a slow reduction in sensitivity across time (half-life = 37.85 min). (**D**) With increasing duration of light, the sensitivity of melatonin suppression to 555-nm light decayed exponentially relative to 460-nm light exposure. All data were analyzed in hourly bins and plotted by midpoint of the binned data. The dashed trace at the lower asymptote (−0.88 log unit) corresponds to the predicted difference in log relative sensitivity at these wavelengths for a photoreceptor with peak sensitivity to 481-nm light.

0.13 SEM; $P < 0.001$, one-sample t test), suggesting that phase-shifting responses to 555-nm light were not mediated by a single short-wavelength–sensitive photopigment (Fig. 3D).

DISCUSSION

Previous studies in mice demonstrated that classical visual photoreceptors are sufficient to entrain the circadian system in the absence of melanopsin (*21*, *22*). To date, however, studies of circadian photoreception in humans have failed to identify a prominent role for cone photoreceptors. We used the differential response of melanopsin and cone photoreceptors to the irradiance, duration, and spectral content of light to evaluate their relative roles in this process. Our data indicate that cone photoreceptors contribute substantially to circadian photoreception for short-duration or low-irradiance light exposures, whereas short-wavelength–sensitive melanopsin cells dominate circadian responses to longer-duration and high-irradiance light exposures.

Cones and melanopsin contribute differentially to melatonin suppression

We found that the sensitivity of melatonin suppression to 555-nm light decayed exponentially relative to 460-nm light during the course of a 6.5-hour light exposure. At the beginning of the intervention, melatonin suppression was equally sensitive to 555-nm light as to 460-nm light, suggesting a substantial contribution from the photopic visual system. By the fourth quarter of light exposure, however, the difference in log relative sensitivity at these wavelengths was consistent with a melanopsin-only response (−0.88 log unit, λ_{max} = 481 nm). On the basis of this short-wavelength shift in spectral sensitivity, we hypothesize that cone photoreceptors provide for temporary suppression of the melatonin rhythm, whereas melanopsin signals light information continuously across long-duration exposure to light. Consistent with this interpretation, a blind individual with no detectable rod or cone function showed a constant level of melatonin suppression across a 6.5-hour exposure to 460-nm light, whereas 555-nm light did not suppress melatonin at all (*4*). Our findings are also similar to "negative masking" responses in mice in which visual photoreceptors drive temporary inhibition of locomotor activity in continuous white light, whereas melanopsin is required for sustained activity

suppression throughout a 3-hour light exposure (*24*). Parallel findings have been reported for the pupillary light reflex in humans and nonhuman primates in response to short-duration exposure (<10 min); visual photoreceptors contribute to pupillary constriction initially, whereas longer steady-state responses and poststimulus constriction appear to be mediated primarily by melanopsin (*25*, *26*). Finally, in response to a short-duration light stimulus (1 or 5 min), mice that lack mid-wavelength–sensitive cones show attenuated phase shifting compared to wild-type mice but normal circadian responses when the light duration is increased (15 min) (*27*). Collectively, these studies suggest that the relative contribution of cones to nonvisual light responses decreases with increasing duration of light, consistent with the present findings in humans. Our data, however, are inconsistent with previous reports in humans showing that melatonin suppression is predominantly short wavelength–sensitive for light exposures ≤90 min in duration (*10*, *14*). These dissimilar findings may be due to methodological differences such as the circadian phase of the light exposure, the light conditions preceding the light intervention, the method of assessing melatonin suppression, and/or the method of fitting and comparing dose-response models.

Although our results are consistent with a gradual reduction in the contribution of cones in driving melatonin suppression, the time course was much longer than that predicted for light adaptation of cone photoreceptors in constant light. Given that we administered the light stimulus near the onset of nocturnal melatonin secretion, the sluggish decay in sensitivity that we observed could be mediated, in part, by simultaneous phase-delay shifting of the melatonin rhythm, as this would delay the recovery of melatonin to baseline values (*28*, *29*). Other physiologic processes that could contribute to the slow time course of recovery include light adaptation of the melanopsin cells (*30*, *31*), the spectral sensitivity and kinetics of melanopsin photoisomerase activity (see below), or complex interactions between melanopsin cells and visual photoreceptors that have yet to be fully elucidated (*32*). Alternatively, the decay in melatonin suppression sensitivity during the night could reflect a circadian decline in the contribution of cone photoreceptors. Therefore, in future studies, it will be important to examine the kinetics of other nonvisual light responses in continuous light and at other circadian phases.

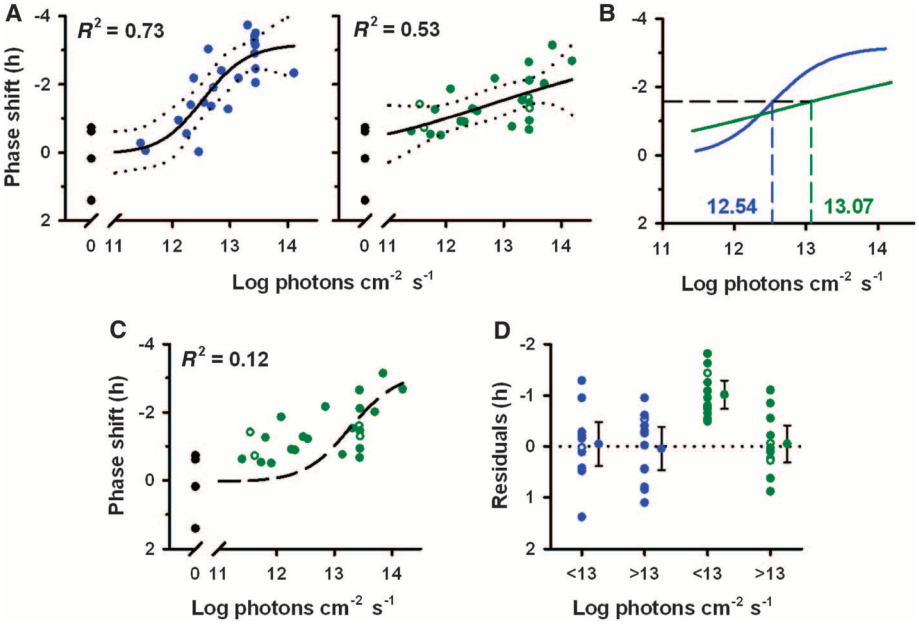

Fig. 3. Circadian phase shifts in response to retinal exposure to 460-nm versus 555-nm light. (**A**) Dose-response curves for circadian phase resetting are shown in response to 6.5 hours of 460-nm light (left, blue circles) versus 555-nm light (right, green circles) exposure. Closed and open circles show phase shifts of plasma or salivary melatonin, respectively, in individual subjects. Black traces show the best-fit dose-response curve with 95% confidence intervals. Black filled circles at 0-log irradiance show phase shifts in response to darkness. (**B**) The dose-response curves are overlaid, demonstrating a difference in relative spectral sensitivity across irradiance levels. The horizontal dashed line indicates the half-maximal phase-shift response, and vertical dashed lines show the corresponding log ED_{50} values, which are indicated on the plot. (**C**) Phase shifts in response to 555-nm light exposure did not match the best-fit univariant dose-response template (black dashed trace). (**D**) At low irradiances (<13 log photons cm^{-2} s^{-1}; ~24 lux for 555-nm light and ~2 lux for 460-nm light), phase-resetting responses to 555-nm light exposure were larger than predicted for a response mediated by melanopsin. Phase-shift residuals are shown relative to the predicted melanopsin-driven response, indicated by the dotted line. The predicted "melanopsin-only" response to 555-nm light exposure was derived by translating the dose-response curve to 460-nm light by the predicted difference in log relative sensitivity at these wavelengths for a photopigment with peak sensitivity to 480-nm light. For each group, the mean is shown with 95% confidence intervals.

Similar to invertebrate opsins, melanopsin photopigment is thought to function as both a photoreceptor and a photoisomerase (*33*–*35*). That is, after activation of melanopsin by light, a different portion of the light spectrum regenerates the chromophore and restores melanopsin photosensitivity. Whereas melanopsin phototransduction is most sensitive to short-wavelength light, recent studies suggest that melanopsin photoisomerase activity may be more sensitive to longer-wavelength light (*26*, *36*, *37*). We found, however, that exposure to 460-nm light alone could maintain melatonin suppression for at least 6.5 hours despite the absence of exposure to any other wavelengths of light that could potentially interconvert "meta"-melanopsin back to its photosensitive form. Short-wavelength light may therefore be sufficient to elicit melanopsin photoisomerase activity even if long-wavelength light is more efficient at restoring photoreceptor function. Alternatively, it is possible that light elicits continuous phototransduction in the melanopsin cells through another biochemical pathway independent of photoisomerase activity (*37*).

Cones contribute to circadian phase shifting at low irradiances

Here, we demonstrated that dose-response curves for circadian phase resetting to 555-nm versus 460-nm light did not fit a univariant model (that is, the curves were not parallel), suggesting that multiple photoreceptor classes mediate human circadian light responses. This

inference is derived from the Principle of Univariance, which specifies that for a response driven by a single class of photoreceptor, dose-response curves to different wavelengths of light have the same shape but differ in relative sensitivity (that is, the log ED_{50} value) (*38*). As melanopsin photopigment may exist in two spectrally distinct states, we cannot rule out the possibility that melanopsin bistability contributed to the nonunivariant behavior we observed for circadian phase resetting (Fig. 3). We consider this possibility unlikely, however, given that the action spectra for melanopsin-driven physiologic responses appear to conform to a univariant photoreceptor model in mice without rod and cone function (*6*, *17*). Our findings are consistent with an irradiance-dependent shift in spectral sensitivity of the human circadian system because of convergent input from visual photoreceptors and melanopsin. Circadian phase shifts were more sensitive to 460-nm light compared to 555-nm light at high irradiances, suggesting a primary role for short-wavelength, light-sensitive melanopsin cells. As irradiance was decreased, however, dose-response curves to 555- and 460-nm light converged such that phase-shift responses to longer-wavelength light were much greater than would be predicted for a melanopsin-only response, suggesting that cones preferentially contribute to circadian photoreception at low-irradiance levels. This interpretation is consistent with the higher sensitivity of cone photoreceptors to light compared to melanopsin-containing ganglion cells (*8*, *20*), the spectral sensitivity of SCN neurons to light pulses in the photopic range (*39*), the preservation of phase resetting in melanopsin-null mice (*6*, *7*, *22*), and the long-wavelength shift in spectral sensitivity for phase-shift responses in mice with intact vision compared to animals without rod and cone function (*6*, *40*). Parallel findings have also been reported for the pupillary light reflex in mice, which is mediated primarily by melanopsin cells at high irradiances and classical visual photoreceptors at low irradiances (*6*, *17*, *41*). Thus, our results extend previous findings in lower mammals and establish a role for visual photoreceptors in human circadian photoreception.

Technical considerations

In an article published while this study was in progress, the spectral sensitivity of human melanopsin cells was defined by examining sustained pupillary constriction after the offset of a light stimulus (*25*). The fitted peak in spectral sensitivity was 482 nm, which is consistent with the spectral tuning of melanopsin cells in the macaque and in lower mammals (*8*, *20*). Therefore, the 460-nm light stimulus that was used in the present study likely elicited strong, but submaximal, stimulation of the melanopsin cells. Similarly, although 555-nm light is best at stimulating the photopic visual system, which is dominated by middle- and long-wavelength–sensitive cones, short-wavelength–sensitive cones respond maximally to ~420-nm light and provide input to the melanopsin cells (*20*). Hence, the short-wavelength stimulus that we used does not provide for complete isolation of the melanopsin-driven response, and we cannot rule out the possibility that rod photoreceptors contributed to the responses. Likewise, the use of 555-nm light does not completely isolate cone function, as melanopsin cells can respond to longer-wavelength light when the irradiance is sufficiently high (*8*, *20*). We hypothesize that in our studies high-irradiance 555-nm light (~14 log photons cm^{-2} s^{-1}) was able to suppress melatonin completely near the end of the 6.5-hour exposure primarily by activating melanopsin rather than cone photoreceptors (Fig. 2). This hypothesis could be tested in blind humans with intact circadian photoreception [that is, in the absence of rod and cone input (*1*, *2*, *4*)] to determine whether high-intensity 555-nm light is sufficient to elicit saturating nonvisual light responses.

Here, we did not examine the potential role of spectral opponency in regulating nonvisual responses, as individual subjects were exposed to single narrow-bandwidth stimuli. Although weak spectral opponent responses have been reported for melatonin suppression (*42*, *43*), other studies suggest that polychromatic light may be as effective, if not more effective, than blue-enriched light at eliciting circadian responses (*44*–*46*). Despite opposing viewpoints on whether adding longer-wavelength light to a short-wavelength stimulus enhances or inhibits circadian responses, these studies are nonetheless consistent with the view that human circadian and neuroendocrine responses receive convergent input from cone photoreceptors. Hence, in future studies, it will be important to determine how the human circadian photoreceptor system integrates and processes complex polychromatic light spectra, especially for light sources commonly used at home and in occupational settings and for lamps used in clinical light therapy applications.

In contrast to results for circadian phase resetting, we did not have the statistical power to detect a difference in the slopes of the dose-response curves for melatonin suppression to 460-nm versus 555-nm light during any quarter of the light exposure (Fig. 2; $F_{3,45} < 2.14$, $P > 0.15$). That these functions can be fit by a univariant model does not contradict our hypothesis that cone photoreceptors and melanopsin contribute to melatonin suppression at the start of the light exposure. Early analytic action spectra studies suggested that the dose response for phase resetting in wild-type animals could be fit with a univariant function (*40*, *47*, *48*), yet it is now well-established that visual photoreceptors and melanopsin contribute to circadian responses in mice (*6*, *7*). It is possible that with larger sample sizes and with adequate sampling across irradiance values, a difference in shape for dose-response curves would emerge for melatonin suppression similar to that described for pupillary constriction in rodless-coneless mice versus melanopsin knockout mice (*41*). Our ability to assess accurately melatonin suppression at the start of the light exposure was limited, in part, by small interindividual differences in the timing of melatonin onset and by the sampling rate of plasma melatonin (every 20 min). This variability was minimized by binning melatonin suppression results over the first hour or more (Fig. 2), but we were unable to compare dose-response curves within the first few minutes of the light exposure when the difference in slopes would be expected to be greatest.

Implications for light therapy

Our findings have implications for the development and optimization of light therapies for a number of disorders, including circadian rhythm sleep disorders (*49*, *50*), seasonal affective disorder (SAD) (*51*, *52*), and dementia (*53*), and the use of light as an alerting stimulus to counter the sleepiness associated with misalignment of circadian phase, particularly during night shift work (*11*, *13*, *54*). It may be important to manipulate the spectrum, duration, and pattern of light dynamically to best stimulate both the melanopsin-driven and the cone-driven photoreceptor systems to maximize the therapeutic potential of light therapy. In taking such an approach, it will be critical to understand the temporal dynamics of the responses of the nonvisual photoreceptor system. Doing so holds the promise of reducing light therapy duration and intensity, thus possibly improving patient compliance and safety (*51*, *52*). The optimal spectral composition of

light for treating SAD and other psychiatric disorders, however, remains undetermined, and it has yet to be shown whether light therapy improves mood through the same sets of photoreceptors that mediate circadian phase resetting and melatonin suppression (*51*, *52*, *55*).

On the basis of the short-wavelength sensitivity of melanopsin, it has been hypothesized that phototherapy can be optimized by using predominantly short-wavelength blue light. Our results indicate that short-duration (<90 min) retinal exposure to narrow-bandwidth 555-nm light (≤24 lux) may be as effective, if not more effective, than an equivalent photon dose of 460-nm light (≤2 lux). Hence, the use of mid-wavelength narrow-bandwidth light early in the exposure period may improve treatment response. Alternatively, assuring that longer-wavelength green light is included in white polychromatic light therapy may be important to an optimal response. Such approaches deserve comparative testing in patients known to respond to light therapy. Our data also raise the possibility that activation of cone photoreceptors in the late evening by relatively low-illuminance light sources, such as liquid crystal display monitors, table lamps, and dimmable lamps, may delay the circadian clock and therefore contribute to the high prevalence of delayed sleep phase disorder (*29*, *56*). Finally, blocking short-wavelength light with blue-blocking goggles may not always be effective in preventing undesired circadian responses (*57*) based on our finding that longer-wavelength light is able to induce robust phase-shift responses.

Designing light therapy to activate optimally melanopsin ganglion cells and visual photoreceptors may be particularly important in a restricted-light environment where bright light may not be available—for example, in submarines, during space and polar missions, or in other poorly lit control rooms, institutions, or environments. Therefore, in the context of everyday life, in which humans are exposed to diverse and variable sources of lighting that vary in irradiance, duration, and spectral content, we hypothesize that the relative contributions of cone photoreceptors and melanopsin to nonvisual light responses vary depending on the nature of the light exposure. The adaptive nature of circadian and neuroendocrine photoreception appears to be analogous to other major sensory systems in mammals, such as image-forming vision and touch, in which multiple receptor subtypes respond differentially to the strength, frequency, and timing of stimuli to ensure appropriate physiologic responses.

MATERIALS AND METHODS

Subjects

Healthy research subjects (n = 66) ages 18 to 30 years were enrolled in a 9-day inpatient study at the Intensive Physiologic Monitoring (IPM) Unit, Brigham and Women's Hospital (BWH; Boston, MA). Physical health was assessed by medical history, physical examination, blood biochemistry and hematology, and electrocardiogram, and mental health was evaluated by interview with a staff psychologist or psychiatrist. Normal sight was confirmed by an ophthalmologic examination and the Ishihara test for color blindness. Sleep and circadian rhythm disorders were exclusionary. For at least 2 weeks before being admitted to the IPM, subjects were required to maintain a regular sleep-wake schedule (8 hours sleep, 16 hours wake), which was verified by continuous actigraphy monitoring (Actiwatch-L, Mini-Mitter). A comprehensive toxicology screen was performed on the day of admission to the IPM to ensure that subjects had refrained from the use of drugs. Of the 66 subjects who were enrolled, 8 subjects were discontinued before being randomized to the experimental light exposure. Four subjects were omitted from the analysis because of equipment failure and subsequent data loss during the light intervention, and two subjects were excluded post hoc because the light exposure was administered at an inappropriate circadian phase (>3.0 hours from melatonin onset). Results from 16 subjects were reported previously by Lockley *et al.* (*12*). Informed consent was obtained from all subjects, and research procedures were approved by the Institutional Review Board at BWH and were in compliance with Health Insurance Portability and Accountability Act regulations and the Declaration of Helsinki.

Protocol design

Subjects lived individually for 9 days (Fig. 1) in an environment free of time cues. During the first 3 days, subjects were scheduled to sleep and wake at their regular prestudy sleep-wake times (8 hours sleep, 16 hours wake). Ambient light was provided by 4100K fluorescent lamps (Philips Lighting). Subjects lived in room light (<190 lux, 0.48 W/m^2 measured in the horizontal plane at 183 cm) until midway through day 3, after which the light was dimmed to <3 lux (<0.01 W/m^2) for the remainder of the study. After awakening on day 4, subjects underwent a 50-hour constant routine procedure consisting of wakefulness enforced by technician monitors, semirecumbent bed rest, and consumption of hourly equicaloric snacks (*58*). After an 8-hour sleep opportunity, subjects awoke in the evening and were administered a 6.5-hour narrow-bandwidth light exposure in a modified Ganzfeld dome (*10*, *12*, *13*). For the light exposure (day 6), a between-subjects design was used in which subjects were assigned to one of two wavelength conditions (460 or 555 nm). In each group, subjects were randomized to 16 irradiances across a broad range of photon densities (2.52×10^{11} to 1.53×10^{14} photons cm^{-2} s^{-1}). These photon densities correspond to approximate illuminances of 0.04 to 27 lux for the 460-nm stimulus and 0.6 to 375 lux for the 555-nm stimulus. Narrow-bandwidth light (half-peak bandwidth = 10 to 14 nm) was generated by a xenon arc lamp and grating monochromator, and the wavelength and bandwidth were verified by measurement with a PR-650 SpectraColorimeter (Photo Research). Before the onset of the light exposure, one drop of 0.5% cyclopentolate HCl was administered in each eye to dilate the pupils (Cyclogyl, Alcon Laboratories). Head position was fixed by a chinrest, and subjects stared at the light continuously for 90 min at a time, followed by a 10-min break during which they could look elsewhere in the otherwise dark room. Subjects were asked to refrain from photophobic behavior (for example, squinting or closing of the eyes), and compliance was monitored by a technician. The light was measured every 30 to 60 min at eye level with an IL1400 radiometer and SEL-033/F/W detector (International Light) to ensure constant irradiance throughout the light exposure. For each wavelength of light, subjects were randomized to an irradiance level just before administration of the light exposure. After completion of the light exposure and an 8-hour sleep opportunity, subjects underwent a second constant routine for 30 hours. After recovery sleep, subjects awoke on day 9 at their habitual wake time and were discharged from the study.

Specimen collection and melatonin assays

On day 2 of the study, an indwelling intravenous catheter was inserted in a forearm vein to allow for continuous collection of blood during

both sleep and wake episodes. During sleep episodes, the constant routine procedures, and the light exposure session, blood was drawn from outside the research suite through a porthole in the bedroom wall. Blood was sampled every 30 min during the constant routine procedures and every 20 min during the 6.5-hour light exposure. Saliva samples were collected hourly during the constant routines and the light intervention, and sample times were digitally time-stamped using a Termiflex system (Warner Power Termiflex). Melatonin concentration was determined by double-antibody radioimmunoassay with the Kennaway G280 antiserum (*59*) by a laboratory blind to condition (V. Ricchiuti, BWH General Clinical Research Center Core Laboratory, Boston, MA). The plasma melatonin intra-assay coefficient of variation (CV) was 10.0% at 1.9 pg/ml and 7.2% at 21.9 pg/ml, and the interassay CV was 12.65% at 3.06 pg/ml and 12.12% at 22.36 pg/ml. The saliva melatonin intra-assay CV was 4.1% at 3.56 pg/ml and 4.8% at 24.2 pg/ml, and the inter-assay CV was 12.15% at 2.37 pg/ml and 10.20% at 19.58 pg/ml.

Melatonin suppression and phase-shift responses

To determine percent suppression of melatonin, we compared the area under the curve (AUC; trapezoidal method) for melatonin during the 6.5-hour light exposure (AUC_{LE}) to the AUC for the melatonin rhythm during the preceding constant routine at the same relative clock times (AUC_{CR1}). Thus, percent melatonin suppression was calculated as $[1 - (AUC_{LE})(AUC_{CR1})^{-1}] \times 100$, whereby higher values indicated stronger suppression of the melatonin rhythm. In five subjects from the 555-nm group, salivary melatonin was used to determine melatonin suppression because there was an insufficient number of blood samples collected during either the constant routine or light exposure. In some subjects, a small negative percent melatonin suppression value was found, which indicated that melatonin levels during the light intervention were slightly higher than those observed during the preceding constant routine. To determine the magnitude of phase-shift responses, we fit the prelight exposure melatonin rhythm during the first constant routine procedure by a three-harmonic regression model to estimate the amplitude. The dim light melatonin onset ($DLMOn_{25\%}$) was defined as the clock time at which the melatonin rhythm crossed a threshold value of 25% of the peak-to-trough fitted amplitude (half the standard amplitude). The phase shift of the melatonin rhythm was calculated as the difference in the timing of $DLMOn_{25\%}$, measured before and after the light exposure intervention using constant routine procedures (days 5 and 7). Phase shifts were determined from plasma melatonin in 46 subjects and from salivary melatonin in 6 subjects (460 nm, $n = 2$; 555 nm, $n = 4$) because of blood sampling difficulties. By convention, phase delays are indicated by negative values, and phase advances by positive values.

Construction of dose-response curves

Dose-response curves were fit with a sigmoidal four-parameter logistic regression model, wherein y_0 is the minimum response, a is the difference between the maximum and minimum response, x_0 is the irradiance that elicits a half-maximal response (the ED_{50} value), and b is the slope parameter:

$$y = y_0 + \frac{a}{1 + \left(\frac{x}{x_0}\right)^b}$$

To determine the set of dose-response curve parameters that resulted in the minimal sum of squares of the residuals, we used the Levenberg-Marquardt method (SigmaPlot 11, Systat Software). The residuals were normally distributed, as determined by the Shapiro-Wilk test for normality (for all dose-response curves, $W > 0.93$ and $P > 0.05$). A global curve-fitting procedure was used to determine the best-fit shared maximum and minimum phase-resetting responses to 460-and 555-nm light exposures. Maximum and minimum phase shifts were −3.19 and 0.034 hours, respectively. These values correspond closely to the saturating phase-shift response to bright polychromatic white light reported previously (−3.24 hours) (*29*) and the average phase shift in response to 6.5 hours of darkness measured in the present study (0.062 hour; $n = 4$). The maximum melatonin suppression response was constrained to 95%, as in our experience melatonin suppression assessed by AUC rarely exceeds this threshold.

To examine the dose response of melatonin suppression across time, we constructed dose-response curves in quarterly (one quarter = 97.5 min) and 1-hour bins across the 6.5-hour light intervention. In the latter analysis, the onset of each bin was spaced at 30-min intervals, resulting in 12 serial dose-response curves. Thus, successive bins overlapped by 30 min each, allowing for smoothing of the data across time. When a melatonin sample did not occur precisely at the onset or offset of a bin, the concentration of melatonin was interpolated linearly from the samples that bracketed the given time point. For each set of dose-response curves shown in Fig. 2C, the log relative sensitivity in Fig. 2D was determined by subtracting the log ED_{50} for the dose response to 555-nm light exposure from the log ED_{50} for the dose response to 460-nm light exposure. The reduction in relative sensitivity across time was modeled by a three-parameter exponential decay function, which was used to calculate the half-life of the difference in relative sensitivity for melatonin suppression in response to 555-nm versus 460-nm light exposure.

Data analysis and statistics

The extra sum-of-squares F test was used to compare dose-response models. This F test allows for comparison of nested models that have a different number of parameters (*60*). To test whether the log ED_{50} or slope parameter differed significantly between dose-response curves, we performed a global curve fit in which the best-fit value for each parameter was shared for dose-response curves to 555-nm versus 460-nm light exposures. The F test was used to determine whether the more complicated model with more parameters (that is, the model with unshared log ED_{50} or slope) resulted in a significant improvement in the difference in sum of squares as compared to the simpler model with fewer parameters (that is, the model with shared log ED_{50} or slope).

To test whether phase-shift responses to 555-nm light exposures were higher than expected for a response mediated by melanopsin, we first derived the predicted absorption spectrum for a vitamin A1–based photopigment with peak sensitivity to 480-nm light exposure using a nomogram procedure (*23*). The predicted univariant dose-response curve to 555-nm light exposure was determined by translating the dose-response curve to 460-nm light by the difference in log relative sensitivity to 555-nm versus 460-nm light exposure for the absorption spectrum template (−0.91 log unit). The observed phase-shift responses to 555-nm light exposure were compared to the predicted melanopsin-driven responses ($\lambda_{max} = 480$ nm) by performing a one-sample t test on the residuals (H_0 = means of residuals = 0; H_A = mean of residuals ≠ 0).

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Since 1985, he has also served as an expert witness on various legal cases related to sleep and/or circadian rhythms. Patents: beneficiary or inventor on several patents related to assessment and modification of the phase and amplitude of the endogenous circadian rhythm, apparatus for delivering high-intensity light to modify circadian rhythms, a method to modify circadian rhythms with short-wavelength light, and a test for evaluating visual function in visually impaired people. S.W.L has received consulting fees from Apollo Lighting and holds a consulting contract with Wyle Integrated Science and Engineering (NASA) to complete an evidence review. He is/was a consultant on federally funded projects at Brigham and Women's Hospital, Thomas Jefferson University and Warwick Medical School. 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