

## Human Melatonin Regulation is Not Mediated by the Three Cone Photopic Visual System

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**ABSTRACT** The aim of this study was to test the hypothesis that the primary visual system is the primary ocular photoreceptive input for human circadian regulation by determining the effects of different wavelengths on light-induced melatonin suppression. Healthy subjects with stable sleep/wake schedules were exposed at night to full-field 505 nm or 555 nm light. The magnitude of melatonin suppression was quantified for melatonin (10 pmol/L) at 0, 10, and 20 min after exposure. The mean pre-exposure melatonin values ( $P=0.001$ ) were significantly lower ( $P=0.001$ ) for the 505 nm exposure ( $8.7 \pm 0.97$ ) compared with the 555 nm exposure ( $9.34 \times 10^{12}$  photons/cm<sup>2</sup>/sec). Furthermore, the 505 nm exposure induced a significantly stronger ( $P<0.01$ ) melatonin suppression than the 555 nm exposure. These results suggest that the cone system that mediates human photopic vision is not the primary photoreceptive system to transduce light stimuli for circadian regulation.

The underlying mechanism of circadian regulation, at least in mammals, is not understood. Together, the results from human and animal circadian studies on different forms of visual blindness suggest that melatonin regulation by light is controlled, at least in part, by photoreceptors which are not part of the visual system. Among these photoreceptors, the rod system has been shown to have a role in circadian regulation, but the cone system may also serve as a photoreceptive input for circadian regulation. The pineal gland secretes melatonin, which is vertebrate circadian time. The pineal gland is innervated by the sympathetic nervous system, and the pineal gland secretes melatonin in a circadian manner. The pineal gland is innervated by the sympathetic nervous system, and the pineal gland secretes melatonin in a circadian manner.

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Curiously, it is not known what photoreceptors transduce light stimuli for circadian regulation. Studies on animals with hereditary or light-induced retinal degeneration have raised the possibility that neither the rods nor the cones used for vision participate in light-induced melatonin suppression, circadian locomotor phase-shifts, or photoperiodic responses (8–12). Furthermore, bilateral removal of the eyes from rod-less, cone-less transgenic mice abolished light-induced circadian phase-shifting and acute melatonin suppression (8, 12). Recently, light-induced melatonin suppression and circadian entrainment have been demonstrated in humans with complete visual blindness (13) and with specific color vision deficiencies (14). The study on humans with color vision deficiencies showed that protanopic and deuteranopic subjects who lacked functioning long wavelength-sensitive cones (red, or L cones), and middle wavelength cone photoreceptors (green, or M cones), exhibited normal light-induced melatonin suppression and entrainment of the melatonin rhythm (14). Thus, by themselves, neither the rod nor green cone system could be

responsible for circadian regulation. Together, the results from human and animal circadian studies on different forms of visual blindness suggest that melatonin regulation by light is controlled, at least in part, by photoreceptors which are not part of the visual system. Among these photoreceptors, the rod system has been shown to have a role in circadian regulation, but the cone system may also serve as a photoreceptive input for circadian regulation.

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The aim of this study was to test the hypothesis that the three cone system which supports photopic (daytime) vision is the primary input for pineal melatonin suppression in humans with normal, healthy eyes. The peak wavelength sensitivity of the photopic visual system is generally believed to be near 555 nm (19). If melatonin regulation is mediated primarily by the three cone photopic visual system, then 555 nm light would be the most potent wavelength for regulating melatonin secretion. Our data show that 505 nm is approximately four times stronger than 555 nm in suppressing melatonin. These results demonstrate that the ocular photoreceptors primarily responsible for pineal melatonin regulation in humans, is not the cone system that is believed to mediate photopic vision. This study is the first test of a specific photoreceptor system for melatonin regulation in humans with healthy, intact eyes.

### Subjects, Materials and Methods

**Subjects.** This healthy females (N=10) and males (N=10) in this study had a mean  $\pm$  SEM age of  $25.7 \pm 0.6$  yrs, demonstrating normal color vision as measured by the Munsell and Farnsworth

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**ABSTRACT** The aim of this study was to test if the three cone photopic visual system is the primary ocular photoreceptor input for human circadian regulation by determining the effects of different wavelengths on light-induced melatonin suppression. Healthy subjects with stable sleeping patterns (wake-up time 7:30 AM  $\pm$  12 min) and normal color vision were exposed at night to full-field 505 nm or 555 nm monochromatic stimuli or darkness for 90 min. Plasma collected before and after exposures was quantified for melatonin. Subjects exposed to 10 irradiances at 505 nm showed no significant differences across mean pre-exposure melatonin values ( $F=0.505$ ). A sigmoidal fluence-response curve fitted to the melatonin suppression data ( $R^2=0.97$ ) indicated that  $9.34 \times 10^{12}$  photons/cm<sup>2</sup>/sec induced a half-saturation response ( $ED_{50}$ ) while  $6.84 \times 10^{13}$  photons/cm<sup>2</sup>/sec induced a saturation melatonin suppression response. Further, a dose of  $4.19 \times 10^{13}$  photon/cm<sup>2</sup>/sec at 505 nm was significantly stronger ( $P<0.01$ ) than an equal photon dose at 555 nm for melatonin suppression. These data demonstrate that the cone system that mediates human photopic vision is not the primary photoreceptor system to transduce light stimuli for melatonin regulation.

### Introduction

The underlying neuroanatomy and neurophysiology which mediate vision have been studied extensively over the past two centuries. More recently, the retinohypothalamic tract (RHT), a distinct neural pathway which supports circadian regulation by environmental light, has been shown to project from the retina to the suprachiasmatic nuclei (SCN) in the hypothalamus (1, 2). A well-defined multisynaptic neural pathway extends from the SCN to the pineal gland which transmits information about light and circadian time for entraining the rhythmic production and secretion of the hormone melatonin (1-4). In addition to synchronizing pineal indolamine circadian rhythms, ocular exposure to light during the night can acutely suppress melatonin synthesis and secretion (5, 6). Light-induced melatonin suppression is a well-defined, broadly used marker for photic input to the RHT and SCN (2, 4, 7, 8).

Currently, it is not known what photoreceptors transduce light stimuli for circadian regulation. Studies on animals with hereditary or light-induced retinal degeneration have raised the possibility that neither the rods nor the cones used for vision participate in light-induced melatonin suppression, circadian locomotor phase-shifts, or photoperiodic responses (8-12). Furthermore, bilateral removal of the eyes from rod-less, cone-less transgenic mice abolished light-induced circadian phase-shifting and acute melatonin suppression (8, 12). Recently, light-induced melatonin suppression and circadian entrainment have been demonstrated in humans with complete visual blindness (13) and with specific color vision deficiencies (14). The study on humans with color vision deficiencies showed that protanopic and deuteranopic subjects who lacked functioning long wavelength-sensitive cones (red, or L cones), and middle wavelength cone photoreceptors (green, or M cones), exhibited normal light-induced melatonin suppression and entrainment of the melatonin rhythm (14). Thus, by themselves, neither the red nor green cone system could be

the primary input for melatonin regulation, at least in humans with color vision deficiencies. Together, the results from human and animal circadian studies on different forms of visual blindness suggest that melatonin regulation by light is controlled, at least in part, by photoreceptors which differ from the photoreceptors that mediate vision.

Recent studies with various vertebrate species have identified several new molecules which may serve as circadian photopigments. These putative photopigments include both opsin-based molecules, such as vertebrate ancient (VA) opsin and melanopsin, as well as non-opsin molecules like the cryptochromes (15-17). Among these new photopigments, only melanopsin has been specifically localized to the human retina (18). The molecular identification of these candidate photopigments and their localization in the retina and/or neural components of the circadian system make them well-suited to act as circadian phototransducers. Functional data confirming their direct role in circadian photoreception, however, are lacking.

The aim of this study was to test the hypothesis that the three cone system which supports photopic (daytime) vision is the primary input for pineal melatonin suppression in humans with normal, healthy eyes. The peak wavelength sensitivity of the photopic visual system is generally believed to be near 555 nm (19). If melatonin regulation is mediated primarily by the three cone photopic visual system, then 555 nm light would be the most potent wavelength for regulating melatonin secretion. Our data show that 505 nm is approximately four times stronger than 555 nm in suppressing melatonin. These results demonstrate that the ocular photoreceptor primarily responsible for pineal melatonin regulation in humans, is not the cone system that is believed to mediate photopic vision. This study is the first test of a specific photoreceptor system for melatonin regulation in humans with healthy, intact eyes.

### Subjects, Materials and Methods

**Subjects.** The healthy females (N=6) and males (N=10) in this study had a mean  $\pm$  SEM age of  $25.7 \pm 0.8$  yrs, demonstrated normal color vision as measured by the Ishihara and Farnsworth

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Munsell D-100 tests (mean FM score:  $64.2 \pm 11.5$ ), had a stable sleeping pattern (mean wake-up time 7:30 AM  $\pm$  12 min), and signed an approved IRB consent document before participating.

**Light exposure protocol.** Each experiment began at midnight when subjects entered a dimly lit room (10 lux). One drop of 0.5% Cyclopentolate HCl was placed in each eye to dilate the pupils, and blindfolds were placed over subjects' eyes. Subjects remained sitting upright in darkness for 120 min. While still blindfolded and just prior to 2:00 AM, a blood sample was taken by venipuncture. During light exposure, each subject's head rested in an ophthalmologic head holder facing a Ganzfeld apparatus that provided a concave, patternless, white reflecting surface encompassing the subject's entire visual field. The subjects were exposed to the light stimulus from 2:00 to 3:30 AM. During this 90 min exposure, subjects sat quietly, kept their eyes open and gazed at a fixed target dot in the center of the Ganzfeld dome. Subject compliance for keeping their eyes open and the subjects' pupil size were monitored by a miniature video camera. At 3:30 AM, a second blood sample was taken. Each subject was exposed to complete darkness from 2:00 to 3:30 AM on their control night and was tested with at least 6 days between each nighttime exposure. Plasma samples were assayed for melatonin by RIA (20). The minimum detection limit of the assay was 0.5 - 2.0 pg/mL. Control samples had 8% and 14% intra-assay coefficients of variation.

**Light production and measurement.** Experimental light stimuli were produced by xenon arc lamps (Photon Technology Int'l, Inc., Princeton, NJ) enclosed in a light-proof chamber and cooled by high-speed fans and water circulation. An exit beam of light from each source was directed by a parabolic reflector, and excess heat in this beam was reduced by a water filter. Monochromatic wavelengths (10-11 nm half-peak bandwidth) were produced by a grating monochromator and light irradiance was controlled by a manual diaphragm. The resulting light beam was directed into the top area of a Ganzfeld dome and reflected evenly off the walls into volunteers' eyes. The entire reflecting surface of the dome was coated with a white surface with a 95-99% reflectance efficiency over the 400 to 760 nm range. Routine measurement of the light irradiance ( $\mu\text{W}/\text{cm}^2$ ) was done with a J16 Meter with a J6512 irradiance probe (Tektronix, Beaverton, OR). Experimental light stimuli reflected from the Ganzfeld domes were measured at volunteers' eye level immediately before and after the 90 min exposure. Additional measures were taken each half hour of the exposure to insure stimulus stability and enable intensity readjustment. Subjects in the 505 nm series were exposed to intensities ranging from 0.011 to 97  $\mu\text{W}/\text{cm}^2$  (a 3 log unit photon density range of  $10^{10}$  to  $10^{13}$  photons/ $\text{cm}^2$ ). Subjects exposed to 555 nm received control or a 15  $\mu\text{W}/\text{cm}^2$  ( $4.2 \times 10^{13}$  photons/ $\text{cm}^2$ ) exposure.

**Statistics.** Two-tailed, Students' t tests were used to assess significance of raw melatonin change from 2:00 to 3:30 AM. These data were then converted to % control-adjusted melatonin change scores as described elsewhere (21). For the 505 nm data, sets of pre-exposure melatonin values and % control-adjusted melatonin change scores were analyzed with one-way, repeated measures ANOVA. Significant differences between groups were assessed with post-hoc Scheffe F-tests; alpha was set at 0.05. For the 505 nm mean % control-adjusted melatonin suppression data, the computer program Origin 6.0 (Microcal, Northampton, MA) was used to fit a fluence-response curve to a 4 parameter model as described elsewhere (22), and to test for goodness-of-fit of the data by coefficient of correlation.

### Results

The full 505 nm data complement, from raw melatonin values to a final fluence-response curve, is illustrated in

Figures 1-3. Across all nights of testing, there were no significant differences ( $F=0.85$ ) between sets of pre-exposure melatonin values indicating that plasma levels were consistent across the different study nights. Figure 1 shows the mean  $\pm$  SEM pre- and post-exposure melatonin values. One-way, repeated measures ANOVA showed a significant effect of light intensity on plasma melatonin % change scores ( $F=17.17$ ,  $P<0.0001$ ). Paired t tests demonstrated that compared to the 0  $\mu\text{W}/\text{cm}^2$  control night, all intensities at or above 5.5  $\mu\text{W}/\text{cm}^2$  significantly suppressed melatonin ( $P<0.05$  or less). In contrast, irradiances of 2.8, 1.4 and 0.011  $\mu\text{W}/\text{cm}^2$  did not suppress melatonin compared to the control night (Scheffe F values: 0.97, 0.01 and 0.02, respectively). As illustrated in Figure 2, all melatonin data were converted to control-adjusted % change scores. As with the melatonin % change scores, ANOVA showed a significant effect of light intensity on plasma melatonin % control-adjusted change scores ( $F=13.59$ ,  $P<0.0001$ ).

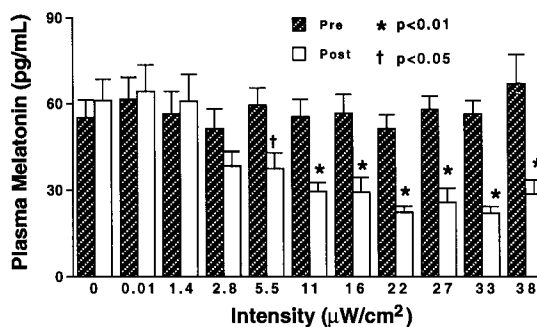


Figure 1: In this graph the bars represent group mean + SEM plasma melatonin values ( $N=8$ ) before and after monochromatic light exposure at 505 nm. There were no significant variations across mean melatonin pre-light exposure values ( $F=0.85$ ). Paired, two-tailed Students' t tests demonstrated which light intensities elicited a significant melatonin suppression.

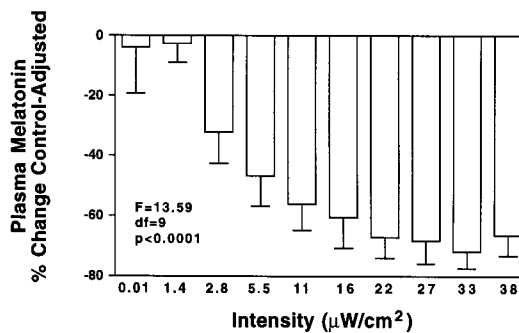


Figure 2: This graph illustrates group mean + SEM % control-adjusted melatonin change values ( $N=8$ ) at 505 nm monochromatic light exposure. The figure shows that progressively higher light irradiance exposure produces increasingly greater melatonin suppression.

Figure 3 illustrates a best fit, sigmoidal fluence-response curve which plots melatonin % control-adjusted scores against stimulus photon density. The specific formula for this curve is included in the figure.

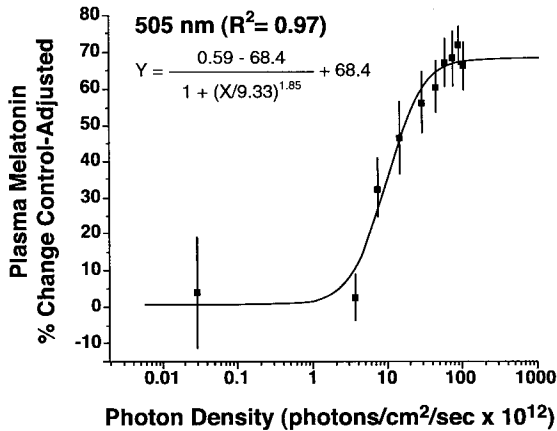


Figure 3: This figure demonstrates the fitted fluence-response curve for photon density and % control-adjusted melatonin suppression (N=8). Each data point represents one group mean ± SEM.

Subjects exposed to 555 nm received both control (0  $\mu\text{W}/\text{cm}^2$ ) and 15  $\mu\text{W}/\text{cm}^2$  ( $4.2 \times 10^{13}$  photons/ $\text{cm}^2$ ) exposures. For the control and light exposure nights, the mean pre-exposure raw melatonin scores were  $64.4 \pm 12.5$  and  $59.6 \pm 6.2$ , while the mean post-exposure scores were  $62.6 \pm 10.5$  and  $49.1 \pm 6.0$ , respectively. The modest drop in melatonin over the 90 min 555 nm light exposure period was not statistically significant ( $t=1.69$ ,  $df=7$ ,  $P=0.14$ ). For comparison of responses to 505 nm and 555 nm, Figure 4 illustrates % control-adjusted melatonin suppression relative at equal photon densities across the two wavelengths. These data reveal that 505 nm is significantly stronger than 555 nm in suppressing melatonin ( $t=-3.04$ ,  $df=14$ ,  $P<0.01$ ).

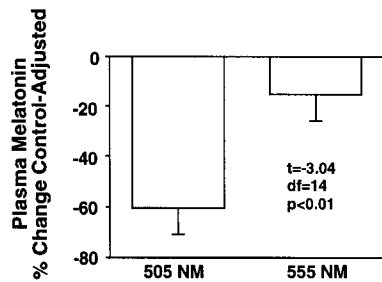


Figure 4: The bars represent group mean + SEM values relative to an equal photon dose of  $4.2 \times 10^{13}$  photons/ $\text{cm}^2$ . These data show that the 505 nm % control-adjusted plasma melatonin suppression is significantly stronger than that for 555 nm.

**Discussion**

The data presented here demonstrate that: 1) there is a clear fluence-response relationship between graded light intensities of 505 nm light and melatonin suppression, and 2) that 505 nm light is significantly stronger than 555 nm light for suppressing melatonin in healthy, human subjects. Previous studies with animals and humans have illustrated fluence-response relationships for melatonin suppression and circadian phase-shifting with exposure to monochromatic light (23-25) as well as white light (22, 26). A recent study on human subjects suggests that a four parameter curve is optimum for modeling light-induced melatonin suppression and circadian phase shifting (27). That contention matches our own earlier animal data (22) as well as the 505 nm data reported here.

The demonstration that 505 nm light is more potent than 555 nm light for controlling melatonin has important basic science and clinical implications. In humans, it is well-established that higher levels of ocular illumination are required for stimulating the circadian system than for supporting vision (6, 25, 30). Consequently, many investigators have considered the three cone photopic visual system to be responsible for stimulating circadian and neuroendocrine responses since this part of the visual system is responsive to "bright" daytime levels of illumination. Over the past 20 years most of the published literature on human circadian responses to light reports light levels in terms of illuminance (lux, lumens) which is a specific measure based on the traditional sensitivity curve of the photopic visual system. The peak wavelength sensitivity of that curve is 555 nm (19). Indeed, some researchers have argued that their data support the notion that the visual cones are involved in circadian phase-shifting in humans (28). If melatonin regulation is mediated primarily by the three cone photopic visual system, then 555 nm light should be the most potent wavelength for regulating melatonin. Our data do not support this hypothesis. On the contrary, 505 nm is significantly stronger, photon for photon, than 555 nm in suppressing melatonin. The clinical implication of this result is that it is not optimum to use photometric values (lux) for quantifying light used therapeutically in patients with certain sleep disorders or circadian disruption due to shiftwork or intercontinental jet travel as is the current standard practice (29). It should be noted, however, that the data presented here involve only the melatonin suppression response in healthy humans. It remains to be determined if there are similar wavelength sensitivities for circadian phase-shifting or light therapy for clinical disorders.

Ultimately, these studies open the door for redefining how light should be measured relative to the circadian system. The best circadian measurement system would match the action spectrum for human circadian regulation. That action spectrum would not only elucidate the relative circadian potencies of different wavelengths, but it should help identify the photoreceptor that initiates signals from the retina to the SCN. Unfortunately, an action spectrum has yet to be established for any circadian response in humans. It remains possible that one of the "classic" visual photopigments such as rhodopsin or the short wavelength sensitive (blue) cone opsin mediates circadian

phototransduction. Alternatively, one of the new opsin or non-opsin molecules that have been proposed as circadian photopigments (15-17) may prove to serve this function. Until the action spectrum for circadian regulation in humans has been clarified, it remains reasonable to continue using photometric terminology and measurements for characterizing light that regulates the circadian system.

In summary, monochromatic 505 nm light suppressed melatonin in a fluence-response manner, and is approximately four times stronger than a 555 nm stimulus at an equal photon dose for melatonin suppression. These data demonstrate that the three cone system that is believed to mediate human photopic vision is not the primary photoreceptor system to transduce light stimuli for melatonin regulation.

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