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Sensitivity of the human circadian system to short wavelength (420 nm) light

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Abstract The circadian and neurobehavioral effects of light are primarily mediated by a retinal ganglion cell photoreceptor in the mammalian eye containing the photopigment, melanopsin. Nine action spectrum studies using rodents, monkeys, and human for these responses indicate peak sensitivities in the blue region of the visible spectrum ranging from 459 nm to 484 nm, with some disagreement in short wavelength sensitivity of the spectrum. The aim of this work was to quantify the sensitivity of human volunteers to monochromatic 420 nm light for plasma melatonin suppression. Adult female (N=14) and male (N=12) subjects participated in two studies, each employing a within-subjects design. In a fluence-response study, subjects (N=8) were tested with eight light irradiances at 420 nm ranging over a four log unit photon density range of 10^{10} to 10^{14} photons/cm^2/sec and one dark exposure control night. In the other study, subjects (N=18) completed an experiment comparing melatonin suppression with equal photon doses (1.21×10^{13} photons/cm^2/sec) of 420 nm and 460 nm monochromatic light and a dark exposure control night. The first study demonstrated a clear fluence-response relationship between 420 nm light and melatonin suppression ($p<0.001$) with a half-saturation constant of 2.74×10^{11} photons/cm^2/sec. The second study showed that 460 nm light is significantly stronger than 420 nm light for suppressing melatonin ($p<0.04$). Together, the results clarify the visible short wavelength sensitivity of the human melatonin suppression action spectrum. This basic physiological finding may be useful for optimizing lighting for therapeutic and other applications.

Key Words melatonin, action spectrum, circadian, wavelength, light, pineal gland, neuroendocrine, photoreception

Introduction

Light can be a potent therapeutic intervention for patients with selected affective and sleep disorders as well as healthy individuals who have circadian disruption due to shift work, transcontinental jet travel, or manned space flight (Commission Internationale de l'Eclairage (CIE), 2004; CIE, 2006; Dijk et al., 2001). The ocular photoreceptive physiology that supports the therapeutic capacity of light, however, has been described only in a nascent fashion.

Two action spectra identified 446-477 nm as the most potent wavelength region for acute plasma melatonin suppression in human subjects (Brainard et al., 2001; Thapan et al., 2001). Data from both studies shared many similarities and suggested that a novel vitamin A retinaldehyde-based photopigment was primarily responsible for this effect. There was poor agreement between these studies, however, on the sensitivity to light at 420-424 nm. One action spectrum tested subjects with 420 nm at two different intensities and calculated that 420 nm would be substantially weaker than the peak wavelength in the melatonin action spectrum (Brainard et al., 2001). In contrast, the other action spectrum tested 424 nm at four intensities and showed that 424 nm was substantially stronger than the peak wavelength of its action spectrum (Thapan et al., 2001).

Recently, there has been an upheaval in understanding the photoreceptive input to the circadian system. A new photopigment, named melanopsin, has been localized in the retinas of rodents, monkeys, and humans (Provencio et al., 2000). Melanopsin is found in a specific subtype of retinal output neuron, the intrinsically photosensitive ganglion cells (ipRGCs) that

project to the suprachiasmatic nuclei (SCN) (Berson et al., 2002; Hattar et al., 2002; Gooley et al., 2001). The ipRGC responses to light appear to parallel those of melatonin suppression and photic entrainment, suggesting that these cells are primary photoreceptors involved in circadian regulation (Berson et al., 2002; Hattar et al., 2002).

Altogether, nine analytic action spectra in humans, monkeys, and rodents have demonstrated the wavelength sensitivity of physiological responses that are mediated by the newly characterized ipRGCs (Brainard et al., 2001; Thapan et al., 2001; Hattar et al., 2002; Hattar et al., 2003; Dacey et al., 2005; Gamlin et al., 2007; Brainard and Hanifin, 2005, for review). Notably, all of the action spectra were fit to single opsin nomograms with high coefficients of correlation and indentify shorter wavelength peak photosensitivities in the blue region of the visible spectrum with calculated peaks ranging from 459 nm to 484 nm. Despite differences in laboratories, physiological endpoints, animal models, and specific techniques, there is a consistent detection of peak responses in the blue spectrum (Brainard and Hanifin, 2005; for review). Together, these studies suggest that a novel ocular photoreceptor system is involved in phototransduction for circadian, neuroendocrine, and other neurobehavioral responses (such as pupil constriction, acute alerting effects, cognitive responses and the like).

Three recent studies have provided compelling evidence that melanopsin is the photopigment that mediates ipRGC phototransduction (Melyan et al., 2005; Qiu et al., 2005; Panda et al., 2005). Specifically, when mouse cells are transfected with the human melanopsin gene they become photosensitive with peak response deeper into the violet – indigo portion of the visible spectrum in the range of 360-430 nm (Melyan et al., 2005). That pattern of photosensitivity encompasses the *in vitro* peak absorption spectrum of melanopsin (Newman et al., 2003). In contrast, human kidney cells transfected with the mouse melanopsin gene are light responsive with peak sensitivity at 479 nm (Qiu et al., 2005). Likewise, the expression of mouse

melanopsin in *Xenopus* oocytes confers peak sensitivity at 480 nm (Panda et al., 2005). These latter two studies identify peaks that are reflective of the action spectra that peak in the blue spectrum (Gamlin et al., 2007; Brainard and Hanifin, 2005, for review). Clearly, there is not complete agreement in specific wavelength sensitivity across the action spectra studies, the study on melanopsin absorption spectrum, and the photic responses of cells transfected with the melanopsin gene. The collective results are consistent, however, in demonstrating a wavelength signature in the short wavelength visible spectrum that appears distinct from the wavelength sensitivity of rod and cone systems that mediate vision.

The aim of this study was to quantify the sensitivity to monochromatic 420 nm for pineal melatonin suppression in humans. The data demonstrate that there is a fluence-response relationship between 420 nm light and melatonin suppression that is consistent with the fluence-response curves for eight other wavelengths (Brainard et al., 2001). A second study shows that 460 nm light is approximately twice as strong as 420 nm light for suppressing plasma melatonin. Together, the results clarify the short visible wavelength sensitivity of the human melatonin suppression action spectrum.

Research Design and Methods

Study designs

In the first study, 8 subjects completed a within-subjects fluence-response experiment that tested eight light irradiances at 420 nm and one dark exposure control night on nocturnal melatonin suppression. In the second study, 18 subjects completed a within-subjects experiment comparing melatonin suppression with equal photon doses of 420 nm and 460 nm monochromatic light and a dark exposure control night.

Subjects

The healthy subjects in both studies had a mean ± SEM age of 24.5 ± 0.6, demonstrated normal color vision by the Ishihara test, had a mean wake up time of 6:54 AM ± 18 min, and signed an approved IRB consent document. All subjects in the fluence-response study also demonstrated normal color vision by their Farnsworth Munsell D-100 color vision score (mean ± SEM of 47.1 ± 6.8). Five females and three males were in the dose response study. Nine females and nine males were in the 420/460 nm comparison study.

Light exposure protocol

As described in detail elsewhere (Brainard et al., 2001), each experiment began at midnight when subjects had their pupils dilated with 0.5% Cyclopentolate, were blindfolded, and remained awake and sitting upright in darkness for 120 min. While blindfolded, a blood sample was taken just prior to 2:00 AM and subjects were then exposed to a 90 min light stimulus until 3:30 AM. During light exposure, each subject sat quietly with their eyes open and their head resting in an ophthalmologic head holder facing a patternless, white Ganzfeld apparatus encompassing their entire visual field. 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 the control night. There were at least 6 days between each nighttime test. Melatonin was quantified with a RIA with a minimum detection limit of 0.5 - 5.0 pg/mL (Brainard et al., 2001). RIA control samples had 14% and 22% interassay coefficients of variation.

Light production and measurement

As detailed elsewhere, monochromatic wavelengths were produced by arc lamps collimated into a grating monochromator (Brainard et al., 2001). The resulting light beam was directed into the top area of a Ganzfeld dome and reflected evenly off the dome surface into

volunteers' eyes. Half-peak bandwidths of the monochromatic stimuli were 14 nm for the dose-response study and 10 nm for the comparison study. Wavelengths at the level of subjects' corneas were measured with a portable spectroradiometer (Ocean Optics S2000). Routine measurement of the light irradiance ($\mu W/cm^2$) was done with both a Tektronix J16 Radiometer/Photometer with a J6512 irradiance probe which was not cosine corrected and an International Light 1400A with an SEL033 #6857 detector head with an F #23102 filter and cosine correction. Each of these meters was calibrated annually and was benchmarked to a reference meter (EG & G Model 580-23A Detector) at the Laser/Optical Radiation Program (Aberdeen Proving Ground, MD). All spectroradiometric and radiometric equipment was calibrated with a standard lamp traceable to NIST. Experimental light stimuli were measured at volunteers' eye level immediately before and after the 90 min exposure. In the 420 nm fluence-response study, intensities covered a 4 log unit photon density range of 10^{10} to 10^{14} photons/cm^2. In the study comparing 420 and 460 nm, the photon density was 1.21 x 10^{13} photons/cm^2/sec. An earlier study, which measured mean transmittance of 36 postmortem lenses of humans aged 20 to 30 years, showed relatively even transmission from 440 to 600 nm but a strong reduction in transmittance below 440 nm (Brainard et al., 1997). Since mean ± SEM % lens transmittance at 420 and 460 nm was 37.23 ± 7.88 and 56.33 ± 10.1 corneal light irradiances at 420 nm were adjusted to compensate for reduced stimulus transmission to the retina in both of the studies.

Statistics

Two-tailed, Students' *t* tests were used to assess significance of raw melatonin change. The melatonin data were then converted to % control-adjusted melatonin change scores (Brainard et al., 2001). 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 Fisher PLSD test with alpha at 0.05. A fluence-response curve was fit to a 4 parameter model for the mean % control-adjusted melatonin suppression data. The formula for this curve includes factors derived from earlier work on the melatonin suppression action spectrum (Brainard et al., 2001). Fit of the data to the curve was assessed by coefficient of correlation.

Results

The fluence-response data are illustrated in Figures 1 and 2. There were no significant differences ($F=0.69$, $df=8$, $p = 0.70$) between sets of pre-exposure melatonin values indicating that 2:00 AM plasma levels were consistent across all of the study nights. Figure 1 shows the mean ± SEM pre- and post-exposure melatonin values. Paired Students' *t* tests showed significant melatonin suppression by retinal irradiances at or above 11 $\mu W/cm^2$. All melatonin data were converted to control-adjusted % change scores (Brainard et al., 2001) and ANOVA showed a significant effect of retinal light intensity on melatonin % control-adjusted change scores ($F=11.74$, $p<0.0001$). Post hoc Fisher PLSD tests demonstrated that compared to the lowest irradiance of 0.016 $\mu W/cm^2$, intensities at or above 4.1 $\mu W/cm^2$ significantly suppressed melatonin. In all cases, irradiances above 4.1 $\mu W/cm^2$ were significantly stronger in suppressing melatonin compared to the irradiances two steps lower. Figure 2 illustrates a sigmoidal fluence-response curve plotting melatonin % control-adjusted scores against photon density. The curve formula is inset in the figure ($R^2 = 0.93$).

= = = Figures 1 and 2 about here = = =

In the wavelength comparison study, for the 420 nm and 460 nm light exposure and the dark control nights, mean pre-exposure raw melatonin values were 73.9 ± 8.7, 68.3 ± 7.6 and 69.8 ± 8.6 pg/mL, respectively. There were no significant differences ($F=1.27$, $df=2$, $p=0.29$)

across these values, indicating that melatonin levels were consistent across all study nights. Mean post-exposure scores were 84.4 ± 10.4, 76.1 ± 9.5 and 56.6 ± 8.8 pg/mL, respectively. Melatonin did not change significantly relative to the 420 nm exposure ($t=0.34$, $df=17$, $p=0.74$), decreased significantly with the 460 nm exposure ($t=2.25$, $df=17$, $p<0.04$), and increased significantly during the control night ($t=-3.32$, $df=17$, $p<0.001$). For direct comparison of responses to 420 and 460 nm, Figure 3 illustrates % control-adjusted melatonin suppression at equal retinal photon densities. These data reveal that 460 nm is significantly stronger than 420 nm in suppressing melatonin ($t=2.3$, $df=17$, $p<0.04$), although five of the 19 subjects had a greater melatonin suppression response to 420 versus 460 nm light. In this study, there was good repeatability in mean ± SEM melatonin suppression responses compared to the fluence-response experiments. In the published 460 nm fluence-response curve (Brainard et al., 2001), 1.21×10^{13} photons/cm^2/sec elicited a 45.3 ± 11.5 % control-adjusted melatonin suppression while in this study that photon dose elicited a 44.4 ± 9.1 % control-adjusted melatonin suppression. Similarly, in the 420 nm fluence-response curve described above and in this study, 1.21×10^{13} photons/cm^2/sec exposure elicited a 22.8 ± 9.7 % and a 20.2 ± 9.1 % control-adjusted melatonin suppression, respectively.

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Discussion

The present data demonstrate a full fluence-response relationship between 420 nm exposure and melatonin suppression in humans. The 420 nm curve is consistent with the fluence-response curves for eight other monochromatic wavelengths in the original melatonin suppression action spectrum (Brainard et al., 2001). In that action spectrum, a 420 nm half-saturation constant of 18.3×10^{12} photons/cm^2/sec was estimated from a very limited data set. That estimate was reasonably consistent but lower than the half saturation constant of 27.4×10^{12}

photons/cm^2/sec derived from the complete fluence-response curve. This new 420 nm data point has a better fit to the original action spectrum model, but does not change the calculated peak (464 nm, 446-477 nm +/- 1 sd) since the curve fitting method is based on the long-wavelength limb of sensitivity of the action spectrum (Partridge and DeGrip, 1991). The within-subjects comparison showed that 460 nm light is significantly stronger than 420 nm light for suppressing melatonin. These results clarify the short visible wavelength sensitivity of the human melatonin suppression action spectrum.

The acute light-induced melatonin suppression response is a broadly used indicator for photic input to the SCN and has been used to elucidate the ocular and neural physiology for circadian and neuroendocrine regulation (CIE, 2004; CIE, 2006; Brainard et al., 1997). Although full analytic action spectra have yet to be developed, a set of studies has confirmed that blue monochromatic light is more potent than other wavelengths for evoking circadian phase shifts and enhancing acute alertness in humans (Lockley et al., 2003; Cajochen et al., 2005). Together, those results are consistent with the nine more fully developed analytic action spectra for circadian and neuroendocrine responses (Brainard and Hanifin, 2005, for review).

The 420 nm results are consistent with the results of two *in vitro* studies in which cells transfected with the melanopsin gene exhibit peak photosensitivities at 479 nm and 480 nm (Qiu et al., 2005; Panda et al., 2005). In addition, studies on amphioxus melanopsin show a peak absorbance near 485 nm (Koyanagi et al., 2005). Together, the nine *in vivo* action spectra and the three *in vitro* studies indicate peak sensitivity in the blue part of the spectrum. In contrast, two *in vitro* studies show peaks in the violet-indigo-ultraviolet parts of the spectrum. Specifically, mouse cells transfected with the melanopsin gene have a peak photosensitivity in the range of 360-430 nm and the direct absorption spectrum for melanopsin showed the strongest activation by 420-440 nm light (Melyan et al., 2005; Newman et al., 2003). This discrepancy

may be due to the difference of *in vitro* melanopsin responsiveness by itself or in a given cell type versus its *in vivo* presence in ipRGCs that are closely connected to other retinal cells. There is increasing evidence that melanopsin may function as an invertebrate-like, bistable photopigment with both sensory and regenerative functions that have differing peaks of wavelength sensitivity (Koyanagi et al., 2005; Mure et al., 2007; Rollag, 2008). Hence, some *in vitro* systems may not match the systemic action spectra due to the blending of different melanopsin isomerization states. Further work is needed to clarify how the ipRGC-melanopsin system supports the wavelength sensitivity of systemic action spectra.

Despite abundant evidence that the melanopsin containing ipRGCs provide primary input for circadian and neuroendocrine phototransduction, the rod and cone photoreceptors still play a role in this physiology. Melanopsin- and cone-knockout mice show that the classical visual photoreceptors can compensate for the loss of melanopsin and, at least partially mediate light-induced circadian, neuroendocrine and neurobehavioral responses (Panda et al., 2002; Lucas et al., 2003; Dkhissi-Benyahya et al., 2007). In contrast, when both melanopsin is knocked out and the rods and cones are compromised, animals lose all visual and nonvisual photoreceptive functions of the eye (Hattar et al., 2003; Panda et al., 2003). Further, cellular recording studies in nonhuman primate retinas have demonstrated that rod and cone cells can directly activate ipRGCs (Dacey et al., 2005). Data from human studies suggest that the visual rods and cones may provide input to the SCN (Hebert et al., 2002; Figueiro et al., 2004; Jasser et al., 2006; Revell and Skene, 2007). It is important to recognize that despite rapid experimental progress on ipRGC physiology, it is currently unknown how these newly discovered photoreceptors work with the classical visual photoreceptors in transducing light in the dynamic, complex polychromatic environments where humans carry out their daily activities.

Importantly, in working with short wavelengths such as 420 nm, there is the potential for significant radiometric measurement error (American National Standards Institute and Illuminating Engineering Society of North America 2001). Special care is required in calibrating and benchmarking meters for accurately quantifying short wavelength visible light. Further, it is critical that the measured light stimuli represent the stimuli reaching the relevant photopigments. Human factors that can modify the measured stimulus include head and eye motion, squinting and eye closure, pupillary reflexes, and ocular media light transduction (Brainard et al., 1997). Most of these factors are controlled in the exposure techniques reported here. In ocular media light transmission, the cornea and aqueous and vitreous humors normally transmit nearly 100% of visible wavelengths to the retina. In contrast, as the human lens ages, it develops pigmentation that attenuates shorter visible wavelength transmission (Brainard et al., 1997; Pokorny et al., 1987). In this study, restricting the age of volunteers to 18-30 years partially controlled this factor. Measurements of transmittance of 36 postmortem human lenses in this age range showed relatively even transmission from 440 to 600 nm. Compared to lens transmission at 460 nm, however, there was a mean 45% reduction in transmission at 420 nm (Brainard et al., 1997). Thus, measured corneal light irradiances at 420 nm were adjusted to compensate for this reduced transmission. Such adjustments are advisable for all studies using short wavelength visible light. One study that used 456 nm light, showed reduced melatonin suppression in older versus younger women (mean ages 57 ± 5 and 24 ± 3 years) suggesting that the sensitivity loss was likely due to age-related changes in subjects' lenses (Herljevic et al., 2005).

Photobiological hazards such as infrared and ultraviolet cataract, photokeratitis, photoretinitis, and ultraviolet erythema have been identified relative to overexposure of the skin and eyes to the ultraviolet, visible and infrared spectra. Whether using short wavelength light experimentally or for pragmatic purposes, it is important to verify that exposures fall within

established national and international safety limits (CIE, 2002; American Conference of Governmental Industrial Hygienists (ACGIH), 2006). The short wavelength stimuli used in this study were well within the established ocular safety limits. Although the ACGIH standards are updated yearly based on the current published literature, some investigators debate if these standards are sufficiently stringent.

A wealth of data published in the past 25 years have demonstrated that light can be a potent biological, behavioral, and therapeutic stimulus in humans (CIE, 2004; CIE, 2006). The data presented here extend our understanding of the wavelength sensitivity of the photoreceptor system that serves as the input system for non-visual, neurobehavioral regulation in humans. Industrialized societies employ light extensively in both public and private buildings to support vision, visual comfort, and aesthetic appreciation within these environments. Since light is also a potent regulator of human circadian and neuroendocrine physiology and different photoreceptive systems mediate visual and neurobehavioral responses, future lighting strategies will need to provide illumination for human neurobehavioral regulation as well as vision. Collectively, lighting manufacturers, lighting designers and architectural engineers have opened the door to understanding this physiology and are considering the development of applications stemming from these discoveries (CIE, 2004; CIE, 2006). Indeed, the aerospace community is exploring how lighting can be used to support vision, circadian regulation and alertness of astronauts in advanced human environments such as the International Space Station and the planned Lunar habitat (Dijk et al., 2001; Gronfier et al., 2007).

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Figure Legends

Figure 1: In this graph bars represent group mean + SEM melatonin values (N=8) before and after monochromatic light exposure at 420 nm. ANOVA and post-hoc Fisher PLSD tests demonstrated which retinal light intensities significantly suppressed melatonin.

Figure 2: This figure demonstrates the fitted fluence-response curve for retinal irradiance photon density and % control-adjusted melatonin suppression on a semilog scale (N=8). Each data point represents one group mean ± SEM. The curve is consistent with the eight fluence-response curves for melatonin suppression with monochromatic light between 440 to 600 nm (Brainard et al., 2001).

Figure 3: The bars represent group mean + SEM values relative to an equal photon dose of 1.21×10^{13} photons/cm^2 of retinal irradiance. These data show that the 460 nm % control-adjusted plasma melatonin suppression is significantly stronger than that for 420 nm.