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Human Melatonin and Alerting Response to Blue-Enriched Light Depend on a Polymorphism in the Clock Gene *PER3*

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Context: Light exposure, particularly at the short-wavelength range, triggers several nonvisual responses in humans. However, the extent to which the melatonin-suppressing and alerting effect of light differs among individuals remains unknown.

Objective: Here we investigated whether blue-enriched polychromatic light impacts differentially on melatonin and subjective and objective alertness in healthy participants genotyped for the *PERIOD3* (*PER3*) variable-number, tandem-repeat polymorphism.

Design, Setting, and Participants: Eighteen healthy young men homozygous for the *PER3* polymorphism (*PER3*$^{5/5}$and *PER3*$^{4/4}$) underwent a balanced crossover design during the winter season, with light exposure to compact fluorescent lamps of 40 lux at 6500 K and at 2500 K during 2 h in the evening.

Results: In comparison to light at 2500 K, blue-enriched light at 6500 K induced a significant suppression of the evening rise in endogenous melatonin levels in *PER3*$^{5/5}$ individuals but not in *PER3*$^{4/4}$. Likewise, *PER3*$^{5/5}$ individuals exhibited a more pronounced alerting response to light at 6500 K than *PER3*$^{4/4}$ volunteers. Waking electroencephalographic activity in the theta range (5–7 Hz), a putative correlate of sleepiness, was drastically attenuated during light exposure at 6500 K in *PER3*$^{5/5}$ individuals as compared with *PER3*$^{4/4}$.

Conclusions: We provide first evidence that humans homozygous for the *PER3* 5/5 allele are particularly sensitive to blue-enriched light, as indexed by the suppression of endogenous melatonin and waking theta activity. Light sensitivity in humans may be modulated by a clock gene polymorphism implicated in the sleep-wake regulation. **(*J Clin Endocrinol Metab* 97: 0000–0000, 2012)**

Human nonvisual responses to light crucially rely on light's wavelength, particularly to short-wavelengths [460–480 nm (blue) light] (1–6). This predominance is such that exposure to 460 nm (blue) monochromatic light during the biological night reduces melatonin secretion and subjective sleepiness (7) and also waking electroencephalographic (EEG) delta-theta activity (4) to a greater extent than exposure to 555 nm (green) monochromatic light. The magnitude of these responses indicates that photoreceptors mediating acute light effects are blue shifted to the visual photopic system (8). Novel data in humans (9) indicate that a polymorphism in the clock gene *PER3* has a differential impact on cognitive brain responses to light. However, it is unknown whether a differential acute response to light *per se* or an altered interaction of the sleep-wake homeostasis and circadian system

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Abbreviations: EEG, Electroencephalographic; rANOVA, repeated-measure ANOVA.

elicited these effects in individuals genotyped for the *PER3* variable-number tandem-repeat polymorphism. Thus, we investigated whether individual differences in the melatonin supression and alerting action of light are modulated by a clock gene polymorphism also implicated in sleep-wake regulation (10).

Subjects and Methods

Participants

Seventy young people (20–31 yr old) were recruited from a pool of participants also enrolled for other studies. Of these, 50 participants completed a general medical questionnaire, Morning-Evening Questionnaire, and Pittsburgh Sleep Questionnaire and provided saliva samples for genomic DNA analysis. Exclusion criteria included extreme chronotypes, smoking, medication or drug consumption, body mass index less than 19 and greater than 28 kg/m^2, shift work, transmeridian flights 2 months before the study, and medical and sleep disorders. The distribution of *PER3* genotypes in the 50 potential candidates was 21 $PER3^{4/4}$ (nine men, 13 women), 10 $PER3^{5/5}$ (nine men, one woman), and 19 $PER3^{4/5}$ individuals (five men, 14 women). Eighteen healthy male volunteers (20–28 yr old; mean ± SEM 25.9 ± 1.2) homozygous for the *PER3* polymorphism (nine $PER3^{4/4}$, nine $PER3^{5/5}$) were carefully matched by age, body mass index, and ethnicity (Supplemental Table 1). Women were not included in this study because we did not have comparable subgroups of $PER3^{5/5}$ and $PER3^{4/4}$ women. All participants gave written informed consent. The study was approved by the local ethics committee (EKBB/Ethikkommission beider Basel, Switzerland) and conformed to the Declaration of Helsinki.

Protocol

A balanced crossover design study was conducted during the winter season (January to April) to minimize the effects of outdoor light levels and comprised three segments separated by 1 wk. The protocol started 10 h after volunteers' habitual wake-up time and ended the next day after usual wake-up time. Sleep-wake schedules were assessed by wrist actigraphy (actiwatch L; Cambridge Neurotechnology Ltd., Cambridge, UK) and self-reported sleep logs. During each protocol, participants underwent 1.5 h under dim light (<8 lux), 2 h under complete darkness, 2 h of light exposure (compact fluorescent lamps with 6500 or 2500 K or incandescent light bulbs at 3000 K), and a post-light episode of approximately 45 min under dim light until habitual sleep time. Each protocol was conducted at the same time of day (evening), same light intensity (ca. 40 lux measured at eye level seated at the desk), and same protocol length (ca. 6 h in total) for each participant. The treatment order (6500 *vs.* 2500 *vs.* 3000 K) was randomized to avoid possible order effects of the light conditions. For detailed information on light settings, please see report published elsewhere (11). The effects of light at 3000 K did not differ from light at 2500 K in either genotype. Thus, here we report the comparison between light at 6500 and 2500 K.

Genotyping

DNA was extracted with the NucleoSpin tissue kit (Marchery-Nagel AG, Oensingen, Switzerland). All genotypes were determined with an allele-specific PCR on an MJ Research PTC-225 thermal cycler (MJ Research/Bio-Rad Laboratories, Reno, NV) using Hot FIREPol DNA polymerase (Solis BioDyne, Tartu, Estonia) and a forward and reverse primer. The *PER3* forward primer was as follows: 5′-TTACAGGCAACAATGGCAGT-3′ and the reverse primer: 5′-CCACTACCTGATGCTGCTGA-3′ [annealing temperature 59 C (25 mM $MgCl_2$)]. The PCR products were analyzed by agarose gel electrophoresis.

Primary outcome measures

The primary phenotypes were salivary melatonin, subjective sleepiness, and waking EEG activity. Saliva melatonin was collected every 40 min during the protocol. A direct double-antibody RIA was used for melatonin assays (validated by gas chromatography-mass spectroscopy with analytical least detectable dose of 0.65 pm/ml; Bühlmann Laboratory, Schönenbuch, Switzerland) (12). The minimum detectable dose of melatonin (analytical sensitivity) was 0.2 pg/ml. Subjective sleepiness was assessed with the Karolinska Sleepiness Scale (13) every 40 min during the protocol. Waking EEG activity was recorded continuously during 6 h of scheduled wakefulness with a Vitaport Ambulatory system (Vitaport-3 digital recorder; TEMEC Instruments BV, Kerkrade, The Netherlands). Eight EEG derivations (F3, F4, C3, C4, P3, P4, O1, O2, referenced against linked mastoids), two electrooculograms, one submental electromyogram, and one electrocardiogram were recorded. All EEG signals were filtered at 30 Hz (fourth order Bessel type antialiasing, low pass filter, total 24 dB/Oct), with a time constant of 1.0 sec before online digitization (range 610 μV, 12 bit AD converter, 0.15 μV/bit; storage sampling rate at 128 Hz). Raw signals were stored online on a Flash RAM card (Viking, Rancho Santa Margarita, CA) and downloaded offline to a personal computer hard drive. EEG were subjected to spectral analysis using a fast Fourier transform (10% cosine 2 sec window), which resulted in a 0.5-Hz resolution. The Karolinska Drowsiness Test (14) was performed hourly during scheduled wakefulness (six times). The 3-min EEG during the Karolinska Drowsiness Test were manually scored for artifacts offline, and absolute EEG power densities were then calculated for artifact-free, 2-sec epochs in the frequency range of 0.5–20 Hz. For data reduction, artifact free, 2-sec epochs were averaged over 20-sec epochs.

Statistical analysis

For all analyses, the statistical package SAS (SAS Institute Inc., Cary, NC; version 9.1) was used. To compare the effects of 2 h light exposure to prelight levels, melatonin, subjective sleepiness, and waking EEG theta activity (5–7 Hz) during light were adjusted to prelight levels for each participant and compared with *t* tests. The time course of salivary melatonin, subjective sleepiness, and waking EEG activity was analyzed with repeated-measure ANOVA (rANOVA) using a general linear model, with the factors light condition, time, genotype, and derivation (for EEG activity). All *P* values derived from rANOVA were based on Huynh-Feldt's-corrected degrees of freedom. Supplemental Table 2 provides main and interaction effects for melatonin, subjective sleepiness, and waking theta activity. The alpha adjustment for multiple comparisons was applied using the Tukey-Kramer test for melatonin, subjective sleepiness, and theta ac-

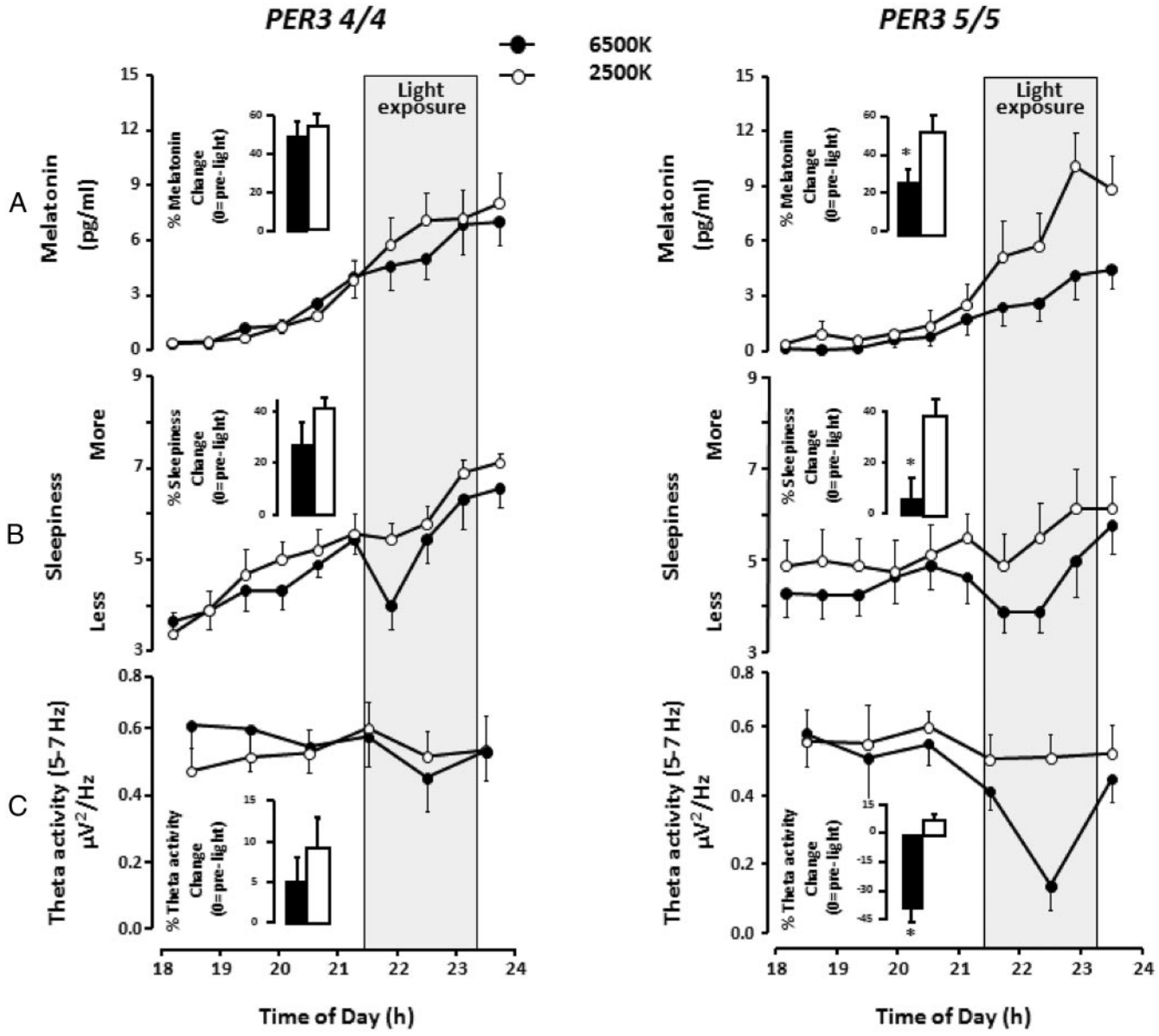

FIG. 1. Light effects on melatonin and subjective/objective markers of alertness in *PER3*$^{4/4}$ (*left panel*) and *PER3*$^{5/5}$ participants (*right panel*). A, Time course of salivary melatonin. *Inset*, Salivary melatonin levels during 2 h of light exposure adjusted to prelight exposure (percentage of melatonin change). B, Time course of subjective sleepiness. *Inset*, Same as for A. C, Time course of waking EEG theta activity (5–7 Hz). Inset, Same as for A. Values as mean ± SEM. *, $P < 0.05$.

tivity due to the multiple time samplings at which these data were collected. To test the degree of melatonin suppression (comparison of absolute melatonin levels during light exposure to prelight) and alerting response to light (comparison of absolute subjective sleepiness levels during light exposure to prelight), we computed the Spearman rank correlation coefficients of these variables.

Results

Melatonin suppression

We found a wavelength-dependent suppression of salivary melatonin modulated by the *PER3* polymorphism, such that melatonin significantly decreased in *PER3*$^{5/5}$ but not in *PER3*$^{4/4}$ individuals after 90 min of blue light [three way rANOVA with factors genotype *vs.* time *vs.* light condition; $P = 0.01$ (Tukey-Kramer test for multiple comparisons)] (Fig. 1A). Blue-enriched light attenuated the evening rise in endogenous melatonin levels significantly more in *PER3*$^{5/5}$ (mean ± SEM 28.1 ± 6.8%) than *PER3*$^{4/4}$ (mean ± SEM 49.6 ± 10.4%) individuals (*t* test; $P = 0.03$) (Fig. 1A, *inset*).

Subjective and objective alertness

Since endogenous melatonin levels in the evening are linked to individual vigilance levels, we tested whether subjective and objective markers of sleepiness also exhib-

ited a genotype dependency. The time course of subjective sleepiness (Fig. 1B) revealed no significant interaction of genotype *vs.* time *vs.* light condition. However, blue-enriched light significantly reduced sleepiness in a genotype-dependent manner, such that $PER3^{5/5}$ were more alert (mean ± SEM 6.7 ± 7.8%) than $PER3^{4/4}$ (mean ± SEM 30.3 ± 8.4%) individuals (*t* test; $P = 0.04$) (Fig. 1B, *inset*). This subjective perception was mirrored by changes in the waking EEG theta activity (5–7 Hz). During blue-enriched light, $PER3^{5/5}$ individuals experienced significantly less theta activity than $PER3^{4/4}$ individuals [two way rANOVA with factors genotype *vs.* condition; $P = 0.01$ (Tukey-Kramer test for multiple comparisons)] (Fig. 1C). Comparison of theta activity during light exposure to pre-light indicated that, during blue-enriched light, $PER3^{5/5}$ had significantly less theta activity (mean ± SEM −34.1.1 ± 5.7%) than $PER3^{4/4}$ (mean ± SEM 5.1 ± 3.4%) individuals (*t* test; $P = 0.01$) (Fig. 1C, *inset*).

Relationship of melatonin suppression and alerting response

Correlations between melatonin suppression and the alerting response to light, as indexed by subjective sleepiness, revealed that, during light at 2500 K, neither $PER3^{5/5}$ ($r = 0.31$; P = ns) nor $PER3^{4/4}$ individuals ($r = 0.29$, P = ns) had significant correlations. During light at 6500 K, $PER3^{4/4}$ individuals had no significant correlation ($r = 0.19$; P = ns), while $PER3^{5/5}$ had a stronger correlation of the degree of melatonin suppression and the alerting response to light ($r = 0.77$; $p = 0.02$) (Supplemental Fig. 1).

Discussion

Nonvisual responses to light, such as melatonin suppression, involve melanopsin-containing intrinsic photosensitive retinal ganglion cells. These cells directly connect via the retinohypothalamic tract to the suprachiasmatic nucleus, which then projects to the pineal gland, the site of central melatonin production (15). This neuroanatomical pathway may explain the reduced melatonin levels during and/or after short-wavelength light exposure, which was stronger in $PER3^{5/5}$ than $PER3^{4/4}$ participants. Recently a functional knockout of *Period3* in mice resulted in altered sensitivity to light, such that the mice deficient for *Per3* had reduced nonvisual responses to light (16), similar to melanopsin knockout mice (17). This goes in line with our observation that a polymorphism in the human clock gene *PER3* may play an important role in the light input of the circadian clock.

Photosensitive ganglion cells project directly and indirectly (via suprachiasmatic nucleus) to brain areas implicated in wakefulness and arousal regulation (15). Recent functional magnetic resonance imaging data (9) show that monochromatic blue light increased brain responses in a left thalamofrontoparietal circuit only in $PER3^{5/5}$ individuals when sleep pressure was high but with no effect on behavior, probably due to the short duration (1 min) light exposure. In this context, our data imply that longer light exposure (2 h) may impact on melatonin suppression and alertness in a *PER3* genotype manner. The *PER3* variable-number tandem-repeat modulates human sleep-wake regulation (10) and also diurnal preference, with increased morning preference in $PER3^{5/5}$ individuals (18). However, because prior wakefulness and circadian phase were similar between the genotypes in our study, the alerting response in $PER3^{5/5}$ cannot be explained only by altered interactions of circadian and sleep-wake homeostatic processes. Rather, increased sensitivity to light, as indexed by more melatonin suppression in $PER3^{5/5}$, may have impacted on brain structures involved in arousal regulation. Nevertheless, without a no-light condition, the exact magnitude of melatonin suppression for each light setting remains uncertain. Our findings indicate that differences in light sensitivity may be modulated by a clock gene polymorphism, which may help to understand the inter-individual variability of the nonvisual responses to light.

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