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METHODS. Thirty-nine participants were exposed to 60 minutes of either continuous light (n = 8) or sequences of 2-millisecond light flashes (n = 31) with different interstimulus intervals (ISIs; ranging from 2.5 to 240 seconds). Melatonin phase shift and suppression, along with changes in alertness and sleepiness, were assessed.

RESULTS. We determined that the human circadian system integrates flash sequences in a nonlinear fashion with a linear rise to a peak response (ISI = 7.6 ± 0.53 seconds) and a power function decrease following the peak of responsivity. At peak ISI, flashes were at least 2-fold more effective in phase delaying the circadian system as compared with exposure to equiluminous continuous light 3,800 times the duration. Flashes did not change melatonin concentrations or alertness in an ISI-dependent manner.

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CONCLUSION. We have demonstrated that intermittent light is more effective than continuous light at eliciting circadian changes. These findings cast light on the phenomenology of photic integration and suggest a dichotomous retinohypothalamic network leading to circadian phase shifting and other NIF photoresponses. Further clinical trials are required to judge the practicality of light flash protocols.

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Introduction

Mammals, including humans, have evolved to adapt their endogenous physiological and behavioral rhythms to be consistent with the Earth's 24-hour light/dark cycle. Such endogenous circadian rhythms range from the behavioral sleep-wake and arousal cycles to the secretion pattern of hormones, such as melatonin, cortisol, and thyroid-stimulating hormone. Circadian rhythms are under the control of a master oscillator located in the anterior hypothalamus just above the optic chiasm called the suprachiasmatic nucleus (SCN). The rhythms of the SCN have a period length that is close to, but not exactly, 24 hours and is entrained (synchronized) to the 24-hour light/dark cycle by light detected by ocular retinal photoreceptive elements (1–3). Inappropriate circadian entrainment is associated with alterations in thermoregulation, cardiovascular function, immune processes, sleep, vigilance, memory, and cognition (4–9).

In the mammalian retina, a small subset of ganglion cells contains the photopigment melanopsin and is intrinsically photosensitive (10–12). These intrinsically photosensitive retinal ganglion cells (ipRGCs) endogenously transduce light but also integrate processed neural signals coming from the traditional rod and cone photoreceptors of the outer retina (13–15). Axons emerging from the ipRGCs project to nonimage-forming (NIF) centers in the mammalian brain to evoke subcortical changes that include, but are not limited to, the resetting of circadian timing (1, 10, 11, 16, 17), suppression of melatonin production (18), activation of the pupillary light reflex (14, 15), change in arousal levels (19, 20) and sleep drive (21), alleviation of seasonal depression (22, 23), and possibly the induction of migraine headaches (24). Given its NIF implications, light has been used for its considerable therapeutic potential in chronobiological disorders such as jet lag and shift work but has also been used to treat sleep and mood disorders as well as cognitive impairment via correction of circadian misalignment and an acute activating response (22, 23, 25–32).

Underlying these NIF responses to light, traditional rod and cones photoreceptors are thought to be implicated in the fast and transient responses, whereas melanopsin is hypothesized to have a predominant effect on the sustainability and persistence of a response (33, 34). To date, however, the integrative physiology

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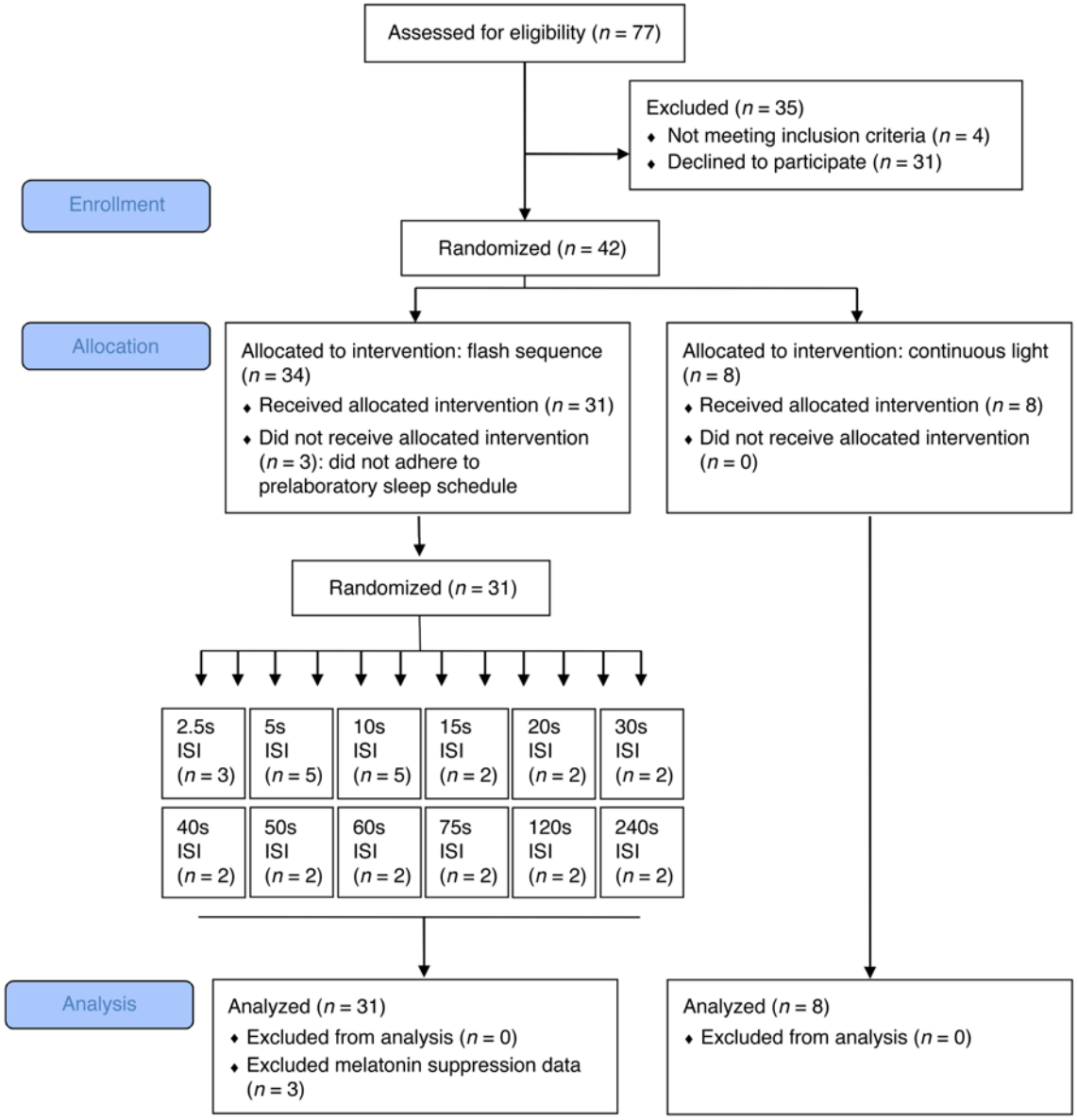


Figure 1. CONSORT flow diagram showing enrollment, allocation, and analysis. Seventy-seven participants were assessed for eligibility. Thirty-five participants were excluded from participation for either not meeting the criteria (n = 4) or declining to participate (n = 31). Forty-two participants participated in the study and were randomized to either a flash sequence or continuous light exposure protocol. Of the 34 participants who were allocated randomly to the different flash sequence light exposures, 3 failed to comply with the prelaboratory sleep schedule and were excluded and 31 were randomly assigned to different sequences of light flashes. Eight participants were allocated to the continuous light exposure. All 39 participants were analyzed. Melatonin suppression values in 3 participants from the flash allocated group were aberrant and had to be excluded.

of NIF photoreception in humans remains incompletely understood, as it has wavelength (35–39), intensity (40), duration (41), and pattern (42–44) responses that are considerably distinct from the traditional perceptual image-forming responses, and most knowledge in this field has been imputed from studies of nonhuman mammals. Understanding NIF characteristics and responses to light is crucial for the development and optimization of light therapy strategies. NIF responses to light in humans have mainly been tested using continuous or intermittent light exposures, ranging from the order of minutes up to more than 6 hours of bright light (41–43). Most of these studies that used intermittent light exposures imply that the circadian system is able to integrate light in a nonlinear fashion. Intermittent patterns of light tested thus far fail, however, to elicit greater changes in outcomes when compared with continuous light exposures. Shorter duration of light exposures in the order of microseconds to milliseconds have been tested in rodents (45–47). While a single 2-millisecond flash of light is insufficient to phase shift the murine circadian system, a sequence of flashes administered once per minute for 60 minutes is sufficient to evoke phase delays (48). This study by Van Den Pol and colleagues, in addition to other published studies in rodents (45–47), established that the mammalian circadian system has the capacity to respond to a sequence of very brief, millisecond flashes of light.

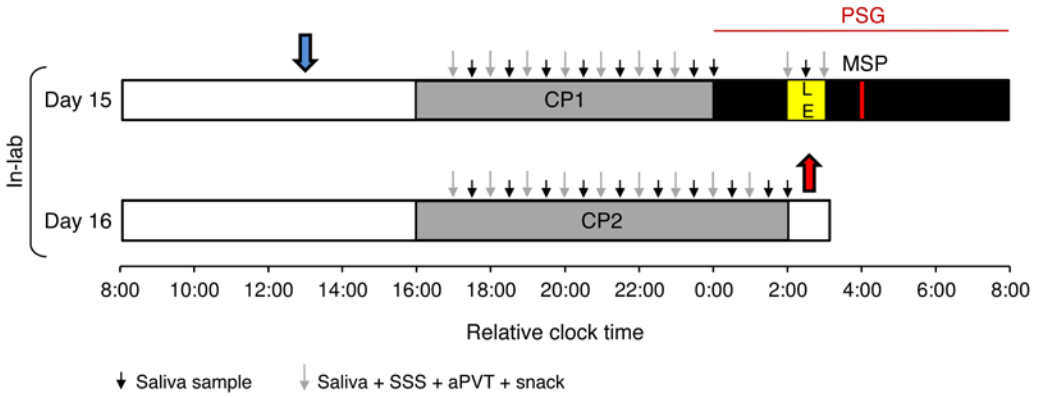


Figure 2. In-lab protocol design. From day 1 to day 14, participants maintained a regular at-home sleep-wake schedule. The approximate clock times in the case of a participant who maintained a regular sleep schedule between 0:00 hours and 08:00 hours from day 1 to day 14 prior to coming to the lab are shown. On day 15, participants came to the sleep lab (blue arrow) and underwent a first CP procedure (CP1) that included multiple assessments of saliva, SSS, and an auditory psychomotor vigilance task (aPVT) in dim light (0.6–1.9 lux). All procedures were timed based on an individual's average MSP from day 1 to day 14. Lights were turned off 4 hours prior to MSP on day 15, and subjects were allowed to sleep (recorded with polysomnography [PSG]). Two hours and fifteen minutes prior to MSP participants were awoken for scheduled light exposure (LE). LE ended 1 hour prior to MSP and consisted of either 60 minutes of continuous light or 60 minutes of a sequence of ultrashort flashes. On day 16, participants were awoken at habitual wake time, provided breakfast and lunch, and then underwent a second CP procedure (CP2) similar to CP1. After CP2, participants were discharged (red arrow) and were offered a taxi ride home.

We have recently shown that a sequence of 60 ultrashort 2-millisecond flashes administered over 60 minutes can elicit a 45-minute circadian phase delay (44). Here, we compare the NIF effect of ultrashort 2-millisecond flashes to that of an equiluminous continuous light 1,250 to 120,000 times the duration of the flashes, present a model of NIF temporal integration of light flashes in humans, and suggest an optimized and selective chronobiological light therapy strategy.

Results

To examine the effects of different temporal patterns of light flashes on the human circadian timing system, we exposed 39 young healthy participants (aged 19 to 36 years) to either a continuous 60-minute light exposure ($n = 8$) or a sequence of flashes of various frequencies from 0.004 to 0.4 Hz (interstimulus intervals [ISI] = 2.5 to 240 s) ($n = 31$) (Figure 1). Both continuous light and flashes were of equal illuminance (1,700–1,805 lux) and composed of similar broad-spectrum white light (Supplemental Figure 1 and Supplemental Table 1; supplemental material available online with this article; doi:10.1172/JCI82306DS1). Light exposure was scheduled to be administered during the early biological night (Figure 2). Post-hoc analysis determined that light administration began 4.70 ± 0.92 hours after the onset of melatonin, a time during which light is expected to evoke delays in circadian timing (49). Acute changes in salivary melatonin concentrations, as well as objective and subjective alertness, were concomitantly measured (Figure 2).

Group differences. There was no difference in age ($U = 104$, $Z = -0.68$, $P = 0.50$, Mann-Whitney test), sex distribution ($\chi^2 = 2.39$, $P = 0.12$, χ^2 test), phase angle of light exposure ($t = -0.52$, df = 37, $P = 0.61$, t test), integral of salivary melatonin between dim light melatonin onset (DLMO) and the onset of light exposure ($t = -0.77$, df = 37, $P = 0.45$, t test), morning or evening preference (Morning-Eveningness Questionnaire, refs. 50, 51, $U = 137.5$, $Z = 0.45$, $P = 0.65$, Mann-Whitney test), or reported prior-month sleep quality (Pittsburgh Sleep Quality Index, ref. 52, $U = 142.5$, $Z = 0.65$, $P = 0.51$, Mann-Whitney test) between the two groups.

Robust circadian phase shift in response to flashes of light. Within the group exposed to 1 hour of continuous light, circadian timing was changed by –0.60 ± 0.34 hours (delay) ($t = -5.04$, df = 7, $P < 0.01$, paired t test). Within the group exposed to flashes, circadian timing changed by –2.99 to 0.30 hours in an ISI-dependent fashion (Figure 3). An initial linear rise to peak responses occurred when flashes were separated by 2.5 to 7.6 seconds of darkness, modeled with a standard linear fit using the formula $y = m \times x$, such that m is the slope of the rise. Following the peak of responsivity of the system, change in circadian phase in response to the light flashes dropped following a nonlinear, power function, as shown by $y = y_0 + A \times x^C$, where y_0 is the response of the system to a similar protocol without light administration (set to –0.11 hours, as this change in circadian phase was observed in a separate cohort of subjects who took part in this protocol but were not exposed to light; ref. 44), A is a scaling factor, and c is the power coefficient describing the decay of the response. The slope of the initial rise (m) was calculated as 0.23 ± 0.07 h/s, the power constant (A) was 5.5 ± 6.0, and the power coefficient (c) was –0.67 ± 0.40.

Flashes of light do not suppress melatonin in a dose-dependent manner. Continuous light suppressed melatonin secretion by 51% ± 40% ($W = 35$, $Z = 2.31$, $P < 0.05$, Wilcoxon signed-rank test) (Figure 4A and Figure 5A). Melatonin suppression was assessed in 28 of 31 participants in the group exposed to flashes, as melatonin concentrations in 3 participants were higher than the assay's maximum sensitivity, even after dilution of the saliva samples. The percentage of melatonin suppression fluctuated under the different flash frequency conditions but did not vary systematically based upon ISI (Figure 4A). If we limited our analysis to the participants exposed to flashes who showed a similar corrected circadian phase shift as those exposed to continuous light (i.e., within the 95% CI of the circadian phase change elicited by continuous

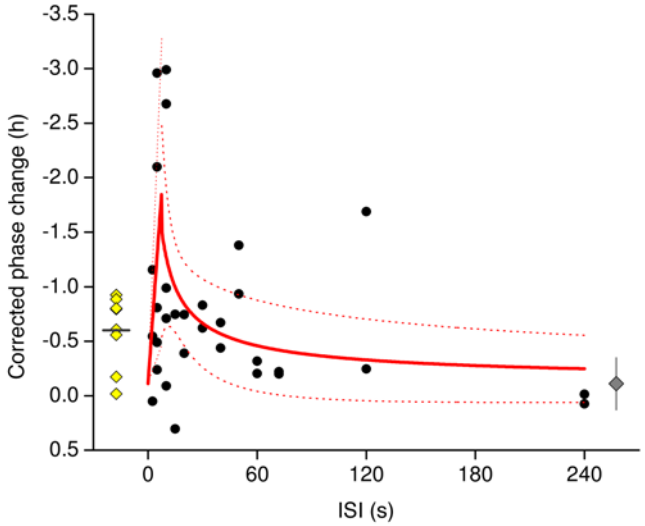


Figure 3. Change in melatonin phase shift as a function of the ISI. An initial linear rise to peak circadian phase change (~1.85 hours phase delay) was observed when flashes were separated by 2.5 to 7.6 seconds of darkness. Thereafter, light-evoked phase shifts dropped following an exponential decay curve as ISI increased (n = 31). Responses of individuals (black circles) are plotted with the modeled curve (red line) and 95% CIs (dotted red lines). The average circadian phase delay of −0.60 ± 0.34 hours to a continuous 60-minute white light exposure is represented a dark gray horizontal bar, while the individual phase shift to continuous light is represented by yellow diamonds. Average phase shift induced by a similar protocol without light administration (data previously published in Zeitzer et al., ref. 44) is represented by a gray diamond with error bars (mean ± SD). In some participants, flashes separated by an ISI of 5 and 10 seconds induced over 2 hours of circadian phase shift. Negative values on the y scale indicate a phase delay.

light: −0.36 to −0.83 h), we still observed that these individuals (n = 7) did not exhibit a significant melatonin suppression after the different flash exposures (W = 12, Z = −0.25, P = 0.81, Wilcoxon signed-rank test) (Figure 5C). Melatonin suppression in this subgroup was significantly lower than that observed in the continuous light group (U = 46, Z = 2.03, P < 0.05, Mann-Whitney test).

Flashes of light do not change objective alertness and subjective sleepiness in a dose-dependent manner. Median reaction time (RT) (t = 4.49, df = 7, P < 0.01, paired t test; Figure 4B and Figure 5B), number of lapses (W = 34, Z = 2.18, P < 0.05, Wilcoxon signed-rank test; Figure 4C), and shift in optimum RT (mean of the 10% fastest RT) (t = 3.62, df = 7, P < 0.01, paired t test) decreased under continuous light exposure. Continuous light did not decrease subjective sleepiness, as assessed by the Stanford Sleepiness Scale (SSS; ref. 53; W = 13.5, Z = 1.5, P = 0.19, Wilcoxon signed-rank test; Figure 4D). Median RTs, numbers of lapses, and SSS scores fluctuated under the different flash frequency conditions; however, there was no consistent increase in alertness or decrease in sleepiness as a function of the ISI (Figure 4, B–D). Median RT (t = −0.02, df = 9, P = 0.98, paired t test; Figure 5D), number of lapses (W = 25.5, Z = −0.15, P = 0.87, Wilcoxon signed-rank test), and shift in optimum RT (W = 16, Z = −1.12, P = 0.27, Wilcoxon signed-rank test) did not change in participants exposed to flashes who showed a similar corrected circadian phase shift as those exposed to continuous light. There was no significant effect of continuous light on electroencephalographic correlates of arousal (Δ: t = −0.54, df = 4, P = 0.6; θ: t = −1.01, df = 4, P = 0.36; α: t = −1.11, df = 4 P = 0.33; paired t test) nor were there associations between these measures and the ISI.

Discussion

This experiment demonstrates that, in direct contrast to image-forming photoreception, the human circadian timing system has the capacity to integrate light over a millisecond time scale. Furthermore, we have demonstrated that a specific temporal pattern of light has the capacity to increase the induction of circadian phase changes to a greater extent than that observed with continuous light. As opposed to the response of the circadian timing system to light flashes, other NIF responses, including increased alertness and suppressed melatonin production, failed to show consistent responsivity to light flashes. This type of sequence of light flashes would be highly improbable to occur naturally and, as such, the response we observed is likely an unintended consequence of the circuitry of the system.

Previous studies in humans have contributed to the elucidation of the characteristics of NIF photoresponses, including wavelength sensitivity (33, 36, 38), timing (49, 54), intensity (40), and photic history (41). A limited number of studies have examined the effect of the temporal pattern of light. Previous studies examining the effect of continuous long- versus short-duration (minutes) intermittent light on the NIF system have implied that the effectiveness of light in changing circadian timing declines rapidly with the duration of the light pulse (42, 43, 55). We have previously shown that much of the effectiveness of light on changing the timing of the circadian pacemaker may, in fact, be mediated within the first few milliseconds of light exposure (44). Our findings suggest that within the NIF photoresponsive structure, the circadian system appears to integrate ultrashort 2-millisecond flashes of light over time in a nonlinear fashion. This temporal integration is such that 0.95 seconds of light delivered as discreet flashes evenly distributed over an hour has the capacity to be more than 2-fold more effective on phase delaying the circadian system than an equiluminous continuous light exposure 3,800 times the duration of the flashes. This is the first demonstration to our knowledge in any species that a sequence of ultrashort intermittent light flashes can be as effective, if not more effective, in changing the phase of the circadian timing system in comparison to a continuous white light of the same spectral composition and illuminance.

Circadian integration renders ultrashort flashes more efficient than continuous light. The responsivity of the circadian system to the flash sequence could be explained by the electrophysiological properties of the ipRGCs, which have a sluggish off response, in that they continue to fire for several seconds after cessation of a light stimulus (10, 56). When flashes are separated by a short ISI, the depolarization of the ipRGCs would become sustained, similar to that evoked by continuous light exposure. The higher effectiveness of flashes on circadian photoreception could arise from the additional firing of cones, which indirectly project to ipRGCs, at light onset and offset, thus generating a continuous response boosted by superimposed multiple spiking responses at each light onset and offset. Flashes seem to offer an ideal stimulus that maximizes inputs from the visual system (cones), which is optimized to be maximally responsive to rapid temporal light fluctuations, and the more sluggish

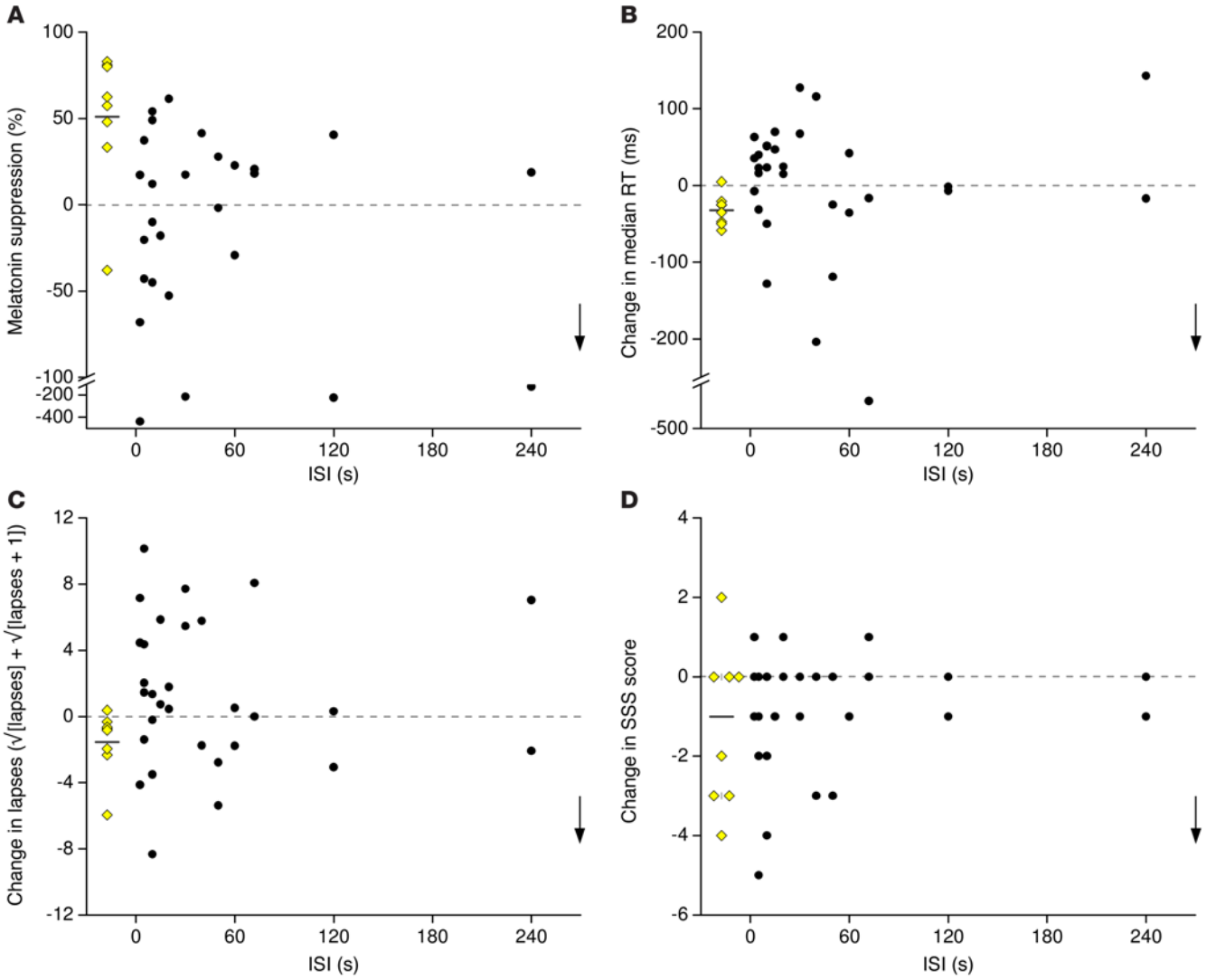


Figure 4. Effect of flashes and continuous light on melatonin suppression, objective alertness, and subjective sleepiness. There was no systematic ISI-dependent change in (**A**) melatonin suppression (n = 28), (**B** and **C**) objective alertness (n = 31), or (**D**) subjective sleepiness (n = 31). Continuous light significantly suppressed melatonin (W = 35, Z = 2.31, P = 0.016, Wilcoxon signed-rank test), reduced RT (t = 4.49, df = 7, P = 0.002, paired t test), and number of lapses (W = 34, Z = 2.18, P = 0.02, Wilcoxon signed-rank test) but did not decrease subjective sleepiness, as assessed by the SSS (W = 13.5, Z = 1.5, P = 0.19, Wilcoxon signed-rank test). (**A**–**C**) Average and (**D**) median changes induced by continuous light are represented as gray horizontal bars; yellow diamonds represent individual data. (**D**) Overlapping yellow diamonds are represented next to each other separated by a vertical red bar. Descending arrows or negative values indicate less melatonin suppression, faster RTs, fewer lapses, and decreased sleepiness.

melanopsin-based system that is hypothesized to integrate light exposure of long durations so as to remove the vagaries of behavior (e.g., walking in a shaded area, weather) on light exposure and its effects on circadian timing. If the flashes are too far apart (e.g., ISI = 240 s), there may be insufficient drive on ipRGCs to evoke a change in circadian timing. Although a rapid dark regeneration of cone opsin is expected after short bleaches (57–59), if the flashes are too close together (e.g., 2.5–7.6 s), there may not be enough time for the bleached fragment of cone opsin to optimally regenerate; therefore, the loss of the superimposed boost in the response renders these sequences similar to continuous light. A similar observation has previously been noted in hamsters exposed to a train of 300-millisecond light pulses separated by 300 milliseconds of dark or 300 seconds of continuous light of equal total photon density (60). While it is a possibility that requires testing, we do not believe that rods have a substantive contribution in flashes subsequent to the first one, given that they are saturated at very low illuminances of light and regenerate at a much slower pace compared with cones.

Integration of light information in the retina, however, does not exclude the possibility of integration of photic drive by neurons in the SCN (61). As with the ipRGCs, SCN neurons exhibit persistent firing, even after cessation of a light stimulation (62), providing a substrate for temporal integration. Alternatively, some neurons in the SCN are activated by phases of darkness (61). While the role of these neurons and their implication in entrainment remain unclear, it is possible that the combination of the excitation by flashes of light with interspersed darkness is able to mediate the observed effects.

A circadian critical fusion frequency. When flashes were separated by 2.5 to 7.6 seconds, circadian phase shift was reduced to a similar magnitude as the shift induced by continuous light. Thus,

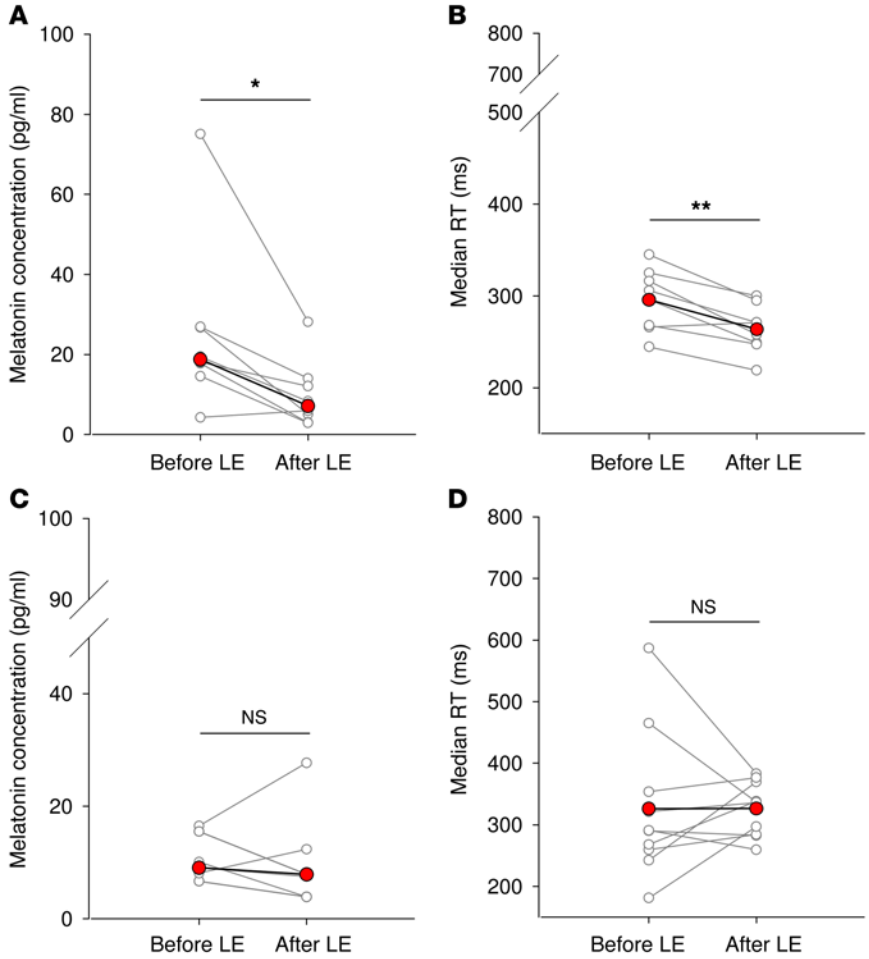


Figure 5. Melatonin suppression and median RT changes associated with a similar circadian phase shift. (**A**) Melatonin concentrations (n = 8, W = 35, Z = 2.31, $^*P < 0.05$, Wilcoxon signed-rank test) and (**B**) median RT (n = 8, t = 4.49, df = 7, $^{**}P < 0.01$, paired t test) are significantly decreased under continuous light exposure. (**C**) Participants exposed to flashes who exhibit a similar circadian phase shift as those exposed to continuous light (i.e., within the 95% CI) do not exhibit a significant decrease in melatonin concentrations (n = 7, W = 12, Z = −0.25, P = 0.81, Wilcoxon signed-rank test) or (**D**) median RT (n = 10, t = −0.02, df = 9, P = 0.98, paired t test) after the different flash exposures. Individual data before and after light exposure are represented as white circles connected with gray bars. (**A** and **C**) Median and (**B** and **D**) average data are represented as red circles connected by black bars.

the circadian system may "perceive" this frequency of stimulation the same as it would continuous light. This is akin to the phenomenon of flicker fusion in image-forming vision, in which scotopic, rod-mediated vision has a critical fusion frequency (CFF) near 15 Hz, while cone-mediated vision reaches a CFF near 60 Hz (63). Based on our findings, we suggest that circadian CFF, after dark adaptation and under a broad band full retinal exposure to a pattern of 2-millisecond stimuli, occurs around 0.13 Hz (7.6 s ISI). As with CFF, we expect circadian CFF to vary across species (64).

A dichotomy in the NIF retinohypothalamic pathways. Given the technical difficulties in assessing the effect of individual 2-millisecond light flashes on melatonin suppression or alertness changes, we cannot exclude that transient changes in these functions have occurred following each brief light exposure. Nevertheless, the absence of an ISI-dependent change in melatonin suppression and alertness over a 60-minute period under different ISIs supports the idea that light-induced circadian phase shifting is not subserved by the same neural pathways as either light-induced melatonin suppression or light-induced changes in alertness. Light-induced changes in melatonin production, alertness, and phase shift are all thought to be communicated to the brain by the ipRGCs (16, 21, 65). While light-induced melatonin suppression is often used as a proxy for the effect of light on the circadian system, our data indicate that there are at least some circumstances in which this is inaccurate. It is possible that distinct subtypes of ipRGCs in the human retina (66, 67) may subserve different NIF functions. The M1 subtype of ipRGCs is implicated mainly in phase shifting the circadian system and has a faster response, a lower intrinsic threshold, and similar extrinsic input compared with other ipRGC subtypes (67). It is therefore possible that temporal integration is limited to M1 ipRGCs and that activation of these ipRGCs with light flashes is insufficient to induce light-induced melatonin suppression or alertness. An alternative possibility is that, as discussed above, the temporal integration of light is occurring at the level of the SCN. While the retinohypothalamic tract, which arises from the ipRGCs, projects to and conveys light information to the SCN, it also projects to other brain nuclei, including the paraventricular nucleus of the hypothalamus (PVN) (68), pretectal area (16), lateral hypothalamic area (LHA) (69), and ventrolateral preoptic nucleus (VLPO) (68, 70). The PVN is involved in the control of melatonin secretion, while the other areas (pretectal area, LHA, VLPO) are all implicated in the regulation of sleep or wake. If temporal integration in the SCN is required for the phase shifting response to flashes, despite whether similar integration occurs in the retina, it is not altogether unexpected that NIF responses, such as light-induced melatonin suppression and changes in alertness, which may not depend on the SCN, are not affected by the flashes in a dose-dependent manner.

Technical considerations. There was considerable variability associated with responses to very short ISIs between the flashes of light. Some of this variability may be explained by difficulties in maintaining eyes open due to sleep inertia or anticipatory blinking, which is more likely to occur when the ISI is very short (e.g., every 2.5 s) rather than long (e.g., every 240 s). We did not record eyelid movement but did discuss with the participants the importance of remaining awake with their eyes open in the Ganzfeld dome. Light is able to penetrate through the closed eyelid in a wavelength-dependent fashion, such that approximately 3% to 14% of light is transmitted (71). We have recently shown that, given sufficient intensity, millisecond light flashes passing through the eyelid have the capacity to induce changes in circadian timing (72). Neverthe-

less, if a participant had his eyes closed, the retinal illuminance of the stimulus would differ from the corneal measured illuminance and could contribute to the variability of the responses to the short ISI. Future studies are necessary to discover, and potentially remove, this source of variability in the response to short ISI.

It is worth mentioning that, even though we fit the observed phase shift as a function of ISI with a linear rise and exponential decay function, it is quite possible that other functions may be more appropriate for these data. This phenomenological fit, however, was chosen as most suitable after multiple tests with various models, based on known retinal and neuronal physiology. Further studies on NIF photoreception are necessary to refine these models and consecutively the fit of our data.

Flashes that are 2 milliseconds in duration, such as those used in this study, are faster than the pupil constriction response (73). While the pupil constricts after the flash occurs, we have found (data not shown) that there is a persistent pupillary constriction for 29.5 ± 3.24 seconds (n = 4) after an individual flash, thereby reducing the overall input to the retina, especially for flashes with an ISI under 15 seconds. Taking into account pupillary constriction induced by both continuous and different sequences of light flashes, continuous light would still contain 850 to 18,000 times more light than the sequences of flashes used in this study. We could have, as many laboratories do, used an antimuscarinic agent to dilate the pupils to avoid the pupillary light reflex and keep it from interfering with the amount of light striking the retina. Given the potential effect of antimuscarinics on the physiologic properties of retinal ganglion cells (74), we feel that the use of such agents would have created greater uncertainty in the interpretation of our results.

A final consideration is that the phase shift observed in our study under continuous light was smaller than that observed in previous studies (41, 49). This difference, however, is likely due to protocol dissimilarities in the intensity of the light exposure (49) and light history of the participants that could lead to a different sensitization of the photoreceptive system (41).

Ultrashort flashes revolutionize light therapy for chronobiological disorders. Whether for mood (23, 28, 75) or circadian disorders (26), light has been shown to be an effective and safe, yet challenging therapy. Multiple modulations have been suggested for the chromaticity (76–78), intensity (79), and pattern (55, 80) of light exposures to render them optimal for clinical and personal use. Here, we show that modulation of the temporal pattern of light at the millisecond time scale can be manipulated to isolate and optimize the effects of light on the circadian timing system. Using modern lighting technologies, such as ultra-bright light-emitting diodes, flashes can also be incorporated into portable mask designs that could be controlled and timed via smart phone software to proactively phase shift and resynchronize jet-lagged travelers to their new time zone, help individuals who want to go to sleep earlier on a regular basis do so (e.g., adolescents), and work in conjunction with daytime light/dark exposure to aid adjusting shift workers. The efficiency of this light treatment is especially pronounced, as flashes have been shown to be able to phase delay the circadian system, even during sleep, when the circadian system is most sensitive to light (54), while the eyelids are closed and without altering sleep (72). This type of light exposure, therefore, opens new treatment possibilities for circadian disorders that will need to be tested in real-world clinical trials as well as possibilities for the alleviation of associated sleep, mood, cognitive, and metabolic declines.

Methods

Participants. Thirty-nine healthy adults (25 males and 14 females; 26.4 ± 5.06 years of age, mean ± SD) were empaneled from August 3, 2012, to August 12, 2015. Participants were enrolled if they reported minimal to no sleep disturbances (Pittsburgh Sleep Quality Index scores: 0–4; ref. 52), alcohol use (Alcohol Use Disorders Identification Test scores: 0–5; ref. 81), or depressive symptoms (Center for Epidemiologic Studies–Depression scores: 0–19; ref. 82) and were of intermediate chronotype (simplified version of Morning-Eveningness Questionnaire scores: 11–26; refs. 50, 51).

Protocol design. Participants took part in a 16-day protocol. From day 1 to day 14 participants maintained a regular sleep time and wake time pattern at home. At-home sleep-wake patterns were monitored using actigraphs (Actiwatch, Philips, or Motionlogger, Ambulatory Monitoring, and self-reported sleep diaries, ref. 83). Participants entered the laboratory on day 15. The midpoint of sleep (MSP), defined as the midpoint of the average sleep period during the prior 14 days (day 1 to day 14), was calculated using the actigraphy data and used as the midpoint of an 8-hour sleep opportunity in the laboratory (e.g., a subject who habitually went to bed at 00:00 hours and woke at 08:00 hours would have a MSP of 04:00 hours and be scheduled to sleep in the lab from 00:00 hours until 08:00 hours). Participants whose bed time or wake time deviated by more than 30 minutes twice across the prior 14 days were not empaneled in the study. All in-lab procedures were individually timed based on the participants' MSP. Upon entry into the laboratory on day 15 until the conclusion of the study on day 16, subjects remained in a customize time isolation suite at the Veterans Affairs Palo Alto Health Care System. Breakfast and lunch were provided to the subjects, and they had ad libitum use of an en suite bathroom. During waking hours and constant posture (CP), room lighting provided by standard overhead fluorescent lamps was dim (0.6–1.9 lux), so as to minimize the effects of background light on the circadian system or melatonin production (40). During the evenings of day 15 and day 16, subjects underwent a CP procedure (Figure 2). The purpose of the CP was to hold constant or remove factors that might otherwise mask endogenous circadian rhythms (84). During the CP, instead of eating dinner, participants were given equicaloric liquid snacks (Ensure, Abbott Laboratories) every 60 minutes, such that the caloric intake that they would have received from their dinner was distributed over either 8 (CP1) or 10 (CP2) hours (85). During the CP, saliva samples were collected every 30 minutes. Alertness and sleepiness levels were monitored every 60 minutes (Figure 2).

Light exposure. During the night between day 15 and day 16, subjects were awakened by the experimenter 2 hours and 15 minutes prior to their MSP (1 hour and 45 minutes after their scheduled bedtime) and kept awake for 1 hour and 15 minutes. During the 15 minutes immediately following arousal into darkness, participants provided a saliva sample and alertness was assessed (see below). During the next hour, they received their experimental light exposure while in a sleeping posture. An additional saliva sample was taken and alertness was reassessed during the final 15 minutes of experimental light exposure. After this hour, they were allowed to return to sleep for an additional 5 hours (Figure 2). Participants were randomly assigned on day 15 to

receive either continuous light (n = 8, aged 27.4 ± 5.0 years) or one of twelve different light exposures that vary by the number of ultrashort 2-millisecond flashes administered during an hour (n = 31, aged 26.1 ± 5.1 years). Flash frequency within the 1-hour light exposure varied between 0.004 and 0.4 Hz (ISI = 2.5 to 240 s). Subjects were randomized such that at least 2 participants were assigned to each of 12 ISI values (Figure 1 and Figure 3). Following an interim analysis, we added an extra 3 participants at an ISI of 10 seconds, 3 participants at an ISI of 5 seconds, and 1 participant at an ISI of 2.5 seconds. Randomization was done by J.M. Zeitzer through a random sequence generator algorithm, and participants were assigned to experimental groups by study personnel. All data assessments were done blind to condition. Flashes were delivered using the xenon flash unit of a ColorDome (Diagnosys). Continuous light was delivered using a broad-spectrum 400 W blue metal halide bulb (Eye Hortilux) with an adapted ballast and a custom-built light box fitted with ultraviolet light filtration (Rosco 03114 2024), neutral density filters (Rosco 3402), and diffusers (Rosco 117). Illuminance of both light exposures was matched to be around 1,800 lux using the ILT1700 radiometer (International Light Technologies). Extensive characterization of the light sources is presented in Supplemental Table 1 and Supplemental Figure 1.

Circadian phase shift and melatonin suppression. Circadian phase was assessed on days 15 and 16 through examination of the endogenous melatonin secretion profile. Saliva (at least 1 ml) was collected every 30 minutes by placing an absorbing swab into the mouth and moving it around slowly over 5 minutes. Next, the swab was put into a tube (Salivettes, Sarstedt). Samples were centrifuged after collection and then frozen and stored at −80°C until assayed. Salivary melatonin was assayed in duplicate according to the manufacturer's instructions using the Bühlmann direct saliva Melatonin ELISA Kits (Alpco) based on the Kennaway G280 anti-melatonin antibody. All samples from a single subject were assayed on the same ELISA plate.

Onset of melatonin secretion was calculated as the time at which salivary melatonin concentrations exceeded a subject-specific dynamic threshold (Supplemental Figure 2). The dynamic threshold was calculated as the mean of the first 3 daytime melatonin concentrations plus twice the standard deviation of these values (86). DLMO was estimated for each participant on days 15 ($DLMO_{15}$) and 16 ($DLMO_{16}$). Circadian phase shift ($\Delta\phi$) elicited by the light exposure was calculated as follows: $\Delta\phi = DLMO_{15} - DLMO_{16}$ (Supplemental Figure 2). Phase delays are represented by convention negative numbers, and phase advances are represented positive numbers. The phase angle (θ) between $DLMO_{15}$ and the onset of the experimental light exposure was also calculated to ensure that the participants were exposed to light at a similar circadian phase (Supplemental Figure 2). Average phase angle of light exposure (4.70 hours) of participants in both groups was plotted on the phase response curve to 1 hour of light, adapted from St Hilaire et al. (49) to obtain an average phase shift (−1.23 hours). Individual deviations from the average phase angle were used to concomitantly obtain a percentage of change from average phase shift. The percentage of change obtained was then applied to each participant's phase shift to obtain a corrected phase shift that takes into account the differences in circadian timing of the light exposure.

Melatonin suppression was calculated as the percentage of change between the sample taken at the end of light exposure and the sample taken immediately prior to light exposure: % melatonin suppression = (baseline [melatonin] − final [melatonin])/baseline [melatonin] × 100.

Monitoring sleepiness and alertness. Subjective sleepiness was monitored by SSS (53). Objective alertness levels were monitored by testing RT over 10-minute segments via the auditory version of the psychomotor vigilance task using the PVT-192 (Ambulatory Monitoring) (87). Auditory psychomotor vigilance task data were analyzed using the manufacturer's software (React, Ambulatory Monitoring). Variables derived from the software included mean and median RT, number of lapses (nonresponse for 400 ms), and shifts in optimum RT (mean of the fastest 10% RTs).

Changes in brain electrophysiological activity were recorded during day 15 by electroencephalography (Siesta, Compumedics). Electroencephalography data derived from each cortical electrode (C3/4, O1/2) were referenced to an electrically neutral/reference auricular electrode. Data were transformed using a fast Fourier transform with a window length of 2 seconds, overlap of 0%, using a Hanning window type with a maximum frequency of 50 Hz, followed by Welch averaging with a mean averaging type on 30 seconds for a threshold of 50% and feature extraction on both power (absolute IU^2 and relative percentage) and frequency (mean Hz and peak Hz). Using this method for each 30-second bin, the absolute power in the different frequency spectra was calculated. Frequency spectra were grouped, as is typical in such analyses, into frequency bands: δ (0.5–4 Hz), θ (4–7.5 Hz), α (8–12.5 Hz), σ (12–14 Hz), β (14–29 Hz), and γ (30–40 Hz). Power spectra bands during the 10-minute auditory psychomotor vigilance task immediately prior to and at the end of the light exposure were analyzed for electrophysiological correlates of arousal (Prana, PhiTools).

Data analysis. Phase shift, percentage melatonin suppression, and changes in the parameters of alertness and sleepiness (after light exposure − before light exposure) were plotted against the ISI (e.g., $\Delta\phi = f[ISI]$; Figure 3). Data were fitted using models, as indicated in the Results. The number of lapses was square root transformed ($\sqrt{lapses} + \sqrt{[lapses + 1]}$) and plotted against ISI for a better clarity of the plot. CI was calculated such as CI = average ± $1.96 \times \sigma/\sqrt{n}$ (where σ is the standard deviation of the data and n is the number of data points). Negative change values for RT, number of lapses, and SSS indicate a faster RT, a decrease in the number or lapses, and a decrease in sleepiness, respectively. Negative percentage of melatonin suppression values indicate less melatonin suppression. Statistics, data fitting, and illustrations were done using OriginPro 8 SR2 (v8.0891, OriginLab). Figure 5 was done using SigmaPlot 11.0 (Systat Software). All data are presented as mean ± SD.

Statistics. Group differences in age, morning or evening preference, and reported prior-month sleep quality were compared using a Mann-Whitney test. Sex distribution between the two groups was compared using a χ^2 test. Phase angle of light exposure between groups and melatonin phase change between nights in the group receiving continuous light were analyzed using a 2-tailed, paired t test after checking for normal distribution of the data. Nonnormally distributed melatonin suppression data and discreet variables of lapses and changes in sleepiness assessed using the SSS were analyzed using the Wilcoxon signed-rank test. Normally distributed continuous data of median RT, shift in optimum RT, and electroencephalographic correlates of arousal were analyzed using a paired t test. All statistical tests used were 2 tailed.

Study approval. The study protocol was reviewed and approved by the Stanford University Institutional Review Board and conforms to the principles expressed in the Declaration of Helsinki. Subjects signed informed consent forms prior to any procedures.

Author contributions

JMZ designed research. RPN performed research and melatonin assays. RPN and JMZ wrote the paper and analyzed the data.

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