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The Circadian Response to the Spectral Properties of Light

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The Circadian Response to the Spectral Properties of Light

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in Physiological Science

by

John Harvey

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ABSTRACT OF THE THESIS

The Circadian Response to the Spectral Properties of Light

by

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Master of Science in Physiological Science

University of California, Los Angeles, 2024

Professor Christopher S. Colwell, Co-Chair

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It is well understood that almost all organisms exhibit daily oscillations in behavior and physiology. These circadian rhythms are capable of being entrained to environmental stimuli, such as light. The circadian system is sensitive to a broad spectrum of light, but mice specifically lack long wavelength sensitive photopigments. I therefore hypothesized that blue-enriched lighting would be better at entraining the circadian system of wild-type (WT) mice compared to red-enriched lighting. My results suggest that under stable phase conditions, the spectral composition of light is of little consequence; however, in response to changes in phase, the spectral properties of light do matter.

The thesis of John Harvey is approved.

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Introduction

The Circadian System

The rotation of the Earth on its axis creates daily rhythmic changes in outdoor lighting intensity and spectrum that we experience as the day and night cycle. Almost all organisms, both plants and animals, have evolved to anticipate and effectively operate under this daily oscillation of light and dark [1]. This evolution is exemplified by the periodic fluctuations in behavior and physiology over the course of the day known as circadian rhythms, which remain essential for survival and general well-being in plants and animals, including humans. Some processes that are under circadian influence include, but are not limited to, hormone secretion, metabolism, immune function, sleep/wake cycle, activity, and core body temperature in endotherms [2-5]. Endogenous cellular networks composed of multiple circadian oscillators generate these rhythms, providing temporal information to the organism's physiological systems; however, in mammals, the part of the nervous system responsible for most circadian behaviors is localized to a bilaterally paired structure in the hypothalamus known as the suprachiasmatic nucleus (SCN). Despite the existence of multiple circadian oscillators, the clock within the SCN exhibits a profound effect on circadian timing and represents the dominant oscillator coordinating daily rhythms throughout the mammalian body [6]. Disruption of the SCN circuit or loss of these cells would produce an arrhythmic sleep/wake cycle, among other desynchronized physiological processes [7,8].

Tau (τ, free-running period) is defined as the time that it takes for the biological oscillator to complete one cycle, i.e., to go from start to finish. In the case of circadian oscillators, Tau is close to, but rarely equal to, 24-hours. For diurnal organisms like humans, Tau is typically longer than 24-hours [9], while for nocturnal organisms, the period is typically less than 24-hours [10]. Genetic factors largely determine the Tau of circadian oscillations [11], although there is evidence for a modest history-dependent regulation [12]. In order for the circadian system to function in a beneficial capacity, it must be capable of synchronizing to the external environment, a process termed entrainment. This typically entails adjusting a biological oscillator with a Tau that is close to 24-hours to the exact 24-hour period (T) of the environment. When entrained, the tau of the biological rhythm equals the period of the entraining stimulus, and, importantly, the two oscillations exhibit a stable and predictable phase relationship. The entraining cue is referred to as a "Zeitgeber'' (translated from German "time giver"). Although any environmental factor that oscillates in a 24-h rhythm can act as a Zeitgeber for different oscillators and under different conditions, light is the Zeitgeber most commonly used by circadian clocks [13]. The reason for this dominant role is that changes in light are highly predicable and omnipresent, and it is, therefore, the primary and most reliable source of information about the "time-of-day".

The curve of the circadian phase response to light depicts the magnitude and direction of phase shifts in response to the biological timing of light stimulation. It has been established that administering light before the midpoint of sleep typically evokes a delay in the circadian phase, whereas light administered after typically advances circadian phase [14]. In other

words: exposure to light during the biological morning or the biological evening, has opposite effects and will, respectively, delay or advance the rhythm [15].

Light information reaches the retina of the eye and can directly influence the circadian system by way of intrinsically photosensitive retinal ganglion cells (ipRGCs) that are responsive to light due to their unique expression of the photopigment melanopsin. This pigment is most sensitive to light in the blue wavelength range (specifically at 480 nm) and is necessary for the normal resetting of behavioral rhythms in most organisms following changes in their light-dark cycle [16]. Additionally, the primary photoreceptors of the retina, rods and cones, can contribute light information through the ipRGCs. Regardless of its origin, the ipRGCs signal to the SCN via their afferent axons that make up the retinohypothalamic tract [17,18]. Animals missing these neurons will present severe disturbances when resynchronizing to light-based phase shifts [18]. Interestingly, melanopsin-containing neurons not only project to the SCN but also to other brain regions where they likely mediate the direct effects of light on mood and arousal [19]. Dysfunction in the ipRGCs would result in an individual that exhibits a free-running rhythm when in an otherwise normal LD cycle, and would be diagnosed as having a non-24-hour sleep-wake disorder.

Light Detection and Entrainment

The circadian system in humans is responsive to a broad spectrum of light encompassing most of what it is perceived as visible light, or light ranging from 380 to 780 nm in wavelength. This is due to the visual pigments of the retina working in tandem with melanopsin to

stimulate the ipRGCs and relay information to the SCN. Including melanopsin, which as mentioned earlier, is sensitive to shorter wavelengths of light, humans have a total of five light-detecting proteins: one class of rod opsin, maximally sensitive to blue-green light (500 nm) and primarily active in dim environments, and three classes of cone opsins, maximally sensitive to red (560 nm), green (530 nm) and blue (430 nm) light [20]. It is important to note that all light receptors are sensitive, albeit to a lesser extent, to a set range of wavelengths around their peak sensitivity (λ_{max}). Additionally, the expression of these light receptors is not equal. In the photoreceptor layer, the prevalence of rods is far greater than that of cones, whilst within the cone population, blue cones make up a small minority (8-12%) [21,22]. These factors must be taken into consideration when attempting to determine how effective any given light source will be at entraining the circadian clock.

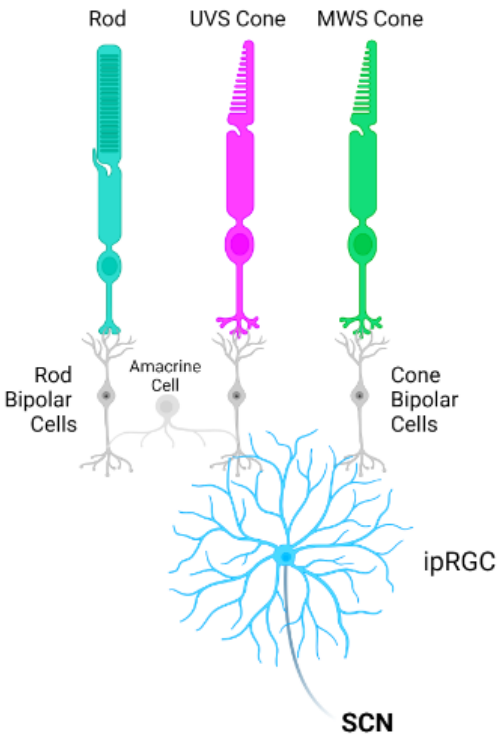


Fig. 1. *Rods, cones and ipRGCs work together to send light information the SCN* Rods and cones provide input to the ipRGCs. Rod input comes via amacrine cells. Cell colors are indictive of their respective λ_{max} values. Figure based on (Peirson et al., 2018) and created with BioRender.com

Given the widespread use of mice to study circadian biology, and their use in the present study, it is important to understand that they possess a different complement of photoreceptors in their retina and, as a result, display different spectral sensitivities. Mouse rod opsin and melanopsin are quite similar to their human counterparts, however, unlike

humans, mice only possess two classes of cone opsin that are middle wavelength (MWS) and ultraviolet sensitive (UVS) with a λ_{max} of 508 nm and 360 nm, respectively [23]. The lack of a long wavelength sensitive opsin makes them far less sensitive to red-enriched light. Given a red-light stimulus at 600 nm of the same intensity, the mouse eye will be approximately 12 times less sensitive compared to the human eye [20]. Furthermore, possibly due to their nocturnal nature, the mouse retina is more rod dominated than the human retina, with cones only accounting for 3% of all photoreceptors [22].

Broadly, these findings indicate that the circadian system is sensitive to a wide spectrum of light [24], and this raises questions about whether the spectral properties of light matter for circadian entrainment. Perhaps the intensity of the illumination is the only parameter biologically important while spectrum does not matter. A variety of studies are beginning to address the issue of whether adjusting the spectral composition of light to reduce melanopic stimulation can be used as a strategy to minimize disruption of circadian rhythms [25-30]. In our own group [31], we found that short-wavelength enriched lighting produced a strong acute suppression of locomotor activity (masking), a robust light-induced phase shift, and increased cFos (a marker for neuronal activation) expression in the suprachiasmatic nucleus in wild-type (WT) mice, while the long-wavelength enriched lighting evoked much weaker responses. Opn4^{DTA} mice, lacking the melanopsin expressing ipRGCs, were resistant to the effects of exposure to dim short-wavelength enriched light at night. Broadly, our findings are consistent with the recommendation that spectral properties of light at night should be considered in order to optimize health in neurotypical as well as vulnerable populations.

An additional and important test on the role of the spectrum is to determine if the wavelengths of light matter for circadian entrainment. This issue has been examined before; for example, the sensitivity of the visual pathway that underlies circadian entrainment was measured in hamsters in some detail [32]. These authors concluded that the circadian system integrates the total number of photons delivered and such integration determines the magnitude of the response. This conclusion suggests that intensity is likely the main determinant of phase shifts. However, this work was done before we had an understanding of the photopigments involved in circadian light detection, thus, we wanted to revisit this issue, taking advantage of the technical advances in our ability to deliver wavelength controlled light exposure using light-emitting diodes (LED). The LED array enabled us to shift the spectral properties of the daylight while keeping the intensity of the illumination at 50 lx.

Hypothesis

Short wavelength (blue) enriched light during the day will be more effective at entraining circadian-controlled activity rhythms of mice compared to long wavelength (red) enriched light.

To test this overarching hypothesis, I carried out a series of experiments comparing the response of WT mice to blue or red-enriched lighting. First, rhythms in activity were measured in groups of mice held on a 12:12 light/dark (LD) cycle until stable entrainment to all 4 differentially enriched-lights (50 lx). Given the multiple ways that light information can be detected, the expectation was that no differences in activity rhythms would be observed under any of the lighting conditions. Next, I examined the effect of rapidly shifting the LD

cycle by advancing or delaying the timing of lights on. For this type of jet lag experiment, the prediction is that the blue-enriched light would result in a more rapid re-entrainment with such effects being more apparent with phase advances. In the last two sets of experiments, I examined the effects of the different enriched lighting on two outcomes broadly used in the circadian field, namely masking behavior and light-induced acute phase shift at CT16. Again, the expectation would be a stronger effect of the blue-enriched lighting on suppressing acute activity during the night (masking) and blue-enriched lighting producing larger light-induced phase shifts of the circadian system in the mice exposed to light at CT 16.

Methods

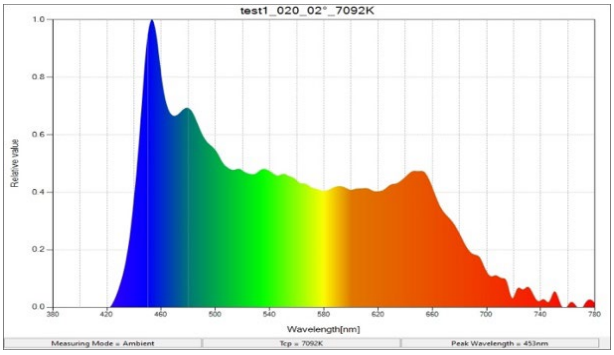
Animals

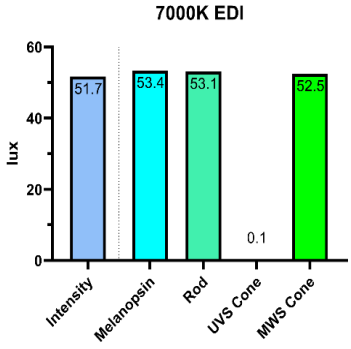
All animal procedures were performed in accordance with the UCLA Animal Care Committee's regulations. Both adult male and female mice (3-4 months old) were used in this study. C57BL/6J mice (stock number 000664; RRID:IMSR_JAX:000664) were obtained from Jackson Laboratory. Cohorts of male and female mice were group-housed prior to experimentation.

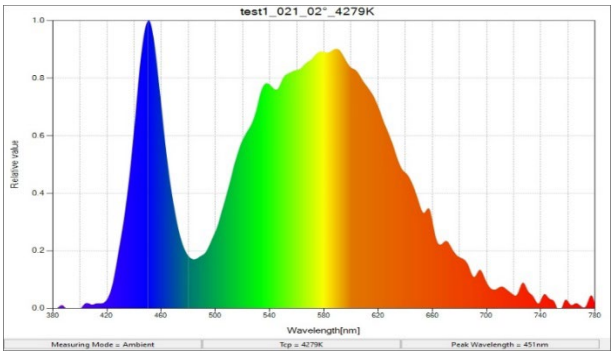
Lighting manipulations

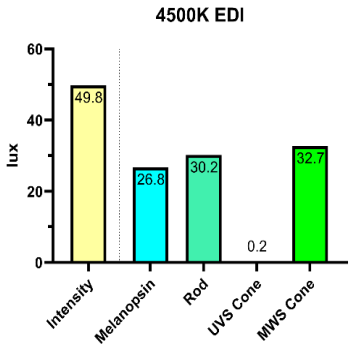
Mice were housed in light-tight ventilated cabinets in temperature- and humidity-controlled conditions, with free access to food and water. All the mice were first entrained for 2 weeks to a normal lighting cycle: 12:12 hr light:dark (LD) before data collection. Light intensity during the day was 50 lx as measured at the base of the cage, and 0 lx during the night. The

time of lights-on defines Zeitgeber Time (ZT) 0 and the time of lights-off defines ZT 12. Following entrainment to normal LD, mice were divided into four cohorts and singly housed under four different lighting conditions for most of the experiment: (1) LD with blue-enriched light (7000K), (2) LD with white light (4500K), (3) LD with red-enriched light (1500K) or (4) LD with pure red light (RED). The kelvin values represent the correlated color temperature (CCT) of the lights, which measures the relative proportions of different wavelengths in a given source of white light, with lower temperatures being rich in red and yellow wavelengths and higher temperatures being rich in blue wavelengths. The 7000K and 1500K lights









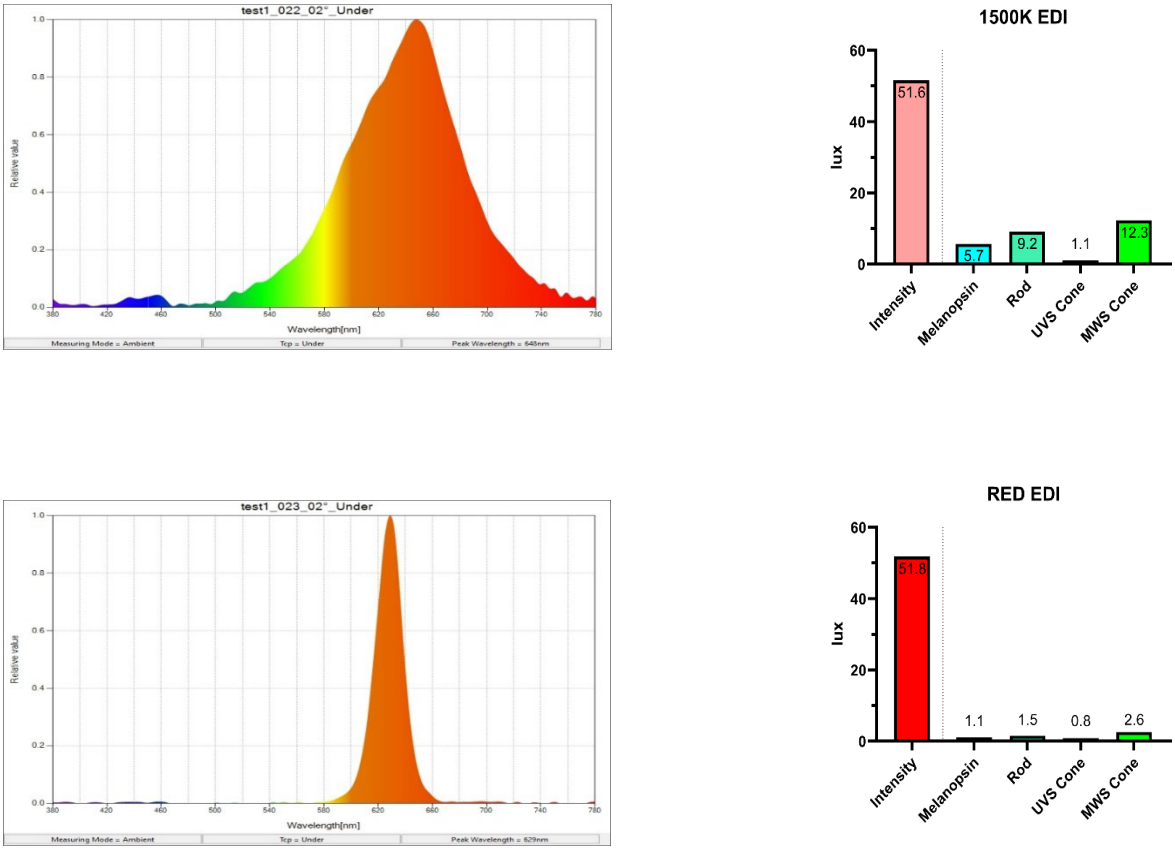


Fig. 2. *Spectral composition and Equivalent Daylight Illuminance for each lighting condition.*
The spectral composition of each lighting condition is shown on the left. On the right is the intensity of each lighting condition (all about 50 lx) as well as the equivalent daylight Illuminance (EDI) for each photopigment in the mouse retina. EDI is a metric used to measure how much daylight is needed to have the same impact as a given light setting. Note the UVS cone EDI remains low in all lighting conditions as none of the LEDs generated light in the violet-ultraviolet range.

were generated by the Korrus Inc (Los Angeles, CA) LED lighting system. The 4500K and RED lights were generated by Lepro and Marreal LED lighting systems, respectively. Following recent recommendations [33], the LED output was measured with a Sekonic spectrophotometer (Model C-7000, Japan) at the level of the cage floor. Using the International Commission on Illumination (CIE) S 026 α-opic Toolbox [34], the melanopic α-

opic irradiance of the 7000K, 4500K, 1500K and RED output was estimated to be at 0.081 W/m^2, 0.041 W/m^2, 0.009 W/m^2 and 0.002 W/m^2, respectively. The melanopic α-opic equivalent dayl ight (D65) illuminance of the 7000K, 4500K, 1500K and RED output was estimated at 53.4 melanopic lx, 26.8 melanopic lx, 5.7 melanopic lx and 1.1 melanopic lx, respectively. The total energy of the 7000K light was measured as an illumination of 51.7 lx, irradiance of 0.20 W/m^2, log photon irradiance of 13.7 s^1/m^2, the 4500K light as an illumination of 49.8 lx, irradiance of 0.15 W/m^2, log photon irradiance of 13.6 s^1/m^2, the 1500K light as an illumination of 51.6 lx, irradiance of 0.26 W/m^2, log photon irradiance of 13.9 s^1/m^2, and the RED light as an illumination of 51.8 lx, irradiance of 0.25 W/m^2, log photon irradiance of 13.9 s^1/m^2. One simple way of comparing light sources is the M/P ratio (melanopic lx divided by photopic lx). For daylight, this will be 1. In our study, the M/P was 1.03 for the 7000K illumination, 0.54 for the 4500K, 0.11 for the 1500K and 0.02 for the RED.

Daily pattern of activity was monitored using a running wheel fixed with a magnetic sensor reporting to a VitalView data recording system (Mini Mitter, Bend, OR). Activity levels with the running wheels are reported as revolutions per minute (rev/min). Cage activity was recorded in 3 min bins, and 14 days of data were averaged for analysis using the using the Clocklab program (Actimetrics, Wilmette, IL; http://actimetrics.com/products/clocklab/). The strength of the rhythm was determined from the amplitude of the $\chi 2$ periodogram at 24 hr, to produce the rhythm power (%V) normalized to the number of data points examined. The periodogram analysis uses a $\chi 2$ test with a threshold of 0.001 significance, from which the amplitude of the periodicities is determined at the circadian harmonic to obtain the rhythm power. The percentage of sleep-phase activity was determined by the relative distribution of

activity during the day versus the night. Fragmentation was determined by the number of activity bouts. Each bout was counted when activity bouts were separated by a gap of 21 min. The onset variability was calculated by first determining the daily onset of activity over 14 days, and then the deviation from the best-fit line drawn through the 14 onset times. To produce the representative waveforms, hourly running averages of the same series of activity data were plotted against time of day.

Lighting schedule advance and delay

The same four cohorts of WT mice (3-4 mo), still individually housed in cages with running wheels fixed with magnetic sensors in light-tight chambers under a 12:12 LD cycle, were used to determine the effects of the four different wavelengths of LED lights on time to entrain to a 6-hr phase advance and subsequent 6 hr phase delay, with 5 days of phase stability between the shifts. Their locomotor activity was recorded per 3-min interval and monitored to ensure the entrainment to the housing LD cycles. Each cohort's lighting schedule was advanced by 6-hrs with the only difference being their respective light sources (7000K, 4500K, 1500K or RED). Activity onsets determined by Clocklab were used to measure the number of days it took for each mouse to entrain to the lighting schedule shift. The same process was repeated for the 6-hr phase delay.

Photic suppression of nocturnal activity (negative light masking)

The same four cohorts of WT mice (3-4 mo) were individually housed in cages with running wheels fixed with magnetic sensors in light-tight chambers under a 12:12 LD cycle to determine the effects of the four different wavelengths of LED lights at night on masking of

nocturnal activity. Their locomotor activity was recorded per 3-min interval and monitored to ensure the entrainment to the housing LD cycles. The animals were exposed to light (7000K, 4500K, 1500K or RED) for 1hr at ZT 14. The activity level during the light exposure was compared to the activity level during the equivalent time (ZT 14 to 15) on the day before the treatment.

Fig. 3. *The experimental timeline.*
WT mice were individually housed in cages with running wheels and entrained for 2 weeks to one of the 4 lighting conditions at a fixed intensity of 50lx (**Fig. 2**). All the following manipulations (phase advance and delay, masking and light-induced phase delay) were carried out on all 4 cohorts of mice, each in their respective lighting condition. Locomotor activity was recorded continuously and monitored to ensure entrainment before and after each light manipulation.

Light induced phase shift of activity rhythms

The same four cohorts of WT mice (3-4 mo) were released into constant darkness (DD) and allowed to acclimate for at least 10 days. The circadian time (CT) of their free-running sleep/wake cycles was monitored with the VitalView. The time of activity onset under DD was defined as CT 12. After the minimum 10-day acclimation period, the mice were exposed to their respective wavelengths of light (50 lx) at CT 16 for 15min, after the treatment, the mice were held in DD for the subsequent 10 days. The best-fit lines of the activity onsets of the sleep/wake cycles before and after the treatment were determined and compared.

Statistics

SigmaPlot (version 14.5, SYSTAT Software, San Jose, CA) or GraphPad Prism (version 10.0.3), GraphPad Software, San Diego, CA) was used to run statistical analyses. One-way analysis of variance (ANOVA) was used to determine the significance of the impact of light exposure on the four assays for the light input to the circadian system in WT mice and other parameters of the activity rhythms. Two-way ANOVA was used to analyse (1) the waveforms of the activity rhythms with time and treatment as factors**.** The Holm-Sidak or Tukey test for multiple comparisons was used when appropriate. Values are reported as the mean ± standard error of the mean (SEM). Differences were determined significant if $P < 0.05$.

Results

No difference in daily activity rhythms among the WT mice under the four lighting conditions.

Lighting cues from the environment are instrumental in entraining the circadian rhythms of animals and humans, including nocturnal WT mice, and are detected by multiple photoreceptors. Still melanopsin expressing ipRGCs are critically involved in circadian entrainment and this photopigment is maximally sensitive to blue/green wavelengths. Therefore, I sought to test the hypothesis that exposure to blue-enriched (7000K) light would produce more stably synchronized daily activity rhythms with less cycle-to-cycle variability than other light sources. Four cohorts of WT mice were first housed under control conditions (LD 12:12), then exposed to either 7000K, 4500K, 1500K or RED light (50 lx) for 2 weeks (**Fig. 2&3**). As expected, all cohorts of WT mice exhibited robust rhythms in activity synchronized to the LD cycle (**Fig. 4A)**. Under the four different lighting conditions, the average waveforms

(1-hr bins) of WT mice were not significantly altered (**Fig. 4B; Table 3**). Note that the RED-light entrained group did appear to start their activity onset earlier with rises detected at ZT 9 although this difference was not significant. Similarly, although not significant, the variability in activity onset was higher in the RED-light group (**Fig. 4G, Table 1**). Other activity rhythm parameters were not expected to change with lighting, and indeed, no differences could be detected (**Fig. 4C-4G; Table 1**). While the sample size needs to be increased, these results suggest that the spectral properties of light used for illumination do not play a role in regulating the daily activity rhythms of WT mice under stable entrainment at an intensity of 50 lx.

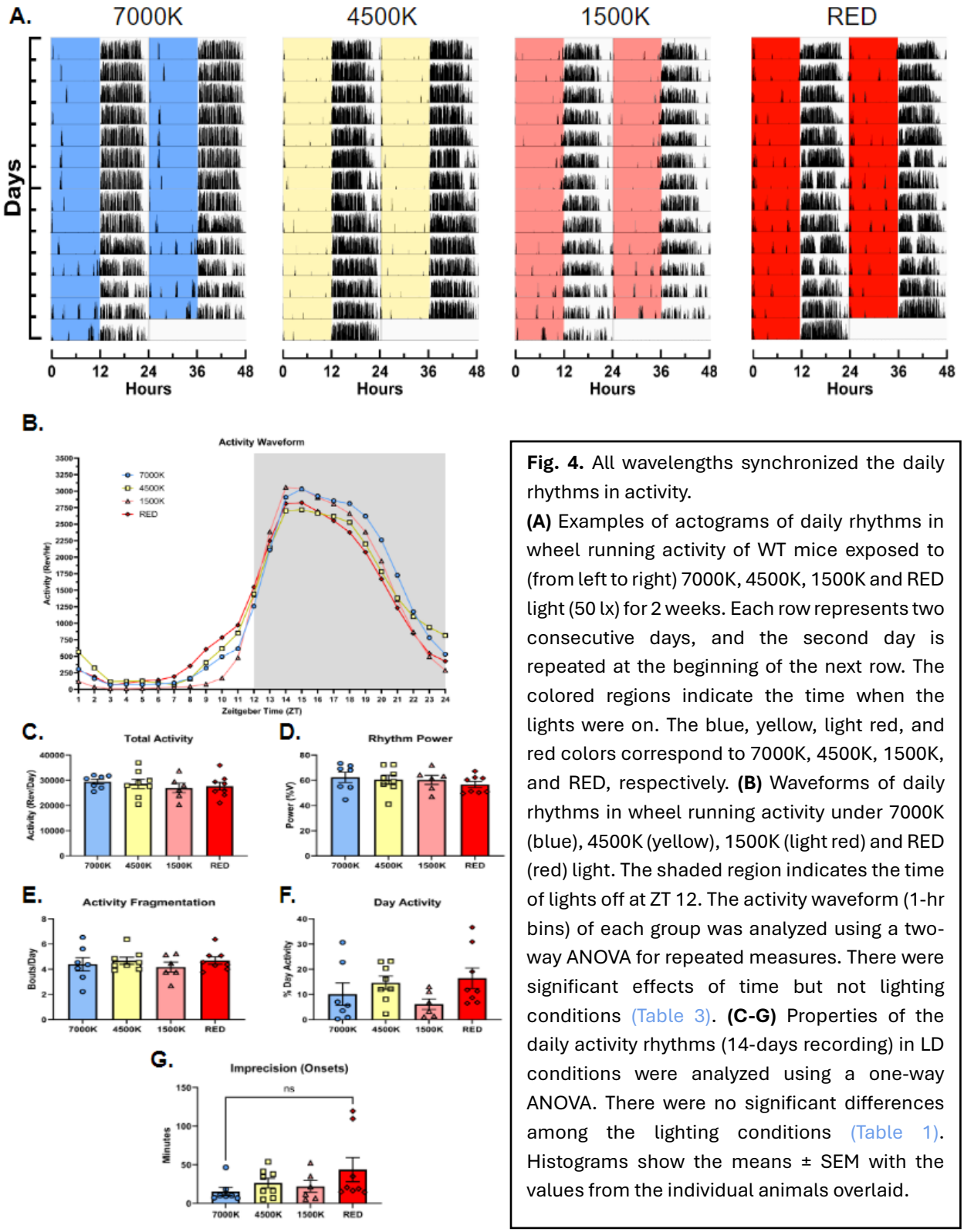


Fig. 4. All wavelengths synchronized the daily rhythms in activity.
(A) Examples of actograms of daily rhythms in wheel running activity of WT mice exposed to (from left to right) 7000K, 4500K, 1500K and RED light (50 lx) for 2 weeks. Each row represents two consecutive days, and the second day is repeated at the beginning of the next row. The colored regions indicate the time when the lights were on. The blue, yellow, light red, and red colors correspond to 7000K, 4500K, 1500K, and RED, respectively. **(B)** Waveforms of daily rhythms in wheel running activity under 7000K (blue), 4500K (yellow), 1500K (light red) and RED (red) light. The shaded region indicates the time of lights off at ZT 12. The activity waveform (1-hr bins) of each group was analyzed using a two-way ANOVA for repeated measures. There were significant effects of time but not lighting conditions (Table 3). **(C-G)** Properties of the daily activity rhythms (14-days recording) in LD conditions were analyzed using a one-way ANOVA. There were no significant differences among the lighting conditions (Table 1). Histograms show the means ± SEM with the values from the individual animals overlaid.

WT mice under 7000K lighting entrain faster to a 6-hr phase advance compared to WT mice under RED lighting.

Next, we also sought to determine if the spectral composition could affect the ability of WT mice to adapt to changes in light timing. The four cohorts of WT mice were subjected to a 6-hr phase advance under their respective lighting conditions (50 lx, **Fig. 2&3**) and given enough time to completely entrain to the new lighting schedule (**Fig. 5A**). In the days immediately following the phase advance, the onset deviation for all animals was measured as the number of minutes after the time of lights off for the beginning of activity to occur. The mice exposed to 7000K did appear to re-synchronize faster to the new LD cycle than the other groups (**Fig. 5B; Table 3**). Similarly, the total number of days to entrain to the phase advance was counted for every animal and averaged based on lighting condition. Again, the mice under 7000K lighting were found to entrain to the phase advance significantly quicker in comparison to the mice under the RED light (**Fig. 5C; Table 2**). Hence, blue-enriched lighting improves the ability of WT mice to adapt to advances in light timing. **5C; Table 2**). Hence, blue-enriched lighting improves the ability of WT mice to adapt to advances in light timing.

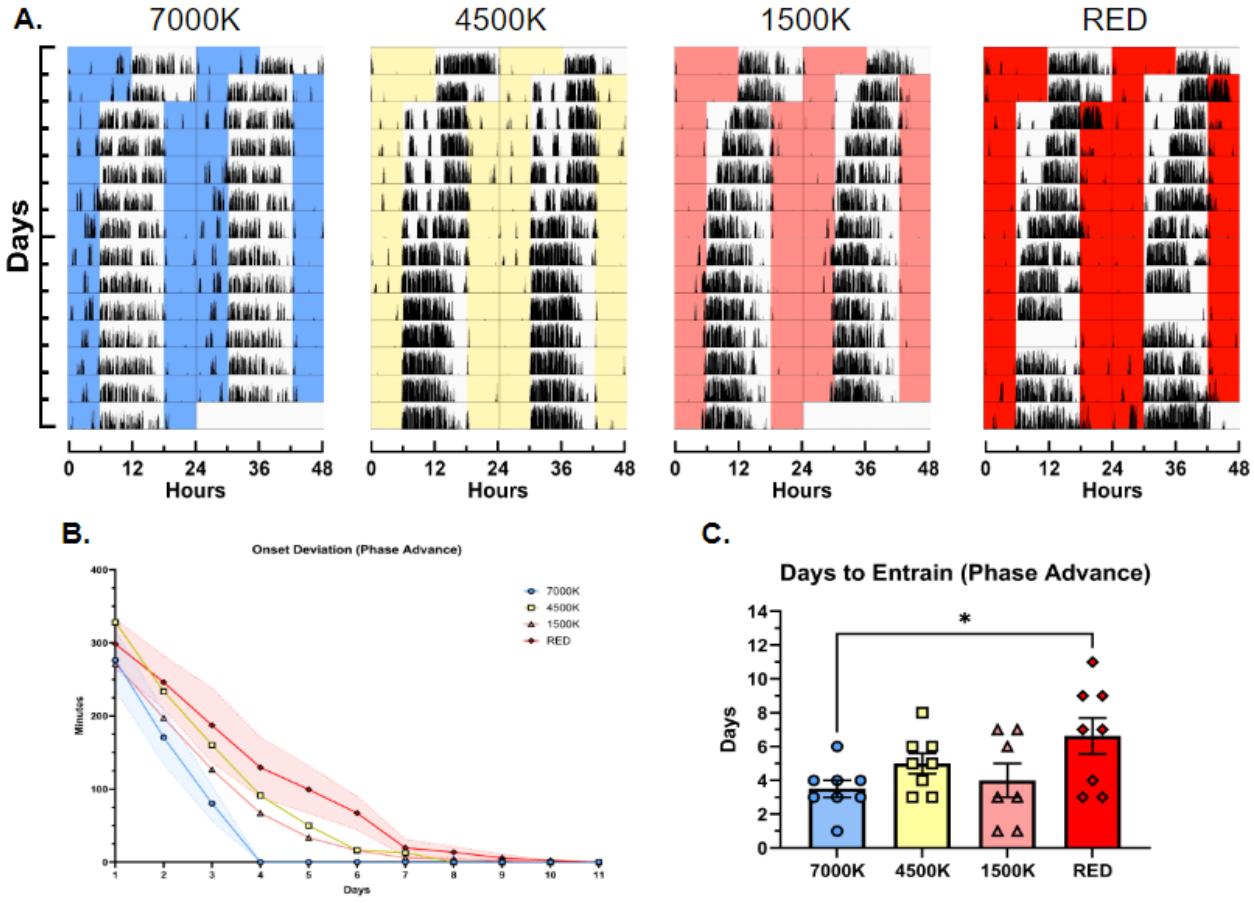


Fig. 5. Mice exposed to blue light (*7000K) re-synchronized faster to a 6-hr phase advance compared to mice under RED lighting*.

(A) Examples of actograms of daily rhythms in wheel running activity of WT mice after being subjected to a 6-hr phase advance (beginning on the 3rd day from the top). **(B)** The average delay in onset of activity compared to time of lights off for each group in the days immediately following the 6-hr phase advance of the lighting schedule, measured in minutes after lights off. The XY plot shows means ± SEM (the shaded regions), note the SEM of the 4500K and 1500K groups were omitted for clarity. **(C)** The average number of days to fully entrain to the 6-hr phase advance by lighting condition. Data were evaluated by one-way ANOVA followed by the Tukey's multiple comparisons test (Table 2). The asterisk indicates significant differences ($P < 0.05$). Histogram shows the means ± SEM with the values from the individual animals overlaid.

WT mice under 7000K, 4500K and 1500K lighting entrain faster to a 6-hr phase delay compared to WT mice under RED lighting.

To further examine the effects of spectral composition on the ability of WT mice to adapt to changes in light timing, the four cohorts of WT mice were subjected to a 6-hr phase delay (**Fig. 3**) under their respective lighting conditions (50 lx) and given enough time to completely entrain to the new lighting schedule (**Fig. 6A**). In the days immediately following the phase delay, the onset deviation for all animals was once again measured, but now as the number of minutes before the time of lights off when the onset of activity occurred. The average onset deviation for the 7000K group was found to be significantly lower by day when compared to the RED group, but not the 4500K and 1500K groups (**Fig. 6B; Table 3**). Lastly, the total number of days to completely entrain to the phase delay was counted for every animal and averaged based on lighting condition. WT mice under 7000K, 4500K, and 1500K lighting all entrained to the phase delay significantly quicker than mice under the RED lighting (**Fig. 6C; Table 2**). Altogether these findings suggest that lighting enriched in shorter wavelengths improves the ability of WT mice to adapt to delays in light timing.

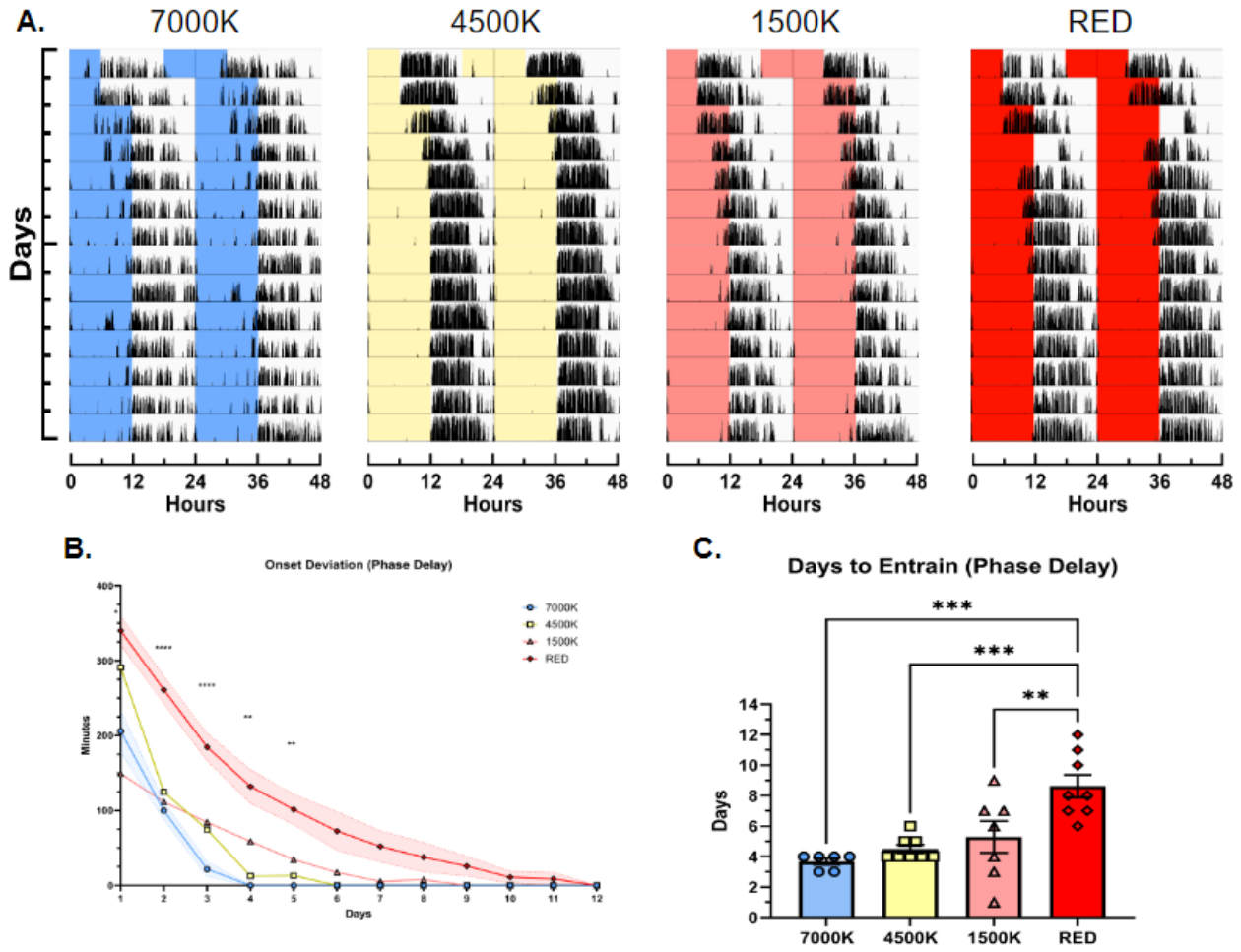


Fig. 6. *Mice under 7000K,4500K and 1500K lighting re-synchronize faster to a 6-hr phase delay compared to mice under RED lighting.*

(A) Examples of actograms of daily rhythms in wheel running activity of WT mice after being subjected to a 6-hr phase delay (beginning on the 3rd day from the top). **(B)** The average advance in onset of activity compared to time of lights off for each group in the days immediately following the 6-hr phase delay of the lighting schedule, measured in minutes before lights off. The asterisks indicate statistical significant differences between the 7000K and RED lighting conditions. The XY plot shows means ± SEM (the shaded regions), note the SEM of the 4500K and 1500K groups were omitted for clarity. **(C)** The average number of days to fully entrain to the 6-hr phase delay by lighting condition. Data were evaluated by one-way ANOVA followed by the Tukey's multiple comparisons test (Table 2). The asterisks indicate significant difference ($P < 0.05$). Histogram shows the means ± SEM with the values from the individual animals overlaid.

WT mice under RED lighting exhibit decreased masking behavior.

Negative masking behavior refers to the light-driven suppression of activity at a time when high levels of activity are typical. While masking itself is not a circadian parameter, its magnitude can offer insight into the sensitivity of mouse photopigments to a given light stimulus. The effects of light-driven masking of activity were measured using wheeling running sensors by exposing each cohort of mice to 1 hour of their respective light source (50 lx) from ZT 14-15, i.e. 2 hrs after lights off and the time of peak activity in WT mice (**Fig. 7A**). The average suppression in activity elicited by RED lighting exposure was less than that observed at the other illuminations (**Fig. 7B; Table 4**), whilst there were no differences between the other wavelengths.

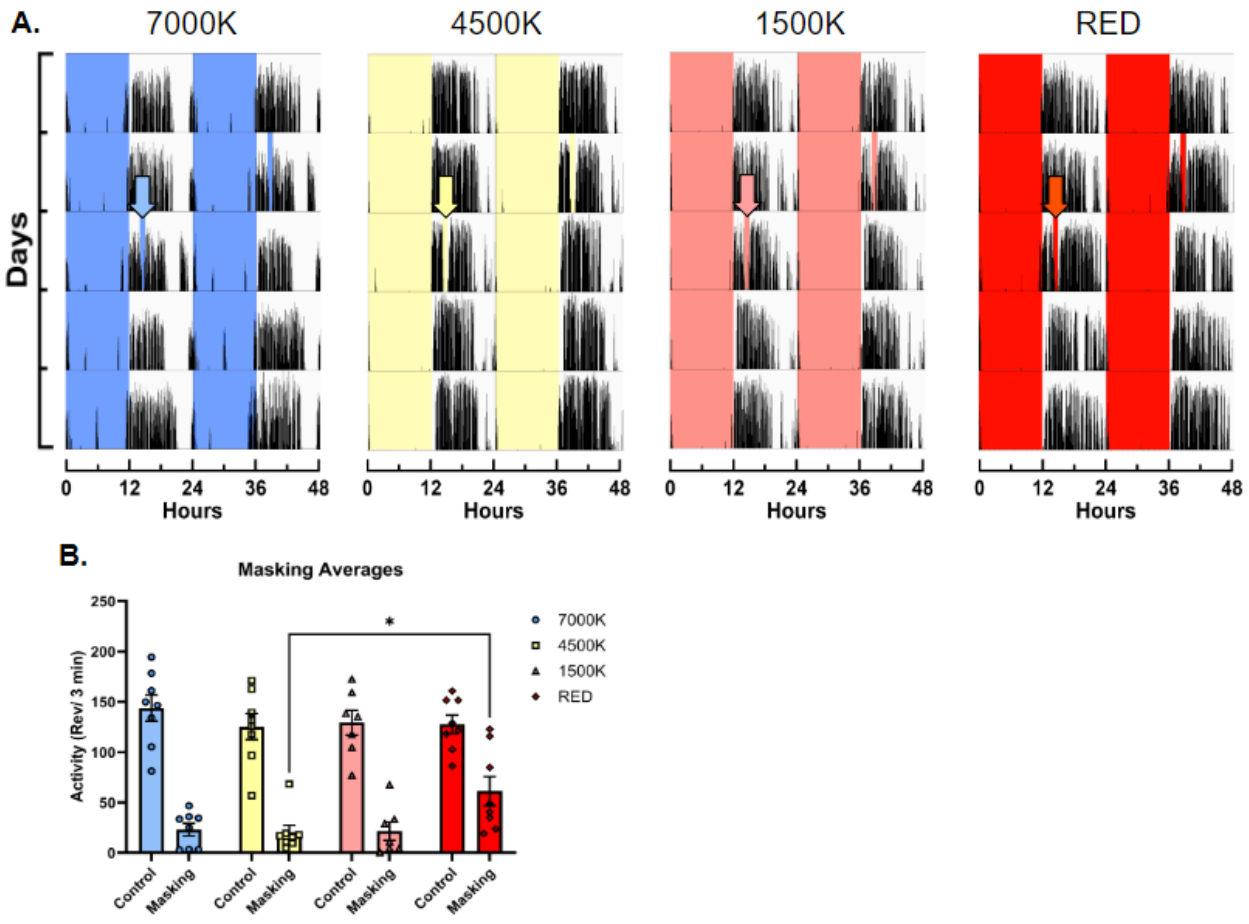


Fig. 7. *RED exposure was less effective in acutely suppressing locomotor activity.*
(A) Examples of actograms of daily rhythms in wheel running activity of WT mice after being subjected to an additional 1-hr of light from ZT 14-15 (beginning on the 3rd day from the top as noted by the colored arrows). **(B)** The average amount of activity suppression as measured by total activity from ZT 14-15 by lighting condition. The control values represent activity from the day before masking, and the masking values represent activity from the day of masking. The difference between the two is indicative of the amount of activity suppression. The masking behavior of each group was analyzed using a one-way ANOVA followed by the Tukey's multiple comparisons test (Table 4). The asterisk indicates significant differences ($P < 0.05$). Histogram shows the means ± SEM with the values from the individual animals overlaid.

The magnitude of the light-induced phase delay is decreased in WT mice when using RED lighting.

Lastly, the impact of the four different lighting conditions was measured on the light-induced phase shift of the circadian system. WT mice, held in constant darkness (DD; **Fig**. 3), were exposed to 15-mins of their respective original lighting condition (50 lx) at their predicted CT 16 times and free-running locomotor activity rhythms measured (**Fig. 8A**). The phase delays elicited by the RED lighting were negligible, while the other lights elicited phase delays upwards of 50-mins in magnitude (**Fig. 8B; Table 4**). The results in **Fig**. **7&8** indicate that while RED light is a less effective regulator of phase, the other wavelengths of light seem to be equally effective, at least at 50 lx.

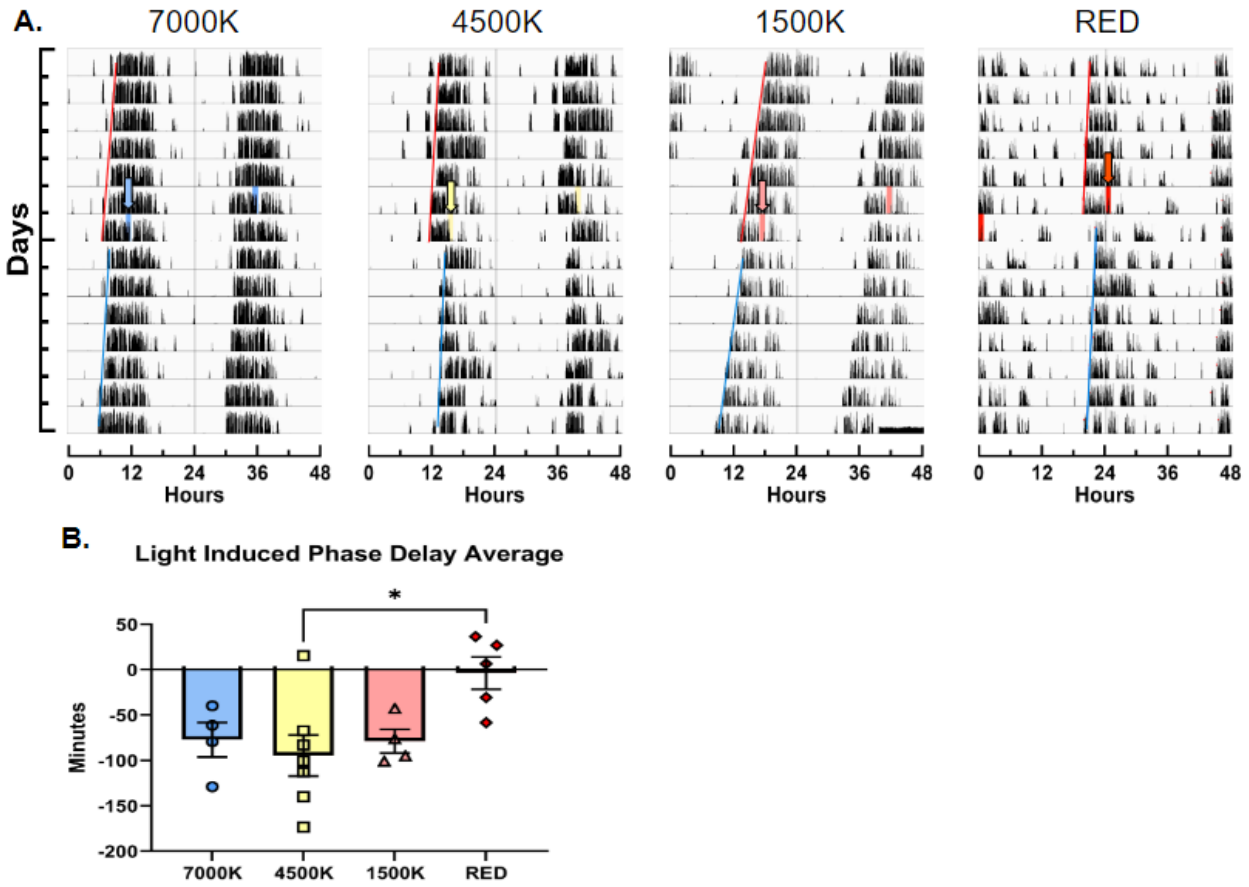


Fig. 8. *The magnitude of the light induced phase delay is decreased in mice exposed to RED lighting while the other wavelengths produced a similar response.*
(A) Examples of actograms of daily rhythms in wheel running activity of WT mice in constant darkness (DD) after being subjected to a 15-min light exposure at CT 16 (on the 7th day from the top as noted by the colored arrows). The red and blue lines on each actogram indicate the change in phase from before and after light exposure, respectively. **(B)** The average phase delay magnitude measured by phase difference in minutes as a result of light exposure at CT 16. The phase delays of each group were analyzed using a one-way ANOVA followed by the Tukey's multiple comparisons test (Table 4). The asterisk indicates significant differences ($P < 0.05$). Histogram shows the means ± SEM with the s from the individual animals overlaid.

	7000K	4500K	1500K	Red	Statistic
Total activity (rev/24 hrs)	29471 ± 986	28586 ± 1862	26979 ± 1888	27685 ± 1604	$F_{(3,28)}$=0.404; P=0.752
Power (% variation)	62.3 ± 4.1	60.5 ± 3.6	60.3 ± 3.5	56.7 ± 2.4	$F_{(3,28)}$=0.489; P=0.693
Activity in day (%)	10.1 ± 4.4	14.1 ± 2.6	6.1 ± 2.1	16.5 ± 4.1	$H_{(3)}$=6.206; P=0.102
Fragmentation (bouts #)	4.4 ± 0.5	4.7 ± 0.3	4.2 ± 0.4	4.7 ± 0.3	$F_{(3,28)}$=0.409; P=0.748
Onset variability (min)	14.2 ± 5.3	26.4 ± 6.3	22.1 ± 7.5	46.2 ± 15.2	$H_{(3)}$=3.736; P=0.291

Table 1: Under stable entrainment to light:dark 12:12 conditions, activity rhythms did not vary with lighting conditions. We compared the impact of 4 lighting conditions on activity rhythms of C57 mice in LD 12:12. An equal number of male and female mice were used. Values are shown as means ± SEM of n=8 animals /light condition for the activity. In cases in which the data showed a normal distribution (Shapiro-Wilk test), and equal variance (Brown-Forsythe), the effects of the different wavelength light was analyzed using a one-way ANOVA. In the cases in which these conditions were not meet, a Kruskal-Wallis One Way Analysis of Variance on Ranks was used.

	7000K	4500K	1500K	Red	Statistic
Advances	3.8 ± 0.3	5.0 ± 0.6	6.3 ± 0.7	6.6 ± 1.1	**$F_{(3,30)}$=2.998; P=0.048**
Delays	3.7 ± 0.2	4.3 ± 0.2	6.0 ± 0.7	8.6 ± 0.7	**$H_{(3)}$=20.044; P=0.001**

Table 2: Time to entrain to a 6-hr phase advance or phase delay varied significantly based on lighting condition. An equal number of male and female mice were used. Values are shown as means ± SEM. In cases in which the data showed a normal distribution (Shapiro-Wilk test), and equal variance (Brown-Forsythe), the effects of the different wavelength light was analyzed using a one-way ANOVA. In the cases in which these conditions were not meet, a Kruskal-Wallis One Way Analysis of Variance on Ranks was used. The time to entrain to the phase delay under RED lighting was significantly different than the other lighting groups; $P < 0.05$.

Parameter	Treatment	Time
waveform	$F_{(3, 695)}$=1.829, P = 0.141	**$F_{(23, 695)}$=152.007, P < 0.001**
Advances (days)	**$F_{(3, 269)}$=13.838, P < 0.001**	**$F_{(10, 46)}$=52.001, P < 0.001**
Delays (days)	**$F_{(3, 237)}$=57.203, P < 0.001**	**$F_{(1, 46)}$=81.543, P < 0.001**

Table 3: Analysis of activity waveforms and onset deviation (phase advance & phase delay) by two-way ANOVA with treatment (7000K, 4500K, 1500K, 1000K) and time (ZT 1-24; days) as factors. Degrees of freedom are reported within parentheses, alpha=0.05. Bold type indicates statistical significance.

	7000K	**4500K**	**1500K**	**Red**	**Statistic**
Masking (% of control)	19 ± 6 (n=8)	10 ± 2 (n=7)	20 ± 7 (n=6)	46 ± 9 (n=8)	$H_{(3)}$=10.144; P=0.017
Phase delay (min)	-75 ± 17 (n=4)	-94 ± 23 (n=7)	-79 ± 13 (n=4)	-4 ± 18 (n=5)	$F_{(3,19)}$=4.115; P=0.024

Table 4: Light evoked masking and light induced phase shifts. Kruskal-Wallis One Way Analysis of Variance on Ranks. Preliminary.

Discussion

Results Summary

While perhaps not entirely unexpected, there did not appear to be any appreciable difference in the daily activity rhythms of mice entrained to a 12:12 LD cycle with four lighting conditions as measured though wheel running activity. The 24-hr waveforms averaged over the course of two weeks did not vary among the 4 cohorts at 50 lx. Additionally, the circadian parameters of total activity, rhythm power, activity fragmentation, day activity and onset imprecision were also analyzed and did not significantly vary between the groups over the same two-week period. Therefore, under stable conditions, the spectral composition of the light does not seem to influence the entrainment of the circadian system. This empirical data is important as there is growing interest in ensuring appropriate lighting in animal facilities [33]. The concern has been raised that inappropriate lighting may compromise animal welfare and cause laboratory animals to be in more variable physiological and behavioral states. Furthermore, proper consideration of light during experimentation is important both when it is explicitly employed as an independent variable or a general feature of the environment. Commonly used animal husbandry guidelines suggest light intensity be at least 50 lx in the day. Our data support that general recommendation if the mice are under stable lighting conditions.

On the other hand, our data suggest that spectral properties of light are more important in determining the speed to re-entrain to rapid changes in the environment. In a pair of experiments that simulated the effects of jet lag or shift work, we compared the time

required to re-entrain to a 6-hr phase shift. The animals exposed to RED light ultimately adjusted to the new phase, it took them many more days. For example, in response to a 6-hr phase advance, the mice exposed to 7000K illumination re-adjusted within 4 days, while the RED-light cohort required 7-8 days. Likewise, the time to entrain to a 6-hr phase delay was within 4 days for the 7000K cohort but was roughly three times longer (11-12days) for the RED-light group. For frequent travelers or those regularly exposed to shift work, these differences would be impactful.

Somewhat surprisingly, the number of days to entrain to the phase delay were generally greater for the phase advance across all groups. Humans typically have a more difficult time entraining to advances; however, this is thought to be the result of humans having a circadian period longer than 24-hrs (~24.5-hrs) [35]. Given that mice have a period shorter than 24-hrs (~23.5-hrs) it would seem that they are better suited for entraining to phase advances. But this difference will need to be explored in future studies.

The RED light exposed group was also less responsive to other classic measures of circadian light response. Masking behavior in the RED cohort was significantly reduced compared to the 4500K cohort, which appears to be in agreement with the RED equivalent daylight illuminance (EDI) values obtained from the spectrophotometer **(Fig. 1)**. The spectral range of this light source was limited to longer wavelengths that only minimally stimulated the mouse photopigments compared to the other 3 light sources. Accordingly, in constant darkness, the RED cohort displayed significantly smaller phase delays after 15-min light exposure at CT 16 compared to the 4500K cohort, further hinting at the limited ability for the

RED light to stimulate the circadian system at 50 lx compared to the other lighting conditions.

Interpretation

These findings indicate that, at 50 lx, the spectral composition of light is of limited consequence to its ability to function as a zeitgeber for WT mice in stable phase conditions. At this recommended intensity, animal holding facilities and research laboratories do not need to worry about the light source for stable entrainment of the circadian system. On the other hand, in response to changes in phase, the spectral properties of light do matter. These differences should be the subject of future work.

Limitations

There are a number of limitations to this work. Perhaps the most obvious is the insufficient sample size to fully address the hypothesis. While the effects of RED light were reduced compared to the other groups, I was unable to differentiate the impact of the 7000K, 4500K and 1500K with my assays. While it is possible that no differences exist, it is hard to rule out the possibility that differences might emerge with an increased sample size. Additionally, the cohorts were not powered for sex difference analyses within or among the cohorts, which are most likely present with regards to the time required to entrain to phase shifts.

Another factor to consider is that the 7000K lights used in the present study, and indeed most LEDs, do not emit any detectable amount of light in the violet-ultraviolet range, leaving the UVS cones of the mice essentially unstimulated. Violet shifted lights would more properly reflect the type of light stimulation that mice receive from daylight [20] and may ultimately

be more effective at entraining the circadian system, making for an interesting follow-up experiment.

Finally, it is worth noting that the light response of nocturnal mice is very different from diurnal humans. The human retina contains a vastly different complement of photopigments compared to mice. On its own, the presence of long wavelength sensitive cones in humans makes us dramatically more sensitive to red light [20]. Additionally, as a diurnal species, the ratio of cones in the photoreceptive layer of the retina is much greater than what is observed in mice, improving our visual acuity in well-lit conditions (photopic vision) and potentially make our circadian system more sensitive to varying wavelengths of light. These factors make mice, and nocturnal rodents in general, less ideal for determining the effects of light wavelength during the day on the circadian system as their vision is rod dominated and they are typically asleep at times when the lights are on.

Future Directions

Going forward, it will be critical to increase the sample size to fully address our hypothesis. It's likely that in dimmer conditions the spectral composition of light will play a larger role in effectively stimulating the circadian system. This may be shown by conducting a similar set of experiments but with a light intensity of 10 lx. Preliminary data suggest that at this intensity, RED light is completely unable to synchronize the circadian system while the other wavelengths are still able to entrain the animals' rhythms in locomotor activity. Furthermore, it would be helpful to probe and associate any behavioral differences with cFos expression in the SCN. There is a strong relation between light evoked cFos expression in the SCN and

circadian entrainment. Finally, all of these experiments were carried out in WT mice. It would be interesting to expand the experiment to include a mouse model for Autism Spectrum Disorder (ASD), for instance the *Fmr1* (*fragile X messenger ribonucleoprotein 1*) *KO* mice, to examine the effects of blue enriched vs red enriched light during the day on ASD aberrant behaviors. More stimulative lighting during the day with minimal disruption during the night may serve to improve circadian deficits as well as symptoms of repetitive behavior and reduced social interaction in *Fmr1 KO* mice [36].

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