Dim light in the evening causes coordinated realignment of circadian rhythms, sleep, and short-term memory

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Light provides the primary signal for entraining circadian rhythms to the day/night cycle. In addition to rods and cones, the retina contains a small population of photosensitive retinal ganglion cells (pRGCs) expressing the photopigment melanopsin (OPN4). Concerns have been raised that exposure to dim artificial lighting in the evening (DLE) may perturb circadian rhythms and sleep patterns, and OPN4 is presumed to mediate these effects. Here, we examine the effects of 4-h, 20-lux DLE on circadian physiology and behavior in mice and the role of OPN4 in these responses. We show that 2 wk of DLE induces a phase delay of ~2 to 3 h in mice, comparable to that reported in humans. DLE-induced phase shifts are unaffected in Opn4−/− mice, indicating that rods and cones are capable of driving these responses in the absence of melanopsin. DLE delays molecular clock rhythms in the heart, liver, adrenal gland, and dorsal hippocampus. It also reverses short-term recognition memory performance, which is associated with changes in preceding sleep history. In addition, DLE modifies patterns of hypothalamic and cortical cFos signals, a molecular correlate of recent neuronal activity. Together, our data show that DLE causes coordinated realignment of circadian rhythms, sleep patterns, and short-term memory process in mice. These effects are particularly relevant as DLE conditions—due to artificial light exposure—are experienced by the majority of the populace on a daily basis.

dim light in the evening | long-day photoperiod | melanopsin | circadian rhythms | short-term memory

Light exerts profound effects on physiology and behavior, synchronizing biological rhythms to the light/dark cycle (LD) as well as directly modulating alertness and sleep (1, 2). In mammals, light detected by the eye is the primary time cue synchronizing circadian rhythms of activity and rest, a process termed entrainment. Exposure to light at dawn and dusk plays a key role, adjusting the phase of the master circadian clock in the hypothalamic suprachiasmatic nuclei (SCN) (3–5). Studies on the photoreceptors mediating circadian entrainment suggested a subset of photosensitive retinal ganglion cells (pRGCs) expressing the photopigment melanopsin (OPN4) (6, 7). These cells have a peak sensitivity at ~480 nm (8, 9), hence differing from the classical visual system, which in humans is most sensitive to light at ~555 nm, corresponding to the red and green cones of the fovea (10). In addition to modulating image-forming responses via local retinal circuitry, OPN4-expressing pRGC axons project to the SCN and different brain areas, setting the circadian clock and driving non-visual responses to light (5, 7, 11).

How does the mammalian brain adapt to changes in daylength? In humans, exposure to long-day photoperiods delays melatonin onset but advances melatonin offset, hence compressing the internal biological night, relative to short-day photoperiods; this is observed in laboratory studies (12, 13) as well as under naturalistic conditions (14, 15). In laboratory mice, the onset and offset of wheel-running activity change dynamically in response to daylength (16). Long-day photoperiods also cause functional reorganization in the SCN. In vivo multiunit recording in mice shows that 16-h light/8-h dark cycles (16:5 LD) weakens phase clustering of SCN neurons (17). Similarly, PERIOD2::LUCIFERASE bioluminescence signals in the mouse dorsal versus ventral SCN are dissociated after 20:4 LD (18). Weakened intercellular coupling in the SCN reflects a form of plasticity, enhancing adaptability of the circadian system to an increase in daylength (19). In addition, 19:5 LD reduces the number of dopamine neurons in the hypothalamus, increasing behavioral immobility and decreasing exploratory activity in rats (20) and mice (21); seasonal variation in photoperiod is also associated with changes in dopamine levels in the human midbrain (22). In the mouse hippocampus, molecular rhythms such as Per1 and Cry1 are blunted under 20:4 LD (23); however, the consequence is complex: it improves object and spatial discrimination in the spontaneous recognition memory task but disrupts context discrimination in the fear conditioning task (23).

Significance

In modern societies, people are regularly exposed to artificial light (e.g., light-emitting electronic devices). Dim light in the evening (DLE) imposes an artificial extension of the solar day, increasing our alertness before bedtime, delaying melatonin timing and sleep onset, and increasing sleepiness in the next morning. Using laboratory mice as a model organism, we show that 2 wk of 4-h, 20-lux DLE postpones rest–activity rhythms, delays molecular rhythms in the brain and body, and reverses the diurnal pattern of short-term memory performance. These results highlight the biological impact of DLE and emphasize the need to optimize our evening light exposure if we are to avoid shifting our biological clocks.

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Aberrant lighting at night may lead to disrupted circadian rhythms and sleep, which are associated with many adverse health outcomes, including impaired concentration and performance, mood disturbances, metabolic diseases, cardiovascular and neurological disorders, and cancer (24–26). Numerous studies have characterized the disruptive effects of dim light at night (DLAN) on metabolic and mood-related processes in rodents. In these studies, animals were exposed to dim light for the entire night (27–35). As such, DLAN is highly relevant to conditions in which low-level light exposure continues throughout the night, such as light pollution. However, DLAN is somewhat different from exposure to artificial electrical lighting as experienced by the majority of the populace, who typically experience higher light levels during the day (though lower than natural daylight) but dim light for a short period in the evening (DLE) (14, 15, 36, 37). In humans, DLE exposure delays melatonin rhythms and sleep timing (14, 15, 37) and reduces alertness on the subsequent day (36); these phase-delaying effects of DLE on the circadian system are found under both natural summer (14) and winter photoperiods (15). As such, DLE combines features of both long-day photoperiods and DLAN. While similar to a long-day photoperiod, the extended light phase is of a lower light intensity and may exert different effects in comparison with the higher light levels during the day. Conversely, unlike DLAN, under DLE the evening light exposure only occurs during the subjective night when the circadian system is most sensitive to light-induced phase delays (38). Although the effects of long-day photoperiods (12–23) and DLAN (27–35) on circadian physiology and behavior have been extensively studied, the effects of DLE—as produced by artificial light exposure—have received less attention. Here, we investigate the effects of 2 wk of DLE in laboratory mice and the role of OPN4 in mediating these responses. Our choice of dim-light level and duration was based upon human studies conducted in nonlaboratory settings (14, 15, 36, 37), which reported that ~3 to 4 h of ~20 to 30 lx artificial lighting exposure increased alertness before bedtime, delayed melatonin timing and sleep onset, and increased sleepiness in the morning. Despite their nocturnality, the mouse phase response curve (PRC) is broadly similar to the human PRC: in both species, light presented during the early night delays circadian rhythms, whereas light presented later at night or early in the morning causes phase advances (5, 38, 39). Our DLE protocol comprised a 12-h light period at 20 lx, during the evening light period at 20 lx, and an 8-h dark phase. Here, we characterize the effects of 4-h, 20-lux DLE on a) locomotor activity rhythms, b) sleep patterns, c) molecular clocks in peripheral tissues, d) short-term memory process, and e) brain cFos signals.

Results

Locomotor Activity Rhythms Are Phase Delayed under DLE. To examine the effects of 20-lux DLE during Zeitgeber times (ZT)12 to 16 on circadian rhythms, locomotor activity phases (onset, midpoint, and offset) were determined from passive infrared sensor (PIR) recording across each 24-h cycle. Among the three phase markers, activity offset is known to show the highest variability (40); this is in agreement with our data under LD. Under DLE, C57BL/6 wild-type (WT) mice exhibited changes in alignment of all three parameters with respect to the light phase (Fig. L). More specifically, activity offsets were delayed by 3.59 ± 0.46 h [main effects of Lighting F(1, 10) = 64.31, P < 0.001], whereas activity midpoints and offsets were delayed by 2.57 ± 0.37 and 2.52 ± 0.83 h [F(1, 10) = 43.94, P < 0.001 and F(1, 10) = 10.64, P = 0.009, respectively]. Changes in activity midpoints and offsets, which occurred hours after the DLE, suggest that these are not the acute effect of activity suppression by DLE. Furthermore, under constant darkness (DD), activity rhythms in mice with prior exposure to DLE free ran at later clock times than mice with prior exposure to LD (n = 8, Albumin-Cre;DbhKO mice per group as described in Molecular Rhythms Are Delayed under DLE), with an overall phase difference of ~4 h [main effects of Prior Lighting: F(1, 14) = 6.77, P = 0.021; F(1, 14) = 6.30, P = 0.025; and F(1, 14) = 6.20, P = 0.026, for onsets, midpoints, and offsets, respectively]. The persistence of DLE-induced phase delay under DD confirmed that these effects were not the result of the suppression of locomotor activity by light.

As the oestrous cycle in female mice (which lasts for 4 to 5 d) has been shown to affect wheel-running activity rhythms (41), we assessed the possibility that the effects of DLE on PIR activity rhythms could vary between sexes. However, there was no Lighting × Sex interaction, indicating that DLE exerted comparable effects in both sexes [Lighting × Sex interactions F(1, 10) = 1.66, P = 0.23; F(1, 10) = 0.06, P = 0.81; and F(1, 10) = 2.68, P = 0.13, for onsets, midpoints, and offsets, respectively].

DLE-Induced Phase Delays Are Unaffected by OPN4 Deficiency. Similar DLE-induced phase delays were observed in OPN4-deficient (Opn4−/−) mice of both sexes. Under DLE, activity onsets, midpoints, and offsets of Opn4−/− mice were delayed by 2.74 ± 0.18, 2.32 ± 0.95, and 1.61 ± 0.61 h [main effects of Lighting F(1, 10) = 215.25, P < 0.001; F(1, 10) = 87.14, P < 0.001; and F(1, 10) = 7.53, P = 0.021, respectively; Fig. 1B]. Although the size of phase shifts appeared smaller in Opn4−/− mice, comparisons between WT controls and Opn4−/− mice indicated that the effects we statistically indistinguishable between genotypes (main effects of Genotype = 0.08; Lighting × Genotype interactions Ps > 0.09). Thus, like other nonvisual responses to light (42–44), the conventional retinal circuitry can support DLE-induced phase delays in the absence of OPN4. Under DLE, the power of ~24 h rhythms persisted in both WT and Opn4−/− mice; however, there was an increase in oscillatory power at ~8 h in both groups as revealed by continuous wavelet transform (45) (SI Appendix, Fig. S1). This is likely to be an artifact of the shortened, 8-h night under DLE rather than reflecting an increase in ultradian rhythms per se.

Sleep Patterns Are Realigned under DLE. To examine changes in sleep patterns, for each 10-s bin of the PIR recording, the mouse’s behavioral state was assigned as either awake (0) or asleep (1), where “1” was defined as sustained PIR inactivity for at least four uninterrupted 10-s bins; if this was not the case, the value of “0” was assigned (Fig. 2A). Sleep proportion was calculated as the number of bins assigned with a value of “1” divided by the total number of 10-s bins during that time window; the duration of a sleep bout was defined as the number of uninterrupted 10-s bins assigned with a value of “1.” It has been validated that ~240 s immobility is highly correlated with electroencephalogram (EEG)-defined sleep times across the 24-h day, with Pearson’s r = 0.94 (46–48).

Total immobility-defined sleep times were unaffected under DLE (SI Appendix, Table S1). However, there were changes in the alignment of sleep patterns under DLE, both in terms of sleep proportion [Lighting × Time of Day interaction in WT: Greenhouse–Geisser corrected F(2,29) = 26.03, P < 0.001; Lighting × Time of Day interaction in Opn4−/−: F(2,33) = 24.17, P < 0.001; Fig. 2B] as well as duration of sleep bouts [Lighting × Time of Day interaction in WT: F(3,14) = 6.40; P = 0.017; Lighting × Time of Day interaction in Opn4−/−: F(3,19) = 14.10, P < 0.001; Fig. 2C]. Crucially, under DLE, sleep proportion was reduced and sleep bouts were shortened in the first 4 h of the light phase (simple effects of Lighting from ZT10 to 4 P < 0.005). Thus, DLE promoted sleep from ZT12 to 16 and postioned the accumulation of sleep pressure in the next morning. Comparisons between WT and Opn4−/− mice did not reveal any genotype difference (main effects of Genotype Ps > 0.08; Lighting × Genotype interactions Ps > 0.1; Lighting × Time of Day × Genotype interactions Ps > 0.2).

As sleep and inactivity are often accompanied by body cooling (49), we examined if there was any change in skin temperature (Tskin) using infrared thermography (SI Appendix, Fig. S2A) as described in our previous study (50). Tskin provides an estimate of
changes in core body temperature over time (50). The phase of $T_{\text{skin}}$ offset was delayed under DLE (SI Appendix, Fig. S2B), corresponding to the delayed PIR activity rhythm and sleep profile. In addition, there was a 2% drop in $T_{\text{skin}}$ (~0.65 °C) during the 4-h DLE period as well as a 0.5 to 1% reduction in $T_{\text{skin}}$ (~0.15 to 0.35 °C) in the light phase (SI Appendix, Fig. S2C).

**DLE Effects Are Weaker Than Effects of a Long Day.** When the intensity of the 4-h evening light is the same as the prevailing light phase, this would become a long-day photoperiod. Dim light in the evening causes coordinated realignment of circadian rhythms, sleep, and lipid/glucose metabolism (Hmger, Hdac3, Ccm4l, Pparγ, Npc1, and Cyp7a1) was also examined. Notably, DLE delayed the circadian phase of the cholesterol synthesis gene Hmger: it peaked at ZT8 under LD (SI Appendix, Fig. S5A) as reported in a previous study (56); however, this was delayed to ZT14 under DLE [Lighting × Time of Day interaction $F(3, 21) = 5.037, P = 0.008$]. The mean $\Delta \phi$ value of all six hepatic genes under investigation

**Molecular Rhythms Are Delayed under DLE.** Misalignment of circadian rhythms is accompanied by changes in clock gene expression throughout the body (51). To investigate this possibility under DLE, we examined clock gene expression (Per2, Bmal1, Rev-erba, Cyp7a1, and Dbp) in the heart, liver, adrenal gland, and dorsal hippocampus at ZT2, ZT8, ZT14, and ZT20. Expression levels of target genes were normalized to the geometric mean of two housekeeping genes, Tbp and Gapdh (52); validation of these housekeeping genes is reported in SI Appendix, Supplementary Methods. Under LD, the phasing of clock gene expression observed was broadly consistent with patterns from previous mouse studies: Per2 peaked at early night, Bmal1 peaked near dawn, Rev-erba peaked during the day, and Dbp peaked before the start of the night (51). Some of these molecular rhythms were delayed under DLE (Fig. 3). Peaks of normalized Bmal1 and Cyp7a1 messenger RNA (mRNA) levels in the adrenal gland were delayed from the dark phase to ZT2 [Lighting × Time of Day interactions $F(3, 21) = 3.37, P = 0.038$ and $F(3, 21) = 3.69, P = 0.028$, respectively]. In the dorsal hippocampus, the peak of normalized Per2 expression was shifted from ZT14 to ZT8 [Lighting × Time of Day interaction $F(3, 21) = 3.17, P = 0.046$]. In addition, centers of gravity (CoG)—providing an estimate of the acrophase ($\phi$) of the molecular rhythm (53–55)—were consistently delayed under DLE; phase shifts ($\Delta \phi$) were expressed as the difference in CoG between DLE and LD (SI Appendix, Fig. S4). When pooled across peripheral tissues, one-sample Student’s $t$ tests (two-tailed) showed that mean $\Delta \phi$ values of Rev-erba ($2.07 \pm 0.57 h$) and Dbp ($1.81 \pm 0.51 h$) were different from the value of 0 [one-sample $t(3) = 3.67, P = 0.035$ and $t(3) = 3.55, P = 0.038$, respectively].

The expression of six hepatic genes involved in adipogenesis and lipid/glucose metabolism (Hmger, Hdac3, Ccm4l, Pparγ, Npc1, and Cyp7a1) was also examined. Notably, DLE delayed the circadian phase of the cholesterol synthesis gene Hmger: it peaked at ZT8 under LD (SI Appendix, Fig. S5A) as reported in a previous study (56); however, this was delayed to ZT14 under DLE [Lighting × Time of Day interaction $F(3, 22) = 5.037, P = 0.008$]. The mean $\Delta \phi$ value of all six hepatic genes under investigation
was 2.35 ± 0.95 h [one-sample t(5) = 2.49, P = 0.055 (two-tailed); SI Appendix, Fig. S5B].

To provide an additional functional measure of hepatic circadian output, we recorded bioluminescence signals from freely moving Albumin-Cre:DbpKo+/- liver reporter mice, which expressed firefly luciferase in hepatocytes under the control of Dbp (57). Animals were kept either under LD or DLE for 1 wk; synthetic luciferin (CycLuc) was then administered in drinking water, allowing hepatic bioluminescence emission to be detected and recorded in DD (Fig. 3B). Under DD, the strength of Dbp bioluminescence rhythm (as indicated by signal-to-noise ratios) and period length were indistinguishable between groups [main effects of Prior Lighting F(1, 14) = 1.84, P = 0.20 and F(1, 14) = 2.11, P = 0.17, respectively]. However, the acrophase of Dbp bioluminescence rhythm was delayed by an average of 2.34 h in mice with prior DLE exposure [main effect of Prior Lighting F(1, 14) = 37.13, P < 0.001; Fig. 3B]. Despite the shift in the hepatic circadian clock and metabolic rhythm, 2 wk of DLE did not affect body weight in C57BL/6 mice (SI Appendix, Fig. S5 C and D).

Short-Term Memory Process Is Altered under DLE in a Sleep-Dependent Manner. Given the DLE-induced delay in circadian physiology of ~2 h, we examined if there was any consequence for short-term memory processes at 2 h into the light phase (ZT2) and 2 h after the light phase (ZT14). We used the spontaneous recognition memory task (Fig. 4A) (58), which is sensitive to effects of aberrant lighting (23, 59–61). Initial analyses confirmed that under LD, there was a day/night difference in short-term object and odor recognition memory performance, with better performance at ZT2 than at ZT14 [main effect of Time of Day F(1, 15) = 6.59, P = 0.021; SI Appendix, Fig. S6A]; this is consistent with our previous findings (60). Repeated recognition assessment 2 wk later did not alter the size or direction of this effect [main effect of Repeated Testing F(1, 15) = 0.038, P = 0.85; Time of Day × Repeated Testing interaction F(1, 15) = 0.60, P = 0.45], demonstrating the stability of this behavioral rhythm under LD.

To examine the effect of DLE, another group of mice first received recognition trials at ZT2 and ZT14 under LD and at the same time points following 2 wk of DLE exposure (Fig. 4A). In these mice, the diurnal pattern of recognition memory performance was reversed under DLE [Lighting × Time of Day interaction F(1, 10) = 9.88, P = 0.01; Lighting × Time of Day × Type of Stimulus interaction F(1, 10) = 0.13, P = 0.73; Fig. 4B]. Under LD, performance was better at ZT2 than at ZT14 [simple effect of Time of Day F(1, 10) = 6.21, P = 0.032]. By contrast, under DLE performance was better at ZT14 than at ZT2 [simple effect of Time of Day F(1, 10) = 4.96, P = 0.05]. This resulted in enhanced performance at ZT14 under DLE [simple effect of Lighting F(1, 10) = 8.67, P = 0.015; Fig. 4B] but not at ZT2 [F(1, 10) = 2.45, P = 0.15]. The improved test performance at ZT14 was due to reduced familiar object exploration [SI Appendix, Fig. S6 B, Right]. In the sample phase, there was a suggestion of elevated exploratory activity at ZT2 but reduced exploration at ZT14 under DLE (SI Appendix, Fig. S6 B, Left). However, these effects did not reach significance [Lighting × Time of Day interaction F(1, 10) = 4.81, P = 0.053; all simple effects Ps ≥ 0.057] and did not relate to the pattern of exploratory activity in the test phase (SI Appendix, Fig. S6B).

We then explored if the reversal of memory performance was related to changes in sleep history under DLE. To address this question, we determined the amount of sleep from time windows of varying widths (from 10 min up to 3 h in 10-min increments) preceding each recognition trial. Adopting a wider time window increased the goodness of the linear fit, R2, between prior sleep and performance, and R2 reached an asymptotic value of ~0.9 at t = ~2 h (Fig. 4 C, Left, data point in magenta). Notably, there was a strong linear relationship between preceding 2-h sleep and performance at the group level (Fig. 4 C, Right). When all individual
Fig. 3. Effects of DLE on molecular clock rhythms. (A) Normalized fold changes in \textit{Per2}, \textit{Bmal1}, \textit{Rev-erbα}, \textit{Cry1}, and \textit{Dbp} mRNA levels in the heart, liver, adrenal gland, and dorsal hippocampus (dHPC) at ZT2, ZT8, ZT14, and ZT20 ($n = 4$ WT mice per condition in most cases, except: $n = 3$ in the heart under LD at ZT2 (\textit{Per2}), liver under LD at ZT2 (\textit{Dbp}), and adrenal gland under LD at ZT8 (all target genes); $n = 2$ in the liver under LD at ZT20 (\textit{Dbp}) and all tissues under DLE at ZT20 (all target genes)). Data are normalized to peak expression values in each lighting condition. Under DLE, \textit{Bmal1} and \textit{Cry1} peak expression in the adrenal gland, as well as \textit{Per2} peak expression in the dHPC, were delayed from the dark phase to the light phase (*Lighting × Time of Day interactions $P_s < 0.05$; †main effects of Lighting $P_s < 0.01$). (B) The mean acrophase of hepatic \textit{Dbp} bioluminescence rhythm in vivo recorded in DD from \textit{Albumin-Cre};\textit{Dbp}^{K\times}\textit{ liver reporter mice expressing firefly luciferase in hepatocytes under the control of \textit{Dbp} (n = 8 mice per Prior Lighting condition). The green trace under the illustration of the mouse liver shows 5 d of in vivo bioluminesence recording under DD from one animal. SE bars are plotted but are within the data symbols. Hepatic \textit{Dbp} rhythm was phase delayed in mice with prior DLE exposure (main effect of Prior Lighting $P < 0.01$). Illustrations of the mouse and mouse liver are created by BioRender.
cases were considered (11 mice × 8 trials per mouse), linear mixed-effects models (62) confirmed the benefits of preceding 2-h sleep on subsequent performance (likelihood ratio $\chi^2 = 7.73, P = 0.005$). The bootstrap 95% CI of the effect of sleep was [0.12, +0.62], which did not overlap with zero. By contrast, sleep history did not bear any relationship to sample exploratory activity (likelihood ratio $\chi^2 = 0.33, P = 0.57$); 95% CIs of these effects, [-58.73, +15.02] and [-0.002, +0.001], respectively, overlapped with zero. Together, our data suggest that prolonged wakefulness at night is associated with a decline, whereas DLE-induced sleep is associated with a facilitation in short-term memory process.

Fig. 4. Effects of DLE on short-term memory process (n = 11 WT mice). (A) Schematic of the spontaneous recognition memory task (object trials) and summary of the order of recognition assessment in subgroup 1 (S1; 6 mice) and subgroup 2 (S2; 5 mice). The light level in the arena was 50 lx on object recognition trials and 0 lx on odor recognition trials. Illustration of the mouse in A is created by BioRender. (B) Recognition ratios from test phases of object and odor trials at ZT2 and ZT14. Higher ratios indicate stronger novelty preference, whereas ratios of 0.5 indicate no discrimination between familiar and novel stimuli. Under DLE, object and odor recognition memory performance were reversed, resulting in enhanced performance at ZT14 under DLE (*simple effect of Lighting $P < 0.025$). (C) Sleep proportion was determined from time windows (t) of varying widths in 10-min increments (t = 10 min, 20 min, 30 min, . . ., 3 h) preceding each recognition trial. (C, Left) Adopting a wider window increased the goodness of the linear fit, $R^2$, between prior sleep and memory performance, and $R^2$ reached an asymptotic value of ~0.9 at t ~2 h (data point in magenta). The dashed line represents the quadratic trend between t and $R^2$. (C, Right) The linearity between preceding 2-h sleep and memory performance at the group level. Short and long dashed lines represent linear regression lines for object and odor trials, respectively.

Dim light in the evening causes coordinated realignment of circadian rhythms, sleep, and short-term memory.
Brain cFos Signals Are Modified under DLE. To assess changes in brain states under DLE, we quantified brain immunofluorescence cFos levels in naïve mice at ZT2 and ZT14. This provides a molecular correlate of neuronal activity within the ~1 to 2 h time window prior to learning in the recognition memory task. We found that the SCN and medial prefrontal cortex (mPFC) were differentially modified under DLE as indicated by the Lighting × Time of Day × Region interaction \[F(1, 8) = 60.23, \ P < 0.001\]. Under LD, there were more cFos+ cells in the SCN at ZT2 than at ZT14 \[simple effect of Time of Day \(F(1, 8) = 39.23, \ P < 0.001\]\, indicating that SCN and mPFC were out of phase under LD. Importantly, the diurnal pattern in the SCN under LD was attenuated but remained significant under DLE \[simple effect of Time of Day \(F(1, 8) = 7.92, \ P = 0.023\]\ due to increases in cFos+ cell count at ZT2 and ZT14 \[simple effects of Lighting \(F(1, 8) = 9.33, \ P = 0.016\) and \(F(1, 8) = 42.30, \ P < 0.001\), respectively; Fig. 5A\]. Moreover, patterns of cFos signals in the SCN dorsal and ventral subregions were dissociated under DLE (SI Appendix, Fig. S7), which may be suggestive of reorganization in the SCN (17–19). By contrast, the nocturnal pattern in the mPFC under LD was reversed by DLE \[simple effect of Time of Day \(F(1, 8) = 75.76, \ P < 0.001\]\: there was an increase in the number of cFos+ cells at ZT2 but reduced signals at ZT14 \[simple effects of Lighting \(F(1, 8) = 65.32, \ P < 0.001\) and \(F(1, 8) = 18.74, \ P = 0.003\), respectively; Fig. 5B\]. The reversal of cFos signals was found in both superficial (layers one to four) and deep layers (layers five/six) of the mPFC (SI Appendix, Fig. S8).

The preoptic hypothalamus (POA) is known to regulate body cooling and promote sleep (63–67), whereas the lateral and
dorsomedial hypothalamus (LHA and DMH) is known to promote wakefulness (68–70). Indeed, cFos signals in these hypothalamic regions were differentially modified by DLE as indicated by the Lighting × Time of Day × Region interaction [F(2, 16) = 12.47, P = 0.001]. In the POA, there was an increase in the number of cFos+ cells at ZT14 (SI Appendix, Fig. S9). By contrast, in the LHA, there was a decrease in cFos+ cell count at ZT14 (SI Appendix, Fig. S10 A and B). In addition, cFos+ cell counts in the LHA and DMH were positively related to each other and to the cortex but not to the POA (SI Appendix, Fig. S10C). Thus, cFos signals in the LHA/DMH versus POA were modified in a way that corresponds to their established roles in regulating wakefulness and sleep, respectively.

**Numbers of Hypothalamic and Midbrain Dopamine Cells Are Unaffected by DLE.** Exposure to long-day photoperiods for 1 to 2 wk is known to decrease the number of dopamine tyrosine-hydroxylase–expressing (TH+) cells in the paraventricular nuclei of the hypothalamus (PVN), increasing behavioral immobility and reducing exploratory activity in rats and mice (20, 21). Similarly, seasonal variation in photoperiod is associated with changes in TH levels in the human midbrain (22). To assess this possibility under DLE, we examined immunohistochemical TH levels in the PVN as well as in the ventral tegmental area (VTA) and substantia nigra pars compacta (SNc), which provide major dopaminergic projections to different parts of the brain. DLE did not reduce the number of TH+ cells in the PVN [main effect of Lighting F(1, 8) = 0.51, P = 0.50; Lighting × Time of Day interaction F(1, 8) = 1.08, P = 0.33; SI Appendix, Fig. S11, Upper] or in the VTA/SNc [main effect of Lighting F(1, 8) = 0.82, P = 0.39; Lighting × Time of Day interaction F(1, 8) = 0.24, P = 0.64; SI Appendix, Fig. S11, Lower].

**Discussion.**

A total of 2 wk of 4-h, 20-lux DLE exerts widespread effects on circadian physiology and behavior in C57BL/6 mice: it 1) delays locomotor activity onset(s), midpoints, and offsets (Fig. 1); 2) promotes sleep and body cooling, postponing the accumulation of sleep pressure in the next morning (Fig. 2 and SI Appendix, Fig. S2); 3) delays molecular clock rhythms in peripheral tissues (Fig. 3); 4) reverses the diurnal pattern in short-term object and odor recognition memory performance, which is associated with changes in sleep history (Fig. 4); and 5) modifies patterns of hypothalamic and cortical cFos signals (Fig. 5). DLE-induced phase delays are unaffected in Opn4−/− mice, indicating that rods and cones are capable of driving these responses in the absence of melatonin as has been described for other circadian responses (42–44). However, these data do not preclude a contribution of melatonin to these responses under normal conditions. It could be argued that DLE-induced phase delays are simply the result of negative masking (i.e., suppression of locomotor activity by dim light) (71). However, this cannot explain phase delays in locomotor activity offset and Tshan offset, both of which should have been masked by the start of the light phase. Moreover, the persistence of DLE-induced phase changes in locomotor activity and hepatic Dhp bioluminescence rhythm under DD indicates that these effects are not the result of negative masking per se.

There are similarities between 20-lux DLE and a long-day photoperiod. Under both conditions, locomotor activity onsets and midpoints are phase shifted, and object recognition memory performance is improved relative to 12:12 LD (23). However, there are also key differences. We do not see a compression of locomotor activity into the dark phase, which is observed under long-day photoperiods (16). And unlike long days (20, 21), 20-lux DLE does not reduce the number of dopamine TH+ cells in the hypothalamus or midbrain. Moreover, in contrast to DLAN (27–30, 32–35), 4-h DLE does not affect body weight despite delaying hepatic circadian rhythms and clock outputs. Thus, the effects of DLE in mice appear to be milder than either long photoperiods or DLAN. A comparison of these paradigms is summarized in SI Appendix, Table S2. However, it should be noted that the effects of long photoperiods on TH reduction in previous studies have used more extreme 19:5 LD conditions (20, 21); as such, longer duration DLE exposure (7 h) may exert comparable effects. It also remains to be determined if longer periods of exposure to DLE (>2 wk) will have more severe consequences for brain dopaminergic signaling or metabolic processes.

Under DLE, circadian rhythms, sleep patterns, and short-term memory processes are realigned, adopting a delayed phase relationship with the extended day. Such coordinated realignment of circadian physiology and behavior is as an adaptive response to environmental changes crucial for survival (19, 72, 73). In fact, the dissociation of SCN subregional cFos signals under DLE (SI Appendix, Fig. S7) is suggestive of some plasticity because weakened dorsal-ventral SCN coupling can maximize adaptability of the SCN to the extended day (17–19). This effect needs to be confirmed in future studies using more direct readings of neuronal activity (17). Under more severe aberrant lighting protocols, such as T7 (3.5-h day/3.5-h night) (59, 61), T20 (10-h day/10-h night) (74, 75), and jet lag (76, 77), sleep and circadian processes fail to adopt a stable phase relationship with the environment. Maladaptation of the circadian system perturbs cardiovascular, metabolic, and memory functions (27–30, 32–35, 51, 59, 61, 74, 76, 77). By contrast, DLE has milder effects: it delays the hepatic circadian rhythms and clock outputs without affecting body weight, and it reverses the day/night pattern of short-term memory performance without causing any behavioral impairment.

While the 20-lux DLE used in this study was based upon human artificial light exposure (14, 15, 36, 37), this is likely to be relatively brighter for mice. For example, the half-maximal effective light intensity (EC50) for human phase-shifting responses to 6.5-h light varies between 12.54 (460 nm) to 13.07 (555 nm) log quanta (78). However, in mice phase-shifting responses to a 15-min light stimulus have an EC50 of 11.41 log quanta (6); this is more than a log unit lower despite the 3-log-unit-shorter stimulus duration. Certainly, due to differences in the geometry of the human versus rodent eye, the 20-lux DLE will result in greater effective retinal irradiance in mice in comparison with humans. As a result, the light level used in this study may be relatively brighter for mice than for humans.

Despite having a higher sensitivity to light, circadian responses of mice are not qualitatively different from those in humans. In both species, light presented during the early biological night delays circadian rhythms, whereas light presented later at night causes phase advance (5, 38, 39). This is evident from our results: the 4-h, 20-lux DLE induces a comparable phase delay of ~2 to 3 h in our mice (Fig. 1) as has been observed in humans (14, 15, 36). The resemblance seems to be at odds with our recent review of the literature, which suggests that humans require brighter (>100 lx) and longer (>30 min) light for phototentrainment, whereas mice can entrain to dimmer (<1 lx) and shorter (15 min) light (5); this difference may partly reflect how visual systems of diurnal and nocturnal species evolved and adapted to their distinct ecological niche (5). However, procedural differences between human and mouse studies may also be important. For example, human studies often adopt the constant routine protocol, which involves keeping participants under hours of dim ambient lighting (<10 lx) prior to the presentation of the phase-shifting light stimulus (79); thus, the participant’s visual system is adapted to dim light beforehand. By contrast, mouse studies usually present phase-shifting light stimuli in total darkness (39), which may evoke a greater relative response. Some support for this hypothesis comes from human studies in which light history has been shown to directly affect the magnitude of circadian responses to light (80, 81). Taken together, a direct comparison of nonvisual responses to light between humans and mice is not
straightforward, and differences in protocols and other extraneous factors must also be considered.

Another potentially relevant difference is the C57BL/6 mouse’s deficiency in pineal melatonin synthesis (82, 83), which is associated with sleep onset at the start of the biological night in humans (84, 85). Nevertheless, despite the lack of pineal melatonin, the output of the C57BL/6 mouse is similar to the human PRC (5, 38, 39). Furthermore, in a previous study comparing melatonin-proficient versus melatonin-deficient mice, these animals were statistically indistinguishable in terms of entrainment to skeleton photoperiods, free-running period length, acute suppression of wheel-running activity by light, and light-induced phase shift (86). While we cannot exclude the possibility of subtle effects of melatonin on circadian responses to light, all available data suggest that the lack of pineal melatonin in C57BL/6 mice will not overly affect our results.

Sleep–wake cycles are regulated by circadian and homeostatic processes (87). Although our results can be explained in terms of a change in phasing of the internal clock (process C), they are equally compatible with an account based on sleep homeostasis (process S); it could be argued that DLE promotes sleep, postponing the accumulation of sleep pressure in the next morning, without affecting the phase of the internal clock. While our data are compatible with a change in either process C or S, in reality, these processes are intricately related (88). For example, adenosine-dependent changes in sleep pressure can affect the SCN (89). On the other hand, the SCN can exert an influence on sleep-regulatory and thermoregulatory neurons in the preoptic hypothalamus via a monosynaptic projection to the subparaventricular zone (SPVZ) and dorsomedial hypothalamic nucleus (DMH) (90–93). Furthermore, in Cry1−/− mice lacking process C, their process S is also compromised (94). Thus, it is likely that processes C and S are both affected under DLE; separating the contributions of these equally important processes may prove challenging (88).

In this regard, both processes C and S are important determinants of behavioral performance. The reversed pattern of performance under DLE could partly be related to the phase shift in the hippocampal molecular clock (Fig. 3), as the hippocampus is involved in some aspects of recognition memory processes (95, 96), and the level of hippocampal Per1 is related to performance (97, 98). In human processes, sleep history could be crucial (88). For example, adenosine-dependent changes in sleep pressure can affect the SCN (89). On the other hand, the SCN can exert an influence on sleep-regulatory and thermoregulatory neurons in the preoptic hypothalamus via a monosynaptic projection to the subparaventricular zone (SPVZ) and dorsomedial hypothalamic nucleus (DMH) (90–93). Furthermore, in Cry1−/− mice lacking process C, their process S is also compromised (94). Thus, it is likely that processes C and S are both affected under DLE; separating the contributions of these equally important processes may prove challenging (88).

Methods

Animals and Housing Conditions. A total of 111 mice were used in this study. In experiments examining home cage activity and sleep, there were 12 WT mice of C57BL/6 background (Opn4+/−) and 12 Opn4−/− mice (equal numbers of male and female) derived from crossbreeding heterozygous Opn4−/− mice (104). Detailed analyses of locomotor activity and immobility-defined sleep under normal and abnormal lighting conditions did not reveal any difference between sexes or genotypes; thus, male C57BL/6 WT mice (Envigo; RRID:IMSR_JAX:000664) were used in subsequent experiments. In the DD experiment in which in vivo hepatic bioluminescence and locomotor activity were examined, 16 Albumin-Cre:Dbplox liver reporter mice (9/9 and 7/7) were used (57).
expressing firefly luciferase in hepatocytes under the control of Dbp were used to measure hepatic bioluminescence. One wallpaper was a chequerboard pattern with 4 × 4 cm black and white squares. The other wallpaper was 25 cm transparent acrylic walls. At least 8 wk before the experiment, all walls were washed with PBS and blocked in normal donkey serum (Jackson ImmunoResearch). The arena, now containing a third replicate of the familiar sample and a novel object, was placed in the center of the arena. The order of recognition assessment is summarized in Fig. 4. The experiment. There were multiple replicates of each object, and different arrangements of the same object were presented in sample and test phases. The arena and objects were wiped with 70% ethanol after each phase.

Mice received 10-min acclimatization trials in the empty arena at ZT8 (midway between ZT2 and ZT14) for 5 d. They then received object and odor recognition trials at ZT2 and ZT14 and at the same ZTs after 2 wk of exposure to DLE. During the sample phase of each trial, two identical replicates of an object were placed in the arena; the sample phase was terminated after 10 min. After a 3-min delay, novelty preference was assessed in the arena, now containing a third replicate of the familiar sample and a novel object; the test phase was terminated after 3 min. For six of the animals (subgroup 1), the order of recognition trials under LD was the following: ZT2 (odor), ZT14 (odor), ZT14 (object), and ZT2 (object); whereas for the remaining animals (subgroup 2), the arrangement was reversed: ZT14 (odor), ZT2 (odor), ZT2 (object), and ZT14 (object). Under DLE, the order of assessment was ZT2 (object), ZT14 (object), ZT2 (odor), and ZT14 (odor) for subgroup 1; for subgroup 2, the order was ZT14 (object), ZT2 (object), ZT14 (odor), and ZT2 (odor). The order of recognition assessment is summarized in Fig. 4A. The identity of novel and familiar stimuli and their spatial positions in the arena at test were counterbalanced in order to take into account any potential stimulus-specific or location-specific bias. Automated tracking of exploratory activity was conducted in ANY-maze (Stoelting; RRID:SCR_002865) and R (RRID:SCR_001905). α = 0.05 was adopted in all analyses unless otherwise specified. To analyze behavioral data (locomotor activity phases, immobility-defined sleep, recognition memory performance, and stimulus exploration times), we conducted within-subjects and split-plot ANOVAs with Lighting, Time of Day, and Type of Stimulus as within-subjects factors and Sex and Genotype as between-subjects factors; for locomotor activity under DD, we conducted between-subjects ANOVAs with Prior Lighting as a factor. To analyze molecular data (clock gene expression and cFos+ cell counts), Lighting × Time of Day × Region split-plot ANOVAs were conducted. Significant interaction effects in most cases were followed up with simple effect analyses in SPSS (116). For main and interaction effects involving within-subjects factors with more than two levels, Greenhouse-Geisser corrections were applied to the degrees of freedom (df) whenever the assumption of sphericity was violated (117); these F values with adjusted df were denoted as F*. To examine the effects of sleep history and sample exploratory activity on test performance, we conducted linear mixed-effects models in R (62), with preceding 2-h sleep proportion or sample exploratory activity as the fixed effect, individual mice as the random effect, and recognition scores as the response variable. The significance of the fixed effect was assessed by examining the change in deviance (~2 × log maximum likelihood) from comparing linear mixed-effects models with versus without the fixed effect of interest. A significant drop in deviance—determined by the likelihood ratio χ² value—indicates that incorporating the fixed effect improves model fitting and explains more variance in the response variable than when it is excluded from the model (118). The 95% CI of the fixed effect (slope) was computed from parametric bootstrap with 10,000 iterations.

Sample Size. Sample size was determined by power analysis (G*Power 3.1, 76). The power analysis was conducted on preliminary data from one experiment (n = 6) in which we observed a significant effect of ZT2 and ZT14 on acute novelty preference. For ZT2, we estimated an effect size of 0.85. The number of animals was determined with the sample size calculator for between-subjects ANOVA with G*Power 3.1 (76). The critical value of α was 0.05, and the power was 0.80. The number of animals was determined to be 6, which was sufficient to detect an effect size of 0.85 at the significance level of 0.05 with 80% power.

Data Availability. Data reported in this manuscript were deposited by S.K.E. on Figshare (September 8, 2021) and can be accessed via https://figshare.com/articles/dataset/Tam_et_al_data.xls/16583834. Supplementary methods, tables S1 to S7, and figures S1 to S11 can be found in the SI Appendix.

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