Comparison of light, food, and temperature as environmental synchronizers of the circadian rhythm of activity in mice

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Abstract Although entrainment (and masking) of circadian rhythms by light has been extensively studied, much less attention has been given to other environmental cycles that can modulate circadian rhythms in mammals. In this study in mice, the entraining strength of different environmental cycles was compared. Running-wheel activity was monitored before, after, and while the animals were under one of four environmental cycles: a full light–dark cycle with 12 h of light and 12 h of darkness each day, a cycle of 1 h of light per day, a cycle of food availability consisting of 80% of the baseline free-feeding amount presented once a day, and an ambient temperature cycle consisting of 23 h at 24°C and 1 h at 12°C each day. Four measures of zeitgeber strength were used: percentage of animals that entrained, rhythm robustness in the entrained state, stability of activity onsets, and stability of acrophases. The results indicate that, at least in mice, a full light–dark cycle is the most powerful modulator of the circadian rhythm of locomotor activity, as a consequence of both entrainment and masking. When entrainment alone is considered, temperature seems to be as strong a modulator as light, while food restriction is a weaker modulator and affects primarily a food-anticipatory component of the activity rhythm.

Keywords Circadian · Locomotor activity · Mus musculus · Nonphotic entrainment

Introduction

The rotation of the Earth around its axis generates daily environmental cycles that over the millennia stimulated the evolution of circadian rhythmicity in living organisms. Although endogenously generated, circadian rhythms are synchronized to the Earth’s rotation through discrete phase shifts caused by exposure to sunlight at particular phases of the circadian cycle [1]. It is generally recognized that, in the presence of full light–dark cycles, the exact timing of animal behavior is determined not only by entrainment of the pacemaker (through parametric and nonparametric mechanisms) but also by acute photic inhibition or stimulation (“masking”) [2].

Although entrainment (and masking) by light has been extensively studied, much less attention has been given to other environmental cycles that can modulate circadian rhythms. Two environmental cycles with clear potential to entrain circadian rhythms are those of ambient temperature and of food availability. Although few in number, studies conducted over the years have demonstrated that animal behavior can be entrained (and masked) by daily cycles of ambient temperature in rats [3], mice [4], Syrian hamsters [5], blind mole-rats [6], palm squirrels [7], marmosets [8], and squirrel monkeys [9], in addition to several ectothermic species for which changes in ambient temperature cannot be adequately distinguished from changes in body temperature [10].

Entrainment by a cycle of food availability (“food restriction”) has received more attention, particularly because it seems to involve two distinct circadian pacemakers. Numerous studies have demonstrated entrainment of animal behavior by daily cycles of food availability in rats [11], mice [12], kowaris [13], sparrows [14], Kuzu rats [15], rabbits [16], squirrel monkeys [17],...
and other species. Because two separate behavioral components were observed when animals were exposed simultaneously to environmental cycles of light–darkness and food restriction, and because the so-called “food anticipatory” component persisted in constant darkness after the main circadian pacemaker in the hypothalamic suprachiasmatic nucleus had been ablated [18], some authors assumed the existence of distinct input pathways to a “light-entrainable pacemaker” and a “food-entrainable pacemaker” [19]. There was no a priori reason why food restriction could not affect both pacemakers, but the notion of distinct input pathways was boosted by the observation that rhythms of gene expression in a peripheral organ such as the liver were affected more rapidly by a change in the cycle of food availability than in the light–dark cycle [20, 21]. Further research demonstrated that the master circadian pacemaker can be entrained by daily cycles of food availability, although entrainment of the master pacemaker in mice is much more difficult than entrainment of the food-entrainable pacemaker, and two distinct components of the activity rhythm can be differentially entrained [22, 23].

Generally, investigators have reported that the light–dark cycle is a stronger zeitgeber than the ambient temperature cycle or the cycle of food availability [4, 9, 13, 14]. However, no quantitative comparison of zeitgeber strength has been conducted so far. The present study provides for the first time a quantitative comparison of the strengths of cycles of light, food, and temperature as modulators of locomotor behavior in mice.

Materials and methods

Animals

Two-month-old, male mice (Mus musculus) of the CD-1 strain were purchased from Charles River Laboratories (Wilmington, MA) and were housed individually in polypropylene cages (24 × 36 × 19 cm) lined with wood shavings and fed Purina rodent chow (Lab Diet 5001) and water ad libitum.

Equipment

The rhythm of running-wheel activity was used as a measure of the state of the circadian pacemaker. A metallic running wheel (12 cm diameter) was attached to each animal cage. Magnetic switches attached to the running wheels were connected to data acquisition boards (Digital Input Card AR-B2001, Acrosser Technology, Taiwan). The data acquisition boards were connected to computers that recorded the number of wheel revolutions in 6-min bins (i.e., 0.1 h intervals). To reduce animal disturbance, cages and water bottles were replaced at monthly intervals, under dim red light if necessary.

For the investigation of entrainment by light and by food restriction, the animal cages were maintained in individual light-tight, ventilated chambers at 24 ± 2 °C. Lighting conditions in each chamber were controlled by a programmable electronic timer (ChronTrol XT, ChronTrol Corp., San Diego, California) that activated white fluorescent bulbs (General Electric F4T5CW) generating an illuminance of approximately 360 lux (range 340–390 lux across chambers), as measured 8 cm above the cage floor. For the investigation of entrainment by cycles of ambient temperature, the animal cages were housed inside a programmable refrigerated incubator (Revco BOD-50, Thermo Fisher Scientific Inc., Waltham, MA) initially set at 24 ± 1 °C.

Procedure

To minimize masking, the animals were maintained in constant darkness (DD), and the environmental cycles consisted of 1 h of stimulation per day. However, because full light–dark cycles are the norm on Earth (even if animals may not be fully exposed to them), an additional group of mice was studied under a full light–dark cycle with 12 h of light and 12 h of darkness each day (LD 12:12).

Ideally, a comparison of the strength of different environmental cycles as synchronizers of circadian rhythms would involve a large number of light intensities (and colors), various arrangements of ambient temperatures, and various conditions of food restriction. As the first systematic study of this type, the current study used a few conditions considered to be representative, as explained below. Reflecting the existence of differences in difficulty to set up the various environmental cycles, the group sizes varied with the experimental conditions, as follows:

A group of 60 mice (in individual isolation chambers) was kept under a full light–dark cycle (LD 12:12) for 1–2 months before being released into DD for at least 2 weeks.

A group of 41 mice (in individual isolation chambers) was kept in DD for at least 2 weeks before being exposed to a cycle of 1 h of light per day for 1–3 months, and then returned to DD for at least 2 weeks. To reduce the time needed for entrainment to occur, the daily light pulse was scheduled to begin slightly before the onset of activity on the first day [24].

A group of 34 mice (in individual isolation chambers) was kept in DD for at least 2 weeks before being exposed (still in DD) to a cycle of food availability consisting of 80 % of the baseline free-feeding amount presented once
every 24 h. The free-feeding amount was pre-determined by measurement of the weight of food placed in the hopper at the beginning of a 6-day interval and the weight of food remaining in the hopper at the end of the interval under an LD cycle. In the study itself, each animal was given 80% of its free-feeding daily intake as a single meal once a day. The mean baseline free-feeding amount was 4.9 g of food pellets per mouse per day. To reduce the time needed for entrainment to occur, the daily food presentation was scheduled to begin slightly before the onset of activity on the first day [25]. The mice were kept under the cycle of food availability for 1–2 months before being returned to ad libitum condition (still in DD) for at least 2 weeks. The body weights of the mice at the end of the study were approximately 90% of the body weight at the beginning of the study.

A group of 20 mice (all of them individually housed) was kept in DD for at least 2 weeks before being exposed (still in DD) to the ambient temperature cycle consisting of 23 h at 24°C and 1 h at 12°C each day. To reduce the time needed for entrainment to occur, the daily cold stimulation was scheduled to begin slightly before the onset of activity on the first day [4]. The change of temperature in the incubator (in both directions) was fully accomplished within 30 min. The mice were kept under the Ta cycle for 1–3 months before being returned to a constant temperature (24°C, still in DD) for at least 2 weeks.

The four environmental cycles used in this study were good exemplars of a full LD cycle, a daily light pulse, a daily cycle of ambient temperature, and a daily cycle of food availability for the following reasons:

An LD cycle with 12 h of light and 12 h of darkness is the standard LD cycle used in laboratories around the world, and an illuminance of 360 lux is above the saturating level for the circadian system of nocturnal rodents [26–28]. For the short light cycle, a daily light pulse of 1 h was intentionally chosen to reduce photic masking, as a 1-h pulse is relatively short but is sufficiently long to evoke a maximal phase shift in the rodent circadian system [29, 30].

Regarding the cycle of ambient temperature, the range of oscillation from 12–24°C approximates that of outdoor temperature in early autumn in various parts of the world, including New York, Beijing, and Tokyo [31], and a temperature cycle with 1 h of cold per day has been previously shown to be as effective as a cycle with 8 h of cold per day [4]. When allowed to choose the temperature of the environment, sedentary mice (without access to running wheels) select an ambient temperature of approximately 26°C at night and 30°C during the day [32], so that, for mice running on wheels, 24°C is probably thermoneutral. Exposure to 12°C is sufficient to evoke an increase in metabolic heat production in mice but is not extreme enough to cause a fall in core temperature [33].

Finally, regarding restricted feeding, anticipatory activity has been observed in animals of many species under a variety of conditions of food delivery, even if entrainment of the second activity component requires hypocaloric food restriction [34]. In the present study, food restriction was hypocaloric at 80% of the free-feeding level.

Data analysis

In all four conditions (LD 12:12, LD 1:23, food restriction, and temperature cycle), the occurrence of entrainment was evaluated by the exhibition of the appropriate circadian period under the environmental cycle (24.0 h) and by the correct initial phase of free-run upon release into DD [2]. These features were analyzed by visual observation of actograms as well as by computer algorithms. By visual observation, circadian period was determined by the slope of the daily onsets, with a straight vertical line in the actogram indicating a 24.0 h period. By computer algorithm, circadian period was computed through the chi-square periodogram procedure in blocks of 10 consecutive days [35]. The free-running period of CD-1 mice is rarely exactly 24.0 h, ranging from 22.7 to 24.6 h with a mean of 23.6 h [24], so that the presence of 24.0-h rhythmicity under an environmental cycle strongly suggests entrainment. The initial phase of free-run was determined by linear regression of onsets during 7 days of free-run projected back to the first day in DD. Onsets were computed according to a computer algorithm that defined an onset as the time point that contains 4 or more wheel turns and that (a) is in a 2-h block that contains activity greater than the daily mean, (b) is preceded by a 2-h block with activity lower than twice the daily mean, and (c) is followed by a 2-h block with activity greater than 1/3 the daily mean. In a few cases in which the computer algorithm failed to reliably identify onsets, only the visual observation method was used.

In records in which entrainment was deemed to have occurred, the four environmental cycles were compared regarding their ability to control the running-wheel activity rhythm. This ability was measured in three different ways: by the robustness of the rhythm, by the stability of activity onsets, and by the stability of acrophases. Rhythm robustness was calculated as the Qp value in the chi-square periodogram procedure expressed as a percentage of maximal rhythmicity [25]. The stability of activity onsets (in min) was estimated by the magnitude of the standard deviation of onsets over 10 consecutive cycles. Onsets were computed according to the computer algorithm described in the previous paragraph. The stability of acrophases (in min) was estimated by the magnitude of the standard deviation of acrophases over 10 consecutive days. Acrophases were computed by the cosinor procedure [36].
Significance tests for differences among group means were conducted by analysis of variance (ANOVA) followed by post hoc tests. Post hoc pairwise comparisons were conducted with Tukey’s HSD test [37].

Results

Records of running-wheel activity of a representative mouse kept under a full light–dark cycle (LD 12:12) before being released into constant darkness (DD) are shown in Fig. 1. The records indicate strong entrainment by the LD cycle, with a robust 24.0-h rhythm showing little day-to-day variation in onset times. After release into DD, the rhythm free-ran with a period of 23.7 h. In this animal, as in about half the number of all animals tested, the onset of activity on the first day in DD was about 90 min earlier than on the last day under LD. The remaining animals started running only a few min earlier on the first day in DD than on the last day under LD.

Representative activity records of mice that were entrained by a daily 1-h light pulse, by food restriction, or by a 1-h daily pulse of cold environment are shown in Fig. 2. Entrainment by the daily light pulse is evinced by the appropriate change in phase and period of the activity rhythm after the introduction of the environmental cycle and by the re-initiation of free-run from the appropriate phase after release into DD (Fig. 2a). The cycle of food availability yielded food anticipatory activity with the appropriate period of 24.0 h (Fig. 2b). The second component of the activity rhythm did not seem to be entrained by food availability in this animal or in most other mice subjected to food restriction in this study. The cycle of ambient temperature (Fig. 2c) produced entrainment of the activity rhythm, as indicated by the appropriate change in phase and period after the introduction of the environmental cycle and by the re-initiation of free-run from the appropriate phase after release into DD under constant ambient temperature. Representative activity records of mice that were not entrained by the environmental cycles are shown in Fig. 3. The animal whose records are shown in panel A had a free-running period only slightly shorter than 24.0 h and drifted slowly over the 15 weeks of the study without ever reaching the point where it could be exposed to the daily pulse during subjective night. The records in Fig. 3b (food restriction) show some anticipatory activity starting around day 40, but this anticipatory component is weak, and the main component of the activity rhythm clearly free-runs through the food-restriction phase. Finally, the records in Fig. 3c show some negative masking in the second half of subjective night, which is noticeable starting around Day 50, but no indication of entrainment.

The numbers of mice that were entrained by each of the environmental cycles are shown in Table 1. Whereas all animals subjected to a full light–dark cycle exhibited entrainment, smaller proportions of animals exhibited entrainment when subjected to the other environmental cycles, and the difference in proportions was statistically significant: \( \chi^2(3) = 27.3947, p < 0.001 \). The two mice that failed to be entrained by a daily light pulse (LD 1:23) had circadian periods very close to 24.0 h and did not drift sufficiently to be exposed to the light pulse during subjective night (Fig. 3a). Three of the mice that were not entrained by the temperature cycle also had circadian periods close to 24.0 h and did not drift sufficiently to be exposed to the daily cold pulse during early subjective night. If the two mice from the light pulse and the three mice from the cold pulse are excluded from the calculations, the differences in proportions of animals entrained in the three groups (with the food-restricted group excluded) are not statistically significant: \( \chi^2(2) = 4.175, p = 0.124 \). Despite individual differences in free-running period, the mean free-running periods (mean ± SEM) of the mice in the three groups before (and after) exposure to the environmental cycles were almost identical (light pulse: 23.81 ± 0.09 h before, 23.88 ± 0.07 h after; food restriction: 23.83 ± 0.06 h before, 23.88 ± 0.08 h after; temperature: 23.81 ± 0.07 h before, 23.90 ± 0.06 h after).

When entrainment did occur, as exemplified in Fig. 2, it was effected in such a way that the mice ran on the wheels immediately after the daily light pulse or the daily cold pulse. Under food restriction, the first component of the activity cycle preceded the daily presentation of food, respectively.

![Fig. 1](image-url)
whereas the second component followed it (or, more commonly, free-ran with or without relative coordination).

Figure 4 shows the mean results of the analyses of rhythm robustness, variability of daily onsets, and variability of acrophases of the activity rhythms of mice that exhibited entrainment under the four environmental cycles. The three indices of strength of entrainment were concordant, except that variability of onsets seemed to be the most sensitive index (revealing the largest number of group differences), followed by variability of acrophases, and
followed by rhythm robustness. As shown in the top panel, there was a significant difference in the robustness of the rhythms under the four conditions, as revealed by ANOVA: $F(3,123) = 9.100$, $p < 0.0001$. Post hoc tests revealed significant differences only between the full LD cycle and the three other conditions.

The middle panel of Fig. 4 shows that there was a significant difference in the variability of onsets under the four conditions, as revealed by ANOVA: $F(3,123) = 34.541$, $p < 0.0001$. The variability of onsets was greater (indicating weaker control by the environmental cycle) under the cycle of food availability than under the other three conditions. Variability of onsets was comparable under the light-pulse and temperature conditions and was smallest under the full light–dark cycle.

The bottom panel of Fig. 4 shows that there was a significant difference in the variability of acrophases under the four conditions, as revealed by ANOVA: $F(3,123) = 18.113$, $p < 0.0001$. The variability of acrophases was greater (indicating weaker control by the environmental cycle) under the cycle of food availability than under the other three conditions. Variability of acrophases was comparable under the light-pulse, temperature, and full LD cycle conditions.

**Discussion**

Entrainment occurred in all four conditions that were studied (full light–dark cycle, 1 h of light per day, food restriction, and 1 h of cold per day). Under a full LD cycle, the well-studied locomotor behavior of mice was observed, with robust daily rhythmicity characterized by concentration of activity during the dark phase of the cycle. After release into DD, the rhythm free-ran with a period shorter than 24.0 h on average. In half the number of all animals tested, the onset of activity on the first day in DD was about 90 min earlier than on the last day under LD, which implies the presence of masking under the LD cycle [38].

As previously noticed by other investigators using mice as experimental subjects, restricted feeding produced strong food anticipatory activity but was not as effective in entraining the main component of the activity rhythm [22, 39–41]. In this study, even the food anticipatory component was entrained (or robustly expressed) only in 44 % of the mice tested. In contrast, entrainment was observed in 100 % of mice exposed to a full LD cycle, 95 % of mice exposed to a daily 1-h pulse of light, and 65 % of mice exposed to a daily 1-h pulse of environmental cold. Although the proportion of animals that exhibited entrainment was higher under the full LD cycle than under the light pulse and temperature pulse conditions, part of the difference was due to the fact that the non-entrained...
animals were not optimally exposed to the environmental cycles. If two non-optimally stimulated mice from the light pulse group and three non-optimally stimulated mice from the cold pulse group are excluded, the differences observed in the proportions of animals entrained in the three groups are not statistically significant.

Three other indices of the strength of the environmental cycles investigated in this study were rhythm robustness, variability of daily onsets, and variability of acrophases. The finding that robustness was greater under a full LD cycle than under a cycle with only 1 h of light per day indicates that photic masking has a non-trivial effect on the control of behavior entrained by light. From the perspective of rhythm robustness, a daily light pulse was as effective as a daily temperature pulse and as 80% restricted feeding presented once a day. From the perspective of day-to-day variability in the acrophase of the activity rhythm, food restriction exhibited weaker control of behavior than the other three environmental cycles. Finally, analysis of the variability in daily onsets revealed that the full LD cycle had the strongest control of behavior, whereas food restriction had the weakest control. The daily light pulse and daily temperature pulse had the same strength of control. The observations that the variability of onsets and acrophases was greater (indicating weaker control by the environmental cycle) under the cycle of food availability than under the other three conditions is not surprising given the fact that only the anticipatory component of the activity rhythm was entrained by the cycle of food availability in this study.

These findings are consistent with previous observations that the light–dark cycle might be a stronger zeitgeber than the ambient temperature cycle or the cycle of food availability [4, 9, 13, 14], but they also provide a more nuanced view of the differences in zeitgeber strength. Combining the evidence from the percentage of entrained animals with the three other indices of behavioral control, it can be concluded that, at least in mice, a full light–dark cycle is the most effective environmental cycle in the control of running-wheel activity only because it adds the effects of masking to the effects of entrainment. When the main effect of masking is removed by presentation of only 1 h of light per day, temperature is just as strong a zeitgeber as light is. Food restriction, however, is a weaker zeitgeber. In this study, food restriction affected only one component of the activity rhythm, produced the smallest percentage of entrainment among the four environmental cycles, and exhibited the greatest variability in daily onsets and acrophases. Why food restriction is a weaker zeitgeber than light and temperature cannot be determined by the data collected in the present study. It is known that, at least in mice and rats, induction of food-anticipatory activity rhythms by daily feeding schedules does not require the master circadian pacemaker in the suprachiasmatic nucleus, and that clock gene rhythms in other brain regions and in peripheral organs can be preferentially synchronized by mealtime, but a food-entrainable pacemaker has yet to be located [42].

A possible confound in the study is that not enough time may have been allowed for the animals to undergo the full effects of the environmental cycles. To a limited extent, this may have been true. The two mice that failed to entrain to the daily light pulses would very likely have experienced entrainment if they had been allowed many months to slowly drift into the zone of influence of the light pulses. More significantly, a larger number of mice subjected to restricted feeding might have experienced entrainment (of either or both activity components) if they had been kept longer under the cycle of food availability. Unfortunately, for reasons of animal welfare, the mice could not be left under 80% feeding for many months. Thus, if the percentage of animals entrained had been the only variable measured, the length of the study might have been an issue. However, group differences in rhythm robustness and stability were clearly observed in animals that were fully entrained, so that the differences observed in zeitgeber strength can be considered to be reliable. It seems appropriate to conclude that, in mice, a full light–dark cycle is the most powerful modulator of the circadian rhythm of locomotor activity, as a consequence of both entrainment and masking. When entrainment alone is involved, however, light and temperature seem to be equally strong modulators, with food restriction coming in a distant third place.

Further research may determine the strength of masking by ambient temperature. Whereas it is clear that the control of behavior by an LD cycle depends on both entrainment and masking, the present study did not evaluate the strength of masking by ambient temperature. Because previous studies suggested that a full LD cycle affects the timing of behavior more strongly than a full ambient temperature cycle does [4, 8, 9], it is likely that full temperature cycles exert a weaker masking effect than LD cycles do. Controlled studies are needed to settle the matter.

Conflict of interest The author declares that he has no conflict of interest.

Ethical approval All applicable international, national, and institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution at which the studies were conducted (University of South Carolina).

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