Entrainment of Circadian Rhythm by Ambient Temperature Cycles in Mice

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Abstract Much is known about how environmental light-dark cycles synchronize circadian rhythms in animals. The ability of environmental cycles of ambient temperature to synchronize circadian rhythms has also been investigated extensively but mostly in ectotherms. In the present study, the synchronization of the circadian rhythm of running-wheel activity by environmental cycles of ambient temperature was studied in laboratory mice. Although all mice were successfully entrained by a light-dark cycle, only 60% to 80% of the mice were entrained by temperature cycles (24-32 °C or 24-12 °C), and attainment of stable entrainment seemed to take longer under temperature cycles than under a light-dark cycle. This suggests that ambient temperature cycles are weaker zeitgebers than light-dark cycles, which is consistent with the results of the few previous studies using mammalian species. Whereas 80% of the mice were entrained by 24-h temperature cycles, only 60% were entrained by 23-h cycles, and none was entrained by 25-h cycles. The results did not clarify whether entrainment by temperature cycles is caused directly by temperature or indirectly through a temperature effect on locomotor activity, but it is clear that the rhythm of running-wheel activity in mice can be entrained by ambient temperature cycles in the nonnoxious range.

Key words circadian, locomotion, entrainment, nonphotic, temperature, CD-1 mouse

Circadian rhythms are generated endogenously but are modulated by environmental cycles. Environmental modulation can be exerted as entrainment (when the period and phase of the rhythm are controlled by the environmental cycle) or as masking (when the environmental cycle enhances or weakens the rhythm without taking control of period and phase). Although entrainment and masking may both be important for the timing of animal behavior in natural settings (Mrosovsky, 1999), entrainment is particularly important because it reflects synchronization of the endogenous pacemaker by the environmental cycle (Johnson et al., 2003).

The mechanism by which daily environmental cycles entrain circadian rhythms has been extensively studied in the case of photic stimuli (i.e., light-dark cycles). Entrainment by light-dark cycles is dependent on the endogenous circadian period, on the period of the light-dark cycle, on the photic response characteristics of the species (as depicted in phase-response curves), and on a number of other factors that are not yet fully understood (Daan and Aschoff, 2001).

The entraining capability of so-called nonphotic stimuli (such as food, exercise, and ambient temperature) has received much less attention than that.
of photic stimuli. In the particular case of ambient temperature (T<sub>a</sub>), the majority of studies that have been conducted have dealt with ectothermic organisms, whose circadian pacemakers can be directly affected by changes in the temperature of the environment (Rensing and Ruoff, 2002). Very few studies of T<sub>a</sub> as a zeitgeber have been conducted in endothermic animals such as birds and mammals. Occasional studies on a few species of rodents (Francis and Coleman, 1988; Goldman et al., 1997; Lindberg and Hayden, 1974; Pohl, 1998; Rajaratnam and Redman, 1998) and primates (Aschoff and Tokura, 1986; Pálková et al., 1999; Tokura and Aschoff, 1983) have indicated that T<sub>a</sub> cycles have the potential to entrain circadian rhythms but are rather weak zeitgebers—as entrainment is not achieved in all subjects, and phase control is weak in subjects that do exhibit entrainment.

The purpose of the present study was to extend the study of entrainment of circadian rhythms by T<sub>a</sub> cycles to the domestic mouse, a species that is particularly suitable for chronobiological research because of the extensive knowledge of the molecular mechanisms of its circadian clocks (Takahashi et al., 2008).

**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.

**Apparatus**

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, Acrosster 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).

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. Lighting conditions were controlled by a programmable electronic timer (ChronTrol XT, ChronTrol Corp., San Diego, CA) that activated white fluorescent bulbs (General Electric F4T5CW) generating an illuminance of approximately 200 lux (range: 180-220 lux across cages), as measured 8 cm above the cage floor. To reduce animal disturbance, cages and water bottles were replaced at monthly intervals, under dim red light if necessary.

**Procedures**

All animals were initially housed under a light-dark cycle with 8 h of light per day (LD 8:16) at a constant T<sub>a</sub> of 24 °C for 2 or more weeks before being released into constant darkness (DD). A short photoperiod (LD 8:16) was used to reduce negative masking caused by photic stimulation.

A group of 10 mice was kept in DD for a month before being exposed (still in DD) to a T<sub>a</sub> cycle consisting of 16 h at 24 °C and 8 h at 12 °C each day (a “neutral-to-cold” cycle). The change of temperature in the incubator (in either direction) was fully accomplished within 30 min. This range of oscillation from 12 °C to 24 °C approximates that of outdoor temperature in early autumn in various cities in the world, including New York, Beijing, and Tokyo (as recorded at www.weather.com). The mice were kept under the T<sub>a</sub> cycle for 4 to 5 months before being returned to a constant temperature (still in DD) for 2 months. A light-dark cycle (LD 8:16) was reintroduced for the last 2 months of the study.

Another group of 10 mice was submitted to a similar protocol, except that the T<sub>a</sub> cycle consisted of 16 h at 24 °C and 8 h at 32 °C each day (a “neutral-to-warm” cycle). When allowed to choose, sedentary mice (without access to running wheels) select an ambient temperature of approximately 26 °C at night and 30 °C during the day (Gordon et al., 1998), so that, for mice running on wheels, 24 °C is probably thermoneutral, and 32 °C is warm. Although the neutral-to-warm cycle was expected to evoke heat loss responses during the warm phase, and the neutral-to-cold cycle was expected to evoke heat conservation responses during the cold phase, the two cycles were chronobiologically comparable in the sense that both involved daily deviations from thermoneutrality.

After the second group of 10 mice completed the protocol, an attempt was made to build a phase-response curve for T<sub>a</sub> pulses. Five mice were kept in DD at 24 °C and were given single 4-h pulses of 32 °C once a week in 5 consecutive weeks. Because the animals were not entrained, the pulses were experienced
at different circadian times by different animals, and data points were obtained for a wide spread of circadian times to allow the construction of a phase-response curve. No attempt was made to build a phase-response curve for cold (12 °C) pulses.

A third group of 10 mice was used for T cycle studies. After being kept in DD at 24 °C for a month, the mice were exposed (still in DD) to a T cycle consisting of 23 h at 24 °C and 1 h at 12 °C each day (T = 24 h) for 3 months, followed by a shorter cycle of 22 h at 24 °C and 1 h at 12 °C (T = 23 h). After a month under T = 23 h, the animals were returned to constant 24 °C (still in DD) for a month and then placed under T = 25 h for 2 months before being returned once again to constant 24 °C. T cycle studies were not conducted for deviations above thermoneutrality.

Data Analysis

For data analysis, running-wheel data were plotted as actograms for visual inspection. Circadian times were determined from the actograms using the daily onset of activity as CT 12. Phase shifts in DD were determined by drawing separate eye-fit lines through the onsets for 5 or 6 days before and 5 or 6 days after the pulse and calculating the time between the two lines on the first cycle following the pulse. Circadian period was computed by the chi-square periodogram procedure (Sokolove and Bushell, 1978) and the Lomb-Scargle periodogram procedure (Ruf, 1999). A preliminary report of the results was presented at the 11th meeting of the Society for Research on Biological Rhythms (Destin, FL, 17-21 May 2008).

RESULTS

Running-wheel activity records of 2 representative mice from the group subjected to a neutral-to-cold (12 °C) T cycle are shown in Figure 1. The animal whose records are plotted in panel A seemed to entrain to the T cycle (hatched vertical rectangle), although only after about 3 months. Entrainment was confirmed by the appropriate phase from which the rhythm free-ran after the environmental cycle was discontinued on day 173. Entrainment to a light-dark cycle (open vertical rectangle) occurred much more rapidly, even though the phase angle of entrainment exhibited small adjustments for more than a month. The animal whose records are plotted in panel B did not entrain to the T cycle but exhibited clear masking (both positive and negative) and perhaps some relative coordination. This animal entrained rapidly to the light-dark cycle. Of 10 mice in the neutral-to-cold group, 6 exhibited entrainment, whereas 4 exhibited some form of relative coordination but not entrainment. All 30 mice used in this study exhibited robust entrainment to the LD cycle.

Running-wheel activity records of 2 representative mice from the group subjected to a neutral-to-warm (32 °C) T cycle are shown in Figure 2. The animal whose records are plotted in panel A was entrained by the light-dark cycle, free-ran with a period shorter than 24 h in DD, exhibited relative coordination for the first 2 months under the T cycle, and finally entrained with a steady phase angle. When released into DD, the rhythm free-ran (now with a period longer than 24 h) from the phase of entrainment. The animal whose records are plotted in panel B was also entrained by the light-dark cycle and free-ran with a period shorter than 24 h in DD, but it did not entrain to the T cycle, even though it exhibited strong relative coordination. Of 10 mice in the neutral-to-warm group, 6 exhibited entrainment, 3 exhibited relative coordination, and 1 exhibited only negative masking.

To evaluate the pattern of entrainment to the T cycles in more detail, waveform plots for each animal were prepared by averaging, bin by bin, the data for 20 consecutive days of entrainment (such as days 141 to 160 in Figure 2A). The results for 4 representative mice are shown in Figure 3. The 2 mice that were entrained by the neutral-to-cold cycle (panels A and B) did most of the running slightly before, during, and slightly after the exposure to the cold. In contrast, the mice that were entrained by the neutral-to-warm cycle (panels C and D) ran mostly before the exposure to the heat (during which negative masking was evident). Negative masking during exposure to the heat is particularly evident in the waveform plots Figure 3C,D), even though it is not always apparent in actogram plots (such as Figure 2A, days 120 to 160). Although the running patterns were different in the neutral-to-cold and neutral-to-warm conditions, it should be noted that in both conditions, the animals did most of the running during the cooler part of the T cycle.

To facilitate visualization of the masking effects of the T cycles, Figure 4 shows average waveform plots for the last 2 days of entrainment (panels A and C) and the first 2 days of free-run after removal of the T cycle (B and D) for a mouse entrained by the neutral-to-cold cycle (A and B) and one entrained by the
Figure 1. Double-plotted actograms of running-wheel activity of 2 representative mice from the group subjected to a neutral-to-cold ambient temperature cycle. In each actogram, time of day is indicated on the horizontal axis and number of days on the vertical axis. The hatched vertical rectangle (single-plotted from day 36 to day 173) denotes the cold segment of the neutral-to-cold cycle. The open vertical rectangle (single-plotted from day 237 to day 302) denotes the light segment of the light-dark cycle. Entrainment to the temperature cycle is seen in panel A but not in panel B.
Figure 2. Double-plotted actograms of running-wheel activity of 2 representative mice from the group subjected to a neutral-to-warm ambient temperature cycle. In each actogram, time of day is indicated on the horizontal axis and number of days on the vertical axis. The open vertical rectangle (single-plotted from day 1 to day 24) denotes the light segment of the light-dark cycle. The hatched vertical rectangle (single-plotted from day 38 to day 161) denotes the warm segment of the neutral-to-warm cycle. Entrainment to the temperature cycle is seen in panel A but not in panel B.
neutral-to-warm cycle (C and D). Removal of the neutral-to-cold cycle (A and B) did not seem to affect the profile of running-wheel activity. Removal of the neutral-to-warm cycle (C and D) confirmed the previous presence of negative masking during late subjective night (between 1000 h and 1600 h in the figure) but did not reveal large changes during early subjective night that might reflect a change in the phase angle of entrainment.

In an attempt to find out the mechanism by which \( T_a \) cycles can entrain the rhythm of running-wheel activity, mice kept in DD were exposed to isolated 4-h pulses of warmth (32 °C). The results are shown in Figure 5. Against expectations, there is no clear function relating the magnitude of phase shifts to the circadian time of stimulation.

As an alternate approach to a phase-response curve, \( T_a \) cycles with only 1 h of temperature displacement per day were used. Out of 10 mice, 8 entrained to a 24-h \( T_a \) cycle (23 h at 24 °C and 1 h at 12 °C each day). After the 24-h \( T_a \) cycle, the animals were exposed to a 23-h \( T_a \) cycle. Figure 6 shows the activity records of 2 mice before and after the \( T_a \) cycle was changed from a period of 24 h to a period of 23 h. Both animals, which were entrained by \( T = 24 \) h, remained entrained under \( T = 23 \) h. Although 8 mice entrained...
to T = 24 h, only 6 remained entrained to T = 23 h. None of the mice entrained to T = 25 h (data not shown). During entrainment to T = 24 h, the animals started running 3 to 7 h before the daily cold pulse, whereas during entrainment to T = 23 h, the animals started running about 1 h before the pulse. Considering that the mice had free-running periods averaging 23.7 h, the phase angles of entrainment under the T cycles suggest that phase delays of at least 18 min (i.e., 0.3 of an hour) can be evoked around CT 17 and that phase advances of at least 42 min (0.7 of an hour) can be evoked around CT 13.

The 20 mice that were successfully entrained by Tn cycles in this study ran an average of 18,589 ± 1075 (mean ± SE) wheel revolutions per 24 h and had a mean free-running period of 23.74 ± 0.04 h in DD prior to the introduction of the Tn cycle. The 10 mice that were not entrained by Tn cycles ran an average of 7120 ± 1670 (mean ± SE) wheel revolutions per 24 h and had a mean free-running period of 23.62 ± 0.12 h in DD prior to the introduction of the Tn cycle. The difference in wheel revolutions between the two groups was statistically significant (t28 = 5.764, p < 0.0001), but the difference in period was not (t28 = 0.937, p > 0.10). On the other hand, the variance of the mean period was significantly larger in the group of mice that were not entrained than in the group of mice that were entrained (F5,19 = 4.50, p < 0.01). Free-running periods ranged from 23.6 to 24.2 h in mice that entrained and from 23.3 to 24.5 h in mice that did not entrain. Within the group of mice that were successfully entrained by Tn cycles, there was not a significant correlation between free-running period and number of wheel revolutions per 24 h (r = 0.259, p > 0.10).

**DISCUSSION**

The results clearly demonstrated that the circadian rhythm of locomotor activity of domestic mice can be entrained by an ambient temperature cycle (Figures 1A and 2A). Although all mice were entrained by a light-dark cycle, only 60% to 80% of the mice were entrained by Tn cycles, and attainment of stable entrainment seemed to take longer under Tn cycles than under a light-dark cycle. This suggests that Tn cycles are weaker zeitgebers than light-dark cycles, which is consistent with the results of previous studies using other mammalian species (Aschoff and Tokura, 1986; Francis and Coleman, 1988, 1990; Goldman et al., 1997; Pálková et al., 1999; Pohl, 1998; Rajaratnam and Redman, 1998; Tokura and Aschoff, 1983).

The characteristics of the Tn cycle are expected to strongly affect the cycle’s ability to entrain the animals’ rhythms. The range of oscillation, the direction of oscillation (above or below thermoneutrality), and the duration of the oscillation are particularly likely to affect the entrainment process. In this study, a temperature of 24 °C was chosen as thermoneutral for mice with free access to running wheels (see Materials and Methods section). The warm cycle oscillated between 24 °C and 32 °C, which covers a nonnoxious warm range of temperatures, and the cold cycle oscillated between 24 °C and 12 °C, which covers a nonnoxious cold range characteristic of early autumn in various areas of the world heavily populated by humans. Approximately 70% of the animals entrained to the Tn cycle regardless of whether it was a neutral-to-warm or neutral-to-cold cycle and regardless of whether the warm/cold phase lasted 8 h or only 1 h. Under the conditions of this study, a neutral-to-cold cycle with a period of 23 h was just as effective as a neutral-to-cold cycle with a period of 24 h, but a neutral-to-cold cycle with a period of 25 h did not produce entrainment. This suggests that the range of entrainment to a neutral-to-cold cycle in mice extends from a period shorter than 23 h to a period that is longer than 24 h but shorter than 25 h. Although the range of entrainment is expected to be dependent on the free-running period (FRP) of the animals, the mean FRP of the mice that entrained to the Tn cycle was not significantly different from the mean FRP of

Figure 5. Phase shifts of the rhythm of running-wheel activity caused by 4-h temperature pulses (32 °C on a 24 °C background) as a function of circadian time of initiation of the pulse. No clear phase-response curve is seen.
the mice that did not entrain. This was so because the mice that failed to entrain had FRPs that were often either shorter or longer than usual, so that the variances of the two groups of mice were significantly different but the means were comparable.

Only $T_4$ cycles in the 12-24 °C range and 24-32 °C range were used in the present study. Possibly, wider ranges of temperature oscillation would provide a stronger zeitgeber, and narrower ranges would provide a weaker zeitgeber, but no attempt was made here to construct an intensity-response curve for temperature perturbations. In a study on Perognathus longimembris, Lindberg and Hayden (1974) found that a range of about 2 °C was sufficient to produce entrainment and that wider ranges (up to 10 °C) did not produce more robust entrainment. In studies on Ammospermophilus leucurus and Mesocricetus auratus, Pohl (1998) found that ranges of 4 °C or 6 °C were weak zeitgebers, whereas ranges of about 10 °C were stronger zeitgebers. In the present study, the ranges were 8 °C (24-32 °C) and 12 °C (12-24 °C).

Close inspection of waveform plots of running activity (Figures 3 and 4) indicated that, when the cold/warm phase was 8 h long, the mice did most of the running slightly before, during, and slightly after the exposure to the cold but ran mostly before the exposure to the heat (during which negative masking was evident). This suggests that the circadian resetting properties of cold and warmth may be distinct, although further experimentation is needed to clarify the distinction. Consistent with the concept of distinct resetting properties of cold and warmth, it has long been known that the cold and warmth senses are psychophysically distinct in human subjects (Stevens and Stevens, 1960; Refinetti, 1990) and are served by distinct sensory receptors and distinct afferent pathways in all species that have been studied (Hensel, 1974; Schepers and Ringkamp, 2009).

An attempt to construct a phase-response curve (PRC) to $T_4$ pulses was unsuccessful in this study (Figure 5). Previous attempts in other species have yielded conflicting results. Although a consistent PRC was described for pocket mice (Lindberg and Hayden, 1974), no clear PRC was identified in common marmosets (Pálková et al., 1999), and inconsistent PRCs were described for the laboratory rat (Francis and Coleman, 1997). As an alternate approach to a phase-response curve, T cycles with only 1 h of temperature displacement per day were used in the present study. No entrainment was observed under $T = 25$ h, but the animals started running 3 to 7 h before the daily cold pulse under $T = 24$ h and about 1 h before the pulse under $T = 23$ h.

At least in Syrian hamsters, PRCs to various environmental stimuli can be grouped into two families: photic PRCs and nonphotic PRCs (Smith et al., 1992). Photic and nonphotic PRCs for the domestic mouse are shown in Figure 7. The photic PRC is simply the average of the PRCs previously constructed in four other laboratories (Benloucif and Dubocovich, 1996; Daan and Pittendrigh, 1976; Kilduff et al., 1998; van der Leest et al., 2009). The nonphotic PRC is an idealized curve inferred from the phase angles of entrainment in three studies in which mouse activity rhythms were entrained by timed physical exercise (Challet et al., 2000; Edgar and Dement, 1991; Marchant and

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Figure 6. Double-plotted actograms of running-wheel activity of 2 representative mice subjected to ambient temperature $T$ cycles with periods of 24 h (days 1 to 19) or 23 h (days 20 to 45). In each actogram, time of day is indicated on the horizontal axis and number of days on the vertical axis. The narrow open rectangles denote the cold segment (12 °C, 1 h) of the $T$ cycle.
forms of nonphotic resetting of circadian rhythms may be modulated by changes in activity levels, so that the changes in activity are the effective resetting stimulus (Mistlberger et al., 2003; Wickland and Turek, 1991). In the present study, the mice that failed to entrain to $T_n$ cycles ran significantly less on their wheels before being subjected to the $T_n$ cycle than did the mice that later entrained to the $T_n$ cycles. Thus, it is possible that the changes in $T_n$ functioned as entraining stimuli only in animals that ran above a certain threshold (about 10,000 revolutions per day in the current study). If so, the entrainment to $T_n$ cycles observed in this study could be attributed to an indirect effect of temperature on running activity, and the resulting modulation of running would be the effective resetting stimulus. Alternatively, only animals that run more than 10,000 revolutions per day might be sensitive to nonphotic resetting by temperature. In a study on Syrian hamsters (Mistlberger et al., 1996), single 3-h cold pulses evoked phase shifts of the activity rhythm in hamsters that were allowed to run during the cold exposure but not in hamsters whose wheels were locked, which suggests that temperature exerted its effect through activity; however, the authors reported also that some of the hamsters phase-shifted in response to cold pulses even though they did not run much (Mistlberger et al., 1996). In a follow-up study, the authors concluded that activity is required for resetting but that the activity levels need not be particularly high (Mistlberger et al., 2003).

Because the master circadian pacemaker can also be entrained by schedules of food availability that involve caloric restriction (Mendoza, 2007), it is possible that the entraining effect of $T_n$ cycles derives from an indirect effect on food intake. It has long been known that homeothermic animals adjust their levels of food intake to compensate for changes in metabolic heat production needed to maintain euthermia following chronic changes in $T_n$ (Hamilton, 1967), and it has been shown that ambient temperature during a meal can affect food intake independently of the temperature at which the animals are maintained before and after the meal (Kraly and Blass, 1976; Refinetti, 1988). Thus, the daily $T_n$ cycle could possibly produce entrainment through a modulatory effect on food intake.

While it is not clear at the present time whether entrainment by $T_n$ cycles in mice is caused directly by temperature or indirectly through a temperature effect on locomotor activity or on feeding, the results of this study clearly demonstrate that the rhythm of locomotor activity in mice can be entrained by ambient temperature cycles in the nonnoxious range.

![Figure 7. Photic and nonphotic phase-response curves for the domestic mouse describing the magnitude of phase shifts evoked by stimuli presented at various circadian times. The abscissa extends to 36 h to facilitate visualization of the curves. The photic curve is the mean of four curves constructed in four different laboratories using light pulses of 15- to 30-min duration and 100- to 300-lux intensity (Benloucif and Dubocovich, 1996; Daan and Pittendrigh, 1976; Kilduff et al., 1998; van der Leest et al., 2009). The nonphotic curve is an estimated curve based on the phase angles of entrainment in three studies in which mouse activity rhythms were entrained by timed physical exercise (Challet et al., 2000; Edgar and Dement, 1991; Marchant and Mistlberger, 1996). The two circles are the present study’s estimates of phase shifts derived from the phase angles of entrainment under temperature $T_n$ cycles (open circle for $T_n = 24$ h; closed circle for $T_n = 23$ h).](image-url)
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