Natural entrainment of circadian systems

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Nonphotic entrainment in a diurnal mammal, the European ground squirrel
(Spermophilus citellus)

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ABSTRACT

Entrainment by nonphotic, activity-inducing stimuli has been investigated in detail in nocturnal rodents, but little is known about nonphotic entrainment in diurnal animals. Comparative studies would offer the opportunity to distinguish between two possibilities. (1) If nonphotic phase shifts depend on the phase of the activity cycle, the phase response curve (PRC) should be about 180° out of phase in nocturnal and diurnal mammals. (2) If nonphotic phase shifts depend on the phase of the pacemaker, the two PRCs should be in phase. We used the diurnal European ground squirrel (*Spermophilus citellus*) in a nonphotic entrainment experiment to distinguish between the two possibilities. Ten European ground squirrels (*Spermophilus citellus*) were kept under dim red light (<1 lux) and 20 $\pm$ 1 °C. During the entrainment phase of the experiment the animals were confined every 23.5 h (*T*) to a running wheel for 3 h. The circadian rhythms of 6 squirrels entrained, 2 continued to free-run and 2 possibly entrained but displayed arrhythmicity during the experiment. In a second experiment, a photic pulse was used in a similar protocol. five out of 9 squirrels entrained, 1 did not entrain and 3 yielded ambiguous results. During stable entrainment, the phase-advancing nonphotic pulses coincided with the end of the subjective day, while phase-advancing light pulses coincided with the start of the subjective day: mean $\psi_{nonphotic}$ = 11.4 h; mean ψ_{photic} = 0.9 h (ψ defined as the difference between the onset of activity and the start of the pulse). The data for nonphotic entrainment correspond well with those from a similar experiments with nocturnal Syrian hamsters where $\psi_{nonphotic}$ varied from 8.09 to 11.34 h (Reebs and Mrosovsky, 1989). This indicates that the circadian phase response to a nonphotic activity-inducing stimulus depends on the phase of the pacemaker rather than on the phase of the activity cycle.

INTRODUCTION

Entrainment of the circadian activity rhythm by nonphotic stimuli inducing locomotor activity (or its correlates) has been demonstrated in several nocturnal mammalian species (Marimuthu *et al*. 1981; Rusak *et al*. 1988; Reebs and Mrosovsky 1989; Edgar and Dement 1991; Hastings *et al*. 1998), and in the crepuscular *Octodon degu* (Kas and Edgar 1999). Entrainment requires phase-dependent sensitivity of the circadian pacemaker expressed in instantaneous phase shifts induced by the stimulus or in modulations of its endogenous period (τ), or both (Beersma *et al*. 1999). The nonphotic PRC for novelty-induced activity in the hamster is such that maximal phase advances are generated by stimuli around ct 4, i.e., early in the subjective day (Mrosovsky *et al*. 1992). The nonphotic PRC is thus out of phase with the light pulse PRC (DeCoursey 1964; Daan and Pittendrigh 1976; Takahashi *et al*. 1984). Consistent with this difference is the fact that scheduled wheel running entrains the circadian system of mice and hamsters to cycles longer than τ in such a way that the scheduled activity falls at the end of the normal daily activity (Mrosovsky *et al*. 1989; Reebs and Mrosovsky 1989; Edgar and Dement 1991; Edgar *et al*. 1991; Marchant and Mistlberger 1996). Activity apparently elicits phase delays around ct 0, while light pulses at this phase cause phase advances. In addition, scheduled wheel running with cycles shorter than τ entrains hamsters when activity occurs at the beginning of their daily activity phase, around ct 12, generating phase advances (Mrosovsky *et al*. 1989).

Nocturnal and diurnal mammals have similar light pulse PRCs (Pohl 1982; Beersma *et al*. 1999). Circadian variations in pacemaker electrical activity are also similar for nocturnal and diurnal mammals with maxima during the subjective day (Sato and Kawamura 1984). These results are in line with the finding that ^{14}C-labeled deoxyglucose uptake is highest during the subjective day in both the nocturnal rat and the diurnal squirrel monkey (Schwartz *et al*. 1983). Yet the behavioral activity of nocturnal and diurnal mammals is 180° out of phase when calculated relative to the phase of the pacemaker. This offers the opportunity to assess whether sensitivity to nonphotic stimuli is related to the pacemaker phase or to the phase of the overt activity rhythm. Different phase responses to photic and nonphotic, activity-inducing stimuli might be a general characteristic of mammalian pacemakers. Alternatively, the relationship between these responses might be adaptively shaped by the particular phase relationship between pacemaker and activity. To distinguish between these alternatives, it would be of interest to learn about nonphotic PRCs in diurnal mammals. The degu (*Octodon degu*s) has been shown to be entrained by scheduled voluntary wheel running activity (Kas and Edgar 1999). This species, however, is better considered crepuscular than diurnal in view of its bimodal activity pattern and its clear preference for wheel running in the subjective night (Kas and Edgar 1998). The European ground squirrel, like other ground squirrel species, is strictly diurnal and avoids above ground activity both at night and during the twilights (DeCoursey 1989; Kenagy 1989; DeCoursey 1997; Hut *et al*. 1999, Chapter 2). We used this species to study phase angles of entrainment to nonphotic and photic zeitgebers. These zeitgebers were presented in cycles of *T*=23.5 h in order to entrain the animals by advances (*T*<τ). We report here that the phase relationship between an entraining nonphotic zeitgeber and the circadian system in diurnal ground squirrels is similar to that in nocturnal mammals.

METHODS

The European ground squirrels (*Spermophilus citellus*) used in this study were captured as juveniles near Vienna (Millesi *et al*. 1999b), or born in enclosures in Haren, the Netherlands (Hut *et al*. 1999b). Experiments were carried out during the summer phase of the annual cycle to avoid problems of periodic torpor. All animals were housed for at least 2 weeks before the experiment in a room with no temperature control and a natural light-dark cycle. Before as well as during the experiment, the ground squirrels were kept individually in lucite cages (lxwxh = 48 x 28 x 50 cm). During the experiment the animals were housed in a climate controlled room (20 ± 1 °C and relative humidity 60%). Continuous dim red light (range: 0.02 - 0.06 lux at cage level, 40x Optometer™, UDT, Santa Monica, CA, USA) was provided as background illumination. Food (rabbit breeding chow, Teurlings®, Waalwijk, the Netherlands) and water were supplied *ad libitum* and replenished every second day at variable times during daytime. Locomotor activity in the home cages was continuously recorded with passive infrared detectors, connected to a PC-based event recording system (ERS) storing the number of events in 2-minute bins.

Experiment 1: Nonphotic stimulus

Ten female ground squirrels (age: 1 to 2 years) were studied in the nonphotic entrainment experiment from April 24 until August 31, 1997. During the first phase of the experiment (19 days), the animals were kept under constant dim red light until all animals showed relatively stable free-running circadian activity rhythms. During the second phase, from May 12 until July 24, periodic nonphotic stimuli were introduced. The stimuli consisted of taking an animal from its home cage by hand and confining it to a running wheel (Ø = 30 cm) for 3 h. This was repeated every 23.5 h until the nonphotic cycle had scanned nearly a full 24-h cycle. The running wheels were located in the same room under similar light conditions (range: 0.02 - 0.07 lux). The total number of revolutions of each running wheel was recorded electronically during each 3-h pulse. In the third phase of the experiment the animals were kept under conditions identical to those in the first phase of the experiment in order to asses free-running periods of activity rhythms.

Experiment 2: Photic stimulus

Nine ground squirrels (8 females, 1 male; age: 2 years) were studied, from April 14 until July 19, 1998. This experiment was similarly designed to the first experiment except for the nonphotic stimulus which was replaced by a 3-h light pulse (TLD 36W/965, Philips, Eindhoven, the Netherlands; mean illumination at cage level: 166 lux, range: 142 - 174 lux). This photic stimulus was also repeated every 23.5 h until the photic pulse had scanned nearly a full 24-h cycle. The animals remained in their home cages during the light pulse.

Period of activity rhythms

Activity data are presented qualitatively in standard actogram format with a resolution of 2 minutes. Free running periods (τ) were estimated for the 10 days preceding the treatment and for days 6 to 15 after the treatment using the χ^2 periodogram analyses on quantitative data
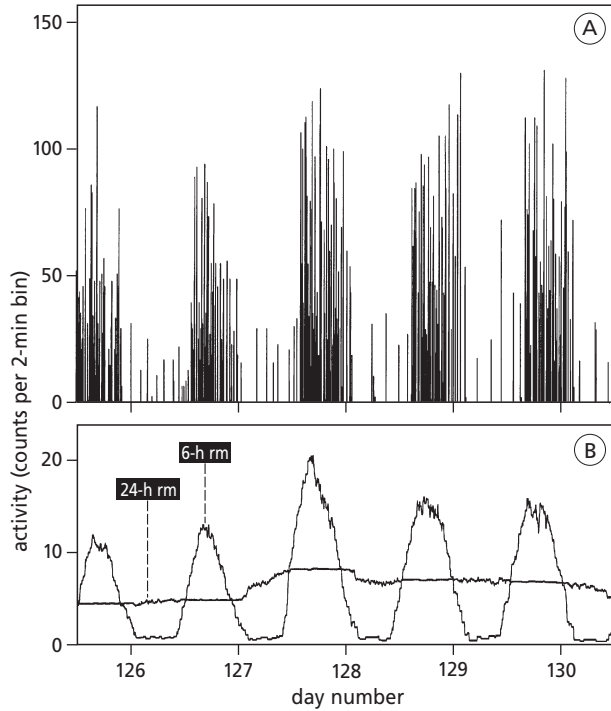
Figure 8.1 Example of determination of reference points for onset and offset of activity based upon the crossings between a 6-hour running mean and a 24-hour running mean (B) of original data (A). A crossing of the 6-h running mean from below the 24-h running mean indicates the onset of activity, the reverse indicates the offset.

(Sokolove and Bushell 1978). The first 5 days after the treatment were skipped to minimize the effect of possible transient cycles after entrainment. The strength of the circadian rhythm is indicated by ΔQ_p, which is the difference between χ^2 periodogram rhythmicity index Q_p and the $\chi^2_{(p=0.05)}$ value for the period of interest (Gerkema *et al.*, 1994). A positive peak value in ΔQ_p around 24 h was considered to represent the period of the free-running rhythm. In addition, the period of the activity rhythm before, during, and after entrainment was estimated by fitting a regression line through the calculated onsets. The χ^2 periodogram analysis is not valid when used to estimate periods of the activity rhythms during the phase of the experiment where the zeitgeber was applied. The missing data during the stimulus are re occurring every 23.5 h and therefore would produce a "significant" rhythm of 23.5 h in the periodogram analyses, even when the activity rhythm itself had a period deviating from 23.5 h.

Phase angle differences

Phase angle differences were calculated as the difference in time between the start of the zeitgeber pulse and the onset of activity. On those days, before and after the treatment, when no zeitgeber pulse was applied, the period of the zeitgeber pulse was extrapolated to calculate virtual phase angle differences. This allowed us to plot the phase of the rhythms before,

during, and after the zeitgeber treatment in the same graph. Phase angle differences were given a positive sign when activity started before the zeitgeber pulse. Onsets of activity were defined as the upward crossing between a 6-h running mean and a 24-h running mean of the original activity counts (Figure 8.1; Meerlo *et al*. 1997). During the 3-h periods when nonphotic or photic zeitgeber pulses were presented, the data were treated as missing. These missing data cause some uncertainty about the accuracy of phase angle difference calculated during stable entrainment. Therefore, in addition to average phase angle differences during the last 20 days of entrainment, we also calculated the time of the first onset after the treatment ended. This estimation is obviously based on fewer data points than the first method, but it has the advantage that there are no missing data that might affect the estimation of the time of activity onset.

RESULTS

Nonphotic entrainment

The ground squirrels in the nonphotic entrainment experiment were separated into 3 categories by visual inspection of the actograms: a) animals that were entrained (n=6; Figure 8.2a and Figure 8.3), b) animals that were possibly entrained but did not show circadian rhythmicity due to multi-day torpor (n=2;), and c) animals that showed no entrainment (n=2; e.g. Figure 8.2b).
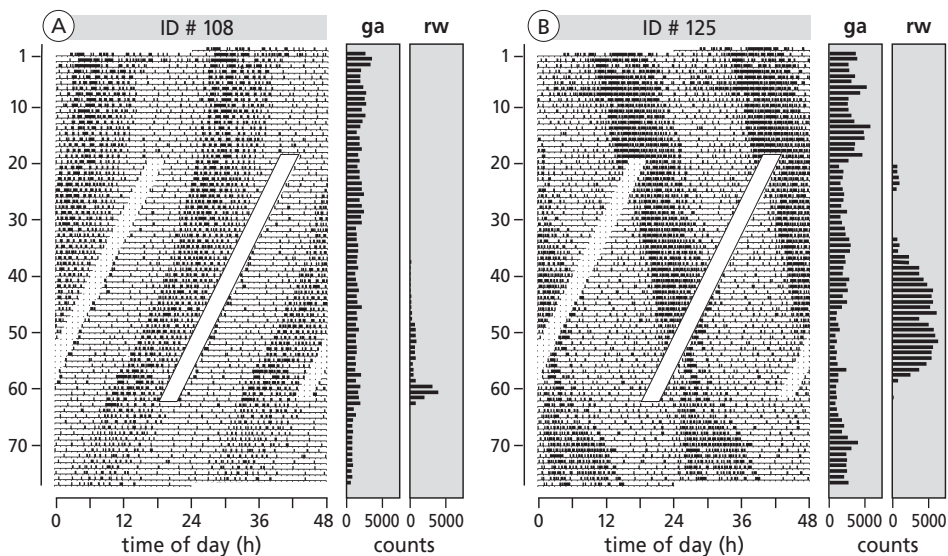
Figure 8.2 Actograms of home-cage locomotor activity of ground squirrels illustrating A) entrainment, and B) lack of entrainment to nonphotic stimuli. Total passive infrared detector counts of general locomotor activity (ga) in the home cage, and amount of running-wheel revolutions during the nonphotic pulse (rw) for each day are indicated at the side of both graphs. Days are indicated vertically from top to bottom, time of day in hours is indicated horizontally. Each horizontal line indicates two consecutive days, activity is indicated by a vertical mark, missing data are indicated by lack of a horizontal line. The 3-h confinement in a running wheel is indicated by a white bar.

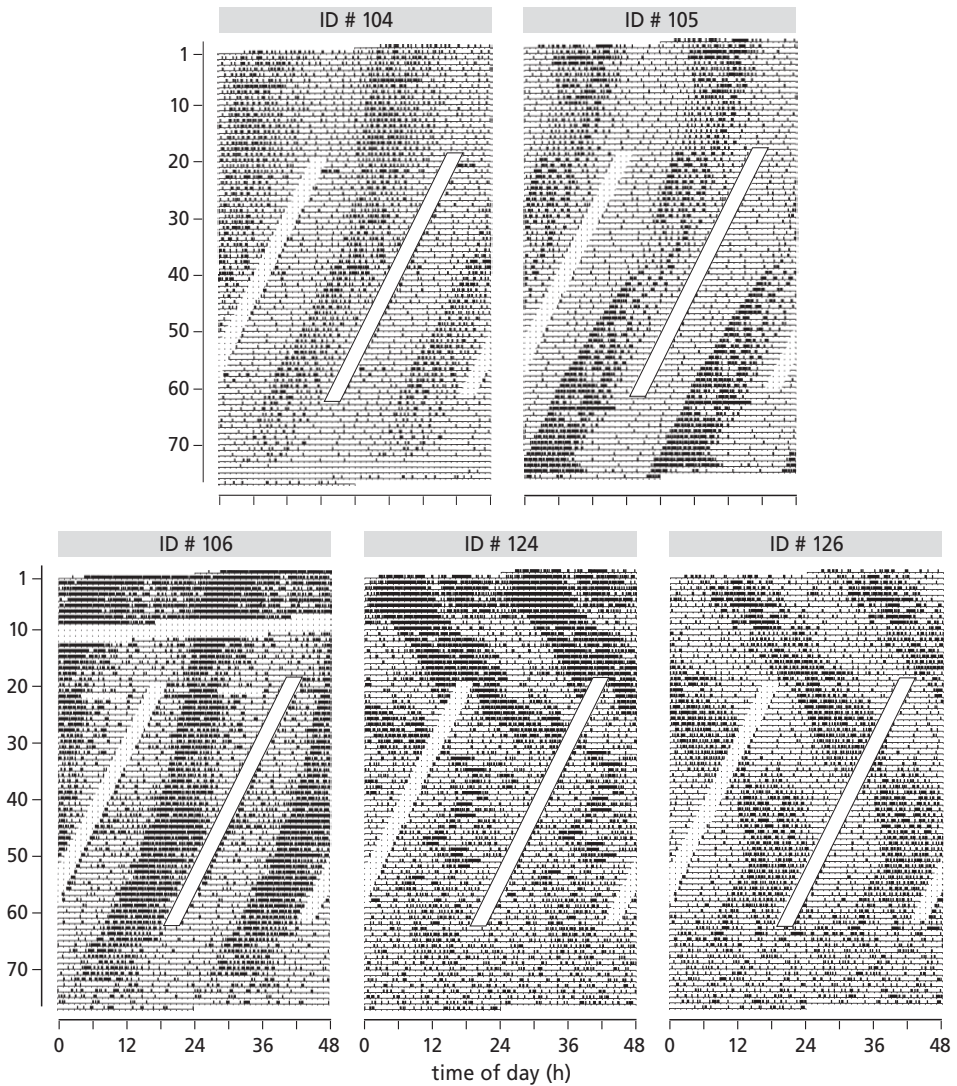**Figure 8.3** Actograms of activity patterns of five ground squirrels that entrained to the nonphotic stimulus, illustrating the consistency of phase angles of entrainment. See Figure 8.2 for information on the actogram format.

Table 8.1 Estimates of τ based on χ^2 peridogram analyses (A), Lomb-Scargle periodogram analyses (B' Ruf 1999), and objectively determined onsets (C) in the 10 days before the treatment (before), the 20 days at the end of the treatment (during), and the 10 days after the zeitgeber treatment (after). The average amount of running wheel revolutions during the nonphotic stimulus is plotted in collum D. Estimations of τ are plotted seperately for the nonphotic and the photic stimulus for those animals that entrained, were possibly entrained, and were not entrained. In 4 cases the free-running period could not be estimated after the zeitgeber treatment because of the occurrence of multi-day torpor. In the case of animal #100 only the last 10 during the treatment were analysed because phase angle differences were not stable before. Activity rhythms were tested for periods between 20 and 30 h. NS indicates the absence of a significant circadian rhythm in the data.

	response	id #	before			during		after			D
			A	B	C	B	C	A	B	C	
nonphotic	entrained	104	23.7	23.9	24.0	23.4	23.5	23.8	23.7	23.6	263
		105	23.9	23.7	23.8	23.5	23.4	23.5	23.5	23.6	5
		106	23.9	23.7	23.9	23.5	23.5	23.4	23.9	23.6	387
		108	23.9	24.0	24.0	23.5	23.5	23.5	23.6	23.7	440
		124	24.9	24.7	24.7	23.7	23.7	NS	21.5	24.3	383
		126	24.6	24.9	24.6	24.0	23.6	NS	25.2	24.8	3545
	possibly entrained	102	23.2	23.2	23.4	24.7	23.5	--	--	--	325
		110	24.4	24.5	24.6	23.6	24.7	--	--	--	386
	not entrained	125	24.4	24.2	24.1	24.1	24.2	24.2	24.2	24.4	4585
		140	23.9	24.2	24.0	24.2	24.1	24.1	24.1	24.2	3542
phutic	entrained	124	24.5	24.5	24.9	23.4	23.6	24.8	26.8	24.2	
		125	23.9	24.1	23.8	23.5	23.5	23.8	24.1	24.0	
		126	24.8	24.6	24.8	23.4	23.4	25.1	25.2	24.7	
		133	23.9	24.0	23.8	23.7	23.6	23.8	24.1	24.3	
		137	24.5	24.5	24.5	23.2	23.3	NS	24.4	24.6	
	possibly entrained	100	24.2	24.2	24.2	23.6	23.7	23.8	24.0	24.0	
		127	25.7	25.3	25.4	23.5	23.4	--	--	--	
		128	24.7	25.5	25.2	23.3	23.4	--	--	--	
	not entrained	136	24.5	24.5	24.5	24.8	24.6	24.7	24.6	24.5	

Figure 8.2 shows representative examples of an entrained and a non-entrained ground squirrel. The 2 animals that did not entrain to the nonphotic stimulus (#125 and #140) displayed a robust circadian activity pattern with high activity levels in the home cage. The number of revolutions in the running-wheel during the nonphotic pulse was considerably higher than in those animals that did entrain to the nonphotic stimulus. In general, high levels of running wheel activity occurred only when confinement to the running wheel coincided with the active phase of the animal. Figure 8.3 shows the other 5 animals that entrained to the nonphotic stimulus. Ground squirrels #104, #105, #106, and #108 had $\tau < 24$ h and all entrained rapidly. Ground squirrels #124 and #126 had $\tau > 24$ h and initially ran free but became entrained on the second opportunity; apparently $T = 23.5$ h was closer to the limits of their range of entrainment. The two animals that did not entrain, #125 and #140, had free-running rhythms with $\tau > 24$ h (Table 8.1).
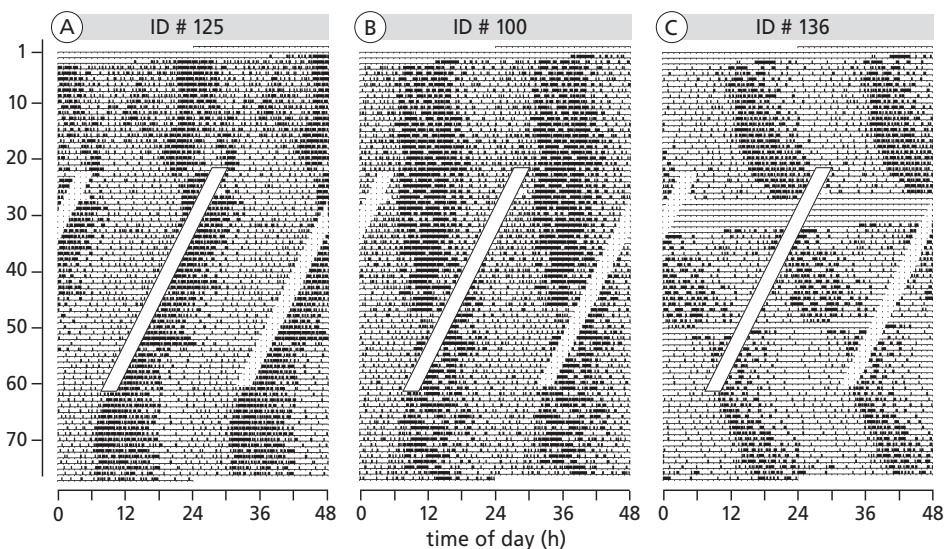
Figure 8.4 Actograms of activity patterns of ground squirrels exposed to the photic treatment. A) Example of an entrained animal. B) Example of an animal that was possibly entrained during the last phase of the treatment; however, the treatment was stopped too early to be conclusive. C) Example of an animal that was not entrained, but showed some relative coordination. To be consitsent with the nonphotic experiment, we omitted activity data during the 3-h light pulse (indicated by a white bar). See Figure 8.2 for further information on the standard actogram format.

Photic entrainment

Three groups were distinguished by visual inspection of the actograms: a) animals that showed entrainment (n=5, e.g. Figure 8.4a), b) animals that were possibly entrained but showed arrhythmicity after the treatment, or the treatment did not last long enough to entrain the animal (n=3; Figure 8.4b), and c) 1 animal that was not entrained but showed relative coordination (Figure 8.4c).

Periods of activity rhythms

For comparing the periods of the activity rhythms before, during, and after the experimental treatment we used the period estimations based on the calculated onsets (Table 8.1, C columns). All animals that entrained to the nonphotic or the photic stimulus showed shortening of the period of the activity rhythm during entrainment (two-tailed paired t-test; τ_{before} vs. τ_{during}; nonphotic: n=6, t=4.76, p=0.005; photic: n=5, t=3.31, p=0.03). During entrainment, the periods of the activity rhythms did not differ significantly from 23.5 h (two-tailed one sample t-test; τ_{during} vs. 23.5 h; nonphotic: n=6, t=0.43, p=0.69; photic: n=5, t=0.5, p=0.64); this indicates entrainment to the 23.5-h zeitgeber cycle. In the nonphotic experiment, the actograms reveal strong after effects. This is reflected in the fact that the free-running periods after entrainment were not significantly different from the activity periods during entrainment (two-tailed paired t-test; τ_{during} vs. τ_{after}; n=6, t=2.19, p=0.08). In the photic experiment, the after effects were less prominent (two tailed paired t-test; τ_{during} vs. τ_{after}; n=5, t=4.88, p=0.008).
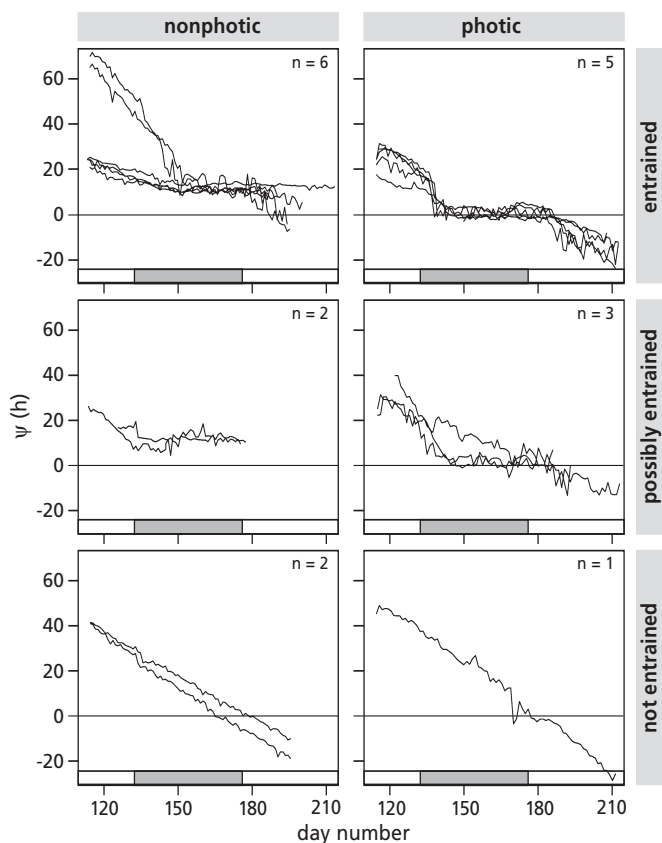
Figure 8.5 Phase angle differences (ψ) in hours between the onset of activity (ct 0) and the start of the zeitgeber pulse (extrapolated to the days before and after the zeitgeber treatment) for the nonphotic and the photic entrainment experiment. Based upon the individual curves and visual inspection of the actograms, the results are divided into three categories: entrained, possibly entrained, and not entrained. The days during which the zeitgeber pulses were given are indicated with a horizontal hatched bar.

Phase angle differences

Figure 8.5 shows the phase angle differences between activity onset and start of the (extrapolated) zeitgeber pulse for all ground squirrels in both the nonphotic and the photic zeitgeber. The results were separated in three groups: animals that a) were entrained, b) were possibly entrained, c) were not entrained.

For calculating stable mean phase angle differences during entrainment, we used two methods: averages for each entrained animal over the last twenty days of the treatment, and first activity onsets after entrainment. Differences between the nonphotic and the photic stimulus (Figure 8.6) were found to be significant with both methods (two-tailed two sample t-test; last 20 days of entrainment: n=9, t=12.05, p< 0.00001; first day after entrainment: n=9, t=10.98, p<0.00001). There were no significant differences between the two methods (two-tailed paired t-test; nonphotic: n=6, t=-0.44, p=0.68; photic: n=5, t=1.11, p=0.3).
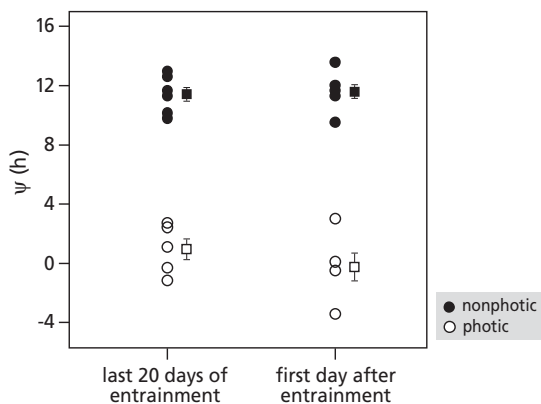
Figure 8.6 Average phase angle difference (ψ) in hours for the nonphotic and the photic entrainment experiments. The last 20 days of entrainment as well as the first day after entrainment were used to estimate the time of onset of activity during entrainment. Circles indicate individual points, squares indicate group averages, and SEM is indicated by error bars.

DISCUSSION

Methodological considerations

Research on locomotor rhythms of diurnal mammals in the laboratory is difficult because their activity patterns tend to be less precise than those of a number of nocturnal rodents. To reduce the problems associated with erratic baselines and definition of phase, we chose to rely on phase angle of entrainment to a repeatedly presented pulse, rather than attempting to quantify phase shifts produced by single pulses.

We used two different methods for analyzing the phase angle difference during entrainment. Since there is no home cage activity during the nonphotic pulse, it is important to use a phase reference point of the activity cycle that is nearly insensitive to missing data. The 6-h and 24-h running mean method for estimating activity onsets (see methods) was found to be rather insensitive to 3 h of missing data when missing data do not occur 3 h before or after the onset of activity. In the nonphotic experiment, the missing data during the pulse do not occur around the onset of activity, therefore, the running mean method for estimating the onset of activity yields reliable results. In the photic experiment, the 3 h of missing data do occur around the onset of activity during the phase of entrainment. The second method, based on the first day after entrainment where no missing data occur, is probably more appropriate for estimating phase angle differences during photic entrainment. Both methods gave similar results; therefore, one may be confident phase angles could be defined during the entrained state.

After effects

Entrainment was defined on the basis of differences between τ before the treatment and the period of the treatment (T), the stability of phase angle during the treatment, and the absence of a phase shift on the first day after the treatment. The finding that after cessation of the treatment τ did not differ significantly from the period of the rhythm during the treatment (Table 8.1) may appear inconsistent with entrainment. However, the post-treatment τ may actually be a manifestation of entrainment (Pittendrigh and Daan 1976a). Moreover, during photic entrainment the τ changes seem to work in the same direction as the phase shifts and therefore contribute to the process of entrainment (Pittendrigh and Daan 1976b,c; Beersma *et al*., 1999). This suggestion is related to the fact that τ changes caused by single light pulses in diurnal mammals result in a similar response curve (τRC) as the phase shifts (PRC) do: phase advances occur around the same circadian time when τ's are shortened while phase delays occur where τ's are lengthened (Beersma *et al*. 1999). However, the τRC as measured with nonphotic pulses in hamsters shows lengthening of τ during the subjective day when the PRC shows advances (Weisgerber *et al*. 1997; Mrosovsky 1993). Nonphotic τ changes would therefore counteract phase shifts during the process of entrainment. In addition, the circadian times where these nonphotic effects on τ occur are advanced for approximately 4 circadian hours when compared with the nonphotic PRC. On the basis of the nonphotic τRC, it is difficult to see how τ changes can add to the process of nonphotic entrainment in the Syrian hamster. However, in an nonphotic entrainment study in Syrian hamsters, Reebs and Mrosovsky (1989) did reveal after effects of the T-cycle presented. The ground squirrels entrain to the nonphotic stimulus with the pulses falling around ct 11.5, a time when the hamster nonphotic PRC shows advances and the τRC shows no change in τ. Since strong after effects are present in the ground squirrels, we would predict shortenings in τ around ct 11.5. The presence of after effects after nonphotic entrainment in both the European ground squirrel and the Syrian hamster suggests that a change of the intrinsic period of the circadian oscillator does contribute to the process of nonphotic entrainment, although this is not clearly predictable from the nonphotic τRC.

Relationship of entrainment to running-wheel activity

In Syrian hamsters, the number of revolutions made during a 3-h confinement to a novel wheel correlates with the subsequent phase shift (Janik and Mrosovsky 1993; Reebs and Doucet 1997; Weisgerber *et al*. 1997). Whether a hamster entrains or not to a repeated wheel confinement is also related to how active it is in that situation (Reebs and Mrosovsky 1989). In the ground squirrels, there was no indication that entrainment was associated with high activity during the stimulus. Both ground squirrels that did not entrain to the nonphotic stimulus showed intense running when confined to the running wheel during their active phase (Table 8.1, e.g., Figure 8.2). In addition, one animal (#105) that did entrain to the stimulus had low or zero activity in the wheel. The number of wheel revolutions appeared to depend on the phase of the circadian cycle and seemed to be unrelated to the probability of entrainment (Figure 8.2). This suggests that the primary entraining stimulus might have been the handling of the animals, possibly inducing a stress response, rather than their levels of induced activity.

Comparison with other diurnal species

In tests with human subjects given 3-h pulses of exercise (Van Reeth *et al.* 1994), illumination from constant light was kept low (<300 lux), presumably to avoid photic effects. Although some delays were obtained in the late subjective night, these were mostly quite small. The main phase delays were obtained when the exercise occurred in the early subjective night. Subsequent to these experiments, it has been found that in some circumstances light as low as 180 lux was capable of resetting circadian rhythms in humans (Boivin *et al.* 1996). It is therefore conceivable that photic input has played a role in the exercise-induced shifts reported by van Reeth *et al.* (1994). Beersma and Hiddinga (1998) found no effect of physical activity on the circadian period of core body temperature rhythms in humans in a forced desynchrony protocol, suggesting that there are no nonphotic phase responses in humans. Their analysis, however, does not rule out the possibility that exercise induced advance and delay shifts canceled out each other. Further attempts to shift circadian rhythms in humans by nonphotic means are reviewed in Redlin and Mrosovsky (1997) and Klerman *et al.* (1998), but there is not enough information to permit construction of a PRC.

Wechselberger (1995) induced activity in marmosets (*Callithrix j. jacchus*) by providing them with a mirror and at the same time playing recorded sounds of conspecific vocalisations. She found that 1 or 2 hours of this procedure produced phase advances when starting at ct 21, and delays when starting at ct 9-15. Ney *et al.* (1998) induced activity by putting marmosets in a novel cage for 2 hours. At ct 20-22, this manipulation resulted in phase advances, at ct 12-14 in phase delays. A phase advance portion in the late subjective night and early subjective day in marmosets is very different from the phase advances in the late subjective day found here with the ground squirrels. Although species might differ in response to a similar stimulus, it is also conceivable that the nonphotic stimulus in marmosets was in fact confounded by photic input. In the experiments of both Wechselberger (1995) and Ney *et al.* (1998), the marmosets were kept in constant light. Therefore, if behavioral activation caused them to open their eyes wider, they may have received an increase in photic input.

It is unlikely that unwanted photic pollution occurred in the present nonphotic experiment for three reasons. First, dim red light was used with light intensities lower than 0.06 lux and light intensities at running wheel level were similar to those at the level of the cage. Second, during stable nonphotic entrainment, the exposure to the novel wheel fell each day in the late subjective day or early subjective night. Any extra photic input at that phase should have resulted in phase delays. But the entrainment was occuring by phase advances because in all cases τ was larger than *T*. Third, when a light pulse was deliberately given on the same cycle of T=23.5 h, the phase angles during stable entrainment were about 12 h different from those with pulses of confinement to the running wheels.

Other experiments on nonphotic effects in diurnal species have been carried out by Reebs (1989) on sparrows. In his study, the animals were kept in continuous darkness and receiving daily 2-h playbacks of conspecific vocalisations. This introduces a further difficulty in arriving at a general account of nonphotic clock phase shifting in diurnal species. In the entrained state, the playbacks fell at the start of the sparrow's subjective day; at this time they exerted a phase advancing effect. With the ground squirrels, phase advances occurred when the nonphotic events were at the end of the subjective day. Therefore, at least with these particular stimuli,

the phase angles of entrainment to nonphotic input are different in sparrows from those found here with ground squirrels, although both species are diurnal.

Altogether, no uniform picture of nonphotic shifting in diurnal animals emerges. The complications that need to be resolved include potential differences between species, potential differences in the types of nonphotic manipulations, and the possibility of photic pollution of nonphotic events.

Comparison between diurnal and nocturnal rodents

The present experiments do show that a diurnal rodent species can be entrained by a periodic nonphotic stimulus. For the reasons given above, this entrainment cannot be attributed to unwanted photic input. Regardless of the extent to which entrainment was brought about by discrete phase shifts or by τ changes, it is clear that diurnal ground squirrels entrain
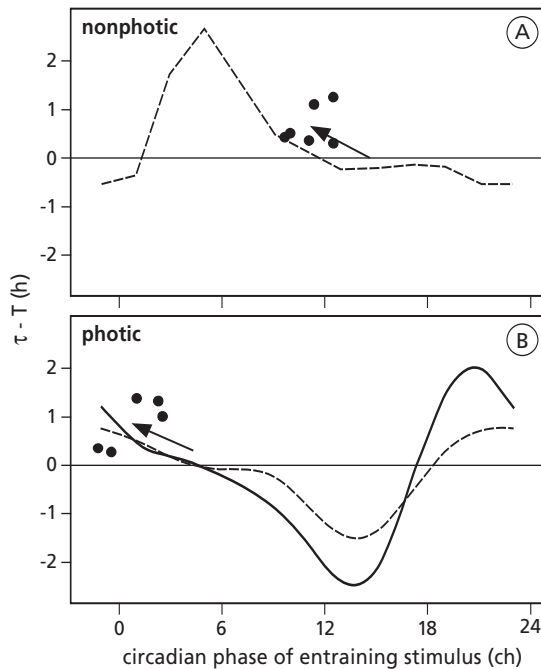

Figure 8.7 Difference between τ during the free run before the treatment and T (in hours) plotted against the circadian phase of the entraining stimulus (in circadian hours) during the last 20 days of stable nonphotic (A) and photic (B) entrainment. In order to indicate the process of entrainment by repeated phase shifts, the nonphotic PRC for novel wheel running in the nocturnal hamster (Mrosovsky *et al*. 1992) (graph A; dashed curve), the average photic PRC for 12 nocturnal mammalian species (graph B; dashed curve)(Johnson 1990; Beersma DGM, Daan S and Hut RA, in prep.), and the average photic PRC for seven diurnal mammalian species (graph B; solid curve)(Johnson 1990; Beersma *et al*. 1999) are plotted. Note that both the nocturnal and the diurnal light pulse PRC's are not corrected for intensity or duration of the used stimulus. The arrows indicate the direction of the entraining stimulus moving through the PRC until stable entrainment takes place.

to nonphotic zeitgebers with similar phase relationships between the circadian pacemaker and the zeitgeber as nocturnal rodents (Kawamura and Inouye 1979; Sato and Kawamura 1984; Kurumiya and Kawamura 1988). This can be predicted using the neurobiological model for photic and nonphotic phase shifts published by Hastings *et al*. (1998), which is based upon a peak in metabolic activity of the supra chiasmatic nucleus (SCN) in the subjective day. We suggest that nonphotic entrainment in rodents is linked to the phase of the circadian pacemaker, rather than to the phase of the activity cycle.

The results from present entrainment experiments reveal information about the diurnal photic and nonphotic PRC in the European ground squirrel, if we assume that entrainment is due to repeated "daily" phase shifts (Pittendrigh and Daan 1976b). The change in period of the activity rhythm from the initial free-run to the entrained state can be used as an indicator for the "daily" phase shift needed for entrainment. These repeated phase shifts are brought about by zeitgeber pulses that affect the circadian oscillator at a certain phase. Both variables, size of the phase shift and phase relationship of zeitgeber pulse with the onset of activity (ct 0), were used to construct points on a photic and a nonphotic PRC (Figure 8.7). During the transient days before actual stable entrainment occurs, the zeitgeber pulses move along the hypothetical PRC. In the present experiments, where $\tau > T$, the zeitgeber pulse moved along the PRC towards an earlier circadian phase every new cycle. During the transient days stable entrainment will eventually occur when the advances increase until the "daily" phase shifts fully compensate for the difference between τ and T. The direction of this process, indicated by the arrows in Figure 8.7, gives additional information on the local shape of the PRC.

Implications and questions

Our findings have potential implications for work on phase shifting the human clock by nonphotic stimuli. For instance, Van Reeth *et al*. (1994) pointed out that in nocturnal rodents induced activity produced phase shifts when given during the usual rest phase. They "hypothesized that the most likely time when exercise may have zeitgeber effects on human rhythms would be during the night" (Van Reeth *et al*. 1994: p. E 965). However, their hypothesis is based on the implicit assumption that nonphotic phase shifts are related to the phase of the activity cycle in both nocturnal and diurnal animals. With the present results in hand, this hypothesis needs to be questioned since nonphotic phase shifting in some diurnal and nocturnal rodents is quite different when expressed relative to the activity cycle. Furthermore, the few nonphotic studies in people indicate a possible delay portion of the PRC in the subjective night (Redlin and Mrosovsky, 1997), which is in line with the nonphotic hamster PRC.

The need for more work on diurnal species has been emphasized as a prelude to extensive studies with humans (Van Reeth *et al*. 1994; Redlin and Mrosovsky 1997). Unfortunately, at the moment it is not obvious what diurnal mammal would be an appropriate species. If marmosets and ground squirrels differ in the relationship of their nonphotic PRCs to rest-activity cycles, then marmosets, as primates and closer to people, would be the appropriate choice. However, before this can be said, it first needs to be resolved to what extent the results with the marmosets depend on photic pollution, and if so whether a purely nonphotic phase shifting situation can be devised for this species.

Finally, if there is any adaptive value in being able to phase shift circadian clocks in response to feedback from locomotor activity, or to behaviorally arousing situations, one might have thought that nonphotic PRCs would be, more or less, 12 hours out of phase in nocturnal and diurnal species. The present work suggests that nonphotic effects on the circdian system in rodents are related to the phase of the circadian pacemaker, not to the activity cycle. This makes an adaptive explanation of nonphotic phase shifting all the more challenging.

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