

The Two-Oscillator Circadian System of Tree Shrews (*Tupaia belangeri*) and Its Response to Light and Dark Pulses¹

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Abstract The wheel-running activity rhythm of tree shrews (tupaia; *Tupaia belangeri*) housed in constant darkness (DD) phase-advanced following a 3-hr light pulse at circadian time (CT) 21. Dark pulses of 3 hr presented to tupaia in bright constant light (LL) did not induce significant phase shifts of the free-running activity rhythm, irrespective of the CT.

In dim LL, tupaia showed simultaneous splitting of their circadian rhythm of wheel-running activity, nest-box activity, and feeding behavior. Light pulses of 6 hr and 2300 lux were presented to 13 tupaia with split wheel-running activity rhythms. These light pulses induced immediate phase shifts in the two components of the split rhythm in opposite directions. No differences were observed between the light-pulse phase response curves of the two components. Equally large immediate phase advances were induced in both components by light pulses of 230 lux, but not by 23 lux. The final phase shifts were small at all CTs. In two tupaia, activity rhythms transiently split and re-fused. Analysis of the relative position of the components in one of these indicates asymmetry in the coupling between the components.

The circadian rhythm of locomotor activity in several species of mammals dissociates into two components during prolonged exposure to constant conditions. This phenomenon of “splitting” typically occurs in bright continuous illumination (LL) in nocturnal species (Syrian hamster—Pittendrigh, 1974; rat—Boulos and Terman, 1979) and in dim LL in diurnal species (ground squirrels—Pittendrigh, 1960; Swade and Pittendrigh, 1967; Pohl, 1972; tree shrew—Hoffmann, 1969). Splitting has been most intensively investigated in the Syrian hamster (Pittendrigh and Daan, 1976b; Earnest and Turek, 1982; Ellis et al., 1982; Turek et al., 1982; Lees et al., 1983; Boulos and Morin, 1986). At the onset of splitting, two components of the circadian activity rhythm often run temporarily with different frequencies, until they reach about 180° antiphase, and a new stable phase relationship is established. Pittendrigh and Daan (1976b) have suggested that the two components reflect the action of two functionally separate oscillators in the mammalian pacemaker. They proposed that these have slightly different light sensitivities, such that in natural conditions of light and darkness, one component primarily locks on to dusk and the other to dawn. The complex system would thereby be capable of adjustment to the annual variations in daylength. Using a simple simulation model, Daan and Berde (1978) showed that the characteristics of splitting as well

1. This paper is dedicated to Dr. Klaus Hoffmann, in recognition of his critical contributions to chronobiology.

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as of photoperiod effects on a two-oscillator system may be due either to different light sensitivity of the two oscillators, or to an effect of illumination on their coupling.

The two-oscillator hypothesis of Pittendrigh and Daan (1976b) has been found consistent with a number of characteristics in the rat pineal *N*-acetyltransferase (NAT) rhythm, where evening rise and morning decline behave as though controlled by two separate oscillators (Illnerová, 1986). However, the two putative oscillators have not been identified so far (see Pickard and Turek, 1982, 1983; Davis and Gorski, 1984), nor has their differential responsiveness to light and dark been established (Boulos and Rusak, 1982a; Boulos and Morin, 1986). Boulos and Morin (1986) have shown that in the split hamster system, either component may lock on to a periodic dark pulse. The high background illumination required to induce splitting in hamsters, however, precluded an analysis of light effects on the different components when separately discernible in antiphase. It is therefore important to extend these studies to diurnal animals, where splitting occurs in low LL. Also, since the two-oscillator model is essentially a functional model, relating putative pacemaker structure to the daily time span over which animals are endogenously programmed to be active, we expect that a comparative approach to diurnal and nocturnal systems should further enhance our insight.

For these reasons, we have decided to follow up on the pioneering work by Hoffmann (1969, 1971) on the circadian system in the diurnal tree shrew (tupaia; *Tupaia belangeri*). Hoffmann showed that tupaia circadian rhythms split predictably into two components when LL intensity is reduced below 1 lux. He further demonstrated hysteresis in the phenomenon, since for re-fusion of the two components, light intensities above 100 lux were required. In this paper, we analyze the effects of single light and dark pulses in unsplit and split tupaia rhythms. We further analyze the interaction between the two oscillators involved in a single case where the components showed relative coordination in an intermediate light intensity without reaching stable antiphase.

METHODS

A total of seven male and six female tupaia, varying in age from 1 to 8 years, were kindly given to us by Prof. Dr. D. von Holst. Most of these animals had been born and reared in his laboratory in Bayreuth, Federal Republic of Germany. The animals were individually housed in cages (65 × 45 × 68 cm, $n = 10$; 40 × 27 × 25 cm, $n = 3$). The first type of cage contained a running wheel with a diameter of 44 cm. To the second cage (the same as used originally by Hoffmann, 1969), a running wheel (diameter 44 cm) and a food box were externally attached. A removable nest box was fastened to both types of cages. Water and food (Altromin-8031 Zucht-/Haltungsdiaät-tupaia) were provided *ad libitum*. A quarter of both an apple and an orange and half a banana were supplied twice a week. The cages were placed in two sound-attenuated rooms at a temperature of 22° ± 1°C. Running-wheel activity was recorded throughout the experiment by an Esterline–Angus event recorder. In addition, feeding behavior and nest-box activity were recorded from three animals by an IR light-beam interruption sensor.

APPLICATION OF LIGHT AND DARK PULSES

Four series of experiments were performed: (1) light pulses presented at circadian time (CT) 21 against a dark background; (2) dark pulses presented against a light background; (3) light

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pulses presented to animals with split rhythms in dim LL; and (4) light pulses with different light intensities applied at CT 12 and CT 24 of the split rhythm.

1. Ten tupaia (five males and five females) were entrained to a light–dark cycle (LD 12:12) for at least 2 weeks and then released into constant darkness (DD). On the seventh day of DD, they received a 3-hr light pulse with the midpoint aimed at CT 21. Activity onset was defined as CT 0. The animals received the light pulse while they were locked up in their nest boxes. During the pulse, the roof of each nest box was replaced by opaque glass. The nest box was placed in a container in which 10 fluorescent light tubes were affixed. The resulting light intensity in the nest box was 2300 lux. A fan in the container prevented the temperature from rising.

2. In the second experiment, seven tupaia (four males and three females) were exposed to bright LL (intensity at the bottom of the cage with the light sensor directed upwards ranged from 465 to 720 lux). Dark pulses with a duration of 3 hr were applied to these animals simultaneously by switching off the room lights. The minimum interval between consecutive dark pulses was 3 weeks.

3. Prolonged exposure to dim LL (0.01–2.8 lux) induced splitting of the free-running rhythms in 13 animals (7 males and 6 females). Light pulses of 2300 lux were presented to animals with split activity rhythms following the same procedure as in unsplit animals. However, the duration of the light pulses was 6 hr instead of 3 hr in order to obtain larger phase shifts, and these light pulses were applied at all phases of the circadian cycle. A minimum interval of 3 weeks between the light pulses was maintained.

4. In 13 animals with split rhythms, the effects of 230- and 23-lux light pulses (6 hr) were investigated. These pulses were presented only at $CT\ 12 \pm 1.5\ hr$ and at $CT\ 24 \pm 1.5\ hr$ —that is, at those CTs at which the largest phase advances and phase delays had been observed for 2300 lux. The light intensities were obtained by placing either one or two neutral density filters on the opaque glass top of each nest box.

DATA ANALYSIS

For animals in DD, the free-running period was estimated by linear regression over activity onsets. Light-induced phase shifts were determined by fitting a regression through the last seven activity onsets prior to the light pulse and a second regression through the activity onsets from cycles 3 to 13 following the pulse. Phase shifts were then estimated by extrapolating both regression lines to the first cycle after the light pulse.

Phase shifts in LL were estimated in a similar way, but the regression lines were now calculated over the last 2 weeks prior to the dark pulse and from cycles 3 to 17 following the pulse. These longer intervals could be used, since the unsplit rhythm appeared more stable in LL than in DD. Furthermore, we used activity offset for designation of phase (CT 12) of the rhythm, since this is the most precise marker of each of the two components in the split condition and at least as precise as activity onset in the unsplit bright-LL condition. If both onset and offset were sufficiently precise, the change in activity time could be calculated (Table 1).

In tupaia with split rhythms, the offset of both components was always much sharper than the onset, and hence only the offset was used to calculate phase shifts. The offset was defined as CT 12 for each component separately in the splitting situation. We attempted to discriminate between the two components by tracing them back to the original initiation of

TABLE 1. Effect of Light and Dark Pulses on the Unsplit Circadian Activity Rhythm in *Tupaia belangeri*

	<i>n</i>	$\Delta\phi$ (SEM)	$\Delta\tau$ (SEM)	$\Delta\alpha$ (SEM)
Light pulses in DD				
CT 21	10	1.94 (0.19)	0.52 (0.08)	
Dark pulses in LL				
CT 0–3	6	0.25 (0.51)	0.03 (0.04)	–0.04 (0.65)
CT 3–6	8	–0.03 (0.22)	–0.06 (0.05)	–0.47 (0.39)
CT 6–9	3	–0.04 (0.56)	0.01 (0.07)	0.37 (0.21)
CT 9–12	3	–0.45 (0.54)	0.00 (0.02)	0.44 (0.61)
CT 12–15	6	–0.69 (0.22)	0.12 (0.08)	0.64 (0.49)
CT 15–18	6	–0.39 (0.35)	0.04 (0.06)	–0.11 (0.66)
CT 18–21	10	–0.36 (0.52)	–0.03 (0.09)	0.51 (0.42)
CT 21–24	3	–0.01 (0.60)	–0.09 (0.12)	0.94 (0.22)

Note. *n*, number of pulses; $\Delta\phi$, steady-state phase shift; $\Delta\tau$, increase in period (τ); $\Delta\alpha$, increase in activity time (α).

the split. The component that originally ran with a slightly longer period than the other was called component 1, and the other component 2.

The effects of light pulses were described for both components separately. The first light pulse was always presented after the animals had displayed a stably split rhythm for at least 4 weeks. Other pulses were presented with a minimum interval of at least 4 weeks. When rhythms were unstable, we did not present a light pulse, and light pulses that were followed by an unclear record were not analysed. Phase shifts were estimated similarly for dark pulses, but the regression line following the light pulse was now calculated from day 7 onwards. Immediate phase shifts were further determined for the first 7 days following a light pulse. For both components, a phase response curve (PRC) was plotted for immediate as well as for final phase shifts. The significance of the phase shifts was tested by analysis of variance. In all figures and calculations, the CTs of pulse administration refer to the midpoint of the light or dark pulses.

RESULTS

LIGHT PULSES AT CT 21 IN UNSPLIT RHYTHMS IN DD

Ten animals were initially stably entrained to an LD cycle. When they were next exposed to DD, all tupaia exhibited a free-running period longer than 24 hr (Fig. 1). After a 3-hr

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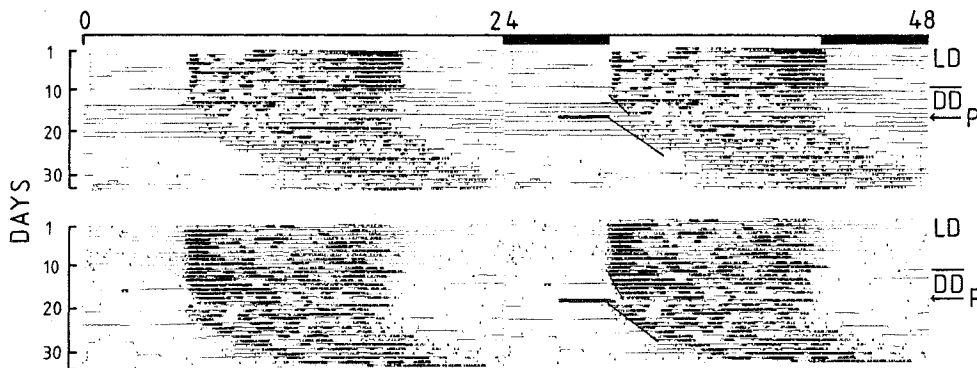


FIGURE 1. Double-plotted actogram of two tupaia. Days are plotted underneath each other. The time of day is indicated above the record. Running-wheel revolutions are marked by a vertical deflection of the pen recorder. During the first 10–13 days, each animal was entrained to LD 12:12. Seven days after release in DD, the animal was exposed to a 3-hr light pulse (indicated on the right by P, and in the activity plot by a black bar).

light pulse at CT 21, the animals showed a significant mean phase advance of 1.94 hr (see Table 1).

Before the light pulse, the mean period was 24.18 hr ($SEM = 0.04$; $n = 10$). After the light pulse, the period length was calculated over those days in which the period appeared stable. During days 3 to 13 following the pulse, the period was 24.70 hr ($SEM = 0.09$). The phase shift calculated was too large to be merely attributable to the spontaneous lengthening of the free-running period, and the increase in period was not correlated with the magnitude of the phase shift. After day 13, the period gradually lengthened until the activity rhythm began to split about 1 week later. This suggests that the change in period documented in Table 1 may not have been due to the light pulse. All animals were then re-entrained to LD 12:12. Subsequent exposure to DD induced splitting in the activity rhythm within 1 week. This rapid splitting made it impossible to investigate the effect of further light pulses on the unsplit free-running activity rhythm of tupaia in DD.

DARK PULSES IN UNSPLIT RHYTHMS IN BRIGHT LL (APPROXIMATELY 600 LUX)

Seven tupaia were exposed to LL ranging from 465 to 720 lux while their running-wheel activity was recorded. The free-running period of most animals was smaller than 24 hr and appeared rather stable (Fig. 2). During the first 20 days in bright LL the mean period was 23.86 hr ($SEM = 0.05$; $n = 7$), while after 3 months the period was 23.95 hr ($SEM = 0.12$; $n = 7$). All animals received seven dark pulses of 3 hr at various phases of their circadian cycle. The minimal interval between these pulses was 3 weeks. In most cases, the onsets and offsets of activity were equally clear. This allowed us to investigate phase shifts in both. We did this only when the beginning or end of activity, or both, were clearly defined; as a result, 12 out of 56 dark pulses were not analyzed.

The mean phase shifts were determined for 3-hr CT intervals (Table 1). At all time intervals, the phase shifts were smaller than 0.5 hr. A change in free-running period was

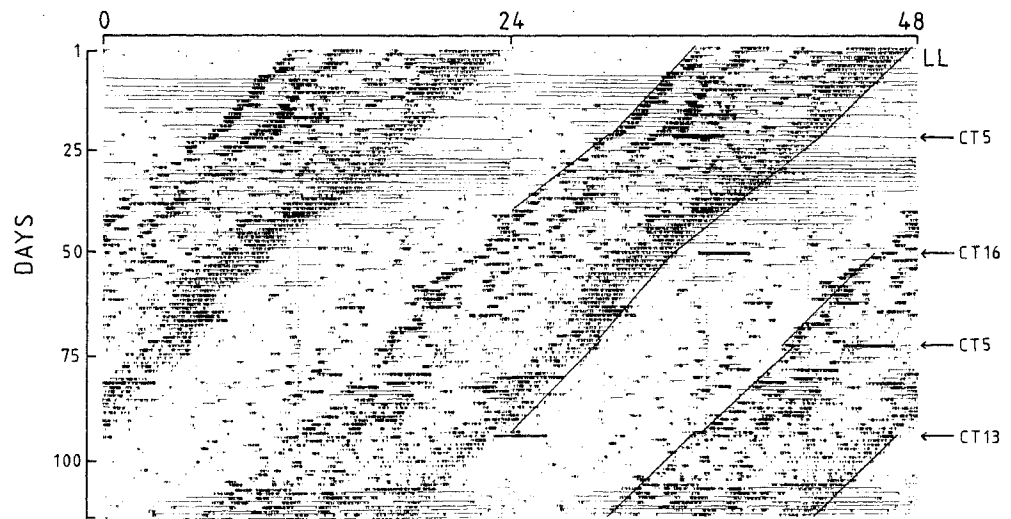


FIGURE 2. Double-plotted actogram of a tupaia in bright LL. The day and CT of 3-hr dark pulses are marked along the vertical axis and in the record.

sometimes observed following a dark pulse (Table 1). However, the mean changes in phase and period were not significant at any interval. The clear onset and offset of activity allowed us also to determine the duration of activity (Table 1). At no CT did dark pulses produce significant changes in activity time.

LIGHT PULSES IN SPLIT RHYTHMS IN DIM LL (APPROXIMATELY 1 LUX)

Thirteen tupaia were kept in dim LL (Fig. 3). Continuous exposure to low light intensities induced splitting of the circadian activity rhythm in all animals within 50 days. In three tupaia, not only running-wheel activity but also nest box activity and feeding behavior were recorded. In both the split and the unsplit conditions, these three rhythms corresponded closely to one another. Thus, during episodes at which an animal ran in its wheel, it also walked in and out of its nest box and approached its food. The activity rhythm appeared more distinct than the other rhythms under all lighting conditions. For these reasons, we decided to continue by recording only running-wheel activity.

The initiation of splitting was qualitatively similar among the animals. There was some variation in the number of days necessary to reach a stable split rhythm (range = 14–50 days). Those animals that did not immediately split when released in dim LL had a range of free-running periods of 23.4 to 23.7 hr. Prolonged exposure to dim LL induced an increase in activity in the middle of the active period, while at the same time, bouts of activity arose just before and just after the main activity time. The duration of the enhanced activity in the middle shortened gradually until the bouts of activity appeared as two distinct components in the record. These components attained a stable and equal period when they were roughly 12 circadian hours apart. The period of the split rhythm appeared constant in the course of the recordings. The mean period was 24.36 hr ($SEM = 0.37$) at the beginning of splitting and 24.39 hr ($SEM = 0.38$) at the end of the recordings, covering an interval of 7–36

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months. The period of the split rhythm was always larger than the period of the previously unsplit rhythm under the same light intensity.

Light pulses of 6 hr were presented to 13 animals with a split activity rhythm (Fig. 4). In split activity rhythms of tupaia, the offset of activity is more sharply defined than the onset. This can also be seen in Hoffmann's (1969, 1971) original records. The CT of light-pulse presentation was therefore expressed relative to activity offset. The offset of activity was defined as CT 12 in the separate analyses for both components.

We discriminated between immediate and final phase shifts of the split rhythm. Light pulses could induce immediate phase delays and advances of the split activity rhythm (Fig. 5). When an immediate phase advance was observed in component 1, an immediate delay was often observed in component 2, and vice versa. The PRCs for immediate shifts are presented in Figures 5A and 5B. Phase delays were obtained with light pulses around the activity offset (CT 12) and phase advances with light pulses around CT 24. Multivariate analysis of variance (MANOVA) over CTs 0–6, 6–12, 12–18, and 18–24 showed a significant phase dependence of the phase shifts in component 1, $F(3, 69) = 13.27, p < 0.0005$. The PRC for immediate shifts of component 2 exhibited the same phase dependence, $F(3, 77) = 11.46, p < 0.0005$. A Student's t test revealed no significant differences between the PRCs at any of the CTs. Moreover, the mean differences between the phase shifts in components 1 and 2 appeared negligible (0.056 hr; $SEM = 0.1205; n = 8$). Because the components were in a split condition about 12 circadian hours apart, they responded differently

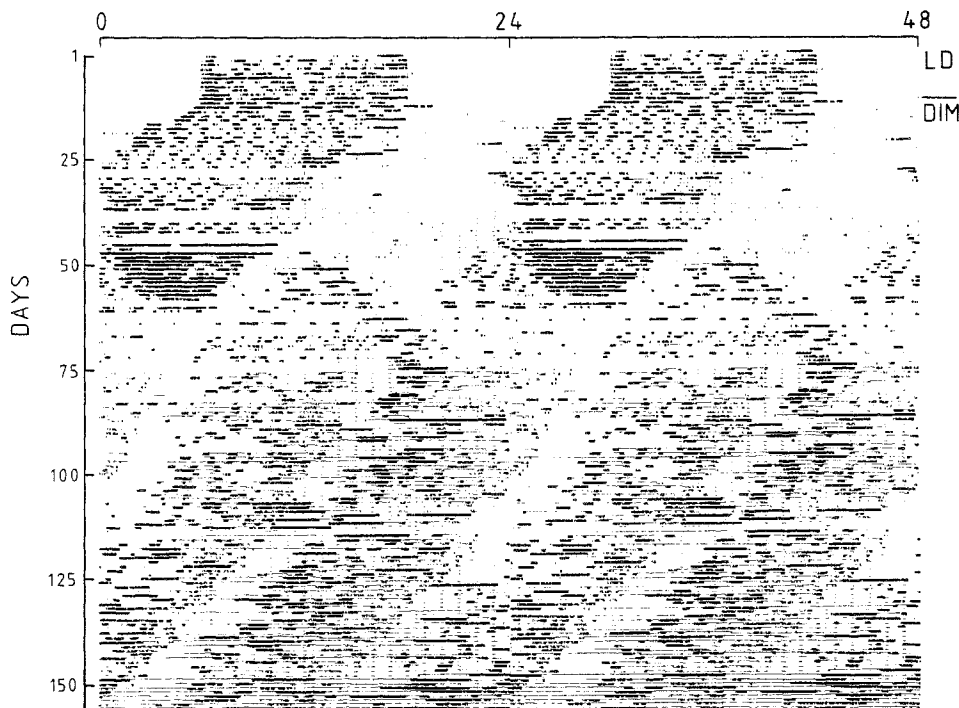


FIGURE 3. Double-plotted actogram of a tupaia in dim LL. From day 1 to day 12, the animal was exposed to LD 12:12. Prolonged exposure to dim LL induced splitting of the circadian activity rhythm.

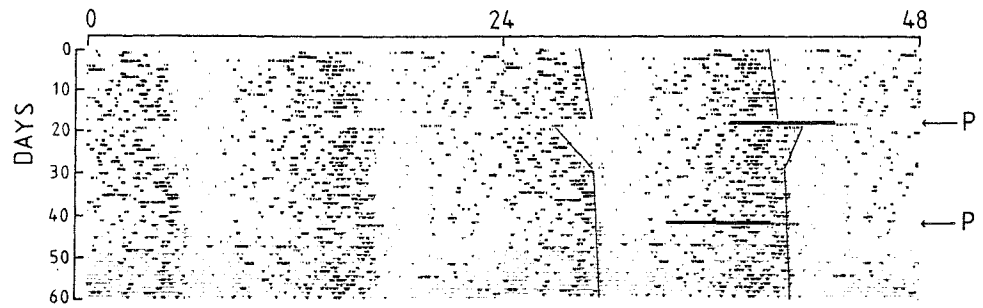


FIGURE 4. Light-induced instantaneous phase shifts of the split activity rhythm of a tupaia in dim LL. Light pulses are indicated by black bars in the activity record. The first pulse hit one component around CT 12 (activity offset) and caused an immediate phase delay in this component. The other component received the light pulse around CT 24 and responded with an immediate phase advance. After about seven transient cycles, the components returned to their prior phase relationship. The second light pulse did not elicit phase shifts in either component.

to a single light pulse. For instance, a pulse that hit component 1 around CT 12, and caused an immediate phase delay, at the same time hit component 2 around CT 24 and caused an immediate phase advance.

Although phase shifts were often substantial during the first few days after a light pulse, they tended to become smaller in the next few days. The mean phase shifts in activity offsets relative to the initial regression were estimated for each single day of the first week. The

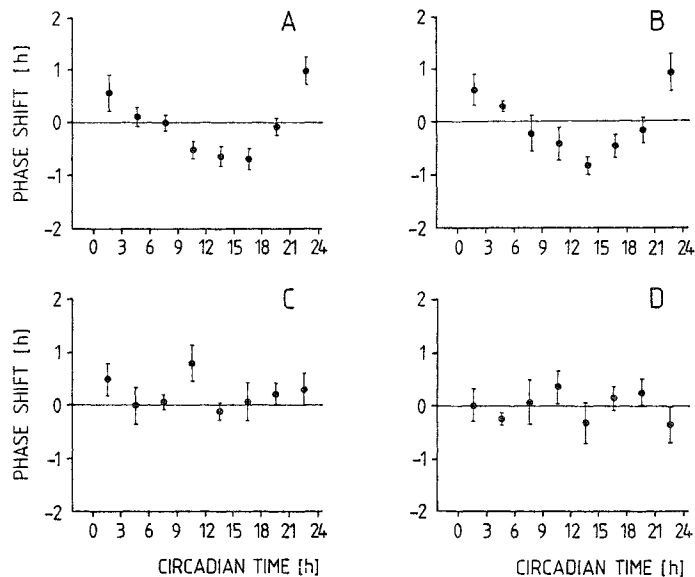


FIGURE 5. PRCs for 6-hr bright light pulses in split tupaia rhythms. (A) Immediate phase shifts in component 1. (B) Immediate phase shifts in component 2. (C) Steady-state phase shifts in component 1. (D) Steady-state phase shifts in component 2. Dots are mean shifts determined for 6-hr pulses centered at 3-hr intervals throughout the circadian cycle. Error bars indicate 1 SEM. The CT of pulse administration refers to the midpoint of the light and dark pulses.

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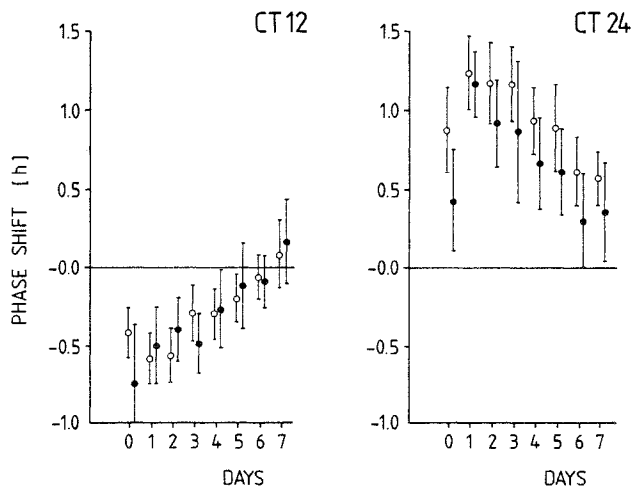


FIGURE 6. Transient resetting by 6-hr bright light pulses in split tupaia rhythms. Mean phase shifts are plotted (\pm SEM) for component 1 (open circles) and component 2 (filled circles) from day 0 until day 7 following a light pulse. Phase shifts were computed as the time difference between activity offset and the extrapolated regression through offsets -14 to -1 before the light pulse. The CT of pulse administration refers to the midpoint of the light and dark pulses. Left panel: Light pulse at CT 12 ± 1.5 hr. Right panel: Light pulse at CT 24 ± 1.5 hr.

results indicated that the phase shifts disappeared gradually within 7 days (Fig. 6). As a result, there was virtually no steady-state phase shift observable in either component (Figs. 5C and 5D), nor were there any significant changes in period length following a light pulse.

The relationship between the intensity of the light pulse and the immediate phase shift was studied by applying light pulses with intensities of 23 and 230 lux at CT 12 (± 1.5 hr) and at CT 24 (± 1.5 hr). The phase shifts obtained by these light pulses were compared with those obtained by 2300-lux pulses at the same CTs (see Table 2). At CT 24, the mean immediate phase shifts for 23-lux pulses were negligible for both components, while for 230-lux pulses the mean phase advances were almost equal to the shifts induced by 2300-lux pulses. At CT 12, phase shifts for 23-lux light pulses were also negligible for both components. At 230 lux, the phase shift of component 1 was about equal to the shift induced by 2300 lux. For component 2, the shift for 230 lux at CT 12 was still very small, compared to the substantial shift for 2300 lux observed in both components. Relative phase shifts were tested at CT 24 because only at this CT were considerable shifts observed. A Mann–Whitney

TABLE 2. Mean Immediate Phase Shifts of Components 1 and 2 in Response to 6-hr Light Pulses of Different Intensity

Intensity of light pulse	CT 12 ± 1.5 hr		CT 24 ± 1.5 hr	
	Component 1	Component 2	Component 1	Component 2
2300 lux	-0.52 (0.15; 19)	-0.62 (0.23; 15)	1.13 (0.25; 12)	1.15 (0.19; 15)
230 lux	-0.56 (0.33; 11)	-0.19 (0.23; 9)	1.21 (0.53; 5)	1.06 (0.37; 8)
23 lux	-0.27 (0.24; 7)	-0.08 (0.15; 10)	0.42 (0.57; 7)	0.15 (0.38; 6)

Note. SEM and *n* are given in parentheses after each entry.

U test indicated no significant differences between 230 and 2300 lux for both components. The difference between phase shifts induced by 23 and 230 lux was significant ($p < 0.025$) for component 2 but not for 1; this was due to one large advance of almost 3 hr.

A CASE OF UNSTABLE SPLITTING

In tupaia T4, which was exposed to an LL intensity of (on average) 2.8 lux, a split of the circadian activity rhythm did not occur until after about 130 days of recording. However, the two split components never reached a stable antiphase during nearly 20 months (Fig. 7). Instead, they appeared to be continuously crossing each other, with a clear expression of “relative coordination” (von Holst, 1939)—that is, with beat phenomena due to modifications in the period of both components, depending on their varying phase relationship. We consider this case, although exceptional among the tupaia records obtained, of particular value in understanding the interaction between the components. In Figure 7, the record has been reproduced in a sixfold plot to facilitate both the illustration of the original record, and our interpretation of the temporal course of both components. Component 2, defined as the one running initially faster than the other, appeared to run for nearly 600 days with a virtually constant period of about 23.6 hr. Component 1 ran more slowly and crossed component 2 five times in the course of the record.

If our interpretation of the position of the two components is correct, it is possible to derive response curves that may reflect the interaction between the two oscillators that created this pattern. These are shown in Figure 8. Activity onset and end for each component were derived from the interpretational lines through them every 10th cycle, and the circadian period in between then plotted as a function of the phase difference (in circadian hours) between the components. Clearly, component 2 was virtually insensitive to the relative phase position of component 1, but component 1 varied systematically in period length. Its period was shortest when approximately in phase with component 2, and longest when approximately in antiphase. It is important here that there was clear asymmetry in the coupling between the components.

The situation in 2.8 lux, with component 2 being dominant (i.e., hardly affected by component 1), changed dramatically when light intensity was further reduced to 0.01 lux on day 650. A slight lengthening of the period of component 1 and a more drastic change in the period of component 2 occurred; these changes were apparently sufficient to let the components lock on in stable antiphase position. The rare continuous crossing of two components in Figure 7 is illuminating for how central circadian oscillators may exert control over locomotor activity. Where overlap occurred, the activity increased markedly. Suppression of activity in one component seemed particularly pronounced in the hours just after activity end of the other component (Fig. 9). Such suppression may lead to a more clearly marked activity offset in both components in the split situation.

DISCUSSION

In several diurnal mammals (*Ammospermophilus leucurus*, *Tamias striatus*, and *Tamiasciurus hudsonicus*), light-pulse presentations at the end of the subjective night produce phase advances of the free-running rhythm (Kramm, 1975, 1976; Kramm and Kramm, 1980; Pohl, 1983). Tupaia is no exception to this. A 3-hour light pulse presented at CT 21 induced

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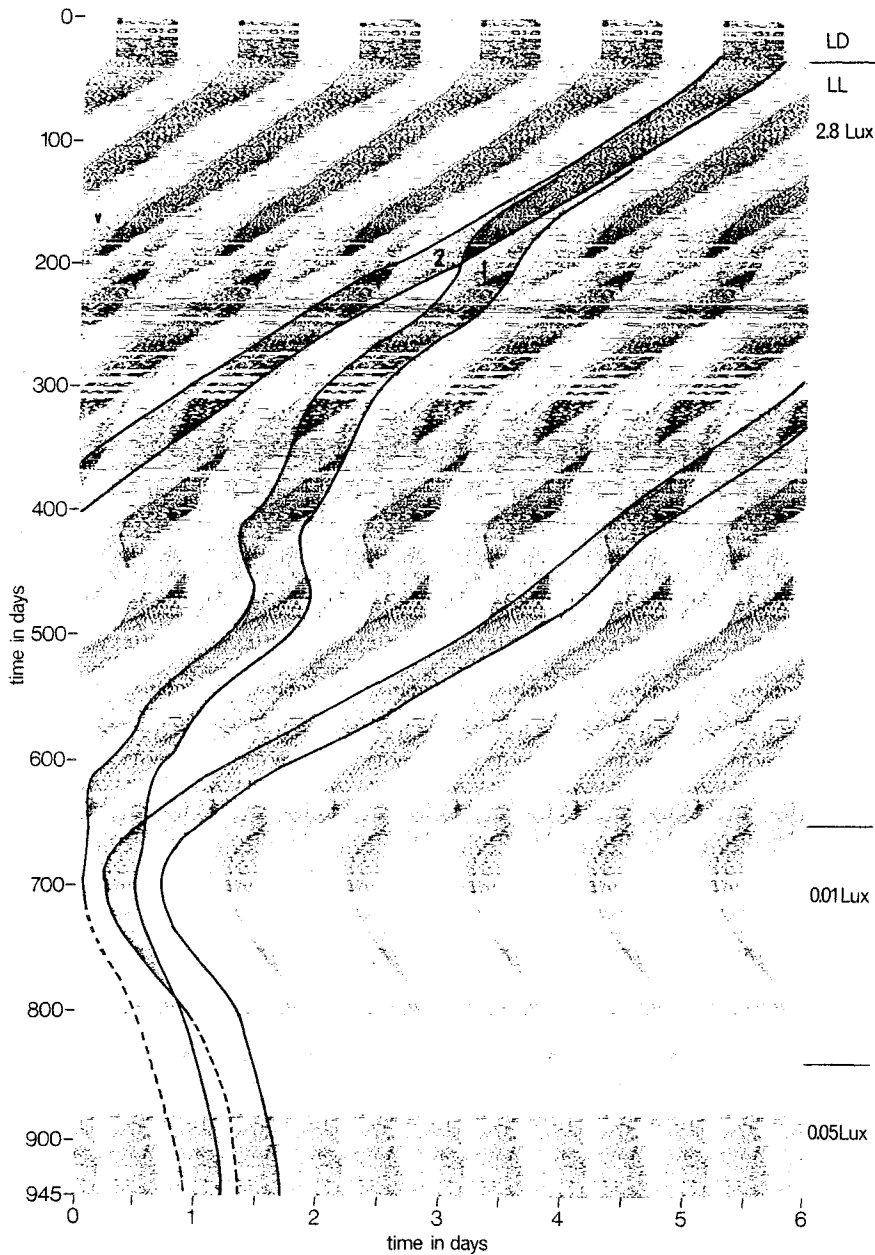


FIGURE 7. Sixfold plot of activity of tupaia T4, studied for 600 days in dim LL (mean intensity 2.8 lux). Drawn lines indicate our interpretation of activity onsets and offsets as controlled by the two components. Notice the stable period (approximately 23.6 hr) of component 2 and the relative coordination in component 1 as it crosses the other five times. After reduction of the light intensity to 0.01 lux, both components reached stable antiphase.

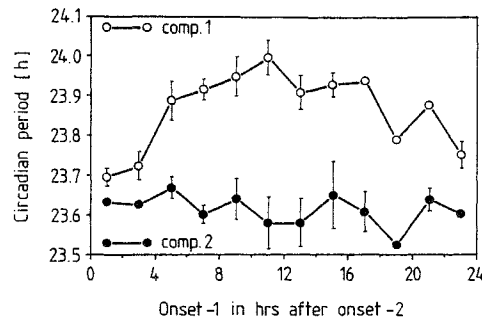


FIGURE 8. Period response curves for the interaction between the two oscillators involved in Figure 7. Symbols represent circadian periods (of 10-cycle sections) of components 2 (filled circles) and 1 (open circles) as averaged over 2-hr bins of phase relationships between the two.

a substantial phase shift in the activity rhythm. Generally, the PRC of diurnal animals has no dead zone, and since large species differences have been observed (Pohl, 1982), it would be worthwhile to determine a complete PRC for *Tupaia belangeri*. However, the free-running rhythm in tupaia splits within 3 weeks after the first release in DD and almost immediately following the second exposure to DD. Such facilitation of splitting as a consequence of prior splitting has also been described for the hamster (Earnest and Turek, 1982). This phenomenon made it impossible to characterize the effects of light pulses in DD at other CTs. In *T. hudsonicus*, *T. striatus*, and *A. leucurus*, phase-advancing shifts are often accompanied by a decrease in period (Pohl, 1982), as in nocturnal rodents (Daan and Pittendrigh, 1976a).

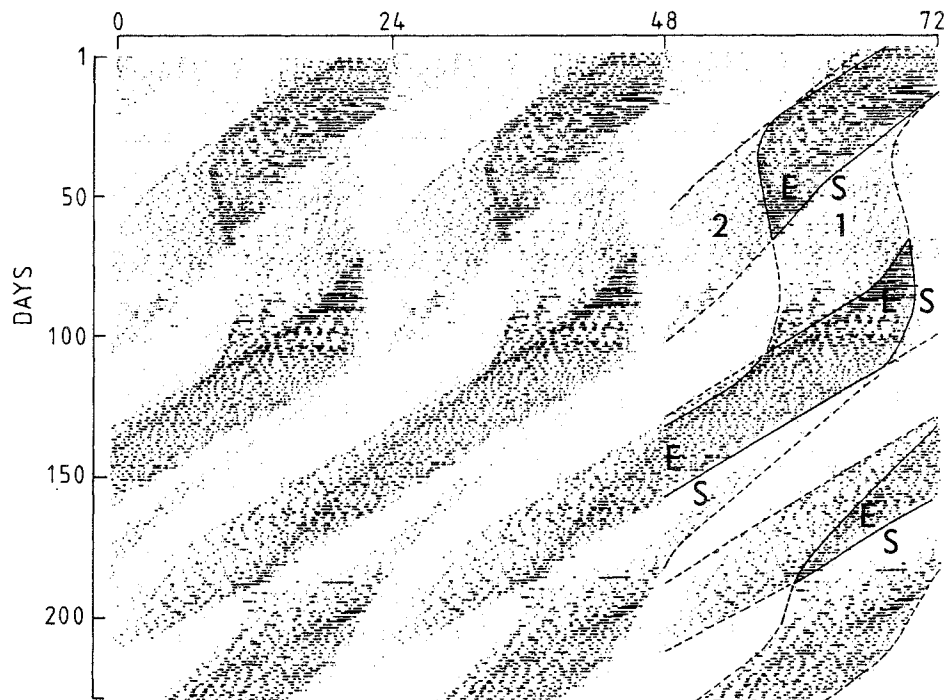


FIGURE 9. Detail from the actogram in Figure 7. Notice the enhancement of activity where activity phases of both components overlap (indicated by E on the right-hand plot) and the suppression of activity of a component when the other has just terminated activity (indicated by S).

In our study, the light-induced phase advance was followed by an increase in period. From our data, it cannot be decided whether the increased period was caused by the light pulse or by the exposure to DD itself.

In nocturnal mammals, light pulses of several minutes are sufficient to phase-shift the circadian system (DeCoursey, 1960; Daan and Pittendrigh, 1976a), while dark pulses of several hours are required to produce equally large shifts (Boulos and Rusak, 1982b). In diurnal mammals, long light pulses are necessary to phase-shift the circadian system in ground squirrels (Pohl, 1982) as well as in tupaia. The effect of dark pulses had not previously been described for a diurnal mammal. In this study, dark pulses were presented against a background intensity of 465 to 720 lux. On the basis of a saturating pulse intensity of 230 lux (Table 2), we surmise that the background intensity was high enough to test the responsiveness to dark pulses. Moreover, the difference in pattern and period between the animals in DD or dim LL and in bright LL indicates that the system was responsive to this intensity. Nevertheless, 3-hr dark pulses did not induce significant shifts of the activity rhythm at any circadian phase. Thus, we conclude that the diurnal tupaia is more sensitive to light against a dark background than to darkness against a light background, which is similar to what is found in nocturnal animals.

The mean free-running period in DD exceeded the free-running period in constant light. This difference between free-running periods under different lighting conditions has been observed in other diurnal animals (e.g., *T. hudsonicus*—Pohl, 1983), but not in all (e.g., *A. leucurus* and *Eutamias sibiricus*—Pohl, 1983). In nocturnal animals, the period often lengthens with increasing light intensities (Daan and Pittendrigh, 1976b; Aschoff, 1979). Typically, splitting of the free-running rhythms is only observed when the nocturnal hamster and rat are placed in LL (Pittendrigh, 1974; Pittendrigh and Daan, 1976b; Albers et al., 1981; Boulos and Rusak, 1982a; Cheung and McCormack, 1983), or when the diurnal *A. leucurus*, *E. sibiricus*, and *T. belangeri* are kept in dim LL or DD (Hoffmann, 1969, 1971; Pohl, 1972, 1983). Thus, although light and darkness affect the free-running period of several diurnal animals in different ways, splitting is only observed in dim LL or DD. This suggests that splitting is not a consequence of lengthening of the period, but of the light intensity to which animals are exposed. This idea is further substantiated by Hoffmann's (1971) data, in which tupaia showed splitting following a decrease in period, and ours, showing splits following either an increase or decrease in period.

In three animals, feeding and nest-box behavior were recorded in addition to running-wheel activity. The unsplit and split rhythms of these functions appeared to run in parallel. In hamsters, running-wheel activity was recorded simultaneously with body temperature (Pickard et al., 1984) or with drinking (Shibuya et al., 1980). In these studies, splitting also occurred simultaneously in different functions, suggesting a common mechanism underlying the generation or coupling of these different rhythms (Pickard et al., 1984). The pattern of splitting was also similar among different animals. A decrease in the duration of activity was always observed at the onset of splitting. At the same time, two new components arose, which finally made up the components of the split rhythm (see also Hoffmann, 1969, 1971). In hamsters, Pittendrigh and Daan (1976b) discriminated between a morning (M) component associated with activity offset at dawn and an evening (E) component associated with activity onset at dusk. In tupaia, both components completely overlapped in the unsplit state. We therefore discriminated between the component with the lowest frequency (component 1) and the one with the highest frequency (component 2).

Bright 6-hr light pulses presented to animals with split rhythms did not result in steady-state phase shifts at any circadian phase (Figs. 6C, 6D). However, light pulses around the activity offset (CT 12) induced immediate phase delays, whereas pulses around CT 24 induced immediate phase advances in both components. Thus the responsiveness of the two components to light pulses was indistinguishable. Since a single light pulse affects the two components at different CT, different instantaneous phase shifts of the two are induced. This changes their phase relationship temporarily. The new phase relation is unstable, since the components regain their previous phase relationship via transients. The mean final phase shift was small at all CTs. The same behavior of the split rhythm has been observed in hamsters following dark pulses (Boulos and Rusak, 1982b; Lees et al., 1983), light pulses (Earnest and Turek, 1986), and carbachol injections (Meijer et al., 1988). All these stimuli could produce immediate delay and advance shifts in both components. However, no consistent steady-state shifts were observed. Although light pulses did not induce reproducible immediate shifts in hamsters, dark pulses and carbachol injections did elicit the same responses in both components. In tupaias, both components also displayed identical responses to 6-hr light pulses. These data further support the view that the two components involved in splitting cannot be distinguished by their responsiveness to external stimuli. Furthermore, since no significant difference between 230-lux and 2300-lux phase shifts was found in either component, whereas 23-lux pulses produced no phase shifts, it is suggested that saturation was attained at 230 lux and that the two components also did not differ in sensitivity.

In two tupaias, activity rhythms transiently split and re-fused. In one of these animals, this process continued to take place over 20 months until the light intensity was lowered to 0.01 lux. Inspection of the actograms suggests that both components can generate activity for a duration that more or less equals activity time in the unsplit state. When the components are 180° out of phase, this animal is continuously active. Thus, both components appear to overlap completely in the unsplit state in this animal. This contrasts with the suggestion by Pittendrigh and Daan (1976b) that one component originates from the onset and the other from the offset of activity.

The amount of activity appears to be determined by the relative position of the components. The rare continuous crossing of two components in Figure 7 is illuminating for how central circadian oscillators may exert control over locomotor activity. It is clear, even without quantitative analysis, that activity is especially intensified during overlap of the two components, and suppressed where they do not overlap. In fact, it is the apparently additive effect of both on locomotor activity that allows one to identify both continuously. This has also been found in crossing circadian activity components in pinealectomized lizards (Underwood, 1981) and in the elegant pacemaker transplantation experiments in cockroaches by Page (1983). However, there appears to be a short time zone of a few hours following each of the two activity bands where the other component does not express itself in activity (see blow-up in Fig. 9). The suppression of activity at the end of component 1 suddenly obscures the onset of component 2 when it moves into this phase position just before reaching stable antiphase. We thus surmise that this activity-suppressing effect at the end of each component oscillator is what obscures the onset of the other, and causes the activity offsets to be so much sharper in splitting than the offsets. This increased precision of activity offset is not specific for tupaias, but appears to be true of hamster splitting as well (e.g., see Fig. 1 in Turek et al., 1982).

LIGHT RESPONSES IN SPLIT TUPAIAS

Also in animals with unsplit rhythms, the pattern of activity might be determined by the relative position of oscillators (Mrosovsky and Hallonquist, 1986). Davis and Menaker (1980) described patterns of activity in hamsters that resemble the pattern in Figure 7 to some extent. These authors suggested that qualitatively different oscillators underlie the activity pattern: One oscillator divides the circadian cycle into a rest time and an active time, but the expression of activity is still dependent on a secondary "bout oscillator." The two oscillators involved in splitting in tupaias each appear to divide the cycle into a rest phase and an active phase.

Thus, while there may be two oscillators involved in splitting in *T. belangeri* circadian rhythms, they seem to have exactly the same functional properties, both with respect to their response to light pulses and with respect to their control over activity and rest of the animal, whether in the unsplit or in the split state. Also, in none of the other mammalian species in which splitting has been observed is there solid evidence for a functional differentiation between the two oscillators. Only in the single case of prolonged relative coordination between the two components (Fig. 7) was there clear dominance of one over the other. This can be deduced from the virtually flat period response curve for the effect of component 1 on 2, and clear phase dependence of the effect of component 2 on 1 (Fig. 8). A relatively minor reduction in background light intensity (2.8 to 0.01 lux) was sufficient to affect this interaction in such a way that a stable antiphase was reached (Fig. 7). This is consistent at least with the proposition that light conditions induce splitting or fusion of the two oscillators by affecting their mutual coupling (Daan and Berde, 1978), rather than through differential light sensitivity in each one of them. It remains open to further inquiry whether the two components represent distinct units in any functional sense or merely arbitrary subsets in a homogeneous population of neuronal oscillators that together form the circadian pacemaker (Boulos and Morin, 1986).

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