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Precision of circadian wake and activity onset timing in the mouse

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Summary. In each circadian cycle, a mouse begins its major activity period with discrete wake onset and activity onset events. The precision with which these events are timed in constant darkness was analyzed using the approach outlined by Pittendrigh and Daan (1976).

1. Negative serial correlations of observed circadian period values (mean $r_1 = -0.471$ for wake data, -0.409 for activity data) imply that deviations in period tend to be compensated by opposite deviations in the following cycle.

2. As a result, precision of the circadian pacemaker must be better than that of observed rhythms. Standard deviation of the pacemaker period $\sigma(\tau)$ was estimated at 5.1 min. Some individual data series had estimates $s(\tau) = 0$, implying a nearly perfect pacemaker.

3. Previous speculation was that wake onset would be under more direct pacemaker control than activity onset, and would therefore be timed more precisely (Pittendrigh and Daan 1976; Richardson et al. 1985). Contrary to this prediction, intervals between successive wake onsets exhibited significantly greater variance than intervals between successive activity onsets. Two possible interpretations of this finding were proposed.

Introduction

A large body of evidence supports the concept of a circadian timing system in mammals, responsible for coordinating the timing of daily rhythms of sleep/wake and rest/activity, among many others (Moore-Ede et al. 1982). The suprachiasmatic nucleus in the hypothalamus is thought to be a biological clock, capable of self-sustained oscillation, which acts as a pacemaker for these rhythms. Little is known, however, about the mechanism by which sleep/wake or rest/activity rhythms are coupled to the central clock.

One approach to studying these coupling mechanisms is to characterize the variability in timing of the rhythms under constant environmental conditions. In constant darkness, mice exhibit rhythms of sleep/wake and rest/activity with periods of approximately 23.5 h (Fig. 1). In approximately half of each cycle (the rest phase), there are only short episodes of wakefulness and virtually no activity. In the other half of each cycle (the active phase), there are typically several hours of consolidated wakefulness with concurrent episodes of activity. Usually, it is easy to define discrete wake onset and activity onset events at the beginning of the active phase. Analysis of timing of these events is a convenient way to characterize the physiological system responsible for sleep/wake and rest/activity rhythms.

We conceptualize the circadian timing of wake and activity onsets as an ordered sequence of events, illustrated schematically in Fig. 2. In this sequence, the central clock generates a wake-up signal once during each cycle. The wake-up signal then initiates a program of physiological responses culminating in wake onset. Once wake onset has occurred, further physiological processes lead to activity onset, independent of previous events or further clock input. This causal chain seems reasonable because (1) wake onset necessarily pre-

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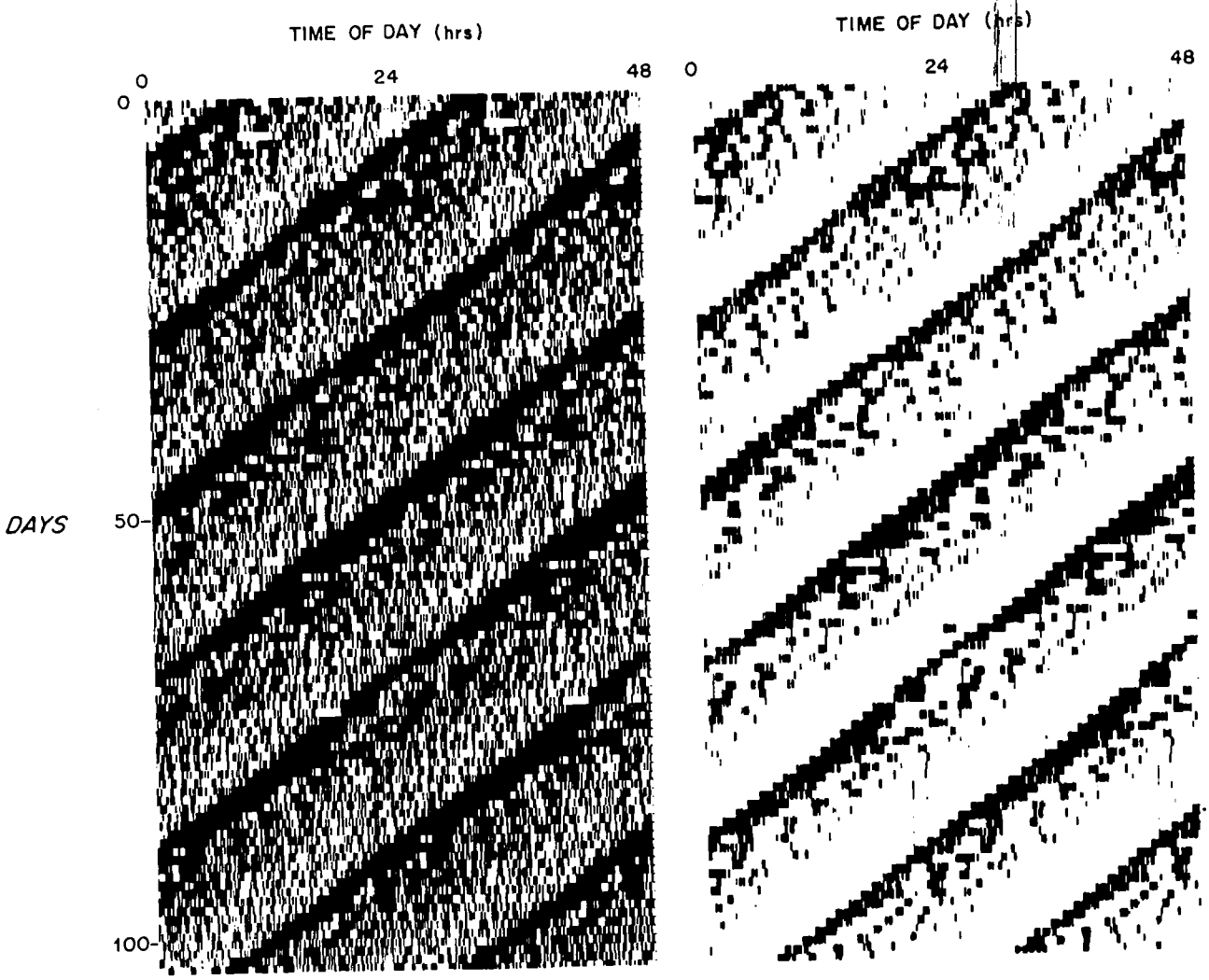

Fig. 1. Plots of circadian wake and activity patterns for a typical mouse in constant darkness. Black area represents occurrence of the specified state, with time of day plotted left to right. Each raster line is 48 h, but the data are double plotted so that successive days are aligned vertically

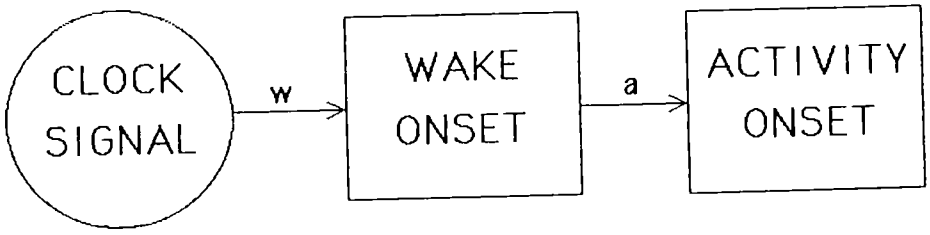

Fig. 2. Schematic diagram of the sequence of events in the circadian timing of wake and activity onsets. During each cycle, a clock signal leads, after a lag time w, to wake onset, and then, after a further lag a, to activity onset

cedes activity onset; (2) the two events usually occur within a few minutes of each other; and (3) sleep, inactive wakefulness, and activity can be thought of as levels along a single axis of arousal. Thus, our model presupposes three discrete events, a clock signal, wake onset, and activity onset, coupled in a linear control pathway by unknown physiological processes.

Unless the coupling is perfect, one must expect that observed variance of wake onset and activity onset timing will arise not only from imprecision of the central clock but also from variability in

lag times between clock signal and wake onset (w) and between wake onset and activity onset (a) (see Fig. 2). Pittendrigh and Daan (1976) outlined a procedure for partitioning variance in the observed period of a rhythm (time between successive wake onsets, or between successive activity onsets) into that part due to the pacemaker, and that part due to the coupling of pacemaker to overt rhythm. The procedure is based on the principle that deviations of the observed period due to loose coupling will tend to be compensated by opposite deviations in the following cycle, while deviations due to pacemaker error will not. Successful partitioning thus depends on estimation of the first order serial correlation (r_1). Using this procedure to analyze the variance in circadian wake and activity onset timing in mice, we attempted to characterize the coupling pathway between pacemaker and overt rhythms, and to determine the precision of the pacemaker itself.

Materials and methods

Experimental animals and surgical procedure. Mice (male *Mus musculus*, C57BL/10J, 30–40 g) were surgically implanted for chronic EEG and EMG recording. The animals were anesthetized by intraperitoneal injection of ketamine hydrochloride (Ketaject; 0.10 mg/g) and xylazine hydrochloride (Rompun; 0.005 mg/g). Each pre-assembled implant consisted of six filisterhead, self-tapping, stainless steel screws (size 00) soldered via shielded phonograph cable to a removable commutator plug. Four of the screws were threaded into holes drilled into the skull, two frontal and two parietotemporal. Dental acrylic was used to insulate the screws and to cement them to the skull. In some animals, the skull was treated with dental fluoride, and the screws were further stabilized by cementing them to a small cylinder of plastic fastened to the skull by cyanoacrylic adhesive. One of the four cortical screws served as ground and was made continuous with cable shielding. The other three were referenced in whichever combination yielded clearest EEG discrimination of wake, NREM sleep, and REM sleep. The two remaining screws were sutured to nuchal musculature for an EMG (electromyogram) recording.

Experimental procedures. After surgery, each mouse was housed individually in a Plexiglas cage equipped with a food bin, water bottle, and running wheel. The implant cable was plugged into a low-torque commutator (Air Precision, Paris, France) mounted in the cage top, which allowed the animal free movement throughout the cage. Each cage was placed in a separate light-tight, thermally insulated, sound-attenuated chamber measuring 61 by 61 by 122 cm. Lighting was provided by externally controlled, water-jacketed fluorescent lamps.

Mice were maintained on a stable LD 12:12 light cycle (12 h light, 12 h dark) beginning at least three weeks prior to surgery. The LD 12:12 schedule was maintained for at least one additional week before release into DD (constant darkness). Recordings were then continued until recording quality deteriorated.

Sleep/wake state and activity determination. Sleep/wake state was determined automatically using a computer program 'SQUEEK' developed for this purpose. The program used template matching techniques to score in 10-s epochs based on EEG alone. Details concerning the algorithm and its implementation may be found elsewhere (Vincent 1978; Richardson et al. 1985).

Wheel-running activity was detected by two magnetic reed switches mounted at opposite positions on a stainless steel wheel. Activity was registered only when both switches were closed within a 10-s epoch (equivalent to at least 180° of wheel rotation). The wheel was tilted and beveled to prevent frictional wear of the recording cable while still providing a horizontal running surface.

Data selection. A total of seventeen mice were recorded. If the level of running wheel activity improved over the course of a recording, the section with poorer activity was eliminated. Data within five cycles of a light pulse or transition from LD 12:12 were also eliminated. The remaining data were divided into uninterrupted sections of at least seven cycles in length. The final analysis using time series techniques was confined to nineteen sections of twenty cycles in length from eight different animals.

Determination of wake and activity onset times. Onset times were usually obvious from inspection of the data (see Fig. 3), but objective criteria were formulated to assure consistency. The particular criteria chosen were those resulting in the greatest regularity of period values in representative samples of data. Beginning at the earliest time of unambiguous activity in each circadian cycle, activity data were followed earlier in time until a gap of at least 25 min, with no minute having more than one epoch of activity, was found. Activity onset was located at the first minute following this gap. Wake data were then followed earlier from the time of activity onset until a gap of at least 2 min, with no minute having more than two epochs of wake, was found. Wake onset was located at the first minute following the gap. Rarely (~1% of events), when there was more than one reasonable choice, and the objective criteria led to a very unusual period value, subjective judgment took precedence over these criteria.

Pittendrigh-Daan procedure. For convenience in presenting the results that follow, we briefly recapitulate the Pittendrigh-Daan method for analyzing the variance of observed period values in a series of consecutive circadian cycles. In these formulae, t = observed circadian period, τ = pacemaker period, l = lag time between clock signal and observed behavioral event, and the subscript i denotes the sequence of the particular circadian cycle to which a value pertains. The derivation assumes independent variation in τ and l, with no serial correlations in either variable. First, by definition,

$$t_i = \tau_i - l_i + l_{i+1} \quad (1)$$

$$t_{i+1} = \tau_{i+1} - l_{i+1} + l_{i+2}. \quad (2)$$

This leads eventually to:

$$\sigma^2(\tau) = (1 + 2\rho_1) \cdot \sigma^2(t) \quad (3)$$

$$\sigma^2(l) = -\rho_1 \cdot \sigma^2(t) \quad (4)$$

where $\sigma^2(t)$ is the variance of t, $\sigma^2(\tau)$ is the variance of τ, $\sigma^2(l)$ is the variance of l, and ρ_1 is the first order serial correlation for t. (For full details, consult Pittendrigh and Daan 1976.)

In our analysis, the series of observed periods t_i were either intervals between successive wake onsets or intervals between successive activity onsets. When wake onsets were used, the lag time l corresponded to w in Fig. 2. When activity onsets were used, l corresponded to the sum $w + a$.

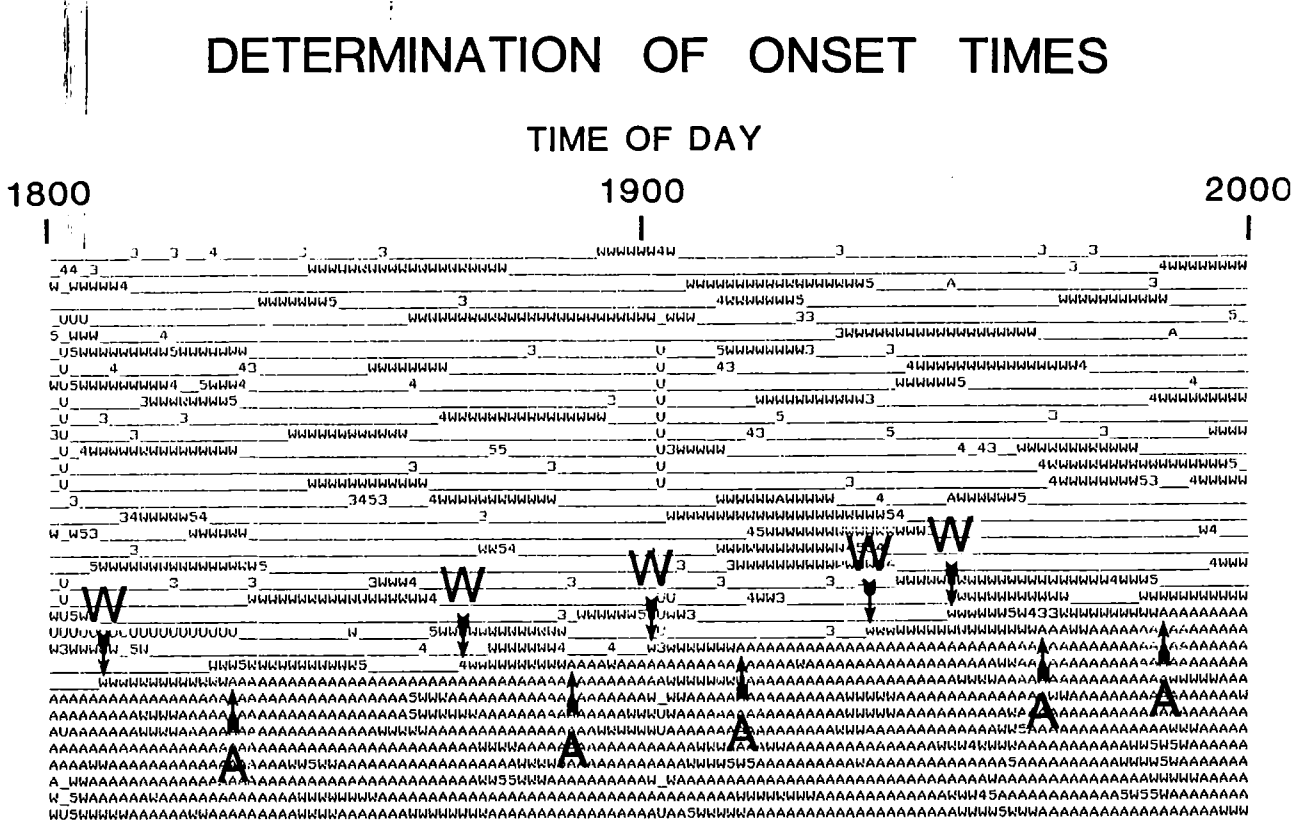

Fig. 3. Two-hour window of data illustrating the method used to determine wake and activity onset times. Each onset time is designated by an arrow and a large letter W (wake onset) or A (activity onset). Each small character represents one minute of data (six 10-s epochs). Time is plotted left to right, and successive days are plotted top to bottom. (Vertically adjacent characters are 24 h apart.) 'W'=all wake; '5'=five 10-s epochs wake; '4'=four epochs wake; '3'=three epochs wake; 'A'=one or more epochs of activity; '–'=two or fewer epochs wake, no activity; 'U'=unscored

Replacing the true variance and serial correlation in formulae (3) and (4) by their usual estimates:

$$s^2(t)=\frac{1}{n}\sum_{i=1}^{n}(t_i-\bar{t})^2$$

$$r_1=\frac{\sum_{i=1}^{n-1}(t_i-\bar{t})(t_{i+1}-\bar{t})}{\sum_{i=1}^{n-1}(t_i-\bar{t})^2} \qquad (5)$$

leads to the estimates of $\sigma^2(\tau)$ and $\sigma^2(I)$, known as 'method of moments estimates', used by Pittendrigh and Daan.

Additional statistical methods. We estimated $\sigma^2(\tau)$ and $\sigma^2(I)$ from formulae (3) and (4) by a more efficient procedure. The Pittendrigh and Daan Eqs. (1) and (2) imply that each series of observed period values follows a moving average process of order one (denoted MA(1)). That is, if $t_1, t_2, t_3, \cdots t_{20}$ denote the observed circadian periods for a given series, then

$$t_i-u_t=e_i-\theta e_{i-1}$$

where u_t and θ are constants, and the e_i's are independent, identically distributed, normal random variables with zero mean and variance σ_e^2 (a process called 'white noise'). (See Chatfield (1975) for an introduction to time series concepts.) Maximum likelihood estimates of θ and σ_e^2, denoted $\hat{\theta}$ and s_e^2, respectively, were obtained using the iterative minimization procedure for fitting ARMA models available in the BMDP Statistical Package. The first order correlation was then estimated by

$$r_1=-\frac{\hat{\theta}}{1+\hat{\theta}^2}$$

and $\sigma^2(t)$ was estimated by

$$s^2(t)=(1+\hat{\theta}^2)\cdot s_e^2.$$

Maximum likelihood estimates are considerably more efficient than the method of moments estimates used by Pittendrigh and Daan (e.g. Priestley 1981).

Comparisons between wake and activity parameters were made using two-sided Wilcoxon signed rank tests for matched pairs.

No problem arises from our use of multiple series from the same mouse. Because the series are separated in time, they are independent under the Pittendrigh-Daan assumption of a MA(1) model.

Results

Results of analysis of each of the nineteen data series of length twenty are presented in Tables 1, 2 and 3. Subscripts of w or a indicate measures from wake or activity data, respectively. Serial cor-

Table 1. Measures from wake onset data. Each data series consists of 20 observed circadian period values $t_{w,i}$ (time intervals between successive wake onsets). r_1 = the maximum likelihood estimate of first order serial correlation of t; $s^2(t_w)$ = the usual estimate of variance of t; $s^2(w)$ = variance of lag time w, estimated by the Pittendrigh-Daan procedure; $s^2(\tau)$ = variance of pacemaker period, estimated by the Pittendrigh-Daan procedure

Series	Mouse	r_1	$s^2(t_w)$ (min^2)	$s^2(w)$ (min^2)	$s^2(\tau)$ (min^2)
1	6	−0.500	243	122	0.0
2	7	−0.498	172	86	0.7
3	7	−0.465	498	232	34.9
4	7	−0.500	394	197	0.0
5	29	−0.498	428	213	1.7
6	29	−0.498	555	276	2.2
7	30	−0.351	203	71	60.5
8	30	−0.438	582	255	72.2
9	30	−0.499	611	305	1.2
10	30	−0.500	738	369	0.0
11	30	−0.465	549	255	38.4
12	30	−0.414	322	133	55.4
13	30	−0.498	259	129	1.0
14	30	−0.499	1056	527	2.1
15	31	−0.498	207	103	0.8
16	39	−0.325	191	62	66.9
17	39	−0.497	155	77	0.9
18	67	−0.499	436	218	0.9
19	69	−0.500	927	464	0.0
Mean		−0.471	449	215	17.9
SD		0.053	258	132	26.9

Table 2. Measures from activity onset data. Each data series consists of 20 observed circadian period values $t_{a,i}$ (time intervals between successive activity onsets). r_1 = the maximum likelihood estimate of first order serial correlation of t; $s^2(t_a)$ = the usual estimate of variance of t; $s^2(w+a)$ = variance of lag time $w+a$, estimated by the Pittendrigh-Daan procedure; $s^2(\tau)$ = variance of pacemaker period, estimated by the Pittendrigh-Daan procedure

Series	Mouse	r_1	$s^2(t_a)$ (min^2)	$s^2(w+a)$ (min^2)	$s^2(\tau)$ (min^2)
1	6	−0.235	110	26	58.3
2	7	−0.497	61	30	0.4
3	7	−0.400	535	214	107.0
4	7	−0.500	293	147	0.0
5	29	−0.339	209	71	67.3
6	29	−0.499	432	216	0.9
7	30	−0.418	135	56	22.1
8	30	−0.441	482	213	56.9
9	30	−0.498	407	203	1.6
10	30	−0.445	371	165	40.8
11	30	−0.325	249	81	87.2
12	30	−0.380	189	72	45.4
13	30	−0.118	94	11	71.8
14	30	−0.474	784	372	40.8
15	31	−0.498	124	62	0.5
16	39	−0.235	120	28	63.6
17	39	−0.500	63	32	0.0
18	67	−0.472	252	119	14.1
19	69	−0.500	846	423	0.0
Mean		−0.409	303	134	35.7
SD		0.112	231	117	34.4

relation magnitudes were smaller for activity data than for wake data (mean −0.409 vs. −0.471, $P<0.01$). Standard deviations corresponding to mean variance values were as follows: $s(t_w)=21.2$ min, $s(t_a)=17.4$ min, $s(w)=14.7$ min, $s(w+a)=11.6$ min, $s(a)=6.8$ min, and $s(\tau)=4.2$ min (wake data) or 6.0 min (activity data). Contrary to expectation, $s^2(t_a)$ was smaller than $s^2(t_w)$ ($P<0.0001$), and $s^2(w+a)$ was smaller than $s^2(w)$ ($P<0.0001$). Both estimates of pacemaker variance were very small, and the two estimates did not differ significantly. The activity lag time (a), measured directly, averaged 16.7 min.

Because $s^2(w+a)<s^2(w)$, it is clear that w and a must be negatively correlated. The magnitude of this correlation $\rho(w, a)$ may be estimated from the data if we assume that lag times are correlated only within the same cycle. Then, from the defining equations

$$t_{w,i}=\tau_i-w_i+w_{i+1}$$

$$t_{a,i}=\tau_i-(w_i+a_i)+(w_{i+1}+a_{i+1})$$

a straightforward calculation gives:

$$\mathrm{cov}(t_w, t_a)=\sigma^2(t_a)+2\mathrm{cov}(w, a)-2\sigma^2(a).$$

This leads to:

$$\rho(w, a)=\frac{\sigma^2(t_a)-\sigma(t_w)\,\sigma(t_a)\,\rho(t_w, t_a)-2\sigma^2(a)}{2\sigma(w)\,\sigma(a)}.$$

All of these parameters are easily estimated from the data, and resulting correlation estimates $r(w,a)$ for each series are presented in Table 3. The correlation values averaged −0.565, with a standard deviation of 0.290.

One key assumption of the Pittendrigh-Daan model is that successive pacemaker periods are uncorrelated, leading to the restriction that t_i values must follow a MA(1) process (defined above). Certain proposed mechanisms for the central clock, such as a multi-oscillator system (Kronauer et al. 1982) or an oscillator with a non-linear restoring force (Wever 1984a) would violate this assumption. Therefore, we considered it crucial to test the assumption using the data at hand.

Preliminary analysis on this point yielded equivocal results. For all series of length seven to ten ($n=126$), we estimated the second order serial correlation (which must equal zero in a MA(1) process) by the analog of formula (5). The r_2 values

Table 3. Measures relating wake and activity data. $\bar{a}$ = mean interval between wake onset and activity onset of the same circadian cycle; $s^2(a)$ = the usual estimate of variance of a; $r(t_w,t_a)$ = the usual estimate of correlation between t_w (the interval between successive wake onsets) and t_a (the interval between successive activity onsets) in the same circadian cycle; $r(w,a)$ = correlation between w (lag time from clock signal to wake onset) and a (lag time from wake onset to activity onset) in the same circadian cycle, estimated using the procedure described in the text

Series	Mouse	$\bar{a}$ (min)	$s^2(a)$ (min^2)	$r(t_w,t_a)$	$r(w,a)$
1	6	17.8	42.2	0.786	−0.717
2	7	17.0	47.1	0.711	−0.833
3	7	16.1	38.8	0.935	−0.133
4	7	7.9	25.0	0.905	−0.460
5	29	17.1	61.3	0.797	−0.665
6	29	13.1	20.5	0.701	0.317
7	30	15.9	33.6	0.786	−0.638
8	30	14.2	24.0	0.964	−0.489
9	30	16.6	39.6	0.949	−0.662
10	30	18.2	42.2	0.910	−0.760
11	30	19.0	45.9	0.939	−0.878
12	30	18.6	33.0	0.917	−0.779
13	30	16.7	64.0	0.682	−0.773
14	30	18.1	65.2	0.899	−0.443
15	31	14.2	27.2	0.826	−0.593
16	39	14.8	28.2	0.730	−0.561
17	39	15.5	29.5	0.840	−0.829
18	67	24.2	84.8	0.732	−0.589
19	69	22.8	134.5	0.791	−0.247
Mean		16.7	46.7	0.832	−0.565
SD		3.5	27.1	0.094	0.290

averaged −0.094 for wake onsets and −0.114 for activity onsets. Both were significantly different from zero by a two-sample, two-tailed t-test. When the ten cycle series were considered alone ($n=73$), however, r_2 values averaged −0.038 for wake onsets and −0.071 for activity onsets, neither of which was statistically significant by a t-test.

The nineteen series of length twenty were subjected to more rigorous testing. First, to see whether the MA(1) models we fitted when computing r_1 were adequate to describe the data, we computed the Ljung-Box overall goodness-of-fit statistic for each series (Ljung and Box 1978), and then combined the resulting P-values using Fisher's method (Littel and Folkes 1971). For wake data, the combined P-value was 0.247 when the first six orders of autocorrelation were taken into account. For activity data, the value was 0.911. These results indicate a very good MA(1) fit for both wake data and activity data.

We also performed another test of the MA(1) assumption in the long series. In a MA(1) model, the method of moments estimate of r_2 asymptotically follows a normal distribution with mean zero and variance $(1+2\rho_1^2)/n$, where $n=20$ is the number of cycles in each series and ρ_1 is the true correlation of order one. (This result is due to Bartlett (1946) and is found in most time series texts.) Hence, we know that

$$20\sum_{i=1}^{19}\left(\frac{r_2^2}{1+2r_1^2}\right)$$

has an approximate distribution of a chi-square variable with 19 degrees of freedom. This test has power against any alternative, while a t-test has no power against certain alternatives. Based on this statistic, we obtained a P-value of 0.456 for wake data and 0.362 for activity data. Thus, even after subjecting the longer series to tests much more demanding than a t-test, we find no evidence to reject the Pittendrigh-Daan assumption of a MA(1) model.

Discussion

Serial correlations of observed circadian period values were in close agreement with previous results. Our r_1 values averaged −0.471 for wake data and −0.409 for activity data. Pittendrigh and Daan (1976) conducted the same analysis of activity data in *Mus musculus*, and reported an average r_1 value of −0.36. Recently, Wever (1984b) analyzed sleep/wake cycle data from humans in time isolation, and found an average r_1 value of −0.402. Wever also found significant r_2 values, however, averaging −0.101. Our r_2 values were close to Wever's but were not statistically significant except in a preliminary analysis of very short series. Further investigation would be required to establish the existence of a second order correlation in the mouse. The negative first order serial correlations indicate that, in both mice and humans, deviations of observed circadian period tend to be compensated by opposite deviations in the following cycle.

Consequently, the estimated pacemaker precision was much better than the precision of the overt rhythms, the standard deviation of τ being on the order of only a few minutes (mean of estimates from wake and activity data = 5.1 min). For several series, estimates of $\sigma^2(\tau)$ were zero, implying a nearly perfect pacemaker. This is in excellent agreement with the results of Pittendrigh and Daan (1976), who estimated $\sigma(\tau)$ at 8.9 min.

A negative second order serial correlation, if established by further work, would indicate that deviations in period are compensated in the second cycle following a deviation as well as in the cycle

immediately following it. This might result in even lower estimates of pacemaker error, but would require a more elaborate model than that proposed by Pittendrigh and Daan (1976).

The most surprising result is that circadian activity onsets were timed more precisely than circadian wake onsets. Sleep and wakefulness are generally considered more fundamental behavioral states than rest and activity. Also, wake onset necessarily precedes activity onset, strongly favoring a model in which wake onset timing is controlled more directly. Thus, it was reasonable for others to speculate that wake onset would be more precisely timed by the pacemaker (Pittendrigh and Daan 1976; Richardson et al. 1985). It may be, however, that precise timing of activity onset has greater survival value than precise timing of wake-up. In the mouse, it may not matter as much when the animal wakes up as when foraging activity begins: there are clear advantages to being the first to forage for food under the cover of nightfall. Thus, the greater precision of activity onset makes sense from an evolutionary perspective.

The finding that activity onset is timed more precisely than wake onset does not necessarily mean that the basic sequence of events shown in Fig. 2 is unreasonable. A putative wake-up signal from the circadian clock must precede wake onset, which necessarily precedes activity onset. Multiple clock signals or more complex coupling processes are possible but unnecessary to explain the data. The finding does mean, however, that the lag times w and a are not independent: they must be negatively correlated (see Table 3; mean calculated $r(w, a) = -0.565$). That is, when the processes intervening between clock signal and wake onset (w) last longer than average, the subsequent lag time to activity onset (a) tends to be shorter than average. Conversely, when w is shorter than average, a tends to be longer. Such a relation has two different biological interpretations.

In the first interpretation, wake onset timing, despite its greater variability, is more directly controlled by the pacemaker than activity onset timing. The physiological processes occurring during the lag time w may facilitate activity onset as well as wake onset. Therefore, when w is relatively long, the system is somehow better prepared for activity onset by the time wake onset occurs, and the subsequent lag time a tends to be shorter. This interpretation preserves the intuitive concept that wake onset is the primary event timed by the pacemaker, with timing of activity onset accomplished indirectly through wake-up.

An equally plausible interpretation is possible, however. Prior to the time of wake onset, a mouse typically alternates frequently between sleep and wakefulness, with wake episodes of varying length (see Figs. 1 and 3). Assuming that an animal is less likely to fall asleep when it is active, the onset of activity during a particular wake episode might greatly prolong the episode and create the appearance of a well-defined wake onset. In this interpretation, activity onset is timed directly by the circadian clock, whereas wake onset is merely the beginning of the particular wake episode during which activity onset happens to occur. Here, the negative correlation $\rho(w, a)$ arises from the gating of activity onset by an ultradian alternation between sleep and wake. This interpretation differs from the first in that circadian timing of wake onset is attributed to an indirect mechanism.

One prediction of the latter interpretation is that there would be no clearly defined circadian wake onset if the amount of activity were reduced sufficiently. A preliminary study in our laboratory, in which activity was limited by locking the running wheel in place, has confirmed this prediction: when the running wheel was locked, the circadian pattern of wakefulness was much less distinct, and circadian wake onsets were very difficult to define. This suggests that the normal circadian variation in expression of wakefulness in the mouse may be largely or even entirely an indirect result of circadian timing of activity.

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