Transient circadian internal desynchronization after light-dark phase shift in monkeys

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Moore-Ede, Martin C., David A. Kass, and J. Alan Herd. Transient circadian internal desynchronization after light-dark phase shift in monkeys. Am. J. Physiol. 233(1): R31-R37, 1977. — In four conscious chair-acclimatized squirrel monkeys (Saimiri sciureus) studied with lights on (600 lx) from 0600 to 2000 h daily (1:12:12), prominent 24-h rhythms in feeding, drinking, activity, body temperature, and urinary potassium, sodium, and water excretion were seen. When the monkeys were subjected to 36 h of darkness followed by 36 h of light each variable demonstrated a circadian rhythm which was not passively dependent on the light-dark cycle. After the 24-h light-dark cycle was abruptly phase-delayed by 8 h, all the rhythms resynchronized with the new light-dark cycle phase, demonstrating that light-dark cycles are an effective zeitgeber. However, the resynchronization of the rhythms of feeding, drinking, activity, and body temperature was 90% complete within approximately 2 days while the 90% resynchronization of the urinary rhythms took approximately 5 days. These results suggest that the circadian timing system in S. sciureus may consist of several spontaneously oscillating units which can become transiently uncoupled during perturbations of environmental time cues.

circadian rhythms; squirrel monkey; light-dark cycle; zeitgeber phase shift

Endogenous circadian oscillations in physiological variables have been demonstrated in organisms ranging from unicellular algae (14) to man (8). There is now considerable evidence to suggest that these physiological rhythms are generated by autonomous oscillators within the organism (21, 25). These circadian oscillators are normally synchronized by environmental time cues (“zeitgebers”), such as the light-dark cycle, but in the absence of such cues, the oscillating system demonstrates free-running periods which are usually different from 24 h.

Any conclusion as to the organization and physiology of these circadian oscillators must be compatible with an important phenomenon known as “internal synchronization.” It has been demonstrated in both unicellular (20) and multicellular (2, 3, 6, 12) organisms that when circadian rhythms in several physiological variables are monitored simultaneously in an individual animal they are usually found to have identical periods. This has been observed whether the organism is synchronized by environmental time cues or has its circadian rhythms free-running under constant conditions. Such internal synchronization either demands that within an organism there must be only one oscillator or “clock” on which all endogenous circadian rhythms are passively dependent, or, if there is more than one oscillator, then the various oscillators must be normally synchronized with one another.

This paper reports studies in which we have manipulated environmental time cues to determine the extent of the coupling between seven behavioral and physiological variables that show circadian rhythmicity in the squirrel monkey. If constant internal phase relationships were maintained between the various circadian rhythms throughout these perturbations then this might suggest that all the rhythms were passively dependent on a single circadian oscillator. On the other hand if certain oscillating functions responded more rapidly than others to perturbations in environmental time cues, so that transient circadian internal desynchronization was observed, then this would suggest the possibility that the circadian timing system in the squirrel monkey is composed of multiple potentially independent oscillators.

Materials and Methods

The studies were performed using four adult male squirrel monkeys (Saimiri sciureus) weighing 600–900 g. For periods of up to 3 wk, continuous urine collections were obtained from unanesthetized monkeys, conditioned to sit in a specially designed metabolism chair. Environmental illumination, temperature, and auditory stimuli were controlled by conducting experiments within an isolation chamber. Once the monkeys were conditioned, they tolerated studies lasting 2–3 wk and showed no ill effects or loss of agility on return to their cages. While in the metabolism chair they behaved normally and maintained body weight.

Metabolism chair. The design of this chair was based on the squirrel monkey chairs used in the behavioral experiments of Kelleher and Morse (17). The monkey sat on a bar and was restrained by a Plexiglas sheet which served as a table around its waist. The space between the table and the monkey was sealed by a soft
rubber waist cuff. The monkey had freedom of movement about the waist. Below the Plexiglas table, it could either squat with its feet on a footrest or sit on the perch.

A lever was provided which the animal could operate to obtain food pellets. Pellets were delivered onto a tray directly in front of the animal from a pellet dispenser (model 11-1, Gerbrands Co., Arlington, Mass.). Drinking water was provided from a calibrated water bottle.

A padded funnel, placed between the monkey’s legs, enabled the collection of urine samples uncontaminated by feces and food debris. Urine passed from the funnel into test tubes within a specially designed automatic fraction collector. The apparatus which contained slots for 24 test tubes (100 x 15 mm) was rotated every 2 h by a stepping motor (Ledex 24-step Digimotor, Ledex, Inc., Dayton, Ohio). The fraction collector was covered by a sheet of Plexiglas which both prevented particles from falling into the test tubes and served as a footrest for the monkey.

Isolation chamber. The monkey, chair, and fraction collector were housed in a temperature-controlled isolation chamber (Forma Scientific, models 12 or 20, Marietta, Ohio). The chamber temperature was monitored by a continuously recording thermometer (Bacharach Instrument Co., Pittsburgh, Pa.). To provide ventilation, the fan on the heating-cooling unit was used to provide a circulation with air from outside the chamber.

A light source within the chamber, yielding approximately 600 lx of white light, was switched on each day in control studies from 0800 to 2000 h and off from 2000 to 0800 h. When the light was off there was less than 1 lx of illumination in the chamber. The animals were thus subjected to a 24-h light-dark cycle (LD 12:12; 600~lx).

The isolation chambers partially attenuated extraneous sounds and a white noise source was used in addition to provide further muffling. The white noise was generated by a Grason-Stadler noise generator (model 901-B, West Concord, Mass). Activities outside the chamber had no discernible effects on the animal’s behavior.

Experimental control and recording systems. The timing and control of the experimental system were accomplished by an automatic switchboard. One section of the switchboard was controlled by a clock which operated switches in electrical circuits every 2 h thus activating the stepping motor of the fraction collector, the timing record on the continuous paper recorders, and the counter and switch which controlled the illumination cycle of the isolation chamber. Another part of the switchboard controlled the food pellet delivery to the monkey. The number of lever operations to gain a pellet was controlled by a counter and the time between pellet deliveries was controlled by an adjustable timer.

Feeding, drinking, and movements in the chair were recorded from each monkey continuously using Harvard C-3 cumulative and six-pen recorders (Gerbrands). Physical activity was monitored by an ultrasound motion detector (Alton Electronics Co., Gainesville, Fla.). Drinking from the water bottle was detected by closure of an electrical circuit between the perch, the monkey, and the water bottle spout. The volume of water consumed each day was determined by measuring the fluid level according to calibrations of the water bottle. The drinking water contained less than 1 meq/l of sodium or potassium and hence contributed insignificantly to the dietary intake of these electrolytes.

Food pellet lever responses and food pellets obtained were also recorded. Electrical pulses were generated from the automatic switchboard by the food lever countdown devices and these were used to activate the recorders. An additional cumulative counter was used to record the total pellets obtained. The 24-h food intake could be read from this counter. By adjusting the number of responses required to gain a pellet it was possible to ensure that the monkey would eat all of the food pellets delivered.

Body temperature. Continuous recordings were made of core body temperature using thermistors built in the laboratory. Prior to implantation, the thermistors were each calibrated by measuring the resistance across a Wheatstone bridge while the thermistor was immersed in a water-filled vacuum bottle at various temperatures. An NBS specification total-immersion celsius thermometer was used to determine the calibration temperatures. The thermistors were implanted using a sterile operative procedure. Anesthesia was induced and maintained with halothane (2-bromo-2-chloro-1,1,1-trifluoroethane, Fluothane) in oxygen. A left paramedial incision was made and the left retroperitoneal space was exposed by blunt dissection. Thermistors, sterilized by soaking in Zephiran (benzalkonium chloride) solution, were implanted in the retroperitoneal space. The thermistor was tied in place with nylon sutures through the abdominal wall muscles and the thermistor leads were brought out to reach the skin surface between the animal’s shoulder blades. The external leads were protected by placing a nylon mesh jacket on the monkey which otherwise allowed the animal freedom of movement. During experiments, body temperature was recorded by connecting a cable to the thermistor leads. The cable was led out of the isolation chamber and connected as one arm of a Wheatstone bridge. The bridge output was amplified and recorded on a Grass Instrument Co. (Quincy, Mass.) polygraph model 7. The Grass paper record was calibrated using the previously determined calibration graph. The thermistors under went no detectable drift in calibration or sensitivity change over the course of the experiments, and this was confirmed by repeat calibrations up to four months later.

Light-dark cycle disruption. A 2-day period of acclimatization to the metabolism chair and isolation conditions was allowed before each experiment. Four monkeys were then studied during a control day with lights off from 2000 to 0800 h and lights on from 0800 to 2000 h (LD 12:12). They were then subjected to 36 h of continuous darkness followed by 36 h of continuous light. During the 4 days of the experiment, urine collections and recordings of feeding and activity and drinking were made as described above. Food and water were available ad libitum throughout both light and dark conditions during this study.

Eight-hour light-dark cycle phase shift. After a 2-day
acclimatization period, four monkeys were studied for 2 control days with lights on between 0800 and 2000 h daily. The animals were then subjected to an 8-h phase delay of the light-dark cycle by adding 8 h of light to the end of the second control day. Thereafter, lights were on from 1600 to 0400 h daily. During the experiment, the monkeys were allowed to feed and drink ad libitum. Care was taken to open the isolation chamber only during the monkey's self-selected activity and feeding periods. Continuous recordings were made of activity, feeding, drinking, body temperature, and urinary excretion rates as described above.

Urine analyses. After the urine samples were removed from the fraction collector they were acidified with two drops of 25% sulfuric acid and refrigerated at 4°C. The volume of urine in each tube was measured, and sodium and potassium concentrations were analyzed by flame photometry (Instrumentation Laboratory, Lexington, Mass.). Urine excretion rates (μeq/h) were then calculated for each electrolyte from the volume of each sample, the concentration of the electrolyte, and the length of time over which the sample was collected.

Data processing. The urinary data was first expressed as a smoothed three-point running mean. This was done by averaging the excretory rate during each 2-h period with the excretory rates of the two neighboring 2-h collections. This procedure reduced the influence of the monkey's irregularly timed micturitions on the excretory pattern without significantly affecting the amplitude of any circadian periodicity in the data.

To define the phases of the rhythms in urinary excretion, the data were expressed as a percentage deviation from a running 24-h mean. This was done by averaging the excretory rate during each 2-h period with the excretory rates of the two neighboring 2-h collections. A similar computation was undertaken to determine the phase of the circadian rhythm in body temperature, while the phases of the circadian rhythms of activity, feeding, and drinking were computed from the daily clock times of commencement and termination of each behavioral activity. This process of phase determination was repeated for each variable for each cycle throughout the experiment. Phase shifts were then computed by comparing the clock time (in hours) of the rhythm zero crossings in each experimental day with the mean clock time of the equivalent zero crossings during the control days of the experiment. Computations were performed using a Hewlett-Packard 2116B computer.

RESULTS

Response to perturbations of the light-dark cycle. Figure 1 presents the mean (± SE) results from four monkeys exposed to the light-dark schedule of 36 h of continuous darkness followed by 36 h of continuous light. Despite manipulations of light-dark cycle each monitored circadian rhythm persisted with a period of approximately 24 h. Monkeys demonstrated their normal 24 h rhythms of activity, feeding, drinking, and urinary potassium, sodium, and water excretion in four squirrel monkeys. After 2 equilibration days (not shown on graph) with lights on from 0800 to 2000 h daily, animals were studied for a control day on the same light-dark cycle followed by a 36-h period of constant darkness and then a 36-h period of constant light. All movements in the chair, feeding, and drinking were confined to the lights-on segment of the 24-h cycle although food and water were continuously available throughout day and night. Urinary potassium excretion fell to a minimum of 66.5 ± 26.2 μeq/h at 0700 h and then rose to a maximum of 203.0 ± 58.1 μeq/h at 1900 h. Urinary sodium excretion similarly showed a circadian rhythm with a minimum of 24.0 ± 19.1 μeq/h at 0900 h and a maximum of 41.2 ±
24.8 μeq/h at 1900 h. Urinary water excretion fell to a minimum of 787.4 ± 223.0 μl/h between 0500 and 0700 h and then rose to a maximum of 1,849.9 ± 408.0 μl/h at 1300 h. 

During the 36 h of constant darkness circadian rhythms of activity, feeding, drinking, urinary potassium, sodium, and water excretion persisted despite the absence of the 12-h light period during the second day of the experiment. Movements in the chair, feeding, and drinking were again virtually restricted to the 12 h between 0800 and 2000 h although the monkeys were in the dark and isolated from other environmental time cues. The amplitudes of all these behavioral rhythms, however, were reduced during the period of constant darkness. In contrast, the circadian rhythms of urinary potassium, sodium, and water excretion persisted with unchanged amplitudes.

When the animals were subjected to the 36 h of constant light during days 3 and 4, the circadian rhythms of the behavioral and urinary variables again continued with little change in pattern. When the lights were left on overnight between 2000 and 0800 h on day 4, there was a small amount of additional activity, feeding, and drinking at the beginning and again at the end of the “night.” However, most activity, feeding, and drinking were confined to the “day” period between 0800 and 2000 h. The circadian rhythms of urinary potassium and water excretion continued with an unchanged amplitude but that of urinary sodium excretion was reduced in amplitude on the last day of constant light.

Response to 8-h light-dark cycle phase delay. A representative response of a monkey to the 8-h phase delay of the light-dark cycle is shown in Fig. 2. Similar responses were seen in all four monkeys studied. During the 2 control days, the monkey confined movements in the chair, feeding, and drinking to the light-on period of the 24 h. The body temperature of the monkey demonstrated a prominent circadian rhythm with an amplitude of more than 2°C. Body temperature fell to a nocturnal minimum at approximately 0600 h and started to rise each day before the lights switched on, and then reached a plateau level at which it remained throughout most of the light-on period. Body temperature then fell at the time of lights off. Urinary potassium excretion fell to a nocturnal minimum at 0500 h and rose to a daily maximum at 1700 h as in the previous experiment. Urinary sodium excretion fell to a minimum at 0300–0700 h and reached a maximum at 1900 h, and urinary water excretion fell to a minimum at 0700 h and rose to a maximum at 1900 h.

After the light-dark cycle phase shift the circadian rhythms of activity, feeding and drinking rapidly readjusted to the new light-on phase of the 24-h cycle. The rhythm of body temperature on the first day after the light-dark cycle phase shift started rising at 0800 h although the lights had not come on and the monkey had not yet started its activity, feeding and drinking. The temperature rose by approximately 1.6°C and then fell back toward the nocturnal level before rising finally when the lights did come on at 1600 h. The body temperature from 1600 to 2000 h remained at a plateau similar to the control day temperature. Then although the lights were not switched off at 2000 h, as they were during the 2 previous days, body temperature started to fall toward the nocturnal level. This phenomenon was seen in each of the four monkeys studied during the light-dark cycle phase shift. The circadian rhythm of body temperature gradually resynchronized with the new light-dark cycle phase but this process occurred more slowly.

The phase shifts of each of the monitored physiological variables were quantified using the “zero crossing” procedure described in Methods. The results for the four animals are presented in Fig. 3. Each circadian rhythm resynchronized with the new phase of the light-dark cycle. However, a transient internal desynchronization was seen with certain rhythms phaseshifting more rapidly than the others.

An exponential function \( \Delta \Phi = -Ae^{kt-C} \), where \( \Delta \Phi = \) the phase shift in hours and \( t = \) the time after the LD phase shift in hours, was fitted to the phase shift data for each variable using an iterative nonlinear least-
squares regression program based on the Marquardt algorithm. The value for the fitted parameters $A$, $k$, and $C$ for each physiological variable, together with the time for the variable to achieve 90% of the light-dark cycle (8 h) phase shift are given in Table 1. While the phase shifts of the circadian rhythms of activity, feeding, drinking, and body temperature were 90% complete within approximately 2 days (51.2 h) after the light-dark phase shift, it took 110.5-132.9 h for the circadian rhythms of urinary potassium, sodium, and water excretion to phase-shift by the same distance. Covariance analysis indicated that the rhythms of the urinary variables took significantly longer to phase-shift ($P < 0.05$) than the behavioral and body temperature rhythms. There was no significant difference between the individual rates of phase shift in the different urinary variables, nor between the rates of phase shift among the nonurinary rhythms.

Table 2 presents the calculated periods of each of the circadian rhythms at intervals after the light-dark phase shift, computed from the instantaneous slopes of the fitted exponential functions for each variable. To achieve a phase delay, each rhythm must transiently show an increase in period. This table demonstrates that the periods of each of these rhythms, although all 24.0 h before and after the phase shift, show different periods during the phase shift because they shift at different rates. They are, therefore, transiently internally desynchronized.

**DISCUSSION**

The squirrel monkeys displayed prominent 24-h rhythms in activity, feeding, drinking, body temperature, and urinary potassium, sodium, and water excretion. These rhythms appeared to be in a steady state during the control periods of these experiments since they had similar phase and amplitude characteristics to those we have observed in longer term (1-2 wk) studies under the same LD 12:12 lighting regimen (11, 23). Furthermore, we have studied the circadian rhythms of activity, feeding, drinking, and the urinary variables in unrestrained animals and have found highly comparable rhythmic characteristics (unpublished data), suggesting that the data obtained from the carefully chair-acclimatized squirrel monkeys used in these experiments was not significantly influenced by the use of chair restraint.

In these experiments we demonstrated that the light-dark cycle was the dominant zeitgeber in these animals, confirming the earlier report of Richter (26); for after the light-dark cycle phase shift each rhythm eventually resynchronized, achieving the same phase relationships with the new phase of the light-dark cycle. However, the rhythms were not passively dependent on the light-dark cycle, because they persisted with an approximately 24-h period when the animals were subjected to 36 h of constant darkness, followed by 36 h of constant light. This evidence, together with our recent demonstration (unpublished data) that each of these rhythms can free-run with periods significantly different from 24 h when the monkeys are maintained in constant environmental conditions, indicates that these rhythms may be termed "circadian.

The purpose of the current study was to determine

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Parameters of the function $\Delta \phi = -Ae^{tk} + C$ fitted to the phase shifts of the monitored circadian rhythms after an 8-h phase delay of the light-dark cycle, where $\Delta \phi$ is the phase shift in hours, $k$ is the time constant, and $C$ is the final steady-state phase shift of the variable in hours. Also given is the time for each variable, in hours, to complete 90% of the light-dark cycle phase shift.
whether changes in the internal phase relationships between circadian rhythms in different variables in the same animal could be induced by zeitgeber manipulations. It was found in the squirrel monkey that this could be achieved when the animals were subjected to an abrupt phase shift of the light-dark cycle. The circadian rhythms of activity, feeding, drinking, and body temperature resynchronized by 90% of the light-dark cycle phase shift within approximately 2 days. However, the circadian rhythm of renal potassium, sodium, and water excretion took approximately 5 days to undergo the same 90% phase shift. There was some suggestion that the circadian rhythm of activity phase-shifted more rapidly than all other variables; however, because of variability in the rates of phase shift between individual animals, this difference was not found to be statistically significant by covariance analysis.

The term internal desynchronization is used to describe a state where different oscillating variables, which normally have identical periods and constant phase relationships within an animal (i.e., are internally synchronized), demonstrate different periods and therefore constantly changing phase relationships (4, 29, 30). The calculation of the period of each monitored circadian rhythm at 24-h intervals after the light-dark cycle phase shift in the current studies (Table 2) demonstrated that the various rhythms had different periods at each instant of time during the resynchronization process. However, once all the circadian rhythms had resynchronized with the light-dark cycle after approximately 7 days, then they all demonstrated an identical period (24.0 h) and their original constant internal phase relationships. Because the internal desynchronization that was observed between the rhythmic variables in the current experiments was observed temporarily between two stable synchronized states, it should be referred to as transient internal desynchronization. In contrast, the term steady-state internal desynchronization is restricted to a situation where two or more rhythmic variables are shown to free-run with different frequencies for a sufficient length of time so that all possible phase angle differences between them have been realized (29, 30).

The transient internal desynchronization that was seen between the urinary circadian rhythms and the other circadian rhythmic variables in the current experiments suggests that the circadian timing system in the squirrel monkey may be composed of more than one potentially independent oscillating unit. After an abrupt change in the phase of the dominant zeitgeber, the various oscillators appeared to become transiently uncoupled. Transient internal desynchronization after environmental zeitgeber phase shifts also appears to occur in rodents (15, 16) and humans (5, 8-10, 18, 19), although the data are harder to interpret. In rodents, the time course of the phase shifts of different rhythmic variables cannot be determined in an individual animal because the rhythms have been determined by killing groups of animals for each data point. In human subjects, studies of zeitgeber phase shifts are complicated by the ability of man to willfully phase shift his activity, feeding, and other behavioral functions with respect to environmental time cues. Thus, the influence of zeitgeber phase shifts cannot be easily separated from the effects induced by simultaneous consciously imposed phase shifts in behavioral patterns. In the present studies, however, we have been able to examine in the squirrel monkey the phase shifts of multiple variables simultaneously in individual animals in response to the manipulation of only one zeitgeber.

The internal phase-angle shifts which occur during resynchronization with a new light-dark cycle phase cannot be taken as conclusive proof that the circadian timing system is composed of multiple-oscillating units. It is possible that there is a single oscillator and that there are major delays in the transmission of phase information to the various tissues which show passive circadian rhythmicity. Such a mechanism, however, would require considerable delays, for the phase shift of the urinary circadian rhythms was not complete until 72 h after complete external resynchronization of the other monitored rhythms had occurred. An alternative interpretation would invoke a scheme where a single self-sustained oscillator could be driving a set of damped oscillators which were not themselves capable of generating self-sustained oscillations. The inertia possessed by these damped oscillators could be sufficient to account for the transient internal phase-angle shifts that were seen during resynchronization with the light-dark cycle. These alternative models have been made untenable however, at least in man, by the demonstration of steady-state internal desynchronization by Aschoff et al. (2-4) and Wever (29, 30). They have shown that more persistent internal desynchronization lasting several weeks occasionally occurs in human subjects isolated in a chamber from all external time cues. In these experiments, circadian rhythms in different physiological variables have been seen to oscillate with independent stable periods so that during the course of an experiment the different variables may cycle past each other and thereby show internal phase-angle shifts of more than 360°. A multiple-oscillator system that is capable of uncoupling must be invoked to explain this observation.

Further evidence suggesting a multiple-oscillator circadian system in multicellular animals is provided from studies where mammalian organs, or even cell suspensions have been maintained in constant culture conditions in vitro, and continue to show persisting circadian or ultradian oscillations (1, 7, 13, 27, 28). Presumably, each isolated tissue in these studies contains one or more self-sustained circadian oscillators which, al-
though normally coupled with the other tissue oscillators within the organism, can oscillate independently when the tissue is removed from the normal coupling influence. In contrast, unicellular organisms such as Gonyaulax appear to possess only one circadian “clock.” McMurray and Hastings (20) have shown that four different circadian rhythms of cellular function (photosynthetic capacity, glow, cell division, and luminescence capacity) continued to have constant phase interrelationships during a variety of experimental manipulations, including a phase shift induced by abrupt alterations of environmental illumination.

We have discussed in greater detail elsewhere (22, 24) the evidence suggesting that the circadian timing system in higher animals may consist of an organization of coupled oscillators, and we have indicated how these concepts provide a useful basis for a search for oscillator locations and their coupling mechanisms. The present studies demonstrate that the renal electrolyte circadian rhythms in the squirrel monkey can be transiently phase-delayed from the other monitored circadian rhythms when the animal is submitted to an abrupt phase shift of the light-dark cycle. Because these urinary rhythms arise from an organ, the kidney, with an anatomically discrete location and with well-defined neural and endocrine communications with other body functions, these studies suggest an opportunity to investigate the physiological nature of the coupling link which maintains the internal synchronization of the renal electrolyte rhythms with other circadian oscillations within the animal.

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