REVIEW

Getting Through to Circadian Oscillators: Why Use Constant Routines?

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Abstract Overt 24-h rhythmicity is composed of both exogenous and endogenous components, reflecting the product of multiple (periodic) feedback loops with a core pacemaker at their center. Researchers attempting to reveal the endogenous circadian (near 24-h) component of rhythms commonly conduct their experiments under constant environmental conditions. However, even under constant environmental conditions, rhythmic changes in behavior, such as food intake or the sleep-wake cycle, can contribute to observed rhythmicity in many physiological and endocrine variables. Assessment of characteristics of the core circadian pacemaker and its direct contribution to rhythmicity in different variables, including rhythmicity in gene expression, may be more reliable when such periodic behaviors are eliminated or kept constant across all circadian phases. This is relevant for the assessment of the status of the circadian pacemaker in situations in which the sleep-wake cycle or food intake regimes are altered because of external conditions, such as in shift work or jet lag. It is also relevant for situations in which differences in overt rhythmicity could be due to changes in either sleep oscillatory processes or circadian rhythmicity, such as advanced or delayed sleep phase syndromes, in aging, or in particular clinical conditions. Researchers studying human circadian rhythms have developed constant routine protocols to assess the status of the circadian pacemaker in constant behavioral and environmental conditions, whereas this technique is often thought to be unnecessary in the study of animal rhythms. In this short review, the authors summarize constant routine methodology and what has been learned from constant routines and argue that animal and human circadian rhythm researchers should (continue to) use constant routines as a step on the road to getting through to central and peripheral circadian oscillators in the intact organism.

Key words chronobiology, sleep, melatonin, temperature, masking

Distinguishing between the contribution of exogenous and endogenous components to observed rhythmicity has been a theme in animal and human circadian research for decades (Aschoff, 1960) and even centuries (deMairan, 1729). The study of organisms under constant conditions has become an accepted paradigm to demonstrate the endogenous nature of observed rhythmicity. Initially, constant conditions were

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defined as constant environmental (external) conditions, hence long-term studies of animals in constant darkness (DD) or constant light (LL). To place animals in a constant environment is still an accepted paradigm to quantify circadian phenotypes, even in this modern era of molecular/genetic analyses. The assumption underlying such experiments is that removal of periodic environmental cycles is sufficient to uncover the contribution of the circadian pacemaker to the observed rhythmicity. This approach follows directly from simple *input-clock-output* schemes. Those long-term experiments under constant conditions were designed primarily to assess the free-running characteristic of endogenous circadian rhythms.

Human circadian rhythm researchers developing constant routine protocols for the assessment of circadian phase hypothesized that removal of periodic environmental cycles was not sufficient to reveal the direct contribution of the clock to circadian rhythmicity. They therefore developed protocols that not only remove periodic influences from the environment but also remove influences due to periodic changes in behavior. This approach followed from the recognition that the periodic sleep-wake cycle influences (or "masks") many different output rhythms. In other words, periodic behavior can feed back onto, and contribute to, observed rhythmicity in many other variables, such as temperature (Fig. 1). Thus, the constant routine expands the notion of "exogenous"—from exogenous to the animal/organism to exogenous to the "core" clock. This scheme implies that measuring a physiological variable in the presence of a sleep-wake cycle does not necessarily reflect the direct contribution of the clock to that variable and may not even reflect the phase of the clock, due to the masking of the underlying endogenous component of the rhythm of interest by the periodic behaviors associated with the sleep-wake cycle. This is particularly true in studies in which the sleep-wake cycle is shifted abruptly, such as in simulated shift work or jet lag studies. Following an early constant routine-like protocol conducted by Aschoff (1947, 1995), Mills et al. (1978) developed the constant routine (CR) protocol recognizing that removal of the masking effects of the sleep-wake cycle was a prerequisite for assessing the status of the clock following abrupt shifts of the sleep-wake/light-dark cycle. Thus, the CR was developed to assess acutely the phase and waveform of endogenous circadian rhythms. The CR method, unlike the use of constant environmental conditions in animal studies, was not developed to assess the period of the human circadian system.

In this brief review, we will describe the general methodology of the constant routine, as well as some variations of the constant routine that are used, and will discuss some of the assumptions underlying use of the constant routine to assess the circadian system. We will present some examples of what has been learned from use of the constant routine that otherwise would not have been revealed and discuss when a constant routine may not be needed or should not be used. Finally, we will argue that periodic feedback from behavior may also affect observed periodicity in animal studies and that the use of protocols that control for this should be implemented by animal researchers as well as human researchers. In fact, we will argue that behavioral cycles need to be removed before it can be concluded that a rhythm in, for example, clock gene expression in particular tissues is endogenous and self-sustained.

WHAT IS THE CONSTANT ROUTINE, AND HOW IS THE CONSTANT ROUTINE DONE?

As outlined above, CRs were developed with the recognition that not only would periodic changes in the external environment potentially interfere with measuring circadian rhythms in output variables, but periodic changes in behavior, especially those behaviors related to the monophasic sleep-wake cycle, might also influence apparent rhythmicity in physiologic variables of interest. The essence of the constant routine is therefore to reduce or eliminate all such periodic changes in behavior, in addition to maintaining a constant environment. Thus, the ideal constant routine should not only keep ambient lighting levels constant and keep room temperature as constant as possible but should also eliminate sleep and the behaviors associated with sleep and distribute nutritional intake across the circadian cycle. The ideal constant routine therefore requires study subjects to remain awake throughout, in a constant posture, restricted to very low activity levels, with nutritional intake distributed throughout day and night. To observe an entire circadian cycle, a CR must also last long enough after allowing for residual changes from beginning the CR to dissipate. Thus, CRs of > 24 hours are required to assess an entire circadian cycle, but how much longer than 24 hours may depend on the variable(s) studied (according to the estimate of Brown and Czeisler, perturbations of the temperature signal by the sleep-wake cycle, postural changes, or

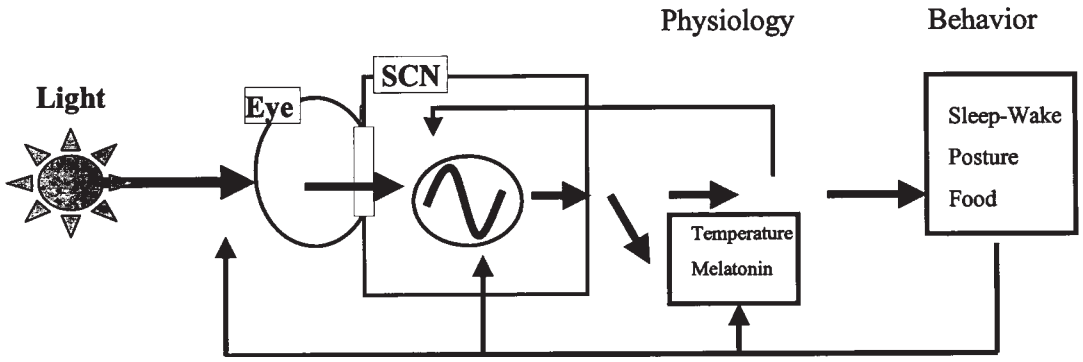

Figure 1. Schematic representation of a multiple feedback model of the circadian system. In this model, a pacemaker (the SCN) drives rhythms in physiology and behavior. Signals from the environment (light) affect the pacemaker; however, the pacemaker is also affected by feedback from physiology and behavior. Physiology is also affected by feedback from behavior and the environment (not indicated). In the constant routine, the status of the pacemaker is assessed after controlling environmental and behavioral rhythmicity.

other factors may last for up to 5 h [Brown and Czeisler, 1992]).

During a CR, rhythms of interest are then measured at frequent intervals to obtain phase and amplitude information. The two most widely measured rhythms in human circadian rhythm research are that of core temperature and melatonin. Core body temperature is often used as a measure of circadian phase because it is easy to collect continuously (even every minute), can be measured without disturbing the subject each time a measurement is taken, and data analysis is not associated with the time delay required for hormonal assay. Melatonin is also widely used as a marker of circadian phase. While not possible to collect as frequently as temperature, its advantage over temperature is that it is thought to be less masked by periodic changes in behavior. Although that remains true, melatonin has more recently been shown to be affected by posture (Deacon and Arendt, 1994; Ritz-De Cecco et al., 2001) and of course is suppressed by light (Lewy et al., 1980), even light of normal room intensity (Zeitzer et al., 2000). Other factors that might potentially affect melatonin levels (such as sleep itself or food and fluid intake) have not been evaluated as extensively, and thus further studies need to be done to determine how carefully controlled such factors need to be to obtain accurate phase estimates using melatonin. Another potential advantage of melatonin as a circadian phase marker that has been advocated is that only a small portion of the circadian cycle (e.g., the onset of nocturnal melatonin secretion) is necessary, thus reducing the duration required for phase assessment. However, the reliability of such an assessment is critically dependent on the assumption that there are no changes in the waveform of the circadian rhythm when comparing data collected under different conditions (such as before and after an intervention) or from different individuals. Unfortunately, this assumption can only be verified by inspecting the entire circadian cycle. The frequency at which a circadian marker can be collected also has important implications for the precision of the circadian phase estimate to be derived from the data; a comparison of the variability of circadian phase markers derived from temperature, melatonin, and cortisol, as well as a discussion of how the frequency of data collection affects the precision of a phase estimate, is the topic of another article in this journal (see Klerman et al., 2002, for details).

The conditions above describe those in an ideal constant routine. The careful reader of the literature must recognize that many variations of the constant routine are in use, and interpretation of those studies must take into consideration how the variations might have affected the results. For example, the version of the CR used by some groups keeps posture constant except for allowing periodic bathroom breaks, thus introducing periodic changes in posture that can affect body temperature (Kräuchi et al., 1997; Dijk et al., 1998), alertness (and thus cognitive performance) (Kräuchi et al., 1997), and hemodynamics (which in turn can affect hormone levels measured in the periphery) for hours following the break. Postural changes introduced during a CR are problematic not only due to their long-lasting effect on the data of interest, but the effects of postural changes can be phase dependent, as we have shown using core body temperature data collected during a forced desynchrony study (Dijk et al., 1998).

Another version of the CR used by some groups keeps posture constant but allows subjects to sleep, either *ad lib* or at their habitual times, thereby introducing periodic changes in sleep-wake state that can affect body temperature, alertness, and hormone levels. It is not easy to quantify the advantages and disadvantages of the CR with sleep versus the CR without sleep. The extent to which sleep per se affects body temperature, hormone levels, and so on has not been firmly established because in most experiments, sleep is associated with changes in posture and light exposure. Recently, Kräuchi and Wirz-Justice (2001) showed that the effects of sleep per se on core body temperature are minimal. Allowing sleep during a CR prevents sleep deprivation, which will affect alertness and performance (see below). However, during sleep, alertness and performance cannot be assessed. Furthermore, sleep itself has an effect on alertness and performance, albeit in a direction opposite to the effects of extended wakefulness. Finally, it should be recognized that during the first hours after awakening, alertness and performance are affected by sleep inertia, thereby introducing another factor that can obscure circadian variation (Jewett et al., 1999).

As discussed briefly below, the conditions under which a CR is carried out should be designed with the outcome measures of circadian phase and amplitude in mind. Thus, if a circadian phase marker is known or suspected to be affected by activity, posture, food, or sleep, those factors should be controlled during the CR.

There may be situations in which an ideal 24+ h CR of constant wakeful bed rest is not feasible. For example, certain clinical populations may not easily tolerate the sleep deprivation of a CR, or there may be a key research question in which the sleep deprivation associated with a CR interferes with the outcome variables. Under such circumstances, modifications to the CR technique should only be made if it is clear that those modifications do not affect the circadian rhythm marker being collected and as long as the interpretation of the results remains cautious.

WHAT HAS USE OF THE CONSTANT ROUTINE TAUGHT US THAT WE WOULD NOT HAVE LEARNED OTHERWISE?

We have learned from the CR that the diurnal rhythms of many physiologic processes are controlled in part by the circadian clock but are also influenced by sleep-wakefulness (and/or the postural changes associated with sleep and wake). Such influences can be either inhibitory or additive and can vary widely in magnitude, making it impossible to know a priori what the direct contribution of the circadian clock to a rhythm of interest might be. Use of the CR has revealed, for example, that the typical diurnal profile of thyroid stimulating hormone (TSH) consists of an endogenous circadian component that is suppressed under entrained conditions by sleep (here sleep and the environmental and behavioral changes associated with it are taken as one) (Allan and Czeisler, 1994) (see Fig. 2). The CR technique has also been used to reveal the underlying circadian variation in plasma glucose tolerance. In that study, a constant glucose infusion was given to subjects for 30 h, and despite this constant level of glucose, plasma glucose levels were elevated during the nighttime hours as compared with daytime hours (Van Cauter et al., 1989). Unmasking the circadian waveform of a particular hormone is a first step in understanding the mechanisms by which the pacemaker generates such waveforms. Understanding the relative influence of the circadian timing system versus the influence of the sleep-wake cycle to daily oscillations in physiological variables will also contribute to our understanding of the health consequences of both sleep deprivation and circadian dysrhythmias.

The CR has also been used to investigate the mechanism(s) by which circadian rhythms in certain variables (e.g., core body temperature) are generated. The CR has shown that postural changes have a direct effect on body temperature and sleepiness (Kräuchi et al., 1997) and that the overt rhythm of body temperature results from the interaction of both heat production and heat loss mechanisms (Kräuchi and Wirz-Justice, 1994).

There is great interest among clinicians about the mechanisms underlying diurnal rhythms in blood pressure and cardiovascular function, primarily due to the prominent influence of time of day on the frequency of myocardial infarction (Muller et al., 1985). While under normal conditions both blood pressure (Kario et al., 1999) and heart rate vary across the 24-h day, under CR conditions, there is a significant endogenous rhythm of heart rate (Kräuchi and Wirz-Justice, 1994), but the diurnal variation in blood pressure is absent (van Dongen et al., 2001). Thus, the CR has revealed that some aspects of cardiovascular function appear to be under circadian control, and the diurnal variation in other aspects of cardiovascular activity are more likely due to changes associated with the sleep-wake cycle.

CRs have also revealed that cognitive processes are not controlled by wake-dependent factors alone but

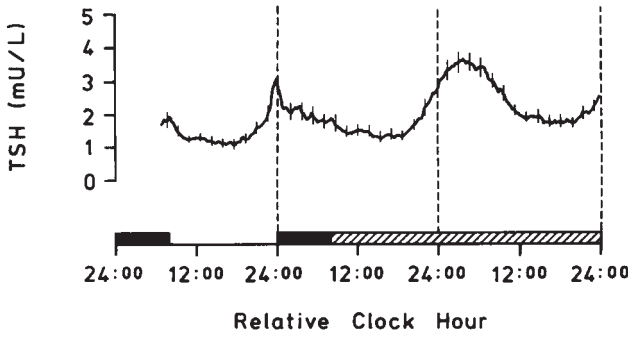

Figure 2. Thyroid stimulating hormone (TSH) under a regular sleep-wake schedule followed by a constant routine. TSH data from 15 young men were averaged (± SEM) with respect to their habitual bedtime and assigned a relative clock hour of 24:00. The solid black bars on the horizontal axis represent the scheduled sleep episodes; the hashed bar represents the constant routine (CR); the vertical dashed lines delineate each successive 24-h segment of the study. Under entrained conditions, there is an increase of TSH prior to habitual bedtime, but TSH begins to decline sharply just after habitual bedtime. Under CR conditions, the evening rise in TSH continues for several hours beyond habitual bedtime, with a more gradual decline. Reprinted with permission from Allan JS, Czeisler CA: Persistence of the circadian thyrotropin rhythm under constant conditions and after light-induced shifts of circadian phase. *Journal of Clinical Endocrinology and Metabolism* 79:508-512, 1994. Copyright The Endocrine Society.

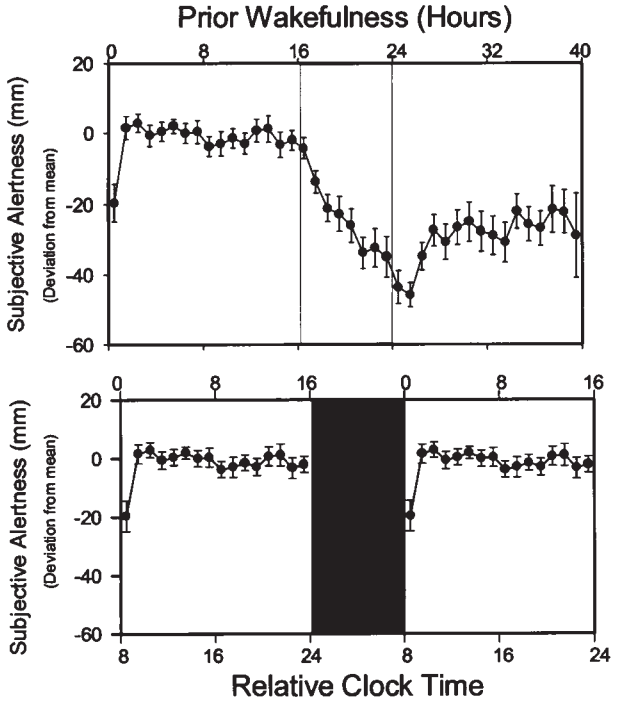

Figure 3. Subjective alertness on a constant routine. Average (± SEM) subjective alertness data from 24 young men collected during a constant routine (CR) are shown in the upper panel. Data were averaged with respect to usual wake time. The open box indicates the timing of the habitual sleep episode. In the lower panel, the first 16 h of the CR are plotted twice, separated by a black box indicating habitual sleep time. Note that the circadian variation in alertness and the timing of the nadir in alertness are only visible under the CR conditions in the upper panel. Reprinted with permission and modified from Dijk D-J, Duffy JF, Czeisler CA: Circadian and sleep- wake dependent aspects of subjective alertness and cognitive performance. *Journal of Sleep Research* 1:112-117, 1992. Copyright 1992 Blackwell Science, Ltd.

that nearly all consist of wake-dependent factors interacting with an endogenous circadian rhythm (Monk et al., 1997). This can be seen in both subjective and objective measures as a decline in performance and alertness over the course of the wakefulness imposed by the CR, superimposed on a 24-h rhythmic component (Dijk et al., 1992) (see Fig. 3). Thus, subjective alertness and performance improve on the second day of a CR relative to the nighttime hours, even though sleep deprivation is greater. Understanding the dynamics of the interaction between circadian and wake-dependent components of alertness and performance is crucial to understanding performance decrements associated with extended wakefulness and night work, to which increasing numbers of people are exposed. Here again, the CR has informed us that the circadian component of most aspects of performance is at its nadir in the early morning hours, typically coincident with or shortly after the nadir of the endogenous body temperature rhythm (see Fig. 3, upper panel). In fact, in the presence of a sleep-wake cycle, both the circadian modulation of alertness/performance and the restorative effects of sleep on those measures are hidden (see Fig. 3, lower panel).

Using the CR, we have also begun to understand entrainment in humans, and the CR has allowed us to identify extreme phenotypes. Under entrained conditions (sleeping at night and awake and ambulatory during the day), masking of the body temperature rhythm can obscure interindividual differences that can be observed under the unmasking conditions of the CR. This is seen in Figure 4, where under entrained conditions, subject 505 appears to have a core body temperature phase similar to the average phase of a group of young men, with a temperature nadir near the middle of her sleep episode (Czeisler et al., 1986). Under CR conditions, her actual phase is revealed to be 6 h earlier than average, occurring near her usual bedtime. Subsequent assessment of circadian period in this subject during a forced desynchrony protocol revealed a shorter than average period of 23.7 h.

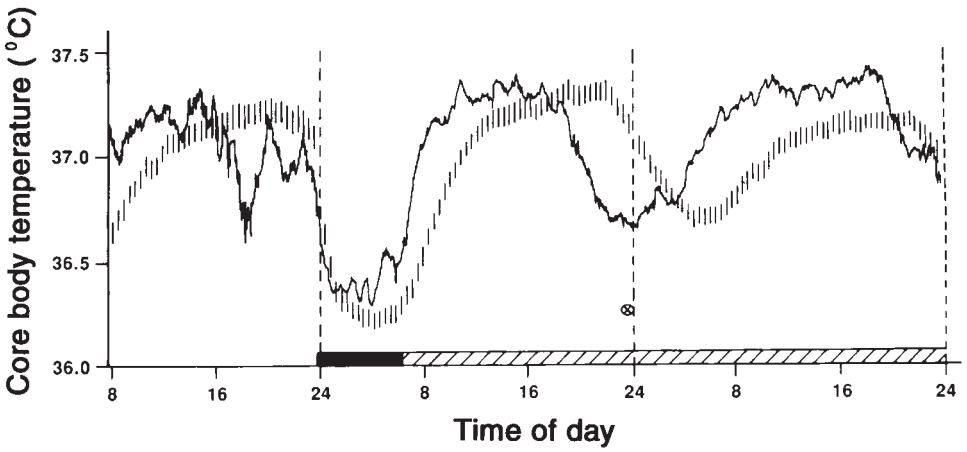

Figure 4. Core body temperature data from a 66-year-old woman (solid line) on a regular sleep-wake schedule followed by a constant routine. Core body temperature data from subject 505 are presented with respect to actual clock hour, and average (± SEM) data from a group of 29 young men are plotted using vertical bars for comparison. The data from the young men were averaged with respect to their habitual bedtime and assigned a relative clock hour of 24:00. The solid black bar on the horizontal axis represents the scheduled sleep episode; the hashed bar represents the constant routine (CR); the vertical dashed lines delineate each successive 24-h segment of the study; the encircled *X* represents the time of the minimum of a waveform fitted to subject 505's CR data, estimated at 23:35. Under entrained conditions, the core body temperature data from subject 505 do not differ markedly from the average data from the young men. Under CR conditions, subject 505 exhibits a markedly advanced temperature rhythm, with a minimum nearly 7 h prior to her habitual wake time. In a subsequent study, this subject was revealed to have a circadian period of 23.7 hours. Reprinted with permission from Czeisler CA, Allan JS, Strogatz SH, Ronda JM, Sánchez RA, Ríos CD, Freitag WO, Richardson GS, Kronauer RE: Bright light resets the human circadian pacemaker independent of the timing of the sleep-wake cycle. *Science* 233:667-671, 1986. Copyright 1986 American Association for the Advancement of Science.

We have observed more subtle differences in entrained phase between young adult "morning" and "evening" types. When we studied morning and evening types under CR conditions, we observed that the phase relationship between their circadian rhythms and their usual sleep-wake cycle was significantly different, such that morning types were waking at an earlier clock hour but a later circadian phase than evening types (Duffy et al., 1999). Although this has also been observed by analyzing temperature data in the presence of sleep (Baehr et al., 2000), these latter data cannot be taken to demonstrate that the changes in internal phase relationship are driven by the circadian pacemaker and are not simply a direct effect of sleep itself. There may be a chronobiological basis for this observed difference in entrained phase, given that we also found that variations in circadian period are correlated with morningness-eveningness and entrained phase in this same age group (Duffy et al., 2001).

In another study, we found significant differences in entrained phase between young and older adults studied under CR conditions (Duffy et al., 1998). Under baseline conditions, the temperature phase of the older subjects appeared to be near the beginning of sleep, while under CR conditions, we found that the temperature phase was in fact significantly closer to wake time in the older subjects. Those differences were not due to the tendency of older subjects to be morning types, because the observed difference in entrained phase in the older subjects was opposite to that found in young morning types (Duffy et al., 1999). The ability to distinguish subtle interindividual differences in entrained phase will be crucial in identifying the genes involved in circadian regulation in humans, as well as the polymorphisms and/or mutations that contribute to extremes of circadian type.

While some researchers have advocated demasking techniques to assess circadian phase from non-CR core body temperature data, we and others have observed that the masking effects of the sleep-wake cycle and those behavioral changes associated with it are phase dependent. This implies that without prior knowledge of circadian phase, one cannot estimate the magnitude of the masking that has occurred. Furthermore, the masking effects caused by the sleep-wake cycle vary widely from person to person (at least 0.35 °C in our study of data from 7 young men) (Dijk et al., 1997), and thus without prior knowledge of the magnitude of the masking effect in an individual, schemes in which a fixed value is added to masked temperature data will be inaccurate (Klerman et al., 1999). Another related issue is that of so-called differential masking:

masking of the underlying circadian component by the sleep-wake cycle differs between variables. For instance, cortisol appears to be only marginally affected by the rest-activity cycle, whereas body temperature is markedly affected. Such differential masking may lead to what appears to be differential rates of reentrainment under conditions, such as jet lag, in which the sleep-wake cycle is shifted abruptly. However, such differential masking does not imply that separate oscillators drive these rhythms. Daan and Beersma (1992) have shown through computer simulations that such patterns can be explained easily by a single pacemaker driving several output rhythms that are differentially masked by the rest-activity cycle. There is a renewed interest in peripheral circadian oscillators and the rate at which they readjust to a phase shift (Yamazaki et al., 2000) and a recognition that behavior may play an important role in the phase control of such peripheral oscillators (Stokkan et al., 2001). The design of experiments and interpretation of data from future studies of the dynamics of central and peripheral oscillators during jet lag and phase-shifting paradigms in humans may benefit from CR protocols in which behavior is controlled. The CR technique can also be used to investigate whether transients, as observed in rest-activity cycles following abrupt phase shifts, are present at the level of the clock itself or are instead a feature of rhythms driven by the clock. By removing the rest-activity cycle and monitoring other output rhythms driven by the pacemaker, the CR can be used to differentiate between these possibilities (see, e.g., Jewett et al., 1997).

Many basic circadian rhythm researchers may have been approached by clinicians who want to know how to assess circadian rhythmicity in a patient. Simply measuring rest-activity cycles and/or ambulatory core body temperature will not, in most cases, reveal circadian rhythm abnormalities. This is even true when such masked data are collected over the course of several days. In a reanalysis of core body temperature data collected over the course of more than 2 months from a free-running blind subject, Klerman et al. (1999) found that phase estimates derived from masked body temperature data could misestimate circadian phase by more than 8 h. It has also been demonstrated in a number of studies of blind subjects that even though the rest-activity cycle appears entrained, the circadian rhythms of cortisol or melatonin can still be free running (Nakagawa et al., 1992; Lockley et al., 1997). These results underscore the fact that in humans, the rest-activity cycle is a poor marker of the circadian clock. In addition, differences in phase angle between the rest-activity/sleep-wake cycle and circadian phase markers in normal subjects can be subtle, and such differences can only be revealed under the unmasking conditions of the CR. It is therefore likely that any abnormality of circadian phase or phase angle in a particular patient would be obscured by measuring ambulatory core body temperature data.

WHEN ISN'T THE CONSTANT ROUTINE USEFUL?

As we have outlined above, the CR is a useful tool in the study of human circadian rhythmicity, and it allows researchers to get closer to the underlying pacemaker by revealing the contribution of the pacemaker to the timing of the output rhythm(s) being studied. This is not to say that the CR should always be the protocol of choice or that there are no potential drawbacks to its use. For example, the CR in its most rigorous form by necessity involves sleep deprivation. Such sleep deprivation will certainly affect any performance/vigilance variable of interest. The sleep deprivation of the CR may also affect other rhythms, such as variations in EEG activity (Cajochen et al., 1999). In addition, it is still unclear whether the CR itself affects circadian phase. There is some reason to suspect that even when carried out in dim light (<20 lux), the continuous exposure to light and sleep deprivation may cause a slight change in phase. A recent study suggests that sleep deprivation alone can cause phase advance shifts of the circadian rhythm of locomotor activity and c-fos expression in the SCN in hamsters (Antle and Mistlberger, 2000).

WHY IS THE CONSTANT ROUTINE "NEEDED" FOR HUMAN RESEARCH BUT NOT ANIMALS?

Whereas the use or usefulness of the CR protocol has become accepted in studies of human circadian rhythms, few hamsters, mice, or rats have participated in such protocols. This may be because animal researchers often rely on the rest-activity cycle or wheel-running activity as their primary marker of the circadian clock, and the CR would remove this marker of circadian phase. Human researchers, however, have concluded that

the rest-activity cycle is a poor marker of the core clock in most situations. Researchers studying human circadian rhythms often assess multiple variables as markers of the clock (e.g., 24-h profiles of melatonin, cortisol, alertness, performance, ocular, and electroencephalographic parameters), while few animal researchers assess multiple output markers of clock function. In fact, sleep and wakefulness are rarely assessed in animal studies. The limited polysomnographically recorded sleep-wake data in rodents reveal that the distribution of sleep and wakefulness across the circadian cycle may be somewhat nearer to uniformity in rodents than in humans or other primates, as would be expected from locomotor activity data. For example, the diurnal rodent *Eutamias sibiricus* sleeps 74% of the time during the dark phase and 27.5% during the light phase (Dijk and Daan, 1989). Similarly, the nocturnal Syrian hamster sleeps 78% in the light period and 51% during the dark period (Tobler and Jaggi, 1987). For rats, these numbers are 69% and 27% (Franken et al., 1991). Thus, the sleep-wake cycle in rodents is highly polyphasic, and the difference in the amount of time spent asleep or awake between the biological night and day is smaller than for humans. From this one might conclude that animal researchers do not need to be as concerned about the masking effects of the sleep-wake cycle as do human researchers. However, reports suggest that indeed animal researchers should be concerned about the effect the sleep-wake cycle has on overt rhythmicity. Franken and coworkers recorded circadian profiles of brain temperature in the rat in the absence and presence of a rest-activity cycle (Franken et al., 1995). They estimated that 84% percent of the observed amplitude of the rhythm of brain temperature could be accounted for by the sleep-wake cycle. That should not be the only concern when studying circadian rhythms in animals allowed to sleep and wake freely. First, in diurnal and nocturnal rodents, the phase relationship between the light-sensitive pacemaker and the rest-activity cycle is reversed. Second, in *Octodon degus*, the phase relationship between the light-sensitive pacemaker and the rest-activity cycle can be reversed abruptly by providing access to a running wheel (Kas and Edgar, 1999). Such findings raise the question of whether there are variations in the phase relationship between the rest-activity cycle and the light-sensitive pacemaker in rodents and indeed if we should assume that the phase relationship is the same in all rodent species.

Assessment of multiple variables, as is common in human studies, is one approach to a more reliable assessment of circadian phase and amplitude in animal studies. However, this does not address the issue of masking, and the alternative is to develop CR-like protocols for use in animal studies. Such an approach was used to demonstrate that the circadian rhythm of electroretinographic responses in iguanas persists when the masking effect of the rest-activity cycle is removed by anesthetizing the animals (Miranda-Anaya et al., 2000). Recently, Aston-Jones et al. (2001) investigated a "circuit for circadian regulation of arousal" in the rat and wondered whether a circadian variation in firing rates (impulse activity) of locus coeruleus (LC) neurons could be part of such a circadian signal. Unfortunately (from a circadian perspective), LC neurons change their firing rate across the three vigilance states, and these investigators recognized that any observed circadian variation in LC firing rates may simply reflect circadian variation in waking, non-REM, and REM sleep. They removed the masking of vigilance state by anesthesia and observed a difference in firing rates of LC neurons between subjective night and day. These examples demonstrate that constant routines or constant routine-like protocols can be used in animal experiments to get through to circadian signals in the intact organism.

The CR is one tool on the palette available to human and animal circadian rhythm researchers. This tool is particularly useful for obtaining information about the phase of a circadian pacemaker in the intact organism on the basis of rhythmicity in variables that are, in part, driven by such a pacemaker but that may also be affected by periodic behaviors. The CR has been used extensively to assess the phase and amplitude of variables driven by the light-entrainable central pacemaker (presumably the SCN) in humans. The CR could also be used to assess the phase of oscillations that may be driven by peripheral oscillators and could even be used to assess whether such oscillations are self-sustained and not merely a direct consequence of periodicity in behavior (Zanello et al., 2000; Bjarnason et al., 2001).

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