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Assembling a Clock for All Seasons: Are There M and E Oscillators in the Genes?

S. Daan,^{*,1} U. Albrecht,[†] G.T.J. van der Horst,[‡] H. Illnerová,[§]
T. Roenneberg,[#] T. A. Wehr,^{**} and W. J. Schwartz^{††}

^{*}Zoological Laboratory, University of Groningen, P.O. Box 14, 9750 AA Haren, the Netherlands,

[†]Max-Planck-Institute for Experimental Endocrinology, Feodor-Lynen-Strasse 7 30625 Hannover, Germany,

[‡]Department of Cell Biology and Genetics, Erasmus University Rotterdam, P.O. Box 1738,
3000 DR Rotterdam, the Netherlands, [§]Academy of Sciences of the Czech Republic, Institute of Physiology,
Videnska 1083, 142 20 Prague 4, Czech Republic, [#]Institute for Medical Physiology, University of Munich,

Goethestrasse 31, D-80336 München, Germany, ^{**}Section on Biological Rhythms NIMH,
Bldg. 10, Room 3S231, 10 Center Drive MSC 1390 Bethesda, MD 20892-1390, USA,

^{††}Department of Neurology, U Mass Medical School, 55 Lake Ave. North, Worcester, MA 01655, USA

Abstract The hypothesis is advanced that the circadian pacemaker in the mammalian suprachiasmatic nucleus (SCN) is composed at the molecular level of a nonredundant double complex of circadian genes (*per1*, *cry1*, and *per2*, *cry2*). Each one of these sets would be sufficient for the maintenance of endogenous rhythmicity and thus constitute an oscillator. Each would have slightly different temporal dynamics and light responses. The *per1/cry1* oscillator is accelerated by light and decelerated by darkness and thereby tracks dawn when day length changes. The *per2/cry2* oscillator is decelerated by light and accelerated by darkness and thereby tracks dusk. These M (morning) and E (evening) oscillators would give rise to the SCN's neuronal activity in an M and an E component. Suppression of behavioral activity by SCN activity in nocturnal mammals would give rise to adaptive tuning of the endogenous behavioral program to day length. The proposition—which is a specification of Pittendrigh and Daan's E-M oscillator model—yields specific nonintuitive predictions amenable to experimental testing in animals with mutations of circadian genes.

Key words evening/morning oscillators, circadian clock genes, pacemaker, photoperiodism, seasonal adjustment

Life on earth faces a dual problem of daily timing. Day and night succeed each other in a cycle of fixed length, but their durations vary systematically with the calendar. How to adjust behavioral programs to this changing day length? This might be achieved by direct or masking effects, for instance, from light on activity or melatonin, but also by adjustment of the endogenous pacemaker-generated rhythm. We have

known for some time that there is a form of memory for day length at the behavioral level. This is clear from *aftereffects* of day length on several aspects of free-running circadian rhythms: aftereffects on period (τ), on activity time (α) (summarized by Pittendrigh and Daan, 1976a), on the phase response curve to light pulses (Pittendrigh, 1981, Fig. 9), and on nocturnal melatonin profiles (Illnerová, 1986, 1991; Wehr et al.,

1. To whom all correspondence should be addressed.

1993; Wehr, 1997). This suggests that the pacemaker is involved in seasonal tuning. Recently, evidence has indeed accumulated showing that circadian pacemakers encode day length: (1) the intrinsic rhythmicity of the SCN in DD is dependent on prior day length (Sumová, Trávníčková, et al., 1995; Sumová, Trávníčková, Peters, et al. 1995; Nuesslein-Hildesheim et al., 2000); (2) when explanted from the body, their rhythm reflects the day length the whole animal had been exposed to before (mammalian SCN: Jagota et al., 2000; avian pineal: Brandstätter et al., 2000). The mechanism by which this information is encoded in the pacemaker is not known. However, a hypothetical principle based on two oscillators was proposed a generation ago. This "clock for all seasons" (Pittendrigh and Daan, 1976b) could simultaneously be exploited to "measure" day length and use this information to adaptively adjust seasonal timing. Recent evidence for double sets of genes involved in the molecular generation of circadian oscillations now calls for a further specification of this hypothesis. We here provide such a specification. It will surely turn out to be incomplete and at best only partly correct. Nevertheless, it may be helpful to generate a series of explicit predictions that may guide some of the next generation of experiments on transgenic and knock-out animals.

EVENING AND MORNING OSCILLATORS

Twenty-five years have passed since the proposition that the mammalian circadian pacemaker consists of a "morning oscillator" (M) locking on to dawn and an "evening oscillator" (E) locking on to dusk (Pittendrigh and Daan, 1976b), yet convincing evidence has accumulated neither to substantiate the E-M model nor to refute it. Research has focused on the phenomenon of "splitting" of the activity rhythm in nocturnal rodents that initially was the main inspiration for the theory. Theoretical exploration of the model (Daan and Berde, 1978) demonstrated that two oscillators have to be nearly identical to have two stable coupling modes—in phase and in antiphase—as expressed in splitting behavior. Studies on diurnal mammals (*Tupaja belangeri*), where splitting occurs in continuous darkness (DD) so that the responsiveness to light pulses can be measured, showed that the two split components indeed represent oscillators with

indistinguishable phase response curves for light (Meijer et al., 1990). Similar evidence for carbachol pulses was obtained in split hamsters in LL (Meijer et al., 1988a). These results suggested that the left and right suprachiasmatic nuclei, which are otherwise indistinguishable, may couple in antiphase in splitting (Daan and Berde, 1978). Similarly, bilaterally distributed insect pacemakers may separate from each other (Koehler and Fleissner, 1978). Indeed, unilateral lesions of the SCN in hamsters led to complete abolishment of one of the split activity components (Pickard and Turek, 1982) or to partial suppression of one component when the lesion was incomplete (Davis and Gorski, 1984). Electrophysiological studies revealed bimodality in the single unit electrical activity of coronal SCN slices of split hamsters (Mason, 1991; Zlomanczuk et al., 1991). These results, though suggestive of a pacemaker origin, not peripheral origin of the split components, were inconclusive concerning the left-right distribution of the electrical activity. Recently, de la Iglesia et al. (2000) found that the two SCN in the brain of split hamsters simultaneously express *Per1* mRNA (normally expressed in the subjective day) in one of the two and *Bmal1* (a marker for the subjective night) in the other. This result now firmly establishes that splitting indeed reflects coupling in antiphase of the left and right SCN.

These developments have compromised the main source of inspiration for the E-M oscillator concept. Pittendrigh and Daan (1976b) viewed splitting as the antiphase between two functionally distinct oscillators, but there is no evidence that there is any functional distinction between the left and right SCN. However, there were other arguments, mostly based on the effects of particular light-dark cycles on activity time. The dual oscillator theory has remained a popular frame of reference with many researchers and has been applied to different aspects of circadian systems: differential responses of onset and end of rodent activity (Pittendrigh and Daan, 1976b; Honma et al., 1985; Elliott and Tamarkin, 1994; Meijer and De Vries, 1995); nonparallel phase shifting of the evening onset and morning offset of (1) melatonin production (e.g., Illnerová, 1991; Illnerová and Vaněček, 1982, 1987) and (2) high c-Fos photoinduction in the rat SCN (Sumová and Illnerová, 1998); sleep-wake and melatonin rhythms in humans exposed to different photoperiods (Wehr, 1995, in press; Wehr et al., 1995). The theory further readily accounts for the bimodality of circadian rhythms often observed (Aschoff, 1966). It

also provides a basis for understanding aftereffects (Pittendrigh and Daan, 1976b) and for maximal rhythm precision at intermediate τ values (Daan and Beersma, in press). From a functional perspective, the main attraction of the model remains that it uniquely accounts both for the adjustment of circadian organization to season and latitude and for the measurement of day length in photoperiodic responses. The mechanism of this “clock for all seasons” (Pittendrigh and Daan, 1976b) has remained elusive, however. Two main sets of facts, emerging from electrophysiological and molecular studies, now simultaneously suggest how the mammalian circadian clock may indeed be built from a morning and evening oscillator.

SCN ELECTROPHYSIOLOGY

The first set of facts is based on a recent serendipitous observation on the electrophysiology of SCN slice preparations from Syrian hamsters (Jagota et al., 2000). Traditionally, such preparations are made from coronal sections through the hypothalamus containing a full dorso-ventral cross section of both SCN (Gillette et al., 1995). These slices produce a single broad circadian peak of high spike frequencies in multiple unit activity (MUA) during the subjective day, alternating with low MUA during the subjective night, but occasionally a slight bimodality can be observed (Mrugala et al., 2000, Fig. 4). Jagota and colleagues (2000) prepared horizontal sections through the same hypothalamic area of Syrian hamsters, containing the full rostro-caudal extent of the SCN, and excluding some of the dorsal margin. In this horizontal slice, the SCN produces two distinct bouts of MUA, one centered around CT 12 and the other 5 to 8 h earlier depending on prior photoperiod (the peak is around CT 7 under LD 8:16, around CT 4 under LD 14:10). The authors proposed that these nonoverlapping bouts of MUA reflect the activity of two component oscillations in the SCN, and they called the CT 4-7 peak M (morning) and the CT 12 peak E (evening).

It is unclear why the expression is so strongly affected by the orientation of the slice. The rise time of the M bout and the fall of the E bout correspond with the rise and fall of the single peak in coronal sections (Jagota et al., 2000). It may therefore be suggested that some (third) influence is missing in the horizontal slice that fills the gap in the coronal slice. In the coronal slice, this influence might either trigger MUA between

the two bouts or bridge the gap by somehow coupling M and E. Be this as it may, we develop the model without further speculating on this issue.

The association of the morning MUA bout with Pittendrigh's M oscillator and the evening bout with the E oscillator is supported by three facts in the study of Jagota et al. (2000). (1) They showed that with expanding photoperiod, M locks on to dawn, whereas E locks to dusk, as suggested by Pittendrigh and Daan (1976b). (2) Glutamate perfusion of the SCN at CT 0—simulating the action of an advancing light pulse on the SCN (Meijer et al., 1988b; Ebling, 1996)—generated an immediate advance shift of M, and no response of E. (3) Glutamate perfusion at CT 16—simulating the action of a delaying light pulse—generated an immediate delay shift of E and no response of M. In the E-M model, M is primarily responsible for phase advances in response to light pulses, and E for phase delays. In the coupled system, both components will eventually be reset by the same amount through the mutual coupling force. We return to phase responses later.

MOLECULAR MECHANISM

The second set of data concerns a number of recent findings on the involvement of mCRY and mPER proteins in circadian oscillations in the mammalian SCN (King and Takahashi, 2000). Their crucial role is summarized here briefly. Both the *mCry* and *mPer* genes are transcribed from nuclear DNA when their promoters are activated by a protein dimer CLOCK/BMAL1. The *mCry* and *mPer* mRNAs are translated in the cytoplasm to mCRY and mPER. As dimeric complexes, these enter into the nucleus. There, mPER2 acts as a factor promoting *Bmal1* transcription, whereas mCRY antagonizes CLOCK/BMAL1 at the promoter regions and thus suppresses *mCry* and *mPer* transcription (Shearman, Sriram, et al., 2000). There are two *Cry* genes in mice (*mCry1* and *mCry2*) as well as two *Per* genes (*mPer1* and *mPer2*). In addition, a third *Per* gene, *mPer3*, has been identified, but its expression is neither sufficient for the maintenance of circadian rhythmicity in the absence of *mPer1* and *Per2* (U. Albrecht, personal communication, November 2000) nor necessary for rhythmicity in their presence (Shearman, Jin, et al., 2000). Shearman, Sriram, et al. (2000) rank *mPer3* among clock-controlled genes rather than among the genes participating in the generation of the circadian oscillation.

So far, the functional implications of the existence of two *mCrys* and *mPers*, both part of the circadian loop in mice, are not clear. We propose that each of the duplicated gene sets plays a nonredundant role in the system. Each of the two sets would be sufficient to sustain rhythmicity, albeit with different dynamics, light responsiveness, and overt expression. A set decelerated by light and accelerated by darkness would thereby lock on to dusk and act as an E oscillator. (Locking on to dusk means that the phase relationship of the entrained oscillator with dusk is better preserved than that with dawn as dawn and dusk move apart with changing photoperiod.) A set accelerated by light and decelerated by darkness would lock on to dawn and act as an M oscillator (i.e., the phase relationship of the oscillator with dawn is better preserved). Specifically, we surmise that *mPer1* and *mCry1* are elements of the M oscillator, whereas *mPer2* and *mCry2* are part of the E oscillator. This is distinct from a molecular dimension to the two-oscillator concept suggested previously by Nusslein-Hildesheim et al. (2000, p 2863), who raised the possibility that "entrainment to different photoperiods changes modifies the phase relationships between light-sensitive *mPer* and light-insensitive *mCry* cycles." Our propositions are based on several aspects of the behavioral and molecular rhythms, in particular, in mice carrying mutations of the respective genes. We review the key features leading to this proposition. We then generate several as yet untested predictions from the hypothesis. Finally, we speculate on the ways in which the two oscillators may be coupled to each other at the molecular level and how they may control behavioral patterns.

To facilitate nomenclature and reduce lengthy descriptions of genes, we will, if not stated otherwise, refer to "*Δgene*" as in a homozygous knockout mouse (deletion on both chromosomes), even if the gene is only partly disrupted, as in the case of the *mper2* mutant.

PERSISTENCE AND FREE-RUNNING PERIOD

In the original version of the rodent E-M oscillator model, the two oscillators were supposed to have different responses of their endogenous period to light intensity. In constant darkness, the "detuning" of the two was assumed to be negative, that is, the E oscillator had a shorter intrinsic circadian cycle length, τ_E ,

than the period, τ_M , of the M oscillator (Pittendrigh and Daan, 1976b; Daan and Berde, 1978). Genetic constructs that lack one of the M components, either *mPer1* (Zheng, Albrecht, Sage, Vaishnav, Sun, Bradley, and Lee, submitted) or *mCry1* (van der Horst et al., 1999; Vitaterna et al., 1999), retain self-sustained circadian rhythmicity, with a rather variable period in DD usually shorter than wild type (Table 1). This is consistent with our model predicting that the E oscillator is less affected in such mutants. In contrast, $\Delta mCry2$ mice have a longer period than wild type (Table 1), interpretable as a predominant expression of the M oscillator. The *mPer2* mutant studied by Zheng et al. (1999) loses stable self-sustained rhythmicity after several days in DD, and thus fits less readily into the scheme than the others. We return to this phenomenon later. Double mutants ($\Delta mPer1$, $\Delta mPer2$, U. Albrecht, personal communication [November 2000], and $\Delta mCry1$, $\Delta mCry2$, van der Horst et al., 1999; Vitaterna et al., 1999) both have lost self-sustained circadian rhythmicity. This is interpreted in the model as both E and M being dysfunctional in each case.

PHASE RESPONSES TO LIGHT

In the original version of the E-M model, the question of whether both oscillators have a complete, bidirectional phase response curve to light was left open:

There is no indication in the available data pointing to a specific shape of the phase response curves for brief light pulses of each oscillator separately. Both may comprise a "complete" PRC, with phase advances and phase delays. Alternatively, one might presume that the PRC of oscillator E has only phase delays (in correspondence with the assumed increasing effect of light on τ_E) while the PRC of oscillator M has only phase advances. (Pittendrigh and Daan, 1976b, p 344)

This need not be a true dichotomy. The oscillators may well have bidirectional PRCs with a larger advance part in M and a larger delay part in E. Data on phase shifting of the rat pineal N-acetyltransferase (NAT) rhythm show that E, reflected in the evening rise in NAT, is instantaneously phase delayed, whereas M, reflected in the morning drop in NAT, is instantaneously phase advanced (Illnerová and Vaněček, 1987; Illnerová, 1991). Phase advances of E are achieved via several transient cycles. The authors

Table 1. Characteristics of circadian rhythmicity in the SCN of wild-type mice and of homozygous mutants of clock-related genes.

Mutant	Wild Type	$\Delta per1$	$\Delta Cry1$	$\Delta per2$	$\Delta Cry2$
τ -DD (h)	23.6 ¹	21.6-23.8 ³	22.5 ⁴	22 instable ¹	24.6 ^{4,10}
$\Delta\phi$ CT 14 (h)	-1.2 ⁵	-1.3 ⁵		0 ⁵	
$\Delta\phi$ CT 22 (h)	+0.6 ⁵	-0.2 ⁵		+1.9 ⁵	
Peak mRNA at CT or ZT					
<i>bmal1</i>	18 ⁶			10 ⁶	
<i>mper1</i>	2 ¹⁰ , 4 ^{7,11,12} , 5 ⁸ , 6 ¹⁶	6 ¹⁵	0 ⁷		6 ¹⁰ , 8 ⁷
<i>mper2</i>	9 ^{8,16} , 10 ^{7,12,14}	9-12 ¹⁵	8 ⁷	6 ¹⁵ -10 ¹	12 ⁷
<i>mCry1</i>	8 ^{10,13} , 9 ⁹ , 12 ^{6,7}	12 ¹⁵		6 ¹⁵ , 8 ⁶	10 ¹⁰
<i>mCry2</i>	-9, 12 ⁷				

1. Zheng et al. (1999).
2. Vitaterna et al. (1994).
3. Albrecht (personal communication, November 2000).
4. van der Horst et al. (1999).
5. Albrecht et al. (2001).
6. Shearman, Jin et al. (2000).
7. Okamura et al. (1999).
8. Field et al. (2000).
9. Kume et al. (1999).
10. Thresher et al. (1998).
11. Shigeyoshi et al. (1997).
12. Takumi et al. (1998).
13. Miyamoto and Sancar (1998).
14. Albrecht et al. (1997).
15. Zheng, Albrecht, Sage, Vaishnav, Sun, Bradley, and Lee (submitted).
16. Shearman, Sriram et al. (2000).

interpreted this as evidence for differential responses of E and M to light. Fully resolving the issue may require genetic deletions destroying the function of either oscillator. Finding identical PRCs in both oscillators would yield equivocal evidence. Finding suppressed advance shifts in homozygous $\Delta mPer1$ or $\Delta mCry1$ mice and suppressed delays in $\Delta mPer2$ or $\Delta mCry2$ mice would provide a strong argument in favor of the E-M concept. Recently, Albrecht et al. (2001 [this issue]) tested the light sensitivity of homozygous *mPer1* and *mPer2* mutants at ZT 14 and ZT 22. They report that $\Delta mPer1$ has lost the capacity for phase advances (at ZT 22) but not for phase delays (at ZT 14), whereas the reverse is true for $\Delta mPer2$. Although full PRCs are required to firmly establish the issue, these results indeed offer support for the proposition that the *mPer1* mutation primarily affects the M oscillator, whereas the *mPer2* mutation has interfered primarily with the E oscillator. Results of Akiyama et al. (1999), showing that antisense phosphotioate oligonucleotide (ODN) suppresses both light-induced *mper1* expression and phase delays, seem to be at variance with this proposition. However, the difference between the suppressive effects of the ODN on *mper1* and *mper2* remains to be tested.

Figure 1. mRNA expression rhythms in the SCN of *mPer1* and *mPer2* (Field et al., 2000) and of *mCry1* and *mCry2* (Okamura et al., 2000). Original average values expressed in deviation from the mean.

PHASE ANGLE DIFFERENCES

All four genes are rhythmically expressed in the SCN of wild-type mice, as demonstrated by in situ hybridization of their mRNAs (review King and Takahashi, 2000). *mPer1* and *mPer2* expression rhythms have markedly different phases (Albrecht et al., 1997; Takumi et al., 1998; Zheng et al., 1999; Okamura et al., 1999; Field et al., 2000; Shearman et al., 2000a): *mPer1* mRNA peaks in the early subjective day, around CT 2 to 6, whereas *mPer2* peaks in the late subjective day, around CT 10 (see Table 1 and Fig. 1). Two earlier reports (Shearman et al., 1997; Zylka et al., 1998) did not observe this difference. The SCN of Syrian (Maywood et al., 1999) and Siberian hamsters (Nuesslein-Hildesheim et al., 2000), as well as of rats (Yan et al., 1999; Miyake et al., 2000), exhibit a similar phase difference as in mice, with *Per1* phase leading *Per2*. This is consistent with the interpretation that *mPer1* reflects the timing of the M oscillator and *mPer2* the E oscillator. Similar phase differences are also found in peripheral tissue (Balsalobre et al., 1998).

It is intriguing to note the correspondence of the peaks in *mPer* expression in the SCN and in MUA in the horizontal SCN slices of Jagota et al. (2000). In both electrophysiological and molecular terms, the M oscillator—characterized as such for different reasons—is expressed maximally around CT 3 to 6, the E oscillator around CT 9 to 12. There is no evidence to suggest a

causal association of *mPer* expression and MUA, especially since the functional proteins lag the respective RNAs by several hours (Field et al., 2000). Yet, the temporal asymmetry with E following M by roughly 90 degrees of the circadian cycle in both cases lends support to our contention that both are manifestations of a two-oscillator structure.

The *mCry1* and *mCry2* rhythms are not as clearly distinguished as those in the *Per* mRNAs. In the study by Okamura et al. (1999), the *mCry1* mRNA peaked around CT 12, whereas *mCry2* displayed a weak oscillation, where no peak is clearly discernible (see Fig. 1). Of two other genes involved, *Clock* and *Bmal1*, the expression of the former does not vary with circadian phase, whereas the latter is expressed maximally from CT 12 to CT 21 (Honma, Ikeda, et al., 1998; Shearman, Jin, et al., 2000; Shearman, Sriram, et al. 2000).

THE HYPOTHESIS

In summary then, we surmise that *Cry1* and *Per1* belong to a part of the oscillating complex that is accelerated by light and constitutes the M oscillator, whereas *Per2* and *Cry2* are a part of the complex that is decelerated by light and constitutes the E oscillator. We propose that each oscillator involves a molecular loop according to the principles reviewed by King and Takahashi (2000): a negative transcription/translation feedback involving both *Per* and *Cry*, possibly enhanced by a positive loop involving at least *Per2* and *Bmal1* (Shearman, Sriram, et al., 2000). E and M are depicted in Figure 2 as being located in the same cell, based on colocalization of *mPer1* and *mPer2* mRNA as reported by Takumi et al. (1998). It is equally possible that different neurons in the SCN oscillate predominantly with either M or E dynamics.

We propose that each of the two main loops controls rhythmic transcription of one or more clock-controlled genes (CCGs). At least one of these genes (indicated by *e* and *m* in Fig. 2) leads after translation to increased neuronal activity and eventually to multiple unit activity as recorded from the SCN. We do not propose that *Cry* or *Per* themselves would trigger MUA of the SCN. On the contrary, the expression of these genes as proteins arrives too late in the circadian cycle for them to be effective signal transducers. There are probably other as yet unidentified genes involved in these pathways. Depending on the timing of the two loops, we can distinguish between an M and an E com-

Figure 2. Outline of the model. In the nucleus, transcription of the clock genes *per* and *cry* is promoted by the CLOCK/BMAL1 complex and suppressed by PER and CRY. The two hypothetical main pathways in the complex model are indicated as separate oscillatory loops, although they interact both via CLOCK/BMAL1 and via multimerization of PER and CRY between the loops. Not shown is the positive feedback of PER2 on *bmal1* transcription (Shearman, Jin, et al., 2000). The two parts of the complex system have slightly different temporal dynamics, leading to differences in the timing of expression of putative clock-controlled genes, indicated here as *m* and *e*. These would eventually lead to two bouts of SCN multiple unit activity, M and E, together suppressing behavioral activity during part of the daily cycle (bottom).

ponent in this activity, with M oscillating with the dynamics of *Per1* and E with *Per2*.

We surmise further that the M oscillator is sensitive to *Per1* expression in response to glutamate (~ light) in the late hours of the subjective night (Albrecht et al., 2001), preceding spontaneous *Per1* expression around CT 3 to 6, and that such exposure leads to a phase advance of the M oscillator. In contrast, *Per2* expression induced in the early subjective night (Albrecht et al., 2001), just after endogenous *Per2* has reached its maximum, would cause a phase delay of the E oscillator. In nocturnal rodents, MUA of the SCN presumably exerts inhibitory effects on behavioral activity. At the bottom of Figure 2, we have, therefore, indicated how activity onset and offset would be under control of the two oscillators. Similar control may be exerted by the SCN on NAT and melatonin production in the pineal. We do not speculate on the specific CCGs involved in these control mechanisms.

The model readily accounts for asymmetries in the transients following single light pulses—or phase

shifts of the zeitgeber—as are well-known at the levels of behavior and physiology. A late subjective night pulse, for instance, immediately advances the morning offset in NAT activity, representing the M oscillator, whereas the daily rise of NAT, supposedly reflecting E, needs several days to catch up (Illnerová and Vaněček, 1987; Illnerová, 1991). Similarly, in transgenic rats in which the *Per1* gene promoter is linked to a luciferase reporter, the advance of the SCN luminescence peak (measured in vitro) is accomplished within 1 day following an advance of the LD 12:12 cycle by 6 h (Yamazaki et al., 2000).

There must be some kind of coupling for the two components of the oscillating system in the SCN to maintain their mutual phase relationship under free-running conditions where both assume the same frequency. In nature, the coupling force will oppose the differential control of each oscillator by light, as it acts to revert to this particular phase relationship from which the light responses pull the system away. Coupling may be envisaged in a large variety of ways, and no strong evidence points to any one of them in particular. If the two oscillators are basically restricted to different populations of neurons, these may couple to each other via one of several ways of neuronal communication (Van den Pol and Dudek, 1993; Miller, 1998; Honma, Shirakawa, et al., 1998). If they are colocalized in the same SCN neurons, coupling might be achieved via differential multimerization of oscillator proteins, via CLOCK/BMAL1 activation of gene transcription in both oscillatory loops, or via still unknown components. In fact, the positive feedback effect of *mPER2* on *bmal1* transcription, with the BMAL1 protein stimulating both *mPer1* and *mPer2* transcription (Shearman, Sriram, et al., 2000), provides evidence for at least one unidirectional coupling pathway from the putative E oscillator on M. The instability of the free-running circadian system in $\Delta mPer2$ mice (Zheng et al., 1999) may actually be attributed to a slightly suppressed *bmal1* rhythm (Shearman, Sriram, et al., 2000), which is possibly involved in both oscillators. Similar instability is caused by deletions of the *clock* gene that is required for the stimulatory action of BMAL1 (Vitaterna et al., 1994). In both $\Delta clock$ and $\Delta mPer2$, coupling can be temporarily restored by a single long light pulse (Vitaterna et al., 1994; Albrecht, unpublished), possibly acting through either *mPer1* induction or BMAL1 degradation (Tamaru et al., 2000).

In the coupling interaction, it is likely that there is some degree of asymmetry in the strengths of the two

oscillators. In mice and rats, several physiological data sets suggest that the E oscillator is often dominant over the M oscillator. One reason to tentatively propose this is that the endogenous rhythmicity appears less stable in $\Delta mPer2$ than in $\Delta mPer1$ mice (Zheng et al., 1999). Another argument derives from a series of studies on the responses of the pineal NAT rhythm to shifts of the light-dark cycle and to single light pulses. Whereas the morning NAT decline, representing a phase of the underlying M, adjusts within one cycle to a delay of the evening NAT rise, representing the underlying E, it takes more transient cycles before the evening NAT rise adjusts to an advance of the morning NAT decline (Illnerová and Vaněček, 1987; Illnerová, 1991). It is not unlikely that the delay:advance ratio in the PRC of mammals reflects this asymmetry. In mice, the delay portion of the PRC for light pulses is much larger than the advance portion (Daan and Pittendrigh, 1976a), and this may well be due to the dominance of the E oscillator.

A current gap in the hypothesis is that the available protein immunoreactivities in or following LD 12:12 mostly appear to peak simultaneously around CT 12 (Hastings et al., 1999; Maywood et al., 1999; Field et al., 2000). The quantification of proteins by immunoreactivity may reflect the complex outcome of a number of processes—protein synthesis, stabilization, and degradation—and has in our view been insufficiently understood to try to explain this discrepancy.

PREDICTIONS

Obviously, our proposition is both a complex and a speculative conjecture. Much remains to be investigated before it can be called a realistic model rather than a working hypothesis. However, the availability of circadian knockout mice now provides a wealth of new possibilities to investigate this complexity. We elaborate on just a few of the predictions to be derived from the hypothesis. These predictions, summarized in Figure 3, primarily concern the behavioral and neurophysiological levels.

At the behavioral level, there is a simple prediction concerning the phase shifting effects of light on the rhythm in the knockout mice. Albrecht et al. (2001) showed that the $\Delta mPer1$ deletion suppresses the phase advance in response to light at ZT 22, whereas in $\Delta mPer2$, phase delays at ZT 14 are eliminated. Full PRCs remain to be measured, but if the result of unidirectional PRCs in these knockout mice holds up, the

Figure 3. Summary of predictions. (A) Phase response curve for brief light pulses in DD. (B) Change of circadian period with increasing fluence rate of constant illumination. Note that both knockout mice (middle and right-hand column) are predicted to remain rhythmic under higher fluence rates than wild types (left column). (C) Aftereffects of prior zeitgeber period: absent or reduced in circadian knockouts compared to wild-type mice. (D) Expression of multiple unit activity of the SCN (see text).

prediction is that also $\Delta mCry1$ will lack phase advances and $\Delta mCry2$ phase delays. This is a rather unique, not otherwise intuitively clear, prediction, in that it is known that *mCry* itself is not expressed in response to light and is also not necessary for the light response of *mPer1* and *mPer2* (Okamura et al., 1999). A less extreme prediction is that $\Delta mPer1$ and $\Delta mCry1$ have suppressed advances and enhanced delays, whereas $\Delta mPer2$ and $\Delta mCry2$ have suppressed delays and enhanced advances (Fig. 3A).

A second prediction about light concerns the dependence of the period of the free-running circadian rhythm on the fluence rate of constant illumination ("Aschoff's rule"). This dependence can be

viewed either as a necessary consequence of the acute phase shifting effect (Daan and Pittendrigh, 1976b; Daan, 1977) or as functionally associated with phase shifts (Beersma et al., 1999). We predict that τ_E should generally increase in $\Delta mPer1$ and $\Delta mCry1$ mice with increasing fluence rate. The same is true for wild-type mice (Daan and Pittendrigh, 1976b), and indeed for most nocturnal rodents (Aschoff, 1979), and the prediction is, thus, not particularly specific for this model (general dominance of E over M in nocturnal animals may indeed have led to the lengthening of τ with increasing light intensity, whereas diurnal animals are often expected to have dominance of M over E). The reverse prediction for $\Delta mPer2$ and $\Delta mCry2$ mice provides a more powerful test. In these constructs, only the M oscillator is supposedly left intact. The period of the M oscillator of these mice, predicted to be accelerated by light, should shorten monotonically with increasing LL fluence rate. This would contrast with all other nocturnal mammals known (Aschoff, 1979). It is not unreasonable to surmise that arrhythmicity in high LL intensities is partly attributable to the opposite influences of light on E and M. If this is true, we may expect that arrhythmicity may require higher light intensities—or even not occur at all—in the mutants where either M or E is lacking (Fig. 3B).

One of the central propositions in the original E-M oscillator model was that with M locking on to dawn and E on to dusk, the phase relationship of E and M in the coupled system will reflect the day length to which an animal has been exposed. Somehow, this phase relationship might mediate the aftereffects of photoperiod on the circadian system, for example, on activity time (α), on pineal NAT and melatonin profiles, and possibly on τ of the free-running rhythm. A general prediction from the model is that none of the homozygous mutants in which either oscillator has been destroyed should show such aftereffects, although aftereffects on τ may also result from interneuronal coupling rather than from coupling between E and M (Fig. 3C).

At the *neurophysiological* level, the predictions are likewise straightforward. If horizontal mouse SCN slices also show two MUA peaks as demonstrated in hamsters (Jagota et al., 2000), we predict that such slices from $\Delta mPer1$ and $\Delta mCry1$ mice lack the M peak, whereas $\Delta mPer2$ and $\Delta mCry2$ lack the E peak (Fig. 3D). If only coronal sections can be studied in mice, or only single peaks are detected, the obvious prediction is that these peaks are reduced in width in the knockouts compared with wild type, with the peaks phase

advanced in $\Delta mPer2$ and $\Delta mCry2$ compared to $\Delta mPer1$ and $\Delta mCry1$.

At the *molecular* level, predictions for knockout mice are not very robust, because neither the generating mechanism nor the coupling pathways are known in sufficient detail. However, predictions can be made for the phase relationship between M and E in wild-type mice in different photoperiods and in constant illumination. With increasing photoperiod, we predict that the phase lead of M over E increases, as M locks on to dawn and E to dusk. It should be possible to observe this in *mPer* gene expression. Although there are no studies on the photoperiodic effects on circadian gene expression in the mouse SCN, a study in Siberian hamsters shows that indeed the phase lag of mPER2 protein relative to mPER1 increases with increasing photoperiod (Nuesslein-Hildesheim et al., 2000, Figs. 4 and 5). In the same species as well as in the Syrian hamster, *mPer1* mRNA expression also retains its phase position relative to lights-on better than to lights-off with increasing photoperiod (Messenger et al., 1999, Fig. 3; 2000, Fig. 3). Similarly, we predict that in constant illumination, as M is accelerated and E is slowed down, the phase lead of M over E increases compared to DD. This change, which in nocturnal mammals would result in LL in the well-known reduction of activity time (α) and in diurnal mammals in increased α (Aschoff, 1960), should likewise be observable in gene expression.

FUNCTIONAL PERSPECTIVE

As envisaged early on (Pittendrigh and Daan, 1976b), two oscillators or components of an oscillating complex pacemaker, coupling both to each other and differentially to dawn and dusk, provide complex organisms with a minimal structure to make their circadian program track day length in the course of the year. We presume that the circadian pacemaker in the SCN of nocturnal rodents generates this flexible program (see Fig. 4). Each of the two oscillators inhibits spontaneous locomotor activity at specific phases of its cycle through increased neuronal SCN activity. The M oscillator responds to early morning light by *per1* expression at CT 16 to 24 (Albrecht et al., 1997; Miyake et al., 2000) and by a phase advance; the E oscillator responds to evening light by *per2* expression at CT 16 (Miyake et al., 2000) and is delayed. Long days thus draw the two apart. Under short days, they revert toward their DD-phase relationship.

Figure 4. Schematic illustration of the control of the M oscillator (solid line) and E oscillator (dashed line) by day length in winter, equinox, and summer, respectively. Lower part of each panel gives the predicted patterns of multiple unit activity and locomotor activity.

The width of the SCN signal, from onset of the morning peak in MUA until the end of the evening peak, would reflect both the prior day length and the "forbidden zone" for activity. In diurnal animals, the same activity of the SCN would then stimulate behavioral activity. The SCN control of behavior becomes particularly clear in split rhythms, where signals from left and right SCN (de la Iglesia et al., 2000), each probably containing the coupled E-M system, drift apart into antiphase with each other. In this situation, behavioral inhibition by each SCN for ca. 10 h leaves only room for two brief bouts of activity. In nocturnal hamsters, these bouts can each be only approximately 2 h (Pittendrigh and Daan, 1976b). In the diurnal tree shrew, they amount to approximately 10 h each (Meijer et al., 1990; Beersma and Daan, 1992), consistent with a stimulatory signal from each SCN.

During the inactive episode of each cycle (in the nonsplit state), the extent of suppression of activity is additionally controlled by homeostatic processes reg-

ulating the need for sleep (Borbély, 1982; Daan et al., 1984) and possibly the fatigue terminating activity. These processes require much better elucidation before we can start modeling the detailed circadian control of behavioral states. The data on human sleep-wake cycles under different photoperiods demonstrate that sleep onset and end lock on to dusk and dawn along with the melatonin profiles, whereas sleep may become bimodal in its nocturnal distributions (Wehr, 1995, in press). They lend confidence that eventually the E-M model will be applicable also to our own species.

Although the E-M model may be naively simplistic in view of the detailed complexity of the generation of circadian oscillations (King and Takahashi, 2000), it has several definite attractions: (1) It unifies data obtained at the molecular, neurophysiological, and behavioral levels; (2) it yields a series of explicit predictions that are readily amenable to experimental verification; and (3) it has obvious implications for the evolution of circadian timing. The rotation of the earth provides two precise timing signals, sunrise and sunset, rather than a single one. We should not be amazed that life that evolved on its surface has developed ways to exploit both signals, for the fine tuning of its daily organization as well as for adjusting its annual functions.

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