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REVIEW

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The mammalian circadian timing system: from gene expression to physiology

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Abstract Many physiological processes in organisms from bacteria to man are rhythmic, and some of these are controlled by self-sustained oscillators that persist in the absence of external time cues. Circadian clocks are perhaps the best characterized biological oscillators and they exist in virtually all light-sensitive organisms. In mammals, they influence nearly all aspects of physiology and behavior, including sleep-wake cycles, cardiovascular activity, endocrinology, body temperature, renal activity, physiology of the gastro-intestinal tract, and hepatic metabolism. The master pacemaker is located in the suprachiasmatic nuclei, two small groups of neurons in the ventral part of the hypothalamus. However, most peripheral body cells contain self-sustained circadian oscillators with a molecular makeup similar to that of SCN (suprachiasmatic nucleus) neurons. This organization implies that the SCN must synchronize countless subsidiary oscillators in peripheral tissues, in order to coordinate cyclic physiology. In this review, we will discuss some recent studies on the structure and putative functions of the mammalian circadian timing system, but we will also point out some apparent inconsistencies in the currently publicized model for rhythm generation.

What are biological clocks?

Most biochemical and physiological processes fluctuate in a temporal fashion, and some of these do so with a relatively constant period length. Cycles with a period length (τ) of approximately 24 h are considered to be circadian, derived from the Latin words *circa diem* (meaning about a day). Rhythms with substantially shorter and longer period lengths are called ultradian and infradian, respectively. This classification is somewhat arbitrary, in that the period length (τ) of ultradian rhythms can range from fractions of seconds to about 20 h, and that of infradian rhythms from about 30 h to decades. Examples of ultradian rhythms are heartbeat frequencies ($\tau \approx 1$ s in man), respiratory oscillations in yeast ($\tau \approx 40$ min, Murray et al. 2001), somite deposition during vertebrate embryogenesis ($\tau \approx 90$ min, Pourquie 2003), and foraging rhythms of the common vole *Microtus arvalis* ($\tau \approx 2.5$ h, Gerkema and van der Leest 1991). Infradian rhythms include female estrus cycles (τ = days to months, depending on the species), circannual mating cycles ($\tau \approx 1$ year), and emergence cycles of some cicada ($\tau \approx 13$–17 years (Hoppensteadt and Keller 1976).

The sole observation of rhythmic behavior or physiology does not necessarily indicate that a biological clock is involved. For example, the circannual sexual cycle of hamsters, which in males manifests itself by a 20-fold oscillation of testicle weight, is controlled by seasonal changes in melatonin secretion (Bartness et al. 1993). In turn, the variations in melatonin secretion are brought about by seasonal changes in day length (photoperiodism). Only processes that continue to oscillate in the absence of external time cues (e.g., temporal changes in light intensity and temperature), are considered to be outputs of biological timekeepers. The same cyclic processes can be controlled by environmental cues in one species and by biological clocks in another species. For example, yearly reproduction cycles are governed by photoperiodism in the Siberian hamster and by a true circannual timekeeper in certain ground squirrels and fruit bats (see Lincoln et al. 2003).

A further complication in studying biological clocks is that they are only detectable if synchronized within an organism or within populations of cells. Thus, if cultures of yeast cells (*Saccharomyces cerevisiae*) are grown in a chemostat that assures a constant supply of oxygen and nutrients, oxygen consumption oscillates with a constant period length of about 40 min (Murray et al. 2001).

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Moreover, under such growth conditions the transcripts of most yeast genes fluctuate in concentration with the same 40 min period length (Klevecz et al. 2004). This ultradian respiratory clock probably also functions in yeast cells grown in normal liquid cultures or on agar plates, but it escapes detection under these conditions because the phases of individual cells do not become synchronized. Likewise, cultured mammalian cells harbor robust circadian oscillators (Balsalobre et al. 1998). However, unless the daily oscillations in gene expression generated by these cell-autonomous timekeepers are recorded in individual cells, they are only noticeable after synchronization by chemical signals (see below) (Balsalobre et al. 1998, 2000b; Nagoshi et al. manuscript in preparation).

Molecular circadian oscillator: model and open questions

Circadian oscillators have been genetically and biochemically dissected in several model organisms. In 1990, Hardin et al. discovered that the Drosophila period protein is required for the cyclic accumulation of its own mRNA and therefore proposed that the period gene is engaged in an autoregulatory feedback loop (Hardin et al. 1990). Since then negative feedback loops of clock gene expression have been uncovered in all genetic model systems for circadian oscillators, including cyanobacteria, neurospora, plants, and mammals. Excellent and detailed reviews are available on all of these systems (Albrecht and Eichele 2003; Froehlich et al. 2003; Golden 2003; Reppert and Weaver 2002; Roenneberg and Merrow 2003; Staiger 2002; Stanewsky 2003); here we will concentrate on a brief discussion of some recent findings about the mammalian circadian timing system.

The identification of mammalian clock genes has greatly profited from genetic studies in the fruit fly *Drosophila melanogaster*, as most essential Drosophila clock genes have orthologs in mammals. Remarkably, however, one essential transcription factor of the circadian oscillator, dubbed CLOCK (for circadian locomotor output cycles kaput) was first identified by a forward genetic approach in the mouse (King et al. 1997).

The cartoon in Fig. 1 displays a simplified version of the mammalian circadian molecular oscillator. The key negative components of this genetic circuitry are the four genes encoding the repressors cryptochrome 1 (*Cry1*), cryptochrome 2 (*Cry2*), period 1 (*Per1*), and period 2 (*Per2*). These genes are activated by the two PAS domain basic helix-loop-helix transcription factors CLOCK and BMAL1, the key positive components of the circadian oscillator. PER and CRY proteins form heteropolymeric complexes of unknown stoichiometry, and once these complexes have reached a critical concentration in the cell nucleus, they interact with the CLOCK-BMAL1 heterodimer and thereby annul the activation potential of these transcription factors. As a consequence *Cry* and *Per* mRNAs and proteins decrease in concentration, and once the nuclear levels of the CRY–PER complexes are insufficient for auto-repression, a new cycle of *Per* and *Cry* transcription can start (Albrecht and Eichele 2003; Reppert and Weaver 2002). Many additional components contribute to the robustness of this molecular clockwork circuitry. For example, the orphan nuclear receptor and repressor REV-ERBα interconnects circadian transcription of the positive and negative "limbs" of the oscillator. *Rev-Erbα* transcription is regulated by the same components that control *Per* and *Cry* transcription, and the resulting circadian accumulation of REV-ERBα leads to periodic repression of *Bmal1* and *Clock* transcription. In turn, this leads to a rhythmic expression of *Bmal1* and (to a lesser extent) *Clock* mRNA that is antiphasic to *Rev-Erbα* expression (Preitner et al. 2002).

Posttranslational mechanisms such as protein phosphorylation also play important roles in generating oscillations of approximately 24 h. For example, casein kinase 1ε (CK1ε), initially identified as an essential Drosophila clock component (Price et al. 1998), phosphorylates PER, CRY, and BMAL1 proteins (Eide et al. 2002; Eide and Virshup 2001; Lee et al. 2004), and hypomorphic *Ck1ε* mutant alleles (dubbed *Tau*) cause a dramatic shortening of the period length in hamsters (Lowrey et al. 2000). In keeping with this observation, an autosomal dominant mutation in the human *Per2* gene that inactivates a CK1ε phosphoacceptor site results in familial advanced sleep phase syndrome (FASPS) (Toh et al. 2001). CK1δ, a close paralog of CK1ε, has also been found to be associated with PER–CRY complexes and may therefore perform a similar function as CK1ε (Lee et al. 2001). Hypophosphorylated PER proteins have a higher metabolic stability than their hyperphosphorylated counterparts, and this may

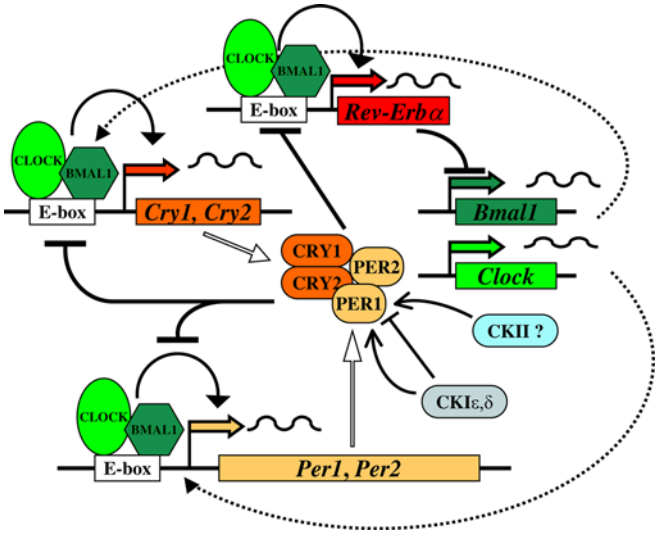

Fig. 1 Simplified model of mammalian circadian oscillator. This model explains several biochemical findings and observations made in mice or hamsters carrying mutations in clock genes. However, as shown in Fig. 2, it does not explain how PER and CRY proteins determine the phase of cyclic *Per* and *Cry* mRNA expression. Phosphorylation by CKIε is believed to render PER proteins less stable (indicated by a *repression bar*). However, it is also conceivable that phosphorylation by CKIε and CKII augments the activity of PER–CRY complexes (indicated by *arrows*, see text)

lead to an increased accumulation of PER proteins. Hence, in both Tau hamsters and human FASPS subjects, threshold levels of PER complexes required for auto-repression are expected to be reached faster than in the corresponding wild-type individuals. As a consequence the period length of the oscillator shortens and its phase becomes advanced. In Drosophila, casein kinase II (CKII) also phosphorylates PER and thereby enhances the repressing activity of this protein (Lin et al. 2002; Nawathean and Rosbash 2004). Given the sequence similarities of mammalian and insect PER and CKII proteins, we consider it likely that mammalian CKII also participates in the modulation of PER activity in the mammalian system. Likewise, glycogen synthase kinase 3 (GSK3) appears to play a role in both the Drosophila and the mammalian circadian oscillator (Harms et al. 2003),

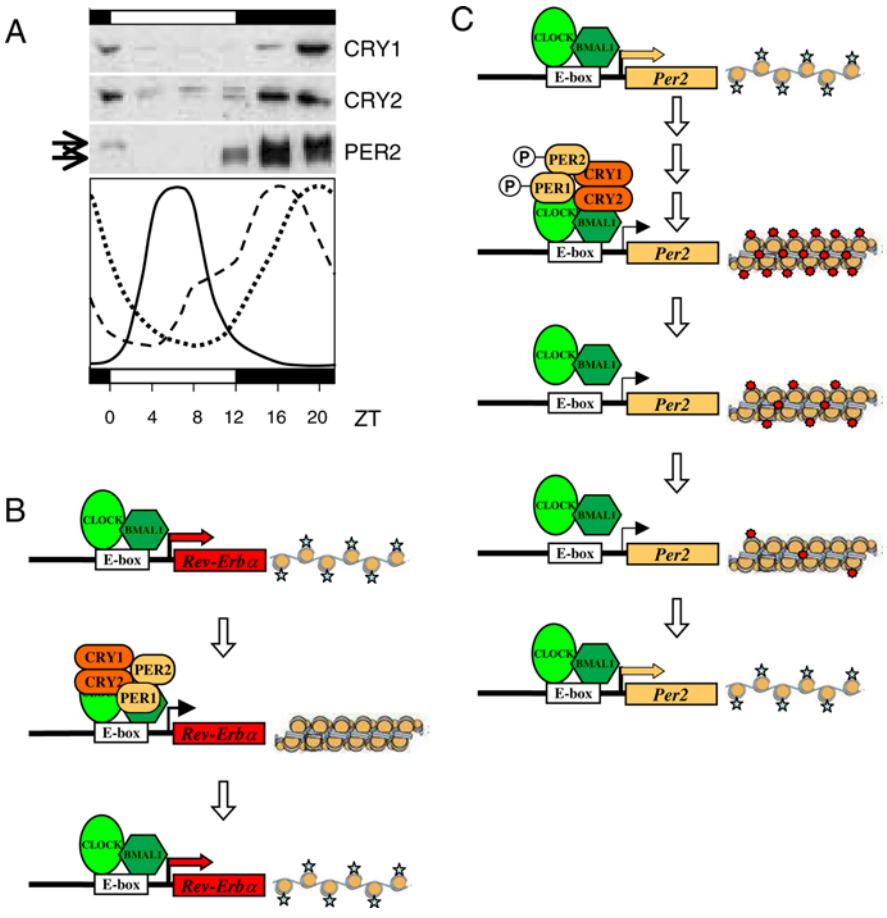

Fig. 2a–c How do CRY and PER proteins determine different phases of circadian gene expression? **a** Circadian clock gene expression in mouse liver. The *three top panels* show an immunoblot of liver nuclear proteins harvested at 4 h intervals around the clock with antibodies raised against mCRY1, mCRY2, and mPER2 proteins. The *bottom panel* displays the temporal accumulation of *Rev-Erbα mRNA* (*solid line*), *Per2* mRNA (*broken line*), and *Cry1* mRNA (*dotted line*) in liver, as measured by TaqMan reverse transcribed polymerase chain reaction (RT-PCR). It can be assumed that the phase of circadian transcription precedes that of circadian mRNA accumulation by about 2 h. The data are adapted from Preitner et al. (2002). **b** Hypothetical model for circadian Rev-Erbα transcription. Circadian *Rev-Erbα* mRNA expression is nearly antiphasic with the circadian accumulation of PER and CRY proteins (see *panel***a**). Hence, the extent of repression of *Rev-Erbα* mRNA transcription correlates closely with the nuclear concentrations of PER and CRY proteins, and a mechanism allowing rapidly reversible transitions between active and inactive chromatin must be operative. Conceivably histone acetylation and deacetylation may be involved (Etchegaray et al. 2003), although this has not yet been demonstrated experimentally for the *Rev-Erbα* locus. *Blue stars* represent putative acetyl groups on nucleosomes of the extended chromatin strings. In the repressed chromatin state, these acetyl groups are assumed to be removed by histone deacetylases recruited by PER–CRY repressor complexes. **c** Hypothetical model for circadian *Per2* transcription. Circadian *Per2* transcription must be regulated by a different mechanism than that described for *Rev-Erbα* mRNA expression in **b**. As shown in **a**, repression starts only toward the end of nuclear PER–CRY accumulation and persists for several hours after PER–CRY has reached trough levels. This delay could be explained if hyper-phosphorylation of PER proteins were required for the initiation of repression of *Per2* transcription, and if the repressed chromatin state outlasted the presence of PER–CRY complexes. In this purely speculative model it is assumed that repression involves the formation of relatively stable heterochromatin domains around the *Per2* gene. Histone modifications, such as methylations and phosphorylations, as well as the recruitment of heterochromatin components, such as HP1, may participate in this process. The heterochromatin-specific signatures are represented by *red symbols* on the nucleosomes of compacted chromatin. After PER–CRY complexes disappear, the heterochromatin-specific histone modifications and/or macromolecular components are gradually removed, and a new round of transcription can ensue. The active state of the chromatin may be associated with histone acetylation (and other histone modifications), as in the model displayed in **b**. Rhythmic Cry1 transcription may be governed by a similar mechanism

and the same may hold true for protein phosphatase 2A (Sathyanarayanan et al. 2004).

Biochemical work by McKnight and coworkers on NPAS2 (a CLOCK paralog in the forebrain) and CLOCK suggests that circadian rhythms could be influenced directly by cellular metabolism (Rutter et al. 2001). At least in vitro, the dimerization of NPAS2 or CLOCK with BMAL1 and/or the binding of the resulting heterodimers to their DNA recognition sequences (E-boxes) are dramatically modulated by the ratio of reduced to oxidized nicotinamide adenine dinucleotides. High [NAD(P)H/NAD(P)+] ratios facilitate the occupancy of E-boxes by NPAS2–BMAL1 or CLOCK–BMAL1 heterodimers while low [NAD(P)H/NAD(P)+] ratios inhibit this process. This opens the exciting possibility that circadian oscillators can adapt their phase to the nutrient state of the cell (see below). It will be interesting to examine whether mutations in the NAD binding sites of NPAS2 or CLOCK will affect the function of these transcription factors in circadian rhythm generation.

Although impressive progress has been made in the genetic and biochemical dissection of mammalian circadian oscillators, the widely advertised oscillator circuitry presented in Fig. 1 should be regarded as a working hypothesis. In fact, many important issues remain unanswered or are even at odds with this oversimplified scheme. For example, the model posits that the cyclic transcription of *Rev-Erbα*, *Cry*, and *Per* is governed by the same mechanism. Yet the phase of circadian *Rev-Erbα* mRNA accumulation differs from that of circadian *Per2* and *Cry1* mRNA accumulation by about 9 h and 11 h, respectively (Fig. 2a). The accumulation of PER and CRY proteins is antiphasic with that of *Rev-Erbα* transcription but almost in phase with *Per* and *Cry* transcription. Thus, while the trough in *Rev-Erbα* expression can readily be explained by the abundance of PER–CRY complexes, more complicated mechanisms must be operative in CRY-mediated and PER-mediated auto-repression. Because PER phosphorylation is considerably retarded with regard to PER protein accumulation, this apparent conflict could be resolved if only CRY–PER complexes containing hyperphosphorylated PER proteins were capable of auto-repression, and if auto-repression were associated with changes in chromatin structure that outlast the presence of PER and CRY proteins. This hypothesis is supported to some extent by the observation that histone acetylation around the *Per1* and *Per2* promoters is rhythmic and parallels the transcription rates of these genes in mouse liver nuclei (Etchegaray et al. 2003). A hypothetical model of how PER–CRY may generate different phases of target gene expression is displayed in Fig. 2b,c. If PER and CRY regulate the cyclic transcription of *Rev-Erbα* and *Per2* (or *Cry1*) indeed by different mechanisms, a signature determining which mechanism is used must exist in each of the PER/CRY target genes. These signatures may be binding sites of yet unknown transcription factors or epigenetic marks on DNA (e.g., CpG methylations) or histones (posttranslational modifications).

In contrast to most biochemical processes, circadian rhythms are temperature-compensated. That is, within the physiological temperature range the period length changes very little if the temperature is increased or decreased. Thus Q_{10}—the ratio between the rates measured for (bio) chemical reactions at two temperatures differing by 10°C within the physiological range—is nearly 1 for the circadian period length (Dunlap 1999). This temperature compensation may be important for poikilothermic organisms, such as fish, amphibians and reptiles, in order to anticipate daytime irrespective of ambient temperature. Surprisingly, however, two recent reports have demonstrated that even mammalian circadian oscillators are temperature-compensated (Izumo et al. 2003; Tsuchiya et al. 2003). The molecular basis for temperature compensation remains elusive, but it is conceivable that synthesis and degradation rates of clock gene products are modulated in the same direction by increasing or decreasing temperature. Moreover, temperature-dependent changes in the accumulation of clock components may be compensated by temperature-dependent interactions between them. For example, at low temperatures the synthesis of *Cry* and *Per* mRNAs and proteins may be reduced, but due to a higher affinity between CRY and PER proteins under these conditions, fewer CRY–PER complexes might be required for repression.

Master and slave circadian oscillators

The suprachiasmatic nucleus (SCN) was positively identified as the master circadian pacemaker in mammals more than a decade ago by an elegant lesion and transplantation experiment. Surgical SCN lesions render laboratory animals completely arrhythmic with regard to wheel-running activity. Remarkably the rhythms can be restored by the transplantation of fetal SCN tissue, and the period length of the rescued rhythmicity is determined by the SCN implant of the donor (Ralph et al. 1990; Silver et al. 1996). Recently, circadian expression of a luciferase reporter gene under the control of the mouse *Per1* promoter has been recorded in real time in individual neurons of SCN explants kept in tissue culture. This elegant experiment has shown that most neurons contain circadian oscillators (Yamaguchi et al. 2003). A similar conclusion has been reached by recording circadian firing frequencies in individual hamster SCN neurons obtained by the enzymatic dissociation of SCN tissue. While all neurons show circadian fluctuations in electrical activity, the periods vary dramatically between different cells. Yet the average period length corresponds to the period length of the locomotor activity of the donor, a clock output directly regulated by the SCN (Liu et al. 1997). Hence, in vivo the SCN neurons must be coupled to synchronize their molecular oscillators. This is probably accomplished by both synaptic communications and paracrine mechanisms. Gene knockout studies in mice suggest that the cell adhesion molecule NCAM-180 and the VIP/PACAP receptor VPAC2 may be involved in the paracrine

coordination of circadian SCN gene expression and outputs (Harmar et al. 2002; Shen et al. 1997).

Although many unicellular organisms have circadian timekeepers, it came as a surprise that vertebrate tissue explants and even immortalized cell lines harbor functional circadian clocks (Balsalobre et al. 1998; Yamazaki et al. 2000). The oscillators in cultured cells can readily be observed after a short treatment with high concentrations of serum or a wide variety of chemicals that induce known signaling pathways, including those involving the activation of protein kinase A (PKA), protein kinase C (PKC), mitogen activated protein kinase (MAPK), glucocorticoid receptor (GR), endothelin, glucose, and retinoic acid receptor (RAR) (Akashi and Nishida 2000; Balsalobre et al. 2000a,b; Hirota et al. 2002; McNamara et al. 2001; Yagita and Okamura 2000; Yagita et al. 2001). Circadian gene expression in tissue culture cells can also be induced by square-wave temperature cycles (e.g., by 12 h 33°C/12 h 37°C). Moreover, body temperature oscillations, recorded by telemetry in the intraperitoneal cavity of mice and simulated in the cell culture medium by a computer-directed incubator, can sustain previously induced cyclic gene expression (Brown et al. 2002). Thus, although body temperature rhythms are not the most important timing cues for peripheral clocks (see below), they probably contribute to the synchronization of these timekeepers.

Until a few months ago, it was thought that peripheral clocks dampen after a few cycles, and that only oscillators in SCN neurons were self-sustained. However, in an elegant study Takahashi and his colleagues have recently shown that circadian timekeepers in liver and lung explants can generate up to 20 (or more) daily cycles of *Per2*-luciferase expression (Yoo et al. 2004). A similar number of daily cycles of luciferase activity could also be recorded in serum-induced NIH3T3 fibroblasts kept in tissue culture when the *Bmal1* promoter drives circadian luciferase expression (Nagoshi et al. manuscript in preparation). Hence, there is little reason to believe that SCN neurons possess more robust clockwork than peripheral cell types or fibroblasts in tissue culture. In spite of this resemblance between central and peripheral clocks, the terms of master and slave oscillators are still justified. Thus, while circadian gene expression persists in peripheral organs of SCN-lesioned mice, their phases are no longer coordinated, neither between the same organs in different individuals nor between different organs in the same individual (Yoo et al. 2004). Therefore, although

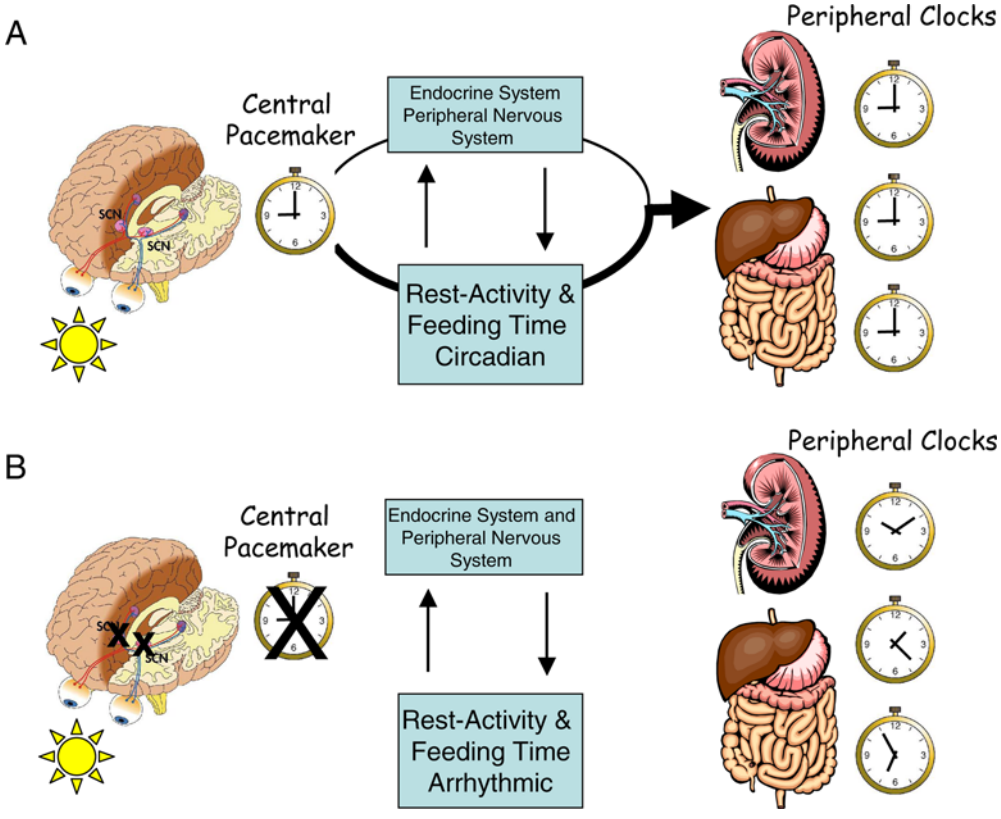

Fig. 3a, b Synchronization of the mammalian timing system. **a** The mammalian circadian timing system is composed of countless self-sustained oscillators that are operative in both suprachiasmatic nucleus (*SCN*) neurons and peripheral cell types. The central pacemaker in the SCN is synchronized every day by the photoperiod via visual and non-visual photoreceptor cells in the retina (see text). Rhythmic neuronal and hormonal SCN outputs drive circadian rest–activity cycles, which in turn limit feeding time to certain time windows during the day. Feeding and/or fasting are the most dominant Zeitgebers (timing cues) for subsidiary clocks in peripheral tissues. However, the SCN also uses more direct timing cues, such as humoral and neuronal signals, to entrain the phases of peripheral clocks (see text). **b** Peripheral clocks continue to oscillate in SCN-lesioned mice, but their phases are no longer coordinated in these behaviorally arrhythmic animals (Yoo et al. 2004)

circadian clocks in the periphery keep ticking in the absence of an SCN, they must be synchronized by the SCN pacemaker (Fig. 3).

Phase entrainment of central and peripheral oscillators

As implied by their name, circadian clocks can measure the day length only approximately. For example, in constant darkness the period length of most laboratory mice is slightly below 24 h. Therefore, the circadian timing system must be corrected every day by a few minutes, in order to stay in harmony with geophysical time. The photoperiod is the key synchronizer of the circadian clock in all well-studied systems. In mammals, photic inputs are perceived by the retina and transmitted in the form of electrical signals directly to SCN neurons via the retino-hypothalamic tract. Neuronal signaling at the synapses between optic nerve endings and SCN neurons involves primarily the neurotransmitters glutamate and PACAP (Hannibal 2002). Recent studies have added an unexpected facet to the photic synchronization of mammalian circadian clocks. Even visually blind mice, whose retinas lack all classic cone and rod photoreceptor cells of the outer layer, can still efficiently phase entrain their circadian pacemaker through light-sensitive ganglion cells in the inner layer (Berson 2003; Freedman et al. 1999). These neurons contain the photopigment melanopsin, and their axons project directly to SCN neurons. Only mice that are deficient in both classic photoreceptors and melanopsin are incapable of adjusting their clock to light–dark cycles (Hattar et al. 2003; Panda et al. 2003). These mice are still rhythmic, but are incapable of phase-shifting their clock in response to light.

Because the phases of peripheral circadian clocks depend on output signals of the SCN, it is sufficient to entrain the phase of the latter to adapt the entire timing system to the physiological needs of the organism. Feeding time is the major Zeitgeber (timing cue) that entrains the phase of peripheral oscillators. Thus, feeding nocturnal rodents such as mice or rats exclusively during the day for a week or longer gradually inverses the phase of circadian gene expression in liver, pancreas, heart, skeletal muscle, kidney, and lung (Damiola et al. 2000; Stokkan et al. 2001). Importantly, feeding time has little influence on the phase of circadian SCN gene expression, and daytime feeding thus completely uncouples the phases of central and peripheral clocks (Damiola et al. 2000; Stokkan et al. 2001). However, as soon as food is offered ad libitum—even after an extended period of daytime feeding—the SCN rapidly reestablishes its dominance and resynchronizes peripheral clocks to the normal, nocturnal mode (Le Minh et al. 2001). Since the feeding time-driven uncoupling of peripheral oscillators from the SCN pacemaker takes much longer (7–10 days) than their re-synchronization to the normal mode, one has to postulate that the SCN can also synchronize peripheral clocks via more direct routes than imposing feeding time. Several observations strongly suggest that glucocorticoid signaling is one of these pathways (Le Minh et al. 2001). For example, circadian corticosterone production/secretion is controlled by the SCN, and the GR agonist dexamethasone is a potent phase shifting agent for peripheral clocks in intact animals and tissue culture cells, but not in the SCN itself. Moreover, daytime feeding inverts the phase of circadian liver gene expression much more rapidly in mice with a liver-specific glucocorticoid receptor gene knockout, while the oscillators in other, GR-proficient tissues of the same animals show slow phase inversion kinetics. Finally, all tissues of adrenalectomized mice display rapid phase inversion kinetics upon daytime feeding. Given the multitude of additional signaling pathways that can affect circadian oscillators in tissue culture cells (see above), it is likely that the phase entrainment of peripheral clocks by hormones, paracrine/autocrine factors, and body temperature rhythms is complex and redundant. Hence the detailed genetic and biochemical dissection of the mechanisms involved in the synchronization of peripheral clocks will be a major challenge in the field.

The benefits of circadian clocks

If cycles in physiology and behavior can be controlled by environmental time cues, what benefits do biological clocks offer to their owners? A key difference between cycles that are driven by external time cues and biological timekeepers is that only the latter can anticipate environmental changes. For example, the circadian clock of plants allows them to produce photo-system I and II components already before sunrise, so that photosynthesis can commence as soon as light energy is available (Harmer et al. 2000). On a similar note, a nocturnal rodent possessing a circadian timekeeper can anticipate dusk in his underground habitat and does not have to forage periodically to examine whether sunset is approaching. Such anticipation may considerably reduce exposure to day-active predators and thus provide a selective advantage to an animal possessing a circadian timing system. The temporal separation of chemically incompatible reactions is another important function of biological timing systems. Cyanobacteria (*Synechococcus elongatus*), in which nearly all genes are transcribed in a circadian fashion, can perform both nitrogen fixation and photosynthesis (Berman-Frank et al. 2001). If these processes were conducted simultaneously, the oxygen generated by photosynthesis would poison the nitrogenase, and nitrogen fixation would be inefficient. The circadian clock solves this problem by separating the phases of photosynthesis and nitrogen fixation. Likewise, circadian oscillators in mammalian liver cells may help to separate glycogen synthesis and degradation in time, limiting the former to the absorptive phase and the latter to the postabsorptive phase (Ishikawa and Shimazu 1976). Some important biochemical reactions can produce harmful side products, and it would be advantageous to limit such activities to a time window during which they are needed. For example, cytochrome p450 enzymes

involved in the hepatic detoxification of xenobiotic substances can generate reactive oxygen species (Bondy and Naderi 1994). This offers perhaps a rational explanation for the circadian expression of many cytochrome p450 genes, with peak expression during the absorptive phase (Furukawa et al. 1999; Lavery et al. 1999).

Considerable insight into the possible functions of the circadian timing system has been gained from transcriptome profiling studies. For example, in liver the accumulation of about 5–10% of all mRNAs undergoes a daily oscillation (Akhtar et al. 2002; Duffield et al. 2002; Panda et al. 2002; Storch et al. 2002). The identification of these cyclic transcripts has led to the conclusion that a large fraction of circadian genes are involved in the processing of food components. Thus, many enzymes playing important roles in the metabolism of proteins, lipids, carbohydrates, and xenobiotic substances appear to be synthesized in a rhythmic fashion. If the anticipation of the absorptive phase is indeed a major task of circadian gene expression, the finding that feeding time is the dominant Zeitgeber for peripheral clocks makes perfect physiological sense, at least for organs such as liver, pancreas, and the gastrointestinal tract.

Transcriptome profiling studies have unveiled two other important aspects of the circadian timing system. First, different circadian transcripts within one tissue can accumulate with many different phases, and secondly, most circadian genes are expressed in a tissue-specific manner. Again, both of these properties are physiologically relevant. As mentioned above, different phases are required for the temporal separation of biochemically incompatible processes (e.g., glycogen synthesis and degradation), and cell type-specific circadian gene expression is in keeping with the concept that different tissues must control the timing of different functions. For example, while our kidney tubules probably play a minor role in circadian food metabolism, they adjust the efficiency of filtration and water-reabsorption to our rest–activity cycles (Ballauff et al. 1991). Many overseas travelers must have noticed that jet lag not only affects the timing of our vigilance state, but also that of urine production.

How do molecular oscillators of the type presented in Fig. 1 govern overt rhythms in physiology and behavior? The transcription of some genes, like the gene specifying the neuropeptide arginine vasopressin, is regulated directly by core components of the oscillator: it is activated by CLOCK–BMAL1 and repressed by CRY–PER complexes (Jin et al. 1999). However, the expression of other clock output genes, such as the ones encoding pyridoxal kinase and some cytochrome p450 enzymes, is controlled by the PAR basic leucine zipper (PAR bZip) transcription factors DBP, TEF, and HLF (Lavery et al. 1999; Gachon et al. 2004). At least *Dbp* is a direct target gene of clock core components, similar to arginine vasopressin (Ripperger et al. 2000). This more indirect pathway, in which clock-controlled transcription factors govern the rhythmic expression of downstream genes, permits the generation of a large number of different phases within the same cell by a transcriptional cascade (Schibler et al. 2003). A recent report suggests that posttranscriptional mechanisms may also be used in establishing cyclic protein accumulation (Baggs and Green 2003). Nocturnin, a mRNA deadenylase, accumulates with a robust circadian rhythm in the retina and many peripheral tissues (Wang et al. 2001). Although nocturnin targets have not yet been identified, the cyclic poly(A) tail shortening of such mRNAs could affect both their stability and translation efficiency in a circadian manner.

Conclusions and perspectives

Since the identification of the first mammalian clock gene in 1997 (King et al. 1997), we have witnessed an explosion of genetic and biochemical studies shedding light on the mammalian circadian timing system. Nevertheless, many enigmas persist, and we are far from understanding the rhythm-generating clockwork circuitry. On the bright side, mathematical modeling has provided proof-of-principle that the auto-regulatory feedback loops proposed for the mammalian clock can generate stable oscillations (Leloup and Goldbeter 2003). However, the kinetic parameters used in such simulations have not been determined experimentally. While genetics has made and will make invaluable contributions to the identification of clock components, we are in urgent need of quantitative biochemical approaches to make further progress. Thus, we will have to purify protein complexes containing the core clock components, identify their subunits, determine their stoichiometry in the complex, estimate the synthesis and decay rates of the central clock components, and determine the equilibrium and rate constants with which they interact. None of these will be easy tasks, but the determination of the rate and equilibrium constants driving macromolecular interactions in living cells will be particularly challenging. Cells are not test tubes, and the effective concentration of a macromolecule cannot simply be obtained by dividing its cellular amount by the cell volume. Indeed, the effective cellular concentrations of macromolecules are influenced dramatically by parameters that are difficult to measure, such as macromolecular crowding (Ellis 2001) and subcellular compartmentalization.

The identification of signaling pathways through which peripheral oscillators are synchronized in the intact organism will be another enticing research area. Feeding time has been shown to have a strong influence on the phase of subsidiary clocks. However, restricted feeding is always associated with restricted fasting, and we do not even know whether it is the former or the latter that synchronizes peripheral clocks. Recent observations made in our laboratory are actually more compatible with a scenario in which periodic starvation is the dominant Zeitgeber. Hence, systemic chemical signals released during the postabsorptive phase might be better candidates for timing cues than signals released during the absorptive phase. Again, the unequivocal identification of such

signals will require both biochemical and genetic strategies.

Last but not least, we wish to emphasize that very little is known about the perhaps most important question, namely whether and how the circadian clock influences the fitness and longevity of an organism. To the best of our knowledge, there are no published studies comparing the life expectancy of intact and SCN-lesioned laboratory animals in a rigorous fashion. *Per2* knockout mice display a high susceptibility to hyperplasias and gamma-ray-induced malignant tumors (Fu et al. 2002). However, it cannot be excluded that PER2 also performs functions that are not related to circadian rhythm generation, and it remains thus unclear whether the tumor proneness is the consequence of a defect circadian oscillator. Serious health problems have also been associated with clock perturbations in man. For example, nurses performing rotating night shift work have been reported to develop breast cancer with an abnormally high frequency (Schernhammer et al. 2001). However, these studies will have to be confirmed by examining larger cohorts. For the moment, jet lag, advanced or delayed sleep phase syndromes, sleep disturbances, and depressions are still the best-known medical consequences of circadian clock perturbations.

All in all, we must admit that the arguments developed above about how circadian clocks may improve the fitness of a mammalian organism are still based on educated guesses. Therefore, we are in urgent need of experimental approaches that rigorously scrutinize the benefits of circadian clocks. Elegant studies with cyanobacteria have shown that such approaches exist, and that the discrimination between the effects of clock gene mutations on clock-related and non-clock-related functions is possible (Ouyang et al. 1998). In these experiments, long and short period mutations in the essential circadian clock gene *kaiC* provide a strong selective advantage to cyanobacteria when these are co-cultured in long and short light–dark cycles, respectively. As the same mutations can have opposite effects under different circadian growth conditions, it must be the resonance of the clock with the photoperiod that improves fitness. As mutations affecting circadian period length are also available for hamsters and mice, such experiments should, in principle, also be feasible for mammalian organisms.

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