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CHAPTER 16

Circadian time keeping: the daily ups and downs of genes, cells, and organisms[☆]

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Abstract: Light-sensitive organisms — from cyanobacteria to humans — contain circadian clocks that produce ~24-h cycles in the absence of external time cues. In various systems, clock genes have been identified and their functions examined. Negative feedback loops in clock gene expression were initially believed to control circadian rhythms in all organisms. However, recent experiments with cyanobacteria and the filamentous fungus *Neurospora crassa* tend to favour protein phosphorylation cycles as the basic timekeeper principle in these species. The study of clock genes in mammals has led to a further surprise; practically all body cells were found to harbour self-sustained circadian oscillators. These clocks are co-ordinated by a central pacemaker in the animal, but they keep ticking in a cell-autonomous fashion when maintained in tissue culture. In mammals, most physiology is influenced by the circadian timing, including rest–activity rhythms, heartbeat frequency, arterial blood pressure, renal plasma flow, urine production, intestinal peristaltic motility, and metabolism.

History of circadian rhythms: from hobby gardening to feedback loops in gene expression

Biological clocks are systems measuring time in the absence of external timing cues such as light or temperature cycles. "Circadian" is derived from the Latin words circa diem (about a day). As the name indicates, circadian timekeepers generate cycles of about — but not exactly — 24 h. Hence, the phase of these oscillators must be corrected by a few minutes every day to keep abreast of geophysical time. The synchronization to the photoperiod is controlled by light inputs in all known organisms, and indeed, circadian clocks are unique to light-sensitive organisms (Dunlap, 1999).

Curiously enough, circadian rhythms were not discovered by biologists, but by a very observant French astronomer, Jean-Jacques d'Ortous De Mairan. In 1729, he noticed daily leaf movements of the mimosa plants cultivated in his backyard and decided to investigate whether these movements were influenced by changes in light intensity or by an endogenous clock. A simple experiment provided the unequivocal answer: when he transferred the plants to pots and kept them in his dark basement, the leaflets continued to fold and unfold in a daily rhythm. Hence, he concluded that the timing of leaf movements was not determined simply by environmental changes (De Mairan, 1729).

De Mairan was not only a good observer, but must have had second sight. He predicted that progress in understanding biological clocks would be slow. Indeed, another 100 years were to pass before the Swiss botanist August de Candolle observed that the leaf movement rhythms of mimosa plants

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were free-running with a period length of 22 h rather than 24 h under constant conditions (Eckardt, 2005). This was an important discovery, since it rendered the possibility unlikely that unnoticed environmental rhythms, for example low amplitude temperature cycles or daily variations in air composition, would drive the leave movement cycles in constant darkness. Yet another 100 years later, Erwin Bünning published a study verifying that the period length of Phaseolum leaf movements has a genetic basis (Bünning, 1932). Compelling evidence for the Mendelian inheritance of circadian rhythms was published by Ronald Konopka and Seymour Benzer in 1971 (Konopka and Benzer, 1971). These authors designed an ingeniously simple mutant screen for the fruit fly *Drosophila melanogaster*, based on the observation that eclosion of flies from pupae is highly circadian. Konopka and Benzer mutagenized male flies using ethyl methane sulfonate. They mated them with females containing fused X-chromosomes and collected those of the male offspring that hatched at unusual times (i.e. during the dark phase). All these males had received their X-chromosomes from their mutagenized fathers, since zygotes with the two fused X-chromosome developed into females. The screening of about 2000 males of the F1 generation resulted in the isolation of three different alleles of one and the same X-linked gene, later now referred to as *period* (*per*). Further analysis revealed that one of these caused arrhythmicity, another period lengthening, and a third one period shortening of both eclosion and locomotor rhythms. In 1987, the groups of Michael Rosbash, Jeff Hall, and Michael Young reported the molecular cloning and transcript analysis of the *per* gene (Bargiello et al., 1984; Reddy et al., 1984). A few years later, Rosbash and co-workers proposed that circadian rhythms might be generated by a feedback loop of *per* gene expression, based on the observations that *per* mRNA abundance followed a daily cycle in *Drosophila* heads and that ectopic overexpression of Per protein attenuated transcription of the resident *per* gene (Hardin et al., 1990; Zeng et al., 1994). The next *Drosophila* clock gene to be cloned was *timeless* (*tim*) (Myers et al., 1995), whose protein product Tim forms multimeric complexes with Per (Gekakis et al., 1995; Rutila et al., 1996).

The year 1997 was a magical year for circadian rhythm research in mammals. Two groups, Tei and coworkers (Tei et al., 1997) and Sun and co-workers (Sun et al., 1997), independently reported on the long-awaited identification of a mammalian homolog of the Drosophila *period* gene. Both groups demonstrated that the mRNA specified by this gene (*Period1*) oscillated in abundance in the suprachiasmatic nucleus (SCN), the master circadian pacemaker in the hypothalamus. In the same year *Clock* (*C*ircadian *l*ocomotor *o*utput *c*ycles *k*aput), the first gene encoding a positively acting transcription factor of the circadian clock, was isolated in mice in a heroic genetic approach by Joseph Takahashi and co-workers (King et al., 1997). One year later, BMAL1 the major dimerization partner of clock was identified in a yeast-two-hybrid screen using *Clock* cDNA as a bait and a hamster hypothalamus cDNA as a prey (Gekakis et al., 1998). In the same year, Drosophila genes encoding orthologues of the mammalian transcription factors CLOCK and BMAL1 were isolated and genetically dissected in the fruit fly (Allada et al., 1998; Rutila et al., 1998). In the years to follow, we witnessed a true clock gene explosion. Several protein kinases (CK1ε/δ, CKII, GSK3, orthologues in both mammals and mammalian species), additional transcription factors, such as Vrille, and Pdp1 in Drosophila (Cyran et al., 2003), and RORα,β,γ (also referred to as NR1F1,2,3, nuclear receptor subfamily1 group F, members 1 to 3) and Rev-Erbα (also referred to as NR1D1, nuclear receptor subfamily1 group D, member 1) in mammals (Preitner et al., 2002; Akashi and Takumi, 2005), RNA-binding proteins (NONO in mouse, NonA in *Drosophila* (Brown et al., 2005b), and a mouse histone methyltransferase-binding protein (WDR5, WD repeat domain protein 5 (Brown et al., 2005b), were added to the repertoire. Thus, while the negative feedback loop model still prevails for metazoan circadian oscillators, its biochemical details are still being modified non-stop. Figure 1 illustrates a currently publicized model for the mammalian circadian clockwork circuitry. In the negative limb, which is the centrepiece of the oscillator, the genes encoding two PER (period) and two CRY (cryptochrome) isoforms are activated by the two

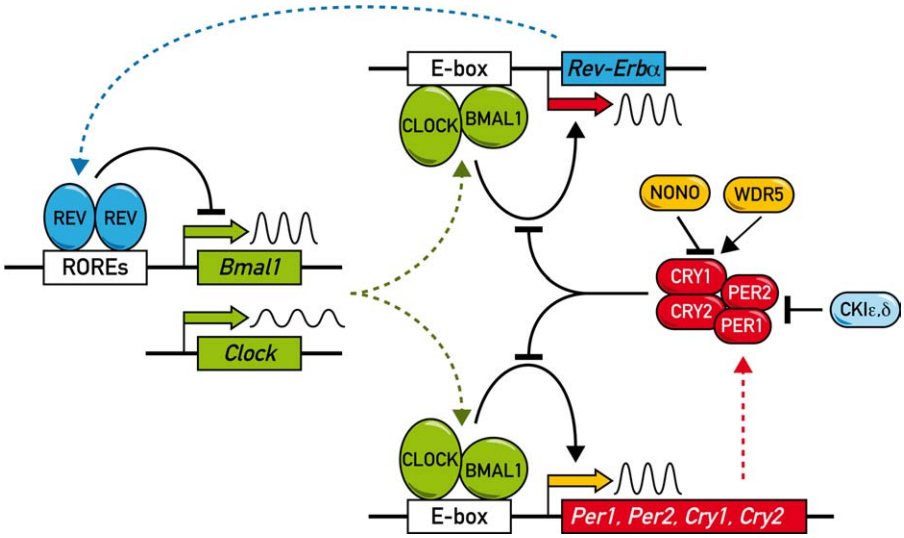

Fig. 1. Hypothetical model of the molecular circadian oscillator. The rhythm generating circuitry is thought to be based on molecular feedback loops within a positive limb (CLOCK, BMAL1) and a negative oscillator limb (PER and CRY proteins) that are interconnected via the nuclear orphan receptor Rev-erbα (see text for details and inconsistencies of this model).

PAS-domain helix-loop-helix transcription factors CLOCK and BMAL1. Once PER:CRY repressor complexes reach a critical concentration, they block the stimulatory action of CLOCK and BMAL1. As a consequence, *Per* and *Cry* genes are silenced, PER and CRY protein concentrations diminish, and a new auto-regulatory cycle of PER and CRY expression can ensue. The transcription of *Rev-erbα* is regulated by the same mechanism, and the periodic accumulation of REV-ERBα elicits the cyclic repression of *Bmal1* (and, to a lesser extent *Clock*). Additional components, such as protein kinases, the RNA-binding protein NONO, and the histone methyl-transferase-binding protein WDR5 are also required for keeping the clock ticking at its normal pace.

A circadian clock in the test tube: protein kinases and phosphatases

Cyanobacteria of the species *Synechococcus elongatus* and the filamentous fungi species *Neurospora crassa* are further examples of systems in which circadian clocks are genetically and biochemically dissected. Although the clock components of these primitive organisms bear no compelling sequence similarity to those of Drosophila or mammalian clocks, negative feedback loops in *clock* gene expression were thought to be the universal rhythm-generating mechanism in all biological systems. However, this view changed dramatically due to Takao Kondo and co-workers' spectacular work on the cyanobacteria clock (Nakajima et al., 2005; Tomita et al., 2005). The three proteins KaiA, KaiB, and KaiC, encoded by the *kaiA/BC* operon, constitute the centrepiece of the cyanobacterium *Synechococcus elongatus*. Like all cyanobacteria genes, *kai* genes are transcribed in a circadian manner. In addition, KaiC, which can act both as an autokinase and an autophosphatase, undergoes robust daily phosphorylation cycles that depend on its physical interactions with KaiA and KaiB. In the absence of photosynthesis, cyanobacteria cannot produce ATP levels that are sufficiently high for transcription and translation. Therefore, RNA and protein synthesis rapidly cease in cyanobacteria kept in constant darkness. However, circadian KaiC phosphorylation continues for several days in the absence of transcription and translation suggesting that variation in protein abundance is not required for circadian rhythm generation (Tomita et al., 2005). Spurred on by this surprising but gratifying result, Kondo and co-workers purified KaiA, KaiB, and KaiC as

recombinant proteins from overexpressing *E. coli* strains and mixed them at the concentration ratios observed in vivo. The addition of ATP to this protein mix triggered 24-h rhythms of KaiC phosphorylation that remained synchronized for several days in the test tube (Nakajima et al., 2005). Hence, protein phosphorylation rather than transcription/translation cycles may be the basic principle of the biological clock in cyanobacteria.

Protein phosphorylation also plays an essential part in generating circadian rhythms in *N. crassa*. In this filamentous fungus, clock protein frequency (FRQ) inhibits the action of the White Collar transcription factors WC-1 and WC-2, which bind to their cognate DNA elements within the *frq* gene promoter as heterodimer complexes (referred to as WCC). Initially, FRQ was thought to act as a transcriptional repressor. However, in 2005, Michael Brunner's group presented compelling evidence that FRQ stimulates the phosphorylation of WCC by an unknown protein kinase, thereby abolishing the DNA-binding ability of this transcription factor complex (Schafmeier et al., 2005). WCC phosphorylation cycles rather than FRQ abundance rhythms may therefore lie at the heart of Neurospora oscillators, and circadian transcription may be a clock output rather than the core mechanism. It will be enticing to investigate whether circadian WCC phosphorylation can be reconstituted in the test tube once the relevant protein kinases and phosphatases will have been identified.

These findings with cyanobacteria and *Neurospora* oscillators strongly suggest that feedback loops in post-translational clock protein modifications are also essential for the oscillator mechanism in insects and mammals. Indeed, the gene expression model presented in Fig. 1 for the mammalian molecular clock suffers from inconsistencies in the phase relationship between PER and CRY mRNA and protein accumulation. Hence, at least in its simplest version, it cannot account for all the experimental observations and theoretical considerations made in the mammalian system. For example, if CRY and PER were the direct transcriptional repressors of their own genes, *Per* and *Cry* transcription cycles should be in antiphase to PER and CRY protein accumulation cycles. However, this prediction has been invalidated by both experimental (Gachon et al., 2004) and mathematical evidence (Schaad, Wanner, and Schibler, unpublished). In fact, antiphasic 24-h accumulation cycles of an mRNA and the protein encoded by it are only possible if temporal protein synthesis and/or stability are regulated independently from temporal mRNA expression. This can be readily demonstrated by numerical solutions of the equation:

$$[protein] = K \int_{-\infty}^{x} e^{[\ln\ 2(t-x)/t_{1/2}]} f(t)[mRNA]\, dt$$

where K is a constant depending on the efficiency of protein synthesis, $t_{1/2}$ the protein half life, t the time of protein synthesis, x the time of protein accumulation, and $f(t)$ the function of time of mRNA concentration, determined by a best-fit equation to the experimentally determined curve. By solving this equation for a series of constant half-lives, we were unable to generate antiphasic protein accumulation cycles from the experimentally measured mRNA accumulation cycles. In fact, even very long protein half-lives that reduce the amplitude of protein cycles to insignificant levels cannot delay the phase of protein accumulation by more than about 4 h from that of mRNA accumulation. Conceivably, transcription cycles play a more important role in clock outputs rather than in the core mechanism of circadian rhythm generation, as has been suggested above for *N. crassa*.

Zeitgeber time, circadian time, and jet lag

As mentioned in the first section, circadian clocks measure daytime only approximately and must be resynchronized daily to keep abreast of geophysical time. Light is the major *Zeitgeber* (German term for "provider of timing cues") for this phase-resetting in all the organisms under investigation. In chronobiology jargon, the time imposed by light–dark cycles is *Zeitgeber* time (ZT). ZT0 is usually defined as the time when the lights are switched on. In contrast, circadian time (CT) is used for the time determined by a circadian oscillator under constant conditions (i.e. in the absence of a synchronizing *Zeitgeber*). For example, mice of the common laboratory strain C57B6 free-run with a period length of 23.77 h (Schwartz and Zimmerman, 1990). Thus, their

circadian clock must be phase-delayed by about 14 min every day. In contrast, human oscillators generate circadian cycles of approximately 24.18 h (Czeisler et al., 1999), and thus have to be phase-advanced by 11 min every day. Since circadian oscillators can be phase-shifted by roughly one to two hours per day, these corrections are not problematic. However, east- and west-bound transatlantic flights cause time-zone differences of several hours which cannot be accommodated in a single day. Hence, for transatlantic travellers it takes several days before circadian time adjusts to *Zeitgeber* time. Since human physiology and behaviour are influenced to a great extent by circadian time, many aspects of daily life style are at odds with the outside world for the first few days after a transatlantic journey. This jet lag affects sleep–wake cycles, as well as the physiology of the kidney and the gastro-intestinal system (see below).

The light-induced phase-shifting of circadian clocks is gated by the oscillator itself. This can be deduced from the phase response curve: light pulses delivered to laboratory mice kept in constant darkness during the subjective day (CT0-CT12) have little influence on the phase, while light pulses delivered during the first half (CT12–CT18) and second half (CT18–CT24) of the subjective night delay or advance the phase respectively. This phase shifting behaviour is probably also involved in the adaptation to seasonal behavioural changes of nocturnal animals: when the days get longer in spring, the phase of activity onset is delayed in the evening and advanced in the morning, leading to a shortened activity phase.

The signalling pathways involved in phase shifting are not yet understood in molecular detail. Nevertheless, I will address some recent observations on how the mammalian circadian timing system is co-ordinated.

The mammalian circadian timing system: a clock in every cell?

The mammalian circadian timing system influences nearly all physiological processes, including sleep–wake cycles, cardiovascular activity, body temperature, acuity of the sensory system, renal plasma flow, intestinal peristaltic motility, hepatic metabolism and detoxification, and many functions of the endocrine system (Schibler et al., 2003). The rhythms of all these clock-controlled processes depend on two tiny aggregates of neurons, called SCN. They were named after their location, immediately above the optic chiasm in the ventral hypothalamus. Bilateral lesions of the SCN renders animals completely arrhythmic. However, behavioural rhythms can be restored in such SCN-lesioned animals by implants of foetal SCN tissue into the third ventricle, close to the position of the ablated SCN (Ralph et al., 1990). The fact that the free-running period length is determined by the donor tissue is even more important: if wild-type hamsters (period length close to 24 h) are SCN-lesioned and grafted with SCN implants of *Tau*-mutant hamsters (period length 20 h), they free-run with a period length of 20 h (Ralph et al., 1990) (Tau is the Greek word for time and is used in chronobiology jargon for period length).

Circadian pacemakers were originally believed to exist only in a few specialized cell types, such as SCN neurons. However, this view has been challenged by the discovery that circadian clocks may exist in most peripheral cell types, and even in immortalized tissue culture cells (Balsalobre et al., 1998). The identification of mammalian clock and clock-output-genes facilitated the examination of circadian rhythmicity in peripheral tissues. These genes were shown to be expressed in daily cycles not only in the SCN neurons, but in virtually all cell types. Nevertheless, the fact that such genes are active in a cyclic fashion in a given tissue does not prove the existence of a circadian clock in this tissue. Indeed, rhythmic gene expression in peripheral organs could be driven simply by oscillating hormones, whose rhythmic secretion is governed by the SCN. Clearly, the unequivocal identification of circadian oscillators in peripheral cell types depends on the demonstration that such cells can generate circadian rhythms in the absence of the SCN master pacemaker. In 1998, Aurelio Balsalobre and co-workers ascertained that circadian cycles of gene expression lasting for several days could be elicited by a serum shock in RAT-1 fibroblasts cultured in vitro (Balsalobre et al., 1998). These experiments were motivated by the observation that most immediate early genes induced by

light in the SCN — including the clock genes *Per1* and *Per2* — are also induced by serum in tissue culture cells. This light-induced immediate-early gene expression correlates with phase-shifting. Subsequently, Emi Nagoshi and co-workers and David Welsh and co-workers measured circadian gene expression in real time and in individual mouse and rat fibroblast to demonstrate that their oscillators function in a self-sustained and cell-autonomous fashion (Nagoshi et al., 2004; Welsh et al., 2004). Since the periods are somewhat variable from cell to cell and from cycle to cycle, the rhythms are not synchronized in untreated cell populations. However, a serum shock transiently synchronizes the oscillations in clock gene expression, such that these can be recorded by studies on cell populations (Fig. 2).

Self-sustained circadian oscillators have also been located in slices from many peripheral organs kept in tissue culture, including liver, kidney, pituitary gland, cornea, and lung. Most body cells probably harbour such clocks (Yoo et al., 2004). In the intact animal, they have to be synchronized to yield coordinated circadian outputs in overt physiology and behaviour. How is this accomplished? As mentioned above, light–dark cycles are the major *Zeitgebers* for the central SCN pacemaker. Light signals required for SCN clock synchronization are not only perceived by classical rod and cone photoreceptors in the outer retina layer, but also by melanopsin-containing ganglion cells in the inner retina layer. These photic inputs are then transmitted as electrical signals to SCN neurons via the retino-hypothalamic tract (RHT). The electrical signalling to the SCN involves the neurotransmitters glutamate and PACAP (also referred to as ADC4AP, adenylate cyclase activating polypeptide) which, when bound to their receptors in SCN neurons, provoke the influx of Ca^{2+}. This results in the activation of several protein kinases (protein kinase A, protein kinase C, mitogen-activated protein kinases), in the phosphorylation of the transcription factor CREB (*C*yclic AMP *R*esponse *E*lement-*B*inding protein), and in the stimulation of immediate-early-gene expression (Albrecht, 2004). *Per1* and *Per2* are among the induced immediate-early genes in all examined species, and the burst in the accumulation of PER proteins probably alters the phase of the molecular clock.

Peripheral oscillators in mammals are not light sensitive and must be phase-entrained by chemical and/or neuronal signalling pathways. It is worthy of note that the circadian oscillators of *in vitro* cultured fibroblasts can be synchronized by a bewildering variety of signalling substances, including those activating nuclear hormone receptors (e.g. glucocorticoid receptor, retinoic acid receptor), G-protein coupled receptors, tyrosine kinase receptors and Ca^{2+} channels (Schibler et al., 2003). Moreover, even low-amplitude body temperature rhythms can sustain the synchronization of peripheral clocks (Brown et al., 2002). The precise molecular mechanisms by which peripheral timekeepers are phase-entrained are not clear, but appear to depend on daily feeding–fasting cycles. Indeed, feeding time, while not affecting the SCN clock, is the most dominant *Zeitgeber* for peripheral oscillators. For example, daytime feeding of nocturnal rodents for a week or longer completely inverts the phase of peripheral oscillators (Damiola et al., 2000).

Our studies have shown that, under certain feeding conditions, glucocorticoid signalling is also used by the SCN to synchronize circadian oscillators in peripheral tissues (Le Minh et al., 2001). One interesting difference between peripheral and central circadian oscillators is that only the latter have a gated phase-shifting behaviour (see above). For example, strong phase-shifting stimuli, such as the glucocorticoid receptor agonist Dexamethasone, can reset the phase of peripheral clocks independently of circadian time, both in vivo and in vitro (Balsalobre et al., 2000; Le Minh et al., 2001). The synchronization of central and peripheral mammalian oscillators is schematically outlined in Fig. 3.

Jet lags, caused by sudden large transitions of time zones that cannot be overcome in a single day, are commonly regarded as sleeping disturbances. However, peripheral organs such as the kidney and the liver are also affected by such time perturbations, and the phase adjustments of their clocks may even lag behind that of the SCN pacemaker. Urination during the night and digestion problems after heavy meals are two typical manifestations of jet lag in the kidney and the gastrointestinal tract. In fact, renal plasma flow and urine production, intestinal peristaltic motility, production of hydrochloric acid by the stomach,

secretion of pancreatic enzymes and bile acids into the gut, and processing of ingested food components and toxins by the liver are all highly circadian (Schibler et al., 2003). These processes require several days to readjust to the new time zone after west- or east-bound journeys.

Human behaviour: larks and owls

Humans can be classified into different chronotypes according to their sleep–wake cycles. For obvious reasons, such innate behavioural differences are generally not apparent during workdays. However, at weekends and on holiday the "true" chronotypes manifest themselves in surprisingly large variations in the timing of rest–activity cycles. As reported by Roenneberg and co-workers (Roenneberg et al., 2003), extreme early birds (larks in chronobiology parlance) wake up and become active when extreme late birds (owls in chronobiology parlance) go to bed. These observations were made on the basis of self-reports of thousands of individuals who were

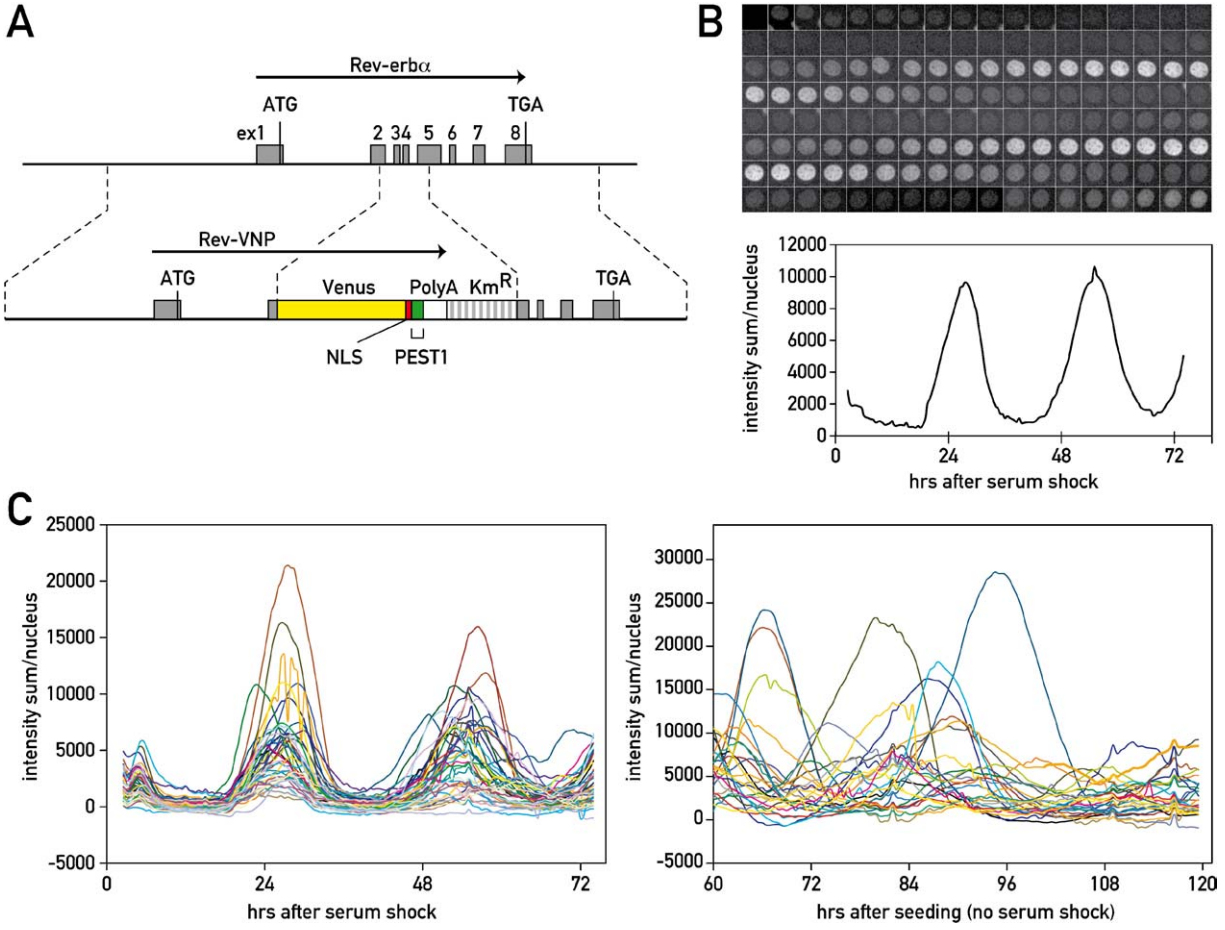

Fig. 2. In vitro cultured fibroblasts contain cell-autonomous circadian oscillators. (A) A Rev-VNP reporter gene was constructed by inserting the open reading frame of Venus, a yellow fluorescent protein, followed by DNA sequences encoding a PEST domain and a nuclear translocation signal (NLS), into a genomic DNA fragment encompassing the *Rev-Erbα* locus. (B) Fluorescent time-lapse microscopy of a Rev-VNP expressing NIH3T3 cell line (1 picture/30 min during 72 h) shows the circadian accumulation of VNP in the nuclei of individual cells. The quantification of the fluorescent signal is shown below the time-lapse recording. (C) VNP accumulation cycles in NIH-3T3 fibroblasts before (right panel) and after synchronization (left panel) by a serum shock. (Reprinted from reference *29* by kind permission of Elsevier Science, Amsterdam, The Netherlands.)

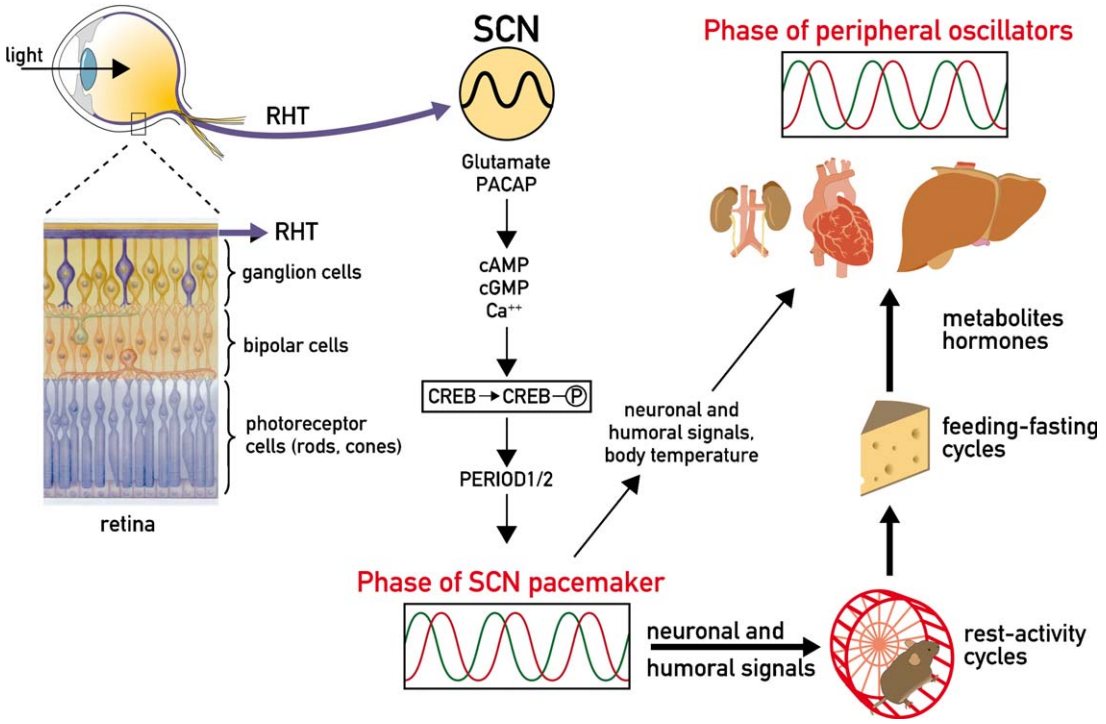

Fig. 3. Synchronization of central and peripheral circadian clocks. The master pacemaker in the SCN is synchronized via light–dark cycles generated by the photoperiod. It then synchronizes peripheral oscillators through behavioural rhythms (rest–activity cycles which engender daily feeding cycles) and neuronal and humoral signals (see text for details). The chemical *Zeitgeber* signals associated with feeding–fasting rhythms and the precise nature of the neuronal signals have not yet been identified.

interviewed about their sleeping behaviour. In a few cases, a hereditary basis for such behaviour could be unequivocally documented. For example, in one case of familial advanced sleep phase syndrome (FASPS), the gene responsible for precocious sleepiness was identified as the circadian clock gene *PER2* (human period 2). Afflicted subjects carry a point mutation in *PER2*, changing a serine into a glycine at amino acid position 662. In the wild-type PER2 protein, serine 662 is phosphorylated by the protein kinase CK1ε, and this phosphorylation reduces the metabolic stability of the protein (Toh et al., 2001). The lack of phosphorylation in the S662G mutant protein could conceivably speed up the oscillator and thereby advance the phase, because the stabilized protein reaches threshold concentrations required for auto-repression earlier during the day.

The systematic recording of free-running human period length means that human subjects must remain under observation for several weeks under laboratory conditions. This is possible in only a limited number of cases. The prohibitively high cost of such experiments does not make experimental recording amenable to large numbers of human chronotypes. However, a recent study by Steven Brown and co-workers may provide a new way of studying human circadian rhythms (Brown et al., 2005a). Fibroblasts from human skin punch biopsies were infected with a lentiviral vector containing a luciferase reporter gene driven from the *Bmal1* promoter. Three to four days after infection, circadian bioluminescence cycles were recorded in real time for four to five days. While the values of different skin biopsies from the same individual were practically identical, skin biopsies from different individuals yielded highly different period lengths, ranging from 22 to 26 h (Fig. 4). Similar experiments with wild-type mice and mice carrying mutations in various clock genes

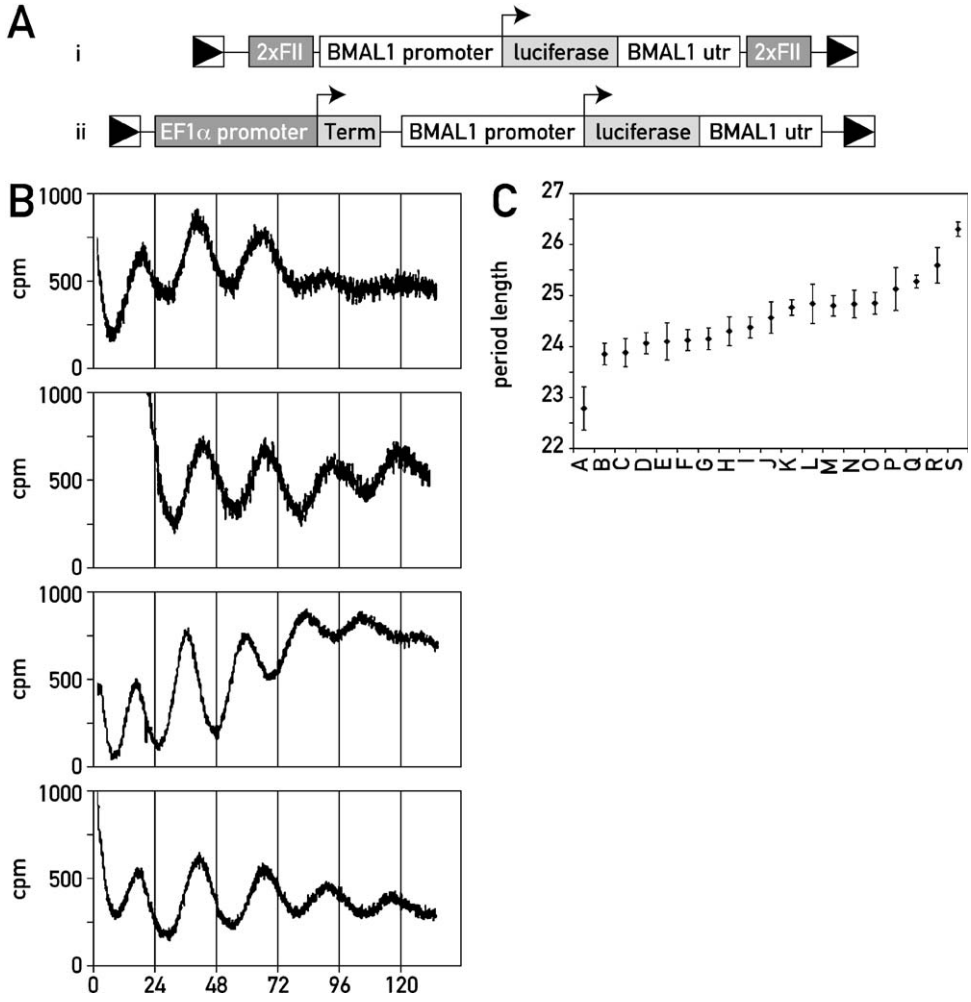

Fig. 4. Circadian bioluminescence in human fibroblasts infected with a lentiviral luciferase expression vector (A) The lentiviral circadian reporter construct contains the mouse *Bmal1* promoter, the firefly luciferase-coding region, and the *Bmal1* 3′UTR, flanked by the long terminal repeats (LTRs) of a lentiviral packaging vector. A DNA segment composed of the *EF1a* promoter and a SV40 terminator is inserted between the upstream LTR and the *Bmal1* promoter, in order to attenuate the influence of transcription regulatory sequences of genomic sequences after viral integration (for details see Brown et al., 2005a). (B) Skin punch biopsies were obtained from 19 individuals. Fibroblasts were isolated from each biopsy, infected with the lentiviral circadian reporter vector shown in panel A, and analyzed by real-time luminometry. Individuals are designated with the letters A–S. (C) Summary of the period lengths of BMAL-luciferase oscillations from all 19 individuals. Each value shows the average plus or minus the standard deviation from two different trials of two different infections of fibroblasts from two to five biopsies per subject. The probability by Student's *t*-test that the most different individuals (A and S) have the same period length is 0.00001; the probability that the second most different (B and R) are equal is 0.004.

suggested that the period values measured in fibroblasts are correlated with those determined for circadian behaviour. Thus, the fibroblasts of mice with behavioural periods longer or shorter than wild-type also display longer and shorter periods respectively (Pando et al., 2002; Brown et al., 2005a). It will be interesting to investigate circadian gene expression in the primary human fibroblasts of different human chronotypes. If, as in mice, a correlation between period length of

fibroblast gene expression and behaviour can be established in human subjects, the inexpensive recording of luminescence cycles from skin biopsies may be used as readouts in genetic linkage studies aimed at the identification of loci that influence human rest–activity cycles.

Perspectives

Although progress in circadian rhythm research has advanced significantly during the past two decades, chronobiologists are unlikely to get bored in the near future. CLOCK input pathways have still to be dissected with regard to the molecular signalling mechanisms by which light–dark cycles synchronize circadian oscillators. Photoreceptors and downstream components required for this process have been identified in several systems, but the path has not yet been paved from capturing of photons to modifying gene expression and resetting the phase. Even less is known about how the SCN synchronizes slave oscillators in peripheral tissues, and about the precise roles of feeding rhythms, hormones, and neuronal signals in this process.

Scientists working on molecular oscillator mechanisms in complex organisms such as plants, insects, and mammals will not rest before they have succeeded in reconstituting a ticking clock in the test tube. As mentioned above, this was recently accomplished in cyanobacteria[21], but even in this simple system the protein–protein interactions generating 24-h KaiC autophosphorylation rhythms in vitro remain obscure. If, as many investigators in the field still maintain, gene expression cycles are at the heart of circadian oscillators in animals and higher plants, the in vitro reconstitution of a ticking clock might well be a "mission impossible". However, it may be possible some day to assemble such a genetic clockwork circuitry in yeast cells, which lack a circadian timekeeping system.

Another fundamental issue is the demonstration in the laboratory that circadian physiology is beneficial to higher organisms. In view of the fact that virtually all light-sensitive organisms possess circadian clocks, there is little doubt that these must provide a selective advantage to their owners. However, compelling experimental evidence in support of this claim has been found only for cyanobacteria and the plant *Arabidopsis thaliana*. Thus, cyanobacteria equipped with clocks whose period length matches that of the light–dark cycles to which they are exposed, rapidly outgrow bacteria with a non-resonating clock (Woelfle et al., 2004). These experiments clearly show that it is the rhythm rather than a crippled gene that is responsible for the growth advantage, since depending on the light–dark cycles the same mutation can be beneficial or deleterious. Likewise, Arabidopsis plants with a resonating oscillator grow more rapidly and are more resistant to environmental insults than plants with discordant clocks (Dodd et al., 2005). In animals, mutations in some clock and clock-controlled genes have been shown to cause severe health problems (Fu et al., 2002; Fu et al., 2005). However, since "resonance experiments" such as the ones described above for cyanobacteria and Arabidopsis have not yet been conducted successfully in animals, we still do not know whether these health problems are the result of perturbed rhythms.

Finally, in spite of the well-funded knowledge that drug metabolism, efficacy, and toxicity are subject to large daily variations (Levi, 2000), attempts to explore these observations clinically are still rather timid. Circadian biologists and physicians will have to collaborate a lot more closely before chronopharmacology and chronotherapy can flourish.

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References

Akashi, M. and Takumi, T. (2005) The orphan nuclear receptor RORalpha regulates circadian transcription of the mammalian core-clock Bmal1. Nat. Struct. Mol. Biol., 12: 441–448.

Albrecht, U. (2004) The mammalian circadian clock: a network of gene expression. Front. Biosci., 9: 48–55.

Allada, R., White, N.E., So, W.V., Hall, J.C. and Rosbash, M. (1998) A mutant Drosophila homolog of mammalian clock disrupts circadian rhythms and transcription of period and timeless. Cell, 93: 791–804.

Balsalobre, A., Brown, S.A., Marcacci, L., Tronche, F., Kellendonk, C., Reichardt, H.M., Schutz, G. and Schibler, U. (2000) Resetting of circadian time in peripheral tissues by glucocorticoid signaling. Science, 289: 2344–2347.

Balsalobre, A., Damiola, F. and Schibler, U. (1998) A serum shock induces circadian gene expression in mammalian tissue culture cells. Cell, 93: 929–937.

Bargiello, T.A., Jackson, F.R. and Young, M.W. (1984) Restoration of circadian behavioural rhythms by gene transfer in Drosophila. Nature, 312: 752–754.

Brown, S.A., Fleury-Olela, F., Nagoshi, E., Hauser, C., Juge, C., Meier, C.A., Chicheportiche, R., Dayer, J.M., Albrecht, U. and Schibler, U. (2005a) The period length of fibroblast circadian gene expression varies widely among human individuals. PLoS Biol., 3: e338.

Brown, S.A., Ripperger, J., Kadener, S., Fleury-Olela, F., Vilbois, F., Rosbash, M. and Schibler, U. (2005b) PERIOD1-associated proteins modulate the negative limb of the mammalian circadian oscillator. Science, 308: 693–696.

Brown, S.A., Zumbrunn, G., Fleury-Olela, F., Preitner, N. and Schibler, U. (2002) Rhythms of mammalian body temperature can sustain peripheral circadian clocks. Curr. Biol., 12: 1574–1583.

Bünning, E. (1932) Ueber die Erblichkeit der Tageszeitperiodizität bei den Phaseolus Blättern. J. wiss. Bot., 81: 411–418.

Cyran, S.A., Buchsbaum, A.M., Reddy, K.L., Lin, M.C., Glossop, N.R., Hardin, P.E., Young, M.W., Storti, R.V. and Blau, J. (2003) vrille, Pdp1, and dClock form a second feedback loop in the Drosophila circadian clock. Cell, 112: 329–341.

Czeisler, C.A., Duffy, J.F., Shanahan, T.L., Brown, E.N., Mitchell, J.F., Rimmer, D.W., Ronda, J.M., Silva, E.J., Allan, J.S., Emens, J.S., Dijk, D.J. and Kronauer, R.E. (1999) Stability, precision, and near-24-hour period of the human circadian pacemaker. Science, 284: 2177–2181.

Damiola, F., Le Minh, N., Preitner, N., Kornmann, B., Fleury-Olela, F. and Schibler, U. (2000) Restricted feeding uncouples circadian oscillators in peripheral tissues from the central pacemaker in the suprachiasmatic nucleus. Genes Dev., 14: 2950–2961.

De Mairan, J.J.d.O. (1729) Observation botanique. Histoire de l'Academie Royale des Sciences, 35–36.

Dodd, A.N., Salathia, N., Hall, A., Kevei, E., Toth, R., Nagy, F., Hibberd, J.M., Millar, A.J. and Webb, A.A. (2005) Plant circadian clocks increase photosynthesis, growth, survival, and competitive advantage. Science, 309: 630–633.

Dunlap, J.C. (1999) Molecular bases for circadian clocks. Cell, 96: 271–290.

Eckardt, N.A. (2005) Temperature entrainment of the arabidopsis circadian clock. Plant Cell, 17: 645–647.

Fu, L., Patel, M.S., Bradley, A., Wagner, E.F. and Karsenty, G. (2005) The molecular clock mediates leptin-regulated bone formation. Cell, 122: 803–815.

Fu, L., Pelicano, H., Liu, J., Huang, P. and Lee, C. (2002) The circadian gene Period2 plays an important role in tumor suppression and DNA damage response in vivo. Cell, 111: 41–50.

Gachon, F., Nagoshi, E., Brown, S.A., Ripperger, J. and Schibler, U. (2004) The mammalian circadian timing system: from gene expression to physiology. Chromosoma, 113: 103–112.

Gekakis, N., Saez, L., Delahaye-Brown, A.M., Myers, M.P., Sehgal, A., Young, M.W. and Weitz, C.J. (1995) Isolation of timeless by PER protein interaction: defective interaction between timeless protein and long-period mutant PERL. Science, 270: 811–815.

Gekakis, N., Staknis, D., Nguyen, H.B., Davis, F.C., Wilsbacher, L.D., King, D.P., Takahashi, J.S. and Weitz, C.J. (1998) Role of the CLOCK protein in the mammalian circadian mechanism. Science, 280: 1564–1569.

Hardin, P.E., Hall, J.C. and Rosbash, M. (1990) Feedback of the Drosophila period gene product on circadian cycling of its messenger RNA levels. Nature, 343: 536–540.

King, D.P., Zhao, Y., Sangoram, A.M., Wilsbacher, L.D., Tanaka, M., Antoch, M.P., Steeves, T.D., Vitaterna, M.H., Kornhauser, J.M., Lowrey, P.L., Turek, F.W. and Takahashi, J.S. (1997) Positional cloning of the mouse circadian clock gene. Cell, 89: 641–653.

Konopka, R.J. and Benzer, S. (1971) Clock mutants of *Drosophila melanogaster*. Proc. Natl. Acad. Sci. USA, 68: 2112–2116.

Le Minh, N., Damiola, F., Tronche, F., Schutz, G. and Schibler, U. (2001) Glucocorticoid hormones inhibit food-induced phase-shifting of peripheral circadian oscillators. EMBO J., 20: 7128–7136.

Levi, F. (2000) Therapeutic implications of circadian rhythms in cancer patients. Novartis Found. Symp., 227: 119–136 discussion 136–142.

Myers, M.P., Wager-Smith, K., Wesley, C.S., Young, M.W. and Sehgal, A. (1995) Positional cloning and sequence analysis of the Drosophila clock gene, timeless. Science, 270: 805–808.

Nagoshi, E., Saini, C., Bauer, C., Laroche, T., Naef, F. and Schibler, U. (2004) Circadian gene expression in individual fibroblasts; cell-autonomous and self-sustained oscillators pass time to daughter cells. Cell, 119: 693–705.

Nakajima, M., Imai, K., Ito, H., Nishiwaki, T., Murayama, Y., Iwasaki, H., Oyama, T. and Kondo, T. (2005) Reconstitution of circadian oscillation of cyanobacterial KaiC phosphorylation in vitro. Science, 308: 414–415.

Pando, M.P., Morse, D., Cermakian, N. and Sassone-Corsi, P. (2002) Phenotypic rescue of a peripheral clock genetic defect via SCN hierarchical dominance. Cell, 110: 107–117.

Preitner, N., Damiola, F., Luis Lopez, M., Zakany, J., Duboule, D., Albrecht, U. and Schibler, U. (2002) The orphan nuclear receptor REV-ERBalpha controls circadian transcription

within the positive limb of the mammalian circadian oscillator. Cell, 110: 251–260.

Ralph, M.R., Foster, R.G., Davis, F.C. and Menaker, M. (1990) Transplanted suprachiasmatic nucleus determines circadian period. Science, 247: 975–978.

Reddy, P., Zehring, W.A., Wheeler, D.A., Pirrotta, V., Hadfield, C., Hall, J.C. and Rosbash, M. (1984) Molecular analysis of the period locus in *Drosophila melanogaster* and identification of a transcript involved in biological rhythms. Cell, 38: 701–710.

Roenneberg, T., Wirz-Justice, A. and Merrow, M. (2003) Life between clocks: daily temporal patterns of human chronotypes. J. Biol. Rhythms, 18: 80–90.

Rutila, J.E., Suri, V., Le, M., So, W.V., Rosbash, M. and Hall, J.C. (1998) CYCLE is a second bHLH-PAS clock protein essential for circadian rhythmicity and transcription of Drosophila period and timeless. Cell, 93: 805–814.

Rutila, J.E., Zeng, H., Le, M., Curtin, K.D., Hall, J.C. and Rosbash, M. (1996) The timSL mutant of the Drosophila rhythm gene timeless manifests allele-specific interactions with period gene mutants. Neuron, 17: 921–929.

Schafmeier, T., Haase, A., Kaldi, K., Scholz, J., Fuchs, M. and Brunner, M. (2005) Transcriptional feedback of Neurospora circadian clock gene by phosphorylation-dependent inactivation of its transcription factor. Cell, 122: 235–246.

Schibler, U., Ripperger, J. and Brown, S.A. (2003) Peripheral circadian oscillators in mammals: time and food. J. Biol. Rhythms, 18: 250–260.

Schwartz, W.J. and Zimmerman, P. (1990) Circadian timekeeping in BALB/c and C57BL/6 inbred mouse strains. J. Neurosci., 10: 3685–3694.

Sun, Z.S., Albrecht, U., Zhuchenko, O., Bailey, J., Eichele, G. and Lee, C.C. (1997) RIGUI, a putative mammalian ortholog of the Drosophila period gene. Cell, 90: 1003–1011.

Tei, H., Okamura, H., Shigeyoshi, Y., Fukuhara, C., Ozawa, R., Hirose, M. and Sakaki, Y. (1997) Circadian oscillation of a mammalian homologue of the Drosophila period gene. Nature, 389: 512–516.

Toh, K.L., Jones, C.R., He, Y., Eide, E.J., Hinz, W.A., Virshup, D.M., Ptacek, L.J. and Fu, Y.H. (2001) An hPer2 phosphorylation site mutation in familial advanced sleep-phase syndrome. Science, 291: 1040–1043.

Tomita, J., Nakajima, M., Kondo, T. and Iwasaki, H. (2005) No transcription–translation feedback in circadian rhythm of KaiC phosphorylation. Science, 307: 251–254.

Welsh, D.K., Yoo, S.H., Liu, A.C., Takahashi, J.S. and Kay, S.A. (2004) Bioluminescence imaging of individual fibroblasts reveals persistent, independently phased circadian rhythms of clock gene expression. Curr. Biol., 14: 2289–2295.

Woelfle, M.A., Ouyang, Y., Phanvijhitsiri, K. and Johnson, C.H. (2004) The adaptive value of circadian clocks: an experimental assessment in cyanobacteria. Curr. Biol., 14: 1481–1486.

Yoo, S.H., Yamazaki, S., Lowrey, P.L., Shimomura, K., Ko, C.H., Buhr, E.D., Siepka, S.M., Hong, H.K., Oh, W.J., Yoo, O.J., Menaker, M. and Takahashi, J.S. (2004) PERIOD2::LUCIFERASE real-time reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues. Proc. Natl. Acad. Sci. USA, 101: 5339–5346.

Zeng, H., Hardin, P.E. and Rosbash, M. (1994) Constitutive overexpression of the Drosophila period protein inhibits period mRNA cycling. EMBO J., 13: 3590–3598.