REVIEW

The Circadian Timing System: Making Sense of day/night gene expression

HANS G. RICHTER¹, CLAUDIA TORRES-FARFÁN², PEDRO P. ROJAS-GARCÍA², CARMEN CAMPINO³, FERNANDO TORREALBA² and MARÍA SERÓN-FERRÉ²

¹ Instituto de Histología y Patología, Facultad de Medicina, Universidad Austral de Chile, Valdivia, Chile

² Unidad de Reproducción y Desarrollo, Departamento de Ciencias Fisiológicas, Facultad de Ciencias Biológicas and ³Departamento de Endocrinología, Facultad de Medicina, Pontificia Universidad Católica de Chile, Santiago, Chile

ABSTRACT

The circadian time-keeping system ensures predictive adaptation of individuals to the reproducible 24-h day/night alternations of our planet by generating the 24-h (circadian) rhythms found in hormone release and cardiovascular, biophysical and behavioral functions, and others. In mammals, the master clock resides in the suprachiasmatic nucleus (SCN) of the hypothalamus. The molecular events determining the functional oscillation of the SCN neurons with a period of 24-h involve recurrent expression of several clock proteins that interact in complex transcription/translation feedback loops. In mammals, a glutamatergic monosynaptic pathway originating from the retina regulates the clock gene expression pattern in the SCN neurons, synchronizing them to the light:dark cycle. The emerging concept is that neural/humoral output signals from the SCN impinge upon peripheral clocks located in other areas of the brain, heart, lung, gastrointestinal tract, liver, kidney, fibroblasts, and most of the cell phenotypes, resulting in overt circadian rhythms in integrated physiological functions. Here we review the impact of day/night alternation on integrated physiology; the molecular mechanisms and input/output signaling pathways involved in SCN circadian function; the current concept of peripheral clocks; and the potential role of melatonin as a circadian neuroendocrine transducer.

Keyterms: biological rhythms, circadian timing system, clock genes, melatonin

Earth rotation imposes 24-hour rhythms to integrated physiological functions

A key evolutionary feature common to all organisms is predictive adaptation to the day/night alternation derived from the Earth's rotation every 24-h (Moore-Ede et al., 1982; Edery, 2000). The 20th century saw the recognition that all living beings, including unicellular organisms, possess a biological clock system that measures time in near 24-h (circadian) units resulting in rhythmic patterns with a period of 24-h, termed circadian rhythms. The tendency of some organisms to sleep at night and some during the day, and the fact that some plants open their leaves during the day and close

them at night, are common observations. The reasonable assumption that these are passive responses to the day/night changes in the environment was proven wrong by a simple yet brilliant experiment performed in the 18th century. In 1729, French astronomer Jean Jacques d'Ortous de Mairan showed that the upright movement of the leaves of the plant *Mimosa pudica* at nighttime and the opening of these leaves during the daytime hours continued over several days when the plant was maintained in constant darkness, indicating that the leaves' movement followed an endogenous 24-h clock (Moore-Ede et al., 1982).

Almost every physiological variable in living organisms shows a circadian rhythm

(for activity/rest, body temperature, and plasma melatonin concentration circadian rhythms in laboratory animals, see Fig. 1). Even complex physiological processes such as childbirth show circadian rhythms; in most women labor begins after midnight and delivery occurs around early morning (Glattre and Bjerkedal, 1983). In individuals exposed to the light:dark (LD) cycle, the phase (i.e. clock time of the peak or trough) of a given rhythm will be similar for different individuals. In the absence of LD signals (by exposure to

constant light or darkness, as well as in the blind), rhythms persist in individuals but with a period close to, but not exactly, 24-h (Enright, 1981). The circadian time-keeping system is actively engaged in the maintenance of normal physiology, not only in adults, but also during development, given that 24-h rhythms in hormones, behavior, and cardiovascular function are present in human, monkey, and sheep fetuses (Serón-Ferré et al., 1993).

The peak and trough of the rhythms for different physiological variables occur at

Figure 1. Photomicrograph of the master circadian clock, the suprachiasmatic nucleus (SCN), and examples of overt circadian rhythms in several mammalian species. *Upper left panel:* coronal section of Nissl stained fetal sheep bilateral suprachiasmatic nuclei (SCN; one is indicated by white arrows); V: third ventricle, bar: 500 μ m. *Upper right panel:* melatonin rhythm in pregnant ewes and their fetuses (closed and open circles, respectively); (mean $\pm$ S.E.; n=4). *Lower left panel:* double plot of locomotor activity rhythm in a rat. Each line represents the recordings of two successive days. Initial recordings were done under *ad libitum* feeding in which the rat shows a nocturnal pattern of activity. The arrow indicates initiation of a restricted pattern of feeding in which food was available from 10:00 to 11:00 hours. Note the shift in the activity rhythm. *Lower right panel:* body temperature rhythm in adult capuchin monkeys (mean $\pm$ S.E.; n=4). Dark bars indicate hours without light.

different clock times within an individual, for instance, in humans under normal LD conditions, cortisol peaks at 0800-h, while temperature peaks at 1400-h and melatonin at 0200-h. In nocturnal animals, such as the rat, corticosterone peaks in the evening and temperature at night, whereas melatonin shows the same phase as in humans and other diurnal animals. The phase relationship between the circadian rhythms of different physiological variables in the 24-h cycle is known as internal temporal order (Moore-Ede et al., 1982; Ederly, 2000), the importance of which becomes clear under normal and pathological conditions in the adult human. In normal subjects, the transient abnormalities derived from intercontinental flights (jet lag) and the adverse effects on working capability after alternate diurnal and nocturnal working schedules are ascribed to an alteration of the internal temporal order (Gold et al., 1992). In clinical practice, it is important to consider circadian rhythms in pharmacokinetics and cell responses to therapy in order to design proper protocols for drug administration (Belanger et al., 1997; Levi, 1997).

In mammals, circadian rhythms are governed by a master clock located in the hypothalamus, which is entrained to environmental signals and commands peripheral oscillators. The use of molecular biology-based approaches, particularly mutational studies, has allowed the identification of a set of genes directly involved in the generation of circadian rhythms, which have been named clock genes. What follows is a review of the components of the circadian time-keeping system, with particular emphasis on molecular mechanisms and the potential role of melatonin as a circadian neuroendocrine transducer.

The mammalian master clock resides in the suprachiasmatic nucleus

The circadian timing system is a supra-physiological system that comprises a hierarchy of biological clocks (peripheral oscillators; see below) commanded by a central nervous system clock (pacemaker).

Such an organization was an early proposal, given that in the 1960s, the existence of circadian rhythms in some physiological variables was demonstrated in dispersed liver cells and in heart, gut, and adrenal explants culture. These and other findings led to a proposal that in mammals and other organisms, the circadian timing system would involve a hierarchy of biological clocks (Moore-Ede et al., 1982).

Systematic lesions of several areas of the hypothalamus in a variety of species that resulted in loss of circadian rhythms, including the locomotor activity rhythm, allowed the identification of the suprachiasmatic nucleus (SCN) of the anterior hypothalamus as a possible site of the biological clock in mammals (Moore and Eichler, 1972; Stephan and Zucker, 1972). A key experiment was the demonstration that activity rhythms were restored by re-grafting the SCN from normal animals (DeCoursey and Buggy, 1989). The definitive finding was the identification of a mutant hamster (tau) displaying a shortened 20-h locomotor activity period that allowed crossed grafting between mutant and normal hamsters with a 24-h locomotor activity period. Thus, lesion of the SCN of a normal animal followed by SCN grafting from a mutant animal restored the locomotor activity rhythm in the recipient animal, but displaying the 20-h period from the donor. This elegant experiment demonstrates that the SCN commands the circadian rhythm of locomotor activity (Ralph and Menaker, 1988; Ralph et al., 1990).

In humans, two clinical correlates showing the importance of the SCN function have been reported. The destruction of the SCN by a tumor resulted in the disappearance of the circadian rhythm of temperature (Schwartz et al., 1986). In another patient, an SCN lesion due to the surgical removal of a hypothalamic tumor resulted in an altered pattern of sleep/wake and body temperature rhythms in the 24-h period. Interestingly, although this patient maintained her intellectual capacities, she showed an impairment of reproducibility in performing the same intellectual tasks when tested on successive days (Cohen and Albers, 1991).

In the rat, each of the bilateral SCNs is formed by a network of approximately 10,000 neurons located on both sides of the third ventricle over the optic chiasm (Moore et al., 2002, and references therein; see Fig. 1, upper left panel, for a coronal section of the fetal sheep SCN). These neurons already exhibit oscillatory activity during fetal life. In rat, non-human primates, and sheep fetuses, the SCN neurons display a day/night rhythm of metabolic activity and *c-fos* expression, which, as in the adult SCN, is higher at noon than at midnight, indicating entrainment of the SCN neurons to the LD cycle (Serón-Ferré et al., 1993). The intrinsic oscillatory capacity of the SCN neurons *in vivo* has been demonstrated by recording electrical activity over 24-h using chronically-implanted electrodes (Kubota et al., 1981; Yamazaki et al., 1998) and also by measuring metabolic activity at discontinuous points in time as 2-deoxyglucose uptake (Schwartz, 1991) and *c-fos* expression (Earnest et al., 1990). The electrical and metabolic oscillatory capacity is maintained for long periods of culture, either as hypothalamic slices (Gillette and Prosser, 1988) or dispersed SCN neurons (Welsh et al., 1995). The latter evidence indicates that the isolated neurons of the SCN are single-cell circadian oscillators.

Suprachiasmatic nucleus entrainment pathways

Light synchronizes the SCN to the 24-h LD cycle, inducing phase shifts in SCN neuronal activity. In several mammals, a bright light pulse applied at early night will delay the phase of the locomotor activity circadian rhythm, whereas at late night it will advance the phase. LD information reaches the SCN neurons through the retinohypothalamic tract. This monosynaptic tract originates primarily from a subset of retinal ganglion cells that express the photopigment melanopsin (Gooley et al., 2003). The retinohypothalamic pathway is anatomically and functionally different from the neural pathway used for pattern vision and uses glutamate and PACAP (pituitary adenylate cyclase-activating polypeptide) to convey

light information (Hannibal, 2002; Gooley et al., 2003). Until recently, light entrainment was thought to rely upon photopigments different from those classically present in rods and cones, such as melanopsin (an opsin-based photopigment expressed in a subset of retinal ganglion cells), because mice lacking rods and cones could be entrained by light (Freedman et al., 1999) and melanopsin-containing retinal ganglion cells are light-sensitive (Berson et al., 2002; Hattar et al., 2002). This hypothesis was tested by analyzing locomotor activity under different lighting conditions in mice with a targeted disruption of the melanopsin gene (Ruby et al., 2002; Panda et al., 2002a). In these mice, light still resets the circadian clock, but the magnitude of phase shifts of the activity rhythms induced by light is decreased. Both reports concluded that melanopsin contributes to, but it is not essential for, resetting the locomotor rhythm at low and medium light levels. Melanopsin may then act in concert with classical photopigments present in rods and cones, which send light information to melanopsin-containing retinal ganglion cells (Ruby et al., 2002; Panda et al., 2002a). Entrainment or synchronization to the day/night cycle requires glutamate and PACAP binding to receptors expressed by the SCN neurons, which evoke second messengers activation that in turn induce expression of the clock gene *Per1* (Hannibal, 2002; see below).

The adult pattern of SCN innervation by the retinohypothalamic tract is attained during late gestation in the human, non-human primates, and sheep (Torrealba et al., 1993; Hao and Rivkees, 1999), whereas it is attained post-natally in the rat and hamster (Müller and Torrealba, 1998). Since only a limited amount of environmental light reaches the fetus, light cannot be an entrainment signal for the fetus (Parraguez et al., 1998). Current evidence suggests that the fetal SCN is entrained by a maternal signal, a possibility that is supported by the identification of melatonin binding sites in the fetal SCN from human and rat (Reppert et al., 1988; Naitoh et al., 1998) and D1 dopamine receptors in the SCN of rat fetuses (Naitoh et al., 1998) and newborn

baboons (Rivkees and Lachowicz, 1997). In fact, it has been shown that periodic administration of melatonin or D1-dopaminergic agonist SKF38393 to pregnant hamsters with SCN lesions are capable of entraining the biological clock in the fetal SCN (Davis and Mannion, 1988; Viswanathan et al., 1994).

Suprachiasmatic nucleus output pathways

The basis of the SCN communication with effectors responsible for the overt physiological rhythms are not well understood. An important question is how the SCN differentially commands a wide range of physiological and behavioral rhythms, such as activity/rest, sleep/wake, body temperature, heart rate, liver and kidney function, up to endocrine rhythms such as those of melatonin, cortisol, gonadotropins and prolactin, among many others. Different peptidergic neuronal subtypes are present in the SCN, which synthesize and release vasopressin (AVP), vasoactive intestinal peptide (VIP), somatostatin, and gastrin-releasing peptide (GRP), etc. Two neurotransmitters are present in a high percentage of synaptic terminals emitted by the SCN neurons,

namely GABA (gamma-aminobutyric acid) and glutamate. Inside the medial hypothalamus, the SCN efferent fibers innervate the medial preoptic area, the subparaventricular nucleus, the dorsomedial nucleus and the paraventricular nucleus (PVN). The neurons innervated by the SCN in these regions belong to one of the following types: endocrine neurons such as GnRH-, TRH- and CRH-containing neurons, autonomic neurons or intermediate neurons. The SCN also projects to extra hypothalamic structures such as the paraventricular nucleus of the thalamus and the intergeniculate leaflet (Kalsbeek and Buijs, 2002). Experiments using retrograde viral tracers have identified multi-synaptic networks connecting the SCN with several organs. The best known of these pathways is the SCN-pineal gland connection, involved in the generation of melatonin rhythm through the innervation network: SCN-PVN-preganglionic neurons from the intermediolateral columns of the spinal chord-sympathetic neurons from the superior cervical ganglion-pineal organ (Fig. 2; Ganguly et al., 2002). An analogous innervation pathway connecting the SCN with the adrenal cortex has been described (Buijs et al., 1999).

Figure 2. Schematic representation of the multisynaptic pathway underlying photic and circadian control of melatonin synthesis in the pineal gland. Glutamate released from the retinohypothalamic tract stimulates the suprachiasmatic nucleus (SCN) GABAergic neurons, which in turn inhibit the stimulatory action of the paraventricular nucleus (PVN) on melatonin secretion by the pineal organ. ILC: intermediolateral columns from the spinal chord; SCG: superior cervical ganglion. The symbol ~ indicates circadian GABA (γ -aminobutyric acid) releasing.

It has been proposed that the connections between the SCN and the autonomous nervous system underlie the signaling of circadian information to the pancreas, liver, heart and even to muscle (Kalsbeek and Buijs, 2002; Terazono et al., 2003) and adipose tissue (Kalsbeek et al., 2001). The importance of the neural pathway between the SCN and the peripheral tissues for the regulation of circadian rhythms has been demonstrated in animals in which lesion of the SCN is followed by SCN grafting. The grafted SCN exhibits a very limited capacity to reinnervate other hypothalamic zones (Silver et al., 1996). Of note, the grafted animals recover the activity/rest rhythm but not endocrine rhythms such as those of adrenal function, plasma melatonin and the gonadotropins cyclic secretory pattern (Meyer-Bernstein et al., 1999), unless neural connections between the graft and the hypothalamus of the host are present (de la Iglesia et al., 2003). These experiments show that the SCN regulates rhythms such as activity by secreting diffusible factors. Recently, prokineticin 2-containing neurons have been described in the SCN, which are apparently involved in the regulation of locomotor activity rhythm (Cheng et al., 2002). In contrast, the SCN control over endocrine rhythms seems to require intact efferent axonal projections from the SCN.

The molecular machinery underlying circadian oscillation involves coordinated expression of clock genes

The analyses of different experimental models (cyanobacteria, *Neurospora*, *Drosophila* and mice) established the molecular interactions that underline the basic self-sustaining oscillatory process in biological clocks. In these species, mutational studies characterized a set of genes that result in the disruption of the normal circadian rhythm of locomotor activity when altered. These genes –called clock genes– are highly conserved between *Drosophila* and mouse. In the latter, the core clock genes are *Per* (*period*; with 2 homologues, *Per1* and *Per2*), *Clock* (*circadian locomotor output cycles kaput*), *Bmal1* (*brain and muscle aryl hydrocarbon*

receptor nuclear translocator [ARNT]-like protein 1), and *Cry* (*cryptochrome*; with 2 homologues, *Cry1* and *Cry2*) (Edery, 2000; Reppert and Weaver, 2002; Okamura et al., 2002). A third *Per* homologue, *Per3*, although expressed in the mouse SCN, may not play a role as a core clock gene as its disruption produces only subtle alterations of the locomotor activity rhythm (Shearman et al., 2000a). In the rodent SCN and peripheral oscillators, the 24-h expression pattern of the *Bmal1* gene is characterized by robust oscillatory levels of *Bmal1* transcripts in antiphase with those of *Per* and *Cry*; whereas the level of *Clock* transcripts remains stable in the 24-h (Reppert and Weaver, 2002; Okamura et al., 2002; Balsalobre, 2002). However this may not be a general rule, because in sheep a robust oscillation of *Clock* mRNA in the SCN was found by *in situ* hybridization (Lincoln et al., 2002). Whether this situation will apply to other diurnal mammals remains to be established. Yu et al. (2002) characterized the genomic structure of the mouse *Bmal1* gene and defined its promoter region. These authors demonstrated that *Bmal1* transcription is activated by CRY1, CRY2, and PER2 proteins, but repressed by CLOCK/BMAL1 heterodimers.

At the protein level, circadian oscillation of clockwork negative factors is well established, as PER1, PER2 and CRY1 accumulate in the nuclei of SCN neurons at the end of subjective day and disappear at the end of circadian night (Maywood et al., 2003, and references therein). There is conflicting evidence on the circadian expression of BMAL1 protein, the dimerization partner of CLOCK (see below). Tamaru et al. (2000), produced polyclonal antibodies against amino acids 154-182 of the rat splice variant BMAL1b and analyzed the SCN of this species by immunoblot. The authors reported circadian oscillation of the BMAL1b protein content with peak at midnight (CT18) and trough at midday (CT06), and a rapid reduction of BMAL1b after exposure to light at early night. Maywood et al. (2003) showed circadian oscillation of the BMAL1 protein in the mouse SCN by immunocytochemistry, immunoblot, and co-immunoprecipitation

studies using commercial antibodies. However, these authors found a BMAL1 content with peak during daytime (CT0-8) and trough during nighttime (CT12-20). In a third report, polyclonal antibodies raised against amino acids 381-579 of the mouse BMAL1 protein (Lee et al., 2001) were used for immunocytochemical and immunoblot analyses (von Gall et al., 2003); but these authors did not detect BMAL1 oscillation and showed that a light pulse at early night does not modify SCN BMAL1 protein content. Hence, further analyses are needed to decipher the actual circadian expression pattern of the BMAL1 protein in the SCN of rodents. In these animals, CLOCK protein shows stable levels in the 24-h cycle; in fact, CLOCK is a nuclear antigen constitutively expressed in the mouse SCN (Maywood *et al.*, 2003; von Gall et al., 2003).

A model of the basic oscillatory process consisting of one transcription/translation negative feedback loop was introduced by Rensing (1997). In the following years, the use of genetic, molecular and biochemical approaches to studying the circadian timing system of mice provided convergent evidence defining a model based on two limbs of interacting positive and negative transcription/translation feedback loops that drive recurrent rhythms in the mRNA and protein levels of key clock components (Dunlap, 1999; Edery, 2000; Hastings 2000; Reppert and Weaver, 2002; Okamura et al., 2002). The constitutively expressed CLOCK has the potential to make temporally specific associations, alternating between BMAL1 and PER/CRY, thus resulting in transcriptional activation or repression, respectively (Maywood et al., 2003). However, according to Lee et al. (2001), circadian rhythmicity is mainly due to coordinated oscillation and timed posttranslational modifications of the negative regulators PER and CRY, which interact with the heterodimer CLOCK/BMAL1 that would remain constitutively bound to the E-box motifs present in the promoter region of clock genes (as shown by chromatin immunoprecipitation assays), thus repressing its positive drive (see below; Lee et al., 2001; Reppert and Weaver, 2002).

The core stimulatory loop, driven by the heterodimer CLOCK/BMAL, upregulates the transcription of the clock genes *Per1-3* and *Cry1-2*, which contain the enhancer sequences known as E-box (canonical core sequence: CACGTG) in their promoter regions (Reppert and Weaver, 2002; Okamura et al., 2002). This positive (feedforward) transcription/translation loop is recurrently counterbalanced by the core inhibitory loop formed by the proteins encoded by *Per1-2* and *Cry1-2* genes. Those PER proteins that escape hyperphosphorylation and degradation, homodimerize in the cytoplasm and, upon translocation to the nucleus, heterodimerize with the other negative elements, CRY1 and CRY2. The PERs/CRYs heterodimers interact with the CLOCK/BMAL1 complex, inhibiting its transcriptional induction on the *Per* genes, thereby closing the negative transcription/translation feedback loop (Reppert and Weaver, 2002; Okamura et al., 2002). In addition, the PER2 protein has a positive effect on *Bmal1* mRNA levels (Shearman et al., 2000b; Yu et al., 2002). Timed accumulation of PER/CRY complexes is regulated by casein kinase I ϵ and δ , which phosphorylate clock proteins and tag them for degradation (Lee et al., 2001). The importance of this process is highlighted by the shorter circadian period displayed by *casein kinase I ϵ* mutant hamsters (tau), as a result of decreased turnover of the negative clock element PER (Lowrey et al., 2000; Reppert and Weaver, 2002; Okamura et al., 2002). As a whole, this clockwork mechanism helps to explain daily robust waves of E-box-containing genes expression. When levels of PER and CRY proteins are high, they interact to repress their own transcription. This results in derepression of CLOCK and BMAL1, therefore allowing a new cycle of E-box-based gene expression to begin (Fig. 3).

The expression of the *Per1* gene is regulated by other transcription factors in addition to the CLOCK/BMAL1 complex. In the mouse, the promoter region of the three *Per* genes contain E-boxes, but only *Per1* and *Per2* contain CREs (cAMP response elements; Travnickova-Bendova et al., 2002). These authors provided evidence accounting

Figure 3. Simplified diagram showing the basic molecular loops that control clock gene expression in a neuron of the suprachiasmatic nucleus. The BMAL1/CLOCK heterodimer binds to E-box DNA motifs at the promoter region of the *Per* and *Cry* genes, activating its transcription (stimulatory loop). The core inhibitory loop, indicated by broken lines, is formed by the PER and CRY proteins. These proteins heterodimerize and the PERs/CRYs heterodimers interact with the CLOCK/BMAL1 complex, inhibiting its transcriptional induction on the *Per* and *Cry* genes. The CLOCK/BMAL1 heterodimer also induces the transcription of other genes containing E-box elements in their promoters, such as vasopressin (AVP; a neuropeptide secreted by the SCN) and albumin gene D-site binding protein (DBP; a transcription factor expressed in central and peripheral tissues). DBP will in turn rhythmically activate the transcription of other genes containing D-site motifs in their promoter regions. See text for further details.

for Ca^{2+} -mediated phosphorylation of CREB (cAMP response element-binding protein) on residues serine 133 and 142 (Ginty *et al.*, 1993 and Gau *et al.*, 2002; respectively), which in turn binds to CREs in the promoters of *Per1* and *Per2*. Interestingly, the responsiveness of the *Per1* promoter region to CREB is remarkably higher than that of *Per2*, suggesting that the apparently functional *Per2* CRE is actually inactivated by the entire promoter context (Travnickova-Bendova *et al.*, 2002). Given that in the SCN, a light pulse triggers phosphorylation of the transcription factor CREB, which in turn quickly induces *Per1* gene transcription, while the *Per2* gene responds more slowly, these authors concluded that the different activation potential of *Per1* and *Per2* CREs

could account for the diverse induction kinetics of these two genes. Overall, current evidence suggests that functional cAMP response elements on the promoter region of the *Per1* gene are the targets to trigger light-induced phase shifting of clock gene expression in the SCN of mammals.

In addition to the E-box and CRE sites, other response elements have been identified in the promoter region of the *Per* genes. The search for additional transcription factors regulating clock gene expression has yielded two PAR (proline and acid amino acid rich) family transcription factors and two basic helix-loop-helix transcription factors. One PAR factor is DBP (named for albumin gene D-site binding protein), which is rhythmically

expressed in several tissues and exhibits high daytime levels (Okamura et al., 2002). DBP knockout mice show disruption of the circadian component of sleep, which is the cyclic tendency to sleep at night in diurnal animals, suggesting that DBP may feedback to the clockwork mechanism (Lopez-Molina et al., 1997). DBP binds to the specific sequence ATTACGTAAC (D-site) located upstream of the second transcription initiation site (1B site) of *Per1* and further increases its transcription rate driven by the CLOCK/BMAL1 complex (Okamura et al., 2002). Another PAR family transcription factor is E4BP4 (named for adenovirus E4 promoter ATF side-binding protein), found by Doi et al. (2001) in the chick pineal gland (an autonomous oscillator in birds). The phase of E4BP4 expression was nearly opposite that of PER2, and these authors identified potential binding sites for E4BP4 in the promoter of *Per2*. When a luciferase construct containing the 5'-flanking region of the *Per2* gene was cotransfected with an E4BP4 expression construct, *Per2* promoter activity was repressed (Doi et al., 2001). Finally, the basic helix-loop-helix transcription factors Dec1 and Dec2 are rhythmically expressed in the mouse SCN with a peak in the subjective day. They are able to repress CLOCK/BMAL1-induced transactivation of the mouse *Per1* promoter (Honma et al., 2002), and therefore may contribute to the downregulation of *Per1*. Evidence indicating that the mRNA levels of *Cry1*, but not of *Per2*, were prematurely elevated in the livers of Rev-Erb α -deficient mice, led Etchegaray et al (2003) to investigate whether Rev-Erb α affected *Cry1* transcription. Using a combination of computer-based analyses, electrophoretic mobility shift assays, and luciferase reporter gene assays, these authors showed that Rev-Erb α acts as a transcriptional repressor of *Cry1*.

Additional mechanisms other than transcription factor binding contribute to the regulation of clock gene expression. Chromatin remodeling complexes might temporally regulate rhythmic gene expression exhibited by clock genes in the circadian feedback loop (Crosio et al., 2000), and histone acetylation is important

in the regulation of clock gene expression in the liver (Etchegaray et al., 2003) and heart (Curtis et al., 2003). The metabolic state of the cell seems to be very important in regulating the expression of clock genes. The McKnight group (Rutter et al., 2001) found that in a purified system, the reduced forms of nicotinamide adenine dinucleotide (NADH/NADPH) cofactors strongly enhance DNA binding of the CLOCK/BMAL1 heterodimer to its recognition sequence, whereas the oxidized forms are inhibitory. This means that the redox state of the cell may be crucial to induce or repress clock gene expression.

All clock gene homologues have been identified in the human genome, and the first mutation of a clock gene has recently been characterized in humans. A mutated phosphorylation site in the hPER2 protein gives rise to a sleep pattern disorder known as familial advanced sleep phase syndrome (Toh et al., 2001); this finding is in agreement with that of a mutant hamster (tau) displaying a shorter locomotor activity period due to a mutation of the gene encoding for casein kinase 1 ϵ that results in reduced phosphorylation of the negative clock element PER2 (see above). On the other hand, polymorphisms of *hClock* (Katzenberg et al., 1998) and *hPer3* (Archer et al., 2003) are associated with morning/evening preferences, i.e. the propensity of some organisms to behave as larks or owls, respectively; whereas polymorphism of *hPer1* is not associated with diurnal preference in normal adults (Katzenberg et al., 1999).

Hundreds of genes are under circadian control in the suprachiasmatic nucleus and peripheral tissues

As discussed, the neurons of the SCN are known to coordinately express clock genes. However, the link between oscillatory expression of clock genes and circadian SCN function as electrical and metabolic rhythmic activity over 24-h, remains largely unknown. By using microarray technology, hundreds of genes displaying a circadian pattern of expression have recently been identified in SCN and liver, in addition to the oscillatory canonical clock genes

(Panda et al., 2002b). These authors estimate that up to 10% of the mammalian transcriptome may be under circadian control. The oscillatory genes found by Panda et al. (2002b) and other authors (see Ueda et al., 2002, for SCN and liver; Storch et al., 2002, for heart and liver; and Kornmann et al., 2001, for liver) are involved in different key cellular pathways such as metabolism, transcription, translation, protein turnover, cell cycle, cell death, vesicle trafficking, ion transport and signal transduction; which clearly underscore the importance of the circadian timing system for integrated physiology (Delaunay and Laudet, 2002).

The question arising from these findings is how the oscillatory expression of only a few canonical clock genes may regulate the oscillation of a high number of clock-controlled genes (CCGs). There is evidence accounting for two mechanisms operating at the transcriptional level. One is the direct action of the CLOCK/BMAL1 heterodimer on CCGs transcription. This first possibility has been demonstrated for the rhythmic transcription of the gene encoding for the neuropeptide AVP in the SCN neurons. The CLOCK/BMAL1 heterodimer binds to E-box DNA motifs located in the AVP gene promoter, thus upregulating AVP transcription (Fig. 3; Jin et al., 1999). However, it must be kept in mind that E-box motifs are also present in the promoter region of a large number of genes that do not follow a circadian pattern of expression. A comparative dissection of the context of the E-box motifs present in the promoter region of the circadian *AVP* and non-circadian *cyclin B1* genes, provided evidence for a strong influence of the E-box-flanking sequences in establishing robust circadian transcription driven by CLOCK/BMAL1 (Munoz et al., 2002). A second mechanism proposed is that the CLOCK/BMAL1 complex may act indirectly through the regulation of other CCGs that are in turn transcription factors. In support of this mechanism, it has been reported that the CLOCK/BMAL1 heterodimer induces expression of the DBP transcription factor, which in turn binds to the D-site located in the promoter region of different genes (Fig.

3). Thus, mice homozygous for a *Dbp* null allele, exhibit an altered circadian expression profile of some genes in the liver, such as steroid 15 β hydroxylase, coumarin 7 hydroxylase and cholesterol 7 β hydroxylase (Ripperger et al., 2000, and references therein). Considering that the CLOCK/BMAL1 heterodimer as well as CCGs such as DBP and probably other proteins (particularly clock-related transcription factors; for instance, Rev-Erb α , E4BP4 and CREB, see above) may bind response elements in the promoter region of several genes, both mechanisms would explain the oscillation of a high number of genes in the SCN neurons and peripheral tissues. Thus, the fraction of the transcriptome that is oscillating at a given time seems to rely upon the coordinated expression and interaction of a number of gene products encoded by clock and clock-controlled genes.

Peripheral clocks are responsible for overt circadian rhythms

The early proposal that the circadian system is a hierarchy of biological clocks commanded by the SCN has been confirmed by recent studies showing *in vivo* oscillatory expression of clock genes in several tissues including other central nervous system components, termed peripheral clocks. There is evidence that SCN lesions might suppress rhythmic oscillation, but not the expression of *rPer2* in the eye, brain, heart, lung, spleen, liver, and kidney (see Sakamoto et al., 1998), which is consistent with the concept of the circadian system being a hierarchical order of biological clocks.

In recent years, several authors have reported antiphase circadian expression of *Bmal1* versus *Per1* and *Per2* mRNAs in eye, heart, kidney and lung sampled from rat at different times of the day (Oishi et al., 1998a,b); whereas in the mouse, other authors described the oscillatory expression of the *Per3* mRNA in liver, skeletal muscle and testis (Zylka et al., 1998) and of the *Per1* mRNA and PER1 protein in pars tuberalis (von Gall et al., 2002). However, the previous evidence of a circadian

oscillation of clock genes in the testis has recently been challenged (Miyamoto et al., 1999; Fu et al., 2002; Alvarez et al., 2003; and Morse et al., 2003). Oscillation of *Per1* and *Per2* clock genes and *Rev-Erb α* , *DBP* and *TEF* (thyrotroph embryonic factor) clock-controlled genes has also been demonstrated in cultured rat fibroblasts (Balsalobre et al., 1998). Using a New-World primate (capuchin monkey; *Cebus apella*) as a diurnal mammal experimental model, we have found expression of the clock genes *Clock*, *Bmal1* and *Per2* in the adrenal gland under *in vivo* and *in vitro* conditions (Richter et al., 2002; Valenzuela et al., 2003). As mentioned above, expression of clock genes has been documented in the heart and liver using microarrays. In peripheral oscillators, not only clock mRNAs, but also the encoded clock proteins have been shown to be expressed following high-amplitude rhythmic patterns over 24-h; for instance, in the liver, PER1, PER2, CRY1, CRY2 and BMAL1 proteins are rhythmically expressed (Lee et al., 2001). A consistent observation is that clock genes in the peripheral oscillators have a phase delay of 4-8-h relative to the SCN rhythm (see Balsalobre et al., 2000; Damiola et al., 2000; and Stokkan et al., 2001). Importantly, under culture conditions, the oscillatory process lasts for weeks in the SCN (i.e. it is self-sustained), whereas it dampens after a few cycles in peripheral tissues (i.e. it is not self-sustained). These findings indicate the existence of unknown but significant differences in the molecular mechanisms of circadian clock in the SCN and in peripheral tissues.

It has been suggested that instead of light, feeding may provide a time cue for some peripheral clocks. An interesting observation is that the phase displayed by clock genes in the liver, pancreas, kidney and heart is modified in rodents subjected to feeding restricted to a few hours every day (Balsalobre et al., 2000; Stokkan et al., 2001; Le Minh et al., 2001; Balsalobre, 2002). These animals increase both locomotor activity and core body temperature in anticipation of the timed daily meal. Such a food-anticipatory activity is controlled by a

circadian clock as it persists when animals are food-deprived after some time of restricted feeding. The observation that a lesion of the SCN does not affect food-anticipatory activity suggests that there is a feeding-entrainable oscillator (FEO). A recent report provides evidence suggesting that NPAS2 (neuronal PAS domain protein 2, also named MOP4) expressed in the forebrain is essential for the full manifestation of food-anticipatory activity (Dudley et al., 2003). NPAS2 is expressed instead of CLOCK in the forebrain as transcriptional partner of BMAL1 (Reick et al., 2001; Dudley et al., 2003). As already mentioned, in previous papers the same group provided *in vitro* evidence for the transcriptional regulation of the clockwork mechanism by intracellular metabolic signals (Rutter et al., 2001). Consistent with the key role of the forebrain in food-anticipatory activity (Green and Menaker, 2003), Davidson et al. (2003) found no evidence of *Per1* expression in the gastrointestinal system in temporal correspondence with food-anticipatory activity. The *in vivo* feeding-derived resetting signal for FEO has not yet been identified, and it is possible that a number of blood-borne factors may act as endogenous *zeitgebers* integrated by the FEO (see Hirota et al., 2002, and references therein for discussion).

It is not known whether the neural connections of the SCN with the autonomous nervous system directly regulate clock gene expression in the pancreas, liver, heart, muscle (Kalsbeek and Buijs, 2002; Terazono et al., 2003) and adipose tissue (Kalsbeek et al., 2001), or whether the regulation is effected through SCN-driven humoral signals (Balsalobre, 2002). Glucocorticoids, a SCN-driven signal, modify the phase of clock genes expression in liver, pancreas, kidney and heart in animals subjected to restricted feeding conditions (Balsalobre et al., 2000) and in rat-1 fibroblasts (Balsalobre et al., 2000; Le Minh et al., 2001; Balsalobre, 2002). In fact, it has been shown that glucose (Hirota et al., 2002; rat-1 fibroblasts) and more generally, the redox state of the cells regulates the expression of clock genes (Rutter et al., 2001).

The only evidence for regulation of a canonical clock gene by a SCN-driven hormone under *in vivo* conditions is the finding that in the mouse pars tuberalis the circadian expression of *mPer1* is dependent upon endogenous melatonin (von Gall et al., 2002). These authors studied the pars tuberalis of pinealectomized mice and found no oscillation of *Per1* expression, whereas the analysis of mice carrying a deletion of the melatonin receptor showed low levels of PER1 protein in the pars tuberalis. These results point to the possibility that melatonin may participate in the entrainment of a peripheral clock.

Melatonin as a neuroendocrine transducer for circadian rhythms

The possibility that melatonin plays a role as a neuroendocrine transducer between the SCN and some peripheral oscillators is being investigated. Plasma melatonin concentration exhibits a robust circadian rhythm. In diurnal and nocturnal species, the plasma melatonin rhythm is characterized by steadily low concentrations during light hours and high concentrations during darkness (Fig. 1; upper right panel). The duration of the daily increase in melatonin reflects the number of hours of darkness in the 24-h cycle and therefore signals the season of the year. Although local melatonin production has been demonstrated in several tissues (retina, digestive tract and testis; Tosini and Fukuhara, 2002; Messner et al., 2001; Tijmes et al., 1996; respectively), the circulating melatonin derives from the pineal organ (Lewy et al., 1980). The daily plasma melatonin rhythm depends on an intact SCN as demonstrated by its disappearance after the lesion of this nucleus (Meyer-Bernstein et al., 1999). A related finding is that a bright light pulse during dark hours quickly lowers the plasma melatonin concentration (Leprout et al., 2001). Also worth noticing is that the rhythm of plasma melatonin persists in some blind subjects and in animals maintained under constant dark conditions (Klerman et al., 2001; Illnerova, 1991; respectively), whereas it is suppressed in

animals maintained in constant light (Torres-Farfan et al., 2004).

Melatonin receptors are present in central and peripheral tissues in the adult and also in the fetus. Melatonin acts through two G protein-coupled membrane-bound receptor isoforms -MT1 and MT2- and maybe on a nuclear receptor from the retinoic acid orphan receptors family, RZR/ROR (Vanecek, 1998; and Carlberg and Wiesenberger, 1995; respectively). In the human, melatonin membrane-bound receptors are found in SCN and pars tuberalis, cerebellum, brain blood vessels, kidney and also prostate (Weaver et al., 1993; Al-Ghoul et al., 1998; Savaskan et al., 2001; Song et al., 1995; Laudon et al., 1996; respectively). A functional role in the regulation of steroidogenesis has been proposed for melatonin receptors in human granulosa cells, and capuchin monkey Leydig and adrenal gland cells (Woo et al., 2001; Valladares et al., 1997; and Torres-Farfan et al., 2003; respectively).

The duration of the nocturnal melatonin peak reflects the duration of the photoperiod, that is, the short days that define winter result in long duration of the melatonin peak. The length of the nocturnal melatonin peak regulates the beginning of the reproductive season (Bartness et al., 1993; Lincoln et al., 2002). In seasonal breeders, such as hamsters and sheep, short days produce the opposite effects on reproduction. Gestation in hamsters takes 2 weeks, and reproduction is stimulated by long days. In contrast, sheep, which have a 21-week gestation period, short days gate reproduction. In this way, both species give birth in spring.

An important variable related to seasonal reproduction is plasma prolactin concentration. In sheep, plasma prolactin concentration is maximal in summer (coinciding with reproductive quiescence) and minimal in winter, in which most animals are already pregnant. The following lines of evidence - derived from *in vivo* experiments - have prompted the suggestion that the impact of melatonin on the pars tuberalis of the pituitary stalk mediate the effects of photoperiod on prolactin secretion in seasonal mammals: prolactin

levels increase in response to reduced melatonin levels; seasonal cycles of prolactin depend on an intact pars tuberalis, but not on the hypothalamic-pituitary connection; and MT1 melatonin receptors are present at a high density in the pars tuberalis (Lincoln et al., 2002, and references therein). The possibility that this region may operate as a transducer of photoperiod information carried by melatonin in an endocrine output signal is reinforced by data on melatonin-dependent secretion of tuberallins (putative pars tuberalis-specific hormones), which in turn may regulate secretion of PRL and other pituitary hormones (Guerra and Rodríguez, 2001, 2002). In the sheep pars tuberalis, analysis of the temporal expression pattern of seven clock genes by means of *in situ* hybridization showed high-amplitude 24-h rhythmic cycles in the expression of *Bmall*, *Clock*, *Per1*, *Per2*, *Cry1* and *Cry2*, but not of *casein kinase 1 ϵ* . Furthermore, the pattern of expression of these genes in other 24-h cycle was different between summer and winter photoperiods (Lincoln et al., 2002). These data agree with the already mentioned observation that in mouse pars tuberalis the circadian expression of the *Per1* clock gene is melatonin-dependent (von Gall et al., 2002).

Whether similar actions of melatonin may take place in other tissues is presently unknown. As mentioned, we have recently reported the expression of functional MT1 melatonin receptors in the adult capuchin monkey adrenal cortex (Torres-Farfán et al., 2003), which is the first evidence for the expression of this receptor in the adrenal gland of a mammalian species. In this report, *in vitro* evidence was obtained for the inhibition of ACTH-stimulated cortisol production by melatonin. We were able to detect the expression of clock genes in this tissue by using semi-quantitative (Richter et al., 2002; Valenzuela et al., 2003) and real-time (unpublished results) RT-PCR and are currently analyzing their oscillatory pattern in the 24-h period under *in vivo* and *in vitro* conditions. We are also currently testing the hypothesis that melatonin may regulate the phase of the

daily expression of these genes in the putative peripheral clock contained in the adrenal gland of adult primates.

The pineal gland of mammalian fetuses, including the human, does not secrete melatonin, although melatonin may play an important role in the entrainment of fetal clocks, given that fetuses are exposed to the maternal melatonin rhythm through the placenta (Fig. 1, upper right panel; Yellon and Longo, 1987; McMillen and Nowak, 1989; Kennaway et al., 1992). This avenue for melatonin to mediate functional interactions between maternal and fetal physiology has been explored at the level of the fetal SCN (Naitoh et al., 1998) and control of fetal circadian rhythms (Houghton et al., 1993; Serón-Ferré et al., 1989, 1993, 2002). Nonetheless, maternal melatonin may be also involved in direct regulation of fetal peripheral clocks given the presence of melatonin receptors not only in the fetal SCN (see above), but also in diverse tissues of the developing sheep (Helliwell and Williams, 1994), as well as in human fetal kidney (Drew et al., 1998) and in non-human primate fetal adrenal gland (Torres-Farfán et al., in press). We have recently detected expression of clock genes (*Bmall*, *Clock*, *Per2* and *Cry2*) in the fetal SCN and adrenal gland of the capuchin monkey (Rocco et al., 2003).

Concluding remarks

Evolution has produced predictive adaptations to take advantage of the reproducible day/night changes imposed by the Earth's rotation. In mammals this adaptation involves the existence of a master clock that induces temporal order in the complex network of physiological and behavioral variables. The recent confirmation of the existence of peripheral clocks in a number of cell types and tissues shed light on the previously inferred hierarchical order of the circadian time-keeping system, and also provided a view of the way in which different circadian physiological and behavioral variables are commanded by the SCN through neural and/or humoral signals. The efforts to understand this circadian timing system have greatly

profited from studies that integrate molecular aspects to the systems and behavioral levels of analysis. A fascinating finding is that disturbed expression pattern of one clock gene results in profound effects at the whole-organism level. The use of genome-wide tools for the analysis of biological clocks has rendered evidence indicating that circadian oscillation is an ubiquitous aspect of cellular regulation and that approximately 10% of the transcriptome is oscillating at any given time. Of note, the vast majority of the hundreds of genes found to follow a circadian pattern of expression in the SCN and peripheral tissues remains to be linked to the clockwork oscillatory mechanism. Further genomic and postgenomic analyses, with particular emphasis on the full characterization of the promoter region of clock and key clock-controlled genes, will help to better understand the connections between clock genes and overt circadian behavior.

A better understanding of the human circadian system will have direct consequences on public health. Human circadian-related sleep disorders are observed as a consequence of jet lag, as well as in shift workers and the blind. Our modern society has imposed rotational shift-working schedules upon some 25% of the population, and particular care must be paid to the prevalence of chronic illness and industrial accidents, which strongly emphasizes our need for temporal stability (Hastings, 2000). In this context, it is disturbing that experiments with flies have shown that constant shifting shortens life expectancy by more than 20% (Aschoff et al., 1971). The development of the circadian time-keeping system during intrauterine life is incompletely understood, and negative implications may arise in pre-term newborns, that abruptly trade a circadian environment controlled by maternal signals for a timeless Intensive Care Unit room. Studies on the circadian system, bringing together all biological levels of analysis, from the molecular to the sociological, will continue to provide a common framework that will help us understand our relation with Mother Earth.

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