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Review

A molecular perspective of human circadian rhythm disorders

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Abstract

A large number of physiological variables display 24-h or circadian rhythms. Genes dedicated to the generation and regulation of physiological circadian rhythms have now been identified in several species, including humans. These clock genes are involved in transcriptional regulatory feedback loops. The mutation of these genes in animals leads to abnormal rhythms or even to arrhythmicity in constant conditions. In this view, and given the similarities between the circadian system of humans and rodents, it is expected that mutations of clock genes in humans may give rise to health problems, in particular sleep and mood disorders. Here we first review the present knowledge of molecular mechanisms underlying circadian rhythmicity, and we then revisit human circadian rhythm syndromes in light of the molecular data.

Theme: Neural basis of behavior

Topic: Biological rhythms and sleep

Keywords: Circadian rhythm; Clock gene; Sleep disorder; Suprachiasmatic nucleus

Contents

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1. Introduction

A large number of physiological variables display 24-h or 'circadian' rhythms. This ensures proper temporal organization of physiological processes, as well as the adaptation of the organism to the rhythmic environment (day/night cycles, seasons, etc.). Although the bases of circadian physiology were laid down half a century ago, the pace of discoveries in this field has increased tremendously in the last several years. Indeed, genes dedicated to the generation and regulation of physiological circadian rhythms have now been identified in several species. Recently, research on the molecular mechanisms of the circadian system has extended beyond the function of the clock itself to more fundamental physiological aspects: how does the molecular clock regulate overt circadian rhythms? How are rhythms in various individual tissues integrated into a coherent whole?

Given the crucial role of the circadian system in timing different physiological processes and in harmonizing them with the daily environmental changes, it is probable that a dysfunction of the clock may have quite important effects on health. It is time to revisit the knowledge acquired by studies on rodents and to apply it to what is known on human rhythms, especially sleep and mood disorders. Here, we first review recent advances on the molecular basis of circadian rhythmicity in mammals, then discuss their implications for human health. For a more detailed description of molecular clock mechanisms in various model organisms, one can refer to recent review articles [11,128,146,197].

2. The circadian system

2.1. The suprachiasmatic nucleus

Circadian rhythms are generated by endogenous cellular clocks, which can function independently from external cues. In mammals, the main circadian clock is located in the suprachiasmatic nuclei (SCN) of the anterior hypothalamus [95,119]. The SCN are situated just above the optic chiasm, on each side of the third ventricle [5,79]. Each of the nuclei is composed of about 10 000 tightly packed neurons in the mouse [5] and slightly more (around 16 000) in the rat [79]. A similar structure has been identified in humans [174]. SCN neurons are not homogenous, however, and have been classified according to their neuropeptide content [5,119]. A simplified view of the SCN in rodents has a core of vasoactive intestinal peptide (VIP) expressing neurons and a surrounding shell of arginine vasopressin peptide (AVP) expressing neurons [5,119].

Lesions of the SCN in rodents abolish locomotor activity rhythms as well as other rhythms [58,140,142], and grafting SCN tissue to a lesioned animal restores its circadian rhythmicity [140,167]. Moreover, restored rhythms have the characteristics of the donor, not those of the acceptor [140]. These observations establish the SCN as a master clock in the organism.

2.2. Input to the suprachiasmatic nucleus

The circadian clock in the SCN can function autonomously, without the need for any external time cue. However, it can be reset by environmental cues, in particular light–dark cycles. This is important as it ensures that the clock is entrained to 24-h cycles, even though its own intrinsic period (or 'free-running period') is not exactly 24 h. The 'core' of the SCN receives photic input from the retina, through the retino–hypothalamic tract [118]. Response to this photic input involves the induction of various genes [9,99,152,161] as well as chromatin remodeling [40] within SCN neurons. In the retina, photoreceptive cells that are involved in entrainment of the SCN clock are distinct from those involved in vision [112], and rather constitute a subset of retinal ganglion cells [26]. The identity of the photopigment is still a matter of debate, although the novel opsin melanopsin [70,133,151] as well as the two cryptochromes (CRY1 and CRY2) [155,187] are likely candidates (reviewed in Ref. [36]). The SCN also receives non-photic input from different parts of the brain, in particular through neuropeptide Y (NPY) projections from the intergeniculate leaflet and serotonergic projections from the median raphe nucleus (reviewed in Ref. [69,122]).

2.3. Output from the suprachiasmatic nucleus

The suprachiasmatic nucleus controls various rhythms, including body temperature, activity and hormone levels, through nervous projections to other nuclei of the hypothalamus and other brain regions (Fig. 1) [32]. In particular, sleep–wake cycles are regulated through projec-

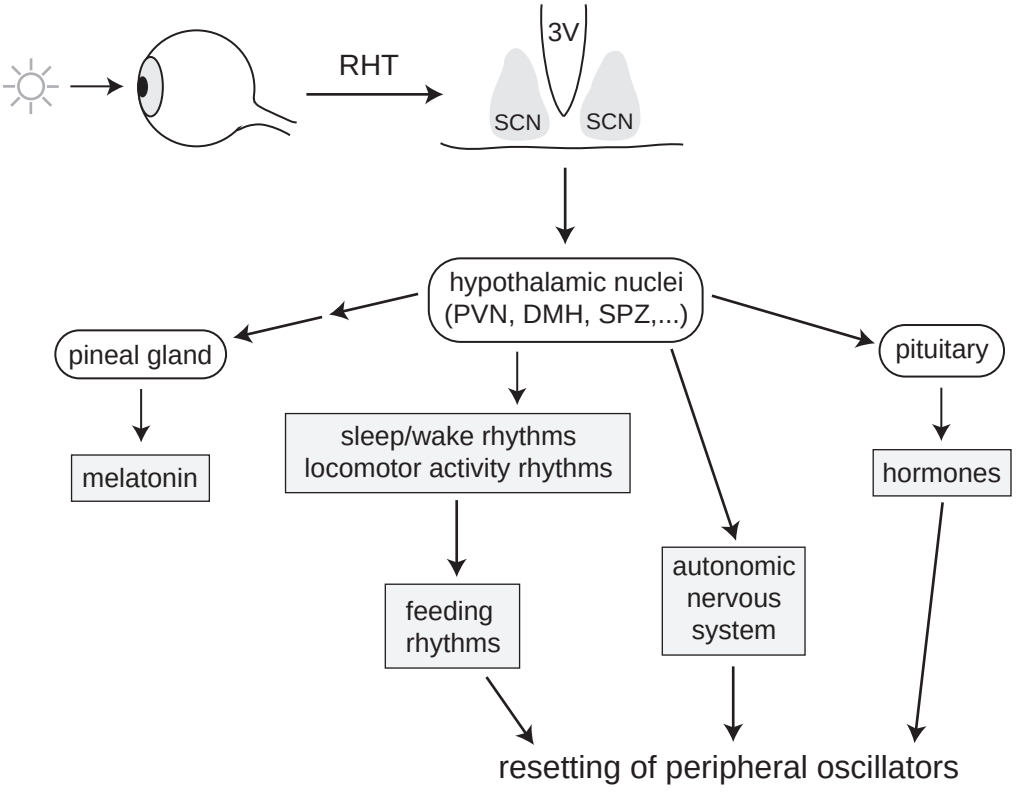

Fig. 1. Resetting of the central and peripheral oscillators in mammals. The central clock of the suprachiasmatic nucleus (SCN) can be ajusted light or day-night cycles of the environment, through the retino–hypothalamic tract (RHT). The SCN can control physiological rhythms and coordinate or reset rhythms of peripheral oscillators by various routes. Many of SCN projections end in other nuclei of the hypothalamus, including the paraventricular nucleus (PVN), the dorsomedial hypothalamic nucleus (DMH) or the subparaventricular zone (SPZ). Rhythmic information is relayed to other part of the brain, to the pineal gland, to the pituitary and to the periphery through the autonomic nervous system. Peripheral oscillators can be entrained by neuronal pathways, hormones and, maybe most importantly, by feeding rhythms (which depend on sleep and activity rhythms). 3V, third ventricle of the brain.

tions from the SCN to the dorsomedial hypothalamus and the posterior hypothalamic area [4,17]. The SCN also sends signals to the periphery through the autonomic nervous system, via the paraventricular nucleus [32,183].

The nature of the signals employed by the SCN to convey rhythmicity to other regions of the brain is still unclear. At least in the case of the control of activity rhythms, a diffusible molecule is involved, since SCN tissue embedded in a permeable capsule is able to restore these rhythms when transplanted into an SCN-lesioned hamster [167]. This diffusible signal may be TGFα, which is synthesized rhythmically by the SCN. This signal abolishes locomotor activity, presumably through EGF receptors of the subparaventricular zone of the hypothalamus [101]. Another possible output signal of the SCN is the protein prokineticin 2, which can inhibit night-time activity in the mouse when injected in brain ventricles [38].

2.4. Communication between neurons within the SCN

Neurons isolated from rodent SCN have been shown to exhibit circadian rhythmicity of firing rate [192], which is based on day–night modulations of calcium currents [137]. These neurons all have slightly different periods and phases, and it is the average of all these individual pacemakers that constitutes the output of the SCN [107,192]. This notion is supported by studies on mouse chimeras in which the SCN is composed of a mixture of wild type and mutant neurons: the resulting circadian rhythmicity of the animals depends on the proportion of wild type and mutant cells in the SCN [110]. What couples SCN neurons? What allows a coherent output? Synaptic transmission between SCN neurons has been proposed to have a role, as well as gap junctions [76,165,166]. Diffusible molecules secreted by SCN neurons may also be involved [38,41,68].

2.5. Peripheral oscillators

Although the SCN is the master circadian oscillator in mammals, many other regions of the brain as well as non-neuronal tissues display circadian rhythms [22,116,127,143,179,202], even when cultured in vitro [3,196]. However, in contrast to the SCN, the amplitude of these peripheral oscillators decreases very rapidly, usually within several days [3,22,196]. The reason why the SCN is a more robust clock is unclear, but it has been suggested

that rhythmicity could be reinforced by intercellular communication, for example through VIP signalling [41,68].

In the organism, peripheral oscillations are sustained by the SCN (Fig. 1) [21,134,153]. The SCN was originally thought to entrain peripheral oscillators through neuronal and hormonal cues, but recent findings suggest a prominent role of feeding rhythms in coupling peripheral oscillators to the central clock [45,134,170]. Indeed, when food availability is restricted to the light period for nocturnal rodents, the phase of peripheral oscillators is shifted while the phase of SCN rhythms is intact [45,170]. Rhythms in peripheral tissues can also be sustained by external temperature cycles [31]. However, the biochemical signals that reset peripheral tissues are still elusive. Glucocorticoids and retinoic acids have both been shown to change the phase of peripheral tissues and are therefore among the candidates [21,116].

3. Cellular circadian oscillators

Different lines of evidence indicate that the basic machinery making up a circadian oscillator is intracellular. For example, individual neurons display circadian rhythms in culture [192], and cultured fibroblasts exhibit molecular circadian rhythms upon treatment with high levels of serum [22] or activation of different signal transduction pathways [6,23]. Moreover, unicellular organisms such as cyanobacteria or fungi also have circadian oscillators (reviewed in Ref. [82]).

3.1. Clock genes and proteins

The first circadian rhythm mutants to be isolated were the *period* mutants in the fruitfly *Drosophila* [97] and *frequency* mutants in the fungi *Neurospora* [57], in the early 1970s. Many years later, the corresponding genes were isolated [16,24,141]. However, it is only in the past several years that a large number of clock genes were discovered, in various models organisms, and that a clearer picture of the molecular clocks appeared (Table 1) [52,147,197]. We call ‘clock genes’ those genes that are important for the generation and regulation of circadian rhythms. For example, the *Period1* (*Per1*) messenger RNA in mammals is at high levels around the middle of the day in the SCN, and then very low during the night. The rhythm in its abundance is delayed by several hours in peripheral tissues [35,127,202].

Table 1
Main clock genes in mammals[a]

Gene	Characteristics of protein product	Function	Effect of mutation[b]	Links to human phenotypes
Clock	bHLH-PAS factor	Activation of clock and clock-controlled genes (with BMAL1)[c]	long period, then arrhythmic[d]	polymorphism linked with ME preference
Bmal1	bHLH-PAS factor	Activation of clock and clock-controlled genes (with CLOCK)[c]	arrhythmic	
Per1	PAS domain	Association with CRYs	short period[e]	
Per2	PAS domain	Association with CRYs	short period, then often arrhythmic[e]	mutation in one case of familial ASPS
Per3	PAS domain	Association with CRY? Output?	short period[e]	polymorphism linked with DSPS
Cry1	similar to DNA photolyases	Association with PERs and inhibition of CLOCK-BMAL1	short period[f]	
Cry2	similar to DNA photolyases	Association with PERs and inhibition of CLOCK-BMAL1	long period[f]	
CKIδ, *CKIε*	casein kinase	Phosphorylation of PERs, CRYs, BMAL1	short period (*CKIε*)[g]	
Rev-erbα	orphan nuclear receptor	Repression of *Bmal1 Clock* and *Cry1*) expression	short period	
Dbp	PAR bZip factor	Output; activation of *Per1*?	short period	
E4bp4	bZip factor	Output; repression of *Per1*?	(not tested)	

Abbreviations: bHLH, basic helix-loop-helix; PAS, Per-Arnt-Sim; bZip, basic leucine zipper; PAR, proline and acidic amino acid rich; *Per*, *Period*; *Cry*, *Cryptochrome*; Dbp, D-site binding protein; ME, morningness-eveningness; ASPS, advanced sleep phase syndrome; DSPS, delayed sleep phase syndrome.

[a] See main text for bibliographic references.

[b] Effect of mutation on locomotor activity rhythms in constant darkness. For the effect on molecular oscillations, refer to references cited in the text. Targeted deletion of the gene, unless otherwise indicated.

[c] Close homologs of *Clock* and *Bmal1* (*Npas2*/*Mop4* and *Bmal2*/*Mop9*, respectively), have been described, but their precise involvement in the clock has not been clarified yet.

[d] Point mutation, leading to skipping of one exon during splicing. The mutant protein has blunted transcriptional activity.

[e] *Per1* and *Per2* knock-out mice have a short period in some reports and a normal period followed by arrhythmicity in another article. Double *Per1*/*Per2* knock-outs are arrhythmic. Double *Per1*/*Per3* or *Per2*/*Per3* knock-outs have a phenotype identical to *Per1* and *Per2* knock-outs, respectively.

[f] Double *Cry1*/*Cry2* knock-outs are arrhythmic.

[g] Point mutation in hamsters, known as the *tau* mutation.

3.2. Feedback loops

A common theme underlying circadian rhythmicity is that oscillations of clock gene transcripts are the consequence of intracellular transcriptional–translational feedback loops. For example, in mammals, the transcription factors CLOCK and BMAL1 heterodimerize and activate the expression of three *Period* (*Per*) genes and two *Cryptochrome* (*Cry*) genes by binding to E-box elements in their promoters (Fig. 2) [56,62,73,180,195]. The protein products of these genes multimerize and translocate to the nucleus, where CRY proteins repress the transcriptional activity of the CLOCK–BMAL1 dimer [63,102], possibly via inhibition of p300 histone acetyl transferase activity [56]. Such a negative feedback loop of CRYs on their own expression is in theory sufficient to generate oscillations. However, additional levels of complexity are superimposed onto the loop to confer accuracy and robustness to the oscillations. First, a positive feedback loop is based on the same negative loop: in mammals, CLOCK and BMAL1 activate the gene encoding REV-ERBα, a transcription factor that can repress the *Bmal1*, *Clock* and *Cry1* genes [56,130,139,182]. Therefore, repression of the activity of CLOCK-BMAL1 by PER-CRY complexes not only inhibits *Per* and *Cry* gene expression, but also *Rev-erbα* gene expression, thus leading indirectly to an activation of the *Bmal1* and *Clock* gene (Fig. 2) [139,158].

The period of the oscillations is controlled by the phosphorylation, degradation and nuclear translocation of the proteins composing the feedback loops [104,185,194]. In particular, casein kinases I (CKI) δ and ε were shown to phosphorylate PER1 and 2, CRYs and BMAL1 [34,55,90,111]. These phosphorylation events target the proteins for degradation [7,34]. CKIε/δ appear to be part of multimeric complexes containing PER and CRY proteins [104].

In fact, we are far from knowing all the parameters that affect the clock, and many clock genes may remain to be discovered. Recent data suggest the involvement of new factors in the core clock mechanism. In addition to REV-

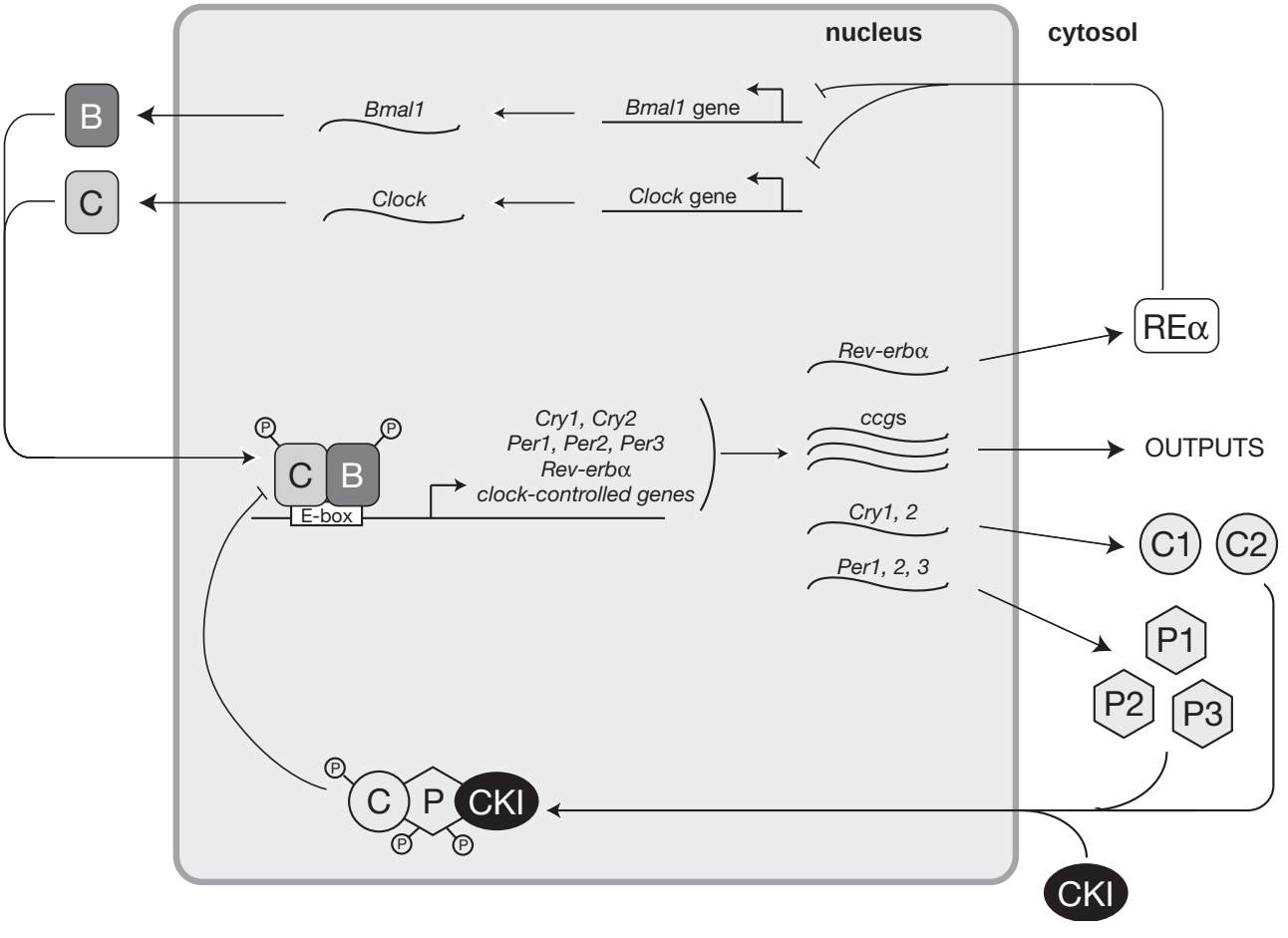

Fig. 2. A schematic model of the molecular clockwork of the circadian clock in mammals. BMAL1 and CLOCK (round rectangles B and C) together activate the expression of clock genes and clock-controlled genes (*ccgs*) through E-box elements in the promoter of these genes. REV-ERBα protein (REα round rectangle) represses *Bmal1* and *Clock* gene expression. REV-ERBα also represses *Cry1* gene expression (not shown). CRY and PER proteins (C circles and P hexagons) multimerize and are phosphorylated by casein kinases I δ and ε (CKI) (which regulates their stability), and CRYs inhibit the transcriptional activity of the CLOCK-BMAL1 dimer. The product of *ccgs* exert output functions. Curved lines represent messenger RNAs and small circled Ps are phosphates.

ERBα, additional factors could bind to the same elements in the *Bmal1, Clock* and *Cry1* promoters, such as REV-ERBβ, RORβ or RORγ, and may also have a role to play in the SCN and/or peripheral tissue clockwork [139,172,182]. Moreover, two basic helix-loop-helix factors, DEC1 and DEC2, were shown to be expressed in the SCN and to inhibit the activity of CLOCK-BMAL1 dimers through association with them and/or competition with the E-box elements in target promoters [75]. In *Drosophila*, recent studies have implicated the kinase GSK-3 [114] as well as Slimb [64,96], a protein involved in targeting proteins to the ubiquitin–proteasome pathway, in regulating the degradation of clock proteins; it will be important to determine whether homologs of these proteins also regulate clock protein turnover in mammals.

3.3. Mutation of clock genes in rodents

Mutation of clock genes leads to abnormal circadian rhythms of locomotor activity (Table 1). In some cases, the mutation or the deletion of a clock gene leads to a shorter or longer free-running period than found in wild type controls. This is observed for *Per1, Per2, Per3, Cry1, Cry2, CKIε, Clock* and *Rev-erbα* mutants [35,111,139,157,177,184,186,199,200]. In other cases, the mutation leads to complete arrhythmicity as soon as the animals are housed in constant darkness: this is observed for *Bmal1* knock-out mice [33], as well as for double *Per1*/*Per2* and *Cry1*/*Cry2* mutants [18,184,187,199]. In two cases, *Clock* mutant and *Cry1*/*Cry2* double-mutant mice, dysregulation of sleep patterns was observed [125,193]. Very close relatives of CLOCK and BMAL1, called NPAS2 (or MOP4) [73,201] and BMAL2 (or MOP9) [74,80,129], respectively, are present in mammals. Both have been proposed to act in some way in the circadian system [73,74,116,143], and NPAS2 physically associates with BMAL1 in vascular tissue [116] and in the forebrain [143]. However, a *Bmal2* knock-out is not yet available, and *Npas2* knock-out mice do not have defects in free-running locomotor activity [61]; moreover, the *Npas2* gene is not expressed in the SCN [160].

In the latter case, however, it should be noted that *Per2* transcript levels are low in the forebrain of *Npas2* mutant mice [143]. This suggests that the ablation of a clock gene may have differential effects on different parts of the circadian system. This is also true for the analysis of *Bmal1* gene expression in mice mutant for the *Clock* gene, which display constitutively low *Bmal1* transcript levels in the SCN yet near peak levels in the periphery [126]. Another example is provided by *Per1* knock-out mice, which display locomotor activity rhythms with a free-running period of about 23 h (slightly shorter than wild type littermates) [35], while embryonic fibroblasts prepared from these mutants have a much shorter period of 20 h [134]. These examples underscore the fact that even though molecular clock mechanisms are similar in the SCN and in the periphery, important differences do exist.

3.4. Clock-controlled genes

The purpose of the regulatory feedback loops described above is the circadian control of clock output. In some cases this control may be quite direct. Since CLOCK–BMAL1 dimer activity varies in a circadian manner (due to the cyclic presence of their inhibitors), any other gene under its control will also be expressed rhythmically (Fig. 3). One example of a gene directly controlled by CLOCK–BMAL1 is the gene encoding AVP in the mouse: its transcript oscillates in the SCN with a phase similar to *Per1*, and the amplitude of the oscillation is reduced in *Clock* mutants [83]. Another well described gene that depends on CLOCK–BMAL1 is *Dbp* [148]. The product of this gene, DBP, is a transcription factor and it itself regulates a number of genes [109,124]. In fact, most clock-controlled genes seem to be regulated indirectly by the clock, via transcription factors themselves oscillating in a circadian fashion (Fig. 3). This allows different clock-controlled genes to oscillate with different phases. In addition to DBP, an example of such clock-controlled transcription factor is E4BP4, which appears to have an opposite function to DBP in the circadian system [117].

Recent studies used DNA array technologies to look on a wide scale at clock-controlled genes in the SCN and in other tissues of mice and rats [8,49,65,94,100,132, 171,182]. The results show that several percent of all genes examined, thus several hundreds of genes, are rhythmically expressed. Comparison between different tissues led to the striking observation that only 5–10% of these circadian transcripts are rhythmic in both the SCN and the liver, or in both the liver and the heart. It appears that besides the genes that must oscillate in various tissues to sustain rhythmicity (including clock genes themselves), a given tissue will also express in a circadian manner a number of different genes tailored to suit its specific needs.

4. Clock genes in humans

4.1. Same genes, similar mechanisms

Although most of the data described above comes from studies on rodents, humans appear to have a similar set of clock genes. Human *PER1, PER2, CLOCK, BMAL1, CRY1, CKIδ*/*ε* have been described [28,34,72,87,88,90, 169,176,178], and other clock genes (*CRY2, BMAL2, NPAS2*) can be found in the public domain human genome sequence. For both human *PER1* and *CLOCK* genes, exon–intron structure is very similar to mouse genes [72,169].

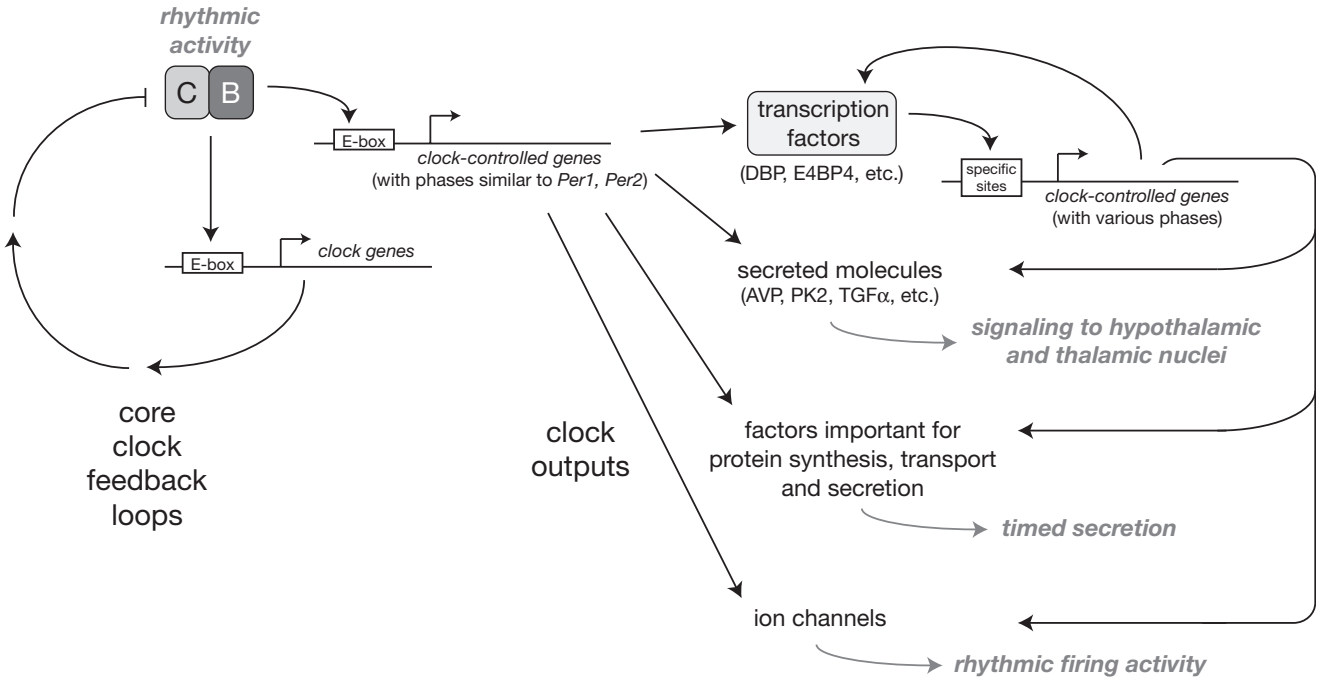

Fig. 3. Clock-controlled genes in the SCN. CLOCK-BMAL1 dimers can regulate through E-box elements the expression of clock genes, as part of the core clock mechanism (see Fig. 2 for details), and of clock-controlled genes, which transmit rhythmicity to the physiology. Clock-controlled genes can encode other transcription factors that themselves regulate other clock-controlled genes in a rhythmic fashion, but with a different phase. The scheme presents some examples of molecules that can be controlled by the clock at the transcriptional level. A similar scheme could apply to other oscillating tissues, but most of the oscillating transcripts are tissue-specific.

4.2. Clock gene expression and function in humans

Knowledge of clock gene expression in humans is of course hampered by the limited possibilities of sampling. Studies must rely on cells that can be easily sampled, or on post mortem samples. Human PER1 and CLOCK are expressed widely in brain regions, non-neuronal tissues and cell lines [72,89,169,198]. In situ hybridization also showed that CLOCK expression in human brain parallels observations made in the mouse [169]. Bjarnason et al. [28] published the first study on circadian expression of clock genes in humans. They looked at the expression of *PER1, CLOCK, BMAL1, CRY1* in the oral mucosa and in the skin. As found in mice, *PER1, BMAL1* and *CRY1* oscillate, while *CLOCK* levels are stable. Moreover, again as in rodents [127], *PER1* and *BMAL1* rhythms are antiphase, with *PER1* reaching peak levels in the early light phase [28]. This is corroborated by studies on human peripheral blood mononuclear cells, where *PER1* transcript levels are higher at 09:00 than at 21:00 h, while *CLOCK* RNA levels appear constant [175]. Human *PER1* expression in the periphery thus occurs during the morning, i.e. at the beginning of the active phase. Curiously, in peripheral tissues of nocturnal rodents, *Per* genes also peak around the beginning of the activity phase [127,202]. Since peripheral oscillators are entrained by feeding rhythms in rodents (cf. Section 2.5), it is tempting to propose that the same occurs in humans, explaining the observed phase of clock gene expression.

Molecular clock mechanisms in humans will probably prove to be very similar to those in rodents. A first clue to support this is the observation that E-box elements of human and mouse *Per1* promoters are all conserved [72]. Moreover, PER proteins are substrates for CKIε both in rodents and in humans [90,111].

5. A genetic cause for human rhythm disorders?

A wide array of physiological variables exhibit 24-h rhythms in humans, and many of these rhythms are controlled by the circadian system (Table 2) [120]. Since mutations of mouse clock genes have profound effects on circadian rhythmicity, it is likely that mutations in human clock genes could give rise to rhythm-related syndromes. Various such syndromes have been described, and in some cases, a genetic origin has been demonstrated.

5.1. Morningness–eveningness preference

Individual differences exist in the preferred timing of rest and activities among the human population and subjects can be described on a scale of morningness–eveningness [77,92]. Differences in the timing of circadian

Table 2
Examples of circadian rhythms in humans

Melatonin production by the pineal gland
Cortisol secretion by the adrenal gland
Core body temperature
Urinary secretion of potassium, sodium, calcium and water
Arterial blood pressure
Hematological variables (hemoglobin, hematocrite, lymphocytes, etc.)
Electroencephalographic (EEG) activity
Rest–activity cycle
Growth hormone secretion
Thyroid-stimulating hormone (TSH)

Note: Extended studies in time isolation revealed that most human subjects would spontaneously desynchronise their rest-activity cycles from their endogenous circadian system. Some rhythms are strongly associated with the endogenous circadian system (e.g. melatonin and cortisol rhythms), whereas others remain linked to the rest–activity cycle (e.g. growth hormone rhythm). Many rhythms are affected by both processes, to different extent.

phase has been reported between morning and evening-type subjects, although it remains unclear whether these differences are a consequence of shifted sleep schedules [20,50,93,103]. Several studies reported that the relationship between the endogenous circadian pacemaker and the sleep–wake cycle is different between young morning and evening-type subjects [19,50,108]. In young, morning-type subjects, the temperature minimum is observed earlier within the sleep episode, which implies that a larger delay portion of the phase-response curve is exposed to evening light. In comparison, young evening-type subjects wake up early in their circadian cycle, which implies that a larger advance portion of the phase-response curve is exposed to morning light. This phase relationship is such that evening-type subjects wake up closer to their maximal circadian drive for sleep. Using the month-long forced desynchrony procedure in 17 young men, Duffy et al. recently found a significant correlation of intrinsic circadian period with self-rated morning–eveningness [51]. Greater morning-like and evening-like preferences were associated with shorter and longer periods, respectively. These results suggest that the intrinsic period influences the phase angle between the circadian system and the light–dark cycle in humans as in other animal species [51,138]. They also suggest that the interindividual variability in the timing of human behavior may be influenced by the circadian period and possibly linked to a genetic predisposition.

Indeed, a study of 260 pairs of monozygotous twins and 50 pairs of dizygotic twins indicates that morningness–eveningness is heritable [78]. A population-based random sample of 410 subjects was enrolled in a comparative analysis of diurnal preference and distribution frequency of two *CLOCK* genes alleles [87]. The polymorphism is a C to T nucleotide substitution in position 3111 of the *CLOCK* cDNA sequence. Morningness–eveningness preferences were based on the Horne–Ostberg questionnaire. Scores were computed for subjects homozygous or hetetozygous for the 3111C allele and for those negative for this allele. Significantly lower scores, consistent with increased eveningness tendency, were observed in 3111C heterozygotes but not in homozygotes. The difference was still significant when the data were pulled for all 3111C carriers compared to non carriers. This last result is expected since it reflects the predominant influence of 3111C heterozygotes ($n=163$) vs. homozygotes ($n=28$). The results from the 3111C carriers remain puzzling due to the lack of significance and, if anything, the slight increase in morningness scores. The authors suggest that the small sample size ($n=28$) explains the negative results and that their results argue against a recessive effect of 3111C on morningness–eveningness. Carriers of the 3111C alleles reported a significant delay in their preferred sleep time that ranged from 10 to 44 min. Considering that very small increases in the intrinsic circadian period can lead to substantial delays in the timing of sleep [51], we would have expected a larger sleep delay in 3111C carriers. When the reverse analysis is done, namely when the frequency of the 3111C allele is determined in function of chronotype, no significant association is found. This was the conclusion of a recent study in healthy and blind subjects by another group [149]. Other studies have shown that a polymorphism in *PER1* [88] and *TIMELESS* gene [136] is not associated with diurnal preferences in healthy subjects.

5.2. Advanced sleep phase syndrome

Advanced sleep phase syndrome (ASPS) was originally reported in older individuals. It is considered infrequent in younger patients unless depression is present [168]. ASPS is characterized by evening sleepiness, sleep onset that is intractably earlier than desired, namely around 18:00–21:00 h, and early morning awakening around 01:00–04:00 h [1,189]. Sleep recordings, when performed at the patient's desired sleep time, are generally normal. However, when performed at later times, early morning awakening and reduced total sleep time are observed and evening sleepiness is reported.

ASPS in younger patients suggest a genetic predisposition. In 1993, Billiard et al. identified a 15-year-old caucasian girl with early evening sleepiness, early bedtime (18:00–20:00) and early morning awakening (04:00–06:00) [27]. The disturbed sleep schedule had been present since childhood and similar symptoms were reported in her mother and maternal grandfather. The diagnosis was confirmed with analysis of actigraphy data recorded over 3 weeks. A polysomnographic recording revealed a sleep onset at 20:09, an abbreviated REM latency of 6 min and an early morning awakening at 01:39 resulting in a reduced total sleep time of 4 h and 49 min.

A familial form of ASPS has been recently described in 29 members of three families, including an 8-year-old child [84]. Affected individuals complain of disabling evening sleepiness and early morning awakening. The transmission of ASPS in these families was consistent with

an autosomal dominant mode. Morningness scores of first-degree relatives revealed a higher morningness tendency as measured by the Horne–Ostberg questionnaire, which suggests the presence of FASPS gene carriers [84]. Minimal levels of depression were found in six patients admitted for a laboratory investigation. However major affective disorders that could realistically account for the ASPS were ruled out. Two nights of polysomnographic sleep recordings were performed in these patients at their preferred sleep times. These recordings revealed a sleep onset around 19:25 h and awakening after a normal sleep episode around 4 h 18 min. Sleep quality and organization were within normal limits. Dim light melatonin onset was advanced to 17:31 h in these patients, thus at a usual temporal position about 2 h prior to sleep initiation. Interestingly, 3-week actigraphic monitoring in their home environment revealed that affected individuals tended to both fall asleep and wake up even earlier on vacation days. This is consistent with a strong physiological tendency to phase advance their behavior. This tendency contrasts with healthy individuals who tend to delay their activities on days off. It is also different from the behavior of unaffected siblings, which indicates that FASPS is not a learned behavior. A 69-year-old female patient was admitted for a 3-week observation period in a time-free laboratory room. After a 3-day baseline period, she was free to eat and sleep whenever she wished, avoiding naps. Core body temperature and activity levels were continuously monitored. A periodogram analysis revealed a free-running activity period of 23.3 h. Under these conditions, healthy individuals would present a period slightly longer than 24 h.

This disorder was later associated in one of the families with a mutation in the *PER2* gene [178]. The replacement of a serine by a glycine results in the inability of CKIε to phosphorylate the PER2 protein. This might induce a faster accumulation of PER2, an acceleration of the clock feedback loops and thus a shorter circadian period (see Section 3.2 and Fig. 2). Interestingly, the phenotype of the familial ASPS is reminiscent of the observations made with *tau* mutant hamsters, which display a mutation in the gene encoding CKIε [111]. The mutated kinase is deficient in PER protein phosphorylation activity.

Another study clarified the pedigree of an additional large family with ASPS [144]. Results of this study suggest that the ASPS phenotype segregates as a single gene with an autosomal dominant mode of inheritance. Altogether, the four cases of familial ASPS suggest that ASPS might be more prevalent than previously believed, such that identification of more affected families is expected. This should lead to the identification of the genetic basis of ASPS.

5.3. Delayed sleep phase syndrome

Delayed sleep phase syndrome (DSPS) is one of the most common circadian rhythm sleep disorders [39], although its prevalence has been estimated to be less than 0.7% in the general population [189]. It is characterized by an inability to fall asleep or to awake spontaneously at the desired times and by a phase delay in the main sleep episode [1]. DSPS patients are entrained to the 24-h day and go to bed at about the same clock time every night. However, they are extreme ‘night owls’ such that sleep onset and wake times are intractably later than desired [43]. Bedtimes are frequently observed around 03:00–06:00 h with wake times around 10:00–15:00 h if the patients are not disturbed. Sleep quality, sleep stage distribution, and sleep duration are normal when patients are not forced to maintain a strict schedule and instead allowed to sleep at their desired times. However, sleep latency is frequently longer than 30 min even if the patient goes to bed at times of their choosing [15,188]. When sleep is planned earlier (e.g. 23:00 h), a significant increase in sleep latency and wake time during the first part of the night is observed [12].

Ebisawa et al. recently recruited 48 patients with DSPS and 30 patients with non-24-h sleep–wake syndrome, all of whom were sighted [54]. The diagnostic criteria of the International Classification of Sleep Disorders were respected [1]. They performed mutation screening of the human *PER3* gene. Their results were compared to those for 100 control subjects with no prior history of sleep or psychotic disorders. An increased frequency of the G647 polymorphism of the H4 haplotype of the *PER3* gene was found in DSPS patients compared to controls. Although significant, this association was weak since 85% of the DSPS patients did not carry the H4 haplotype. The authors proposed that DSPS is genetically heterogeneous and that unknown genetic factors would confer susceptibility.

The mechanisms linking the G647 polymorphism to an increased susceptibility to DSPS have yet to be elucidated. The authors proposed that this polymorphism, with or without another polymorphism, might alter the CKIε phosphorylation of hPER3. It could also affect interaction with PER1/2 or be involved in an output pathway of the SCN. The authors then expanded their database to 59 patients with DSPS, 37 with non-24-h sleep–wake syndrome, and 109 healthy controls [54]. They performed mutation screening of the complete coding region of the CLOCK gene and analyzed the distribution of the T3111C polymorphism, which has been associated with morning–evening preference (see Section 5.1 above). No polymorphisms in the coding region of the CLOCK gene were more frequent in patients with either DSPS or non-24-h sleep–wake syndrome. Despite the expectations, a reduced frequency was observed in the occurrence of the T3111C allele, although these results were not significant after Bonferroni’s correction for multiple comparisons. Further studies will thus be necessary to clarify what genes are susceptible to DSPS.

A pedigree of the extended family of a proband with DSPS has been constructed [13]. The preference for

eveningness was much higher in that family than reported in the general population. No simple pattern of genetic transmission was identified, although the tendency to eveningness was often passed from parents to children.

5.4. Non-24-h sleep–wake syndrome

Non-24-h sleep–wake syndrome is extremely uncommon in sighted subjects living under normal conditions. Lack of entrainment is more frequently observed in blind patients or in astronauts and sub-mariners living under artificial light–dark cycles [91]. It can also be associated with psychiatric conditions such as schizoid or avoidant personality disorders [30]. Patients with non-24-h sleep–wake syndrome often report irregular sleep–wake cycles and periodic insomnia mixed with daytime sleepiness. In the disturbed periods, the patient suffers from severe sleep-onset insomnia and difficulty awakening in the morning. Sleep onset tends to be delayed by 1–2 h from 1 day to the next. Sleep–wake log and wrist actigraphy monitoring for several consecutive weeks reveal longer than 24-h rest–activity cycles that may be interrupted by periods of relative coordination to the 24-h day [30]. Periods of long days with 24–40 h without sleep followed by 14–24 h of uninterrupted sleep may also occur [181,188]. Serial polysomnographic recordings will reveal a pattern of cyclic sleep disruption if they occur at the same clock time each time.

It has been proposed that in sighted individuals, the hypernychthemeral syndrome might be a more severe form of DSPS [191]. Indeed, some cases of DSPS have converted to a non-24-h sleep–wake syndrome after chronotherapy using a 27-h day [85,131]. A reduced sensitivity of the human circadian system to light, as assessed by nighttime melatonin reduction, was reported in a sighted 41-year-old man with hypernychthemeral syndrome [115].

So far, mutation screening analyses of human *PER3* and *CLOCK* genes have not revealed an association with specific haplotypes [54]. However the prevalence of the R54W and the A157V variants of the human melatonin 1a receptor gene was several times more frequent in patients with non-24-h sleep–wake syndrome [54]. This difference was not significant and its role on the etiology of the disorder is unclear.

5.5. Seasonal affective disorder

The condition coined in 1984 by Rosenthal and colleagues as seasonal affective disorder (SAD) is a recurrent affective disorder tied with the changes of seasons [150]. Typically, patients diagnosed with SAD experience recurrent depressive episodes during either the fall–winter months (winter form) or the spring–summer months (summer form), while recovering spontaneously outside of these seasons. Atypical symptoms such as increased carbohydrate craving, increased weight gain, increased sleepiness, and reduced energy levels are present. Seasonal variations in mood and behavior have been documented in epidemiological studies in the general population [113]. Within the winter months, subjects frequently change their sleep habits, possess less energy, and experience variations in temperament. It has been proposed that human tolerance to changes in the lighting conditions, within seasons, varies according to a spectrum that includes at its end clinical (e.g. seasonal affective disorder or SAD) or sub-clinical syndromes (e.g. sub-SAD), which have a genetic basis, at least for the winter forms [113].

The cause of seasonal affective disorder (SAD) is still unknown but several hypotheses involving the circadian system have been proposed such as the phase delay, the melatonin, the photoperiod, and the reduced amplitude hypotheses (reviewed in Ref. [29]). In a recent study of 55 SAD patients and 55 controls, Wehr et al. reported a significant increase in the duration of melatonin secretion in the winter vs. summer in SAD patients [190]. No significant difference with seasons was observed in healthy volunteers. However, in this study, the timing of sleep episodes was not reported. It thus remains difficult to determine whether the longer melatonin secretion is a consequence of longer sleep/darkness episodes linked to the onset of depression or whether changes in the circadian timing system play a role in the appearance of depressive symptoms. The authors proposed that the retina or retinal projections to the circadian system could be less responsive to light in SAD patients than in healthy controls. Differences in the retinal sensitivity to light are supported by a few studies [71,145].

The possibility that the circadian signal is weakened in SAD and that bright light exerts its antidepressant effect by enhancing circadian amplitude has been proposed [42]. Until recently, there has been no convincing evidence to support this hypothesis [37,53,105]. In a recent study [98], seven SAD patients and matched controls were enrolled in a 120-h forced desynchrony procedure, using 20-h days. This revealed a reduction in the amplitude of the circadian rhythm of core body temperature in depressed patients in winter relative to control subjects. This finding was associated with greater temperature values and suggests a disturbance in thermoregulatory processes in winter depression. However, the sample size was small, the changes were still observable in remitted patients and the results do not necessarily reflect a reduction in the strength of the circadian oscillation.

Despite these concerns, the endogenous circadian system is most probably involved in the pathogenesis of winter depression. Namely, the interactions of the sleep–wake cycle and circadian system with neurotransmitter systems such as dopamine or 5-HT, and with neurohormones remain potential avenues for future research. A genetic defect at the level of clock regulation by afferent pathways involving NPY and serotonin is also possible

[135]. A genetic polymorphism at the CLOCK gene locus was not associated with major depression [47]. To our knowledge, no mutation screening of the circadian clock genes have yet been carried out in SAD.

5.6. More loci, more disorders

Human rhythm syndromes could be due to a mutation in core clock genes, but also in genes important for input and output pathways (as discussed for SAD, above). In this regard, it is important to mention that in addition to clock gene mutants [10,159,187], mice knock-out for other genes can display defects in circadian photoreception: this is the case for the gene encoding the PAC1 receptor [67], whose ligand, PACAP, is a neurotransmitter found in the retinohypothalamic tract [66]. It is also the case for *Crx*, a homeobox gene expressed in the retina [60], whose mutation is associated with three different human retinal diseases. The gene encoding melanopsin, a novel opsin suspected to be involved in circadian photoreception, has been deleted in the mouse: these knock-out animals display normal circadian rhythms, but the phase shifts of the rhythms and gene induction in the SCN in response to light are reduced [133,151]. A degeneration of the retinal ganglion cells that express melanopsin, or even mutations in the melanopsin gene in humans, could possibly lead for affected individuals to problems in synchronizing to environmental light–dark cycles.

The VPAC2 receptor is of special interest because both light responses and clock function are drastically affected in mice lacking this protein [68]. The effects of the gene deletion are difficult to interpret because the receptor can bind both PACAP (a peptide present in the retinohypothalamic tract) and VIP (expressed by SCN neurons), but it is tempting to propose that VIP signaling within the SCN is essential for the coupling of SCN neurons and for sustaining robust circadian rhythmicity [41,68]. Mice mutant for NCAM cell adhesion molecule [162], tyrosine kinase fyn [163], and ras-associated binding protein Rab3a, which is involved in synaptic vesicle trafficking [86], also present circadian rhythm defects, and these proteins may be involved in intra-SCN communication, or in output pathways. About the latter protein, it is interesting to note that X-linked non-specific mental retardation can be a consequence of mutations in the *GDI1* gene, which encodes a protein involved in the recycling of Rab GTPases, including Rab3a [44].

An intriguing possibility is that mutation of a gene that is important for an output pathway of the clock may cause a defect specific to the regulation of this pathway (for example sleep, food intake or others). An example of this is the *dfmr1* gene in *Drosophila*: disruption of this gene abolishes locomotor activity rhythms, while leaving eclosion rhythms unaffected [48,81,121]. The *dfmr1* gene is the homolog of the human X-linked gene *FMR*, whose mutation results in mental retardation (fragile X syndrome). It is noteworthy that the behavior of fragile X patients includes sleep problems [81,121].

The most striking demonstration that many as yet uncharacterized genetic loci have an influence on circadian rhythms comes from quantitative trait locus and genetic interaction analyses. These analyses, aiming at defining loci that contribute to variations in circadian behavior between mouse strains, identified many loci, of which none corresponded to the map position of known clock genes [164,173].

6. Clock gene mutations: effects beyond sleep and rhythm syndromes

6.1. Other roles for clock genes?

The clock controls a wide variety of pathways. Clock mutations thus impact significantly on many physiological processes. In addition to this, a dysfunction of the clock in humans may have implications that extend beyond circadian biology. The analysis of clock gene mutants in animals reveals that some clock genes may have functions in addition to timekeeping, and suggests that circadian capacity may be essential for many functions previously unsuspected to be linked to the clock.

Clock genes or clock-controlled genes activated by the transcription factor CLOCK (for example *Per1, Dbp* or *Avp*) display a blunted level of expression in *Clock* mutant mice [83,148]. A broader comparison of wild type and *Clock* mutant mice demonstrated that the CLOCK protein affects a large number of genes [132]. Interestingly, most of these genes do not cycle [132], suggesting that CLOCK may have roles outside the clock mechanism. On the other hand, while CLOCK was thought to regulate circadian expression in all tissues, recent data strongly suggest that this is not the case in the testis: in this organ, *Per1* and *Clock* genes are not expressed in the same cells, *Per1* transcript levels seem to be developmentally regulated and they remain elevated in *Clock* mutant mice [123].

In *Drosophila*, mutation of clock genes can have very diverse effects, such as perturbation of the homeostatic response to sleep deprivation [156], reduction of fertility [25] and elimination of sensitization to repeated cocaine exposures [14]. In the latter case, similar observations have been made in *Per1* knock-out mice, while *Per2* mutant mice exhibit the opposite phenotype, a hypersensitized response to cocaine [2].

6.2. Links with cancer

The tolerance to and efficacy of cancer treatments can be greatly improved by scheduled administration of the drugs in a circadian time frame (reviewed in Ref. [106]). This is due to normal circadian rhythms in cell function and proliferation. However, two recent articles suggest that

the connection between circadian rhythmicity and cancer extends well beyond chronotherapy. In one study, Filipski et al. compared the growth of a tumor implanted in mice with a SCN lesion and in sham-lesioned controls [58]. Tumor growth is accelerated by 2- to 3-fold in SCN-lesioned animal. This suggests that the SCN can play an antitumor role. This conclusion is supported by experiments on *Per2*-mutant mice [59]. These mice spontaneously develop salivary gland hyperplasia and malignant lymphomas, and they are more sensitive to γ radiation than their wild-type littermates. These effects appear to be due to the dysregulation of proto-oncogenes and subsequent genomic DNA damage and inhibition of apoptosis. These two reports shed new light on clinical studies showing an increased risk of breast cancer in women working at night [46,154].

7. Perspectives

Chronobiology is entering an exciting phase in which strong ties between basic and clinical research will be established. As human rhythm disorders become better defined, and familial cases identified, work on animal systems will be a precious aid in understanding the cause of the observed symptoms. Mutation screening and attempts to link polymorphisms to circadian rhythm disturbances will become more and more common in the near future. The possibility of monitoring clock gene expression in human samples will provide new circadian variables to study human circadian system in various clinically and socially relevant situations, including shiftwork, jetlag, aging and psychiatric disorders. Moreover, as was done with rat or mouse cultured cells, the induction of circadian rhythms in human cells by a serum shock may allow the study of human clock mechanisms. Such an experiment was reported with human vascular smooth muscle cells [116]. Using a similar approach on primary cultured cells collected from a human subject could allow rapid testing for genetic defects in the clock of a experimental subject. For example, cells from a FASPS patient would present short-period oscillations after a serum shock in culture. Ultimately, molecular circadian studies in humans, in addition to extending further our understanding of circadian and sleep disorders, will help to design specific drugs and treatments for these health problems.

Note added in proof

Johansson and coworkers found a significant difference in genotype distribution between SAD patients and controls, for a polymorphism in the *NPAS2* gene (471 Leu/Ser) [203]. They also linked a *PER3* polymorphism to diurnal preference (same polymorphism as in ref. [54]).

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