Review Article

Basic Science Review on Circadian Rhythm Biology and Circadian Sleep Disorders

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Abstract

The sleep-wake cycle displays a characteristic 24-hour periodicity, providing an opportunity to dissect the endogenous circadian clock through the study of aberrant behaviour. This article surveys the properties of circadian clocks, with emphasis on mammals. Information was obtained from searches of peer-reviewed literature in the PUBMED database. Features that are highlighted include the known molecular components of clocks, their entrainment by external time cues and the output pathways used by clocks to regulate metabolism and behaviour. A review of human circadian rhythm sleep disorders follows, including recent discoveries of their genetic basis. The article concludes with a discussion of future approaches to the study of human circadian biology and sleep-wake behaviour.

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Introduction

The sleep-wake cycle, with its characteristic intervals of activity alternating with restfulness that recur with a periodicity approximating the 24-hour day-night cycle, is the prototypical example of a behaviour that demonstrates a circadian rhythm. Circadian (from the Latin "*circa diem*" – "about a day") rhythms are also discernible in physiological and biochemical properties of the human body, such as core body temperature and corticosteroid and melatonin levels. Sleep and wakefulness, however, possess the practical advantage that they can be monitored non-invasively; thus, temporal alterations in activity have provided opportunities for elucidating the mechanism that engenders the circadian rhythm in animals such as fruitflies (*Drosophila melanogaster*),[1] golden hamsters (*Mesocricetus auratus*),[2,3] mice and rats[4] and humans.[5-7] This article will review our current understanding of mammalian circadian biology and human circadian disorders and discuss approaches for advancing our knowledge.

Endogenous Clocks

To generate a circadian rhythm, a system whose state oscillates with a period (τ) of 24 hours is essential. Based on its condition at any moment, the system signals the appropriate time-specific response, be it biochemical, metabolic or behavioural. The synchrony achieved between an organism's metabolism and its environment confers a survival advantage[8,9] that has spurred the development of such systems in evolutionary history. This timekeeping system, termed the endogenous clock, has evolved into a free-running entity that produces circadian rhythms even when time cues (*zeitgebers*) are absent. At its core, the clock consists of interacting molecules whose levels repeatedly fluctuate every 24 hours. A time point is specified by the concentrations of these molecules in the nucleus and cytoplasm.

Numerous experiments in mammals confirm that the suprachiasmatic nuclei (SCN) in the hypothalamus function as "master clocks" that reset and synchronise the circadian rhythms of peripheral tissues.[10] The SCN consist of neuron clusters whose electrical potential frequency fluctuates spontaneously with an approximate 24-hour periodicity that peaks during the biological daytime of diurnal and nocturnal species. The average human SCN free-runs with a τ slightly exceeding 24 hours.[11] To maintain clock-environment synchrony, external *zeitgebers* induce changes in the concentrations of the molecular components of the clock to levels consistent with the appropriate stage in the 24-hour cycle, a process termed entrainment. The ease with which clocks are entrained imposes a requirement for strict

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experimental conditions to detect and/or confirm circadian rhythm disorders (constant darkness for experimental animals, time isolation facilities for humans).[5]

Molecular Components of the Endogenous Clock

Figure 1 illustrates a working model of the mammalian circadian clock that is based on experimental results in mice and rats. Expression of the clock component genes yields ribonucleic acid (RNA) molecules that undergo post-transcriptional processing in the nucleus. The messenger RNAs are exported to the cytoplasm where ribosomal translation forms proteins. Kinase-mediated protein phosphorylation occurs in the cytoplasm, affecting their stability. After a time delay, sufficient protein molecules accumulate in the cytoplasm and form heterodimers that are imported into the nucleus, where they regulate gene expression through closure of positive and negative feedback loops. Interaction among the loops adds more complexity.

The CLOCK-BMAL1 heterodimer is a transcriptional activator which recognises and binds to the nucleotide sequence CACGTG, termed the E-box, that is upstream of the *Period* (*Per*) and *Cryptochrome* (*Cry*) genes and the genes for 2 orphan nuclear receptors, *Retinoid-related Orphan Receptor* (*Ror*) and *Rev-Erb*.[12,13] Three *Per* (*Per1*, *2* and *3*), 2 *Cry* (*Cry1*, *2*), 3 *Ror* (α, β and γ) and 2 *Rev-Erb* (α, β) homologues exist in mammals.[4,14] After transcription and translation, these proteins gradually accumulate in the cytoplasm. Cytoplasmic phosphorylation of PERIOD (PER) by several isoforms of casein kinase I (CKI), including CKI delta and CKI epsilon[15,16] and possibly other kinases, regulates its stability in a complex fashion.[17] Eventually, PER attains a critical level, permitting dimerisation with CRY and nuclear translocation. There, CRY suppresses CLOCK-BMAL1-induced transcription of *Per*, *Cry*, *Ror* and *Rev-Erb*, thus completing a negative feedback loop. Recently, it has been shown that binding of CRY to CLOCK-BMAL1 requires that BMAL1 first be acetylated at a specific lysine residue (K537) by CLOCK, which has intrinsic acetyltransferase activity.[18] ROR and REV-ERB translocate independently to the nucleus where they compete for binding to the orphan nuclear receptor target sequence, called the RORE sequence (AAAGTAGGTCA), in the *Bmal1* promoter. Their antagonistic effects (ROR activates *Bmal1* transcription, REV-ERB inhibits it) generate a rhythmic level of BMAL1 and thus CLOCK-BMAL1.[12,14] Additionally, PER2 activates *Bmal1* transcription in a positive feedback loop through a mechanism that is currently unknown.[19]

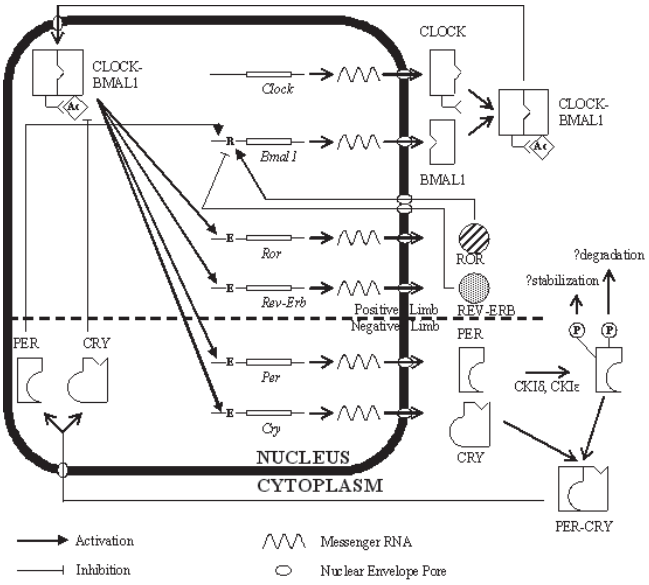

Fig. 1. A working model of the mammalian endogenous circadian clock, displaying the interlocked positive and negative feedback loops. Following convention, genes are displayed in italics while proteins are displayed in Roman letters. It is currently unsettled whether BMAL1 acetylation proceeds in the cytoplasm or is confined to the nucleus. Among the PER homologues (Table 1), only PER2 activates *Bmal1* transcription. The specific amino acids involved in stabilisation and destabilisation of PER are also uncertain.

Ac: acetyl; CKI: casein kinase I, CRY: cryptochrome; E: E-box sequence (CACGTG); P: phosphate; PER: period; R: RORE sequence (AAAGTAGGTCA); ROR: Retinoid-related Orphan Receptor

Most clock component messenger RNAs and proteins oscillate with a τ of 24 hours, except for CLOCK, CKIδ and CKIε. Nuclear levels of PER and CRY, which form the negative limb of the oscillator, reach a maximum at the normal transition from day to night. BMAL1, in contrast, reaches a peak intranuclear concentration at the transition from night to day. ROR and REV-ERB maxima occur between that of BMAL1 and PER-CRY. The CLOCK-BMAL1 complex constitutes the positive limb of the oscillator, whose activity is modulated by ROR and REV-ERB.

To achieve the fluctuations observed, it is crucial that nuclear entry of translated proteins is delayed precisely. This mechanism still remains obscure. PER phosphorylation alters its stability, delaying its nuclear entry and that of CRY (which must bind to PER to gain access to the nucleus).[17] However, the identities of all kinases involved and the exact order and timing of the amino acid phosphorylation are still unknown. Additional genes (*Npas2/Mop4*, *Arntl2/Mop9* and *Fbxl3*) have been proposed as clock components[4,20] but their roles remain undefined.

Known and putative human circadian clock components are listed in Table 1, together with their loci. As circadian rhythm abnormalities have only been associated with a minority of them, it is still uncertain if they are all non-redundant functional clock components.

Table 1. A List of Known and Putative Human Circadian Clock Genes and Their Chromosomal Loci.

Gene	Locus	Associated circadian rhythm disorder
hPer1	17p13.1	
hPer2	2q37.3	Advanced sleep phase disorder (causative mutation)
hPer3	1p36.2	Delayed sleep phase disorder (predisposition)
hCry1	12q23.3	
hCry2	11p11.2	
hClock	4q12	Evening preference in sleep-wake behaviour
hBmal1	11p15.2	
hCKIδ	17q25.3	Advanced sleep phase disorder (causative mutation)
hCKIε	22q13.1	Delayed sleep phase disorder (protection)
hRev-Erbα	17q21.1	
hRev-Erbβ	3p24.2	
hRorα	15q22.2	
hRorβ	9q21.13	
hRorγ	1q21.3	
hNpas2/Mop4	2q11.2	
hArntl2/Mop9	12p11.23	
hFbxl3	13q22.3	

Genes known to be associated with specific circadian rhythm disorders or altered sleep preference are indicated.

Afferent and Efferent Limbs

To coordinate time-appropriate metabolic responses in peripheral tissues, the rhythm of SCN neuronal activity must be entrained by the daily environmental cycle.

The major *zeitgeber* is light intensity. The circadian system relies on a small population of intrinsically photosensitive ganglion cells in the retina to detect variations in illumination.[21] These neurons contain the photosensitive pigment melanopsin, project directly to the SCN via the retinohypothalamic tract and utilise glutamate and pituitary adenylate cyclase-activating polypeptide (PACAP) as neurotransmitters.[22,23] Entrainment entails the sudden shift in concentration of one or more clock components, accomplished through a combination of altered gene expression and protein stability. The pathways that regulate gene transcription in the SCN neurons in response to retinal signals include mitogen-activated protein (MAP) kinase cascades, intracellular calcium and calmodulin, c-Fos protein and nitric oxide.[24-26] In addition to the CKI isoforms mentioned previously, protein kinase A and C (PKA and PKC) also phosphorylate PER.[27-29]

The SCN also receives input through the geniculo-hypothalamic tract from the intergeniculate leaflet of the thalamus and serotonergic input from midbrain raphe neurons. These may allow non-photic entrainment cues, such as feeding, exercise and social cues, to influence the SCN.[30-32]

The output pathways from the SCN neurons are incompletely elucidated at present. At the intracellular level, the target sequences (E-box and RORE) of clock components are present upstream of other genes as well. Conceivably, these clock-controlled genes (CCGs) form the first in a series of expanding transcriptional cascades. Three examples of CCGs that possess the E-box in their promoters are arginine vasopressin (*Avp*) (a neuropeptide), D-element binding protein (*Dbp*) (a transcriptional factor) and prokineticin 2 (*Pk2*) (a regulator of diverse biological functions, including sleep).[33-35] The recent discovery that CLOCK has intrinsic histone acetyltransferase activity suggests that CLOCK-BMAL1 may also regulate the transition between transcriptionally active euchromatin and inactive heterochromatin.[36]

Each SCN neuron functions autonomously, generating its own spontaneously oscillating electrical potential.[10] Synchrony of neuronal activity is partially achieved through the mediator vasoactive intestinal polypeptide (VIP) acting through its receptor, VPAC2.[37] The SCN then regulates the circadian expression of genes in peripheral tissues through a mixture of neural pathways, neurohormones and neuropeptides.

SCN neurons project directly to the medial preoptic, paraventricular and dorsomedial nuclei and the subparaventricular zone of the hypothalamus and the basal forebrain and midine thalamus; secondary projections fan out to the neocortex, limbic system, hippocampus, anterior pituitary, hypothalamus and reticular activating system (RAS) to modify wakefulness (RAS), thermoregulation and feeding (hypothalamus), memory and learning (hippocampus), mental performance (neocortex) and endocrine secretion (pituitary).[38]

The SCN neurons comprise distinct sub-populations that contain VIP, gastrin-releasing peptide (GRP), arginine vasopressin (AVP), gamma-aminobutyric acid (GABA) and doublecortin, neuropeptides that could potentially be used in paracrine signalling.[31,37,39] Additional molecules that are used in output pathways are prokineticin2 (PK2) and, indirectly, the neuropeptides and neurohormones produced in the hypothalamus, pituitary and pineal.[40,41] PK2 is especially relevant to the sleep-wake cycle as it has been postulated as one of the output molecules that transmit behavioural circadian rhythms.[41] Melatonin levels typically rise in late evening; this dim light melatonin onset (DLMO) is used as a position marker for the circadian clock,

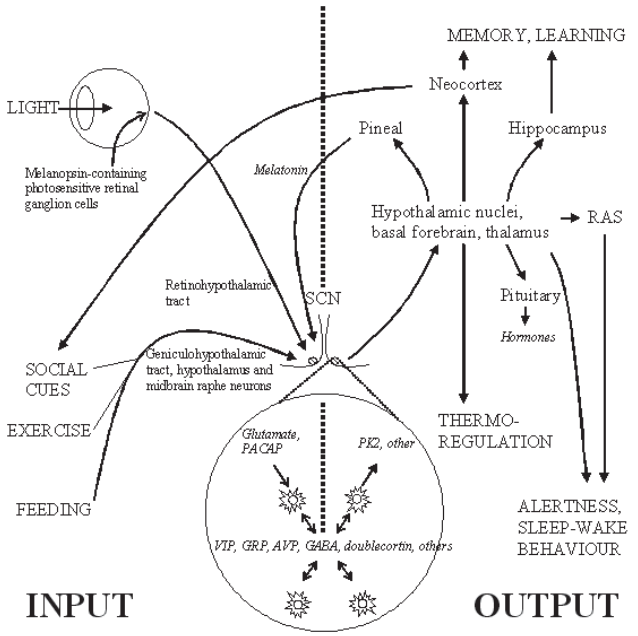

Fig. 2. The pathways leading to (input) and from (output) the suprachiasmatic nuclei (SCN). Chemical signals are displayed in italics. Note that behavioural and chemical changes brought about by output pathways from the SCN can themselves entrain the nuclei.

AVP: arginine vasopressin; GABA: gamma-aminobutyric acid; GRP: gastrin-releasing peptide; PACAP: pituitary adenylate cyclase-activating polypeptide; PK2: prokineticin2; RAS: reticular activating system; SCN: suprachiasmatic nuclei; VIP: vasoactive intestinal polypeptide

correlating with the onset of sleepiness and decline in core body temperature.[42] It has also been used therapeutically to treat circadian rhythm disorders such as jet lag and shift work disorder.[43]

Figure 2 summarises our current knowledge of the pathways leading to and from the SCN.

Human Circadian Rhythm Disorders

Human circadian rhythm disorders are classified according to the phase relationship between the internal clock and the external day-night cycle. As this asynchrony invariably interferes with the sleep-wake cycle, the scheme devised by the American Academy of Sleep Medicine to categorise intrinsic circadian rhythm sleep disorders (CRSDs) is a useful guide.[44]

In advanced sleep phase disorder/syndrome (ASPD), the major sleep episode is advanced in relation to the desired clock time, leading to an early sleep onset and a final awakening that is earlier than desired. The reverse occurs in delayed sleep phase disorder/syndrome (DSPD) where sleep onset and final awakening are delayed with respect to the desired clock time. The sleep-wake cycles in both ASPD and DSPD maintain a stable entrainment in relation to the 24-hour day-night cycle. In contrast, free-running disorder (FRD), also known as non-24-hour-sleep-wake syndrome, demonstrates a chronic, progressive pattern of 1- to 2-hour delays in sleep onset and wake times. Irregular sleep-wake rhythm/pattern (ISWR) patients have temporally disorganised and variable episodes of sleeping and waking behaviour with short sleep periods of a few hours that are distributed randomly through the day and night. Two other CRSDs (jet lag disorder and shift work disorder) are recognised but these are extrinsic disorders that occur after a voluntary or imposed shift in the timing of sleep and are not reflective of circadian clock dysfunction. Of the 4 intrinsic CRSDs, DSPD is the most commonly encountered (83%), followed by ISWR (12%). ASPD and FRD are rarely diagnosed, accounting for fewer than 2% each of CRSD patients.[45]

The majority of CRSDs are associated with medical conditions such as stroke, mania, depression, intracranial infection, head injury and the use of central nervous system stimulants or depressants. ASPD is more prevalent in the elderly, FRD is common in those who are totally blind while ISWR is observed mainly in the mentally retarded and the demented. Primary CRSDs in healthy individuals are, however, rare.

A major breakthrough in correlating these disorders with endogenous clock function was achieved through the study of a family showing autosomal dominant inheritance of ASPD.[5] A volunteer from this family, studied in a time-isolation facility, had a significantly shorter than average τ of 23.3 hours. ASPD seems to result from a substantially shortened τ, coupled with normal entrainment; DSPD patients presumably have a lengthened τ, although this remains unconfirmed. Based on its prevalence among the totally blind, FRD may reflect a failure of entrainment; the progressive delay in sleep and wake times is due to the human τ exceeding 24 hours slightly. ISWR sufferers probably have dysfunctional internal clocks as their sleep behaviour mimics that of experimental mammals which have undergone SCN ablation.[46]

The genetic basis of familial advanced sleep phase syndrome (FASPS) has been elucidated in 2 kindreds. In the first family, FASPS was due to a missense mutation altering the serine residue at position 662 of hPER2 to glycine (S662G).[6] A threonine-to-alanine mutation (T44A) that impairs hCKIδ function underlies FASPS in a second kindred.[7] This is analogous to the arginine-to-cysteine mutation (R178C) that causes a short-period variant in a golden hamster model of FASPS.[2] R178C results in a hypofunctioning CKIε. Based on these discoveries, it was proposed that defective CKIδ/ε-mediated phosphorylation at S662 stabilised hPER2, allowing earlier nuclear accumulation of PER-CRY heterodimers and closure of the negative feedback loop. To test this hypothesis,

phosphorylation of the mouse homologue (mPER2) was analysed and mice were targeted with wild-type and mutant versions of hPER2.[17,47] The results showed that CKIδ phosphorylates mPER2 at multiple sites other than S659 (equivalent to S662 in hPER2) and, contrary to expectation, the primary consequence of S662G is neither altered degradation nor nuclear localisation of PER, but, rather, reduced *Per* transcription. A corollary of these findings is that PER phosphorylation at a site other than S662 promotes its degradation. It is obvious that a satisfactory explanation for the shortening of τ in FASPS has not been accomplished.

Our understanding of the genetics of other CRSDs is even more rudimentary in the absence of families with Mendelian inheritance of these traits. A single nucleotide polymorphism (SNP) (3111 T $\rightarrow$ C) in the 3'-untranslated region of *hClock* and a valine-to-glycine mutation (V647G) in *hPer3* are associated with DSPD while a serine-to-asparagine mutation (S408N) in *hCKIε* appears to protect against DSPD.[48-50] The Smith-Magenis syndrome (SMS), which results from an interstitial deletion in the 17p11.2 chromosomal band, has an associated circadian rhythm abnormality that resembles ISWR.[51,52] Melatonin rhythms are inverted with peaks noted in daylight hours. Analyses of patients with the SMS phenotype who lack the typical deletion have demonstrated point mutations in the retinoic acid-induced 1 (*RAI1*) gene, suggesting that haploinsufficiency of this gene results in SMS.[53] This hypothesis is biologically plausible as *RAI1* may be downstream of *Ror* and *Rev-Erb* (circadian clock components) in a signalling cascade.

Future Directions

Now that the initial categorisation of the constituents of the circadian clock is practically completed, attention has shifted to the problem of generating a self-sustaining circadian oscillator with a τ that approximates 24 hours. We are still working out the mechanisms involved, including details of gene transcription regulation, post-translational modification (phosphorylation and acetylation), protein stability and nuclear transport.[54] At the organism level, work has focused on clarifying the entrainment pathways of the SCN, and the interactions between its component neurons that lead to coordinated function and signalling to peripheral tissues. The gaps in our current knowledge are particularly obvious for humans as ethical considerations preclude the use of mutagenesis and gene targeting, which were successfully applied to the study of circadian mutants in experimental mammals. Although specific human genes can replace their mouse counterparts in gene targeting experiments in mice, doubts remain whether the resulting behavioural change observed in nocturnal mice is wholly applicable to diurnal humans.

How, then, can we enhance our knowledge of human circadian biology, especially sleep behaviour? One way is to pursue the study of hereditary sleep variants. The main limitation of this approach is the rarity of familial CRSDs. A second obstacle is the possible failure of identification of the causative gene(s).

Observation of the sleep preferences of normal individuals suggests that, for the majority, sleep onset and wake times display a continuum that ranges between the extremes typical of ASPD and DSPD. As in other quantitative traits, several genes with small additive or subtractive effects may determine sleep preference. If sleep preference is approached thus, then the techniques used successfully in the study of polygenic diseases with a high heritability rate could be applied.[55] The responses of individuals to the Horne-Östberg Morningness-Eveningness Questionnaire can be used to gauge and classify them based on their sleep time preference.[56] Association studies can be performed on 2 contrasting groups of "morning larks" and "night owls", initially with SNPs in the vicinity of clock component genes (Table 1) and subsequently on a genome-wide basis using commercially available SNP panels.

As the circadian oscillator appears to contain a network of interwoven feedback loops, tinkering with individual components may yield limited information because of redundancies or excessive disruption. Mathematical modelling may provide better insights into the intricacies of oscillator function. This approach has previously been used to speculate on the organisation of the circadian system.[57] A recent mathematical analysis of the mammalian circadian clock concluded that the PER2 negative feedback loop is most responsible for setting the period of oscillation.[58] The validity of any conclusions, however, rests on the assumptions made in the construction of the model and the values assigned to the various parameters. Ultimately, experimental testing is required for confirmation of any inferences.[59]

DNA microarray technology permits the analysis of expression of several thousand genes simultaneously at any time point in the circadian day.[60] When applied to the study of tissues of organisms sacrificed at specific time points, the fraction of the genome in any tissue that demonstrates circadian rhythmicity can be identified. The order in which the genes are expressed would also make plain the CCG cascades involved in circadian clock output pathways.

Better methods are also needed to delineate the neural basis of sleep and wakefulness. The ideal method is one that surveys the brain functionally in real-time as a subject falls asleep or awakens, but this is technically difficult with the present generation of noisy equipment that might alter the sleep patterns of the test subjects.

The ultimate goal of this enterprise is a complete

understanding of the circadian system, and its influence on sleep, that would enable a practitioner of sleep medicine to precisely adjust his therapies to achieve the desired sleep onset time. The practical application of this knowledge will benefit many people who are currently suffering from the extrinsic CRSDs, namely, jet lag and shift work disorder.

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