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THE MAMMALIAN CIRCADIAN CLOCK: A NETWORK OF GENE EXPRESSION

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1. ABSTRACT

The circadian timing system provides a temporal structure across an organism to modulate and synchronize biological function. The mammalian circadian system is composed of many individual clocks. Circadian clocks are found in individual cells that have to be synchronized by a central pacemaker. This pacemaker can be viewed as a managing director who coordinates temporal physiology and behavior in the organism. In this review I will discuss the current understanding of the clock mechanism at the molecular level, how it adjusts to environmental changes and what the implications of a defect in the circadian clock are on mammalian physiology.

2. INTRODUCTION

In mammals, including humans, most vital processes undergo daily rhythms. The sleep-wake cycle, secretion of many hormones, locomotor activity, heart beat, renal blood flow, response of the immune system to antigens and many other physiological activities fluctuate with a period of about 24 hours (1). Under constant conditions these fluctuations are circadian and persist under constant conditions as exemplified in volunteers that were kept in a bunker without experiencing daily time cues (1). Hence these circadian fluctuations or rhythms must be controlled by an endogenous timing system, the so called circadian clock. The pacemaker of this clock is thought to be located in the suprachiasmatic nuclei (SCN) in the anterio-ventral part of the hypothalamus just above the optic chiasm. As indicated by the term 'circadian' the clock can tell time only approximately (the period in humans is slightly longer than 24 hours) and has therefore to be reset every day by the environmental light-dark cycle through an input pathway to the clock in the SCN (Figure 1). This resetting of the circadian clock is accomplished via the retinohypothalamic tract (RHT), which transmits light information from the retina directly to a subset of SCN neurons. The oscillations generated in the SCN are translated into overt rhythms in behavior and physiology through output pathways (Figure 1) that probably involve both electrical and chemical signals.

The circadian clock was originally believed to exist only in a few specialized cell types such as the SCN neurons. However, it was discovered that circadian clocks exist even in immortalized cell lines (2). Now it is believed that most peripheral cells contain circadian oscillators with a molecular makeup very similar to that of the SCN neurons. Peripheral clocks are synchronized by the SCN clock (3). The synchronization of the peripheral clocks (Figure 1) is thought to be achieved through humoral outputs from the SCN or alternatively by external signals such as food ingestion and processing. Examination of the molecular mechanisms underlying circadian clocks in different species has revealed that the general mechanism is conserved and can be described as an autoregulatory feedback loop. However, the molecular components that make up the clock in different species can differ considerably (reviewed in (4) (5)).

3. MUTATIONS OF THE CLOCK IN MICE AND HAMSTERS

Mutant mice and hamsters that display aberrant circadian wheel running behavior have been investigated in order to begin to decipher the molecular mechanism of the mammalian circadian clock (6, 7). Additionally, targeted mutations and deletions of candidate circadian clock genes have led to a better understanding of the function of clock genes in the mouse. Loss or mutation of specific clock genes can lead to altered period length (*Cry1, Cry2, Per1, Per3, CkIε*) and slow (*Clock, Per2*) or immediate loss (*Bmal1*) of circadian rhythmicity under constant conditions. *Cry1/Cry2* (15, 16) and *Per1/Per2* double mutant animals (8, 9) lose circadian rhythmicity immediately under constant conditions, confirming the importance of these genes in the clock mechanism. However, *Per3* seems to be dispensable for the clock because deletion of *Per3* has only

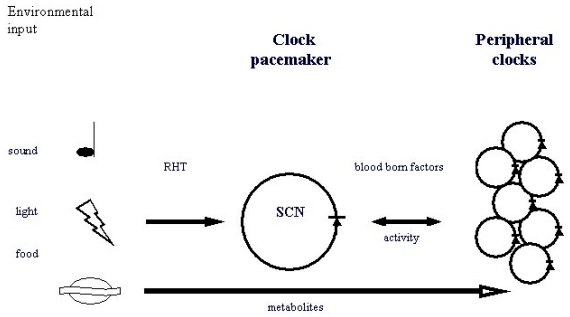

Figure 1. Diagram of the circadian system. The circadian clock pacemaker located in the suprachiasmatic nuclei (SCN) is adjusted by environmental stimuli and synchronizes peripheral oscillators (e.g. liver or kidney). Peripheral oscillators can also be influenced directly by environmental cues such as food. Peripheral oscillators (or clocks) can feed back to the clock pacemaker or other peripheral oscillators establishing a complicated network of clock interactions.

a weak effect on circadian wheel running activity (10) and *Per1/Per3* and *Per2/Per3* double mutants display the phenotype of *Per1* or *Per2* mutants, respectively (8). *Per2/Cry* double mutants revealed interesting phenotypes, indicating interactions between those genes. In particular, it seems that Cry2 interacts with Per2 *in vivo* (11). The array of phenotypes observed after the disruption of the main molecular clock components suggests that not all clock gene products are equally important for the maintenance of circadian rhythmicity.

The only nonredundant gene in the clock known so far is *Bmal1* (*Mop3*). Deletion of this gene leads to immediate arrhythmicity in constant darkness. On the molecular level there is no expression of *Per1* and *Per2* in the SCN, which indicates that *Bmal1* positively regulates *Per* gene expression (12) (Figure 1). Given the phenotype of the dominant negative *Clock* mutation, the effects of *Bmal1* on *Per* gene expression are consistent with an activator role of the CLOCK/BMAL1 transcription factor complex (13, 14) .

Mice lacking both *Cry1* and *Cry2* are behaviorally arrhythmic (15, 16) and show constant high non-cycling expression of the *Per1* and *Per2* genes in the SCN and peripheral tissues (17). This indicates that the *Cry* genes act as negative regulators of *Per* gene expression (Figure 2). Mice missing either the *Cry1* or the *Cry2* gene are rhythmic but display altered period lengths. *Cry1*$^{-/-}$ mice have a period shorter than 24 hours, whereas the period of *Cry2*$^{-/-}$ mice is longer than 24 hours. In constant darkness the clock of these mice runs faster or slower, respectively, supporting the notion that the clock consists of two opposite acting oscillators (18,19).

Mice with targeted deletions in *Per1* and *Per2* are behaviorally arrhythmic comparable to the phenotype of *Cry1/Cry2* mice (8, 9). This demonstrates the necessity of these genes in the clock mechanism. A mutation in *Per2* alone leads to a gradual loss of circadian locomotor activity, a reduced expression of *Per1* and *Cry1,* and a shift in *Bmal1* peak expression (20, 11). *Per3* and *Clock* expression, however, seem to be unaffected (21). These findings are consistent with a role of *Per2* as a positive regulator of circadian gene expression (20, 21) (Figure 2). This positive regulation appears to be the result of the double negative effect of *Per2* on *Bmal1* expression via its regulation of CLOCK/BMAL1 and *Rev-erbα* (22) (Figure 2). Mice lacking *Per1* display unaltered mRNA rhythms of *Per2*, *Cry1* and *Bmal1* (8, 9). However, in peripheral tissues total PER2 protein accumulates in liver cells (9) but in the nucleus of SCN cells less PER2 protein is detected (8). Assuming that the clock mechanism in liver cells and SCN cells is the same, these findings are consistent with a role for PER1 in regulating PER2 posttranscriptionally and thus influencing nuclear transport of PER2 (Figure 2). A reduction or loss of PER2 protein in the nucleus leads to a shift in *Bmal1* transcription (20, 11) and sets the synchronized oscillating expression of clock components out of phase, leading either to an unstable period length (9) or eventually to a loss of circadian rhythmicity (8) in *Per1* mutant mice. Under constant light conditions the period length of *Per1* mutants is longer than 24 hours, whereas for *Per2* mutants it is shorter and no loss of rhythmicity is observed in either mutant (23). This indicates that under constant light conditions a lack of *Per1* makes the clock slower, whereas a lack of a functional *Per2* accelerates the clock. This lends further support to the view that the clock consists of two opposite acting oscillators (18, 19).

Posttranslational regulation of clock components is essential to prevent accumulation of their proteins in the cell. Accumulation would lead to an equilibrium in transcriptional activation of clock genes and thus to a loss of rhythmicity in gene expression. One gene product that is thought to regulate clock genes posttranslationally is casein kinase Iε (CKIε). This gene codes for a protein kinase that is involved in phosphorylation of *Per* gene products. A mutation in the *CKIε* gene of hamsters, the so called tau mutation, leads to a very short period length (24, 6), demonstrating the importance of posttranslational regulation of clock components.

4. BASIC MECHANISM OF THE CIRCADIAN CLOCK IN MAMMALS

The data presented above gives rise to the following model for the intracellular clock mechanism in mammals. It involves interacting positive and negative transcriptional and post-transcriptional feedback loops driving recurrent rhythms in mRNA and protein levels of clock genes (Figure 2). Two basic helix-loop-helix (bHLH)-PAS (Period-Arnt-Single minded)-containing transcription factors, CLOCK and BMAL1(=MOP3) heterodimerize and bind to E box enhancer elements present in the promoters of clock genes such as *Period* (*Per*) *Cryptochrome* (*Cry*) and *Rev-erbα.* PER and CRY proteins interact and inhibit the CLOCK-BMAL1 driven transcription of their own genes in the nucleus, thereby establishing a negative feedback. This negative feedback loop is modulated by CKIε and CKIδ which phosphorylate

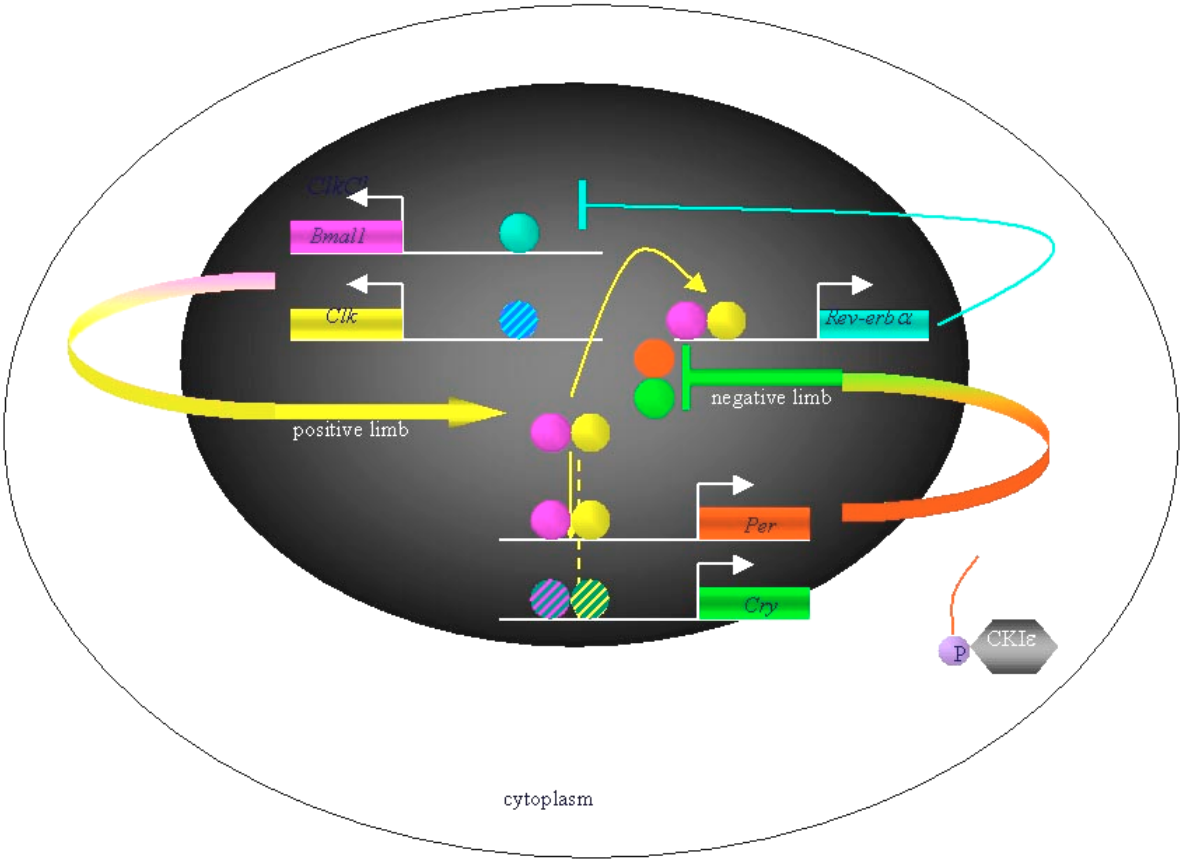

Figure 2. Simplified model of the molecular mechanism driving the circadian clock in a cell. Clock (yellow) and Bmal1 (purple) proteins (positive limb) drive the expression of *Per*, *Cry* and *Rev-erbα* genes in the nucleus (dark blue). The Per (red) and Cry (green) proteins in turn inhibit CLOCK/BMAL1 action and thereby downregulate their own and *Rev-erbα* (cyan) expression. Absence of Rev-erbα protein allows the *Bmal1* (and maybe also *Clock*) gene to be transcribed to produce new CLOCK/BMAL1 transcription factors to start a new circadian cycle. Posttranslational modifications of all proteins might occur. Such a modification mediated by casein kinase Iε (CKIε) for PER2 is indicated.

PER1, PER2, CRY1, CRY2 and BMAL1, respectively, and thus critically regulate the temporal structure of the clock. The orchestrated temporal program of posttranslational clock protein modification contributes to the 24 hour kinetics of the clockwork, which requires coordinated activity of several kinases, phosphatases and ubiquitinases.

The positive feedback loop involves the rhythmic expression of *Bmal1* which is expressed 12 hours out of phase relative to *Per* and *Cry* mRNA. The positive feedback loop is generated by the activation of the nuclear orphan receptor *Rev-erbα* through the binding of the CLOCK-BMAL1 heterodimer on its E-box containing promoter (22). The REV-ERBα protein then represses *Bmal1* transcription by acting through Rev-erb/ROR response elements present on the *Bmal1* promoter. This results in the decline of *Bmal1* mRNA levels, whereas *Per* and *Cry* levels rise. The PER and CRY proteins that enter the nucleus to inhibit their own gene expression through inhibition of the CLOCK-BMAL1 heterodimer also inhibit *Rev-erbα* transcription, resulting in an activation of *Bmal1* transcription. Thus the positive and negative feedback loops are co-regulated by CLOCK-BMAL1 heterodimers (Figure 2). The rhythmic mRNA expression of *Per* genes depicted in Figure 2 is synchronous to rhythmic histone H3 acetylation and RNA polymerase II binding at the promoters of *Per1* and *Per2*. This is probably mediated through an interaction of CLOCK with the histone acetyltransferase p300 (25).

5. SYNCHRONIZATION OF THE CLOCK TO THE ENVIRONMENT

The circadian clock has an intrinsic period that is not exactly 24 hours. Therefore, the clock has to be reset periodically to ensure synchronization of the organism's physiology to the environmental light-dark cycle. The dominant regulator of this entrainment is light (26). Light information is transmitted from the retina via the retino-hypothalamic tract (RHT) directly to the SCN. Light elicits glutamate release at the synapses of the RHT, which activate glutamate receptors that mediate influx of calcium ions (27). This leads to the activation of several signal transduction pathways evoking chromatin modifications (28), modification of clock proteins (29), activation of immediate early genes, such as *cfos*, NGFI-A (30, 31), and

the clock genes *Dec1, Per1* and *Per2* (32) (33, 34, 35). The behavioral response induced by light (or glutamate) exposure during night is a phase delay or phase advance of the clock (resetting), depending whether light is sensed during the early or late night, respectively (36). Several signal transduction mechanisms seem to be involved in the clock's resetting mechanism such as the cyclic AMP responsive element binding protein (CREB) driven transcription pathway (37, 38, 39), cyclic GMP mediated pathways (40, 41) and others (reviewed in (42)). Mutations in the *Per1* and *Per2* genes in mice indicate that *Per* genes play a role in resetting the circadian clock in response to light (43). Studies using the promoter of the *Per1* gene have shown that forskolin, agonists of adenylate cyclase (which increase cAMP producion), phorbol esters and growth factors (which activate protein kinase C and the MAP kinase signalling pathway) and calcimycin (which increases the intracytoplasmic calcium concentration) regulate *Per1* gene induction (44, 45, 46, 47, 48, 49). This indicates that many different factors are able to activate, at least, the *Per1* gene expression and thereby potentially contribute to synchronization between cells.

Mammals sense light probably exclusively through the eyes. However, the clocks of peripheral mammalian oscillators are not light sensitive and have to be synchronized to the major clock in the SCN probably through diffusible factors (50). Recently, candidates for diffusible factors that have the potential to synchronize clocks outside the SCN have been identified: TGFα and prokineticin 2 (PK2) (51, 52). Both polypeptides are produced and secreted by the SCN in a circadian fashion and suppress locomoter activity when infused into the third ventricle of the brain. These properties make TGFα and PK2 good candidates for diffusible signals by which the SCN can synchronize other oscillators. These two polypeptides may not only affect hypothalamic tissue but also tissues distal from the SCN. That there are probably more synchronizers than TGFα and PK2 is suggested by the fact that PK2 receptors are only found in gastrointestinal muscle cells, but not in other peripheral tissues that necessitate synchronization. Individual SCN neurons contain a variety of neuropeptides such as vasopressin, vasoactive intestinal peptide (VIP), gastrin-releasing peptide and somatostatin (80). In addition, electrophysiological studies have shown that glutamate is also an SCN neurotransmitter (81) that conveys circadian signals to hypothalamic target structures (82). The presence of all these transmitters in different combinations endows SCN neurons with a rich diversity of substances to transmit its signals. For example, the circadian peak of blood corticosterone is controlled by the SCN indirectly through the paraventricular nucleus, where neurons containing corticotropin releasing hormone (CRH) regulate the secretion of adrenocorticotropic hormone (ACTH) from the pituitary (82, 83). Taken together, these findings suggest that an intricate network of neuronal and hormonal pathways produce these rhythmic output signals.

Serum inducibility of clock genes in rat-1 fibroblasts (2) suggests that blood-born factors must stimulate signal transduction pathways that influence the molecular oscillators in cells of peripheral tissues. Glucocorticoids, which are hormones secreted into the blood stream in daily cycles, can reset the circadian time by changing *Per* gene expression in peripheral tissues (53). Interleukin-1 can also affect *Per1* gene expression (47), and in the vasculature, retinoic acid has been shown to phase shift *Per2* mRNA rhythmicity in vivo and in serum-induced smooth muscle cells in vitro (54). The finding that interferon-α can disrupt the rhythm of locomoter activity, body temperature and clock gene expression in mice (55) reinforces the importance of blood-born factors within the circadian system.

The findings described above indicate that environmental cues other than light can influence the mammalian circadian clock . For example the mole rat (spalax ehrenbergi) is able to uncouple the light input pathway from the clock (56) and probably uses cues such as temperature or humidity to synchronize its clock. Environmental temperature cycles appear to participate in the synchronization of at least the peripheral clocks in mammals (57). Interestingly, restricted feeding can lead to an uncoupling of the phase of peripheral oscillators from the SCN clock (58, 59) and, as a consequence, peripheral oscillators in the liver can display an opposite circadian cycle of clock gene expression compared to that of the SCN. How this uncoupling of peripheral clocks from the SCN clock works is not understood. However, feeding has an impact on metabolic pathways and hence also on the electron transport chain in mitochondria. There is evidence that heterodimerization of clock components that drive circadian transcription is influenced by the redox state of the cell (60) and the concentration of carbon monoxide (61). These findings suggest that the metabolic state of the organism can also influence clock function.

6. IMPACT OF THE CLOCK ON PHYSIOLOGY

In mammals, many tissues show circadian oscillations (3) which are driven by their own autonomous oscillators and are synchronized by a central pacemaker in the SCN. The SCN imposes temporal structure across the brain and peripheral organs *via* neural and endocrine outputs. Disruption of this temporal programming leads to changes in physiology and behavior, as observed during shift work, jet lag and aging (5). Therefore it is of great importance to identify the relationships between the SCN and peripheral tissues. Several research groups have taken on this challenge using differential display and microarray technology comparing expression of genes at different time points in cells (62, 63) and tissues such as the liver (64, 65, 66, 67), SCN (66) and the heart (67). These studies revealed that the circadian clock regulates many aspects of the cells molecular machinery. Diverse daily programs of gene expression, including transcriptional regulation, protein turnover and cell signaling were identified. Interestingly, circadian expression of genes seems to be tissue specific, illustrating the different demands of the body on diverse tissues.

If the clock is defective one would expect that this would have adverse effects on the organism. Support

for this view comes from mice mutant in the *Per2* gene. These mice are more cancer prone and it seems that the *Per2* gene plays a role in tumor suppression and DNA damage responses by regulating the temporal expression of genes involved in cell cycle regulation, such as *c-Myc*, *Cyclin D1*, *Cyclin A* and *Mdm-2* (68). This uncovers a relationship between the circadian clock and the cell cycle, another clock in which feedback loops constitute an essential device for regulating many molecular components similar to the circadian clock. These findings will have implications in chemotherapy of cancer patients, because the efficiency and/or toxicity of antiproliferative drugs depends on the circadian timing of treatment.

The molecular mechanism of the circadian clock is a cellular property (2). Hence, individual cellular clocks will synchronize by cell-cell communication within a specific tissue and tissue-tissue communication within an organism. Evidence how cell-cell communication might regulate the molecular clockwork in mice has emerged (69). An inactivation of the receptor for vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating polypeptide (PACAP) leads to a loss of normal circadian rest/activity behavior accompanied by a failure to express core clock genes such as *Per1*, *Per2* and *Cry1* in a circadian fashion, as well as a failure of circadian expression of the clock-controlled gene arginine vasopressin (AVP) in the SCN. This highlights the role of intercellular neuropeptidergic signaling in maintenance of circadian function in the SCN. Neuropeptidergic signaling is probably affected in *Per1* and *Per2* mutant mice, as evidenced by the abnormal sensitization of these mice to cocaine (70) and alteration in expression of molecules important for neuronal communication in *Per2* mutant mice (71).

The sleep/wake cycle exhibits a daily rhythm and is entrained to a 24 hour period by the day/night cycle (72). Sleep is thought to be regulated by two processes: a homeostatic process, which maintains a balance between sleep duration and sleep intensity, and a circadian process, which determines the times of high or low sleep propensity (73). How the circadian clock and its molecular components influence the behavior of sleep has been investigated by using mice mutant in molecular components of the circadian clock. Mice mutant in the gene *Clock* did not show altered slow-wave activity during baseline or after sleep deprivation (74). The pattern of sleep is differentially affected in *Per1* and *Per2* mutant mice, reflecting distinct roles of these genes in the circadian system. In contrast, the homeostatic regulation of sleep is preserved in these mutants (75). These findings indicate that *Per* genes have an influence on the circadian but not on the homeostatic component of sleep. Consistent with this view is the finding that a mutation in a phosphorylation site of the human Per2 protein leads to familial advanced sleep phase syndrome (76). A connection between the circadian clock and the homeostatic process of sleep regulation has come from the analysis of *Cry1/2* double mutant mice. These mice exhibit high non-REM sleep which implicates the cryptochrome genes in the homeostatic regulation of sleep (77). Taken together it seems that the circadian clock influences both the homeostatic and the circadian aspects of sleep regulation.

7. PERSPECTIVES

In the past few years the progress in understanding the molecular basis of the circadian clock has been tremendous. The identification of clock genes and the finding of peripheral oscillators has advanced our understanding of the circadian timing system. However, additional clock components await to be discovered. Understanding how known clock components function and are regulated has just started and results in a model presented in figure 2. The regulation of many clock components is not understood, e. g. how is transcription of *Bmal1* initiated, how are the proteins of clock components regulated and what is the mechanism of nuclear transport ? Is regulation at the RNA level occuring in mammals as it does in other phyla (78, 79) ? These are just a few questions addressing the clock mechanism at the molecular level.

At the cellular level future work will concentrate on the communication between cells and the synchronization of cellular circadian rhythms to generate an overt rhythm in a tissue. Which signaling molecules are used to coordinate individually ticking cellular clocks? How do such overt rhythms finally translate into behavioral and physiological rhythms? Understanding the coupling and interaction of the network of clocks will advance medical diagnosis and treatment of clock related pathologies as manifested in shift workers that have great tendency to develop heart problems, mental illness and alcohol abuse.

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