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# The circadian clock in mammals

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**Abstract** The basic physiological and anatomical basis for circadian rhythms in mammalian behaviour and physiology is introduced. The pathways involved in photic entrainment of the circadian clock are discussed in relation to new findings that identify the molecules that are involved in signalling between the environment and the clock. The molecular basis of endogenous cycles is described in the mouse, and compared to the mechanism that is present in the fly. Finally we speculate on the relationship between circadian physiology and pain.

**Key words** Molecular chronobiology • Circadian • Mammals • Genes • Cluster headache • Pain

## Introduction

In the course of biological evolution on our planet, living organisms have adapted to the daily rotation of the earth on its axis. One such adaption arose through the acquisition of endogenous circadian clocks that can be synchronized to the daily and seasonal changes in external time cues, the most important of which are light and temperature. Thus life forms are able to anticipate environmental transitions, so as to perform activities at biologically advantageous times during the day, and undergo characteristic seasonal responses. The importance of such a mechanism becomes dramatically manifest with the well-known effects of jet lag and shift work. These are clearly related to perturbations in the endogenous clock(s), which regulate much of our physiology and behaviour. Moreover, malfunctions in the human circadian timing system are implicated in several clinical manifestations, including chronic sleep disorders in the elderly, manic-depression, and seasonal affective disorders (SAD or winter depression) [1].

In multicellular organisms, circadian clocks are organised into multitissue systems, which function as biological timing mechanisms that regulate the activities of the organism in relation to environmental cycles, while providing internal temporal cues. Circadian clocks in vertebrates have been localised to neural structures such as the suprachiasmatic nucleus (SCN) of the anterior hypothalamus, the reti-

na and the pineal [2–4]. However, the role that these structures play in the organisation of the circadian system has been shown to vary even among similar species [5]. In birds, circadian rhythms are regulated by an oscillatory system consisting of the retina, the pineal and the SCN, while in other non-mammalian vertebrates circadian control is provided principally by the pineal and the retina. In mammals, the SCN is considered to be the master circadian pacemaker, while the role of the pineal is marginal, and the retinal clock is mainly involved in the control of circadian rhythms present in the eye.

## The suprachiasmatic nucleus: function and morphology

The major mammalian oscillator is located in the SCN of the anterior hypothalamus and drives circadian rhythms in physiology and behaviour during the circadian light-dark (LD) cycle via projections to the hypothalamus and other brain centres involved in the co-ordination of neuroendocrine, autonomical and behavioural regulation of homeostasis. These rhythms are entrained to the LD cycle, either by photic or non-photic input to the SCN, by phase-shifting the temporal alignment of the rhythms. Experimentally, phase-shifts of the SCN pacemaker activity can be induced by exposure to light, the direction and intensity of the phase-shift depending on the time point at which light is administered during the circadian cycle. Light pulses administered during the subjective day (circadian time (CT), 0–12) have little or no effect. At these circadian times only non-photic stimuli, such as circulating melatonin, physical activity and food availability, have been reported to produce phase advances [6]. On the other hand, light pulses can cause phase delays and advances during the early subjective (CT 12–18) and late subjective night (CT 18–0), respectively.

Morphologically, the rat SCN can be divided into two main parts: a dorsomedial part of the caudal SCN (dmSCN), or 'shell' of the SCN; and a ventrolateral part (vlSCN) or 'core' SCN [7]. The differences in arrangement and morphology of the constituting neurons in these two parts suggest that they may play different roles in the generation and regulation of circadian rhythmicity. This idea is supported by the partially different neuropeptide content in these two areas. In the rat, most of the neurons in the dmSCN synthesize vasopressin (VP), whereas neurons in the vlSCN synthesize vasoactive intestinal peptide (VIP), peptide histide isoleucine (PHI) and/or gastrin releasing peptide (GRP). A smaller proportion of somatostatin-producing neurons is found in between these two cell populations. In addition, gamma amino-butyric acid (GABA) is present in most, if not all SCN neurons [7]. Neurons in the vlSCN or core SCN receive glutamatergic (Glu) input from the retina via the RHT, neuropeptide Y (NPY) input via the GHT, and serotonergic (5-hydroxytryptamine, HT) input from the raphe nuclei. Neurones in the dmSCN receive non-photic input from the cortex, basal forebrain and hypothalamus [8].

## Photic entrainment in the SCN

Photic input is conveyed to the SCN along two pathways that originate in the retina: (1) the direct, retinohypothalamic tract (RHT) and (2) the indirect, retinogeniculate tract (RGT). The RGT forms synapses in the intergeniculate leaflets (IGL) of the lateral geniculate thalamic nucleus, where non-photic input from other brain areas is integrated. From the geniculate thalamic nucleus, the geniculohypothalamic tract (GHT) descends to the SCN. The main projection for non-photic input is from the raphe nucleus to the SCN. Photic entrainment of circadian rhythms during the subjective night depends on the release of glutamate (Glu) from RHT nerve terminals in the SCN. It is generally believed that phase-shifts result from an intracellular cascade that is activated by an NMDA-receptor-mediated $Ca^{2+}$ influx. $Ca^{2+}$ induces phosphorylation of $Ca^{2+}$/cAMP responsive element (CRE)-binding proteins (CREBs) by activation of the calmodulin (CaM)-CaM-kinase pathway. In addition, nitric oxide synthase (NOS) is thought to have a positive modulatory effect on phosphorylation. Phosphorylated CREB enters the cell nucleus where it activates the transcription of immediate early genes such as *c-fos* and *jun-B*, by binding to CRE promoters [9]. Light-induced *c-fos* and *jun-B* mRNA synthesis can only be detected during the subjective night, so that onset and offset of transcriptional activation might be ultimately responsible for phase-shifts. The fact that CREB protein concentrations are constant during the circadian cycle and that CREB can only be phosphorylated in response to light during the subjective night, suggests that gating may occur upstream of the phosphorylation step [10]. More recently however, Obrietan et al. [11] have shown that phosphorylated CREB (CREB-P) cycles in the SCN, even in constant darkness. Thus the circadian clock must feed into the kinases that modulate the activity of CREB.

Structural properties implicate the GHT in the regulation of circadian entrainment. Firstly, GHT neurones arise in the IGLs, in which photic and non-photic input is integrated, and secondly, in contrast to the excitatory effects of Glu released from the RHT, the main neurotransmitter in the GHT is GABA, which inhibits SCN neurones. Thus, GABA could be involved in the control of the extent of light-induced phase-shifts. Recently a role for histamine (HA) as the final neurotransmitter in the entrainment of circadian rhythms, instead of Glu has been proposed [12]. Thus release of HA from neu-

rones of the tuberomamillary nucleus, that terminate in the SCN, may be regulated by Glu, released from RHT neurones, and GABA, released from neurones of the GHT. By interacting with $GABA_B$ receptors, GABA can inhibit the release of Glu by neurones of the RHT and thus inhibit HA release indirectly. Furthermore, following the activation of $GABA_A$ receptors on histaminergic neurones in the SCN, GABA can inhibit HA release directly.

Circadian rhythmicity generated in the core neurones of the SCN is synchronized through a network of interneuronal connections, and is entrained by direct and indirect retinal input. The non-retinal input pathways can modify the response of the core pacemaker to retinal input. Projections from the core to the shell neurones guarantee the synchrony of all SCN neurones. The circadian rhythm that is generated in the SCN influences physiological functions, including body temperature, locomotor activity, sleep, oestrous cycle, oxygen utilization, water and food intake, adrenal corticosterone production and pineal melatonin synthesis. The best described of these clock-controlled pathways is a multisynaptic pathway by which the SCN controls the diurnal synthesis and secretion of the pineal hormone melatonin. Melatonin can in turn influence the phase of the rhythm via feedback inhibition of SCN neuronal activity [13]. The role of melatonin in circadian behaviour varies among species. Thus, while in hamsters the hormone mediates seasonal variation in reproductive behaviour, in man it has a role as a 'sleep-promoting hormone' [14].

## Molecular chronobiology

The most widely accepted model describing the molecular mechanism leading to the generation of circadian rhythmicity in the mammalian SCN neurones is similar to that already described for *Drosophila*, albeit with some important differences [15, 16]. In mammals there appears to be a duplication of resources, due to the presence of three *period (Per)* genes, and two *cryptochrome (Cry)* genes [17]. Another major difference in the mammalian clock regards the role played by murine *timeless* (*mtim*). In particular, *mtim* is a homologue of the *Drosophila tim2* gene [18, 19] and is not the mammalian homologue of the 'true' *Drosophila* clock gene *timeless* (now re-baptized *tim1)*. Accordingly, *mtim* shows no evidence of rhythmic circadian regulation, mTIM protein is not degraded by light (as in *Drosophila*) and, furthermore, mTIM possibly does not dimerize with PER as happens in *Drosophila* [17].

Our current knowledge regarding the workings of the mammalian system is summarised in Fig. 1. Transcription of the two *mCry* (*mCry1* and *mCry2*) genes is driven by the positive elements CLOCK:BMAL1, and the mCRY proteins then feedback to turn off the transcription of their respective genes in an autoregulatory negative feedback loop. The same positive elements (CLOCK:BMAL1) initiate the transcription of the three *mPer* (*mPer1*, *mPer2* and *mPer3*) genes. In this case, however, transcription is not turned off

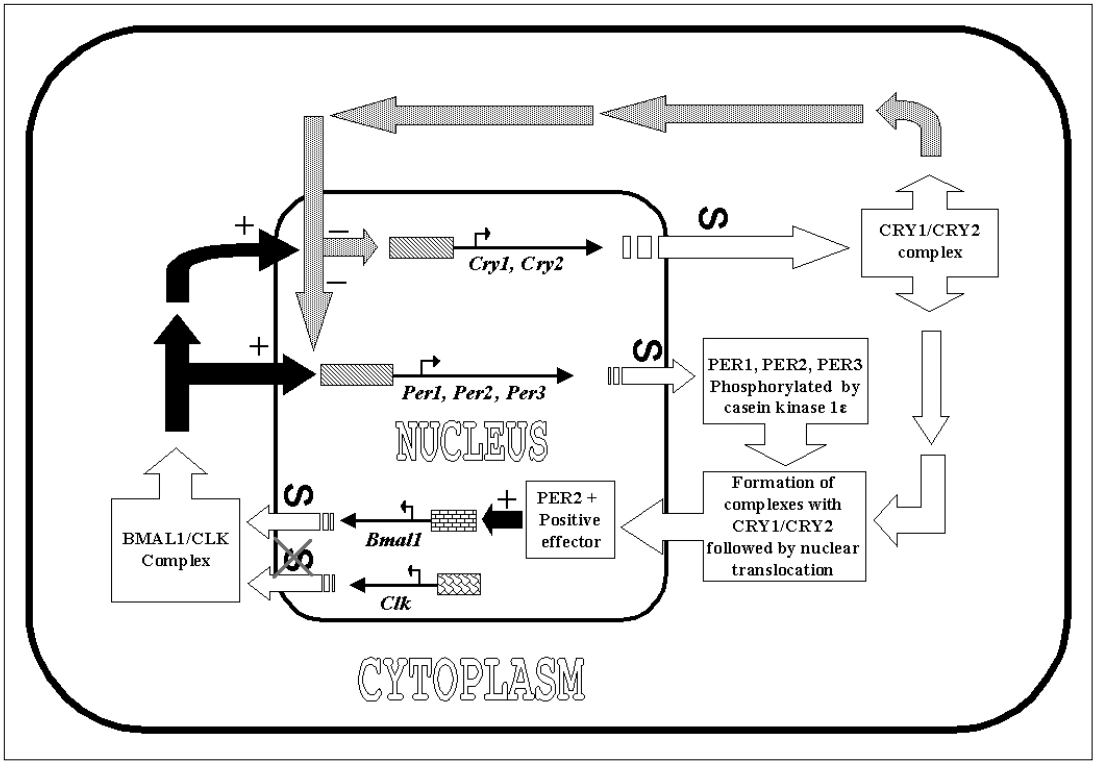


**Fig. 1** Molecular mechanism for the mammalian circadian clock. Clock genes are shown in the nucleus. *Patterned boxed*, promoters; *horizontal arrows*, transcribed regions; *bent arrows*, start codons. Positive (+) and negative (-) regulators. **S**, cycling transcripts

by their respective mPER gene products, but by the mCRY proteins [20]. Thus the *mCry*s are not only negative regulators of the *mPer* genes, but also of themselves. The mPER2 protein instead, acts as an effector of *Bmal1* transcription via a positive transcription factor (as yet unidentified) that interacts with cycling mPER2, generating in turn, cycles of *Bmal1* transcription [20]. In this way there is a physical interlocking of *Bmal1, mPer,* and *mCry* transcription rhythms. The roles of mPER1 and mPER3 have yet to be clarified.

The above model implies that at the start of the circadian day, *mPer* and *mCry* transcription are driven by accumulating CLOCK:BMAL1 heterodimers acting through E-box enhancers. Following a delay, the mPER and mCRY proteins are synchronously expressed, and translocated to the nucleus, where the mCRYs directly interact with CLOCK:BMAL1, thus inhibiting *mPer1-3* and *mCry1-2* transcription. At the same time that the mCRYs negatively regulate CLOCK:BMAL1-mediated transcription, mPER2 could: (a) be involved in translocating a transcriptional activator(s) into the nucleus or (b) participate in a complex to enhance *Bmal1* transcription. In this way, the *Bmal1* RNA rhythm would generate a 4–6 hour delay in the BMAL1 protein rhythm. Such a delay would ensure the availability of sufficient CLOCK:BMAL1 heterodimers at the appropriate circadian time. This would in turn guarantee the correct temporal alignment in the transcription of the mulitple *mPer* and *mCry* genes, thereby reinitiating the cycle.

A further fascinating aspect of the mammalian circadian system regards the nature of the cell type(s) and of the photopigment(s) involved in photic entrainment. In this respect it has recently been shown that the retinal photoreceptors (i.e. the rods and cones) are probably not necessary for this task [21]. The anatomical determinants of photic entrainment have nonetheless been shown to lie within the eye itself, since enucleated animals (from which the eyes have been surgically removed) are not entrainable to photic stimuli [22]. Thus, when the *Cry1* and *Cry2* genes were discovered in mice, it was reasonable to suppose that the corresponding proteins could be involved in mediating photic entrainment [23], as had been shown to be the case for the single *Drosophila cry* gene product [24]. Preliminary experiments provided support to this idea [25], but recent studies have implied that CRY1 and CRY2 are probably not involved in photic entrainment [26, 27]. As a consequence, attention is being devoted to other classes of photopigments, such as opsins, in the search for a true 'circadian photopigment'. In this respect, a very recent study has reported the identification of a novel mammalian opsin (named melanopsin) that is expressed in cells of the inner retina [28]. Interestingly, the anatomical distribution of retinal cells that contain melanopsin is very close to the distribution of the cells known to project from the inner retina to the SCN, suggesting that these cells (and melanopsin) actually mediate photic entrainment of circadian rhythms.

## Circadian rhythms and pain

Circadian rhythms in pain sensitivity are well-known [29]. One of the most dramatic examples of the relationship between pain and rhythms is seen with the cluster headache syndrome (CH). It has been noted previously in these pages that the striking circadian pattern of CH and the hypothalamic activation that accompanies these episodes [30] suggest a rather direct physical relationship between the SCN and pain [31]. We doubt there is a causal relationship between circadian rhythms and CH, as discussed briefly in [31], but this intriguing rhythmic disability provides us with opportunities to study the possible genesis of this apparent neurovascular disorder [30]. A mammalian model of CH would be very useful, and the advent of new molecular technologies such as the gene expression array, could be very useful in examining the transcriptional profile in a limited brain area, during an induced CH attack. In this way, the changes in gene expression during the various phases of any painful episode could be investigated. This approach needs not be limited to CH, but to any painful syndrome that can be restricted to a specific organ, for which a model organism can be utilised. CH could provide a useful initial avenue for the molecular exploration of pain in general, which is clearly a complex and multifactorial molecular process [32]. Such an analysis could generate new pathways that could be targeted for more specific alleviation of pain. We have the technology, now give us the model.

## References


1. Bunney WE, Bunney BG (2000) Molecular clock genes in man and lower animals: Possible implications for circadian abnormalities in depression. Neuropsychopharmacology 22:335–345
2. Moore RY, Eichler VB (1972) Loss of a circadian adrenal corticosterone rhythm following suprachiasmatic lesion in the rat. Brain Res 42:201–206
3. Takahashi JS, Menaker M (1984) Multiple redundant circadian oscillators within the isolated avian pineal gland. J Comp Physiol 170:479–489
4. Tosini G, Menaker M (1996) Circadian rhythms in cultured mammalian retina. Science 272:419–421

5. Menaker M, Tosini G (1996) The evolution of vertebrate circadian systems. In: Honma K, Honma S (eds) Circadian organization and oscillatory coupling. Hokkaido University, Sapporo, pp 39–52
6. Mistlberger RE, Antle MC, Glass JD, Miller JD (2000) Behavioral and serotonergic regulation of circadian rhythms. Biol Rhythm Res 31:240–283
7. van Esseveldt LKE, Lehman MN, Boer GJ (2000) The suprachiasmatic nucleus and the circadian time-keeping system revisited. Brain Res Rev 33:34–77
8. Moore RY, Speh-JC (1993) GABA is the principal neurotransmitter of the circadian system. Neurosci Lett 150:112–116
9. Edelstein K, Beaule C, D'Abramo R, Amir S (2000) Expression profiles of *JunB* and *c-Fos* proteins in the rat circadian system. Brain Res 870:54–65
10. Ginty DD, Kornhauser JM, Thompson MA, Bading H, Mayo KE, Takahashi JS, Greenberg ME (1993) Regulation of CREB phosphorylation in the suprachiasmatic nucleus by light and a circadian clock. Science 260:238–241
11. Obrietan K, Impey S, Smith D, Athos J, Storm DR (1999) Circadian regulation of cAMP response element-mediated gene expression in the suprachiasmatic nuclei. J Biol Chem 274:17748–17756
12. Jacobs EH, Yamatodani A, Timmerman H (2000) Is histamine the final neurotransmitter in the entrainment of circadian rhythms in mammals? Trends Pharmacol Sci 21:293–298
13. Kalsbeek A, Cutrera RA, Van Heerikhuize JJ, Van der Vliet J, Buijs RM (1999) GABA release from suprachiasmatic nucleus terminals is necessary for the light-induced inhibition of nocturnal melatonin release in the rat. Neuroscience 91:453–461
14. Zhdanova IV, Wurtman-RJ (1997) Efficacy of melatonin as a sleep-promoting agent. J Biol Rhythms 12:644–650
15. Dunlap JC (1999) Molecular bases for circadian clocks. Cell 96:271–290
16. Zordan M, Costa R, Macino G, Fukuhara C, Tosini G (2000) Circadian clocks: What makes them tick? Chronobiol Int 17:433–451
17. Reppert SM, Weaver DR (2000) Comparing clockworks: mouse versus fly. J Biol Rhythms 15:357–364
18. Benna C, Scannapieco P, Piccin A, Sandrelli F, Zordan M, Rosato E, Kyriacou CP, Valle G, Costa R (2000) A second *timeless* gene in *Drosophila* shares greater sequence similarity with mammalian *tim*. Curr Biol 10:512–513
19. Gotter AL, Manganaro T, Weaver DR, Kolakowski LF Jr, Possidente B, Sriram S, MacLaughlin DT, Reppert SM (2000) A time-less function for mouse *Timeless*. Nat Neurosci 3:755–756
20. Shearman LP, Sriram S, Weaver DR, Maywood ES, Chaves I, Zheng BH, Kume K, Lee CC, van der Horst GTJ, Hastings MH, Reppert SM (2000) Interacting molecular loops in the mammalian circadian clock. Science 288:1013–1019
21. Freedman MS, Lucas RJ, Soni B et al (1999) Regulation of mammalian circadian behavior by non-rod, non-cone, ocular photoreceptors. Science 284:502–504
22. Foster RG (1998) Shedding light on the biological clock. Neuron 20:829–832
23. Miyamoto Y, Sancar A (1998) Vitamin B2-based blue-light photoreceptors in the retinohypothalamic tract as the photoactive pigments for setting the circadian clock in mammals. Proc Natl Acad Sci USA 95:6097–6002
24. Stanewsky R, Kaneko M, Emery P et al (1998) The *cry$^b$* mutation identifies cryptochrome as a circadian photoreceptor in *Drosophila*. Cell 95:681–692
25. Thresher RJ, Vitaterna MH, Miyamoto Y et al (1998) Role of mouse cryptochrome blue-light photoreceptor in circadian response. Science 282:1490–1494
26. Okamura H, Miyake S, Sumi Y et al (1999) Photic induction of *mPer1* and *mPer2* in *Cry*-deficient mice lacking a biological clock. Science 286:2531–2534
27. van der Horst G, Muijtjens M, Kobayashi T et al (1999) Mammalian Cry1 and Cry2 are essential for maintenance of circadian rhythms. Nature 398:627–630
28. Provencio I, Rodriguez IR, Jiang G et al (2000) A novel human opsin in the inner retina. J Neurosci 20:600–605
29. Guney HZ, Gorgun CZ, Tunctan B, Uludag O, Hodoglugil U, Abacioglu N, Zengil H (1998) Circadian-rhythm-dependent effects of L-N-G-nitroarginine methyl ester (L-NAME) on morphine-induced analgesia. Chronobiol Int 15:283–289
30. May A, Bahra A, Buchel C, Frackowiak RSJ, Goadsby PJ (1998) Hypothalamic activation in cluster headache attacks. Lancet 352:275–278
31. Kyriacou CP (2000) Molecular chronobiology. J Headache Pain 1:5–10
32. Luo ZD (2000) Molecular dissection of pain mediators. Pain Rev 7:37–64