Coordination of circadian timing in mammals

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Time in the biological sense is measured by cycles that range from milliseconds to years. Circadian rhythms, which measure time on a scale of 24 h, are generated by one of the most ubiquitous and well-studied timing systems. At the core of this timing mechanism is an intricate molecular mechanism that ticks away in many different tissues throughout the body. However, these independent rhythms are tamed by a master clock in the brain, which coordinates tissue-specific rhythms according to light input it receives from the outside world.

Circadian rhythms, as exemplified by the sleep/wake cycle, are the outward manifestation of an internal timing system. The full force of genetic, molecular and biochemical approaches, complemented by precise behavioural observations, has rapidly advanced our knowledge of circadian timing in mammals. The focal point of this system is a master clock, located in the suprachiasmatic nuclei (SCN) of the anterior hypothalamus, which orchestrates the circadian programme[1]. Principal advances in understanding the molecular and biochemical basis of circadian timing have provided a rapidly evolving model of the underlying 'clockwork'. Recent developments have also revolutionized our view of SCN input and output mechanisms. These include the discovery of a new visual pathway from retina to the SCN that entrains (synchronizes) circadian rhythms to the solar day, and the elucidation of ways in which the SCN clock ultimately generates output rhythms in physiology and behaviour.

Defining the molecular basis of circadian timing in mammals has profound implications. In terms of fundamental brain mechanisms, the circadian system is among the most tractable models for providing a complete understanding of the cellular and molecular events connecting genes to behaviour. Thorough dissection of the

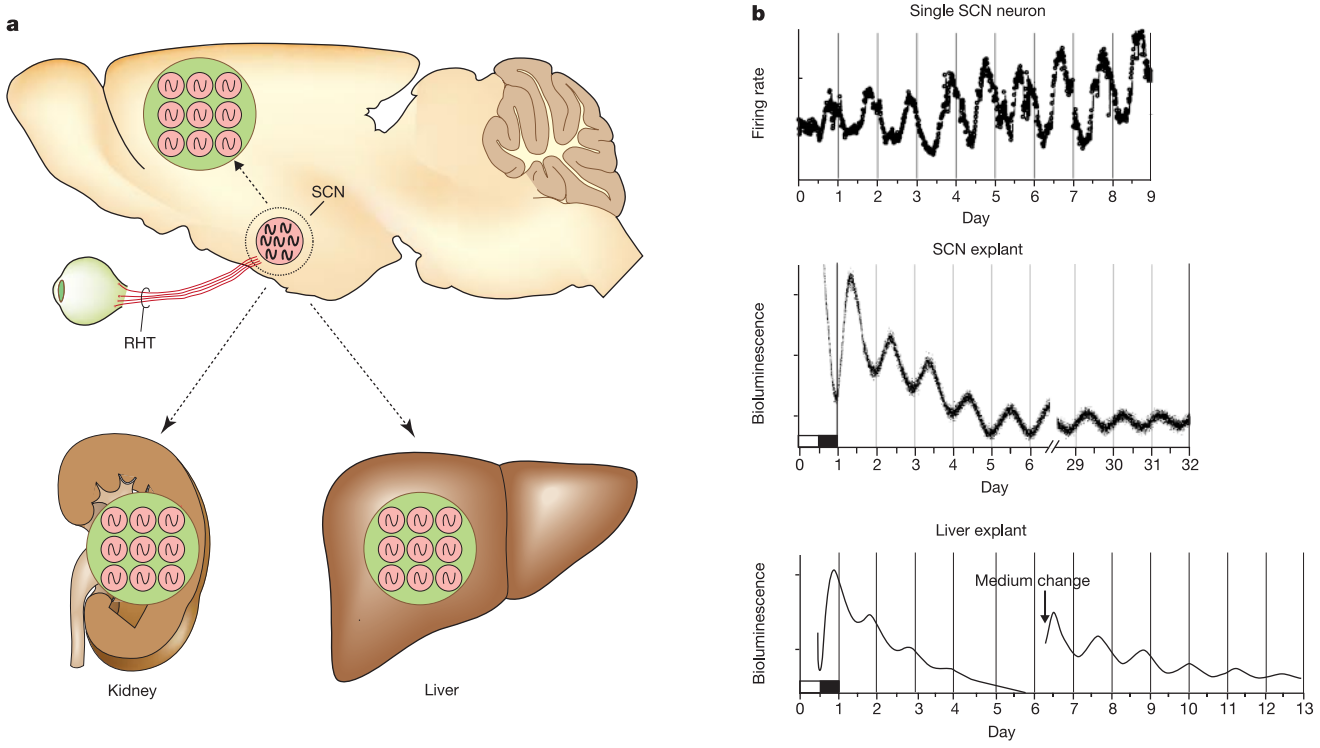

Figure 1 The mammalian circadian timing system is a hierarchy of dispersed oscillators. **a**, The master clock in the SCN is composed of numerous clock cells. The SCN receives light information by a direct retinohypothalamic tract (RHT) to entrain the clock to the 24-h day. The entrained SCN, in turn, coordinates the timing of slave oscillators in other brain areas (for example, cortex) and in peripheral organs (for example, kidney and liver). **b**, A single SCN neuron in culture expresses robust circadian rhythms in firing rate over 9 days of study, proving that the core clock mechanism is contained within single cells (adapted from ref. 83). SCN and liver explants from transgenic rats expressing a *mPer1*-driven luciferase reporter gene exhibit bioluminescence rhythms in culture; the black and white bars along the *x* axis indicate the light–dark cycle at the time of tissue collection (adapted from ref. 9). The SCN explant rhythm persists for weeks in culture, whereas the liver explant rhythm dampens. A medium change on day 7 restarts the liver oscillation, showing that the dampening was not due to tissue death.

genetic basis of circadian behaviour may help to decipher this connection for more complex behaviours. Understanding the molecular clock could increase our knowledge of how gene mutations of the molecular clock contribute to psychopathology (for example, major depression and seasonal affective disorder)[2]. Similarly, such understanding should lead to new strategies for pharmacological manipulation of the human clock to improve the treatment of jet lag and ailments affecting shift workers, and of clock-related sleep and psychiatric disorders.

A hierarchy of distributed oscillators

Circadian timing in mammals is organized in a hierarchy of multiple circadian oscillators (Fig. 1a). The oscillatory machinery of the master clock is contained within single neurons[3] (Fig. 1b), and it is possible that most of the approximately 20,000 neurons that comprise the bilateral SCN are 'clock cells'. Molecular evidence is beginning to emerge for functionally distinct populations of clock cells within the SCN[4–6].

Intriguingly, there are also circadian oscillators scattered throughout the body, as the genes involved in the intracellular SCN clock mechanism have been found to be rhythmically expressed in other brain areas, in peripheral organs, and even in immortalized cell lines in culture[7–9]. The extra-SCN, 'slave' oscillators expressed *in vivo* can only sustain 24-h oscillations for a few days without input from the master clock (Fig. 1b). For orchestrated circadian timing, the collective SCN synchronizes the timing of slave oscillators, each of which is a multioscillatory entity. Synchronized slave oscillators, in turn, regulate local rhythms in physiology and behaviour. A hierarchical multioscillatory system seems to confer precise phase control and stability on the widely distributed physiological systems it regulates[10].

Analysis of clockwork function in slave oscillators (such as the liver and cultured cell lines) has been invaluable for defining biochemical principles important for the core clock mechanism, as rigorous biochemical analysis of a small brain structure like the SCN is difficult. Indeed, genetic studies have shown that the molecular composition of the timing mechanisms in the SCN clock and slave oscillators is very similar[11]. The actual mechanism that distinguishes the self-sustaining oscillatory function of the master clock from the damped oscillation of slave oscillators is unknown, but the mechanism may have more to do with global differences in clock protein levels and/or kinetics than the existence of a specific element (gene/protein) expressed only in the SCN.

Transcriptional feedback loops

Knowledge of circadian clock mechanisms in the fruit fly *Drosophila melanogaster* has greatly aided the formulation of mammalian clock mechanisms[12]. Homologues of most of the genes involved in the fly circadian clock have been cloned in mammals, and the general core clock mechanism of interacting transcriptional feedback loops is similar between flies and mice. However, there has been a shuffling of specific functions between several structurally disparate components, and gene duplication has led to increased complexity among mammalian clock genes[1,12]. Even though the principal clock genes may have been identified (Table 1), genome-wide complex trait analysis in mice has revealed the presence of yet-to-be discovered clock-modulating genes[13].

The intracellular clock mechanism in the mouse involves interacting positive and negative transcriptional feedback loops that drive recurrent rhythms in the RNA and protein levels of key clock components (Fig. 2). Rhythmic transcriptional enhancement by two basic helix–loop–helix (bHLH)-PAS (Period–Arnt–Single-minded)-containing transcription factors, CLOCK and BMAL1 (also called MOP3), is essential for clockwork function and provides the basic drive to the system[14–17]. CLOCK–BMAL1 heterodimers activate transcription by binding to E box enhancers and are highly selective for those with the nucleotide sequence CACGTG[15,16]. Negative feedback involves rhythmic inhibition of the CLOCK–BMAL1 drive by negative regulators (Fig. 2). Specifically, CLOCK–BMAL1 heterodimers activate the rhythmic transcription of three *period* genes (*mPer1–mPer3* in the mouse) and two *cryptochrome* genes (*mCry1* and *mCry2*). The resultant mPER and mCRY proteins translocate back into the nucleus where the mCRY proteins act as negative regulators by directly interacting with CLOCK and/or BMAL1 to inhibit transcription, closing the negative feedback loop[18–21].

The positive feedback loop involves the rhythmic regulation of *Bmal1* transcription, whose RNA levels peak 12 h out of phase relative to *mPer* and *mCry* RNAs[21,22]. Recent studies provide a revised view of the regulation of the positive feedback loop (Fig. 2): to generate the positive loop, CLOCK–BMAL1 heterodimers, while activating *mPer* and *mCry* transcription, also activate transcription of the orphan nuclear receptor gene *Rev-Erbα*[23]. The REV-ERBα protein then represses *Bmal1* transcription by acting through Rev-Erb/ROR response elements in its promoter[23,24]. As a result, *Bmal1* RNA levels fall, whereas *mPer* and *mCry* RNA levels rise. When the mCRY proteins enter the nucleus to inhibit *mPer* and *mCry* transcription (through actions on CLOCK–BMAL1), they also inhibit *Rev-Erbα* transcription resulting in a de-repression (activation) of *Bmal1* transcription[23,25]. mPER2 may provide the positive drive on *Bmal1* transcription when *Rev-Erbα* expression is inhibited[21]. Thus, the positive and negative transcriptional feedback loops are co-regulated by CLOCK–BMAL1 heterodimers, with differences in the loop dynamics coming from the differential activities of the co-regulated protein products (Fig. 2).

mPER and mCRY outputs of the negative feedback loop are

Table 1 Components of the mouse clock mechanism

Proteins		Mutations*	
Family	Member	Gene(s)	Behavioural phenotype†
bHLH-PAS	CLOCK‡	*Clock*§	Long period, then arrhythmic
	BMAL1 (MOP3)	*Bmal1 (Mop3)*	Arrhythmic
PER-PAS	PER1	*Per1* or *Per1* + *Per3*	Short period, then arrhythmic
	PER2	*Per2* or *Per2* + *Per3*	Short period, then arrhythmic
	PER3	*Per3*	Short period
		Per1 + *Per2*	Arrhythmic
Flavoproteins	CRY1	*Cry1*	Short period
	CRY2	*Cry2*	Long period
		Cry1 + *Cry2*	Arrhythmic
Casein kinase	CKIε	*CKIε*‖	Short period
Orphan nuclear receptor	REV-ERBα	*Rev-Erbα*	Short period

*Unless otherwise noted, the mutations listed are deletion mutations induced by targeted mutagenesis.
†The most severe phenotypes of homozygous mutant animals studied under constant conditions are listed.
‡A CLOCK-like role has been described for MOP4 (also known as NPAS2)—a bHLH-PAS transcription factor closely related to CLOCK—in the cerebral cortex[82] and in the vasculature[41].
§Ethylnitrosourea-induced semidominant autosomal mutation. The mutation is a nucleotide transversion in a splice donor site causing exon skipping and deletion of part of the transactivation domain[14].
‖Spontaneous semidominant autosomal mutation described in the Syrian hamster (the *tau* mutation). The mutant enzyme is deficient in its ability to phosphorylate PER[35].

essential for maintaining a functioning circadian clock, as disruption of either the *mPer1* and *mPer2* genes together or both *mCry* genes causes immediate behavioural arrhythmicity when the double-knockout animals are placed in constant conditions[19,26–28] (Table 1). There is partial compensation of function between family members, as the clock continues to oscillate after single gene mutations, albeit for variable periods of time in constant conditions depending on the gene mutated[19,26–30] (Table 1). mPER3 does not have a critical role in the maintenance of the core clock feedback loops, as molecular and behavioural rhythms are preserved in *mPer3* null mutant mice, and there is no synergy with either the homozygous *mPer1* or *mPer2* mutants[26,31] (Table 1). Instead, mPER3 probably functions as an output signal.

Post-translational control of the clockwork

Circadian patterns of clock protein abundance, phosphorylation, interactions and subcellular location each contribute to building time delays into the 24-h clockwork. Although translational control may also contribute to a time delay between *mPer* RNA and protein rhythms, post-translational processes have been the most well studied mechanisms for post-transcriptional control.

Clock protein phosphorylation

CLOCK, BMAL1, mPER1 and mPER2 undergo temporal changes in phosphorylation *in vivo*, with maximal phosphorylation correlating with the time of negative feedback on *mPer*/*mCry* transcription[32] (Figs 2 and 3). Ironically, transcriptional activation occurs when levels of CLOCK and BMAL1 in the nucleus are at their lowest[32], suggesting that the phosphorylation status of the transcription factors is important for the transcriptional competency of the heterodimer[33,34]. Phosphorylation may also be important for the translocation of CLOCK and BMAL1 into the nucleus, and the formation of protein complexes that inhibit CLOCK–BMAL1-mediated transcription (see below).

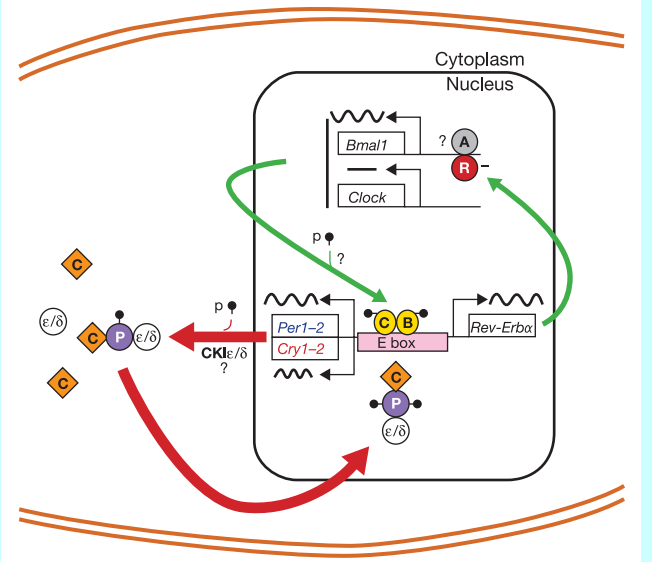

Figure 2 Mammalian circadian clockwork model. The clock mechanism comprises interactive positive (green) and negative (red) feedback loops. CLOCK (C, oval) and BMAL1 (B, oval) form heterodimers and activate transcription of the *Per*, *Cry* and *Rev-Erbα* genes through E-box enhancers. As the levels of PER proteins increase (P, blue circle), they complex with CRY proteins (C, diamond) and CKIε/CKIδ (ε/δ, circle), and are phosphorylated (p). In the nucleus, the CRY–PER–CKIε/CKIδ complexes associate with CLOCK–BMAL1 heterodimers to shut down transcription while the heterodimer remains bound to DNA, forming the negative feedback loop. For the positive feedback loop, increasing REV-ERBα levels (R, circle) act through Rev-Erb/ROR response elements in the *Bmal1* promoter to repress (−) *Bmal1* transcription. CRY-mediated inhibition of CLOCK–BMAL1-mediated transcription de-represses (activates) *Bmal1* transcription, because REV-ERBα-mediated repression is inhibited. An activator (A, circle) may positively regulate *Bmal1* transcription (?) alone or by interacting with mPER2. There are probably kinases (?) other than CKIε and CKIδ that participate in phosphorylation of clock proteins.

So which kinases are important for the mammalian clockwork? Casein kinase Iε (CKIε) is an important clock modulator in Syrian hamsters, because a defect in the hamster CK1ε gene corresponds to the short-period *tau* mutation[35]. CKIε can phosphorylate PER1 and PER2, and the mutant hamster kinase has a lower rate of phosphorylation[35]. In addition, a human genetic disorder characterized by shortened circadian period and advanced sleep phase is associated with a missense mutation in human *PER2*, and the mutant protein is less effectively phosphorylated by CKIε *in vitro*[36]. Of note, the degree of PER1 and PER2 phosphorylation is not appreciably altered in homozygous *tau* mutant hamsters[32]. With the recent finding that CKIε can also phosphorylate mCRY1, mCRY2 and BMAL1, it is possible that alterations in the temporal phosphorylation of one of these proteins may contribute to the altered clock phenotype in *tau* mutant hamsters[33].

In vivo evidence suggests that CKIδ is a second kinase important for the mammalian clockwork[32]. CKIδ is highly homologous to CKIε (76% identical at the amino acid level in humans) and phosphorylates mPER1, mPER2, mCRY1, mCRY2 and BMAL1 *in vitro*[33,37]. Mitogen-activated protein kinase can also phosphorylate BMAL1 *in vitro* (ref. 34). Recent work in *Drosophila* suggests that glycogen synthase kinase-3 and related kinases should be examined for their involvement in the mammalian clockwork[38]. The kinases responsible for rhythmic phosphorylation of CLOCK have not been identified. Clearly, the orchestrated temporal programme of clock protein phosphorylation contributes to the 24-h time kinetic of the clockwork, requires the coordinated activity of several kinases, and probably involves phosphatases.

Regulated nuclear entry

Nuclear entry of the mPER and mCRY proteins is a vital checkpoint for progression of the clockwork cycle. *In vivo* studies of the liver oscillator show that the mPER proteins are rate limiting for the mPER–mCRY interactions in cytoplasm that, in turn, are necessary for nuclear accumulation of the complex[32]. Accordingly, the robust oscillation in mPER protein abundance drives the clock mechanism forward, as it brings clock protein complexes into the nucleus at the proper time for negative transcriptional feedback. There is also a co-dependency between the mPER and mCRY proteins for effective nuclear accumulation. This helps explain why the molecular clock continues to cycle with single gene mutations (*mPer1*, *mPer2*, *mCry1* or *mCry2*) but abruptly stops cycling in double-knockout animals in which either *mPer1* plus *mPer2* or *mCry1* plus *mCry2* genes are targeted (Table 1). The balance between the nuclear import and export of clock proteins also contributes to their cellular location and may provide a point for fine-tuning circadian cycle length[39,40].

Rhythms in subcellular localization of CKIε and CKIδ are synchronous and are driven by association with mPER and mCRY occurring in the cytoplasm, which enable subsequent accumulation of multimeric complexes in the nucleus[32] (Fig. 2). PER proteins, which have distinct binding sites for mCRY and CKIε/CKIδ, act as bridge proteins that are not only required for the trimeric protein assembly, but may also be required for phosphorylation of the mCRY proteins by the casein kinases[33,37].

Of note, the mCRY proteins are essential for the stability of phosphorylated mPER2, probably through direct mPER2–mCRY association[32]. This interaction seems to protect phosphorylated mPER2 from ubiquitination and subsequent degradation in the proteosome[40]. On the other hand, the mCRY proteins are not necessary for the stabilization of phosphorylated mPER1 (refs 21,

32). It is not known how the differential effects of mCRY protection contribute to the roles of mPER1 and mPER2 in the clock mechanism.

A nuclear timesome

Once in the nucleus, mCRY–mPER–CKIε/CK1δ complexes associate with CLOCK and BMAL1 (ref. 32). This 'timesome' negatively regulates transcription of *mPer*, *mCry* and *Rev-Erbα* genes through disruption of activity of the transcriptional complex (Fig. 3). Chromatin immunoprecipitation experiments show that CLOCK and BMAL1 are both constitutively bound to *mPer1* E boxes over the circadian cycle in the liver oscillator[32]. It is the rhythmic binding of negative regulators to the DNA-anchored CLOCK–BMAL1 heterodimers that generates the rhythm in transcriptional activity[32]. In slave oscillators in vascular tissue, the activation of the nuclear hormone receptors retinoic acid receptor-α (RARα) and retinoid-X receptor-α (RXRα) seems to act cooperatively with the mCRY proteins to negatively regulate CLOCK–BMAL1-mediated transcription[41]. This result reveals potential complexity provided by the involvement of numerous co-regulators. The constant binding of CLOCK and BMAL1 to DNA argues against a major role of redox state in modifying the binding of CLOCK–BMAL1 heterodimers to E boxes *in vivo*[42,43].

The hyperphosphorylated state of the timesome probably targets the clock protein complex for degradation at the end of the inhibitory phase, perhaps through the ubiquitin-proteasome pathway[37,40]. The continued presence of CLOCK–BMAL1 heterodimers bound to DNA at the end of the negative phase of transcriptional regulation probably reflects a mixed population of newly synthesized and 'older' transcriptional complexes, which differ in functional activity.

Photic input to the SCN

A 'circadian' photoreceptor

A critical feature of circadian timing is the ability of the clockwork to be reset by environmental stimuli. In mammals, light is the most potent entraining signal, with the retinohypothalamic tract (RHT) being the principal retinal pathway through which entraining information reaches the SCN[44,45]. Recent studies build a strong case for the involvement of the photopigment melanopsin in circadian photoreception.

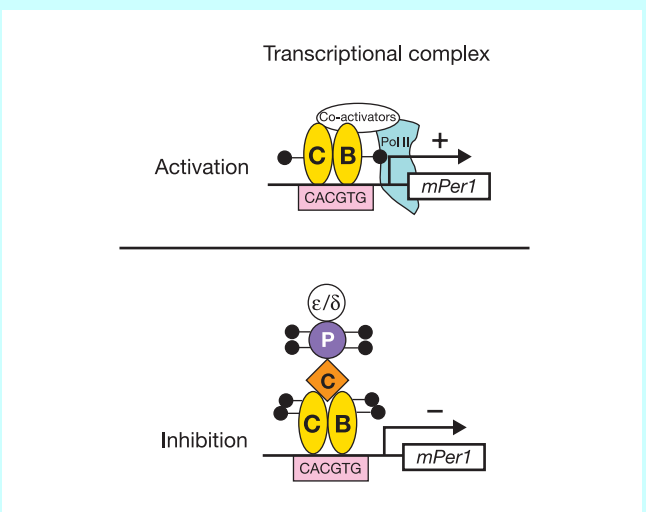

Figure 3 CLOCK–BMAL1 heterodimers remain bound to E boxes over the circadian cycle. During transcriptional activation, CLOCK (C) and BMAL (B) are bound to CACGTG E boxes in the *mPer1* promoter, and co-activators are recruited to activate transcription. During transcriptional inhibition, the CRY-containing complex of negative regulators binds to the CLOCK–BMAL1 heterodimer, and may inactivate the co-activator complex. At the time of transcriptional inhibition, CLOCK, BMAL1 and the mPER proteins are hyperphosphorylated.

Classical retinal photoreceptors, the rods and cones, with their opsin-based visual pigments are necessary for the conscious perception of light; however, they are dispensable for several light responses[45]. Light-induced phase shifts of the SCN clock, inhibition of nocturnal melatonin production, inhibition of activity by light (negative masking), and pupillary constrictor reflexes are all mediated by a non-rod, non-cone system. These responses have similar properties, including high threshold and the ability to integrate photic input over substantial periods[45]. Isolation of novel opsin-like molecules fuelled speculation that a new photoreceptive molecule present within the inner retina mediates these responses.

Localization of melanopsin gene expression and melanopsin immunoreactivity within a widely dispersed population of retinal ganglion cells suggests that melanopsin is a circadian photoreceptor[46–48]. This special population of retinal ganglion cells is unique in neurochemical phenotype (containing pituitary adenylate cyclase-activiting peptide (PACAP) as well as melanopsin), has the appropriate morphology (with large dendritic fields), and projects directly to the SCN and intergeniculate leaflet[47,49,50]. Most importantly, melanopsin-positive ganglion cells projecting to the SCN respond directly to light, even when physically isolated[47,51]. Although these studies do not exclude that another molecule within the melanopsin-positive cells is responsible for photosensitivity, the most parsimonious view is that the non-rod, non-cone system conveying light to the SCN is mediated by melanopsin action within this special subset of ganglion cells (Fig. 4).

However, it is not clear whether melanopsin is sufficient for circadian photoreception. The melanopsin pathway to the SCN may be complemented by the classical photoreceptor system and/or retinal cryptochromes[52,53], resulting in functional redundancy. Even though the mCRY proteins bind flavin, a light-sensing role has not been shown[54]. Rather, as detailed previously, the primary function of the mCRY proteins is in negative regulation within the clock feedback loops.

Transducing retinal input

SCN neurons downstream of retinal ganglion cell terminals receive and process photic input. The principal neurotransmitters of the retinohypothalamic tract are glutamate and PACAP. Photic and non-photic input also reaches the SCN indirectly through the intergeniculate leaflet and midbrain, with GABA (γ-aminobutyric acid), neuropeptide Y and serotonin having principal roles. The neurochemistry of input to the SCN has been reviewed elsewhere[1].

How does activation of retinal ganglion cells lead to alterations in the molecular clock in SCN neurons? Gene expression of *mPer1* is rapidly induced after exposure to light at either the beginning or the end of night[55]. Whereas *mPer2* induction is robust after exposure to light early in the night, exposure later in the night does not lead to a readily detectable induction[8]. These data focus attention on *mPer2* as a mediator of early-night responses (phase delays) and on *mPer1* as a mediator of phase advances. One study of mice with targeted disruption of either *mPer1* or *mPer2* is consistent with these proposed roles[56]. Another study using a similar strategy, however, shows that the *mPer1* gene is not necessary for light-induced phase shifts[30]. The precise roles of the *mPer* genes in light-induced clock resetting need clarification.

Analysis of clock proteins after nocturnal light exposure indicates that the number of nuclei immunoreactive for mPER1 and mCRY1 is increased in the SCN several hours after a phase-resetting light pulse[57]. The finding that mCRY1 is increased along with mPER1, despite the absence of an effect of light on *mCry1* RNA levels, suggests that more mCRY is translocated to the nucleus owing to increasing mPER levels. Thus, PER-dependent nuclear accumulation of CRY may be key for resetting circadian phase, as well as

controlling the central clock mechanism[32,40].

The mechanisms by which light leads to an increase in *mPer1* gene expression are better understood. Photic induction of *mPer1* and *mPer2* gene expression seems to be mediated by chromatin remodelling[58] and the binding of phosphorylated CREB (cyclic AMP responsive element-binding protein; pCREB) to a cAMP-responsive element (CRE) in the respective promoters[59] (Fig. 4b). Notably, the photic induction is largely independent of E-box-mediated enhancement, as light induces *Per* expression quite effectively in *Clock/Clock* mutant mice[60]. Conversely, activation of *mPer1* and *mPer2* gene expression by CLOCK–BMAL1 heterodimers (presumably acting on E boxes) is independent of the CRE elements present within these promoters[59]. One unique feature of the circadian response is that CREB phosphorylated on serine 142 (rather than the more widely studied Ser 133) seems to be primarily responsible for photic induction of *mPer1* RNA[61].

SCN to slave oscillators to local rhythms

Sodium-dependent action potentials provide the primary means by which the SCN transmit circadian outputs to other brain areas and are essential to its role as a pacemaker[1]. There are at least two ionic mechanisms that are under clock control in the SCN—an L-type Ca^{2+} current and a K^+ current that is essential for maintaining membrane potential[62]. The summed activity of these currents may contribute to the striking firing rate rhythm exhibited by SCN neurons[3]. But how does the core clock mechanism regulate these ionic events? Channel subunits could be regulated rhythmically at the transcriptional or translational level. Channel activity could also be regulated by a rhythm in post-translational processes, such as phosphorylation and/or interactions with regulatory proteins. These regulator proteins could be clock-controlled genes (CCGs) that contain E-box enhancers and are therefore directly controlled by CLOCK–BMAL1 heterodimers (so-called first-order CCGs), or downstream CCGs indirectly controlled by CLOCK–BMAL1 heterodimers.

Signalling molecules from SCN efferents include neurotransmitters and secreted factors. An output role for secreted factors comes from the results of SCN transplant studies that suggest that the alternating activity of SCN-derived 'inhibitory' and 'activating' factors drives locomotor activity rhythms (and hence rest/activity and sleep/wake cycles) in rodents. Two neuropeptides, transforming growth factor-α (TGF-α) and prokineticin-2 (PK2), have recently been identified as candidate output factors. TGF-α is a potential inhibitory substance, as its infusion into the third ventricle inhibits locomotor activity[63]. The growth factor is expressed in the SCN and retina and appears to inhibit locomotor activity by action on receptors in the hypothalamic subparaventricular zone (SPZ), a major relay station for SCN efferents. The most marked effect of growth factor signalling, however, is regulating light-induced suppression of locomotion, independent of circadian control[63]. By mediating this light-induced suppression (so-called 'masking'), TGF-α reinforces circadian control of the noctural activity profile.

PK2, a peptide whose RNA levels are highly and rhythmically expressed in mouse SCN, contributes more directly to the circadian control of behavioural activity levels[64]. *PK2* is a first-order CCG in the SCN, as its RNA levels are regulated by CLOCK and BMAL1 acting on E-box enhancers in the gene's promoter. Receptors for PK2 are expressed in many SCN target sites, as well as in the SCN itself, but notably not in the SPZ. Intracerebroventricular infusion of PK2 at night, when peptide levels are normally low, markedly reduces the nocturnal increase in locomotion[64]. The PK2-induced decrease in locomotor activity is consistent with a role of the endogenous peptide in suppressing locomotion during the day, when mice are normally inactive. Thus, clock-controlled PK2 expression sculpts the daily activity profile and may provide a key link between the circadian clock and behavioural outputs (including sleep).

What are the mechanisms whereby the SCN pacemaker controls slave oscillators in tissues outside of the brain? There are neural outputs from the SCN to peripheral organs by means of the

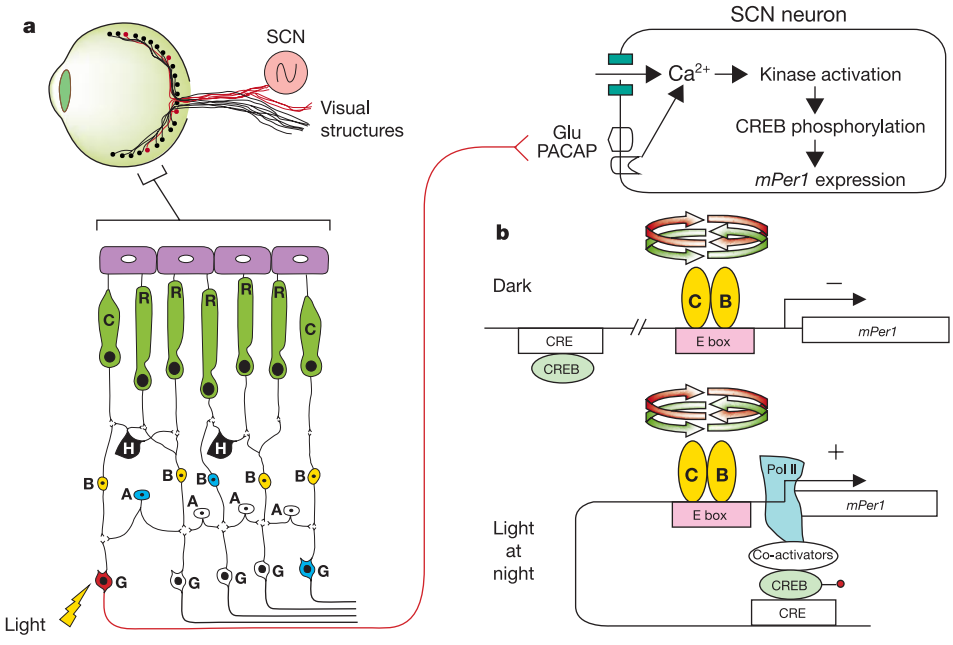

Figure 4 New visual pathway from retina to the SCN. **a**, A small population of widely dispersed, melanopsin-positive ganglion cells (red) form the retinohypothalamic tract that projects to the SCN. These ganglion cells (G) are directly light responsive. They also receive input from rods (R) and cones (C) through bipolar (B) and amacrine cells (A), some of which may contain crytochromes (blue). The precise anatomy of inputs to the melanopsin-positive neurons remains to be established. Glutamate (Glu) and PACAP mediate light effects on the *mPer* genes in SCN neurons. **b**, Light at night activates *mPer1* expression through phosphorylated CREB, independent of CLOCK–BMAL1 E-box control. Red and green arrows indicate interacting negative and positive loops of the clockwork, respectively.

autonomic nervous system, supporting direct (multisynaptic) neural control[65]. Hormonal signals are capable of entraining peripheral oscillators, as glucocorticoid agonists can effectively shift peripheral oscillators in mice[66]. There is also a more indirect way by which the SCN control the phase of slave oscillators in peripheral tissues. The SCN entrain some slave oscillators (for example, liver) by regulating the rest/activity cycle and thereby the timing of feeding behaviour[67,68]. Thus an artificially imposed feeding schedule in rodents can completely uncouple peripheral oscillators from SCN control. Feeding-induced signals, antagonized by glucocorticoids, entrain these peripheral oscillators[69]. The complex interaction of neural, hormonal and behavioural outputs may regulate the timing of slave oscillators by converging on *Per1* and *Per2* gene expression in peripheral tissues (Fig. 5a).

Once the timing of slave oscillators is coordinated by the SCN, there needs to be a mechanism to transduce the synchronized molecular oscillation into local rhythms. One way this could happen is through local first-order CCGs[70,71] (Fig. 5b). D-element binding protein (DBP) is the prototype of such a CCG, as it regulates the rhythmic transcription of key enzymes involved in hepatic metabolic processes[72]. Recent evidence indicates that DBP works together with the related basic leucine zipper transcription factor E4BP4, whose rhythm phase is opposite that of DBP, to affect differentially the same *cis*-acting element and thereby drive rhythmicity in responsive genes[73]. E4BP4 rhythmicity is probably regulated by the repressor REV-ERBα, similar to the way *Bmal1* rhythmicity is regulated in the core clock mechanism[24].

The combination of DNA microarray technology and the sequencing of the genomes of humans and rodents provides a powerful way to identify the multitude of CCGs in mammalian tissues[74]. Microarray analysis in fact has been applied to the study of circadian gene expression in serum-shocked fibroblast cultures[75,76] and in various tissues in rats and mice[24,77–80]. Collectively, these studies show that between 2 and 10% of the analysed genes exhibit circadian oscillations in steady-state RNA levels. Circadian gene expression is remarkably tissue-specific (less than 5% overlap between tissues) and, in many cases, involves rate-limiting steps that are distinct for the functions of that organ[78,79]. Unexpectedly, only a small subset of rhythmic genes seems to be under direct transcriptional control by CLOCK–BMAL1 heterodimers[79]. These first-order CCGs (such as PK2 and DBP), along with the core clock proteins, thus seem to be critical mediators of circadian information, as they are the most direct way through which the core oscillation can be transduced to regulate downstream events. The microarray data provide direct evidence for the ubiquitous role that circadian timing has in regulating diverse physiological events in mammals.

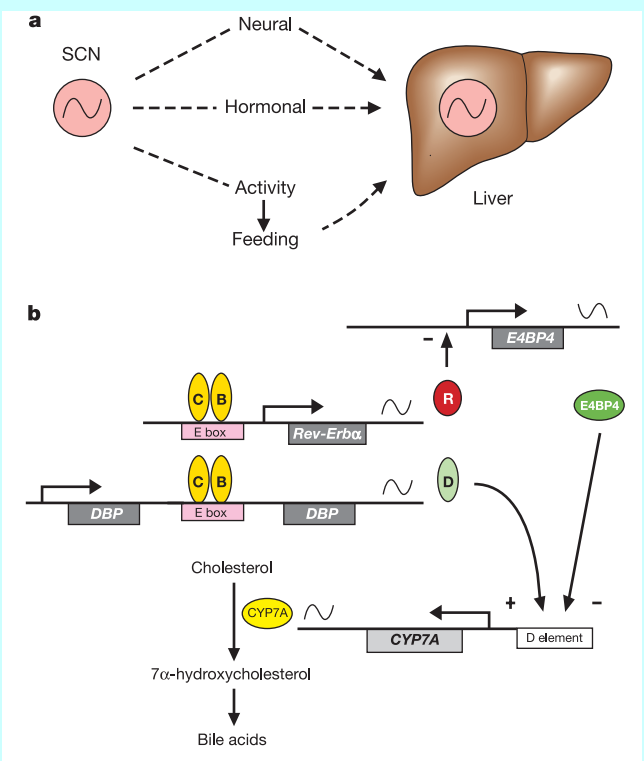

Figure 5 Mechanisms from SCN to clock-controlled gene expression in liver. **a**, Multiple output mechanisms from SCN to liver. Adapted from ref. 69. **b**, DBP-regulated local output gene in liver. DBP (D) rhythmicity is driven directly by CLOCK–BMAL1 heterodimers through an intronic E-box enhancer. E4BP4 rhythmicity is antiphase and driven by the repressor REV-ERBα (R). DBP (+) and E4BP4 (−) act through a D element to coordinate the rhythmic transcription of cholesterol 7α-hydroxylase (*CYP7A*), which is the rate-limiting step for bile acid production.

What's next?

Although we can assemble the cloned clock genes and their protein products into a coherent clockwork model, the model continues to evolve (for example, a revised view of the regulation of the positive feedback loop; see Fig. 2). Moreover, it is likely that large parts of the mammalian clockwork remain to be unravelled. A case in point is the unexpected loss of robust behavioural and molecular rhythmicity in mice with targeted disruption of the $VPAC_2$ receptor[81]. Intercellular signalling apparently has an important paracrine role in reinforcing intracellular circadian oscillations. Understanding the mechanisms by which single oscillators interact to form a functional oscillator at the tissue level remains one of the important challenges for future studies.

Recent DNA microarray data suggest that direct regulation of rhythmic gene expression by the core feedback loop is a rare, but critical, event controlling the distinctive temporal profiles of gene expression in peripheral tissues. The use of chromatin immunoprecipitation combined with DNA microarray analysis of the regulatory regions of mammalian genes provides a focused approach for discovery of first-order CCGs directly under CLOCK–BMAL1 control in the SCN and in slave oscillators. Fertile research also lies in elucidating the cascading interactions of networks of CCGs that connect the clockwork to the expressed rhythms. Finally, work is needed to assess the importance of locally controlled, rhythmic gene expression in physiology and how loss of circadian control contributes to disease states at the organ and systemic levels. □

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