Central and peripheral circadian oscillator mechanisms in flies and mammals

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Summary

Circadian oscillators are cell-autonomous time-keeping mechanisms that reside in diverse tissues in many organisms. In flies and mice, the core molecular components that sustain these oscillators are highly conserved, but the functions of some of these components appear to have diverged significantly. One possible reason for these differences is that previous comparisons have focused primarily on the central oscillator of the mouse and peripheral oscillators in flies. Recent research on mouse and *Drosophila* peripheral oscillators shows that the function of the core components between these organisms may be more highly conserved than was first believed, indicating the following: (1) that central and peripheral oscillators in flies do not necessarily have the same molecular mechanisms; (2) that mammalian central oscillators are regulated differently from peripheral oscillators; and (3) that different peripheral oscillators within and across species show striking similarities. The core feedback loop in peripheral oscillators might therefore be functionally well conserved, and central oscillators could be specialized versions of a basic oscillator design.

Key words: Circadian clock, *Drosophila*, Mouse, Molecular mechanisms, Genetics

Introduction

Circadian clocks are molecular time-keeping mechanisms that reside in a diverse range of cell types in a variety of organisms. The primary role of these cell-autonomous clocks is to maintain their own ~24 hour molecular rhythm and to drive the rhythmic expression of genes involved in physiology, metabolism and behavior. The key features of a circadian clock are its ability to synchronize (entrain) to environmental time cues, or zeitgebers, such as light-dark or temperature cycles, and to maintain rhythmic function when placed in constant conditions.

In mammals the central 'master' clock is located in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus (Klein et al., 1991). The SCN is entrained to light-dark (LD) cycles by a distinct set of photosensitive retinal ganglion cells that project to the SCN through the retinohypothalamic tract (Berson et al., 2002; Hattar et al., 2002; Moore et al., 1995; Provencio et al., 2002). The SCN in turn activates rhythms in behavior (e.g. locomotor activity), by secreting factors (e.g. TGFα and prokineticin) that act locally within the hypothalamus (Cheng et al., 2002; Kramer et al., 2001), and entrains subservient circadian oscillators in peripheral tissues (e.g. liver and kidney) via humoral signals (e.g. glucocorticoids) (Balsalobre et al., 2000a; Balsalobre et al., 2000b; Oishi et al., 1998; Ueyama et al., 1999). Such peripheral oscillators can, however, become uncoupled from the SCN if their specific needs dictate – as occurs in liver, lung and skeletal muscle after entrainment by food (Yamazaki et al., 2000). The SCN maintains robust (>2 week) rhythms when entrained to LD cycles in vivo and cultured in vitro, whereas peripheral oscillators lose rhythmicity after just 4-5 days under the same conditions (Yamazaki et al., 2000).

In flies the central clock is located in a group of 5-6 bilaterally symmetric small ventral lateral neurons (sLN_vs) situated in the lateral brain close to the optic lobes (Helfrich-Forster, 1996). As in the SCN, sLN_vs receive light input from retinal photoreceptors in the compound eyes and extra-retinal photoreceptors within the brain; however, they can also be entrained directly by light that penetrates the cuticle (Helfrich-Forster et al., 2001; Stanewsky et al., 1998). In constant dark (DD) conditions, sLN_vs maintain robust rhythms in gene expression and locomotor activity (Ewer et al., 1992; Frisch et al., 1994; Helfrich-Forster, 1998; Zerr et al., 1990). Peripheral oscillators in flies (e.g. antennal clock cells and Malpighian tubules) can also maintain robust (>7 day) rhythms in cell culture, which suggests that fly peripheral oscillators depend less on the sLN_vs than do their mammalian counterparts on the SCN (Emery et al., 1997; Giebultowicz and Hege, 1997; Plautz et al., 1997). Indeed, precisely how much influence the sLN_vs have over fly peripheral oscillators is not known, given that light penetrating through the cuticle appears to entrain peripheral clocks, thus negating the requirement for a 'master' clock to synchronize other oscillators in the fly. Despite this difference, the sLN_vs can be regarded as a central oscillator because, like the SCN, they drive behavioral rhythms in locomotor activity.

The core components involved in central and peripheral clocks of flies and mammals are largely conserved and in both they form interlocked feedback loops in transcription/translation (Fig. 1, Table 1). In flies, most of the information regarding the oscillator mechanism has derived from studies of mRNA and protein levels in whole head extracts. As such, the majority of information concerns oscillators in head peripheral tissues (i.e. photoreceptors and antennae), because the sLN_vs

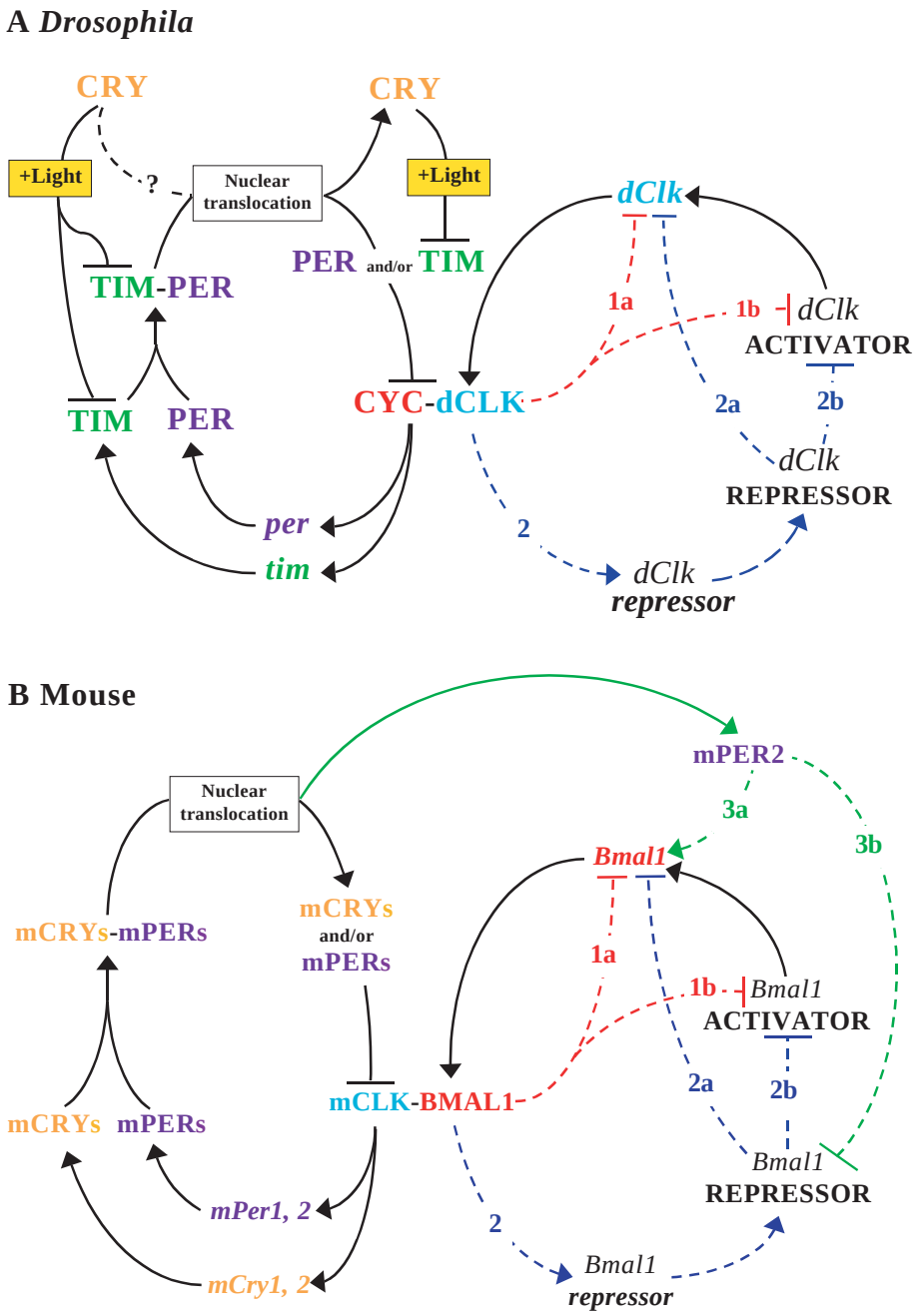

Fig. 1. Interlocked feedback loop model for *Drosophila* and mouse circadian oscillators. (A) *Drosophila*. The *per*/*tim* loop (left) and *dClk* loop (right) are shown. The model is based on data from peripheral oscillators. dCLK-CYC heterodimers activate *per* and *tim* transcription, and PER and TIM monomers accumulate in the cytoplasm and form heterodimers. PER-TIM then enters the nucleus, binds dCLK-CYC and inhibits the activation of *per* and *tim*, thus completing the *per*/*tim* feedback loop. dCLK-CYC dimers inhibit *dClk* transcription either directly (1a/1b) or indirectly via activation of a *dClk repressor* (2). Repression might occur through protein-DNA interaction (1a/2a) or protein-protein interaction (1b/2b). Repression of *dClk* is relieved when PER-TIM binds to dCLK-CYC, thus preventing this negative-feedback, and ensuring that *per*/*tim* and *dClk* mRNA transcripts cycle in anti-phase. Light input occurs through CRY, ultimately leading to the degradation of TIM. CRY can bind to TIM and PER, but whether CRY translocates to the nucleus with the PER-TIM dimer is not known. *dClk* repressor, gene that represses *dClk* transcription; *dClk* REPRESSOR, protein that represses *dClk* transcription; *dClk* ACTIVATOR, protein that activates *dClk* transcription. (B) Mouse. The *mPer*/*mCry* loop (left) and *Bmal1* loop (right) are shown. The model is based on data from the SCN (black and green lines) and peripheral tissues (black, red and blue lines). mCLK-BMAL1 dimers activate *mPer1*, *mPer2*, *mCry1* and *mCry2* transcription, which is followed by the accumulation of mPERs and mCRYs in the cytoplasm. The mPERs bind to mCRYs and translocate to the nucleus. mCRYs and/or mPER2 then bind and inhibit mCLK-BMAL1, completing the *mPer*/*mCry* negative-feedback loop. Repression of *Bmal1* transcription in peripheral tissues is mCLK-BMAL1 dependent. mCLK-BMAL1 might repress *Bmal1* either directly through protein-DNA (1a) or protein-protein (1b) interaction, or indirectly by activating a *Bmal1 repressor* (2). The *Bmal1* REPRESSOR may operate via protein-DNA (2a) or protein-protein (2b) interaction. Repression would then be relieved by mPER-mCRY-mediated inhibition of mCLK-BMAL1. In the SCN, mPER2 might co-activate *Bmal1* by binding the *Bmal1* ACTIVATOR (3a) or the *Bmal1* REPRESSOR (3b). The figure is primarily based on in vivo data, since it is becoming increasingly evident that the interpretation of cell culture experiments is complicated by the presence of endogenous clock components (either expressed naturally or resulting from experimental manipulation). *Bmal1* repressor, gene that represses *Bmal1* transcription; *Bmal1* REPRESSOR, protein that represses *Bmal1* transcription; *Bmal1* ACTIVATOR, protein that activates *Bmal1* transcription.

make up only a small percentage (<1%) of the total number of oscillators in the head. In mammals, however, previous work focused primarily on the central SCN oscillator because of the desire to link it to the behavioral rhythm in locomotor activity. Daily fluctuations in mRNA and protein levels can also be readily monitored in the SCN, whereas a similar examination of most clock components in the fly sLN_vs is not possible. Thus, previous comparisons between these species have largely compared fly peripheral oscillators with the mouse central oscillator.

Although the two systems show striking similarities, they also show some equally important differences. Here we outline current understanding of the fly peripheral and mouse central oscillators, paying particular attention to the differences between the two. We then consider more recent findings that suggest that peripheral oscillators of mammals share more in

Table 1. mRNA and protein profiles for clock genes in *Drosophila* and mouse

Drosophila		*dClock*	*cycle*	*cryptochrome*	*period*
Peripheral					
Whole head	ZT/CT	23-05 (×4)	Constitutive	04-10 (×2)	14-15 (×10)
	ZT/CT	24-05 (×4)	Constitutive	Accumulates in the dark	20-22 (×10)

Mammal		*mClock*	*Npas2 Mop4*	*Bmal1 Mop3*	*mCry1*	*mCry2*	*mPer1*	*mPer2*
Peripheral								
Vasculature	CT	Constitutive	01-05 (×4)	23-05 (×5)	23-04 (×4)	n/d	n/d	13-17 (×10)
Heart	ZT	21-03 (×1.5)	Low/absent	22-03 (×19)	15-24 (×3)	12-21 (×3)	11-13 (×5)	12-17 (×8)
	CT	n/d		n/d	n/d	n/d	09-12 (mod)	12-15 (high)
Forebrain	CT	Low/absent	Expressed, cycling n/d	22-02 (high)	14-24 (low)	n/d	10-15 (mod)	14-18 (high)
Kidney	CT	n/d	Low/absent	n/d	n/d	n/d	09-15 (mod)	12-15 (high)
Skeletal muscle	CT	n/d	n/d	n/d	18-21 (×2)	06-12 (×1.5)*	09-15 (mod)	12-18 (high)
Liver	ZT/CT	21-03 (×2)	Low/absent	21-03 (×15)	14-17 (×2)	08-10 (×2)*	08-16 (×20)	10-18 (×20)
	CT	Bi-phasic?	n/d	15-03 (×4)	21-24 (×3)	15-21 (×2)	15-18 (×10)	15-18 (×10)
Central								
Suprachiasmatic nucleus (SCN)	ZT/CT	Constitutive	Low/absent	13-21 (×1.5)	08-16 (×2)	08-16 (×2)*	04-08 (×10)†	06-12 (×15)†
	CT	n/d	n/d	13-21 (×2)	12-18 (×2)	12-16 (×2)	10-14 (×10)	10-14 (×10)

Central and peripheral oscillator components in mammals are aligned with each other and with their respective homologs in *Drosophila* peripheral oscillators. Black letters, mRNA levels; red letters, protein levels. The time of peak transcript or protein abundance is shown in hours, followed by the amplitude (peak to trough) of the oscillation in parenthesis. ZT, Zeitgeber time (light-dark conditions); CT, circadian time (constant dark conditions); ZT/CT, measurements made under both conditions. Where mRNA and protein levels have been determined from several studies showing differences in peak phase, we have accounted for all studies by broadening the peak. Where amplitudes vary between studies we have quoted the highest fold cycle. Where relative mRNA levels have not been precisely quantified (e.g. mouse forebrain) we have subjectively used high, moderate (mod) or low to give an indication of their relative amplitudes with respect to one another; n/d, not determined or not done. All SCN data are derived from mRNA in situ hybridization and immunocytochemistry in mice. All peripheral tissue data are from RNase protection assays or westerns on *Drosophila*, mice or rats. The table is based on data from the following: *Drosophila* (Allada et al., 1998; Bae et al., 1998; Darlington et al., 1998; Egan et al., 1999; Emery et al., 1998; Hardin et al., 1990; Ishikawa et al., 1999; Rutila et al., 1998); mammalian vasculature (McNamara et al., 2001); heart (Cermakian et al., 2001; Young et al., 2001; Zhou et al., 1997); forebrain (Garcia et al., 2000; Reick et al., 2001; Zhou et al., 1997); kidney (Cermakian et al., 2001; Zhou et al., 1997); skeletal muscle, (Cermakian et al., 2001; Kume et al., 1999; Miyamoto and Sancar, 1999; Zylka et al., 1998b); liver (Balsalobre et al., 1998; Bunger et al., 2000; Lee et al., 2001; Miyamoto and Sancar, 1999; Okamura et al., 1999; Zhou et al., 1997; Zylka et al., 1998b); mammalian SCN (Bae et al., 2001; Bunger et al., 2000; Field et al., 2000; Jin et al., 1999; Kume et al., 1999; Miyamoto and Sancar, 1999; Okamura et al., 1999; Shearman et al., 2000; Shearman et al., 1997; Zheng et al., 2001).

*Also reported to be constitutive.

†Amplitude also reported to be much lower at ~1.5-fold.

common with those of the fly than the mammalian central oscillator and, hence, that the central oscillators of both organisms may have acquired novel regulatory features that make them specialized versions of a more basic design.

The fly peripheral oscillator

Two interlocked transcriptional/translational feedback loops operate in the *Drosophila* circadian oscillator: a *period*/*timeless* (*per*/*tim*) loop and a *dClock* (*dClk*) loop (Fig. 1A; Table 1). These feedback loops have recently been reviewed in great detail (Allada et al., 2001; Meyer-Bernstein and Sehgal, 2001; Young and Kay, 2001); thus we provide only a brief overview. In the *per/tim* loop, *per* and *tim* transcription is activated by dClock-Cycle (dCLK-CYC), a basic-helix-loop-helix (bHLH)/PAS (PER-ARNT-SIM) protein heterodimer that binds canonical E-box sequences (CACGTG) in the *per* and *tim* promoters (Allada et al., 1998; Darlington et al., 1998; Hao et al., 1997; Rutila et al., 1998). The Period (PER) and Timeless (TIM) proteins accumulate in the cytoplasm as PER-TIM heterodimers after a substantial phosphorylation-dependent delay and then translocate into the nucleus (Gekakis et al., 1995; Kloss et al., 1998; Martinek et al., 2001; Price et al., 1998; Saez and Young, 1996; Vosshall et al., 1994). Nuclear PER-TIM then bind to dCLK-CYC, thereby repressing *per* and *tim* transcription (Bae et al., 2000; Lee et al., 1999). Once PER and TIM are degraded, another cycle of *per* and *tim* activation can begin. In the *dClk* loop, *dClk* transcription is repressed by dCLK-CYC (Glossop et al., 1999), probably indirectly since there are no canonical E-boxes in or around *dClk* (N.R.J.G. and P.E.H., unpublished). Repression of *dClk* is relieved when PER-TIM binds to dCLK-CYC, which (indirectly) promotes *dClk* transcription. The opposing effects of dCLK-CYC and PER-TIM on transcription ensure that *per*/*tim* and *dClk* mRNA transcripts oscillate out of phase (Table 1). In DD conditions the oscillations in *per*/*tim* transcription persist for a week or more (Brandes et al., 1996; Stanewsky et al., 1998). *dClk* mRNA oscillations in DD have not been extensively studied, although *dClk* mRNA does cycle after 2 days in constant darkness (Bae et al., 1998). *cyc* mRNA and protein levels are constant throughout the circadian cycle (Bae et al., 2000; Rutila et al., 1998; Wang et al., 2001).

Light entrains the oscillator through a blue-light-responsive pterin/flavin-binding protein, cryptochrome (CRY) (Fig. 1A; Table 1). CRY can bind TIM in response to light, ultimately causing TIM degradation and predictable phase advances or delays in the oscillator (Hunter-Ensor et al., 1996; Lee et al., 1996; Myers et al., 1996; Saunders et al., 1994; Zeng et al., 1996). Transcription of *cry* is also under control of the oscillator (Table 1). The *cry* mRNA profile reflects that of *dClk*, albeit delayed by several hours (Egan et al., 1999; Emery et al., 1998; Ishikawa et al., 1999). *cry* and *dClk* mRNA levels

are similarly perturbed in several clock mutants. In $dClk^{Jrk}$ and cyc^{01} mutants, *dClk* and *cry* mRNAs are constitutively high, whereas in per^{01} and tim^{01} mutants *dClk* and *cry* are constitutively low (Emery et al., 1998). This suggests that *cry* and *dClk* are under similar transcriptional regulation. Unlike the robust *per*/*tim* oscillations, *cry* mRNA oscillations dampen quickly, tending to trough levels by the third day in DD (Ishikawa et al., 1999).

Although CRY can entrain peripheral oscillators in the fly tissue-autonomously (Emery et al., 2000b), light input to the sLN_vs can also occur through retinal and extra-retinal photoreceptors (Helfrich-Forster et al., 2001). As such, the sLN_vs can still entrain in flies that contain a single amino acid mutation that disrupts the flavin-binding domain of CRY (cry^b). Entrainment in these flies to pulses of light is, however, impaired compared with the wildtype (Stanewsky et al., 1998).

The mammalian central oscillator

In mammals, two interacting transcriptional/translational feedback loops also operate (Fig. 1B; Table 1): an *mPer/mCry* loop and a *Bmal1* loop (reviewed by Allada et al., 2001; Meyer-Bernstein and Sehgal, 2001; Young and Kay, 2001). The mammalian homolog of dCLK, mCLK, forms heterodimers with BMAL1 (the mammalian CYC homolog) and drives rhythmic transcription from E-boxes in three mammalian *Period* genes (*mPer1*, *mPer2* and *mPer3*) (Gekakis et al., 1998; Hogenesch et al., 1998; Jin et al., 1999). Of these genes, *mPer1* and *mPer2* are further involved in the oscillator; *mPer3* appears to be involved in output rhythms (Bae et al., 2001). mCLK-BMAL1 is also thought to activate transcription of the two *cry* homologs in mammals, *mCry1* and *mCry2*. Activation of *mCry1* presumably occurs through a canonical E-box situated ~300 bp upstream of the *mCry1* promoter (Kume et al., 1999). No such E-box has been identified at the *mCry2* locus, but *mCry2* is believed to be regulated similarly to *mCry1* and the *mPers* given that they exhibit similar mRNA cycling phases (Table 1) and that *mPer* and *mCry* transcript levels are low in *mClk* mutant mice (Shearman et al., 2000).

As in *Drosophila*, mPER1 and mPER2 proteins undergo a substantial phosphorylation-dependent delay in accumulation with respect to their mRNAs (Akashi et al., 2002; Lowrey et al., 2000; Takano et al., 2000). mPER1 and mPER2 peak in concert with the mCRYs (Table 1), form mPER-mCRY complexes, and translocate to the nucleus (Griffin et al., 1999; Kume et al., 1999; Vielhaber et al., 2001; Yagita et al., 2002). mCRYs (perhaps in conjunction with mPERs) then act as potent negative regulators by directly interacting with the mCLK-BMAL1 heterodimer (Griffin et al., 1999; Kume et al., 1999; Lee et al., 2001). mPER2 has also been shown to positively regulate *Bmal1* transcription, similarly to dPER positive regulation of *dClk*. However, mPER2-dependent activation of *Bmal1* is thought to occur indirectly via co-activation or nuclear shuttling of an activator rather than inhibition of a repressor as in the case of *dClk* (Shearman et al., 2000). This mechanistic difference explains why low levels of *Bmal1* transcript are seen in the SCN of *mClk* mutant mice: activators and repressors of *Bmal1* transcription are both dependent on mCLK-BMAL1.

Light entrains the SCN by inducing transcription of *mPer1* and *mPer2* (Albrecht et al., 1997; Albrecht et al., 2001; Shearman et al., 1997; Shigeyoshi et al., 1997; Zylka et al., 1998b). Analysis of mCRYs in the SCN initially suggested that they do not play a role in entrainment analogous to that of CRY in *Drosophila*: the *mPer* genes are still activated in response to light in *mCry* double-knockout mice (Okamura et al., 1999). However, the *mCrys* were recently shown to be involved in entraining the SCN to light (Selby et al., 2000; Thompson et al., 2001), but this role is carried out in the retina, where opsin-based photoreceptors also function to entrain the SCN (Berson et al., 2002; Freedman et al., 1999; Hattar et al., 2002; Provencio et al., 2002).

Mammalian peripheral oscillators

The circadian oscillator mechanism in peripheral tissues appears to be different from that in the SCN. In peripheral tissues, *Bmal1* regulation is similar to that of *dClk* in the fly, and different from *Bmal1* regulation in the SCN, since *Bmal1* mRNA is constitutively high in *mClk* mutant mice (Oishi et al., 2000). This result implies that mCRY (and perhaps the mPERs) stimulate *Bmal1* expression by relieving mCLK-BMAL1-dependent repression of *Bmal1*. This mCLK-BMAL1-dependent repression can occur directly, through interactions with either the *Bmal1* promoter or the *Bmal1* activator protein, or indirectly by activation of a *Bmal1* repressor that interacts with the *Bmal1* promoter or the *Bmal1* activator itself (Fig. 1B). Intriguingly, mouse *Bmal1* knockouts have low levels of the mutant *Bmal1* mRNA in liver (Bunger et al., 2000). It is difficult to reconcile this result with the high levels of wild-type *Bmal1* mRNA in the liver of *mClk* mutant mice, although loss of positive regulatory elements, alteration of mutant *Bmal1* mRNA stability, or transcriptional interference from the selectable marker could lead to low levels of mutant *Bmal1* mRNA in the *Bmal1* knockout. Even though genetic elements involved in mCLK-BMAL1-dependent repression are clearly present at the *Bmal1* locus, a different mechanism operates in the SCN, because *Bmal1* transcripts are tonically low in *mClk* mutant mice. Thus, positive regulation of *Bmal1* by mPER2 in the SCN must occur through a separate, or redundant, mechanism.

Recent in vivo studies in mouse liver have shown that mPER1 and mPER2 are rate limiting for the nuclear translocation of mCRYs and, in the SCN, mPERs require mCRYs to achieve and maintain nuclear translocation (Shearman et al., 2000; Vielhaber et al., 2001; Yagita et al., 2002). The low levels of *Bmal1* mRNA in *mCry*-deficient mice and the dependence of mPERs and mCRYs on each other for nuclear translocation suggest, therefore, that nuclear mPER and/or mCRY is necessary for the positive regulation of *Bmal1*. Whether it is nuclear mPER, mCRY or both, the positive regulatory effect of these factors on *Bmal1* transcription (via relief of mCLK-BMAL1-dependent repression) ensures that the *mPer*s and *Bmal1* cycle in anti-phase with one another (Table 1).

Functional divergence: TIMs and CRYs in flies and mice

An obvious difference between the fly and mammalian oscillators is that mCRYs appear to have taken over the role of TIM in facilitating mPER nuclear localization and, indeed, it is now thought that a mouse TIM homolog (mTIM) does not

operate in central or peripheral mammalian oscillators (Field et al., 2000; Zylka et al., 1998a). So, if mCRYs have replaced mTIM in the mouse circadian oscillator, how do the functions of mCRYs and dCRY differ?

A role for *Drosophila* CRY as an inhibitor of dCLK-CYC (analogous to that of mCRY in inhibiting mCLK-BMAL1) has not been investigated. However, in LD cycles, CRY starts to accumulate in the dark at a time when *per*/*tim* transcription (by dCLK-CYC) first starts being repressed (Table 1). Given the role of mCRYs in the mammalian SCN, re-examination of previous results in the fly also suggests that mCRY and dCRY are more functionally conserved than was previously believed. For example, in *Drosophila* cell culture, CRY nuclear localization is dependent on both PER and TIM (CRY and TIM alone are insufficient) and appears to be induced by light (Ceriani et al., 1999). However, CRY-TIM interactions are also evident in the dark (Ceriani et al., 1999; Rosato et al., 2001). Further, flies containing the cry^b mutation have constitutive intermediate levels of *per* and *tim* transcripts and PER and TIM protein in whole heads (Stanewsky et al., 1998). In the photoreceptors of these mutants TIM protein, and presumably PER and PER-TIM, remains predominantly cytoplasmic (Stanewsky et al., 1998). These phenotypes (i.e. cytoplasmic TIM and intermediate levels of *per* and *tim* mRNAs) precisely mimic those of per^{01} flies (Hunter-Ensor et al., 1996), which suggests that CRY and PER cooperatively translocate to the nucleus in fly peripheral clocks. One caveat is that nuclear TIM, and presumably PER, is present in the renal Malpighian tubules of cry^b mutants (Ivanchenko et al., 2001). It will be interesting to see whether, as is the case for mPER in *mCry*-deficient mice, such staining is actually peri-nuclear (Shearman et al., 2000).

In *Drosophila* peripheral oscillators CRY might therefore facilitate PER-TIM nuclear translocation, or vice versa, just as mPERs and mCRYs require each other for nuclear entry in mammals. Such a conserved role is supported by the recent finding that dCRY can engage in protein-protein interactions with dPER independently of TIM (Rosato et al., 2001). Thus, in addition to mediating PER nuclear localization in flies, TIM might also facilitate movement of CRY into the nucleus. In mammals, mPERs and mCRYs have bypassed this requirement for TIM, thus rendering TIM redundant. Hence, although the roles of TIM in flies and mice have clearly diverged, the functions of the PERs and CRYs might still have been largely retained in fly and mouse peripheral tissues and the SCN, but not the sLN_vs, where CRY is not critical for oscillator function (Emery et al., 2000a; Emery et al., 2000b; Stanewsky et al., 1998).

Transcript cycling of the positive factors: *dClk* and *Bmal1*

A second difference between oscillators in the mouse SCN and fly periphery concerns the transcriptional activator that is rhythmically expressed: *dClk* in flies (*mClk* is constitutively expressed) and *Bmal1* in mammals (*cyc* is constitutively expressed). Currently, the activators of *dClk* and *cyc* and of *mClk* and *Bmal1* are not known; thus any explanation for this 'switch' remains speculative. Interestingly, however, *dClk* transcription is repressed by dCLK-CYC (Glossop et al., 1999), although this repression is probably indirect owing to the absence of consensus E-boxes in or around *dClk* (N.R.J.G. and P.E.H., unpublished). Recently, Yu et al., have shown in vitro that mCLK-BMAL1 heterodimers can, either directly or indirectly, repress *Bmal1* transcription (Yu et al., 2002). Further, a functional analog of mCLK, NPAS2, that can take the place of mCLK in some mammalian peripheral oscillators (e.g. forebrain) can also repress *Bmal1* transcription in cell culture when co-transfected with BMAL1 (Reick et al., 2001). Importantly, these in vitro results accurately reflect *Bmal1* regulation in vivo in peripheral tissues (Oishi et al., 2000). These findings suggest that negative feedback loops from the positive elements of the fly and mouse oscillators (i.e. dCLK-CYC repression of *dClk* and mCLK/NPAS2-BMAL1 repression of *Bmal1*) operate through similar, but as yet unknown, mechanisms.

So why is *dClk* rhythmically expressed in flies and *Bmal1* rather than *mClk* rhythmically expressed in mammals? *dClk* and *cyc* are both bHLH-PAS-protein-encoding genes that presumably derived from a single common ancestor and were once under the same regulatory control. Thus, after activation, both genes would have been subject to dCLK-CYC-dependent repression. Since the cyclic accumulation of the dCLK-CYC dimer would require only one of these proteins to cycle, regulation of the other could be more flexible. Hence, elements concerned with *cyc* repression were lost, and the fly oscillator appears to have chosen cyclic regulation of *dClk* over *cyc*. Conversely, in mammals, *Bmal1* was chosen over *mClk*, and so *mClk* regulation became more flexible. In support of this theory is the fact that in all mammalian clock tissues examined thus far, *Bmal1* is always present and cycles at the mRNA level (Table 1). *mClk* and *Npas2*, by contrast, show variability in their spatial expression and cyclic nature. For instance, *mClk* expression in the SCN is constitutive, whereas *Npas2* expression cycles in the vasculature (see Table 1 and below).

Do *dClk* and *Npas2* share any similarities in their regulation? Recent in vivo work on the mouse vasculature has shown that *Npas2* mRNA transcripts cycle. This suggests that, unlike its constitutively expressed analog *mClk*, *Npas2* regulation is more like that of *dClk*. *Bmal1* transcripts also cycle in the vasculature, peaking at the same time as those of *Npas2* (Table 1). This observation is particularly interesting because it shows that the oscillatory phases of both positive elements in the mouse vasculature are more similar to those of the fly than to those of the mouse SCN.

Phase relationships between central and peripheral oscillators

A third difference between fly peripheral and mouse central circadian oscillators is the anti-phase cycling of the core transcripts: *mPer1* and *mPer2* expression peaks at CT 04-10 hours whereas *Drosophila per* peaks at CT 14-15 hours, and *Bmal1* peaks at CT 12-21 hours whereas *dClk* peaks at CT 23-05 hours (see Table 1)[†] Note that, in a variety of mammalian peripheral tissues, *Per1* and *Per2* transcripts reach peak levels in the early night (ZT/CT 09-17 hours) (Table 1); moreover,

[†]Zeitgeber time (ZT) refers to time in hours during LD cycling conditions, where ZT 0 is lights on and ZT 12 is lights off. Circadian time (CT) refers to time in hours during constant environmental conditions (typically constant darkness) after entrainment to LD cycles, where CT 0 is the time when lights would have come on and CT 12 is the time lights would have gone off.

luciferase reporter gene assays in rats show that *mPer1* oscillations in DD in cultured peripheral tissues peak in the mid to late night – in anti-phase with the SCN oscillations (Yamazaki et al., 2000). Likewise, the patterns of *dClk* and *Bmal1* cycling are also similar in the fly and mouse periphery: *dClk* mRNA peaks at ZT/CT 23-04 hours, whereas *Bmal1* transcripts peak at ZT/CT 21-05 hours (*Npas2* mRNA in the vasculature also peaks at ZT/CT 01-05 hours). The phases of the *Per* and *Bmal1* oscillations in mammalian peripheral tissues therefore closely reflect those in the fly and are dramatically different from those in the SCN, which peak much earlier. The entrainment of fly and mammalian peripheral oscillators is necessarily different since, in flies, light acts directly through CRY (Emery et al., 2000a; Emery et al., 2000b; Stanewsky et al., 1998), but in mammals it acts indirectly via the SCN. This indirect action, mediated by humoral factors, imposes a delay that is likely to account for the difference in the phases of mammalian central and peripheral oscillators.

Cultured mammalian fibroblasts also contain a circadian clock that is set by various pharmacological agents (e.g. serum shock, dexamethasone and endothelin 1) thought to mimic endogenous humoral factors (Balsalobre et al., 2000a; Balsalobre et al., 1998; Yagita et al., 2001). The oscillator in these cells has properties similar to those of that in other mammalian tissues: *Per* mRNA cycles in antiphase to *Bmal1* mRNA; PER1 and PER2 accumulation is delayed compared with their peak mRNA levels; clock gene expression can be entrained by SCN cells; and clock function is dependent on *Cry* genes (Allen et al., 2001; Balsalobre et al., 1998; Yagita et al., 2001). The oscillator mechanism in cultured fibroblasts is presumed to be like that in other peripheral tissues, but regulation of the *Bmal1* loop has not been tested to determine whether this is the case. Moreover, the phase of clock gene expression is expected to be similar to that in other peripheral tissues, but it is difficult to assess the phase of expression relative to an LD cycle since the clock is set by pharmacological agents.

Interestingly, peripheral oscillators in other vertebrates have phases closer to the SCN than to peripheral tissues of mammals. In the *Xenopus* eye, *xPer1*, *xCry1*, *xCry2a* and *xCry2b* mRNAs peak at ~CT 18-02 hours (Zhu and Green, 2001; Zhuang et al., 2000) and *Bmal1* peaks at CT 11-15 hours (C. Green, personal communication). A similar situation exists in the chicken pineal gland: *cCry2* and *cPer2* mRNAs peak at CT 22-02 hours and *cBmal1* and *cBmal2* mRNAs peak at CT 10-14 hours (Bailey et al., 2002; Fukada and Okano, 2002; Yamamoto et al., 2001). Zebrafish peripheral oscillators (e.g. those in the liver, eye, pineal gland, kidney and an embryonic cell line) are unusual because they are directly light entrainable, as is the case with *Drosophila* oscillators, but phase shifts are mediated by the light-induced expression of *mPer2*, as in the mammalian SCN (Pando et al., 2001; Whitmore et al., 2000). The phase of zebrafish peripheral oscillators is similar to those of *Xenopus* and chickens: *zfPer1*, *zfPer2* and *zfPer3* peak at ZT 00-06 hours and *zfClk*, *zfBmal1* and *zfBmal2* peak at ZT 12-18 hours (Cermakian et al., 2000; Pando et al., 2001; Whitmore et al., 1998). Thus, in non-mammalian vertebrates, the phase of rhythmically expressed clock genes in peripheral tissues is a few hours earlier than the mammalian SCN and almost 12 hours earlier than mammalian peripheral tissues. In each case, the rhythmic repressors are essentially antiphase to the rhythmic activators, owing to the interlocked feedback loop mechanism.

mCRY verses dCRY revisited

Previous comparisons between the roles of mCRY and dCRY suggested that their functions had diverged significantly. mCRY appeared to be a core clock component that has no photoreceptive properties, whereas dCRY seemed to be a cell-autonomous photoreceptor that conveys light information to the oscillator without being intimately embedded in the core workings.

Recent work has shown that, in the fly, CRY may serve both roles. In the central oscillator it serves as the predominant photoreceptor but is dispensable within the core clockwork: the c*ry*^{b} mutant has wild-type free-running locomotor activity in constant light conditions that render wild-type flies arrhythmic (Emery et al., 2000a). In peripheral oscillators, however, CRY may well serve as an integral clock component. Two key experiments have shown this. The first relies on the fact that the circadian clock in flies can be entrained by temperature cycles, thus, allowing the photoreceptive role of CRY to be uncoupled from its oscillator function. When wild-type flies are entrained in DD to temperature cycles and then transferred to constant temperature they show clock-driven behavioral rhythms in locomotor activity (which are LN dependent) and in electroantennogram (EAG) responses to odorant (an antennal output that is thought to be driven by oscillator cells in the antennae) (Krishnan et al., 2001; Wheeler et al., 1993). When the c*ry*^{b} mutant is treated similarly it retains a functional sLN_v oscillator but loses EAG rhythms, which shows that the peripheral antennal clock is non-functional (Krishnan et al., 2001). Importantly, loss of the EAG output rhythm in temperature-entrained *cry*^{b} flies results from a non-functional oscillator, because rhythmic *per* and *tim* mRNA transcription in antennae is severely crippled compared with that in temperature-entrained wild-type flies (Krishnan et al., 2001). Although it remains possible that CRY is also a thermoreceptor, the most parsimonious interpretation of this result is that the non-functional antennal clock results from a dependence of the oscillator on CRY. The second experiment focused on the renal Malpighian tubules (Mts) and the oscillating nature of the clock proteins PER and TIM in this tissue. Ivanchenko et al. showed that, in c*ry*^{b} flies, the oscillations of PER and TIM in Mts are disrupted in LD conditions and abolished in DD, whereas PER/TIM oscillations in the sLN_vs are essentially wild-type in LD and DD (Ivanchenko et al., 2001). Taken together, these data indicate that CRY is an essential component of the oscillator in peripheral, but not central, clocks.

In mice, a similar problem of dissecting the role of mCRYs as photoreceptors was encountered. Because mCRYs are a core component of the oscillator, mCRY-double-knockout mice exhibit arrhythmic behavior (i.e. the central oscillator is non-functional) (van der Horst et al., 1999; Vitaterna et al., 1999). mCRYs were not believed to have photoreceptive properties, because in the SCNs of these mice, light pulses administered in vivo can induce transcription of core clock genes: *mPer1* and *mPer2* (Okamura et al., 1999). The interpretation was that photic input to the oscillator can occur in the absence of mCRYs. Freedman et al. and Selby et al., have shown that mice

lacking either classical opsin-based photoreceptors [*retinal degeneration* (*rd*/*rd*) mutants missing rods and most cones] or *mCry1* and *mCry2* can in fact entrain to LD cycles (Freedman et al., 1999; Selby et al., 2000). However, mice lacking both photoreceptor types have severely impaired ability to entrain, which indicates that opsin and mCRY have redundant roles in the transduction of light information (Selby et al., 2000). Further support for mCRYs involvement in light transduction comes from experiments eliminating opsin-based photoreception by ocular retinaldehyde deprivation, which leaves photic signaling to the SCN intact (Thompson et al., 2001). The discovery of another mammalian photoreceptor, melanopsin, in retinal ganglion cells that project to the SCN also suggests that, in common with the sLN_vs in *Drosophila*, the SCN has redundant mechanisms for entrainment (Hattar et al., 2002; Provencio et al., 2002).

These studies suggest that dCRY and mCRYs function similarly in flies and mammals. In both, they serve as core clock components and/or as photoreceptors depending on the tissue. A similar situation exists in zebrafish, but the six *zfCry* genes in this species have become specialized, functioning as either core clock components or as photoreceptors (Cermakian et al., 2002; Kobayashi et al., 2000). Since photoentrainment of zebrafish peripheral tissues is dependent on blue-light photoreceptors, and therefore probably cryptochromes (Cermakian et al., 2002), it will be interesting to determine how CRY photic signaling has evolved from a TIM-degradation-based light entrainment mechanism in *Drosophila* to a *zfPer2*-induction-based mechanism in zebrafish.

Conclusions

As studies of circadian oscillators are rapidly progressing three important features are becoming clear. (1) Central and peripheral oscillator mechanisms are different in flies. The sLN_vs do not require CRY for oscillator function, whereas peripheral oscillators do. A further example of central versus peripheral differences is found in the moth, in which PER nuclear cycling is absent in the brain even though it cycles in peripheral tissues much in the same way as it does in *Drosophila* (Sauman and Reppert, 1996). Thus, even between species as closely related as flies and moths, the central oscillator has acquired species-specific differences. (2) Central and peripheral oscillator mechanisms in mammals are different. The *Bmal1* negative-feedback loop in the SCN is regulated differently than that in peripheral tissues. (3) Peripheral oscillators in flies and mammals show more similarities within their molecular workings than they do with their respective central oscillators. This is most evident when one considers the requirement for CRY and the analogous regulation of the *dClk*/*Bmal1* negative-feedback loops, which suggest that the function of core clock components is highly conserved. Thus, in order to advance our understanding of the 'general oscillator', comparisons between species should focus on peripheral oscillators. From this, it should then be easier to identify the species-specific modifications of the central oscillator that have arisen.

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