

# The ins and outs of circadian timekeeping

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Recent research in *Drosophila* and in mammals has generated fascinating new models for how circadian clocks in these organisms are reset by light and how these clocks, in turn, direct circadian outputs. Though light perception by the central clock is ocular in mammals, it probably proceeds via a mechanism separate from traditional visual transduction. In *Drosophila*, one mechanism is non-ocular and is in fact present in many different tissues. In both organisms, the cryptochrome family of photoreceptor-like molecules plays a role in the circadian clock, though their function is incompletely understood. Moreover, although a master clock resides in the brain, a functional clock appears to reside in most cells of the body. In these tissues, at least some output genes are controlled at the transcriptional level directly by clock proteins; others appear to be regulated by cascades of circadian transcription factors. Taken together, these observations are reshaping thinking about inputs and outputs of metazoan circadian clocks.

### Addresses

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### Abbreviations

**cry** cryptochrome  
**SCN** suprachiasmatic nucleus  
**WC** White-collar

### Introduction

Circadian clocks have been conserved in many instances throughout the evolution of living organisms, allowing bacteria, animals, and plants to adapt their physiological needs to the time of day in an anticipatory fashion. These pace-makers regulate a plethora of processes. In cyanobacteria, for example, the majority of promoters of metabolic genes appear to exhibit cyclical expression [1]. In mammals, physiological processes like sleep–wake cycles, body temperature, heartbeat, and many aspects of liver, kidney, and digestive tract physiology are all under circadian control [2]. The fashion in which clocks control such diverse functions in metazoan organisms remains a largely unexplored question in the field of chronobiology, and will be one focus of our review.

Circadian clocks continue to ‘tell time’ in the absence of environmental time cues such as changes in light intensity. The period length (‘Tau’ or ‘ $\tau$ ’ in clock parlance) of each cycle is approximately, 24 hours under constant conditions (e.g. constant darkness). Hence, in order for the circadian clock to tell time accurately, it must be readjusted (or phase shifted) every day by the light regimen (or photoperiod).

The mechanism by which light signals entrain the clock is another topic of intense interest, and will be our other focus.

### Central clock mechanisms: a brief summary

In all cases examined to date, circadian clocks have been cell-autonomous: a single cell can generate and maintain self-sustained circadian oscillations. The molecular basis for these rhythms may rely on a negative feedback loop in which clock proteins negatively regulate their own abundance or activity. This regulation may occur both at the transcriptional and at the post-transcriptional level. For example, in the bread mold *Neurospora crassa*, the Frequency protein negatively regulates its own transcription by interfering with the ability of the White-collar-1 and White-collar-2 (WC-1 and WC-2) proteins to activate *frequency* transcription [3]. Similarly, the clock from the cyanobacteria *Synechococcus* consists of a single cluster of three genes — *kaiA*, B, and C — whose mRNA levels oscillate in a circadian fashion and whose protein products govern expression of the *kai* locus [4].

Similar negative feedback loops appear to operate in metazoans. From *Drosophila melanogaster* to mammals, striking similarities in clock mechanism have been observed. In *Drosophila*, the clock genes *period* and *timeless* are transcribed in circadian fashion. This rhythm is affected by negative feedback from the Period and Timeless proteins, as mutations in *period* and *timeless* affect the timing of their transcription. Indeed, the transcriptional rhythm is thought to be generated by Period and Timeless themselves, which heterodimerize and translocate to the nucleus in order to repress their own transcription [5]. They probably do this by interfering with the ability of a protein heterodimer composed of the Clock and Cycle proteins to stimulate transcription via an E-box motif [6,7]. Such E-box sequences are found in both the *period* and *timeless* promoter regions. Circadian post-transcriptional regulation is also believed to play a crucial role in clock function. For example, the protein Double-time phosphorylates Period protein, which in turn probably enhances the proteolytic degradation of Period [8,9]. As shown in Figure 1, virtually all of the *Drosophila* proteins and sequence motifs discussed above possess one or more mammalian homologs that have been suggested to play similar roles in mammalian function. A possible exception is the recently discovered mammalian homolog of *timeless*, whose mRNA expression — low-level and constant — is quite different from the cyclically expressed *Drosophila timeless* gene, and whose *in vivo* functions remain unclear [10,11].

### Communication between the central pacemaker and peripheral clocks

Complex multicellular organisms appear to have central clocks that reside in discrete ‘pacemaker tissues’ in the

central nervous system. In these tissues, individual independent clocks in each cell cooperate to generate an as-yet-unknown timing signal used by the body as a whole. In *Drosophila*, these are composed of eight lateral neurons in the brain [12]. In lower vertebrates, the pineal gland can play a similar role, and in mammals the suprachiasmatic nucleus (SCN) of the hypothalamus is the primary pacemaker tissue. Physiological studies led to the simple model that signals from the pacemaker tissues direct circadian output responses in peripheral tissues areas [13].

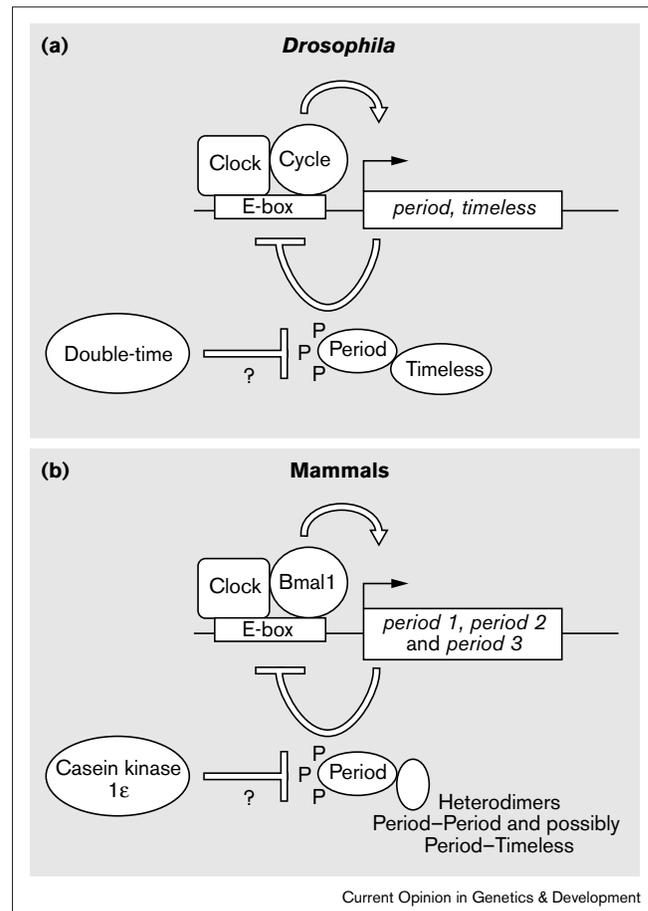
Recent evidence, however, suggests that the actual mechanism may be more complicated. In flies, fish, and mammals, circadian clocks independent of canonical pacemaker tissues have been found to be much more widespread. Circadian rhythms of melatonin synthesis were found in cultured retinas of many vertebrate species [14,15] and circadian rhythms of Period protein synthesis were discovered in cultured *Drosophila* pupal ring glands [16]. Independent clocks were then found in explanted tissues throughout the *Drosophila* body, including head, thorax, and abdominal structures [17], and in isolated zebrafish organs such as kidney and heart [18••]. Finally, circadian rhythms of clock gene expression were found to be induced by a serum shock administered to serum-starved immortalized rat fibroblasts in culture [19••]. Thus, current evidence suggests that most cells may have a circadian clock.

At first glance, the utility of separate clocks in individual peripheral cells is difficult to explain. It is possible, however, that this redundancy has evolved for more efficient control of circadian outputs. Different outputs are activated at different times. In order to control all of them individually, the SCN (or other central pacemaker tissues) would have to emit separate controlling signals for each differently phased output. By contrast, if each cell has its own clock, the pacemaker needs to send only one synchronizing signal periodically and peripheral clocks might then be able to function autonomously to regulate circadian outputs. Such a synchronizing signal is probably humoral rather than neuronal as circadian rhythms in expression of the Period 2 gene have been detected in cells that are not connected to the peripheral nervous system, such as leucocytes [20]. Figure 2 illustrates two alternative models by which central pacemakers could direct overt biochemical outputs in the periphery. Obviously, these two models need not be mutually exclusive.

### Regulation of circadian clock outputs

Recent studies have also shed light on the molecular mechanisms by which the central clock may control circadian output genes. Some of these downstream genes appear to be regulated by the same *cis*-acting DNA elements and transcription factors that govern the expression of the central clock genes themselves. The arginine vasopressin gene, expressed in SCN neurons, provides an excellent example

**Figure 1**

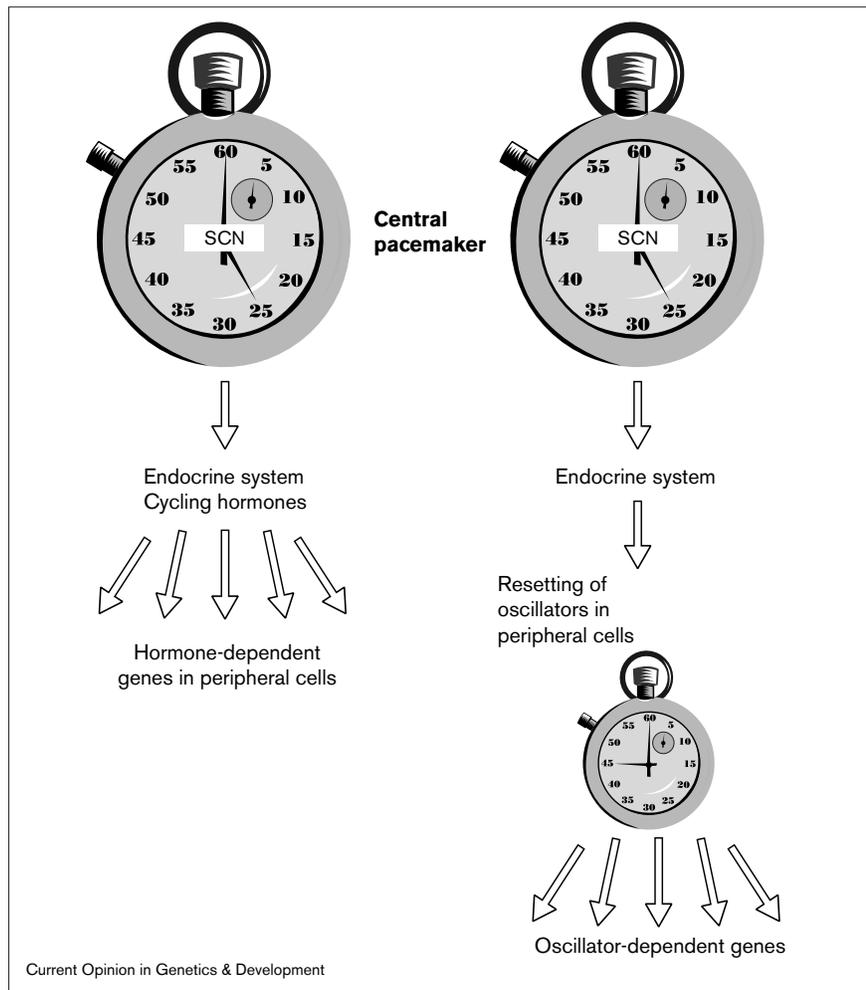


Clock molecules and models of clock mechanisms in (a) *Drosophila* and in (b) mammals. In both cases, Period proteins heterodimerize (in *Drosophila*, with Timeless protein; in mammals, the relevant partners remain to be established) to repress their own transcription. This transcription is activated by heterodimers of Clock and Cycle (in *Drosophila*) or Clock and Bmal1 (in mammals), which act through E-box motifs in the promoter region. Accumulation of Period protein in *Drosophila* is attenuated by phosphorylations effected by the Double-time protein, a homolog of the mammalian casein kinase 1ε.

of such a strategy. The product of this gene is responsible for the circadian regulation of salt and water balance in the bloodstream. The vasopressin promoter region harbors an E-box motif similar to the one thought to mediate transcriptional activation of the *period* genes via heterodimers of the Clock and Bmal1 proteins, and vasopressin transcription is drastically reduced in homozygous *clock* mutant mice [21]. Moreover, transient co-transfection studies show that the expression of reporter genes carrying the vasopressin promoter are activated by Clock and Bmal1 and repressed by Period. Thus, vasopressin appears to be regulated exactly like the *period* genes themselves [22••].

Some circadian genes, however, are regulated only indirectly. If an output gene that is directly hard-wired to the cell's circadian oscillator encodes a transcription factor, the rhythmic accumulation of this transcription factor

Figure 2



Strategies for SCN regulation of peripheral circadian outputs. Peripheral outputs demonstrate many different phase angles. The SCN might direct the secretion of multiple hormones to either activate or repress different outputs in circadian fashion. Alternatively (or in addition), the SCN might direct the secretion of hormone(s) that synchronize independent cellular clocks in the periphery. These clocks might in turn activate or repress circadian outputs at an intracellular level.

may control the circadian regulation of downstream genes. The genes encoding the transcription factor DBP (albumin D-box binding protein) and two cytochrome P450 enzymes, testosterone hydroxylase and coumarin hydroxylase, are an example of this type of transcriptional cascade. The most upstream member of this cascade, the DBP gene, is expressed with stringent circadian rhythmicity in most tissues, and its promoter region contains E-box motifs that could be direct targets for clock regulation. The *DBP* gene, in turn, probably regulates both hydroxylases [23].

Thus, the available evidence indicates that clock-controlled genes may be regulated by the central clock either directly by the same genes that drive central clock function or indirectly by cascades of circadian transcription factors. Evidence exists, however, that this regulation is not entirely unidirectional. Some output genes can feed back to influence certain parameters of the central clock itself. For example, as measured by wheel-running activity, *dbp*<sup>-/-</sup> animals have a central clock period that differs by 30 minutes from isogenic wild-type animals [24].

Most clock-controlled outputs remain poorly understood at the molecular level, but this situation is rapidly changing. In *Drosophila*, the *lark* gene has been shown to be involved in the circadian regulation of pupal eclosion [25]. In mammals, other members of the PAR (proline and acid rich) family of transcription factors (of which DBP is a member) are also under circadian control [26,27]. Altogether, many circadian processes have been characterized, and are summarized in Table 1. Some, such as locomotor activity and melatonin secretion, may be regulated by direct innervation rather than by humoral factors. For example, the cyclic-AMP cascade gene *CREM* (cAMP response element modulator) and its regulator ICER (inducible cAMP early repressor) are under circadian control via adrenergic signals [28]. *CREM*, in turn, is a regulator of a key enzyme in the pathway for the synthesis of melatonin in the pineal gland [29]. Interestingly, patients with cervically severed spinal cords demonstrate loss of circadian melatonin synthesis, demonstrating a neural pathway from the SCN to the pineal gland via the brainstem [30]. Several hormones are also regulated in circadian fashion via the SCN–hypothalamic–pituitary axis

[31]. Nevertheless, the mechanisms that govern the regulation of most of these processes remain enigmatic.

### Light as an input to the circadian clock

How light changes the phase of the circadian clock — or ‘entrains the clock’, in chronobiological terms — is another subject of intense recent investigation. In *Drosophila*, not only do autonomous clocks exist in many peripheral tissues, but they are also light-entrainable in isolated body parts [17]. Moreover, ablation of the eyes has only a minor effect on photo-entrainment in this organism [32]. Taken together, these observations strongly suggest that *Drosophila* clocks are reset by light via non-ocular mechanisms. Non-ocular entrainment also occurs in some birds and reptiles, in which a central pacemaker appears to reside in the pineal gland [33]. For example, pinealocytes cultured *in vitro* can reset their clocks in response to light and must thus contain all of the necessary photopigments and downstream signaling components required to convey light inputs to the molecular oscillator [34].

In mammals, the scenario seems to be different. Blind rodents with enucleated eyes or severed optic nerves are unable to entrain their clocks, so it is presumed that the path of light entrainment must proceed via ocular mechanisms only [35]. Nevertheless, mice with retinas lacking the traditional rod and cone photoreceptor cells are still light-entrained [36••,37••]. Thus, the mammalian photoreceptor must reside in another type of retinal cell. One recent study [38] suggested that shining light on the back of the knee can phase-shift the human circadian clock but another [39] could find no evidence for non-ocular light entrainment in hamsters. Therefore, non-ocular light entrainment is well-established in animals but remains controversial in mammals.

What is the nature of the circadian photoreceptor molecule itself? A great deal of attention has recently been focused upon the mammalian and *Drosophila* homologs of the plant cryptochrome blue-light photoreceptors, which affect growth [40], flowering time [41], and phototropism [42]. In *Drosophila*, ectopic overexpression of the cryptochrome gene (*cry*) product results in enhanced circadian photosensitivity as measured by the ability of light to phase-shift the clock, and light decreases the abundance of the Cry protein. Nevertheless, a likely null mutation in this gene does not abolish entrainment of the clock by 12 hour light-dark cycles. In light of these results, it is likely that cryptochromes are major but not the only circadian photoreceptors in *Drosophila* [43••,44••].

Two mouse cryptochrome gene homologs have been found, *cry1* and *cry2*, and their mRNAs are expressed in a circadian fashion, like the mRNA of their *Drosophila* homolog [45]. Knockout of either gene resulted in a change in central clock period length — also observed in the *cryB* loss-of-function mutant in *Drosophila* — and double knockout mice were completely arrhythmic in constant darkness.

**Table 1**

#### Circadian phenomena and molecular bases.

##### Circadian processes

Sleep–wake cycles  
Some clock gene RNAs/proteins  
Body temperature  
Heartbeat  
Blood pressure  
Liver metabolism  
Liver blood flow  
Glomerular filtration  
Renal plasma flow  
Urine production  
Acid secretion into gastrointestinal tract  
Gastric emptying time  
Locomotor activity (also in *Drosophila*)  
Pupal eclosion (only in *Drosophila*)

##### Rhythmically expressed substances and gene products

Some clock gene RNAs/proteins (e.g. Period1–3, Bmal1, cry1, cry2 in mammals; Period, Timeless, Clock, Cry in *Drosophila*)  
Some output gene RNAs/proteins (e.g. DBP, TEF, HLF, CREM/ICER in mammals; lark in *Drosophila*)  
Hypothalamus–pituitary–adrenal axis hormones (CRF, ACTH, glucocorticoids)  
Some other hormones (e.g. TSH, thyroid hormone, parathyroid hormone, testosterone, vasoactive intestinal peptide)  
Liver metabolic enzymes (e.g. cytochrome P450 hydroxylases and conjugases)

This surprising result suggests that mouse Cry1 and Cry2 are essential for the maintenance of circadian rhythms. Paradoxically, the amplitude of light-induced phase shifts was increased rather than reduced in *cry2* knockout mice. Similar experiments have not yet been published for *cry1* knockout mice. As *cry1/cry2* double knockout animals were arrhythmic, the sensitivity of their circadian system to light could not be measured. In fact, the locomotor rhythms of these mutant mice were essentially ‘hard-wired’ to periods of light and darkness, an observation consistent with total loss of clock function [46,47••].

With these fascinating new results, it appears necessary to envisage different roles for cryptochromes in different animal species. In mammals, gene-deletion experiments confirm a role for cryptochromes in the central clock whereas data on their ability to directly transduce light signals remain ambiguous. It remains possible, if not likely, that the light signal in mammals is mediated by another as-yet-unidentified photopigment.

Light also has an effect upon clock gene products other than cryptochromes. Indeed, it has been known for several years that the *Drosophila* Timeless protein is rapidly degraded in response to light [48–51]. Recent genetic studies place *cry* epistatic to *tim* in this respect [43••]. In mammals, though Timeless protein levels have not yet been reported, *timeless* RNA levels remain unchanged by light. By contrast, *period1* and *period2* mRNA levels in the SCN are rapidly induced by

light [52,53]. Interestingly, this effect is neuron-specific: ventrolateral SCN neurons are strongly light-responsive but have only weak autonomous mRNA rhythms; in contrast, dorsolateral neurons exhibit vigorous cycling of *period1* and *period2* RNA but fail to show light-induced expression [54]. The differences between these neural populations probably result from the fact that ventrolateral SCN neurons connect most directly to the retino-hypothalamic tract. Nevertheless, qualitative and quantitative differences in aspects such as the composition of the receptor population could also be relevant. Indeed, recent evidence indicates that specific neuronal receptors are involved in light-mediated clock entrainment. In particular, pharmacological studies have implicated glutamate receptors in light-mediated phase shifting. This class of receptors is believed to mediate calcium influx to corresponding neurons [55], and the neuronal ryanodine subclass is believed to specifically mediate light-induced phase delays [56]. Studies such as these, as well as differential RNA screening approaches to directly examine light-induced RNAs, will hopefully continue to elucidate the relevant mechanisms. Indeed, one such differential screen has already identified four RNAs, including those from the previously-identified *c-fos*, *nur77*, and *egr3* genes [57].

## Conclusions and perspectives

This review has attempted to simplify the discussion of circadian timing systems by dividing it into three parts: an input pathway leading to the clock, a pacemaker, and an output pathway emanating from it. Research performed during the last few years, however, has shown that these components are not completely distinct. For example, cryptochrome genes, thought to be photoreceptors, were found to be vital to clock function in mammals; and the DBP output gene was found to change the period of the central clock by an unknown feedback mechanism.

Future circadian research will have to address a number of unresolved issues relevant to all three clock parts. For example, the precise signal transduction cascade operative during light entrainment remains unknown in all circadian model systems. Similarly, the proposed negative feedback loops generating the circadian oscillations in various organisms are still lacking important elements. For example, cyclic Period protein accumulation in *Drosophila* lags about six hours behind cyclic *period* mRNA expression. From these data, mathematical modeling suggests that the rate of *period* mRNA translation and/or that of Period degradation must also be under circadian control [27]. In mammals, another particularly intriguing problem is how the SCN pacemaker synchronizes clocks in cells of peripheral tissues. The different phase angles of biochemical oscillations in the SCN and those in the periphery suggest that this entrainment is accomplished by yet-unidentified humoral factors. In short, despite the breathtaking speed at which progress has been made, in this field of research most remains to be done.

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