

CIRCADIAN RHYTHMS FROM MULTIPLE OSCILLATORS: LESSONS FROM DIVERSE ORGANISMS

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Abstract | The organization of biological activities into daily cycles is universal in organisms as diverse as cyanobacteria, fungi, algae, plants, flies, birds and man. Comparisons of circadian clocks in unicellular and multicellular organisms using molecular genetics and genomics have provided new insights into the mechanisms and complexity of clock systems. Whereas unicellular organisms require stand-alone clocks that can generate 24-hour rhythms for diverse processes, organisms with differentiated tissues can partition clock function to generate and coordinate different rhythms. In both cases, the temporal coordination of a multi-oscillator system is essential for producing robust circadian rhythms of gene expression and biological activity.

The temporal coordination of internal biological processes, both among these processes and with external environmental cycles, is crucial to the health and survival of diverse organisms, from bacteria to humans. Central to this coordination is an internal CLOCK that controls CIRCADIAN RHYTHMS of gene expression and the resulting biological activity (BOX 1). Despite disparate phylogenetic origins and vast differences in complexity among the species that show circadian rhythmicity, at the core of all circadian clocks is at least one internal autonomous circadian OSCILLATOR. These oscillators contain positive and negative elements that form autoregulatory feedback loops, and in many cases these loops are used to generate 24-hour timing circuits^{1,2}. Components of these loops can directly or indirectly receive environmental input to allow ENTRAINMENT of the clock to environmental time and transfer temporal information through output pathways to regulate rhythmic clock-controlled gene (CCG) expression and rhythmic biological activity.

Whereas a self-contained clock in single-celled organisms programmes 24-hour rhythms in diverse processes,

multicellular organisms with differentiated tissues can partition clock function among different cell types to coordinate tissue-specific rhythms and maintain precision. Now that individual molecular circadian oscillators have been sufficiently described, it has become possible to go beyond single oscillators to try and understand how multiple oscillators are integrated into circadian systems. Evidence accumulated in recent years indicates that the intracellular oscillator systems of single-celled organisms might be more complex than those of higher eukaryotes, whereas the complexity of circadian outputs in multicellular organisms is an emergent property of intercellular interactions. In this review, we discuss the complexity of the circadian clocks on the basis of molecular genetic and genomic comparisons of circadian mechanisms among five instructive model systems: the cyanobacterium *Synechococcus elongatus*, the filamentous fungus *Neurospora crassa*, the fruitfly *Drosophila melanogaster*, mammals (such as mice) and birds.

We begin with a brief overview of the molecular aspects of circadian oscillators, but because this is not our main focus we refer readers to other reviews that cover this topic in depth³⁻⁹. This is followed by

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doi:10.1038/nrg1633

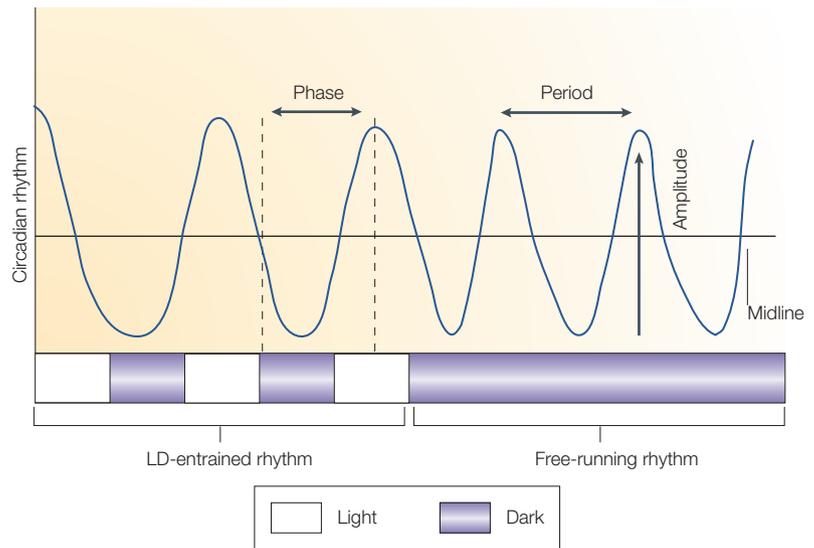
Published online 10 June 2005

Box 1 | **Some key principles of circadian biology**

In diverse organisms, biological processes that occur within many cells and tissues have the capacity to oscillate with a wide variety of periodicities; that is, they show peak-to-peak intervals, or periods, of activity. The molecules, cells and tissues involved are known as oscillators. Some oscillators, known as circadian oscillators, express periods of approximately 24 hours and form the circadian biological clock. Some of the key properties of a circadian oscillator are

shown in the figure. Circadian oscillators can be entrained to local time through the detection of an environmental cue, known as a zeitgeber, such that the endogenous timing of peaks and troughs stably corresponds to an environmental reference point. This property is known as the relative phase. The dominant zeitgeber for most species is the light:dark (LD) cycle, and specialized photoreceptive and phototransductive mechanisms have evolved in all biological-clock systems. Stably entrained oscillators or populations of oscillators that respond to zeitgebers can, in turn, regulate downstream oscillators, and therefore function as pacemakers to synchronize downstream rhythmic events to the environment.

Circadian rhythms in all organisms share defining properties. These properties include: a rhythm with a periodicity of about 24 hours, even in the absence of an environmental cycle (called a free-running rhythm); the ability of the clock to be entrained in a time-dependent manner by environmental stimuli; and compensation of period length for changes in an organism's natural environment. For example, when an organism is placed in varying temperatures within its physiological range, the period of the rhythm does not change significantly, which is indicative of a buffering in the system to compensate for changes in rates of biochemical reactions. Here, the rhythm is said to be 'temperature compensated'. The amplitude of a rhythm is a measure of the level of expression, and is measured from the midline (indicated in the figure) to either the peak or the trough. These are the key properties of a biological timing mechanism that responds rapidly to several environmental cues to maintain an appropriate phase relationship with environmental cycles.



CLOCK

A circadian clock is a 24-hour timing mechanism that is composed of molecular oscillators.

CIRCADIAN RHYTHM

A biological rhythm with a ~24-hour period that persists in constant conditions.

OSCILLATOR

A system of components that interact to produce a rhythm with a definable period length. A circadian oscillator can drive a rhythmic output, but requires other oscillators (pacemakers) for its entrainment and/or function. A circadian oscillator can therefore be self-sustained, but cannot operate properly independently of other oscillators.

ENTRAINMENT

The process by which an environmental rhythm, such as the light–dark cycle, regulates the period and phase relationship of a self-sustained oscillator.

PACEMAKER

An oscillator that drives an output and/or entrains another oscillator. A circadian pacemaker is a specialized oscillator that operates independently of other oscillators to drive rhythmic outputs, either directly or through other oscillators, and is entrained by environmental cues.

SUPRACHIASMATIC NUCLEUS

A small region of the brain that sits on top of the optic chiasm in the anteroventral region of the hypothalamus. Each of the bilaterally paired nuclei that comprise the SCN contains 8,000–10,000 cells packed together.

a discussion of the evidence for multiple oscillators in the single-celled cyanobacteria *S. elongatus* and *N. crassa*, and then evidence for the partitioning of oscillators in different cell types in multicellular mammals, birds and flies. All the systems that are discussed in this review use at least one specialized **PACEMAKER** oscillator that responds to environmental signals and coordinates rhythmic outputs, either directly or through other oscillators. Such a network of coupled oscillators is thought to add to the precision and stability of the clock, while providing the ability of individual oscillators within cells or tissues to control different rhythmic outputs.

Cell-autonomous oscillatory mechanisms

A cell-autonomous circadian oscillatory mechanism has been known for many years to be the source of endogenous circadian rhythmicity in unicellular organisms. This is exemplified by demonstrations of various circadian rhythms in isolated cells from species such as the marine dinoflagellate *Gonyaulax polyedra*, as well as in *N. crassa* and *S. elongatus*¹⁰. A

crucial question early on in the field was whether or not cell-autonomous circadian oscillators also exist in multicellular organisms. The first demonstration of cell-autonomous circadian-rhythm generation in such organisms came from studies in the snail *Bulla gouldiana*, in which individual neurons in the base of the retina were shown to express circadian rhythms in membrane conductance for at least two days in culture¹¹. In other landmark experiments in mammals, Welsh *et al.*¹² established that the oscillatory machinery in rats functions within individual cultured **SUPRACHIASMATIC NUCLEUS** (SCN) neurons (discussed in detail later). The cell-autonomous nature of circadian oscillators in multicellular organisms has also been demonstrated in diverse tissues in *D. melanogaster*^{13,14}, in cultured mammalian tissues and immortalized cell lines^{15–22}, in cells from the retinas of amphibians and birds^{23–27}, and in the pineal glands of birds, reptiles and fish^{28–34}.

What is known about the mechanism of a cell-autonomous oscillator? In recent years, great strides have been made by using genetic techniques to identify

key components of core oscillator mechanisms in bacteria and diverse eukaryotes, and in understanding the basic principles of circadian-oscillator function. Cell-autonomous circadian oscillators comprise positive and negative elements that form feedback loops^{1,2,35–39} (FIG. 1). In many of these oscillators, the positive elements of the loop activate the transcription of so-called ‘clock genes’ that encode the negative elements of the system. As a result, the concentrations of the negative elements rise, and they physically interact with the positive elements to inhibit their activity. This inhibition reduces transcription of the genes that encode the negative elements. Phosphorylation-induced decay of the negative elements decreases their concentrations,

which leads to reactivation of the positive elements, allowing the cycle to start again. The negative elements also activate the expression of one or more of the positive elements to form interlocking positive and negative-feedback loops that are important for maintaining the stability and robustness of the oscillator. All of these events impose time delays within the core feedback loop, such that the molecular cycle takes ~24 hours. Rhythmic transcription seems to be required for circadian-oscillator function in most organisms, although it is not a universal requirement^{3,40}. In fact, oscillations in the abundance of clock proteins might not be essential in cyanobacteria, even for basic circadian rhythmicity⁴¹.

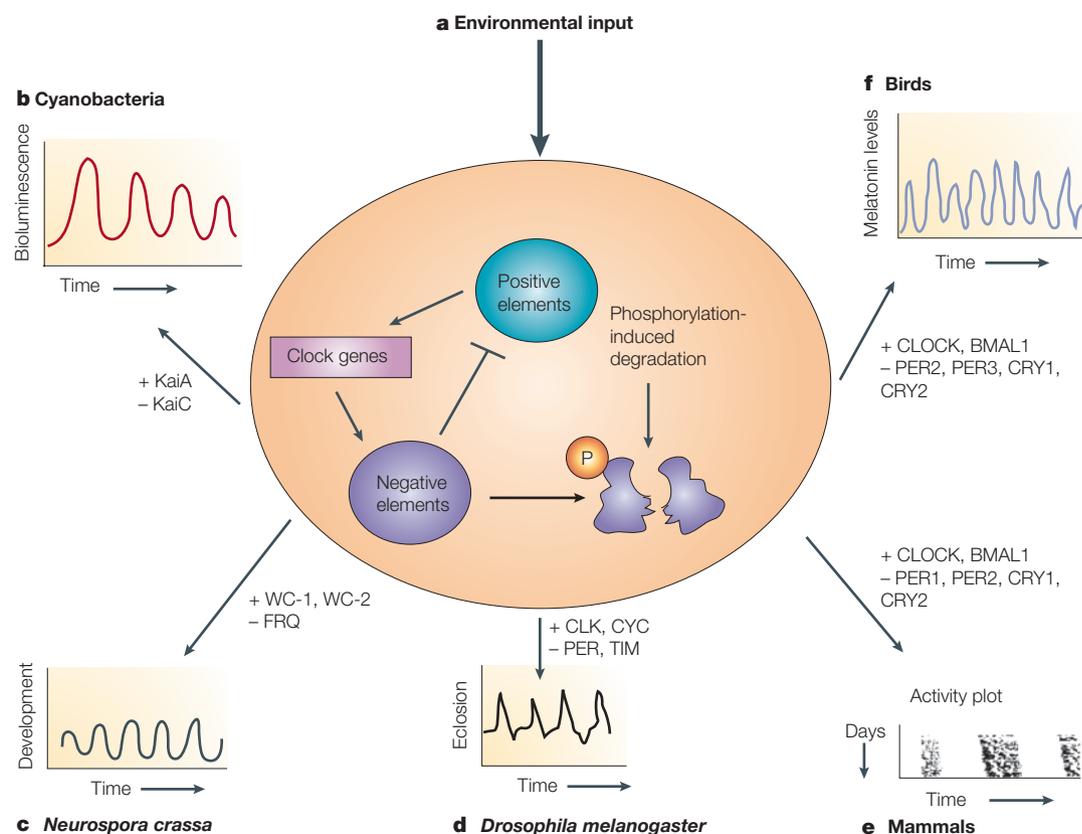


Figure 1 | Circadian oscillators are controlled through a common mechanism. a | Most circadian systems use a clock mechanism involving oscillators that are composed of positive and negative elements, which form feedback loops. In these loops, the positive elements activate the expression of the clock genes. The clock genes, as well as driving rhythmic biological outputs, encode negative elements that inhibit the activities of the positive elements. Phosphorylation of the negative elements leads to their eventual degradation, allowing the positive elements to restart the cycle. Clock genes can sometimes also function positively to increase the expression of the positive elements (not shown). **b–f** | Although the same basic mechanism is present, the components vary in different organisms. The core oscillator components are indicated for the model organisms discussed in this review (positive elements (indicated by ‘+’ symbols): KaiA, WHITE COLLAR-1 (WC-1), WHITE COLLAR-2 (WC-2), CLOCK (CLK in *Drosophila melanogaster*), CYCLE (CYC), and brain and muscle Arnt-like protein 1 (BMAL1, also known as MOP3 and ARNT1); negative elements (indicated by ‘-’ symbols): KaiC, FREQUENCY (FRQ), period (PER), timeless (TIM), cryptochrome (CRY)). Examples of circadian activities that are commonly experimentally assayed in these organisms are also shown. These oscillators receive environmental input and, either alone or coupled to other oscillators, send signals through an unknown mechanism to the rest of the organism to control rhythmic behaviours. In cyanobacteria (**b**), rhythmic output is measured by fusing the promoters of rhythmic genes to a luciferase reporter gene to monitor the resulting bioluminescence. In *Neurospora crassa* (**c**), rhythmicity in the development of asexual conidiospores is monitored. In flies (**d**), mammals (**e**) and birds (**f**), rhythms in locomotor activity can be monitored using automated equipment. Another rhythmic event in flies is eclosion (**d**), which is the emergence of adult flies from their pupal case. For mammals (**e**), activity (dark lines) is shown as a vertical stack (in chronological order, with each horizontal row representing activity for one day) and double plotted for clarity. In addition, rhythms in gene expression and biochemical activities, such as those shown for melatonin levels in birds (**f**), provide further measures of rhythmicity.

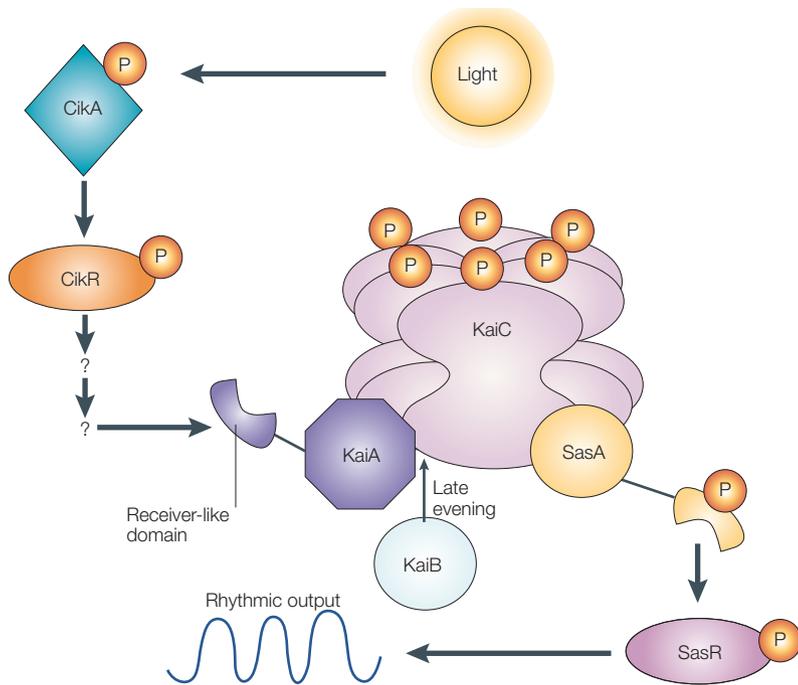


Figure 2 | The cyanobacterial periodosome model. Environmental information, such as daylight, is transduced through the phosphorylation and activation of Circadian input kinase A (CikA). CikA in turn phosphorylates and activates its predicted binding partner, Circadian input kinase R (CikR). Information is then transferred through protein–protein interactions to the receiver-like domain of the circadian-clock protein KaiA. KaiA interacts with KaiC and stimulates autophosphorylation of KaiC, which is hexameric. In the phosphorylated state, KaiC hexamers can form a complex with other clock components. Synechococcus adaptive sensor A (SasA) joins the complex and is thereby stimulated to phosphorylate its predicted binding partner, Synechococcus adaptive sensor R (SasR). Phosphorylated, active SasR sends temporal information from the periodosome to the rest of the cell to activate rhythmic gene expression, either directly or indirectly. Late in the evening, another protein, KaiB, binds to KaiC and inhibits KaiA-stimulated phosphorylation of KaiC. The complex then dissociates into its individual components (not shown) and ends the cycle. The molecular events that reactivate the cycle in constant environmental conditions have not yet been described.

Recently, progress has also been made in understanding how components of circadian oscillators signal to output pathways to regulate CCGs and biological rhythms. This advance is attributable to the recent use of microarray technology for the identification of rhythmically expressed genes^{42,43}, and to the biochemical description of the oscillator components themselves. For example, in mammals the basic helix-loop-helix (bHLH)-containing transcription factors **CLOCK** and **BMAL1** (brain and muscle ARNT-like protein 1; also known as MOP3 and ARNT1) of the oscillator loop can directly affect the circadian regulation of components of the output pathways. They achieve this by binding to consensus E-box sequences in the promoters of output genes and thereby regulating their transcription^{3,43–45} (FIG. 1). Furthermore, microarray studies have identified several putative direct targets of the positive elements of the feedback loop in *N. crassa* and *D. melanogaster* that contain consensus binding sites for the positive factors^{46–49}. Many of these direct targets are transcription factors, signaling components or hormones that can in turn affect the rhythmic expression of downstream CCGs.

In animals, the core clock genes are conserved, and our understanding of the makeup of the molecular oscillator in mammals actually results from a detailed description of homologous oscillator components in *D. melanogaster*². Interestingly, although orthologues of most of the genes involved in the fly oscillator have been cloned in mice, and the general feedback-loop mechanism is similar, there are differences in specific functions between orthologues for several of the components. Furthermore, gene duplication has led to increased complexity among vertebrate clock genes¹, and more core oscillator components will probably be identified⁵⁰. Despite this, it is clear that certain steps in the basic circadian-oscillator mechanisms are remarkably conserved. Therefore, understanding the molecular basis of the circadian oscillator in one organism has taught us about oscillator function in others. Emerging knowledge about differences in circadian organization between diverse model organisms is also likely to be informative. This will allow existing models of individual oscillators to be expanded to understand the coordination of a circadian network that consists of multiple oscillators.

Oscillator networks in unicellular species

In unicellular organisms, mounting evidence indicates the existence of more than one oscillator within a single cell, each of which is predicted to consist of different components. For example, in *G. polyedra*, two oscillators that respond to different wavelengths of light are thought to differentially regulate rhythms of bioluminescence and phototaxis⁵¹. These oscillators might include so-called ‘slave oscillators’ that are normally synchronized by a pacemaker, or might themselves be pacemakers that function to regulate distinct outputs. Pittendrigh suggested that slave oscillators might regulate certain aspects of output, while leaving the circadian programme open to the adjustment of one component without interference with the others⁵². In addition, he suggested that slave oscillators need not have all the circadian properties that are ascribed to the pacemaker, such as the ability to be directly entrained by light, because they will normally be coupled to and entrained by the pacemaker. These early theoretical insights into circadian organization have inspired an awareness of the potential for multiple oscillators within cells, as recently observed in cyanobacteria and *N. crassa*.

The circadian clock in cyanobacteria. In *S. elongatus*, a pacemaker that is based on the products of the circadian clock genes *kaiA*, *kaiB* and *kaiC* orchestrates a global rhythmic regulation of gene expression, and controls the timing of cell division^{53,54}. However, the circadian-timing mechanism itself is independent of the cell cycle⁵⁵. Although the Kai clock components form a molecular feedback loop that is similar to that described in eukaryotes⁵⁶, the fundamental timekeeping mechanism involves homotypic and heterotypic interactions among clock proteins, rather than transcriptional control. This culminates in the formation

Table 1 | **Examples of processes that are controlled by pacemakers and oscillators in model organisms**

| Organism | Oscillator or tissue with pacemaker function | Processes regulated by pacemaker | Other oscillators present? | Processes regulated by other oscillators |
|----------------------------------|--|---|---|---|
| <i>Synechococcus elongatus</i> | Kai periodosome | Cell division, photosynthesis, carbohydrate synthesis, gene expression, amino-acid uptake | Predicted, but not yet described | Gene expression? |
| <i>Neurospora crassa</i> | FRQ/WC oscillator | Conidiation, gene expression | FLO Other oscillators (not yet described) | Gene expression Gene expression, conidiation? |
| <i>Drosophila melanogaster</i> * | Ventral lateral neurons Olfactory sensory neurons Autonomous oscillators in other tissues? | Locomotor activity Odour-dependent electrophysiological responses Gene expression, other rhythms? | N/A | N/A |
| Mammals | SCN | Locomotor activity, electrical firing, cytosolic calcium levels, 2-deoxyglucose uptake, neuropeptide secretion, gene expression | Heart Lung Liver Kidney Fibroblasts Pineal gland | Heart rate, systolic blood pressure, vasodilation, gene expression Gene expression Metabolism, vesicular trafficking, detoxification, gene expression Gene expression Gene expression Melatonin levels |
| Birds | Retina SCN Pineal gland | Melatonin levels Noradrenaline levels, electrical firing, sympathetic tone Melatonin levels | Predicted, but not yet described | Gene expression? |

*Oscillators in flies do not meet the classic criteria of a pacemaker; however, they are not dependent on other oscillators for their entrainment or function, and autonomously control rhythmic outputs — therefore they have pacemaker properties. FLO, FRQ-less oscillator; FRQ/WC, *frequency/white-collar* oscillator; SCN, suprachiasmatic nucleus.

of a high-molecular-weight clock complex known as the periodosome, the assembly and disassembly of which might define the circadian PERIOD (FIG. 2). Recent data show that a temperature-compensated circadian rhythm of KaiC phosphorylation can be achieved *in vitro*, with only KaiA, KaiB, KaiC and ATP as components of the clock⁵⁷. So, these proteins inherently possess the ability to undergo a series of interactions that can account for oscillator function, and other periodosome components link the clock to fundamental cellular processes.

Many avenues of research have provided the tantalizing suggestion that the periodosome interacts directly with the bacterial NUCLEOID to affect DNA supercoiling^{9,58}. This could account for the fact that the entire genome seems to be under clock control at the transcriptional level. Several laboratories are testing this hypothesis using different approaches. However, different sets of genes are expressed with distinct PHASE relationships, indicating that there are also other layers of control⁵⁹. The periodosome also seems to be intimately linked to the metabolic status of the cell. For example, Light-dependent protein A (LdpA) is an iron-sulphur cluster protein that is sensitive to the redox state of the cell, and is involved in input to the clock and co-purifies with periodosome components⁶⁰. A subpopulation of KaiA molecules that co-purify with LdpA at SUBJECTIVE MIDDAY runs with a higher molecular weight on SDS-PAGE gels, which is indicative of modification by LdpA that might affect its function. So, in reduced conditions, in which LdpA is active, it is predicted that LdpA binds to the

periodosome and affects its activity. By sampling redox status, the clock could therefore measure the metabolic status of the cell, which in cyanobacteria depends not only on light intensity, but also on other environmental factors, such as nutrients and temperature⁶⁰.

Mutant alleles of any of the kai genes that change circadian period do so globally for all genes that are examined⁶¹. However, in some mutant backgrounds, different periods can be observed for different genes. This is most striking in mutant strains that lack one of the GROUP 2 SIGMA FACTORS, such as Sigma factor C or RNA polymerase D3. In these backgrounds, the circadian period of expression from the photosystem II reaction centre protein AI (*psbAI*) promoter (and some others that have similar circadian patterns) is altered, whereas that from the *kaiBC* promoter, which is also expressed in a circadian pattern, is unaffected⁶². Moreover, in wild-type cells, the period of *psbAI* expression shows more variability than that of *kaiBC*, such that at a given light intensity, different periods are recorded from the two genes. In the future, new methods for monitoring the expression of two genes in the same cell might provide further insights into the extent to which different periods can be sustained, and for how long. However, the available data indicate that more than one oscillator is likely to function within the cyanobacterial cell, although there is currently no evidence for a genuinely Kai-independent oscillator. Therefore, it is reasonable to assume that the Kai oscillator is a pacemaker, and that the other oscillators represent potential slaves (TABLE 1).

PERIOD

The time after which a defined phase of an oscillation (such as a peak or trough) recurs.

NUCLEOID

A structure in a prokaryotic cell that is composed of chromosomal DNA and its associated chromatin-like scaffolding proteins.

PHASE

The instantaneous state of an oscillation relative to a reference point.

SUBJECTIVE MIDDAY

The portion of a circadian day that is spent in constant darkness, which corresponds to the midday phase of a light-dark cycle.

GROUP 2 SIGMA FACTORS

Members of a family of sigma factor proteins that are responsible for conferring promoter-specific contacts on the RNA polymerase enzyme of eubacteria, thereby allowing specific genes to be transcribed.

For almost a decade, the method of choice for monitoring circadian rhythms in *S. elongatus* has been the measurement of bioluminescence from a population of cells that carry luciferase reporter genes. Remarkably, the bioluminescence that emanates from a population of 100,000–10,000,000 cells has a very precise rhythmicity, which persists in constant light (LL) conditions for at least two weeks after the last synchronizing dark-to-light transition⁵³ (BOX 1). This indicates either a high level of precision within individual cells or coupling among cells. One might imagine that, even with a fairly precise mechanism, the rhythm in each cell would run slightly shorter or faster than in other cells. This would lead to an eventual loss of synchrony that would be indistinguishable from arrhythmia of individual cells. However, recent imaging of individual cells during growth and division has shown extraordinary heritable stability of the phase relationships and periods of clonal progeny, and there is little evidence for strong coupling among physically associated, non-clonal cells⁶³. A weaker coupling cannot yet be ruled out, but the current evidence indicates that individual cyanobacterial cells do possess clocks of high precision, which can be ‘remembered’ through many generations after the last phase-setting cue is received.

Together, these data indicate that the clock within each *S. elongatus* cell is precise and self-reliant but flexible, allowing it to adjust to localized growth conditions with differential effects on outputs to multiple

behaviours. This precision and flexibility probably involves the periodosome pacemaker that is linked to other oscillators within the bacterial cell.

The circadian clock in *Neurospora crassa*. Studies in *N. crassa* have helped to understand many of the basic mechanisms that underlie circadian rhythms, including negative feedback and light and temperature entrainment, which are common to all clock systems^{64–66}. The *N. crassa* clock controls several rhythmic processes, the most frequently assayed of which is the daily production of asexual CONIDIOSPORES.

The well-studied FRQ/WC oscillator (FWO) is composed of the negative-feedback loop element that is encoded by *frequency* (*frq*), and positive elements that are encoded by *white collar-1* (*wc-1*) and *wc-2*. Mutations in any of these genes can alter period, temperature compensation, or entrainment of *N. crassa* circadian rhythms⁶⁷. However, strains that lack a functional FWO still show rhythmic behaviour, indicating that there are other oscillators. For example, Δfrq strains (which carry null alleles of *frq*) show a conidiospore-development rhythm in constant darkness (DD), although the period is variable, ranging from 12–35 hours^{68,69}. This period can be stabilized through an unknown mechanism by adding farnesol or geraniol, two intermediates of the sterol synthesis pathway, to the growth medium⁷⁰. The Δfrq conidiation rhythm lacks some canonical clock properties, including temperature compensation, but can be reset by temperature pulses. These results indicate the presence of a temperature-responsive oscillator, referred to as an ‘FRQ-less oscillator’ (FLO), that is activated or upregulated in response to farnesol or geraniol and feeds into the conidiation pathway.

Robust conidiation rhythms are also observed in Δfrq strains in cultures that are subjected to temperature cycles over a 5°C range in LL or DD⁷¹; however, this rhythm was later shown to be the result of a direct effect of temperature on development rather than being due to the effects of a FLO⁷². Other rhythms have been observed in the absence of FWO components, including rhythms in nitrate reductase activity⁷³, diacylglycerol levels⁷⁴ and gene expression⁴⁷. Together, these results indicate the presence of multiple oscillators within the *N. crassa* cell that respond to different input signals, but that at least some of these potential FLOs are slaves to the FWO. These slaves require the FWO to show all the properties that characterize circadian rhythms, including stable rhythmicity and temperature compensation (TABLE 1).

Three evening-specific *N. crassa* CCGs that show a daily rhythm in mRNA accumulation in the absence of FRQ were recently identified using microarrays⁴⁷. One of these, W06H2 (now called *clock-controlled gene-16*, *cgc-16*), was confirmed by northern assays to show rhythmic expression in the absence of FRQ and under conditions in which the conidiation rhythm is abolished, such as during growth in LL (Z. A. Lewis *et al.*, personal communication). In wild-type backgrounds, the FLO that is responsible for generating the *cgc-16*

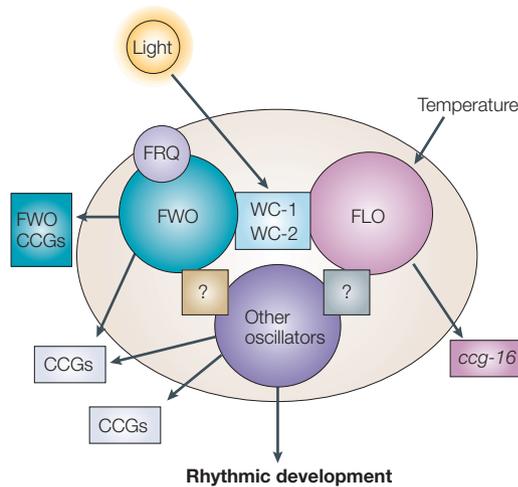


Figure 3 | Multiple oscillators in the *Neurospora crassa* cell. The FRQ/WC oscillator (FWO), consisting of FREQUENCY (FRQ), WHITE COLLAR-1 (WC-1) and WC-2, receives light signals from the environment to the blue light photoreceptor WC-1. The components of the FWO transfers this temporal information to other molecules to control the rhythmic expression of clock-controlled genes (CCGs). The FWO is also coupled to another oscillator, called a FRQ-less oscillator (FLO). This FLO responds to temperature and directs the rhythmic expression of distinct CCGs, including *clock-controlled gene-16* (*cgc-16*). Genetic experiments have uncovered other oscillators in the cell that are independent of the FWO under certain growth conditions. However, all the oscillators that are shown might communicate with each other to coordinately regulate some rhythmic processes, such as rhythmic development.

CONIDIOSPORES
Asexually produced haploid fungal spores that are formed on a specialized aerial hypha — the conidiophore — that rises above the substratum.

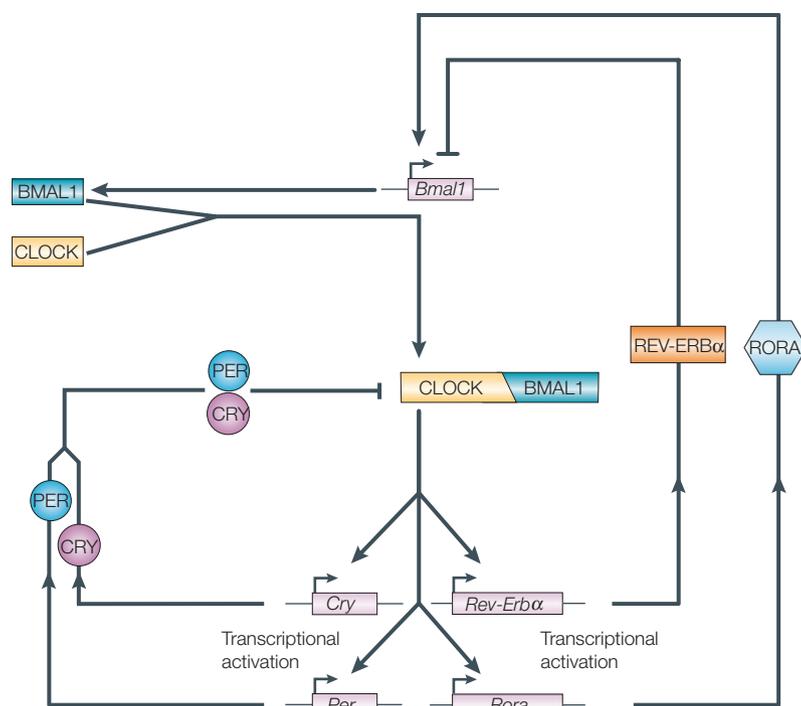


Figure 4 | Molecular interactions in mammalian circadian-feedback loops. CLOCK and BMAL1 (brain and muscle ARNT-like protein 1, also called MOP3 and ARNT1) form heterodimers and activate transcription of the genes *period* (*Per*) and *cryptochrome* (*Cry*), the retinoic acid receptor-related orphan receptor gene *Rora* and the orphan nuclear receptor REV-ERB (NR1D) group member gene *Rev-Erbα* (also called *Nr1d1*). PER and CRY proteins slowly accumulate as heterodimers and feed back to inhibit CLOCK–BMAL1-dependent transcription. REV-ERB α accumulates quickly and inhibits *Bmal1* transcription, then RORA, which accumulates more slowly, activates *Bmal1* transcription. This oscillator is composed of interlocking feedback loops that regulate the abundance and activity of transcription factors. These transcription factors are, in turn, thought to control the expression of genes in the output pathways from the oscillator, resulting in behavioural and physiological rhythms.

rhythm responds to both temperature and light cues for synchronization; however, in the absence of the FWO, the FLO responds better to temperature cues. So, although the FLO responsible for *cgc-16* rhythms can function autonomously, it is coupled to the FWO, and this is mediated by the WC-1 and WC-2 proteins. In addition, another autonomous circadian oscillator was recently identified through the study of mutations that can function in DD or LL in the absence of components of the FWO to regulate rhythmic conidiation (K. Seo and D.B.-P., unpublished observations). All the canonical circadian clock properties are exhibited by this oscillator: the rhythm-free runs in constant conditions, the rhythm is temperature compensated and light pulses can reset the phase of the rhythm.

Together, these studies show that the cellular circadian clock of *N. crassa* is a network composed of coupled circadian oscillators (the pacemaker FWO and one or more FLOs), and at least one other autonomous circadian oscillator (a second potential pacemaker), that respond differently to environmental inputs and can direct diverse outputs (FIG. 3). The presence of several oscillators probably contributes to the diverse rhythmic processes that are under clock control (for example, conidiation and the expression of genes that

are unrelated to development^{47,75}). Similar to the multi-oscillator system in cyanobacteria, coupling between the oscillators is likely to increase the stability and precision of the clock.

Oscillator networks in multicellular species

So far, there is no evidence for the presence of multiple oscillators within single cells in multicellular eukaryotes as there is in microbes. Instead, circadian complexity in these species arises from the presence of molecular oscillators, which share the same molecular machinery, in various cell types. How then do oscillators in individual cells in multicellular organisms become coordinated to produce circadian rhythms? In other words, is there a specialized pacemaker that responds to input from the environment and, through an ability to communicate temporal information, 'sets the pace' of the network of cellular oscillators in various tissues in these organisms?

We begin this discussion with mammals, in which the circadian system seems to consist of a light-entrainable pacemaker in the brain that coordinates the rhythms of peripheral oscillators. We then discuss the situation in birds, which have a complex clock system that is composed of several coupled pacemakers in the brain. Finally, we discuss the clock of *D. melanogaster*, which has multiple light-responsive oscillators throughout the head and body that have pacemaker properties, but unlike mammals and birds, seems to lack a centralized pacemaker in the brain.

The circadian clock in mammals. The SCN has been shown to be a circadian pacemaker in mammals. Ablation of the mammalian SCN eliminates circadian patterns of behavioural activity, endocrine output, and many biochemical processes throughout the organism⁷⁶. Furthermore, transplantation of SCN tissue to SCN-lesioned rats restores circadian behavioural rhythmicity⁷⁷. An important property of the SCN is that individual neurons can generate self-sustained molecular and physiological oscillations^{12,18,78} (TABLE 1). This indicates that the SCN contains a collection of cell-autonomous oscillators that are coupled to each other to form the complete SCN pacemaker that is responsible for setting the phase and period of biological rhythms throughout the organism. Consistent with the idea that the circadian pacemaker resides in the SCN, it receives photic input through the retino-hypothalamic tract (RHT)⁷⁹, which is required for the entrainment of mammalian circadian rhythms to LD cycles.

The mammalian intracellular circadian oscillator requires the activity of several components: the negative elements period homologues 1 and 2 (PER1 and PER2) and cryptochrome 1 and 2 (CRY1 and CRY2) and the positive-acting proteins CLOCK and BMAL1 (REFS 4,46,80,81) (FIG. 4). Although the SCN is crucial for the generation of biological rhythms throughout the organism, the expression and rhythmic regulation of mammalian clock genes is not unique to the SCN. Instead, rhythmic expression of the same clock genes

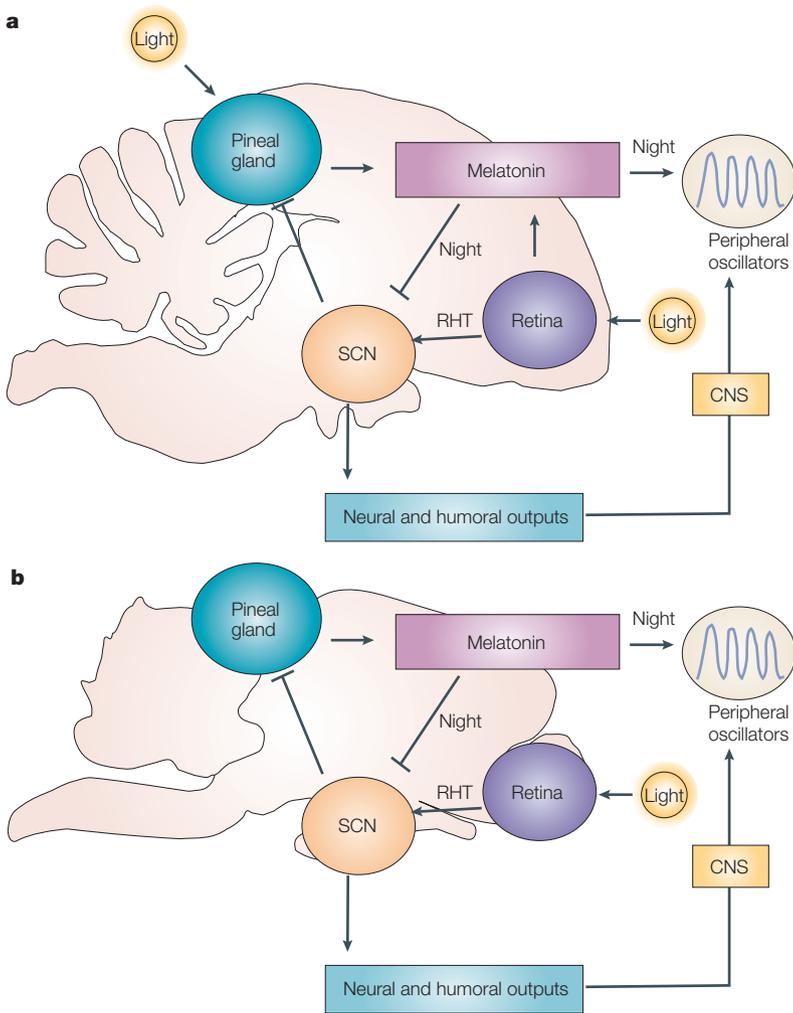


Figure 5 | A comparison of avian and mammalian pacemaker organization. a | The neuroendocrine-loop model of avian pacemaker organization. This representation of a generalized avian brain shows the locations of the pineal gland, retina and suprachiasmatic nucleus (SCN; consisting of the visual SCN (vSCN) and the medial SCN (mSCN)) each of which are damped circadian pacemakers that rely on mutual interactions to maintain rhythm stability and amplitude. For simplicity, the vSCN and the mSCN are shown as a single unit. The roles of the pineal gland and retina in circadian organization vary between species; the pineal gland is crucial to circadian rhythms in passerine (perching) birds, such as the sparrow, whereas the retina has a more important role than the pineal in chickens and quails. In sparrows and chickens, the SCN is active during the subjective day and inhibits melatonin biosynthesis in the pineal gland, so that it is only produced during the night. Therefore, neither the vSCN or mSCN secretes melatonin directly, but lesions of the vSCN affect pineal secretion of melatonin. In addition, humoral and neural outputs from the SCN affect the CNS and peripheral sites to which the CNS projects. During the night the pineal gland secretes melatonin into the bloodstream. Among other targets, melatonin inhibits activity within the SCN through specific melatonin receptors and restricts the SCN's output to the subjective day. This output coordinates downstream oscillators in peripheral tissues that are responsive to melatonin. In chickens and quails, the retina secretes melatonin into the bloodstream at night to inhibit SCN activity and regulate melatonin-responsive peripheral oscillators. The vSCN, but not the mSCN, receives light signals from the retina through the retinal hypothalamic tract (RHT). **b** | The mammalian pacemaker system differs from that in birds primarily in the number of tissues that make up the centralized pacemaker. In mammals, the SCN alone serves as a pacemaker that receives light signals from the retina through the RHT (whereas the light-perceptive pineal gland and retina, together with the SCN, form the pacemaker system in birds), and directly regulates pineal melatonin biosynthesis as an output of the clock. In both mammals and birds, pineal melatonin secretion is restricted to the night and, through melatonin receptors expressed in the SCN, inhibits night-time SCN activity. Similar to birds, rhythmic melatonin levels regulate sleep-wake cycles, and along with other neural and humoral outputs from the SCN, is thought to coordinate peripheral oscillator function. In both panels, interactions show overall effects only, as not all steps in the pathways involved are shown.

that make up the SCN oscillator is widely distributed among many peripheral cells and tissues, including the liver, endocrine tissues, the heart and the skeletal muscles^{20,82,83}. Furthermore, forskolin or corticosteroid treatment of fibroblast cell lines induces stable rhythms of core clock-gene mRNAs *in vitro*^{15,84}. These findings indicate that peripheral oscillators are similar to the SCN pacemaker in terms of basic molecular organization, raising the important question of what sets the SCN pacemaker oscillator apart from other peripheral oscillators.

The foremost difference is that the SCN contains the only known mammalian oscillators that can be entrained by light; there is currently no reproducible data to indicate that peripheral tissues in mammals receive direct photic input. This suggests a hierarchical model in which the SCN pacemaker provides a crucial link between the outside world and the internal circadian time-keeping mechanism. This is consistent with observations that clock-gene rhythms in the SCN are entrained more rapidly in response to light than they are in peripheral oscillators²⁰. Peripheral oscillations are also phase-delayed by 4–12 hours relative to the circadian patterns observed in the SCN, indicating that this is the time taken for a signal to be sent from the SCN to the periphery to entrain peripheral oscillators⁸³.

Peripheral oscillators also show other differences from the SCN pacemaker oscillator. Using a transgenic rat model, Yamazaki *et al.*²⁰ showed that cultured non-SCN tissues express circadian rhythms of luciferase (*Luc*) expression that is driven by the *Per1* promoter that dampen (show a progressive decrease in amplitude of the rhythm) after several cycles, whereas cultured explants of the SCN continue to cycle for many weeks. These data indicated that peripheral oscillators are not as robust as the pacemaker oscillator of the SCN. However, isolated peripheral tissues from *Per2-Luc* knock-in mice showed rhythms that were equally as robust as those in the SCN¹⁹, perhaps owing to regulation by the full complement of transcriptional regulatory elements. *Per2-Luc* rhythms persisted in SCN-lesioned mice, but were desynchronized within and between animals¹⁹, indicating that one role of the SCN is to synchronize self-sustained circadian oscillators among cells and tissues⁸⁵. Conversely, oscillators in peripheral tissues were shown to be unnecessary for circadian rhythms in activity⁸⁶.

The most crucial distinction between the SCN pacemaker and peripheral oscillators is that SCN cells, but not peripheral cells, have the capacity to confer behavioural rhythmicity to SCN-lesioned rodents *in vivo* (as described above)^{77,87} and to other cells *in vitro*. Immortalized rat SCN2.2 cells retain the endogenous oscillatory and pacemaker properties of the SCN *in situ*⁸⁸, and can generate self-sustained rhythms of gene expression and glucose metabolism. They also restore behavioural rhythmicity when transplanted into SCN-lesioned hosts^{89,90}. Because transplantation studies provide a reliable test of pacemaker function, it is noteworthy that viable transplants of non-SCN cell

types do not restore behavioural rhythms to arrhythmic, SCN-lesioned rodents⁸⁹. Furthermore, co-culture models have demonstrated that SCN cells, but not fibroblasts, confer metabolic and molecular rhythms to co-cultured cells⁹¹. Therefore, mechanisms for intercellular communication are presumably unique to the SCN and necessary for its function in synchronizing rhythmicity in downstream oscillators.

Together, these data show that the SCN pacemaker functions as the coordinator of peripheral tissues that also have inherent circadian properties¹⁹. The crucial question still remains as to how the SCN controls rhythms in peripheral tissues. This probably involves the production of one or more signals by the SCN that are received by peripheral tissues to synchronize their oscillators. In potential contrast to this model, restricted feeding has been shown to entrain circadian oscillators in peripheral tissues, whereas the SCN pacemaker seems to be unaffected^{92,93}. However, it is likely that under natural conditions peripheral oscillators require SCN input to set the timing of feeding (possibly through control of the sleep–wake cycle⁹⁴), which is consistent with SCN pacemaker function in insuring appropriate phasing of downstream oscillators. Although some progress has been made in identifying candidates for the signal or signals that are produced by the SCN^{4,95,96}, more work is necessary to verify their role in synchronizing peripheral oscillators.

There has currently been no demonstration that oscillations in clock-gene expression in peripheral tissues are directly linked to physiological rhythms. However, evidence to indicate that different oscillators contribute to the regulation of distinct outputs comes primarily from the use of high-density microarrays to identify rhythmically expressed genes^{6,42,43,47,97–103}. In mammals, up to 10% of the transcriptome shows a circadian rhythm in mRNA accumulation in any given tissue, whereas less than 1% shows a circadian expression pattern across multiple tissues^{6,42,104}. These data indicate that most oscillator output is tissue-specific and reflects distinct functions of different organs (TABLE 1). Furthermore, several genes cycle in the SCN but not in peripheral tissues (such as genes that encode peptide neurotransmitters), whereas other genes cycle in the peripheral tissues, but not in the SCN (for example, neuronal PAS domain protein 2, *Npas2*). Further analysis of microarray data will not only provide insights into the rhythmic outputs that are controlled by various tissue-specific oscillators, but will also provide important clues to the identity of candidate signals that are specific to the SCN and confer its pacemaker function.

The circadian clock in birds. The avian circadian system is even more complex than that of mammals, involving pacemakers that regulate peripheral tissues that are present in the pineal gland, the retina, and the SCN (which in birds consists of two structures, the visual [v]SCN¹⁰⁵ and medial [m]SCN¹⁰⁶). However, the contribution of these pacemakers to the clock varies considerably among avian species^{26,107}.

A ‘neuroendocrine-loop model’ for avian circadian organization has been proposed to explain interactions between the components of the pacemakers in this complex system¹⁰⁸. The premise of this model is that the system is composed of circadian oscillators that reside within the SCN, the retina and the pineal gland. These oscillators are damped oscillators, in that the amplitude of the rhythm becomes reduced over time, but are capable of self-sustained oscillation in the presence of photic input and/or neural or endocrine input from the rest of the system (FIG. 5a). A related model, the ‘internal resonance model’, was later proposed by Gwinner¹⁰⁹, and suggests that oscillators in the pineal and SCN stabilize and amplify each other through the secretion of a periodic signal by both the SCN and pineal that is perceived by the other tissue (resonance). These two models are not mutually exclusive. In both, each pacemaker directly receives photic input: the pineal gland contains several photopigments and phototransduction systems that affect melatonin biosynthesis, and the vSCN receives photic input from the RHT.

Each pacemaker in the avian circadian system might independently affect downstream processes (TABLE 1). The pineal gland influences the CNS and peripheral sites through the secretion of melatonin during the night, and tissues that express melatonin receptors are affected by this pacemaker directly. The SCN pacemaker affects output through several pathways. A HUMORAL output is thought to affect local HYPOTHALAMIC function^{90,110}, and a neural output through SCN afferents affects CNS and peripheral sites to which they project. Among the neural output pathways is the regulation of autonomic SYMPATHETIC NERVOUS SYSTEM activity¹¹¹, which can affect a broad range of peripheral physiological functions, including regulation of the pineal gland itself¹¹². So, coordination of avian circadian outputs through the pacemakers in the pineal gland and SCN might affect many physiological outputs. Peripheral rhythms have not been explored in birds as extensively as in mammals. However, many peripheral tissues express oscillator genes^{113–116}, and this is an important area for future research.

The dual regulation by pacemakers in the pineal and SCN is fundamentally different from the regulation of central and peripheral oscillators expressed by *D. melanogaster* (see below). However, it differs from mammals only in degree (as opposed to kind), as despite the importance of the SCN, the mammalian pineal gland also participates in circadian organization, only to a lesser extent⁹². Rhythmic melatonin levels, an output of the mammalian SCN, regulates sleep–wake behaviour and peripheral function, which is similar to the situation in birds¹¹⁷ (FIG. 5b).

Several groups have identified avian orthologues of the mammalian clock genes^{115,116,118–120}. Recent functional genomic analyses of the chick pineal gland⁹⁷ and retina⁹⁸ showed that, although both tissues express such genes, they are differentially regulated between these two pacemaker tissues

HUMORAL

Pertaining to elements that are dissolved in the blood or body fluid, typically serum.

HYPOTHALAMUS

The part of the brain that lies below the thalamus, forming the main portion of the ventral region of the diencephalon and functioning to regulate bodily temperature, certain metabolic processes and other autonomic activities.

SYMPATHETIC NERVOUS SYSTEM

Refers to a part of the autonomic nervous system that generally has excitatory function and regulates heart rate and blood pressure.

within the same animal. For example, the positive components *BMAL1*, *BMAL2* and *CLOCK* are expressed rhythmically in the pineal gland, with peak levels in the late subjective day. By contrast, in the retina, only *BMAL1* is rhythmically expressed. Furthermore, whereas the negative elements *PER2*, *PER3*, *CRY1* and *CRY2* are all expressed rhythmically in the pineal gland, only *PER3* and *CRY1* are rhythmic in the retina. In addition, the phases of peak expression of these genes are not consistent with the mammalian model. For example, the mRNAs for *PER3* and *CRY1*, which are both thought to encode negative elements of the oscillator-feedback loop, are expressed 180° out of phase, with *PER3* peaking at night and *CRY1* peaking during the day. At the very least, analysis of non-mammalian models such as birds opens questions about the universality of the oscillator-feedback-loop model among vertebrate classes. Further work at the protein level in these and other species will tell us more about the dynamics of clock-gene expression and the common mechanism(s) underlying circadian clocks.

Why is the avian pacemaker system apparently more complex than that of mammals? Birds have extra-ocular photoreceptors and, correspondingly, have independently regulated pacemakers, whereas mammals have neither of these. Some researchers have suggested that the loss of extra-ocular photoreceptors and multiple pacemakers might be related to a nocturnal common ancestor^{121–123} for all extant mammals¹²⁴. Evidence for such an ancestor for mammals includes the predominance of nocturnal behaviour among extant mammals and of primarily nocturnal species in early mammalian fossil records, as well as the relatively recent parallel evolution of cone OPSINS among mammals, as calculated on the basis of molecular-sequence analysis. Therefore, early nocturnal mammals, similar to their extant nocturnal descendants, would have experienced sunlight primarily at dawn and at dusk, at intensities that are far below the detection abilities of intracerebral and peripheral extra-ocular photoreceptors. The evolutionary pressures that lead to loss of function are an area of great debate in the evolutionary biology literature. However, one hypothesis is that lack of use relieves selective pressure to maintain a function, and through mutation and genetic drift, genes that encode such processes might be lost over long periods of time¹²⁵. This could have caused the loss of extra-ocular photoreceptors in mammals, and because circadian pacemakers are associated with photoreceptors, these might also have been lost.

The circadian clock in Drosophila melanogaster.

Perhaps unsurprisingly, the hierarchical model of the mammalian and avian circadian-clock system does not hold for organisms such as *D. melanogaster*, in which peripheral oscillators can be directly entrained by cyclic environmental cues. In *D. melanogaster*, the cell-autonomous circadian timekeeping mechanism comprises two interlocked transcriptional feedback

loops^{3,126–131} (FIG. 6a), and the clock genes that comprise these feedback loops are expressed in various tissues throughout the head, thorax and abdomen¹³² (FIG. 6b). Almost all these tissues show rhythmic clock-gene expression, and therefore contain circadian oscillators. These oscillators are roughly divided into the ‘central’ oscillator, which comprises several groups of neurons in the brain that control locomotor-activity rhythms, and ‘peripheral’ oscillators, which comprise all other oscillators in the head and body¹³³ (TABLE 1).

Although circadian oscillators are found in many tissues, only two rhythmic outputs have been identified in *D. melanogaster* adults, the most extensively studied of which is locomotor activity. A group of 4–5 small ventral lateral neurons (sLN_vs) in each hemisphere of the brain is both necessary and sufficient to drive robust locomotor-activity rhythms^{134–136}. The other rhythmic output is in olfactory responses, which are measured by assaying odour-induced electrophysiological responses in the antennae, called electroantennaegrams (EAGs)¹³⁷. EAG responses to ethyl acetate are rhythmic in wild-type flies under constant darkness but not in *per*⁰¹ or *tim*⁰¹ null mutants, therefore demonstrating that this rhythm is a *bona fide* circadian rhythm¹³⁷. These olfaction rhythms require peripheral oscillator function¹³⁷, and a more detailed analysis has shown that odorant-receptor neurons in the antennae are both necessary and sufficient for EAG rhythms¹³⁸.

As sLN_vs and odorant-receptor neurons are not known to control other oscillators, they do not meet the classic definition of a pacemaker. However, these light-entrainable oscillators (see below) function independently of other oscillators to set the pace of rhythmic outputs, and therefore function as peripheral pacemakers. Given the presence of autonomous circadian oscillators in many other head and body tissues, it is likely that these tissues mediate as yet undiscovered metabolic, physiological and behavioural rhythms and also function as pacemakers.

Studies using a luciferase reporter driven by the *per* promoter show that, unlike the mammalian system, circadian oscillators in isolated peripheral *D. melanogaster* tissues (for example, wings, legs and antennae) function autonomously and are directly entrainable by light, further indicating that each oscillator might function as a pacemaker¹³. For example, *per-luc* rhythms in detached antennae can be reset by light¹³, and rhythms in the MALPHIGHIAN TUBULES are phase-shifted with the same kinetics in intact and headless flies¹³⁹. These results indicate that the *D. melanogaster* circadian system is organized as a distributed set of autonomous oscillators with pacemaker function, which contrasts with the hierarchical nature of the mammalian and avian circadian system of a centralized pacemaker and peripheral oscillators^{1,19}. However, it is possible that not all *D. melanogaster* oscillators function autonomously; recent studies indicate that the neuropeptide Pigment-dispersing factor (PDF) might mediate communication between

OPSINS

A family of visual pigments.

MALPHIGHIAN TUBULES

The part of an insect's gastrointestinal tract that excretes nitrogenous waste and maintains ionic balance.

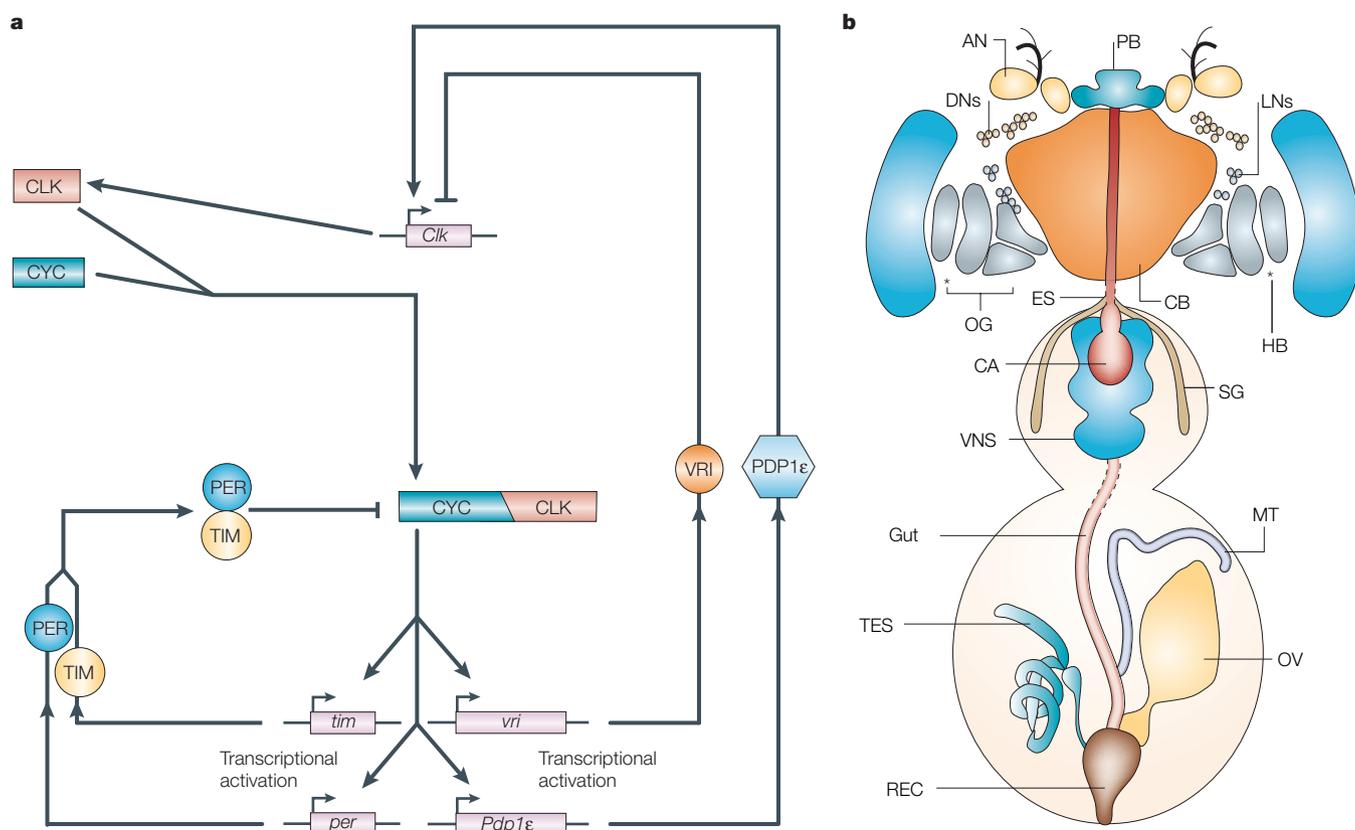


Figure 6 | The *Drosophila melanogaster* circadian system. a | Molecular interactions in *Drosophila melanogaster* circadian feedback loops. Clock (CLK) and cycle (CYC) form heterodimers and activate *period* (*per*), *timeless* (*tim*), *vri* and *PAR domain protein 1* (*Pdp1ε*) transcription. PER and TIM proteins slowly accumulate as heterodimers and feed back to inhibit CLK–CYC dependent transcription. VRI accumulates quickly and inhibits *clk* transcription, then the slower accumulating levels of PDP1ε activate *clk* transcription. **b** | The complex, multi-tissue oscillator system of *D. melanogaster*. In *D. melanogaster*, all the indicated tissues, except the ovary, are thought to contain autonomous oscillators, which are based on the PER–feedback loop (a), and some of these, if not all, have pacemaker function. Although *per* and *tim* are expressed in the ovary, their expression is not rhythmic. AN, olfactory sensory neuron; CA, cardia; CB, central brain; DN, dorsal neuron; ES, esophagus; HB, Hofbauer–Buchner cells, indicated by asterisks; LN, lateral neuron; MT, Malpighian tubules; OG, optic ganglia; OV, ovary; PB, proboscis; REC, rectum; SG, salivary gland; TES, testes; VNS, ventral nervous system.

central oscillator neurons in the brain, thereby allowing the coordinated control of locomotor-activity rhythms^{134,140,141}.

Similar to *D. melanogaster*, peripheral oscillators in zebrafish (*Danio rerio*) and plants can be directly entrained by light. This indicates that they might not need a centralized pacemaker, as a principal function of such a pacemaker in mammals is to convert light entrainment signals from the eye into humoral signals that entrain peripheral tissues^{142,143}. However, the potential for central oscillator function does exist in zebrafish, as pineal melatonin is produced in this species³¹. Whether or not this is the case, an advantage of autonomous light entrainable oscillators is that they would permit tissue-specific specialization of circadian timing.

Conclusions

Circadian clocks in diverse organisms are composed of multiple oscillators, but these are coordinated in different ways. In cyanobacteria and fungi, at least one oscillator is directly linked to the environment

for entrainment, and might therefore serve as the pacemaker for slave oscillators through direct or indirect coupling. Furthermore, genetic data indicate that other independent oscillators exist in *N. crassa* cells, and these potentially have pacemaker activity. Identification and characterization of the key components of these oscillators will be needed to address questions of pacemaker function and the mechanisms of entrainment.

In multicellular organisms in which peripheral tissues are not directly entrained by light, such as rodents and birds, a centralized pacemaker system in the brain seems to be essential for converting photic entrainment signals into downstream signals that entrain peripheral tissues. This idea is consistent with the apparent lack of a centralized pacemaker in *D. melanogaster* in which peripheral oscillators can be directly entrained by light. An important goal is to identify the mechanisms and components that allow communication between oscillators and their coordination by a pacemaker, which together results in the precise timing of stable rhythmic biological activities.

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Acknowledgements

The authors are members of the Center for Research on Biological Clocks at Texas A&M University, Houston, USA. The work described in this review from the authors' laboratories was funded by the US National Institutes of Health, including an NINDS Program Project Grant and an NIEHS Center Grant.

Competing interests statement

The authors declare no competing financial interests.

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