Molecular mechanisms and physiological importance of circadian rhythms

Article *in* Nature Reviews Molecular Cell Biology · November 2019

DOI: 10.1038/s41580-019-0179-2

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Molecular mechanisms and physiological importance of circadian rhythms

Alina Patke, Michael W. Young and Sofia Axelrod*

Abstract | To accommodate daily recurring environmental changes, animals show cyclic variations in behaviour and physiology, which include prominent behavioural states such as sleep–wake cycles but also a host of less conspicuous oscillations in neurological, metabolic, endocrine, cardiovascular and immune functions. Circadian rhythmicity is created endogenously by genetically encoded molecular clocks, whose components cooperate to generate cyclic changes in their own abundance and activity, with a periodicity of about a day. Throughout the body, such molecular clocks convey temporal control to the function of organs and tissues by regulating pertinent downstream programmes. Synchrony between the different circadian oscillators and resonance with the solar day is largely enabled by a neural pacemaker, which is directly responsive to certain environmental cues and able to transmit internal time-of-day representations to the entire body. In this Review, we discuss aspects of the circadian clock in *Drosophila melanogaster* and mammals, including the components of these molecular oscillators, the function and mechanisms of action of central and peripheral clocks, their synchronization and their relevance to human health.

Circadian rhythmicity
A physiological or behavioural oscillation with a period of ~24 h, which is sustained in constant conditions and entrainable by external cues such as light.

Laboratory of Genetics, The Rockefeller University, New York, NY, USA.

**e-mail: young@rockefeller.edu*

https://doi.org/10.1038/s41580-019-0179-2

Most organisms anticipate daily changes in their environment, including light, temperature and food availability for optimal fitness[1–5]. Prominent daily behavioural and/or physiological rhythms have been observed in animals, plants, fungi and bacteria. These rhythms are referred to as circadian, stemming from the Latin '*circa diem*' or about a day, and are the result of an autonomous, intrinsic timekeeping system called the circadian clock[6,7]. The circadian clock is able to keep running even under constant environmental conditions with an approximately 24-h periodicity. In a process referred to as entrainment, the phase of the circadian clock, meaning its stage in the cycle relative to external time, is determined by environmental cues termed zeitgebers. The response of the circadian clock to zeitgebers depends both on the strength of the stimulus and on the circadian phase during which it is applied. Consequently, zeitgebers can advance or delay the circadian clock, thereby ensuring its synchrony with the solar day. In normal conditions, these principles form the basis of the adaptive advantage that circadian clocks convey to an organism by optimizing the timing of fundamental cellular and physiological processes and behaviours. Yet erroneous exposure to zeitgebers, which is common in contemporary society, can disrupt circadian homeostasis and have detrimental effects on human health[8].

The circadian clock is genetically controlled, and mutations in so-called 'clock genes' can change rhythmic behaviour in animals, including insects and humans, and in plants, fungi and bacteria[9]. In essence, the circadian clock constitutes an autoregulatory succession of expression, accumulation and degradation of clock gene products that forms an autonomous molecular oscillator. Delays built into discrete stages of this cycle are crucial to its timing, although it is still unclear how the overall 24-h periodicity is achieved. In animals, the molecular clock controls the expression of output genes throughout the body, thereby temporally controlling the activity and function of different cells and organs[10]. Normal circadian physiology is created by a hierarchical network of central and peripheral clocks. In both vertebrates and invertebrates, a dedicated set of neurons controls circadian behaviour and can convey time-of-day information to 'downstream' clocks in peripheral tissues and organs.

In this review, we focus on the fruit fly *Drosophila melanogaster* and on mammals as representative and complementary model systems that have been key to advancing our understanding of the molecular and cellular composition of circadian clocks. First, we discuss the organization of circadian clocks at the molecular level and how circadian rhythmicity is established and

the period length is controlled. We then discuss how circadian activity contributes to the optimal function of tissues and organs, to organismal physiology and to disease aetiology.

The molecular circadian clock

At the heart of the molecular circadian clock in animals is a transcription–translation feedback loop (TTFL), which takes approximately 24 hours to complete. In this section, we describe the chief components of the molecular oscillators in *D. melanogaster* and in mammals, and discuss how recent findings have improved our understanding of these molecular clocks.

The molecular clock in D. melanogaster

***The first mutants displaying altered circadian behaviour were found in D. melanogaster*[11].** A genetic screen for the timing of eclosion, which occurs predominantly in the morning in wild-type flies, yielded an arrhythmic strain named *period*^{0} (*per*^{0}) and two additional mutants named *period short* (*per*^{S}) and *period long* (*per*^{L}), which shortened or lengthened the period to 19 h and 28 h, respectively. Genetic tests suggested that all three mutations are alleles of the gene *period*, whose molecular identity was subsequently determined[12,13]. In the following years, screens for locomotor activity, which peaks at dusk and dawn, which were aided by the short generation time and powerful genetics of *D. melanogaster*, uncovered a network of circadian 'clock genes'[14]. Biochemical and genetic studies ultimately revealed a TTFL in which two transcriptional inhibitors, Period (PER) and Timeless (TIM)[15], physically associate and translocate to the nucleus, where they repress the transcription of their own genes by suppressing a pair of transcription activators, Clock (CLK)[16] and Cycle (CYC)[17] (FIG. 1). CLK and CYC accumulate constitutively in the nucleus and form a heterodimer, which binds to E-box-containing enhancers upstream of the promoters of *per* and *tim*. The levels of *per* and *tim* mRNAs peak at the end of the day, whereas their protein levels are highest in the second half of the night (FIG. 2).

As PER and TIM accumulate in the nucleus, they increasingly inhibit CLK–CYC function. Light-dependent TIM degradation occurs during the day through the activity of the photoreceptor protein Cryptochrome (CRY) and the E3 ubiquitin ligase Jetlag (JET), which also degrades CRY[18–20] (FIG. 1). In the absence of TIM, PER is destabilized by Double-time (DBT)[21,22], which is the fly orthologue of mammalian casein kinases 1(CK1) δ and ε, and by the E3 ubiquitin ligase supernumerary limbs (SLIMB)[23,24]; the concomitant loss of PER and TIM restarts the circadian cycle. The transcriptional targets of CLK–CYC include downstream clock output genes, whose cyclic expression confers circadian rhythmicity to cell and tissue function[25–33]. A second TTFL controls the expression of the *clk* mRNA. CLK–CYC bind to E-boxes in the enhancers of the genes encoding the transcription factors Vrille (VRI) and PAR domain protein 1ε (PDP1ε), which control *clk* transcription[34] (FIG. 1). VRI binds to VRI/PDP1ε-binding boxes in the *clk* enhancer and represses *clk* transcription, whereas PDP1ε activates it later in the night, thus resulting in rhythmic *clk* mRNA expression. However, modulating the phase in which the *clk* mRNA is expressed does not affect behavioural rhythms[35] and CLK protein does not oscillate, so the role of rhythmic *clk* mRNA expression remains unclear. Nevertheless, PDP1ε is essential for rhythmicity[36], possibly by controlling the expression of the neuropeptide pigment-dispersing factor (PDF), which is required for behavioural rhythms[37].

Regulation of the molecular clock in D. melanogaster. What regulates the precise timing for the TTFL to ensure near 24-h rhythmicity? On the transcriptional level, the degree of feedback repression of the transcription of *per* and *tim* is a key regulator of the circadian clock. Overexpression of CLK–CYC, increasing their activity at *per* and *tim* E-boxes[38,39], or overexpression of *per* under its own promoter shorten the circadian period, whereas a reduction of *per* levels[40] or disruption of clockwork orange (CWO), which has a dual role as a competitive inhibitor of CLK–CYC and as a suppressor of CLK target genes, lengthen the period[41–43].

At the post-transcriptional level, regulation of protein synthesis, stability and accumulation all contribute to the precise timing of the circadian clock by introducing crucial delays into the TTFL. The synthesis of the proteins CLK, CWO and TIM is inhibited by the microRNAs *bantam*[44], *let-7* (REF.[45]) and *mir-276a* (REF.[46]), respectively (FIG. 1), and overexpression of either microRNA causes period lengthening[44,45] or arrhythmicity[46]. The stability of the *tim* mRNA depends on the activity of the deadenylase POP2 (REF.[47]), whereas Twenty-four[48] and its activator Ataxin 2 (REFS[49,50]) facilitate the translation of the *tim* and *per* mRNAs by promoting their binding by polyadenylate-binding proteins type 1. The protein turnover of PER, TIM and CLK is regulated by different kinases and phosphatases. DBT phosphorylates PER[21,22] and CLK[51,52], both in the cytoplasm and in the nucleus (FIG. 1). Once PER is phosphorylated by the kinase NEMO[53], it is phosphorylated by DBT, thereby enabling subsequent downstream PER phosphorylation events, which mark it for SLIMB-mediated proteasomal degradation[23,24]. Mutations in *dbt* that affect its kinase activity yield flies with short or long periods (*dbt*^{S} and *dbt*^{L}, respectively[21]) or arrhythmic flies (*dbt*^{AR})[54].

As mentioned above, the PER–TIM heterodimerization retards DBT-dependent PER phosphorylation and degradation. Although DBT is expressed constitutively, rhythmic assembly of the PER–TIM–DBT complex, along with the DBT binding partner Bride of double-time (BDBT)[55,56], creates rhythmicity of PER degradation. DBT is also required for CLK inactivation, probably by providing a scaffold for phosphorylation by an unknown kinase[52] and subsequent PER-mediated disengagement from DNA[51,52]. The stability of PER and TIM is also affected by two phosphatases: protein phosphatase 2A stabilizes PER by antagonizing its DBT-mediated phosphorylation and subsequent degradation[57], whereas protein phosphatase 1 stabilizes TIM[58] (FIG. 1). TIM is also subject to sequential phosphorylation by Shaggy and CK2, which promote its accumulation in the nucleus, a process that also depends on importin-α[59–62]. Although

Eclosion
The emergence of an insect from the pupal case.

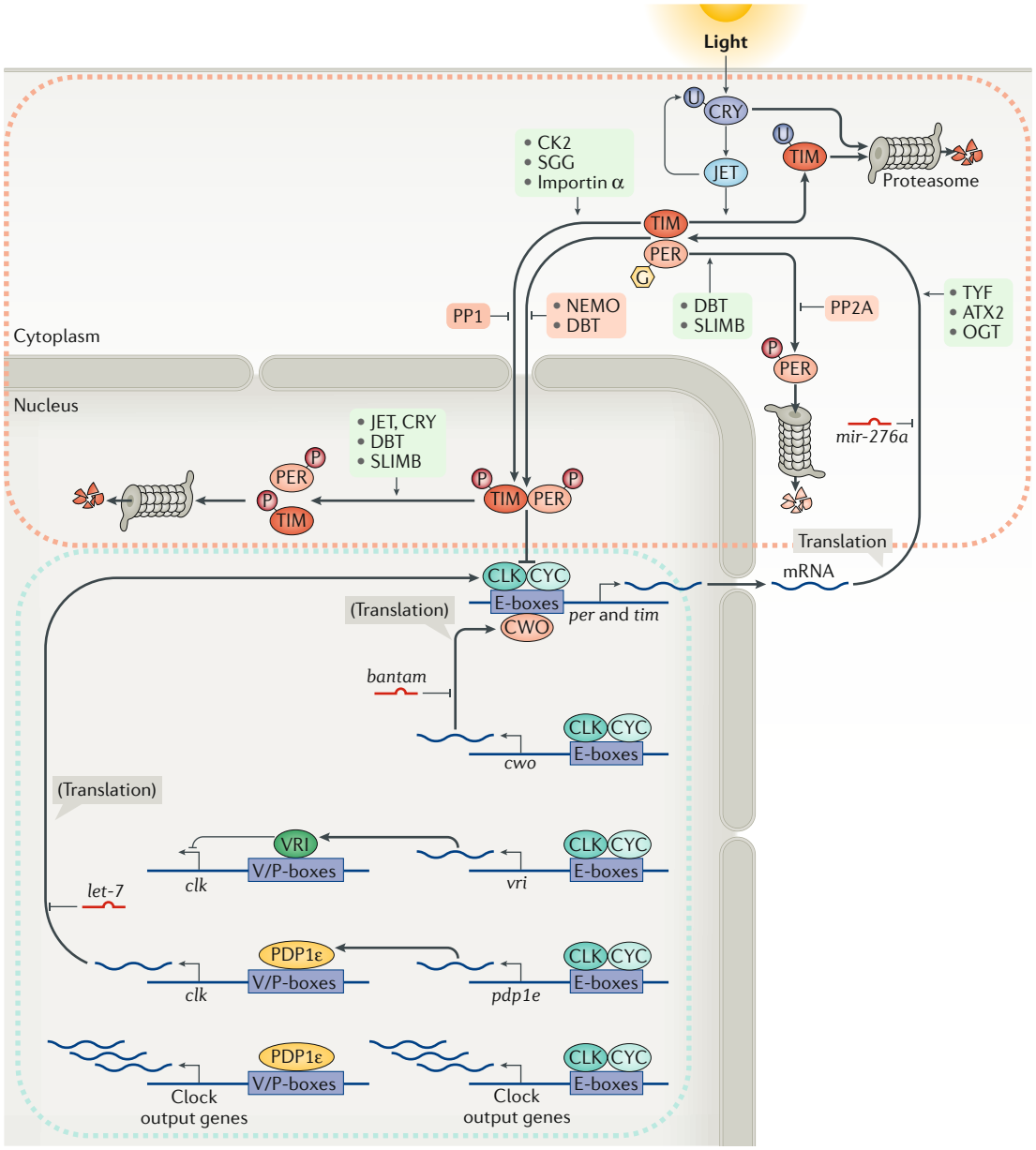

Fig. 1 | **The *Drosophila melanogaster* molecular clock.** The transcriptional repressors Period (PER) and Timeless (TIM) inhibit the transcription activators Clock (CLK) and Cycle (CYC), forming the first transcription–translation feedback loop (TTFL; dashed orange line). When PER and TIM are degraded, CLK and CYC can activate the transcription of the *per* and *tim* mRNAs, thereby restarting the circadian cycle. PER and TIM accumulation, translocation into the nucleus and degradation in the cytoplasm and in the nucleus are regulated by several post-translational modifiers including the kinases Double-time (DBT; also known as casein kinase 1), Shaggy (SGG), casein kinase 2 (CK2) and NEMO; protein phosphatase 1 (PP1) and PP2A; and O-GlcNAc transferase (OGT), the RNA-binding protein Ataxin 2 (ATX2), the nuclear import factor importin-α and the E3 ubiquitin ligase supernumerary limbs (SLIMB), which ubiquitylates phosphorylated PER. These factors, together with a second, interlocked feedback loop (dashed green line) consisting of the transcription factors PAR domain protein 1ε (PDP1ε) and Vrille (VRI), which control the cycling levels of the *clk* mRNA, and of Clockwork orange (CWO), which exhibits dual roles as a competitive inhibitor of DNA binding by CLK–CYC and suppressor of CLK target genes, control the total duration and stability of the TTFL that regulates the circadian clock. By binding to E-box enhancer elements, CLK–CYC regulate transcription of genes that confer circadian rhythmicity to target-gene expression. Light resets the TTFL through the degradation of TIM and Cryptochrome (CRY) by the E3 ubiquitin ligase Jetlag (JET), both in the cytoplasm and in the nucleus. Translation of different clock mRNAs is inhibited by microRNAs, as indicated. G, O-linked β-D-*N*-acetylglucosamine; P, phosphate; TYF, Twenty-four; U, ubiquitin; V/P-boxes, VRI/PDP1ε-binding boxes.

PER and TIM dissociate prior to nuclear entry[63], PER requires TIM for nuclear translocation and is retained in the cytoplasm by DBT and by *O*-GlcNAcylation[64], which consequently reduces PER binding to CLK[65].

The molecular clock in mammals

Although the central features and principles of the molecular clock are conserved from insects to mammals, some notable differences exist[9]. As in *D. melanogaster*,

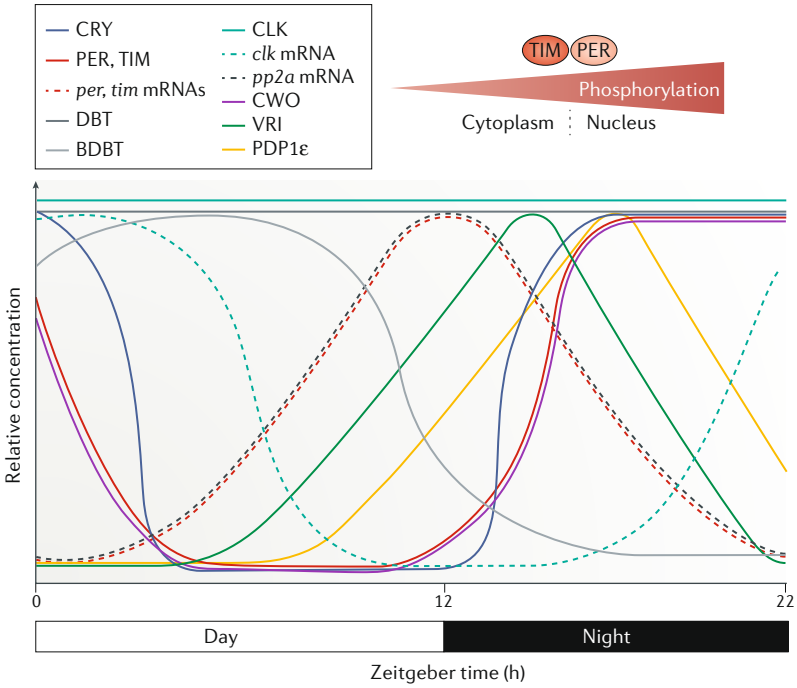

Fig. 2 | **The expression of several circadian clock genes oscillates over the day–night cycle.** Shown are approximate relative concentrations of key circadian clock factors: mRNAs are denoted as dashed curves and proteins as solid curves. Top panel: phosphorylation of cytoplasmic Period (PER) and Timeless (TIM) increases over the course of the night promoting their nuclear accumulation, which is highest in the second half of the night. BDBT, Bride of Double-time; CLK, Clock; CRY, Cryptochrome; CWO, Clockwork orange; CYC, Cycle; DBT, Double-time; PDP1ε, PAR domain protein 1ε; pp2a, protein phosphatase 2A; VRI, Vrille.

the cell-autonomous molecular circadian clock in mammals consists of interlocking TTFLs (FIG. 3). In the main loop, the positive elements that drive the circadian cycle are heterodimers of the basic helix–loop–helix (bHLH)–Per-Arnt-Sim (PAS) transcription factors BMAL1 (also known as ARNTL; orthologue of fly CYC) and CLOCK (orthologue of fly CLK). CLOCK–BMAL1 activate the transcription of target genes that contain E/E′-box elements in their promoter and/or enhancer regions. These genes include the negative elements that attenuate the main loop — members of the mammalian PER and CRY protein families. PER1, PER2 and PER3 are orthologues of the *D. melanogaster* single PER protein, and CRY1 and CRY2 are structurally related to the fly CRY. Although different PER and CRY paralogues can to some extent compensate for loss of another paralogue, their roles in the mammalian clock are not completely redundant. For example, circadian rhythmicity is only abolished upon inactivation of both CRY1 and CRY2, whereas their individual loss shortens or lengthens the circadian period, respectively[66]. At a later stage of the cycle, complexes containing PER and CRY proteins inhibit the activity of CLOCK–BMAL1, effectively preventing their own continued production. Once PER and CRY levels sufficiently drop, CLOCK–BMAL1-mediated transcription can resume, thus completing the cycle.

In addition to the *PER* and *CRY* genes, CLOCK–BMAL1 target genes include the nuclear receptors REV-ERBα and REV-ERBβ (REV-ERBα/β), which together with retinoid-related orphan receptor α (RORα), RORβ and RORγ (RORα/β/γ) form a second loop that ensures the rhythmic expression of BMAL1, analogous to the regulation of fly *CLK* by VRI and PDP1ε (REFS[67,68]). REV-ERBα/β and RORα/β/γ compete for binding of REV-ERB–ROR response elements in the promoter and enhancer regions of target genes, including *ARNTL*, and inhibit or activate their transcription, respectively[69] (FIG. 3). Another CLOCK–BMAL1 target gene, D-box binding protein (DBP), and its related proline and acidic amino acid-rich–basic leucine zipper (PARbZip) transcription factors TEF and HLF, compete with NFIL3 to activate or inhibit, respectively, the expression of clock genes from D-box-containing promoters[70,71].

A notable difference between the *D. melanogaster* and mammalian clocks is the role of the CRY proteins. Whereas the fly CRY (dCRY) is not a component of the core TTFL but feeds into it through its light-dependent control of TIM stability (FIG. 1), the mammalian CRY proteins have assumed the role of TIM and act as the main transcriptional repressor of CLOCK–BMAL1 (FIG. 3). The primary function of the closest mammalian TIM homologue appears to be the protection of stalled replication forks[72]. However, as mammalian TIM can interact with mammalian CRY proteins and its absence alters the circadian period, TIM has a sustained, if not entirely conserved, role in regulating the mammalian circadian clock[73,74].

A recent addition to our understanding of CLOCK–BMAL1 regulation has been the identification of CHRONO (also known as circadian-associated repressor of transcription), which, like the CRY proteins, inhibits CLOCK–BMAL1 on E-boxes, but does so through a different epigenetic mechanism[75–77]. Remarkably, CHRONO is the gene rhythmically expressed in the greatest number of tissues in a diurnal primate, surpassing even the better-known core clock components[78]. The biological function of another recently identified CLOCK–BMAL1 repressor, PASD1, appears to be the dampening of molecular clock oscillations. This is consistent with its narrow expression profile only in tissues that do not show circadian rhythmicity, such as the germline and oncogenically transformed somatic tissues[79]. Notably, PASD1 is broadly conserved in mammals except the murine lineage. Clearly, there is still much to be learned about the nature and function of the cell-autonomous circadian oscillator, especially in non-traditional model organisms, including humans.

Regulation of the mammalian molecular clock. Transcriptional regulation of mammalian clock genes lies at the very core of the cell-autonomous circadian oscillator and is a highly regulated process. Genomic profiling studies have revealed a transcriptional cycle that proceeds from a poised state to activation, active transcription and repression[80]. Each of these states is distinguished by a unique combination of chromatin-bound clock proteins, recruitment and activation of RNA polymerase II and distinct sets of histone modifications. It is important to note that although this transcriptional cycle can be seen globally in the liver, it does not necessarily predict the phase in which an individual

E/E′-box
The DNA element CACGT(T/G), which is bound by the basic helix–loop–helix transcription factors CLOCK–BMAL1.

D-box
(DBP response element). A DNA element (TTATG(C/T)AA) bound by transcription regulators of the proline and acidic amino acid-rich–basic leucine zipper family (DBP, TEF, HLF) and E4BP4 (also known as NFIL3).

Basic leucine zipper
(bZip). A protein domain common in many DNA-binding proteins.

Topologically associating domains
Genomic regions with extensive internal chromatin interactions (such as between promoters and distal enhancers) and fewer contacts with neighbouring regions.

target gene will be expressed[81]. Rather, the phase appears to be primarily defined by the rhythmic activity of intergenic enhancers bound by clock proteins[82]. Recent technical advances in the analysis of genome topology have enabled the unbiased assessment of enhancer–promoter interactions, and found rhythmic compaction in select subregions of topologically associating domains[83]. The circadian regulation of promoter–enhancer proximity is functionally important to the clock, as shown by period shortening upon deletion of an intronic enhancer region in the *CRY1* gene[84]. Furthermore, the transcriptional repressive function of REV-ERBα can be attributed at least in part to its suppression of functional promoter–enhancer interactions[83]. Thus, circadian genome-topology dynamics have emerged as a new regulatory layer in the molecular clock cycle.

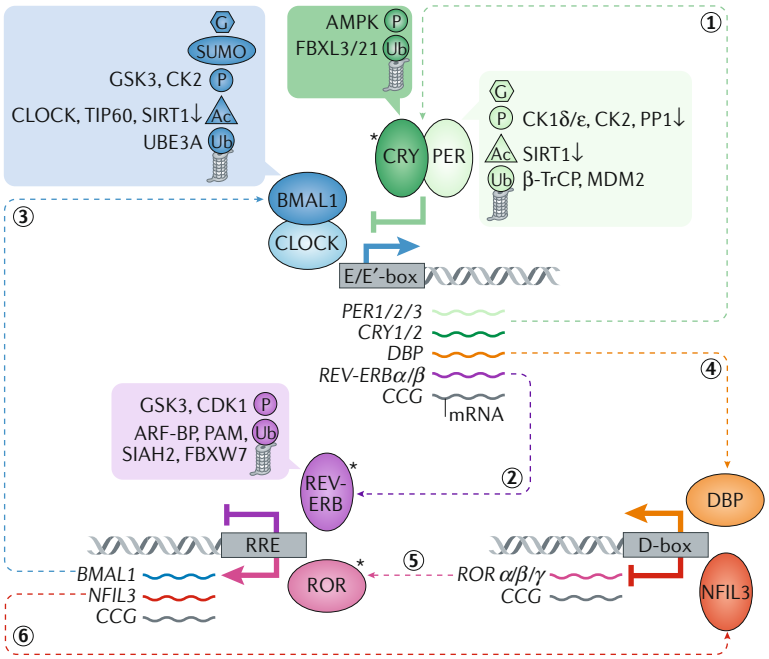

Fig. 3 | **The molecular circadian clock in mammals is formed by interlocking transcription–translation feedback loops.** In the main loop, the transcription factors CLOCK–BMAL1 induce the expression of their own negative regulators, the Period (PER) and Cryptochrome (CRY) proteins (step 1). By inhibiting the transcriptional activity of CLOCK–BMAL1, PER and CRY repress their own expression. Once PER and CRY levels have sufficiently dropped, a new cycle of transcription by CLOCK–BMAL1 can begin. CLOCK–BMAL1 also induce the expression of the nuclear receptors REV-ERBα and REV-ERBβ (REV-ERBα/β) (step 2), which oppose retinoid-related orphan receptor α, β and γ (RORα/β/γ)-mediated BMAL1 expression (step 3), and the expression of D-box binding protein (DBP) (step 4), which activates transcription from D-box-containing genes including *RORα/β/γ* (step 5). D-box-dependent transcription is inhibited by NFIL3, itself a REV-ERB and ROR target gene (step 6). All loops also control the expression of clock-controlled genes (CCG), which mediate circadian output. Selected factors that mediate post-translational modifications and degradation of specific clock proteins are shown in matching colours. Downward arrows indicate the removal rather than addition of a post-translational modification by the respective factor. Targets of small-molecule modulators being explored for the treatment of sleep, mood and metabolic disorders are marked with an asterisk. For clarity, the translocation of clock factors between the cytoplasm and nucleus, which is a key regulatory step, and additional regulatory DNA elements that contribute to the accurate phase of clock gene expression have been omitted from the diagram. For simplicity, *BMAL1*, *REV-ERBα* and *REV-ERBβ* are used in place of the official gene names *ARNTL*, *NR1D1* and *NR1D2*, respectively. Ac, acetylation; AMPK, AMP-activated protein kinase; ARF-BP1, also known as HUWE1; BMAL1, also known as ARNTL; CDK1, cyclin-dependent kinase 1; CK1, casein kinase 1; CK2, casein kinase 2; E/E′, E/E′-box; G, addition of *O*-linked β-D-*N*-acetylglucosamine; GSK3, glycogen synthase kinase 3; P, phosphorylation; PAM, also known as MYCBP2; PP1, protein phosphatase 1; RRE, REV-ERB/ROR response element; SIRT1, sirtuin 1; SUMO, sumoylation; β-TrCP, also known as F-box/WD repeat-containing protein 1A; Ub, ubiquitylation.

Following transcription, RNA N^6-adenosine methylation regulates the translocation of mature *PER2* and *BMAL1* transcripts from the nucleus to the cytoplasm[85]. Once clock proteins are synthesized, there is extensive regulation at the level of post-translational modification and protein stability (FIG. 3). A general trend among findings so far is that the circadian period length in mammals is exquisitely sensitive to alterations in the phosphorylation state of the PER proteins and the stability of the CRY proteins. PER proteins are subjected to successive phosphorylation events of multiple residues by CK1δ and CK1ε and by CK2 (REFS[86–94]), which controls their susceptibility to proteasomal degradation mediated by the E3 ubiquitin ligase β-TrCP (also known as F-box/WD repeat-containing protein 1A)[95,96], although the outcomes of the phosphorylation events are complex. Whether phosphorylation promotes protein stability or degradation depends not only on the modified residue but also on subtle differences in the responsible kinase. For example, phosphorylation by an alternatively spliced variant of CK1δ has opposite effects on PER2 stability and on circadian period length compared with phosphorylation by the canonical CK1δ (REF.[97]). *O*-GlcNAcylation of PER2 can also compete with its phosphorylation to further modulate clock cycling[98]. PER2 degradation is also promoted through its de-acetylation by sirtuin 1 (SIRT1)[99]. Notably, the oncoprotein and E3 ubiquitin ligase MDM2 can promote PER2 degradation regardless of its phosphorylation state[100].

The stability of CRY1 and CRY2 can also be affected by phosphorylation[101] (FIG. 3). Unlike the PER proteins, however, CRY phosphorylation by AMP-activated protein kinase (AMPK) is not part of a complex multisite phosphorylation sequence. Phosphorylated CRY1 and CRY2 become a target for the E3 ubiquitin ligases FBXL3 and FBXL21, and targeting of the CRY1–FBXL3 complex to the proteasome is facilitated by JMJD5 (REFS[102–107]). The circadian period length closely tracks CRY protein abundance when manipulated genetically or pharmacologically, although additional features of the CRY proteins, such as the carboxy-terminal tail, can also modulate it[108–115]. Surprisingly, CRY proteins can also act as cofactors in targeting other proteins such as the proto-oncogene MYC for FBXL3-mediated ubiquitylation and degradation[116,117].

Other clock proteins are also regulated through post-translational modification and degradation. BMAL1 acquires a wide variety of modifications throughout its life cycle (FIG. 3). Phosphorylation by CK2α promotes its nuclear accumulation, whereas phosphorylation of different residues by glycogen synthase kinase 3 (GSK3; orthologue of the fly protein Shaggy) leads to instability[118–120]. BMAL1 turnover is further regulated through conjugation of SUMO by a yet to be specified SUMO E3 ligase and through ubiquitylation, which can involve the E3 ligase UBE3A (REFS[121–123]). Finally, the acetylation state of BMAL1 has been linked to its transcriptional activity, although different mechanisms have been proposed:

BMAL1 acetylation by CLOCK promotes its interaction with CRY1 and is reversed by SIRT1, whereas acetylation of the same residue by TIP60 enables productive transcription elongation[124–126]. REV-ERBα is another reported GSK3 target, although in this case the modification protects it from degradation mediated by the E3 ligases ARF-BP1 (also known as HUWE1) and PAM (also known as MYCBP2)[127,128]. REV-ERBα degradation mediated by another E3 ligase, SIAH2, affects the circadian period length, whereas its degradation by the E3 ubiquitin ligase FBXW7 following CDK1-mediated phosphorylation controls circadian amplitude, meaning the difference between the peak and trough values of the oscillation[129,130].

The negative elements of the core molecular clock assemble into large multi-protein complexes that contain all three PER proteins, both CRY proteins and CK1δ, into which CLOCK and BMAL1 are incorporated in the nucleus[131]. When purified from liver nuclear extracts, all of these proteins appear to be part of the same 1.9-MDa complex. However, other studies have suggested the existence of alternative clock-repressive complexes in the nucleus on the basis of the differential ability of PER and CRY proteins to repress CLOCK–BMAL1-mediated transcription[132–136]. We recently observed that an altered form of CRY1 with an internal deletion of 24 residues owing to a splice site mutation, which predisposes to delayed sleep phase disorder, enhances CRY1 binding to CLOCK–BMAL1 but not to PER2 and acts as a stronger transcriptional inhibitor than wild-type CRY1 (REF. [114]).

Clock functions throughout the body

The master (central) pacemaker is comprised of a set of neurons and glia in the brain, which are necessary for entrainment to external zeitgebers and transmit temporal information to downstream peripheral clocks, which are located in other brain areas or throughout the body. The degree to which the central pacemaker is required for circadian rhythmicity of different cells, tissues, physiological functions and behaviours varies between species and tissues. To achieve synchrony between central pacemaker cells, they are coupled to each other through neurotransmitters and neuromodulators. Synchronization of central and peripheral clocks is coordinated by the nervous system, hormones and body temperature.

The central pacemaker in D. melanogaster

The expression of clock genes in a small number of neurons is necessary and sufficient to maintain functional behavioural rhythms in constant darkness devoid of external zeitgebers[137]. In *D. melanogaster*, this neuronal pacemaker network consists of ~150 neurons subdivided into five bilateral clusters, which according to their location in the fly brain have been named large ventral lateral neurons, small ventral lateral neurons, dorsal lateral neurons, lateral posterior neurons and dorsal neurons. Not all clock neurons have the same function, and in fact there is a subdivision into neurons responsible for different aspects of daily locomotor rhythms. Furthermore, recent studies showed that a robust locomotor rhythm is an emergent property of a combination of different rhythms in different clusters of clock neurons, and that selectively activating or inactivating those clusters causes predictable changes in the activity patterns of flies[138–140]. Such differences in rhythmicity of clock neurons are a function of differential expression of clock genes[25] and downstream factors, and of differential coupling to other clock cells through neuromodulators — notably PDF[141] — and through diurnal circuit remodelling of synaptic connections (reviewed in[142,143]). In fact, clock neurons and glia cells change their synaptic partners rhythmically across day and night, suggesting that the brain circuitry itself undergoes circadian plasticity[144,145]. Glia themselves have a well-documented yet relatively understudied role in the central pacemaker (reviewed in[146]). Different clusters of clock neurons have been implicated in regulating different rhythmic behaviours, including eclosion, locomotion, feeding, mating, courtship and temperature preference (see below)[147]. The multitude of clock cell populations and additional genetic factors are believed to create a robust circadian rhythm, which allows animals to optimally anticipate cyclical changes in their environment yet is able to adapt to seasonal changes in day length[148].

The central pacemaker in mammals

The circadian physiology of mammals is based on a hierarchical network of central and peripheral oscillators. The master, central pacemaker is located in the suprachiasmatic nuclei (SCN) of the hypothalamus, as proved by the striking exchange of the behavioural circadian period between normal hamsters and a short-period mutant following reciprocal SCN transplantations[149]. Further transplantation studies demonstrated that a diffusible signal from the SCN is sufficient for controlling the circadian period of the recipient[150], although we know now that a complex network of humoral and neuronal signals and of body temperature rhythms mediates circadian control downstream of the SCN (reviewed in[151]). In this way, time-of-day information is transmitted to the entire body to affect daily cycles of physiology and behaviour.

The molecular makeup of the cell-autonomous core circadian oscillator is conserved between cells in the SCN and elsewhere in the body, except for a potential subtle difference in the ability of NPAS2, a CLOCK homologue, to compensate for the loss of CLOCK[152,153]. What sets the SCN apart from the peripheral oscillators and allows it to maintain a stable circadian rhythm essentially indefinitely without dampening is the intercellular coupling between its neurons. Anatomically and functionally, the SCN can be divided into a 'core' region and a 'shell' region, which differ in their neuropeptide profiles, efferent projections and ability to maintain intercellular synchrony. Circadian activity across the SCN proceeds in a highly stereotypical fashion with progressive differences in phase and amplitude reminiscent of a tidal wave. This striking pattern can be beautifully visualized through long-term bioluminescence recordings from organotypic SCN slices of mice expressing a PER2–luciferase fusion protein and depends on electrical coupling[154]. SCN neurons exhibit pronounced circadian rhythms in membrane potential and spontaneous firing rate, which directly affect circadian behaviour as shown recently through optogenetic manipulation[155]. Several recent reviews (for example, REF. [156]) provide an

Delayed sleep phase disorder
A circadian rhythm sleep disorder characterized by a delay in the major sleep episode relative to the desired sleep time.

Efferent projections
Axons exiting from a particular region such as the suprachiasmatic nuclei.

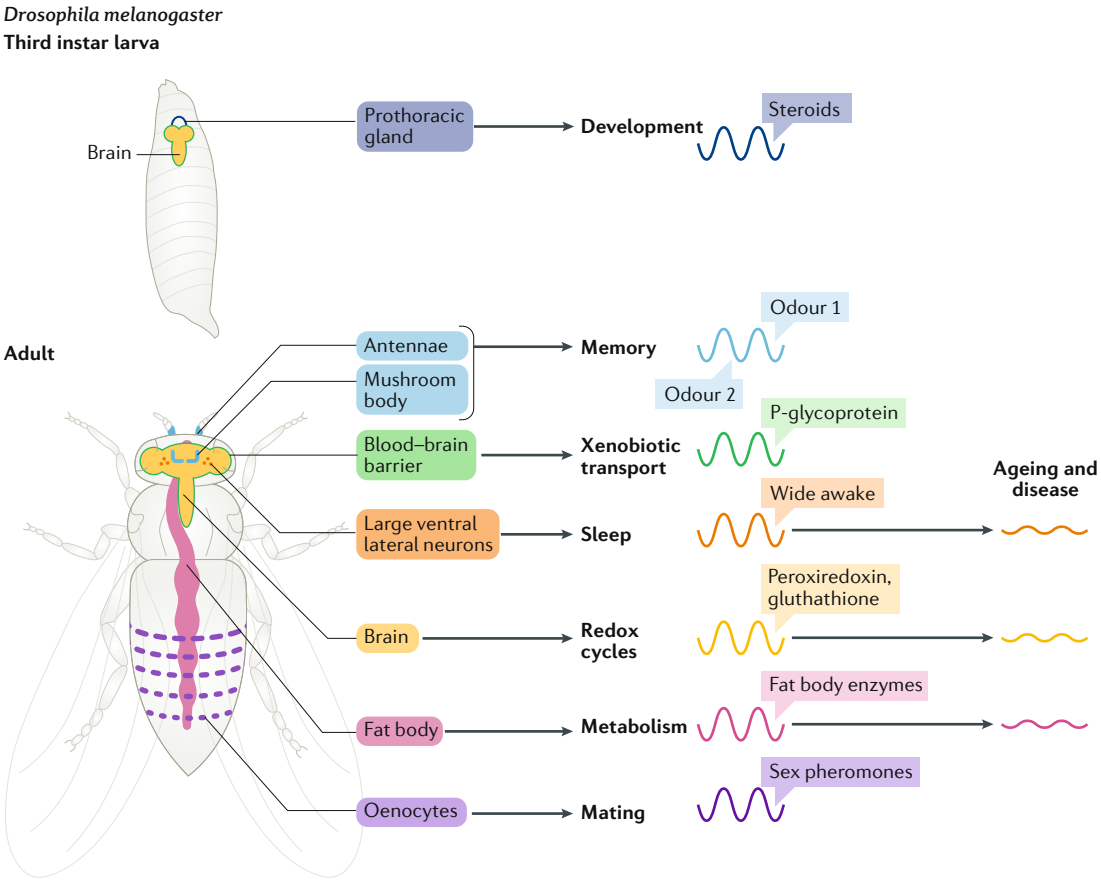

Fig. 4 | **Peripheral clocks in *Drosophila melanogaster*.** Circadian clocks control various physiological processes through rhythmic expression of molecules in different tissues, for example steroids during development[165], time-of-day memory associating with two different odours[185], sex pheromones during mating[183], P-glycoprotein expression in the blood–brain barrier for xenobiotic transport[186], sleep-promoting factors[175], fat body enzymes[33] and redox enzymes[291,292]. During ageing or in disease, the amplitude of sleep, metabolic and redox rhythms can be reduced[283,347,348], potentially contributing to health problems.

Prothoracic gland
An endocrine gland in certain insects regulating moulting by secretion of steroid hormones such as ecdysone.

Oenocytes
Pheromone-producing secretory cells found in most insects.

Malpighian tubules
An excretory and osmoregulatory system used by some invertebrates; Malpighian tubules are functionally similar to the mammalian kidney.

Proboscis
A flexible and tubular mouth part used by many insect species for feeding.

Short neuropeptide F
A signalling molecule released by subpopulations of neurons including some clock neurons; orthologue to mammalian neuropeptide Y.

excellent, comprehensive overview of our current mechanistic understanding of the SCN, so we will not discuss it in further detail here. One notable recent update to the canonical model of SCN function has been the contribution of non-neuronal cell types. Circadian rhythms in SCN-resident astrocytes have been shown to be sufficient, although not required, for modulating circadian behaviour and can even confer oscillations to clock-less SCN neurons[157–159]. Thus, normal pacemaker function in the intact SCN results from interplay of the neuronal network with local non-neuronal clocks.

Peripheral clocks in D. melanogaster

In *D. melanogaster*, most tested tissues show circadian rhythms in gene expression levels and peripheral oscillators have important roles in development, metabolism and behaviour (FIG. 4). Depending on the tissue, between 50 and 2,000 genes have been estimated to be rhythmically expressed in a diurnal manner[25–33]. Although oscillation in some tissues, including in the prothoracic gland and in oenocytes, depends on input of neuropeptides from the central pacemaker[160], other tissues including the Malpighian tubules, antennae and proboscis function independently of the central pacemaker and in direct response to environmental stimuli[161–164].

Eclosion, which was the first behaviour to be tested in fly screens, is mediated by a clock in the prothoracic gland, which produces the moulting steroid hormone ecdysone. The clock regulates steroid synthesis and communicates time-of-day information to the prothoracic gland through short neuropeptide F signalling from the small ventral lateral neurons[160]. Interestingly, removing the clock only from the prothoracic gland is lethal, presumably due to acute desynchronization of steroid signalling from developmental signals originating in other areas of the body[165].

An increasingly important area of circadian research concerns the circadian regulation of metabolism[166]. Flies have an organ called the fat body, which performs the functions of the human liver and adipose tissue. A study analysing gene expression in the fat body reported over 100 cycling transcripts, which are largely related to metabolic function, immunity and reproduction[33]. The fat body clock is sufficient to drive cyclic expression of several clock target genes[33], whereas others depend on input from the brain clock, specifically the neuropeptide F-expressing dorsal lateral neurons cluster[167]. Malpighian tubules, which serve as the fly's kidney, exhibit cell-autonomous and light-entrainable clock function, likely regulating water homeostasis in a circadian fashion[162,168].

One of the most apparent outputs of the circadian clock is the sleep–wake cycle. Timing of sleep is determined by two factors: the need to sleep — also called sleep pressure — and the circadian clock[169]. Sleep timing and duration are highly sensitive to the internal and external state of the animal, which is controlled by the coordinated action of neuronal and glial circuits, including the central pacemaker, intracellular factors, neurotransmitters and neuromodulators, and can be influenced by the immune system and metabolism (reviewed in[170]). Clock-less flies can have normal total amounts of sleep, despite it being randomly distributed across day and night[171]. Conversely, sleep mutant flies can exhibit normal rhythmicity despite overall reduced sleep[172–174]. Nevertheless, separating the two processes genetically can be complicated owing to some genes having roles in both processes, as a substantial proportion of flies in some sleep mutant populations also appear to be arrhythmic[172–174]. For example, the sleep gene encoding Wide awake, whose disruption reduces the duration of daily sleep, is expressed in and affects clock neurons in a circadian manner, thereby showing the interconnectedness of mechanisms controlling sleep and circadian rhythms[175]. Reciprocally, various manipulations of circadian genes and neurons have an effect on sleep[176]. Notably, mutants of the clock gene *cyc* display reduced rebound sleep after sleep deprivation[177,178]. Like mammals, hungry *D. melanogaster* sleep poorly; this starvation-induced sleep deprivation is mediated by *clk* and *cyc*, pointing towards regulatory integration of two homeostatic behaviours — feeding and sleep — by the circadian clock[179]. The DN1 subset of clock neurons regulates both wake and sleep at different times of the day[180,181], and recent work showed that these neurons also integrate temperature to regulate the timing of sleep[182].

The circadian clock also regulates mating by influencing the production of sex pheromones in secretory cells called oenocytes. The oenocyte clock functions cell-autonomously; however, the neuropeptide PDF, which is released by master clock neurons in the central brain, is required to set the correct phase of the oenocyte clock and thereby of sex hormone production[183]. Memory formation is an example of a neuronal process that, although being located in the brain, is downstream and therefore, by our definition, peripheral to the central clock. Similar to what was described for honeybees at the beginning of the twentieth century[184], flies remember a specific odour stimulus learned at a certain time of day, and reproduce the correct stimulus–time pair the next day. In this form of appetitive learning, a functioning circadian clock is only needed to encode time-of-day information, not for memory formation per se: clock-less *D. melanogaster* are still able to learn a specific stimulus, but time-of-day information is lost[185]. A novel addition to the list of processes and organs regulated by the clock is the blood–brain barrier. A recent study showed that *D. melanogaster* perineurial glia, which form the outermost layer of the brain, need a functioning clock to modulate diurnal oscillations of a xenobiotic transporter, which prevents import of toxic substances into the brain in a time-of-day-dependent manner[186].

Peripheral clocks in mammals

The discovery of circadian rhythms in cultured fibroblast cell lines some 20 years ago led to the realization that, contrary to long-standing belief, the molecular clock of mammals operates not just in the SCN, but in virtually all the tissues and cells of the body[187,188]. To date, peripheral clocks have been described in the liver, lung, kidney, heart, skeletal muscles, adipose tissue and many other tissues (FIG. 5). A notable exception are embryonic stem cells and induced pluripotent stem cells, which do not exhibit a functional molecular clock cycle although circadian rhythms in glucose utilization are still observed[189–191]. There is ample evidence that peripheral clocks in different organs are essential for their function. For example, mice manipulated to have the circadian clock operate only in the brain show normal rhythms but not overall levels of locomotor activity; conversely, normal activity levels and body weight are achieved by a having a functioning circadian clock only in muscles, despite exhibiting behavioural arrhythmicity[192]. Other examples of the necessity of peripheral clocks in mammals include the control of glucose homeostasis by the liver and pancreatic clocks, ovulation by the ovarian clock, wound healing by the skin clock and energy expenditure by the ventromedial hypothalamus clock[193–197] (FIG. 5). In addition to these not entirely unexpected examples, some surprising relationships have also emerged whose mechanistic basis is still unclear, such as control of sleep by BMAL1 in skeletal muscle rather than the brain[198].

Circadian physiology is generally the result of cyclic expression of clock-controlled genes downstream of the core molecular oscillator. Although clocks are ubiquitous throughout the body, the nature, number and phase of rhythmically expressed genes is highly tissue-specific in mice, non-human primates and humans[10,78,199,200]. This limited overlap may be explained at least in part by organ-specific needs of circadian output. For example, in the heart, cyclic expression of ion channels and metabolic enzymes enables diurnal variations in cardiac electrical properties and metabolism, which match daily fluctuations in energy demand and nutrient availability[201–204]. In the skin, clock-controlled expression of cell cycle and DNA repair genes mediates rhythmic proliferation and sensitivity to ultraviolet-induced DNA damage[205,206], whereas in the kidneys, circadian oscillations in the rate of glomerular filtration and in ion excretion coincide with rhythmic expression of membrane transporters[207].

Within the largely organ-specific circadian transcriptome, the core clock genes represent the group of genes cyclically expressed in the highest number of different tissues and with the most consistent phase across all tissues, suggesting the existence of an overall shared molecular circadian oscillator in different organs, despite their unique profiles of clock output genes. This apparent paradox can in fact be explained by diverse manners in which molecular clocks and cell-type-specific transcription regulators are functionally integrated. First, some core clock components have well-defined functions in addition to their role in the molecular clock, and these pleiotropic functions can

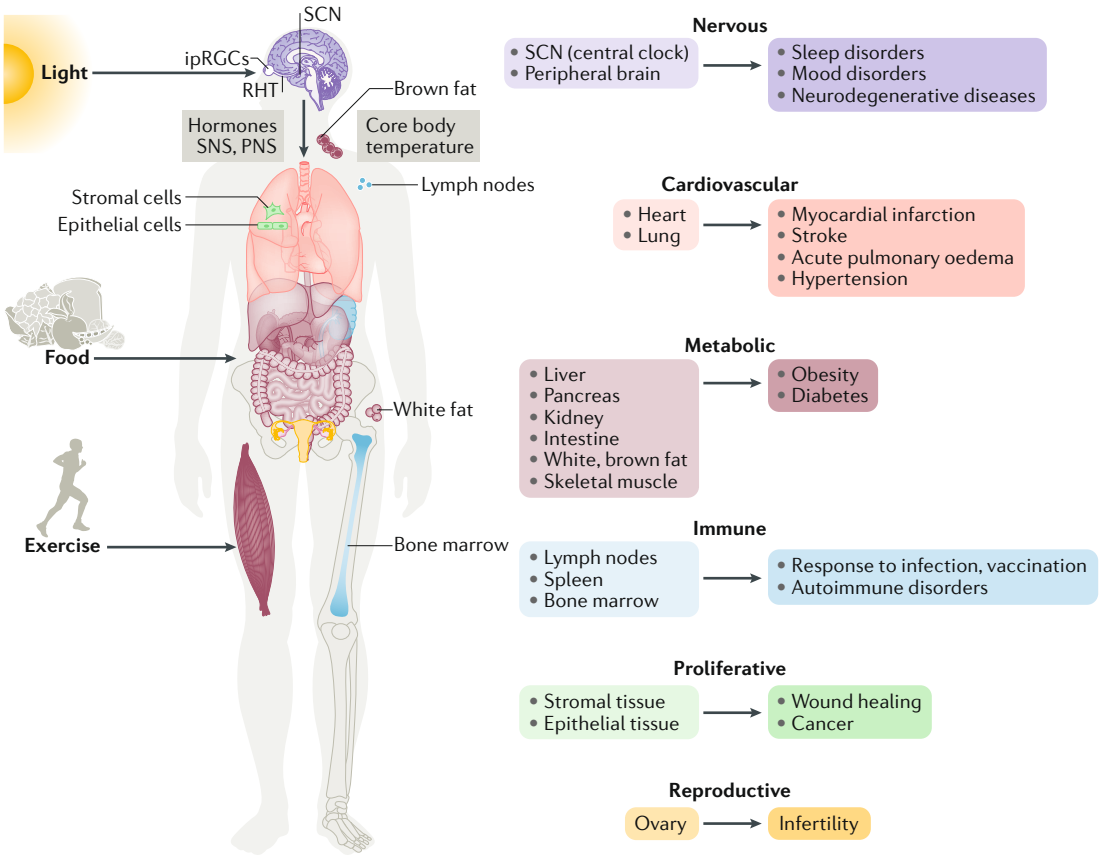

Fig. 5 | **The central clock and selected peripheral clocks in humans.** The central circadian pacemaker is found in the suprachiasmatic nuclei (SCN); this receives time-of-day information from light detected by intrinsically photosensitive retinal ganglion cells (ipRGCs) and transmitted through the retinohypothalamic tract (RHT). Internal time-of-day representations are relayed to the rest of the body through hormones, the sympathetic nervous system (SNS), the parasympathetic nervous system (PNS) and the core body temperature. Additionally, the phase of select peripheral body clocks that contribute to metabolic homeostasis is set by the timing of food intake and exercise. Physiological domains with circadian rhythmicity are distinguished by colour; for each domain, representative organs in which local circadian oscillators operate are shown, along with select pathophysiologies that have been linked to circadian dysfunction. More in-depth information on such pathophysiologies can be found in Supplementary Box 1, and in the selected references for the nervous[287,349,350], cardiovascular[351], metabolic[352,353], immune[354], proliferative[355] and reproductive[356] tissues.

provide direct links to tissue-specific transcriptional programmes. For example, REV-ERBα can silence gene expression not just through direct DNA binding of REV-ERB–ROR response elements, but also indirectly through binding to DNA-tethered cell-type-specific transcription factors such as hepatocyte nuclear factor 6 in the liver or Krüppel-like factor 15 in the heart[208–210]. Another prominent example is the interaction of CRY1 and CRY2 with a wide variety of nuclear receptors, including steroid hormone receptors and lipid-sensing peroxisome proliferator-activated receptors[211–213]. CRY-mediated repression of these nuclear receptors limits the expression of their target genes in the liver and muscle, respectively, and contributes to glucose homeostasis and exercise capacity. Thus, such 'dual-use' factors have the capacity to function as keystones in bridging the core molecular clock cycle with tissue-specific transcription programmes.

Second, CLOCK–BMAL1 activity can be affected by other bHLH transcription factors, whose abundance and activity vary across tissues. Among them are DEC1 (also known as bHLHe40) and DEC2 (also known as bHLHe41), which themselves oscillate and regulate a subset of CLOCK–BMAL1 target genes (reviewed in[214]). The master regulator of cellular oxygen homeostasis hypoxia inducible factor 1α (HIF1α) is another bHLH–PAS protein, whose genomic binding sites overlap to a large extent with those of BMAL1, and there is even evidence for physical dimerization of the two proteins[215,216]. This contributes to a tight reciprocal relationship between the circadian clock and hypoxia responses, which, for example, controls exercise-induced metabolic switches in skeletal muscles[217]. Of note, CRY1, which is the main inhibitor of CLOCK–BMAL1 transcriptional activity, has recently emerged as a direct negative regulator also of HIF1α (REF.[218]). Upstream stimulatory factor 1 is another bHLH protein whose cistrome intersects with that of CLOCK–BMAL1 to the extent that it can suppress the circadian rhythm disruptions of the classical CLOCK$^{\Delta 19}$ mutant[219]. Synergistic interactions have also been observed, for example between CLOCK–BMAL1 and the master regulator of muscle differentiation, myogenic differentiation 1, in the regulation of muscle-specific oscillating target genes[220]. Finally, in cancer cells,

Cistrome
The genome-wide set of *cis*-acting targets of a *trans*-acting factor, for example, the in vivo genome-wide binding locations of a transcription factor.

expression of the bHLH transcription factor and oncoprotein MYC attenuates circadian cycling through down-regulation of BMAL1, although the exact mechanism remains to be clarified[221,222].

Third, cell-type-specific variations in genome topology and chromatin accessibility can contribute to the expression of unique sets of clock output genes in different organs. For example, liver-specific chromatin loops mediate the recruitment of clock-bound distal enhancers to relevant promoters[200]. Although CLOCK–BMAL1 have been proposed to function as pioneer-like transcription factors, which promote DNA accessibility through nucleosome removal, more recent data suggest that BMAL1 rather binds to already-accessible DNA sites, thereby conceivably increasing their exposure to other transcription factors[223–225]. Although exactly how BMAL1 is recruited to genomic target regions remains to be clarified, the very limited overlap between the BMAL1 cistromes in different tissues suggests a role for cell-type-specific transcription regulators in this process.

The size of the tissue-specific circadian transcriptome can be greatly amplified through an intermediate layer of differentially expressed transcription regulators, which are directly controlled by the core molecular clock and in turn mediate the circadian expression of further downstream targets. Examples of this include the PARbZip transcription factors DBP, TEF and HLF, which show different expression patterns in the SCN as well as many peripheral tissues[70,226,227]. The PARbZip proteins are direct transcriptional targets of CLOCK–BMAL1 while controlling the rhythmic expression of genes containing D-box elements in their regulatory regions. At least in the liver, PARbZip targets include many major detoxification enzymes, thereby illustrating the importance of circadian considerations in drug metabolism[228].

Last, it should be noted that despite an overall shared molecular clock identity throughout the body, some tissue-specific adaptations to the canonical molecular clock do exist. For example, alternative forms of CLOCK–BMAL1 inhibition — through hepatocyte nuclear factor 4A and PASD1 — have been described in liver and intestinal cells and in germline tissue, respectively[79,229].

Entrainment of circadian clocks

Central and peripheral clocks are aligned with the phase of external zeitgebers through entrainment. Zeitgebers include light, temperature, food, exercise and mechanosensory stimulation, and entrain circadian rhythms by acting either on the central pacemaker or directly in peripheral tissues.

Clock entrainment in D. melanogaster

Overall, entrainment of peripheral oscillators in insects is less dependent on a master pacemaker compared with mammals, by virtue of their direct responsiveness to external zeitgebers such as light and temperature[230]. The sensitivity of the circadian clock to a zeitgeber varies with the phase during which it is applied, as described in so-called phase-response curves. When different groups of flies are exposed to 15-min light pulses at different zeitgeber times, a phase shift in the circadian locomotion with respect to the original phase is observed at times of increased sensitivity, such as after dusk and before dawn, but not during 'dead zones', when light has no effect on the circadian phase[231,232]. In general, a light pulse at the beginning of the night results in a phase delay due to the transient degradation of cytoplasmic TIM, whereas a light pulse in the early morning leads to phase advances owing to nuclear TIM degradation. A light pulse during the day has no impact on phase resetting, as during this 'dead zone' TIM levels are too low to be affected by light-induced degradation.

The *D. melanogaster* photoreceptor CRY, which is a flavoprotein similar to DNA photolyases[233], is expressed in most clock-containing tissues. Upon excitation with light at a wavelength of 450 nm (the most potent zeitgeber in *D. melanogaster*[160,234] and in mammals[235]), CRY binds to TIM[18], leading to recruitment of the E3 ubiquitin ligase Jetlag[19] and rapid degradation of both CRY and TIM[19,236] (FIG. 1), thereby resetting the clock[231,237,238]. However, in the absence of CRY, light resetting can still occur through the fly visual system, specifically through the rhodopsin 1–7 photoreceptors[239]. Rhodopsin 7 has a higher excitation maximum than the other rhodopsins and CRY, thereby facilitating entrainment of the circadian clock to longer wavelengths in *D. melanogaster*[240].

In addition to light, temperature cycles can also entrain the fly circadian clock (reviewed in[142]), with differences as small as 2 °C being sufficient for robust entrainment. Ionotropic receptors in the chordotonal organ sense temperature, which is transmitted to the large ventral lateral neurons clock in the brain for entrainment of temperature cycles[241]. Other zeitgebers include vibration cycles, which are sensed through proprioceptive organs and can affect circadian locomotor rhythms[242], and feeding rhythms, which entrain circadian rhythmicity of insulin-producing cells to match food availability[243]. The fat body clock is also entrained by feeding and, as in mammals, the provision of food at odd times phase-shifts the fat body, but not the brain clock, resulting in the two tissues being in a state of jetlag relative to each other. If food availability is in sync with the normal activity patterns of flies, limiting food access to certain daytime hours improves heart function during ageing, sleep duration[244] and reproduction[33], resulting in a positive effect on health. Notably, the extended lifespan attributed to caloric restriction[245] functions through clock-independent processes[246].

In *D. melanogaster*, the ubiquitous bacterial endosymbiont *Wolbachia* has recently been shown to affect locomotor rhythms, raising the question of whether the microbiome can act as a zeitgeber[247]. The existence of different zeitgebers shows that the clock uses multiple routes of entrainment, which are integrated in the brain. If animals are presented with multiple zeitgebers such as light and temperature, which are out of phase with each other, light usually dominates due to the presence of CRY in clock neurons[248] — but such conflicts can reduce the amplitude of the molecular clock and change behaviour rhythms[249], thereby illustrating that aligning different cues is optimal for clock robustness.

Ionotropic receptors
Ligand-gated ion channels, which form pores for specific ions in the plasma membrane upon binding of a specific extracellular ligand.

Chordotonal organ
A sensory organ found along the body wall of insects and crustaceans, which operates as an auditory organ, a position and movement sensor or a sensor of wind, gravity or temperature.

Clock entrainment in mammals

Although light also acts as the primary zeitgeber in mammals, including in mice and humans, they can only perceive it through the eye. Thus, unlike in *D. melanogaster*, the core molecular oscillator present in tissues and cells throughout the body is not directly regulated by light. In fact, the mammalian homologues of the main fly circadian photoreceptor, CRY1 and CRY2, have lost photosensitivity and adopted a novel function as transcriptional repressors[250]. Consistent with a light-independent role, this transcription repression function does not require residues that mediate light responses in *D. melanogaster*[251]. Instead, in mammals, the central pacemaker receives photic time-of-day information from intrinsically photosensitive retinal ganglion cells expressing the photopigment melanopsin (reviewed in[252]). Through the retinohypothalamic tract, intrinsically photosensitive retinal ganglion cells couple directly to the SCN, which subsequently conveys phase information to peripheral oscillators elsewhere in the body (FIG. 5).

Peripheral clocks are responsive to phase-adjustment signals from the SCN, which are essential for maintaining optimal phase relationships between the central and different peripheral oscillators[188]. Direct SCN output pathways include efferent projections to other, mostly hypothalamic, brain regions and humoral signals, including oscillations in glucocorticoid and melatonin[151]. Circadian rhythms in core body temperature offer another way to modulate peripheral oscillations. Whereas the SCN itself is resistant to temperature changes, peripheral clocks can be reset by physiological temperature cycles or short-term heat pulses[253–256]. This process depends on the transcription factor heat-shock factor 1 (HSF1), whose activity oscillates in a circadian manner[257]. Temperature cycles also mediate the rhythmic expression of cold-inducible RNA-binding protein (CIRP), which increases the amplitude of circadian gene expression[258]. Circadian rhythms in blood and tissue oxygenation have also been described and cycles in oxygen and carbon dioxide concentrations in the physiological range can reset the circadian clock of cultured cells[259,260]. For this form of entrainment, the interactions discussed above between molecular clock components and HIF1α-mediated hypoxia signalling provide a fitting mechanistic explanation. The existence of a systemic blood-borne factor that can entrain at least a subset of peripheral oscillators has been deduced from parabiosis experiments between SCN-lesioned mice and normal mice[261]. Although the exact nature of this signal remains elusive, an oscillating activity was found in serum that induces rhythmic changes in actin dynamics and in the activity of the transcription factor serum response factor (SRF), whose target genes include PER2 (REF.[262]).

The SCN can also affect peripheral clock synchronization indirectly, by modulating behavioural rhythms in rest–activity and food intake. This has been most prominently demonstrated in the liver, where cycling of only a small fraction of the circadian transcriptome can be sustained by systemic signals in vivo in the absence of the local oscillator[263]. Yet circadian cycling in the liver is highly sensitive to the timing of food intake, to the extent that it can in large part be maintained by time-restricted feeding even in the absence of a functional clock[264–268]. Indeed, it has long been known that a food-entrainable oscillator can reset the phase of peripheral clocks, albeit not of the SCN[264,269]. One of the food-induced synchronization signals has been suggested to be insulin, which can mimic feeding-induced clock resetting and shift the phase of select peripheral oscillators, but not of the SCN[270–272]. More generally, the activity of several clock-modifying enzymes, including AMPK, SIRT1 and PARP1, depends on metabolic states, thereby conceivably enabling clock resetting through food intake[99,101,273]. Analogous to the relationship between food and the liver clock, timed exercise can reset the circadian clock in skeletal muscles[274]. Although the mechanism underlying this phenomenon remains to be clarified, it is conceivable that exercise-induced transient hypoxic stress and induction of HIF1α could modulate clock cycling as described above. Organ-specific microenvironments can also have different effects on the respective peripheral oscillators. For example, the mammary circadian clock is sensitive to mechano-chemical stiffness of the extracellular matrix, but shows opposite responses in different cell types: whereas a stiff microenvironment dampens circadian oscillations in epithelial cells, it strengthens them in fibroblasts, and vice versa[275,276]. The net result of the functions of all these mechanisms in normal physiological conditions is an optimal internal phase relationship between the central and various peripheral clocks in the body. This is crucial for sustaining the many cyclic variations in neurological, metabolic, endocrine and cardiovascular function that are essential to human health.

Circadian dysfunction diseases

The molecular circadian clock operates in most cells of the body and exerts temporal control over the physiological activity of different tissues and organs, thus leading to cyclic variations in gene expression and tissue function. Whereas normal circadian regulation of these processes promotes physiological homeostasis, circadian dysfunction can in turn adversely affect them and lead to various neurological, metabolic, endocrine, cardiovascular and immune function comorbidities.

Diseases and ageing in D. melanogaster

Altered circadian rhythms in flies have been linked to various pathologies, including immune and neurodegenerative dysfunction and ageing-related diseases. Survival of bacterial infection depends on the precise timing of infection: night-time exposure generally improves survival[277]. Clock mutations alter the ability of flies to fight certain infections, which is related to the impaired generation of antimicrobial peptides and the ability to stage a phagocytic response[277–279]. *D. melanogaster* sleep more after infection in a time-of-day-dependent manner, which is required for recovery. Mutants of the antimicrobial peptide-encoding gene *nemuri* are deficient in the acute increase in sleep that follows infection, which is linked to reduced survival[280]. Flies serve as models for various neuropsychiatric pathologies, which have been linked to disturbances of the circadian clock, and vice versa. The fly orthologue of Ataxin 2, mutations in which cause spinocerebellar ataxia type 2 in humans, is

Parabiosis
The surgical joining of two organisms to form one shared physiological system.

Chronotype
The intrinsic preference of an individual with regards to the timing of rest and activity during a 24-h period, including early (also referred to as morningness), intermediate or late (also referred to as eveningness).

required for *per* expression in clock neurons, thereby linking Ataxia with the function of the molecular clock[49,50]. In a fly model of Huntington disease, overexpression of mutant Huntingtin causes arrhythmia in a heat shock protein-dependent manner[281], whereas lowered expression of the DBT regulator Spaghetti is linked to shortened lifespan and age-related locomotor deficits in a fly model of Alzheimer disease[282].

During ageing, sleep is fragmented and the amplitude of locomotor rhythms is reduced along with period changes despite the persistence of molecular oscillations[283] (FIG. 4), which is believed to be due to progressive desynchronization of different groups of pacemaker neurons[284]. Adding the zeitgeber temperature, reducing PKA signalling[284] and CRY overexpression[285] can improve rhythms in aged flies. A newly discovered class of genes, many of which have a role in the oxidative stress response, exhibits de novo cycling during ageing[286]. Oxidative stress is also a shared feature of neurodegenerative disorders, which incidentally have a strong association with circadian dysfunction[287]. Interestingly, *period* null flies have lowered resilience to oxidative stress and heightened neuronal degeneration[288], and, in a sensitized background, reduced longevity[289]. In mice, inactivation of BMAL1 also reduces the lifespan, which can be partially reversed by lifelong treatment with antioxidants[290]. Antioxidant enzymes show diurnal oscillations[291,292], suggesting that time-of-day removal of toxic free radicals might link the clock to ageing.

Box 1 | **The evolutionary benefits of maintaining circadian clocks**

Circadian clocks are thought to confer an adaptive advantage based on three independent lines of evidence. First, circadian clocks are ubiquitous in nature and have likely evolved independently multiple times, because the core clock genes in animals, plants and fungi are not clearly related and an even lower level of conservation is seen between bacteria and eukaryotes[325]. Second, misalignment of internal periodicity with the environmental rhythm is deleterious to fitness and, in fact, having a mismatched clock is worse than having none at all. Growing bacteria[3], plants[326], insects[4,327,328] and mammals[1,329,330] with different intrinsic period lengths in non-matching light–dark cycles reduces their lifespan, whereas resonance of internal and environmental periods promotes longevity. Last, in environmental conditions that favour specific variations of the circadian rhythm, both in nature or in experimental settings, these rhythms are selected for and the resulting circadian clocks are altered to fit the respective external settings[331–334].

In principle, an intrinsic circadian clock could confer an adaptive advantage in two different ways. In what is referred to as the 'extrinsic advantage', having an internal clock would allow an organism to anticipate daily-recurring environmental changes in light, temperature, availability of food or mating partners and presence of predators. In support of this model, colonization of *Drosophila melanogaster* at different latitudes is accompanied by modifications of the circadian clock as an adaptation to different day lengths[334–336] and, in chipmunks and squirrels with lesions in the suprachiasmatic nuclei (where the master circadian pacemaker is located), increased mortality has been attributed to increased susceptibility to predator attacks[337]. By contrast, an 'intrinsic advantage' could arise from optimal temporal coordination of different physiologic processes. Indeed, the persistence of circadian rhythms in populations of *D. melanogaster* bred under constant environmental conditions for more than 50 years[338] as well as in some cave-dwelling[339] and polar[340] animals can be interpreted as evidence for an intrinsic adaptive value of the circadian clock, even in the absence of diurnal environmental changes. In primordial cells, circadian clocks could have provided many benefits, including minimizing DNA photo-damage by limiting DNA replication to night-time[341,342] and energy conservation by temporally separating conflicting metabolic pathways[343,344] and by transiently downregulating costly cellular processes such as gene expression[345]. Although lack of a circadian clock does not limit the lifespan in *D. melanogaster* in laboratory conditions, circadian arrhythmicity is associated with reduced fecundity[346]. Taken together, the available data suggest that circadian clocks have evolved to aid organisms to efficiently organize their temporal relationship with the environment and their internal physiological processes.

Diseases in mammals

In humans, misalignment between endogenous circadian rhythms and environmentally imposed rest–activity cycles is associated with a wide variety of diseases, most prominently metabolic, cardiovascular and mental disorders and cancer. The adverse effects of mismatched internal and external cycles on human health align with observations in hamsters, flies and cyanobacteria, which indicate that an organism's optimal fitness requires resonance between the endogenous circadian rhythm and environmental cycles[1–5] (BOX 1). Although this evolutionary conservation underscores the immutability of the underlying biological principles, contemporary lifestyles in modern societies routinely infringe on this rule. This phenomenon is referred to as social jetlag and is associated with many of the same metabolic, cardiovascular and psychiatric risks that have been found in shift workers[293–296]. Social jetlag is especially pronounced in late chronotypes, which is consistent with their higher susceptibility to many of the above-mentioned common circadian comorbidities[297–299]. The widespread use of light-emitting electronic devices, which have become nearly ubiquitous, appears to further exacerbate this trend[295,300,301]. Thus, health hazards associated with circadian dysfunction, which were originally discovered in shift workers, may indeed pose a risk for a much larger segment of the population. The epidemiological and experimental evidence for major circadian comorbidities, including metabolic, cardiovascular and mental disorders and cancer, as well as their potential clock-related aetiology are discussed in Supplementary Box 1.

The clock as a pharmacological target

The nuclear receptors REV-ERBα and REV-ERBβ are not only key components of the molecular circadian clock but also function as major regulators of metabolism and mood[210,302,303]. REV-ERB agonists show remarkable efficacy in mice in maintaining wakefulness[304–306], reducing anxiety[305], alleviating adverse metabolic effects of diet-induced obesity[306], inducing selective cancer cell death[307] and reducing neuroinflammation[308]. REV-ERB antagonists[309], on the other hand, promote mania-like behaviour in mice when applied to the ventral midbrain[303] and show cardioprotective potential for aortic valve replacement surgery performed at high-risk times of day[310]. Pharmacological activation of RORα and RORγ, molecular antagonists of REV-ERBα and REV-ERBβ in the molecular clock, strengthens circadian oscillations[311,312] and shows therapeutic potential in mouse models of Alzheimer disease and Parkinson disease, depression and obesity[311,313–315]. Stabilizers of the CRY proteins lengthen the circadian period and improve glucose tolerance[110,316], whereas inhibitors of their transcriptional repressive activity slow the growth of a breast cancer cell line[317]. Although it remains to be seen which of these therapeutic effects will be preserved in human trials, clearly there is great potential for small molecules targeting molecular clock factors for treating circadian

disorders and their associated comorbidities. One drug that clearly alters the circadian cycle and has already been clinically used for decades is the mood stabilizer lithium, which lengthens the circadian period[318–320]. Although lithium is known to inhibit the clock modifier GSK3, other means of decreasing GSK3 activity have in fact the opposite effect on the circadian period[319,321,322]. Thus, additional studies will be required to characterize the molecular mechanisms through which lithium modulates the circadian clock.

Conclusions and future perspective

Understanding how circadian clock genes work together has provided a direct view of the relationship between genes and specific behaviours, in particular the sleep–wake cycle. Next, the intercellular circuitry and external input signals that affect circadian clocks are being elucidated. Together these advances are showing us how circadian clocks connect different environmental and internal stimuli, and inform an organism, including its different organs, tissues and cells, about the time of day. Finally, we have arrived at a time where the profound effects of circadian clocks on our body have fully penetrated into mammalian and human research, showing that circadian disruption can have considerable effects on human health. Many in our field advocate a rewriting of medical practice, to include the advances in circadian biology into the treatment of patients. Chronotherapy, light therapy and circadian intervention, among others, are all part of a new circadian medicine[323,324], which will hopefully become a safe and low-cost standard intervention for many pathologies. The accelerating progress in these research areas over the past 50 years provides a stunning example of how fundamental research can generate new insights into biology and suggest important new applications for improving human health.

Published online: 25 November 2019

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Acknowledgements
This work was supported by grants from the National Institutes of Health (GM054339 and NS053087) and Calico Lifesciences LLC to M.W.Y.

Author contributions
M.W.Y. established the scope of the review. A.P. and S.A. researched data and wrote the subsections on mammals and flies, respectively, and jointly contributed to the remaining subsections. All authors made substantial contributions to the discussion and revision of the manuscript prior to submission.

Competing interests
Research in the laboratory of M.W.Y is partly funded by Calico Lifesciences LLC.

Peer review information
Nature Reviews Molecular Cell Biology thanks Felix Naef, Satchidananda Panda and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.

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Supplementary information
Supplementary information is available for this paper at https://doi.org/10.1038/s41580-019-0179-2.