Signaling components that drive circadian rhythms
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In the past year, knowledge of how information is relayed in the regulation of circadian rhythms has advanced considerably. Experiments using genetic knockout animals suggest that circadian photoreception consists of an integration of multiple signaling pathways. Versatility of clock proteins is seen in terms of their function in the central pacemakers versus the periphery. This versatility also extends to previously identified molecules, such as retinoid receptors, redox factors and mitogen-activated protein kinase, that have newly identified roles in circadian signaling pathways. Advances in circadian research over the past year include the common themes of redundancy and plasticity.

Introduction
The physiological properties of most organisms display a daily or circadian — Latin for ‘about a day’ — pattern of activity, which is regulated by an endogenous circadian clock. Models of the internal system that maintains circadian rhythms incorporate three different components [1]. The first is an input pathway, which transmits information from external stimuli to the internal clock and thus synchronizes it with the environment. The second component is the pacemaker itself, which generates the endogenous daily rhythm. This central clock is dependent on the rhythmic (~24 hours) expression of autoregulatory proteins that repress their own transcription by interacting with their transcriptional activators (Figure 1) [2,3]. In Drosophila melanogaster and mammals — the animal models highlighted in this review — the transcriptional activators are CLOCK (CLK) and BMAL1 (also known as CYCLE in Drosophila). The repressing factors are PERIOD (PER) and TIME-LESS (TIM) in flies; in mammals the repressing factors are the products of the cryptochrome (cry) genes, mCry1 and mCry2, although some of the three mammalian homologs of PER may also modulate negative regulation [3–5]. In addition, interlocked positive regulatory loops exist whereby the repressive factors also positively regulate the transcription of some of the activators [6,7]. Mutations of the activators and repressors alter the period of the clock or lead to arrhythmia. The last component is a circadian output pathway, which conveys information from the central oscillator to effect the daily changes in an activity or physiological process.

Redundant input pathways
In all organisms where the molecular mechanisms of entrainment have been studied, light changes the levels of a clock component, thereby resetting the clock to a different time of day. Thus, in Drosophila, TIM is degraded by light, whereas in mammals and Neurospora, mPer1 and mfrq mRNA levels increase in response to light. Work in Drosophila indicates that redundant pathways mediate circadian entrainment (Figure 2a). The visual system is not required for light entrainment, as anatomical mutants lacking eyes and norpA mutants — which lack the visual transduction protein phospholipase C — are able to entrain to light [8–10]. However, a critical factor required for circadian photoreception is cry. Flies mutant for this gene, cryb, are partially ‘circadian light blind’, as they cannot shift their clocks in response to light pulses and remain rhythmic in constant light, whereas wild-type flies become arrhythmic in constant light [11,12]. Indeed, in whole head extracts and in malphigian tubules from cryb flies, the acute response of TIM to light is abolished [13,14]. CRY itself is degraded in response to light through a process that involves electron transport [13].

Yet, cryb mutants are not totally circadian blind, as they maintain an entrainable molecular clock in their lateral neurons (LNs) — the Drosophila central pacemakers — and can entrain their rest–activity behavior to new light:dark (LD) cycles [11]. It appears that the external eyes, cry and at least one other pathway contribute to circadian photoreception. glass60 mutants — cryb double mutants — which lack CRY as well as all known internal and external structures of the eye, are circadian-blind [15•].
The data indicate that some extraocular photoreceptor located in one of the anatomical structures ablated in *glass60j* mutants participates in circadian entrainment. Two candidate sites for this anatomical location are the Hofbauer–Buchner (H–B) eyelet, found underneath the retina of the compound eye, and the DN1 dorsal neurons [15•]. Both structures project to LNs and are eliminated by the *glass60j* mutation [15•,16,17]. The use of redundant input pathways for entraining rhythms may be conserved through evolution. Mice show deficits in entrainment to LD cycles only when both *cry* genes and all rods and most cones are knocked out (Figure 2b) [18]. Interestingly, a substantial behavioral response to light remains in these mice, suggesting the presence of an additional photoreceptor for entrainment. However, the role of mammalian CRY in circadian photoreception is still unclear, because the action spectrum of CRY does not match that of circadian resetting [19].

**Central pacemaker versus peripheral clocks**

Although CRY does not appear to play a role in central pacemaker activity in *Drosophila*, it may function as a clock component in peripheral clocks that drive rhythms of local
tissue-specific functions. For instance, a circadian rhythm in olfactory activity in *Drosophila* antennae can be observed, as measured by electroantennogram (EAG) responses [20]. *cry* mutants show no rhythmic EAG output under constant conditions after entrainment to either an LD or a temperature cycle [21•]. Cultured *cry* antennae also show reduced molecular rhythms in both LD and constant darkness (DD) [21•]. Such effects on the functioning of the core antennal oscillator indicate that CRY is vital to this clock [21•].

In mammals, an analogous picture emerges where a homologous protein assumes the job of a core clock component in the periphery. Although the mechanisms of clock function in the periphery appear to be the same as those in the mammalian pacemaker (the suprachiasmatic nucleus [SCN]) [22], some of the proteins involved are different. MOP4 is highly homologous to CLK and can similarly heterodimerize with BMAL1 to activate transcription of *clock* genes [23,24]. Because MOP4 is not found in the SCN, it apparently does not play a role in the central clock [7,25,26]. However, MOP4 is expressed in the vascular smooth muscle of mice and, surprisingly, *Mop4* mRNA, as well as *bmal1*, *mCry1*, and *mPer2* mRNAs cycle in this tissue [27•]. In addition, MOP4 is expressed in and contributes to clock function in the basal forebrain [28]. Thus, although the central pacemaker and peripheral clocks both use autoregulatory feedback to maintain rhythmicity, they also seem capable of using different components.

**MOP4 modulates peripheral clocks**

Both MOP4 and CLK interact with the retinoic acid receptor (RAR) and retinoid X receptor (RXR) [27•]. Coexpression of RXRα or RARα abrogates DNA binding by CLK–BMAL1 and MOP4–BMAL1 heterodimers and inhibits transcriptional activation from a luciferase reporter, in a ligand-dependent manner [27•]. This ligand-dependent abolition could be a mechanism by which steroid hormones and/or vitamins affect peripheral clocks (Figure 3a). Indeed, retinoic acid delays *mPer2* rhythms in both cultured human vascular smooth muscle cells and in the heart and aorta of mice, when injected intraperitoneally [27•]. Glucocorticoids also reset peripheral clocks, although the mechanisms for this are unknown [29].

Metabolic state may be another regulator of clock function (Figure 3a). Changes in the ratio of oxidized and reduced forms of nicotinamide adenine dinucleotide (NAD and NADH, respectively) alter the binding affinity of both CLK–BMAL1 and MOP4–BMAL1 to their DNA recognition sites [27•].

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**Figure 2**

Models for circadian input. (a) At least three different input pathways transmit light information to the central *Drosophila* pacemaker. One depends on CRY, which is degraded in response to light in a manner dependent on electron transport. TIM is the clock component that is responsive to light. TIM’s light-induced degradation is mediated by CRY and leads to resetting of the clock. The visual system (i.e. compound eyes) is not necessary for circadian entrainment, but does contribute to entrainment. A third extraocular pathway exists, which may be mediated by the H-B eyelet or the DN1 dorsal neurons. It is unknown if the visual system and the extraocular pathway promote clock resetting through TIM. (b) A model for circadian input in mammals. The light responsive clock component in mammals is *mPer1*. Transcription of *mPer1* is induced by light. The eyes are required for mammalian circadian entrainment, but mutant mice lacking all rods and most cones can still entrain. The cryptochromes, which are expressed in other retinal layers, are thought to play a role in circadian photoreception, in addition to their role as core clock components. An alternate pathway, using an unidentified photoreceptor (possibly opsin-based), may also exist.
Sites [30**]. The reduced form (NADH) promotes DNA-binding of the heterodimer, but if the oxidized form (NAD) predominates, then BMAL1 homodimers, which do not involve their PAS (Per/Arnt/Sim) domains, which are likely mediated by MAPK signaling.

**Developmental kinases and the clock**

Post-translational modifications, such as phosphorylation of clock proteins, are crucial for maintaining the circadian oscillations that underlie clock function. In Drosophila, tim as well as per RNA cycling are dispensable for behavioral rhythmicity [33–36]. Behavioral rhythms were restored in tim01 mutants by a transgene containing the native tim promoter, although RNA encoded by this transgene cycled weakly if at all [35]. Moreover, constitutive expression of both per and tim rescued behavior in per01/tim01 double mutants [36]. Cyclic expression of the clock proteins continued in the small LNs despite noncycling RNAs and was responsible for maintaining behavioral rhythmicity.

**Signalling pathways to peripheral clocks.** (a) Secreted signals from the SCN modulate some circadian outputs. Hormones and vitamins alter transcriptional activity in peripheral clocks. Clocks in peripheral tissues may also be influenced by feeding and/or the redox state. Although feeding does not feed back on the central clock, an entraining role for redox activity is possible. Secreted signals from SCN cells can drive clock function in fibroblasts, but it should be noted that transplant experiments indicate that some SCN-driven rhythms may also require synaptic activity [5]. The mechanism by which the SCN confers rhythmicity to peripheral clocks is not known, but likely involves regulation of MO4–BMAL1. TGFα is a secreted factor that influences locomotor activity rhythms and timing of the sleep–wake cycle. In Drosophila, circadian locomotor activity requires PDF, which is rhythmically released by the lateral neurons. Effects of PDF are likely mediated by MAPK signaling.

Stability of clock proteins may be regulated by phosphorylation [37–39]. The double-time (dbt) gene encodes a casein kinase Iε (CKIε) homolog that is integral for PER phosphorylation and is also involved in cell proliferation and survival in imaginal discs [40–42]. Non-lethal mutant dbt alleles can shorten or lengthen the period of locomotor activity [40,43]. DBT is thought to phosphorylate PER and destabilize it. Thus, its actions on PER are opposed by those of TIM, which stabilizes PER [44]. DBT appears to play a similar role in mammals. In cell culture, mammalian and human CKIε can phosphorylate and destabilize the mouse and human homologs of per, mPer1 and hPer1 [45,46]. Hamster tau mutants, which have a short-period phenotype due to a missense mutation in the hamster CKIε gene, show greatly reduced PER kinase activity in vitro [47]. Finally, the CKIε phosphorylation site of hPer2 is mutated in a family afflicted with the circadian disease, familial advanced sleep phase syndrome (FASPS) [48**].

Although levels of Drosophila DBT do not cycle, subcellular localization of DBT follows the circadian pattern of PER [49]. Because PER and DBT are in a complex at all times tested, it is unlikely that TIM stabilizes PER by preventing its association with DBT. Rather, TIM may block phosphorylation of PER, so that maximally phosphorylated forms of PER are found only after TIM is degraded at daybreak [49]. DBT activity also affects nuclear localization of PER [49,50].

A second kinase, SHAGGY (SGG), the fly homolog of glycogen synthase kinase 3 (GSK3) and a component of the Wingless (Wg) signaling pathway, affects phosphorylation of TIM. sgg overexpression results in a short-period phenotype and increased TIM phosphorylation, whereas sgg hypomorphs exhibit a longer period (~26 hours) [51*]. Nuclear localization of PER occurs ~2 hours earlier in sgg overexpression larvae versus wild-type larvae. Thus, both SGG and DBT may regulate period length by controlling nuclear translocation of the PER/TIM heterodimer [51*].

**Signalng pathways that mediate output**

Although a great deal of research has focused on understanding circadian input systems and the central clock, only recently have we gained greater insight into the mechanisms by which the central pacemaker regulates peripheral clocks. The generation and timing of a signal to regulate peripheral clocks and circadian output may result from the assimilation of numerous signals from individual SCN cells.

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Signaling components that drive circadian rhythms

Wang and Sehgal

Using chimeric mice created from wild-type and clock mutant embryos showed that the SCN integrates the periods of individual cell oscillators, both mutant and wild-type, to generate a period for the animal as a whole [52]. The independent variability of period and amplitude of circadian rhythms in the chimeras suggests that different cell types determine these components of circadian rhythms.

An immortalized SCN cell line can induce rhythms of per gene expression and circadian metabolic activity in cocultured NIH/3T3 fibroblasts [53]. The conferred rhythms were phase-delayed 4–12 hours relative to those seen in the SCN cells, a delay similar to that seen between the SCN and peripheral tissues in vivo [54–56]. A diffusible signal from the SCN cells is likely required for inducing rhythmicity, because the two populations of cells were separated by a semi-permeable membrane, so as to prevent physical contact. Although the SCN factors that drive rhythms in peripheral oscillators are not known, activity rhythms are driven, at least in part, by transforming growth factor (TGFα), which is secreted rhythmically by the SCN [57**].

In Drosophila, a secreted factor that is required for rhythmic rest–activity is the neuropeptide pigment-dispersing factor (PDF) [58]. Effects of PDF on activity rhythms involve components of the mitogen-activated protein kinase (MAPK) signaling pathway (Figure 3b). This was demonstrated through studies of the Drosophila homolog of the neurofibromatosis-1 (nf1) gene, which encodes a Ras-GTPase activating protein, neurofibromin [59]. nf1 null mutants display arrhythmic locomotor activity in DD [60*]. Molecular cycling of both per and tim RNA and proteins is preserved in nf1 mutants, indicating that neurofibromin functions downstream of the clock. nf1 mutants have increased levels of activated MAPK, and nf1-induced arrhythmia is rescued by loss-of-function mutations in the Ras-MAPK signaling pathway [60*]. Interestingly, the arrhythmic clock mutants per01, tim01, and pdf01 mutants have decreased levels of activated MAPK. Levels of phosphorylated MAPK cycle near nerve endings that are thought to rhythmically release PDF [60*,61].

MAPK also plays a role in circadian output in other organisms. Activity of MAPK is high at night in embryonic chicken photoreceptors and is likely responsible for an increase in the affinity of cGMP-gated ion channels, which are important for coupling photon absorption to neural processing [62]. The activated forms of components of the classical Ras-MAPK pathway, such as MAPK, MAPK/Erk (extracellular regulated kinase) kinase, Raf-1, and Ras are also expressed in a circadian manner in the chick pineal gland, with a peak occurring at night [63]. In addition, MAPK plays a role in input pathways in mammals as well as in chicks [63–66].

Conclusions and future directions

Signaling is important for all components of the circadian system (Figure 4). The importance of transmitting light information to the central clock can be seen in the redundancy of input pathways. Plasticity in circadian rhythms has previously been noted [67] and is manifest in many ways. The same protein plays different roles in different systems. For example, the mouse CRY proteins seem to have taken over the role of Drosophila TIM as binding partners for the mammalian PERs; also, CRY is a clock component of peripheral, but not central oscillators in Drosophila. The role of central clock proteins can be taken over by homologous proteins in the periphery (e.g. MOP4).

Reports from this past year detail how well-known signaling pathways (e.g. Wg, MAPK) and proteins and molecules involved in other functions and diseases (NAD/NADH, RXR, RAR, CKIε, TGFα, and neurofibromin) are intimately involved in maintaining circadian rhythms. Expanding the search for genes involved in clock function by looking at genes involved in other known signaling pathways has clearly been fruitful. Recent genome studies

Figure 4

Signaling molecules of each of the components of the circadian system. Circadian input uses redundant pathways that include dedicated circadian photoreceptors and the visual system to transmit information from environmental stimuli to the clock. CRY plays a role in the core clock in mammals and may also play a role in peripheral clock function in Drosophila. In the central clock, dedicated clock genes exist as well as genes that are not solely dedicated to the clock, such as DBT and SGG (note that SGG is, thus far, implicated only in Drosophila rhythms). To date, the only known role of PDF is mediating circadian rhythms in Drosophila. The other factors that mediate output are not dedicated to the clock.

Using chimeric mice created from wild-type and clock mutant embryos showed that the SCN integrates the periods of individual cell oscillators, both mutant and wild-type, to generate a period for the animal as a whole [52]. The independent variability of period and amplitude of circadian rhythms in the chimeras suggests that different cell types determine these components of circadian rhythms.

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on cycling genes and gene expression in clock mutants [68,69] should further expand our knowledge of the mechanisms modulating circadian rhythms.

**Update**

Phosphorylation of mammalian clock proteins may involve CK1ε in addition to CK1δ [70]. Breakthroughs have recently been made, identifying circadian photoreceptive mechanisms in mammals. A small subset of ganglion cells in the mouse retina that have axons projecting to the SCN also contain a photopigment known as melanopsin [71–73]. Electrophysiology experiments indicate that these ganglion cells are also directly responsive to light [74**]. These data make melanopsin a leading candidate as a mediator of circadian photoreception, along with the visual pathway and the circadian rhythms (Figure 2b). Finally, recent progress has elucidated output mechanisms. Diurnal modulation of calcium currents appears to underlie the rhythm of spontaneous firing rate in the SCN [75].

**References and recommended reading**

Papers of particular interest, published within the annual period of review, have been highlighted as:

* of special interest
** of outstanding interest

16. The authors demonstrate that, in addition to CRY in the LNs and the compound eyes, an extracellular photoreceptor mediates light input. The H-B eyelet and DN1 dorsal neurons both project to the LNs and are candidate sites for extracellular photoreception.
22. The authors present evidence that although CRY does not appear to be required for central clock function in Drosophila, it contributes to clock activity in a peripheral tissue, the antenna.
28. McNamara P, Seo SB, Rudic R, Sehgal A, Chakravarti D, Fitz Gerld GA: Regulation of CLOCK and MOP4 by nuclear hormone receptors in the vasculature: a humoral mechanism to reset a peripheral clock. Cell 2001, 105:877–893. MOP4 is a component of a vascular clock. Interaction of the nuclear receptors RAR and RXR with CLOCK and MOP4 blocks transcriptional activity of CLOCK-BMAL1 and MOP4-BMAL1 in a ligand-dependent manner. This provides a mechanism by which hormones could signal peripheral clocks.
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Signaling components that drive circadian rhythms

Wang and Sehgal


74. Berson DM, Dunn FA, Takao M: Phototransduction by retinal ganglion cells that set the circadian clock. Science 2002, 295:1070-1073. This paper shows that some retinal ganglion cells are directly light responsive. This is the first demonstration of photic sensitivity in a non-rod, non-cone cell type in the retina.