Published in final edited form as:

Trends Neurosci. 2012 September ; 35(9): 574–585. doi:10.1016/j.tins.2012.05.007.

Speed control: cogs and gears that drive the circadian clock

Xiangzhong Zheng¹⁾ and Amita Sehgal^{1),2),*}

¹⁾Department of Neuroscience, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA 19104

²⁾Howard Hughes Medical Institute, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA 19104

Abstract

In most organisms, an intrinsic circadian (~24 h) timekeeping system drives rhythms of physiology and behavior. Within cells that contain a circadian clock, specific transcriptional activators and repressors reciprocally regulate each other to generate a basic molecular oscillator. A mismatch of the period generated by this oscillator with the external environment creates circadian disruption, which can have adverse effects on neural function. Although several clock genes have been extensively characterized, a fundamental question remains: how do these genes work together to generate a ~24 h period? Period altering mutations in clock genes can affect any of multiple, regulated steps in the molecular oscillator. In this review, we examine the regulatory mechanisms that contribute to setting the pace of the circadian oscillator.

Keywords

circadian period; kinase; phosphatase; phosphorylation; stability; nuclear localization

Introduction

An intrinsic circadian timing system enables organisms to adapt to daily light:dark and temperature cycles on earth. In mammals, the central circadian pacemaker is the suprachiasmatic nucleus (SCN) that consists of 20,000 heterogeneous neurons in the hypothalamus, above the optic chiasm. This central pacemaker orchestrates a range of rhythmic processes, the most prominent being the ~24 h sleep:wake cycle, through its direct regulation of downstream neurons, but also by synchronizing cell-autonomous oscillators throughout the body. Within clock cells, a molecular clockwork sustains the rhythmic expression of clock genes. How this molecular oscillator generates a ~24 h period that couples to the daily environmental cycle has been a subject of extensive investigation over the past two decades.

Studies of circadian clocks in mammals, flies, fungi and cyanobacteria have revealed a remarkably conserved molecular framework for circadian timing. Although extreme circadian disruptions are rare in humans, a large proportion of the human population may

© 2012 Elsevier Ltd. All rights reserved.

*Corresponding author: Department of Neuroscience, HHMI University of Pennsylvania Perelman School of Medicine TRC 10-136 3400 Civic Center Blvd, Building 421 Philadelphia, PA 19104 Tel: 215-573-2985 Fax: 215-746-0232 amita@mail.med.upenn.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

have internal circadian periods that do not synchronize appropriately with the daily light:dark cycle, or with work or social schedules. Accumulating evidence demonstrates that mismatches of our body clock with the environment have adverse health effects [1-3]. Thus understanding the basic mechanisms of circadian period determination is of great interest to researchers and clinicians alike. In this review we examine the contributions of multiple temporally gated events in the molecular clock, such as circadian transcription/translation, clock protein phosphorylation and degradation, nuclear retention and suppression of transcription in setting the speed of the circadian clock in fruit flies and mammals. We also discuss how hardwired components of the clock machinery integrate with intracellular signaling pathways and components of energy metabolism to regulate circadian period.

Transcriptional regulation of circadian period

The basic molecular circadian oscillator in eukaryotes consists of a transcriptional activator and a repressor, such that the activator drives transcription of the repressor mRNA, and accumulated repressor protein feeds back to inhibit the activator (Fig 1A). While we still do not know exactly how this feedback loop is maintained and how it generates a ~24 h period, the thinking is that it requires delays between various steps of the cycle. It is known that post-transcriptional and post-translational mechanisms generate a delay between repressor mRNA and protein accumulation in the cytoplasm, and a delay in the nuclear expression of the repressor protein, which results in sequential activation and repression of clock gene expression (Fig 1B). In addition to this major negative feedback loop, an additional feedback loop positively and negatively regulates the transcription of the activator gene (Fig 1A). Importantly, positive and negative feedback regulation of clock genes also exists in prokaryotes such as cyanobacteria, although transcriptional mechanisms are not necessary for rhythm generation in cyanobacteria [4].

In *Drosophila*, the activator is a heterodimeric complex of CLOCK (CLK) [5] and CYCLE (CYC) proteins [6]. Both CLK and CYC are constitutively expressed in the nucleus [7-8], and they heterodimerize and bind to E-box enhancer elements (motif cacgtg) in the promoters of repressor genes, *period* [9-12] and *timeless* [13-15], to activate their transcription during the day [5-6, 16-17] (Fig 2 and 3). Expression of PER and TIM is delayed relative to their RNAs, such that RNA expression peaks at the end of the day and protein peaks in the middle of the night. Increased accumulation of PER-TIM in the nucleus inhibits CLK-CYC activity, and hence repression of transcription in the late night and early morning [17-19]. Degradation of PER and TIM during the day allows a new cycle to begin (Fig 3). Critical to this feedback loop is the sequential activation and repression of clock gene expression (Fig 1B). Lack of either one of the four genes mentioned abolishes molecular and behavioral rhythms under constant dark conditions. This basic organization of the molecular circadian clock is well conserved in other organisms (Fig 2).

Clk mRNA exhibits robust circadian expression as a result of another feedback loop in *Drosophila*, where CLK-CYC activate the expression of a transcriptional repressor, *vrille* (*vri*) and an activator, PAR domain protein 1e (*Pdp1e*) [20] (Fig 2). A yet unknown mechanism delays the peak expression of *Pdp1e*, so that accumulation of *Pdp1e* lags behind that of *vri*. Since VRI and PDP1e bind to the same regulatory element in the *Clk* promoter, this temporal expression profile ensures tandem repression and activation of *Clk* transcription [20-21]. The significance of such regulation is not clear, since CLK protein is not expressed cyclically [8, 22] and reversing the phase of *Clk* mRNA encoded by a transgene (on top of the endogenous *Clk* profile) does not affect free-running behavioral rhythms, even though CLK protein starts to cycle in these transgenic flies [23]. Nonetheless, expression of CLK and a neuropeptide, pigment dispersing factor (PDF), which is required for rest:activity rhythms [24], is diminished in the lateral neurons of *Pdp1e* mutants [25],

underscoring the important role of PDP1 ϵ in sustaining behavioral rhythms. Similar positive and negative feedback regulation is present in the mammalian circadian clock (Fig 2), where nuclear receptors RORs activate *cyc* ortholog, *Bmal1* [26-28], and REV-ERBs repress *Bmal1* and *Clock* [29-31]. As in *Drosophila*, RORs and REV-ERBs are themselves targets of CLOCK-BMAL1 and negatively regulated by the repressors, Cryptochrome (CRY) 1 and 2 and PER, predominantly PER2 [28-29, 32]. As in case of *Clk* in *Drosophila*, cycling of *Bmal1* mRNA levels is not necessary for the rhythmic expression of *Per2* [31].

Based upon the model described above, one would postulate that activity levels of the circadian activator (CLK-CYC in *Drosophila* and Clock-BMAL1 in mammals) are important determinants of circadian period. In fact, removing one copy of wild type *Clk* or *cyc* gene in flies slows down *per* and *tim* mRNA accumulation and lengthens circadian period [5-6]. Similarly, heterozygous mice carrying a dominant negative mutation of *Clk* have lengthened period [33-35]. In line with this prediction, overexpression of *vri* causes long period behavioral rhythms, presumably due to reduction of *Clk* mRNA [21] and consequently slower *per* and *tim* accumulation [20, 36]. Also, loss of *rev-erbs* (α and β) renders a short circadian period in mice, likely due to increased *Bmal1* and *Clock* expression [32].

These data suggest that occupancy of CLK-CYC on the promoters of their target genes is rate-limiting [19]. Indeed, CLK is present at limiting amounts in *Drosophila* [7]. However, flies carrying multiple copies of wild-type *per* (apparently including its own promoter) display short circadian periods [37]. Transgenic rescue of the *per*⁰¹ mutation also revealed that levels of *per* mRNA are inversely correlated with period length [38-39], consistent with the notion that reducing or increasing dosage of *per* lengthens or shortens circadian period respectively [37, 40]. Thus, CLK-CYC binding to the *per* promoter is either not rate limiting or *per* overexpression also increases expression of *Clk*. However, when *per* or *tim* is constitutively overexpressed under the control of a heterologous promoter, some flies exhibit a long circadian period [41]. This long period phenotype is likely due to prolonged suppression of CLK-CYC activity by constitutively expressed PER/TIM. Thus a short period only occurs when *per* is overexpressed by its endogenous promoter, presumably because in this scenario the higher dose of *per* feeds back rapidly to curtail CLK-CYC activity on its own promoter and thereby prevents further accumulation of PER protein [40-41]. This was supported by other studies in which *Clk* was overexpressed or the viral transcriptional activator VP16 was fused to CYC to generate a more transcriptionally active CLK-CYC-VP16 complex. In either case these flies displayed a short period [42-43], which depended upon the presence of the native *per* promoter. Mammalian data are consistent with this idea. Overexpression of a *Clock* transgene in mice also shortens period length [44], whereas transgenic rats that constitutively express *Per1* have long circadian period [45].

It should be noted that there is a limit to the extent period will change with alterations in *per* levels. Constitutive overexpression of either *per* or *tim*, to levels that eliminate protein cycling, renders flies arrhythmic. Also, the increase in period in flies that retain protein and behavioral rhythms under these conditions is relatively small [41]. Under these conditions, period may be regulated by translational/post-translational control or other transcriptional mechanisms. In fact, in arrhythmic flies that overexpress *per*, *tim* mRNA levels are also elevated [41], consistent with the idea that PER-TIM not only suppress CLK-CYC activity, but also promote expression of *Clk* and its target genes [46]. This interlocked feedback regulation presumably adds resilience to circadian pacemaking. Another transcription factor, Clockwork orange (CWO), cooperates with PER to repress CLK-CYC mediated transcription of target genes, including *per*, *tim*, *vri*, *Pdp1 ϵ* and *cwo* itself [47-48] (Fig 2-3). *cwo* mutant flies have a long period, consistent with the idea that weaker feedback repression, as with a *per* deficiency [37], extends the circadian cycle [48-49]. Mammalian

homologs of *cwo* (*Dec1* and *Dec2*) have a similar function in modulating expression of some BMAL1-Clock target genes. However, their effect on circadian period is still controversial [50-51].

Translational regulation of circadian period

Circadian period may also be regulated at the translational level. The Twenty-four (TYF) protein, which interacts with poly(A)-binding protein (PABP) interacting protein, specifically interacts with *per* and *tim* transcripts to promote their translation in central pacemaker neurons [52]. *tyf* mutants have reduced PER levels in these pacemaker neurons and these flies have a long period, consistent with the prediction that reduced feedback repression of CLK-CYC activity lengthens circadian period. Interestingly, overexpression of *tyf* also causes a long period [52]. In this case, elevated translation of PER phenocopies the effect of PER overexpression by a heterologous promoter, perhaps due to elevated PER levels at a time when PER normally declines in wild-type flies. Thus either weaker feedback repression by PER or prolonged expression of PER, can lengthen circadian period. No mammalian homolog of TYF has been identified so far. Nevertheless, translational regulation of circadian period in mammals has been reported. Overexpression of the RNA binding protein LARK increases translation of *Per1* to cause a long period; conversely, knockdown of *Lark* results in a short circadian period in NIH 3T3 cells [53]. The longer period may result from a similar mechanism suggested above for the effect of *tyf* overexpression on circadian period. Intriguingly, in flies, *lark* does not affect molecular oscillations of PER in the central pacemaker cells, the small ventral lateral neurons (s-LN_vs), nor does it affect rest:activity rhythms, although it alters nuclear localization of PER in the large LN_vs [54]. However, loss of *lark* advances the phase of the eclosion rhythm without affecting circadian period [55]. Thus the effect of LARK on the circadian system seems to be cell type dependent.

Stability of the circadian repressor is a major determinant of circadian period

From the data discussed above, one can surmise that the accumulation and duration of expression of the repressor affects period. Predictably, factors that affect the stability of the repressor, such as phosphorylation/dephosphorylation, modulate circadian period.

Casein kinase regulates PER stability in *Drosophila*

Rhythmic expression of PER is a regulated process of temporal accumulation and degradation. In *Drosophila*, one critical regulator of this event is the Doubletime (DBT) protein, a kinase that is most closely related to human casein kinase 1 ϵ (CK1 ϵ) [56]. Mutations in *dbt* cause long (*dbt^L*), short (*dbt^S*) or arrhythmic (*dbt^{arr}*) circadian behavior. DBT physically interacts with PER and promotes PER phosphorylation and subsequently, proteasomal degradation (Fig 3). A severe hypomorphic mutation of the *dbt* gene (*dbt^P*) results in constitutively high levels of hypophosphorylated PER proteins [57]. Because PER expression is low in flies that lack TIM [58-59], it is believed that TIM stabilizes PER protein, partly by interfering with DBT in the cytoplasm. This regulation of PER stability by DBT and TIM contributes to the delay between the accumulation of *per/tim* mRNA and that of the PER-TIM complex (Fig 3).

dbt^P mutants do not survive to adulthood, which limits its use in molecular and behavioral studies. In contrast, the other three *dbt* alleles (*dbt^S*, *dbt^L*, *dbt^{arr}*) are viable and they all have dominant effects on period [57, 60], suggesting that they all interfere with the normal function of DBT. Overexpression of *dbt^L* or *dbt^S* transgene in flies phenocopies the corresponding endogenous alleles [61]. In addition, overexpression of the kinase-dead form

DBT^{K/R} produces long periods or arrhythmicity [61]. The DBT^{ar} protein has reduced enzymatic activity towards PER [62], which may account for the constitutively high levels of PER in *dbt^{ar}* flies [60]. DBT^L protein also has reduced enzymatic activity [62-63] and produces less efficient degradation of PER [64]. Thus, PER levels are higher in *dbt^L* mutants during its normal degradation phase in the morning, which results in a long period [65]. Interestingly, DBT^S has reduced activity (towards casein) [63, 66], but the rate of PER degradation induced by DBT^S is similar to that of wildtype DBT [64]. In fact, DBT^S phosphorylates PER slightly faster than does DBT^{WT} [62]. In the *dbt^S* mutants, total PER protein levels peak and disappear earlier than wildtype flies [57]. However, nuclear accumulation of PER protein in the photoreceptor cells is delayed in the night [67]. Thus it appears that a shorter duration of PER expression in the nucleus is sufficient to explain the short period of *dbt^S* mutants.

Interestingly, two short period mutations of *per* (*per^S* and *per^T*) restore behavioral rhythmicity in *dbt^{ar}* flies, suggesting that PER^S and DBT^{ar} affect the same regulatory event that controls PER turnover [60]. The *per^S* mutation (S589N) is a point mutation in a “PER short” domain (named because several mutations in this region cause a short period phenotype). DBT phosphorylation of this PER short domain (residues 585-601) suppresses phosphorylation of a downstream domain (residues 604-629) (Fig 4), leading to a more stable but less active PER; conversely, inhibiting phosphorylation of the PER short domain (by deletion or mutations, such as PER^S) promotes phosphorylation of the downstream domain, which renders PER a highly active repressor, but unstable [66, 68]. Thus, levels of PER^S protein decline prematurely in the late night [69], and flies carrying a *per* transgene (*per^{ΔS}*) lacking the PER short domain phenocopy *per^S* mutants [66]. Further analysis of this PER short domain revealed that another kinase NEMO (NMO) targets S596 to stimulate DBT phosphorylation of neighboring sites [70]. Following multi-site phosphorylation, the “PER short” domain acts as a resistor to slow down DBT phosphorylation of PER at other sites (Fig 4), including sites required for recognition by the E3 ligase, Supernumerary limbs (SLIMB) [62, 71] and consequent proteasomal degradation [70]. Reduced expression of *nmo* is expected to fasten PER degradation, and hence shorten circadian period [70, 72]. On the other hand, overexpression of *nmo* is expected to strengthen this resistor and thereby delay PER degradation; this would result in prolonged expression of PER, and consequently a long period. However, two recent studies reported inconsistent results: while one observed no effect of *nmo* overexpression on period [70], the other found a period lengthening effect [72]. Intriguingly, CLK levels increase when *nmo* expression is reduced, whereas CLK levels decrease when *nmo* is overexpressed [72]. The alteration of CLK levels may contribute to the observed change of circadian period, but this has not been addressed. Importantly, it is still unknown as how this resistor is regulated to allow progressive phosphorylation of PER that eventually leads to SLIMB-dependent degradation of PER [73].

Casein kinase regulates PER stability in mammals

In mammals, CK1 phosphorylates mammalian PER (mPER) proteins and stimulates their proteasomal degradation [74-76]. Consistent with the idea that increased stability and, therefore, prolonged expression of PER lengthens circadian period, CK1Δ/ε deficient cells/tissues exhibit a long period molecular oscillation [77-79]. Chemical inhibition of CK1Δ/ε-dependent phosphorylation of PER also produces a dramatic period lengthening effect [78]. In addition, CK1α stimulates phosphorylation and degradation of PER1 [80]. Of these, it appears that CK1Δ is the dominant regulator of circadian period [81-83].

A short period mutation, *tau*, discovered 20 years ago in the Syrian hamster [84] is caused by a point mutation in CK1ε. The *tau* mutation was initially reported to reduce CK1ε enzyme activity (towards casein or phosvitin) [85]. However, it was later found to be a gain-

of-function mutation that increases PER phosphorylation [86] and accelerates PER degradation [75, 86-87]. It appears that CK1 ϵ^{tau} preferentially targets some PER sites for degradation [86]. This may also be true of DBT S in flies.

Human subjects that suffer from familial advanced sleep phase syndrome (FASPS) display extreme early sleep and wake times, which can be associated with a short circadian period [88]. A T44A mutation in CK1 Δ , which reduces enzymatic activity, was found in one FASPS kindred. Consistent with clinical observations, transgenic mice expressing this mutant CK1 Δ^{T44A} display a short circadian period. Interestingly, transgenic flies expressing the human CK1 Δ^{T44A} exhibit a long period of behavioral rhythms [83], whereas those expressing CK1 ϵ^{tau} display a short period, similar to that in mammals [89]. Since human CK1 ϵ cannot replace DBT in *Drosophila* [90], CK1 data obtained from this heterologous system are difficult to interpret.

In another kindred of FASPS, affected individuals carry a S662G mutation in human PER2 (hPER2) that affects phosphorylation by CK1 ϵ [91]. Phosphorylation of S662 by an unknown kinase facilitates CK1 phosphorylation of neighboring sites and increases hPER2 stability. Thus, the S662G mutation reduces hPER2 protein levels, while a change at the same site to an aspartate residue (S662D) increases hPER2 [92-93]. Phosphorylation of S659 (the corresponding site of S662 in hPER2) also affects stability, and perhaps even nuclear retention, of mouse PER2 [94].

Although conflicting in some aspects, data discussed above converge on the idea that CK1 has multiple roles in circadian period regulation. Mutations in PER or CK1 may differentially affect phosphorylation of specific sites and some of these sites have opposing function. Interestingly, a similar mechanism also exists in *Neurospora* (Fig 4).

Protein Phosphatase activity stabilizes clock proteins

Temporal phosphorylation of PER in *Drosophila* does not appear to be regulated by rhythmic expression of *dbt* mRNA or protein. Instead, it seems to be a consequence of rhythmic interaction of PER-TIM-DBT proteins [57, 95]. In addition, rhythmic expression of protein phosphatase 2A (PP2A) may cause temporal de-phosphorylation of PER. mRNA expression of *twins*, a gene encoding a PP2A regulatory subunit, is regulated by the circadian clock [96]. While PP2A stabilizes PER by counteracting DBT mediated degradation [96], protein phosphatase 1 (PP1) primarily affects stability of TIM through de-phosphorylation in flies [97] (Fig 3). Inhibition of PP1 by overexpressing a nuclear inhibitor of PP1 slows down nuclear accumulation of TIM and consequently, a long period [97]. In mammals, PP1, rather than PP2A, appears to be the primary phosphatase that acts in concert with CK1 Δ/ϵ to regulate PER phosphorylation and stability [98-99]. Since CK1 expression does not cycle, it is possible that rhythmic interaction of PER/CRY/CK1/PP1 generates circadian oscillations of phosphorylation [100]; alternatively, an as yet unknown regulatory subunit of PP1 may be expressed cyclically to regulate phosphatase activity [99]. This balanced action of kinases and phosphatases on circadian clock protein also exists in *Neurospora* [101].

CRY stability influences circadian period

Like TIM in flies, CRY is an obligate partner of PER in mammals. Although PER and CRY stabilize each other, it appears that different mechanisms regulate PER and CRY stability [102]. An F-box protein of the SCF ubiquitin ligase complex, FBXL3, targets CRY for degradation [103-105]. *Fbxl3* mutant mice have reduced expression of PER1/PER2, whereas CRY1/CRY2 levels are unchanged. However, the rate of CRY degradation is reduced. Thus delayed degradation of CRY is sufficient to slow down the molecular oscillator, and

consequently a long circadian period of about 27 h. It is possible that slower degradation of CRY prolongs nuclear retention of PER [100]. Consistently with this idea, loss of *Fbx13* widens the nadir of activity of a PER2-LUC reporter, but a CK1e^{tau} mutation reverses this effect by promoting clearance of nuclear PER [102].

Regulation of nuclear accumulation and activity of circadian repressors

Not only is the circadian accumulation and turnover of PER and TIM (or CRY) important for period determination, temporally regulated nuclear localization, and duration of nuclear retention of the circadian repressor may also be critical in setting the pace of the molecular oscillator. Both PER and TIM are cytoplasmic proteins when independently expressed in insect S2 cells. Upon coexpression, direct interaction of PER and TIM promotes their nuclear localization [106-107]. However, it remains a mystery as how this event is regulated. It appears that nuclear entry is preceded by transient dissociation of PER and TIM, followed by sequential nuclear entry of PER and TIM in a circadian cycle [108-110]. Thus coordinated nuclear localization of PER and TIM is required for a normal circadian period.

TIM regulates nuclear localization of PER

TIM is constantly exported from the nucleus by exportin-mediated mechanisms [40]. While the molecular signature in TIM that regulates this export has not been characterized, it is clear that a nucleus localization signal (NLS) in TIM regulates its nuclear localization. Mutating an NLS in TIM prolongs cytoplasmic accumulation of TIM and PER but reduces their nuclear accumulation, resulting in a long circadian period in transgenic flies [111]. Several long period mutations of *tim* or *per* exhibit delayed nuclear accumulation of TIM and PER. TIM^{L1} has a mutation in the PER interaction domain and is delayed in nuclear entry [112]; likewise, PER^L has a mutation in the TIM interaction domain, and also exhibits delayed nuclear localization [113]. Recently, a new mutation of TIM (P116L) was found to sequester both TIM and PER in the cytoplasm. While *tim*^{PL} homozygotes are arrhythmic, heterozygous *tim*^{PL} mutants display a long period, likely due to less efficient nuclear accumulation of both TIM and PER [114]. In *tim*^{UL} flies, which have a 33h period length, TIM and PER accumulation in the evening is delayed, and they also display extended nuclear localization in the morning [115]. Interestingly, the *per*^S mutation, which produces a protein that exhibits normal nuclear localization in the late night [113], but faster decline in the early morning [69, 116], rescues the period length of *tim*^{UL} back to 24 h [112]. It is possible that the PER^S-TIM^{UL} complex gets degraded earlier in the morning.

The above discussion does not address an important issue: is TIM required for nuclear accumulation of PER under normal conditions? A direct answer to this question requires assays of PER protein in a *tim*⁰¹ null mutant, which has been a challenge due to the low PER levels in this mutant. However, when PER is stabilized (by virtue of a mutation at one DBT target site, serine 47 to alanine, that impairs SLIMB-dependent degradation [73]), it localizes to the cytoplasm in *tim*⁰¹ mutants [114]. Thus, TIM is necessary for nuclear accumulation of PER.

DBT and PP2A regulate nuclear accumulation of PER in *Drosophila*

PER may be held in the cytoplasm by DBT: loss of *dbt*^P results in PER accumulation in the nucleus [57] and, in fact, this can occur in the absence of TIM [117]. On the other hand, DBT is a nuclear protein when independently expressed in S2 cell or in *per*⁰¹ null mutants, but it translocates to the cytoplasm when it is coexpressed with *per* in S2 cells [110]. These findings suggest that newly synthesized PER and DBT trap each other in the cytoplasm, and an interaction of PER/TIM promotes nuclear localization of PER/TIM/DBT proteins. As

with *dbt^P* mutants, PER is hypophosphorylated and stabilized in the nucleus throughout the daily cycle when the PP2A catalytic subunit is overexpressed [96]. Thus de-phosphorylation of PER by PP2A stabilizes it in the nucleus. It is not clear if PP2A counteracts DBT in the cytoplasm to promote nuclear import of PER, or it simply counters the destabilizing effect of DBT on PER in the nucleus. Regardless, as discussed above, stabilization of PER alone is not sufficient to promote nuclear accumulation of PER. In support of this idea, it was recently found that *O*-GlcNAcylation stabilizes PER in the cytoplasm, but this modification delays nuclear accumulation of PER [118]. It is unknown how rhythmic phosphorylation and *O*-GlcNAcylation act in concert to regulate circadian period.

CK1 regulation of mammalian PER

Whether CK1 regulates nuclear expression of PER in mammals is still unclear. Liver-specific disruption of CK1 Δ increases daytime levels of nuclear PER1 and PER2, which produces a lengthened period, as measured by a molecular reporter [77]. However, in cultured CK1 Δ /e double deficient fibroblasts, PER1 and PER2 proteins are constitutively cytoplasmic, in contrast to their nuclear localization in wildtype cells [98]. Unlike PER2, PER1 is a predominantly nuclear protein when expressed alone in human embryonic kidney (HEK) 293 cells and coexpression of PER1 and CK1e leads to phosphorylation-dependent cytoplasmic retention of both proteins [119]. In contrast, CK1e and CK1 Δ promote nuclear translocation of PER3, such that mutations of potential CK1 phosphorylation sites in PER3 impair its nuclear localization [74]. It appears that different cell types and assays result in different observations.

CK2 regulation of PER

While CK1 plays a prominent role in regulating the stability and nuclear localization of PER in *Drosophila*, another casein kinase, CK2, is also important for circadian period determination. A *Ck2a* mutation (*Tic*) decreases CK2 activity, delays PER nuclear entry and lengthens circadian period [120]. A point mutation in CK2 β (*Andante*) results in a long period that is associated with delayed nuclear translocation of PER and TIM, even though PER and TIM proteins accumulate to abnormally high levels in these flies [121]. Consistent with the fly data, knockdown of *Ck2* by RNA interference (RNAi) in *Drosophila* S2 cells leads to more cytoplasmic accumulation of PER [68]. Also, in mammalian cell culture, overexpression of *Ck2a* shortens circadian period [122], whereas inhibition of CK2 activity lengthens circadian period [123].

Role of GSK3/SGG in regulating circadian period

Like PER, TIM undergoes rhythmic phosphorylation [124-125]. Since TIM coordinates nuclear localization of PER as discussed above, phosphorylation events on TIM are expected to influence circadian period. Currently the only known kinase that phosphorylates TIM is glycogen synthase kinase (GSK) 3 β [Shaggy (SGG) in flies]. Genetic evidence clearly demonstrates that SGG regulates circadian period in *Drosophila*: while reduced *sgg* expression decreases TIM phosphorylation and lengthens period, increased expression of *sgg* shortens period, which was attributed to premature nuclear localization of PER/TIM proteins [126]. Since SGG/GSK3 β was subsequently found to phosphorylate PER [97, 114, 127], it is unknown as how much of the effect of SGG on circadian period is dependent on TIM.

The exact role of mammalian GSK3 in circadian period determination is controversial. Treatment with lithium, a presumed inhibitor of GSK3 β , lengthens circadian period of firing rate rhythms of cultured SCN neurons [128], and delays the phase of circadian gene expression in NIH 3T3 cells [129]. Similarly, kenpaullone or lithium inhibition of GSK3 β induces a phase delay of *Per2* transcription in mouse embryonic fibroblasts (MEFs) [130].

Genetic depletion of GSK3 (α and β isoforms) in MEFs also lengthens period of *Per2* oscillations [130]. On the other hand, overexpression of *Gsk3 β* advances the phase of the circadian oscillation, likely due to advanced nuclear entry of PER2 [129]. However, GSK3 β may also act on REV-ERB α [131] or Clock [132] to affect the circadian clock. Knocking down *Gsk3 β* with small interfering RNA or inhibiting it with small molecule inhibitors has opposite effects from the genetic manipulations described above [133]. In support of the latter effect, a genomewide RNAi screen found that knock-down of positive regulators of insulin signaling (which inhibits GSK3 β activity) lengthens circadian period [134]. Since the specificity of RNAi and chemical inhibitors is uncertain, future studies designed to knock out *Gsk3* in a tissue specific manner may clarify its exact function in period regulation.

Intracellular signaling pathways regulate circadian period

The molecular framework discussed above is likely the primary determinant of circadian period within a clock cell (Fig 1-4). However, intracellular/extracellular signals or environmental factors may also contribute to timekeeping. Studies of known signaling pathways have indicated that some of these regulate the speed of the circadian oscillator. In addition, such pathways are thought to be used by the clock to transmit time-of-day signals to cells that directly control behavior.

cAMP-PKA signaling

In *Drosophila*, the central pacemaker lateral neurons produce the neuropeptide PDF [24], which acts through a cAMP coupled receptor [135-137], in widespread regions of the central brain [138]. While PDF likely also signals to neurons outside the clock neuron circuit, thus far studies have focused on its function in synchronizing clock neurons. Overall PDF signaling seems to lengthen the overt circadian period, since flies lacking PDF or PDF receptor have a slightly shortened period [24, 137, 139]. Interestingly, lack of PDF signaling shortens the period of the clock in some neurons and lengthens it in other neurons [139]. This different effect of PDF signaling on period is probably due to divergent cAMP signaling targets in these cells [138-139]. Reducing G_s protein signaling to cAMP in the central pacemaker neurons is sufficient to lengthen circadian period [140], whereas global loss of *dunce*, a gene encoding for a phosphodiesterase that inhibits cAMP production, results in a short circadian period [141]. On the other hand, administration of caffeine, a non-specific inhibitor of phosphodiesterase, lengthens the circadian period of behavioral rhythms in flies [142] and mice [143]. Due to the promiscuous nature of caffeine action, the mechanism underlying the effect of caffeine on circadian period is unknown.

AMPK and TOR-S6K signaling

In mammals, some components of cell survival/growth signaling and of energy metabolism regulate circadian period. For example, glucose deprivation or treatment with aminoimidazole carboxamide ribonucleotide (AICAR) activates the energy sensor AMP activated protein kinase (AMPK), which in turn phosphorylates and destabilizes CRY1 and lengthens circadian period in cultured MEFs [144]. These data conflict with the aforementioned finding that stabilization of CRY1/2 through loss of *Fbx13* also lengthens period. It is possible that CRY1 and CRY2 have different functions [145], or, AMPK preferably destabilizes CRY1 during the accumulation phase, which delays feedback repression by CRY on Clock-BMAL1, whereas loss of *Fbx13* stabilizes CRY1/2 in the degradation phase, thus prolonging protein expression. Alternatively, AMPK may act on additional clock components. Indeed, activation of AMPK through injection of metformin (a drug commonly used to reduce blood sugar levels) into mouse peripheral tissues increases CK1 ϵ dependent PER2 degradation, and shortens circadian period [146].

As in mammals, *Drosophila* AMPK may regulate CK1 activity and/or, it may influence circadian period through its role in the nutrient sensing Target of rapamycin (TOR) signaling pathway. Indeed, increased activity of TOR-S6K in central pacemaker neurons lengthens circadian period, likely by inhibiting GSK3 β /SGG [147]. The insulin-AKT cascade stimulates TOR activity, and also acts in parallel with TOR-S6K signaling to phosphorylate and deactivate GSK3 β /SGG [147-149]. In support of a role for insulin signaling in circadian period regulation, *Akt* mutant flies display a short circadian period, while loss of *Pten*, a negative regulator of insulin signaling, results in a long period [147]. Similarly, *Pten* mutant mice have a small but significantly lengthened period [150]. In addition to these genetic studies, feeding mice a high-fat diet, which potentially elevates mTOR activity, also alters expression of clock gene and lengthens the period of behavioral rhythm [151]. Furthermore, a p90 ribosomal S6 kinase (S6KII) downstream of RAS/MAPK signaling regulates circadian period through its interaction with CK2 in *Drosophila* [152]. These findings thus reveal an important converging point of nutrient sensing, energy metabolism and cell growth signaling on circadian timekeeping, not only in metabolic tissues, but also in central pacemaker neurons.

Concluding remarks

Studies over the past few decades have provided us with tremendous knowledge of the circadian timing system. Identification of genes that are essential for circadian clock function has uncovered basic cogs and gears that drive the circadian clock (Fig 2-4). However, a fundamental question remains: how are these cogs and gears assembled to generate a 24 h period? As illustrated in Figure 5, we suggest that multiple steps contribute to the overall period length of a transcriptional feedback oscillator. Unraveling the real time dynamics of clock gene transcription/translation, protein accumulation / degradation / interaction, nuclear localization and transcriptional activity, is key to testing this idea.

An altered circadian period interferes with normal daily lives, as evidenced by FASPS patients [153]. While occurrence of such severe genetic disorders is rare, some cases of morning larks and evening owls may result from altered intrinsic circadian period [154]. Different chronotypes in human populations [154] could arise from a misregulated step in any part of the molecular clock mechanism. In such cases, adjustment of phase preference for early morning or late night does not address the potentially adverse consequences of a mismatch of internal period with the environmental cycle (Box 1). A better understanding, on the very basic level, of how circadian period is generated could eventually help in the development of therapies for circadian disorders.

Acknowledgments

This work is supported by a grant from the National Institutes of Health (2R01NS048471-06A1) to A.S. and a 2010 National Alliance for Research on Schizophrenia and Depression (NARSAD) Young Investigator Award to X.Z.

References

1. Foster RG, Roenneberg T. Human responses to the geophysical daily, annual and lunar cycles. *Curr Biol*. 2008; 18:R784–R794. [PubMed: 18786384]
2. Bechtold DA, et al. Circadian dysfunction in disease. *Trends in Pharmacological Sciences*. 2010; 31:191–198. [PubMed: 20171747]
3. Benca R, et al. Biological rhythms, higher brain function, and behavior: Gaps, opportunities, and challenges. *Brain Research Reviews*. 2009; 62:57–70. [PubMed: 19766673]
4. Mackey SR, et al. The itty-bitty time machine: genetics of the cyanobacterial circadian clock. *Adv Genet*. 2011; 74:13–53. [PubMed: 21924974]

5. Allada R, et al. A mutant *Drosophila* homolog of mammalian *Clock* disrupts circadian rhythms and transcription of *period* and *timeless*. *Cell*. 1998; 93:791–804. [PubMed: 9630223]
6. Rutila JE, et al. CYCLE Is a second bHLH-PAS clock protein essential for circadian rhythmicity and transcription of *Drosophila period* and *timeless*. *Cell*. 1998; 93:805–814. [PubMed: 9630224]
7. Bae K, et al. dCLOCK Is present in limiting amounts and likely mediates daily interactions between the dCLOCK-CYC transcription factor and the PER-TIM complex. *J Neurosci*. 2000; 20:1746–1753. [PubMed: 10684876]
8. Houl JH, et al. *Drosophila* CLOCK is constitutively expressed in circadian oscillator and non-oscillator cells. *J Biol Rhythms*. 2006; 21:93–103. [PubMed: 16603674]
9. Konopka R, Benzer S. Clock mutants of *Drosophila melanogaster*. *Proc Natl Acad Sci USA*. 1971; 68:2112–2116. [PubMed: 5002428]
10. Bargiello T, et al. Restoration of circadian behavioural rhythms by gene transfer in *Drosophila*. *Nature*. 1984; 312:752–754. [PubMed: 6440029]
11. Reddy P, et al. Molecular analysis of the period locus in *Drosophila melanogaster* and identification of a transcript involved in biological rhythms. *Cell*. 1984; 38:701–710. [PubMed: 6435882]
12. Zehring WA, et al. P-element transformation with *period* locus DNA restores rhythmicity to mutant, arrhythmic *Drosophila melanogaster*. *Cell*. 1984; 39:369–376. [PubMed: 6094014]
13. Myers M, et al. Positional cloning and sequence analysis of the *Drosophila* clock gene, *timeless*. *Science*. 1995; 270:805–808. [PubMed: 7481771]
14. Sehgal A, et al. Loss of circadian behavioral rhythms and *per* RNA oscillations in the *Drosophila* mutant *timeless*. *Science*. 1994; 263:1603–1606. [PubMed: 8128246]
15. Sehgal A, et al. Rhythmic expression of *timeless*: a basis for promoting circadian cycles in *period* gene autoregulation. *Science*. 1995; 270:808–810. [PubMed: 7481772]
16. Hao H, et al. A circadian enhancer mediates PER-dependent mRNA cycling in *Drosophila melanogaster*. *Mol Cell Biol*. 1997; 17:3687–3693. [PubMed: 9199302]
17. Darlington TK, et al. Closing the circadian loop: CLOCK-induced transcription of its own inhibitors *per* and *tim*. *Science*. 1998; 280:1599–1603. [PubMed: 9616122]
18. Lee C, et al. PER and TIM inhibit the DNA binding activity of a *Drosophila* CLOCK-CYC/dBMAL1 heterodimer without disrupting formation of the heterodimer: a basis for circadian transcription. *Mol Cell Biol*. 1999; 19:5316–5325. [PubMed: 10409723]
19. Menet JS, et al. Dynamic PER repression mechanisms in the *Drosophila* circadian clock: from on-DNA to off-DNA. *Genes Dev*. 2010; 24:358–367. [PubMed: 20159956]
20. Cyran SA, et al. *vrille*, *Pdp1*, and *dClock* form a second feedback loop in the *Drosophila* circadian clock. *Cell*. 2003; 112:329–341. [PubMed: 12581523]
21. Glossop NRJ, et al. VRILLE feeds back to control circadian transcription of *Clock* in the *Drosophila* circadian oscillator. *Neuron*. 2003; 37:249–261. [PubMed: 12546820]
22. Yu W, et al. PER-dependent rhythms in CLK phosphorylation and E-box binding regulate circadian transcription. *Genes Dev*. 2006; 20:723–733. [PubMed: 16543224]
23. Kim EY, et al. *Drosophila* CLOCK protein is under posttranscriptional control and influences light-induced activity. *Neuron*. 2002; 34:69–81. [PubMed: 11931742]
24. Renn SCP, et al. A *pdf* neuropeptide gene mutation and ablation of PDF neurons each cause severe abnormalities of behavioral circadian rhythms in *Drosophila*. *Cell*. 1999; 99:791–802. [PubMed: 10619432]
25. Zheng X, et al. An isoform-specific mutant reveals a role of PDP1e in the circadian oscillator. *J Neurosci*. 2009; 29:10920–10927. [PubMed: 19726650]
26. Akashi M, Takumi T. The orphan nuclear receptor ROR α regulates circadian transcription of the mammalian core-clock *Bmal1*. *Nat Struct Mol Biol*. 2005; 12:441–448. [PubMed: 15821743]
27. Nakajima Y, et al. Bidirectional role of orphan nuclear receptor ROR α in clock gene transcriptions demonstrated by a novel reporter assay system. *FEBS Letters*. 2004; 565:122–126. [PubMed: 15135064]
28. Sato TK, et al. A functional genomics strategy reveals Rora as a component of the mammalian circadian clock. *Neuron*. 2004; 43:527–537. [PubMed: 15312651]

29. Preitner N, et al. The orphan nuclear receptor REV-ERB α controls circadian transcription within the positive limb of the mammalian circadian oscillator. *Cell*. 2002; 110:251–260. [PubMed: 12150932]
30. Crumbley C, Burris TP. Direct regulation of *CLOCK* expression by REV-ERB. *PLoS ONE*. 2011; 6:e17290. [PubMed: 21479263]
31. Liu AC, et al. Redundant function of REV-ERB α and β and non-essential role for *Bmal1* cycling in transcriptional regulation of intracellular circadian rhythms. *PLoS Genetics*. 2008; 4:e1000023. [PubMed: 18454201]
32. Cho H, et al. Regulation of circadian behaviour and metabolism by REV-ERB- α and REV-ERB- β . *Nature*. 2012; 485:123–127. [PubMed: 22460952]
33. Herzog ED, et al. Clock controls circadian period in isolated suprachiasmatic nucleus neurons. *Nat Neurosci*. 1998; 1:708–713. [PubMed: 10196587]
34. Nakamura W, et al. *Clock* mutation lengthens the circadian period without damping rhythms in individual SCN neurons. *Nat Neurosci*. 2002; 5:399–400. [PubMed: 11953751]
35. Vitaterna MH, et al. Mutagenesis and mapping of a mouse gene, *Clock*, essential for circadian behavior. *Science*. 1994; 264:719–725. [PubMed: 8171325]
36. Blau J, Young MW. Cycling *vri* expression is required for a functional *Drosophila* clock. *Cell*. 1999; 99:661–671. [PubMed: 10612401]
37. Smith RF, Konopka RJ. Effects of dosage alterations at the *per* locus on the period of the circadian clock of *Drosophila*. *Mol Gen Genet*. 1982; 185:30–36.
38. Baylies MK, et al. Changes in abundance or structure of the *per* gene product can alter periodicity of the *Drosophila* clock. *Nature*. 1987; 326:390–392. [PubMed: 2436052]
39. Hao H, et al. The 69 bp circadian regulatory sequence (CRS) mediates *per*-like developmental, spatial, and circadian expression and behavioral rescue in *Drosophila*. *J Neurosci*. 1999; 19:987–994. [PubMed: 9920662]
40. Ashmore LJ, et al. Novel insights into the regulation of the Timeless Protein. *J Neurosci*. 2003; 23:7810–7819. [PubMed: 12944510]
41. Yang Z, Sehgal A. Role of molecular oscillations in generating behavioral rhythms in *Drosophila*. *Neuron*. 2001; 29:453–467. [PubMed: 11239435]
42. Zhao J, et al. *Drosophila* Clock can generate ectopic circadian clocks. *Cell*. 2003; 113:755–766. [PubMed: 12809606]
43. Kadener S, et al. Circadian transcription contributes to core period determination in *Drosophila*. *PLoS Biol*. 2008; 6:e119. [PubMed: 18494558]
44. Antoch MP, et al. Functional identification of the mouse circadian *clock* gene by transgenic BAC rescue. *Cell*. 1997; 89:655–667. [PubMed: 9160756]
45. Numano R, et al. Constitutive expression of the *Period1* gene impairs behavioral and molecular circadian rhythms. *Proc Natl Acad Sci USA*. 2006; 103:3716–3721. [PubMed: 16537451]
46. Glossop N, et al. Interlocked feedback loops within the *Drosophila* circadian oscillator. *Science*. 1999; 286:766–768. [PubMed: 10531060]
47. Lim C, et al. *clockwork orange* encodes a transcriptional repressor important for circadian-clock amplitude in *Drosophila*. *Current Biology*. 2007; 17:1082–1089. [PubMed: 17555964]
48. Kadener S, et al. Clockwork Orange is a transcriptional repressor and a new *Drosophila* circadian pacemaker component. *Genes Dev*. 2007; 21:1675–1686. [PubMed: 17578907]
49. Matsumoto A, et al. A functional genomics strategy reveals *clockwork orange* as a transcriptional regulator in the *Drosophila* circadian clock. *Genes Dev*. 2007; 21:1687–1700. [PubMed: 17578908]
50. Nakashima A, et al. DEC1 modulates the circadian phase of clock gene expression. *Mol Cell Biol*. 2008; 28:4080–4092. [PubMed: 18411297]
51. Bode B, et al. Genetic interaction of *Per1* and *Dec1/2* in the regulation of circadian locomotor activity. *J Biol Rhythms*. 2011; 26:530–540. [PubMed: 22215611]
52. Lim C, et al. The novel gene twenty-four defines a critical translational step in the *Drosophila* clock. *Nature*. 2011; 470:399–403. [PubMed: 21331043]

53. Kojima S, et al. LARK activates posttranscriptional expression of an essential mammalian clock protein, PERIOD1. *Proc Natl Acad Sci USA*. 2007; 104:1859–1864. [PubMed: 17264215]
54. Huang Y, et al. Altered LARK expression perturbs development and physiology of the *Drosophila* PDF clock neurons. *Mol Cell Neurosci*. 2009; 41:196–205. [PubMed: 19303442]
55. Newby LM, Jackson FR. A new biological rhythm mutant of *Drosophila melanogaster* that identifies a gene with an essential embryonic function. *Genetics*. 1993; 135:1077–1090. [PubMed: 8307324]
56. Kloss B, et al. The *Drosophila* Clock gene *double-time* encodes a protein closely related to human casein kinase I ϵ . *Cell*. 1998; 94:97–107. [PubMed: 9674431]
57. Price JL, et al. *double-time* is a novel *Drosophila* clock gene that regulates PERIOD protein accumulation. *Cell*. 1998; 94:83–95. [PubMed: 9674430]
58. Suri V, et al. TIMELESS-dependent positive and negative autoregulation in the *Drosophila* circadian clock. *EMBO J*. 1999; 18:675–686. [PubMed: 9927427]
59. Price J, et al. Suppression of PERIOD protein abundance and circadian cycling by the *Drosophila* clock mutation timeless. *EMBO J*. 1995; 14:4044–4049. [PubMed: 7664743]
60. Rothenfluh A, et al. Short-period mutations of *per* affect a *double-time*-dependent step in the *Drosophila* circadian clock. *Current Biology*. 2000; 10:1399–1402. [PubMed: 11084344]
61. Muskus MJ, et al. *Drosophila* DBT lacking protein kinase activity produces long-period and arrhythmic circadian behavioral and molecular rhythms. *Mol Cell Biol*. 2007; 27:8049–8064. [PubMed: 17893330]
62. Ko HW, et al. Role for Slimb in the degradation of *Drosophila* Period protein phosphorylated by Doubletime. *Nature*. 2002; 420:673–678. [PubMed: 12442174]
63. Preuss F, et al. *Drosophila doubletime* mutations which either shorten or lengthen the period of circadian rhythms decrease the protein kinase activity of casein kinase I. *Mol Cell Biol*. 2004; 24:886–898. [PubMed: 14701759]
64. Syed S, et al. Kinetics of doubletime kinase-dependent degradation of the *Drosophila* Period protein. *J Biol Chem*. 2011; 286:27654–27662. [PubMed: 21659538]
65. Suri V, et al. Two novel *doubletime* mutants alter circadian properties and eliminate the delay between RNA and protein in *Drosophila*. *J Neurosci*. 2000; 20:7547–7555. [PubMed: 11027213]
66. Kivimäe S, et al. Activating PER repressor through a DBT-directed phosphorylation switch. *PLoS Biol*. 2008; 6:e183. [PubMed: 18666831]
67. Bao S, et al. The *Drosophila* double-time^S mutation delays the nuclear accumulation of *period* protein and affects the feedback regulation of *period* mRNA. *J Neurosci*. 2001; 21:7117–7126. [PubMed: 11549722]
68. Nawathean P, Rosbash M. The Doubletime and CKII kinases collaborate to potentiate *Drosophila* PER transcriptional repressor activity. *Mol Cell*. 2004; 13:213–223. [PubMed: 14759367]
69. Marrus SB, et al. Effect of constant light and circadian entrainment of *per^S* flies: evidence for light-mediated delay of the negative feedback loop in *Drosophila*. *EMBO J*. 1996; 15:6877–6886. [PubMed: 9003764]
70. Chiu JC, et al. NEMO/NLK phosphorylates PERIOD to initiate a time-delay phosphorylation circuit that sets circadian clock speed. *Cell*. 2011; 145:357–370. [PubMed: 21514639]
71. Grima B, et al. The F-box protein Slimb controls the levels of clock proteins Period and Timeless. *Nature*. 2002; 420:178–182. [PubMed: 12432393]
72. Yu W, et al. NEMO kinase contributes to core period determination by slowing the pace of the *Drosophila* circadian oscillator. *Current Biology*. 2011; 21:756–761. [PubMed: 21514156]
73. Chiu JC, et al. The phospho-occupancy of an atypical SLIMB-binding site on PERIOD that is phosphorylated by DOUBLETIME controls the pace of the clock. *Genes Dev*. 2008; 22:1758–1772. [PubMed: 18593878]
74. Akashi M, et al. Control of intracellular dynamics of mammalian Period proteins by casein Kinase I ϵ (CKI ϵ) and CKI α in cultured cells. *Mol Cell Biol*. 2002; 22:1693–1703. [PubMed: 11865049]
75. Meng Q-J, et al. Setting clock speed in mammals: The CKI ϵ *tau* mutation in mice accelerates circadian pacemakers by selectively destabilizing PERIOD proteins. *Neuron*. 2008; 58:78–88. [PubMed: 18400165]

76. Eide EJ, et al. Control of mammalian circadian rhythm by CKI ϵ -regulated proteasome-mediated PER2 degradation. *Mol Cell Biol.* 2005; 25:2795–2807. [PubMed: 15767683]
77. Etchegaray J-P, et al. Casein kinase 1 δ regulates the pace of the mammalian circadian clock. *Mol Cell Biol.* 2009; 29:3853–3866. [PubMed: 19414593]
78. Isojima Y, et al. CKI ϵ / Δ -dependent phosphorylation is a temperature-insensitive, period-determining process in the mammalian circadian clock. *Proc Natl Acad Sci USA.* 2009; 106:15744–15749. [PubMed: 19805222]
79. Lee H, et al. Essential roles of CKI Δ and CKI ϵ in the mammalian circadian clock. *Proc Natl Acad Sci USA.* 2009; 106:21359–21364. [PubMed: 19948962]
80. Hirota T, et al. High-throughput chemical screen identifies a novel potent modulator of cellular circadian rhythms and reveals CKI α as a clock regulatory kinase. *PLoS Biol.* 2010; 8:e1000559. [PubMed: 21179498]
81. Meng Q-J, et al. Entrainment of disrupted circadian behavior through inhibition of casein kinase 1 (CK1) enzymes. *Proc Natl Acad Sci USA.* 2010; 107:15240–15245. [PubMed: 20696890]
82. Walton KM, et al. Selective inhibition of casein kinase 1 ϵ minimally alters circadian clock period. *J Pharmacol Exp Ther.* 2009; 330:430–439. [PubMed: 19458106]
83. Xu Y, et al. Functional consequences of a *CK1A* mutation causing familial advanced sleep phase syndrome. *Nature.* 2005; 434:640–644. [PubMed: 15800623]
84. Ralph MR, Menaker M. A mutation of the circadian system in golden hamsters. *Science.* 1988; 241:1225–1227. [PubMed: 3413487]
85. Lowrey PL, et al. Positional syntenic cloning and functional characterization of the mammalian circadian mutation *tau*. *Science.* 2000; 288:483–491. [PubMed: 10775102]
86. Gallego M, et al. An opposite role for *tau* in circadian rhythms revealed by mathematical modeling. *Proc Natl Acad Sci USA.* 2006; 103:10618–10623. [PubMed: 16818876]
87. Dey J, et al. The *tau* mutation in the Syrian hamster differentially reprograms the circadian clock in the SCN and peripheral tissues. *J Biol Rhythms.* 2005; 20:99–110. [PubMed: 15834107]
88. Jones CR, et al. Familial advanced sleep-phase syndrome: A short-period circadian rhythm variant in humans. *Nat Med.* 1999; 5:1062–1065. [PubMed: 10470086]
89. Fan J-Y, et al. *Drosophila* and vertebrate casein kinase I Δ exhibits evolutionary conservation of circadian function. *Genetics.* 2009; 181:139–152. [PubMed: 18957703]
90. Sekine T, et al. Casein kinase I ϵ Does Not Rescue double-time Function in *Drosophila* Despite Evolutionarily Conserved Roles in the Circadian Clock. *J Biol Rhythms.* 2008; 23:3–15. [PubMed: 18258753]
91. Toh KL, et al. An *hPer2* phosphorylation site mutation in familial advanced sleep phase syndrome. *Science.* 2001; 291:1040–1043. [PubMed: 11232563]
92. Shanware NP, et al. Casein Kinase 1-dependent phosphorylation of familial advanced sleep phase syndrome-associated residues controls PERIOD 2 stability. *J Biol Chem.* 2011; 286:12766–12774. [PubMed: 21324900]
93. Xu Y, et al. Modeling of a human circadian mutation yields insights into clock regulation by PER2. *Cell.* 2007; 128:59–70. [PubMed: 17218255]
94. Vanselow K, et al. Differential effects of PER2 phosphorylation: molecular basis for the human familial advanced sleep phase syndrome (FASPS). *Genes Dev.* 2006; 20:2660–2672. [PubMed: 16983144]
95. Kloss B, et al. Phosphorylation of period is influenced by cycling physical associations of double-time, period, and timeless in the *Drosophila* clock. *Neuron.* 2001; 30:699–706. [PubMed: 11430804]
96. Sathyanarayanan S, et al. Posttranslational regulation of *Drosophila* PERIOD protein by protein phosphatase 2A. *Cell.* 2004; 116:603–615. [PubMed: 14980226]
97. Fang Y, et al. Post-translational regulation of the *Drosophila* circadian clock requires protein phosphatase 1 (PP1). *Genes Dev.* 2007; 21:1506–1518. [PubMed: 17575052]
98. Lee, H.-m., et al. The period of the circadian oscillator is primarily determined by the balance between casein kinase 1 and protein phosphatase 1. *Proc Natl Acad Sci USA.* 2011; 108:16451–16456. [PubMed: 21930935]

99. Gallego M, et al. Protein phosphatase 1 regulates the stability of the circadian protein PER2. *Biochem J.* 2006; 399:169–175. [PubMed: 16813562]
100. Lee C, et al. Posttranslational mechanisms regulate the mammalian circadian clock. *Cell.* 2001; 107:855–867. [PubMed: 11779462]
101. Diernfellner ACR, Schafmeier T. Phosphorylations: making the *Neurospora crassa* circadian clock tick. *FEBS Letters.* 2011; 585:1461–1466. [PubMed: 21453704]
102. Maywood ES, et al. Tuning the period of the mammalian circadian clock: additive and independent effects of CK1 ϵ^{Tau} and Fbx13 A^{fh} mutations on mouse circadian behavior and molecular pacemaking. *J Neurosci.* 2011; 31:1539–1544. [PubMed: 21273438]
103. Godinho SIH, et al. The After-hours mutant reveals a role for Fbx13 in determining mammalian circadian period. *Science.* 2007; 316:897–900. [PubMed: 17463252]
104. Busino L, et al. SCFFbx13 controls the oscillation of the circadian clock by directing the degradation of Cryptochrome proteins. *Science.* 2007; 316:900–904. [PubMed: 17463251]
105. Siepka SM, et al. Circadian mutant *overtime* reveals F-box protein FBXL3 regulation of cryptochrome and period gene expression. *Cell.* 2007; 129:1011–1023. [PubMed: 17462724]
106. Saez L, Young MW. Regulation of nuclear entry of the *Drosophila* clock proteins Period and Timeless. *Neuron.* 1996; 17:911–920. [PubMed: 8938123]
107. Vosshall L, et al. Block in nuclear localization of period protein by a second clock mutation, *timeless*. *Science.* 1994; 263:1606–1609. [PubMed: 8128247]
108. Meyer P, et al. PER-TIM interactions in living *Drosophila* cells: an interval timer for the circadian clock. *Science.* 2006; 311:226–229. [PubMed: 16410523]
109. Shafer OT, et al. Sequential nuclear accumulation of the clock proteins Period and Timeless in the pacemaker neurons of *Drosophila melanogaster*. *J Neurosci.* 2002; 22:5946–5954. [PubMed: 12122057]
110. Saez L, et al. A PER/TIM/DBT interval timer for *Drosophila*'s circadian clock. *Cold Spring Harb Symp Quant Biol.* 2007; 72:69–74. [PubMed: 18419263]
111. Saez L, et al. A key temporal delay in the circadian cycle of *Drosophila* is mediated by a nuclear localization signal in the Timeless protein. *Genetics.* 2011; 188:591–600. [PubMed: 21515571]
112. Rothenfluh A, et al. Isolation and analysis of six *timeless* alleles that cause short- or long-period circadian rhythms in *Drosophila*. *Genetics.* 2000; 156:665–675. [PubMed: 11014814]
113. Curtin KD, et al. Temporally regulated nuclear entry of the *Drosophila period* protein contributes to the circadian clock. *Neuron.* 1995; 14:365–372. [PubMed: 7857645]
114. Hara T, et al. Post-translational regulation and nuclear entry of TIMELESS and PERIOD are affected in new *timeless* mutant. *J Neurosci.* 2011; 31:9982–9990. [PubMed: 21734289]
115. Rothenfluh A, et al. A TIMELESS-independent function for PERIOD proteins in the *Drosophila* clock. *Neuron.* 2000; 26:505–514. [PubMed: 10839368]
116. Zerr D, et al. Circadian fluctuations of period protein immunoreactivity in the CNS and the visual system of *Drosophila*. *J Neurosci.* 1990; 10:2749–2762. [PubMed: 2117644]
117. Cyran SA, et al. The Double-Time protein kinase regulates the subcellular localization of the *Drosophila* clock protein Period. *J Neurosci.* 2005; 25:5430–5437. [PubMed: 15930393]
118. Kim EY, et al. A role for O-GlcNAcylation in setting circadian clock speed. *Genes Dev.* 2012; 26:490–502. [PubMed: 22327476]
119. Vielhaber E, et al. Nuclear entry of the circadian regulator mPER1 is controlled by mammalian casein kinase I ϵ . *Mol Cell Biol.* 2000; 20:4888–4899. [PubMed: 10848614]
120. Lin J-M, et al. A role for casein kinase 2 α in the *Drosophila* circadian clock. *Nature.* 2002; 420:816–820. [PubMed: 12447397]
121. Akten B, et al. A role for CK2 in the *Drosophila* circadian oscillator. *Nature Neurosci.* 2003; 6:251–257. [PubMed: 12563262]
122. Maier B, et al. A large-scale functional RNAi screen reveals a role for CK2 in the mammalian circadian clock. *Genes Dev.* 2009; 23:708–718. [PubMed: 19299560]
123. Tsuchiya Y, et al. Involvement of the protein kinase CK2 in the regulation of mammalian circadian rhythms. *Sci. Signal.* 2009; 2:ra26. [PubMed: 19491384]

124. Myers MP, et al. Light-induced degradation of TIMELESS and entrainment of the drosophila circadian clock. *Science*. 1996; 271:1736–1740. [PubMed: 8596937]
125. Zeng H, et al. A light-entrainment mechanism for the *Drosophila* circadian clock. *Nature*. 1996; 380:129–135. [PubMed: 8600384]
126. Martinek S, et al. A role for the segment polarity gene *shaggy*/GSK-3 in the *Drosophila* circadian clock. *Cell*. 2001; 105:769–779. [PubMed: 11440719]
127. Ko HW, et al. A hierarchical phosphorylation cascade that regulates the timing of PERIOD nuclear entry reveals novel roles for proline-directed kinases and GSK-3 β /SGG in circadian clocks. *J. Neurosci*. 2010; 30:12664–12675. [PubMed: 20861372]
128. Abe M, et al. Lithium lengthens the circadian period of individual suprachiasmatic nucleus neurons. *Neuroreport*. 2000; 11:3261–3264. [PubMed: 11043560]
129. Iitaka C, et al. A role for glycogen synthase kinase-3 β in the mammalian circadian clock. *J Biol Chem*. 2005; 280:29397–29402. [PubMed: 15972822]
130. Kaladchibachi S, et al. Glycogen synthase kinase 3, circadian rhythms, and bipolar disorder: a molecular link in the therapeutic action of lithium. *J Circadian Rhythms*. 2007; 5:3. [PubMed: 17295926]
131. Yin L, et al. Nuclear receptor Rev-erb alpha is a critical lithium-sensitive component of the circadian clock. *Science*. 2006; 311:1002–1005. [PubMed: 16484495]
132. Spengler ML, et al. A serine cluster mediates BMAL1-dependent CLOCK phosphorylation and degradation. *Cell Cycle*. 2009; 8:4138–4146. [PubMed: 19946213]
133. Hirota T, et al. A chemical biology approach reveals period shortening of the mammalian circadian clock by specific inhibition of GSK-3 β . *Proc Natl Acad Sci USA*. 2008; 105:20746–20751. [PubMed: 19104043]
134. Zhang EE, et al. A genome-wide RNAi screen for modifiers of the circadian clock in human cells. *Cell*. 2009; 139:199–210. [PubMed: 19765810]
135. Lear BC, et al. A G protein-coupled receptor, groom-of-PDF, is required for PDF neuron action in circadian behavior. *Neuron*. 2005; 48:221–227. [PubMed: 16242403]
136. Mertens I, et al. PDF receptor signaling in *Drosophila* contributes to both circadian and geotactic behaviors. *Neuron*. 2005; 48:213–219. [PubMed: 16242402]
137. Hyun S, et al. *Drosophila* GPCR Han is a receptor for the circadian clock neuropeptide PDF. *Neuron*. 2005; 48:267–278. [PubMed: 16242407]
138. Shafer OT, et al. Widespread receptivity to neuropeptide PDF throughout the neuronal circadian clock network of *Drosophila* revealed by real-time cyclic AMP imaging. *Neuron*. 2008; 58:223–237. [PubMed: 18439407]
139. Yoshii T, et al. The neuropeptide pigment-dispersing factor adjusts period and phase of *Drosophila*'s clock. *J Neurosci*. 2009; 29:2597–2610. [PubMed: 19244536]
140. Dahdal D, et al. *Drosophila* pacemaker neurons require G protein signaling and GABAergic inputs to generate twenty-four hour behavioral rhythms. *Neuron*. 2010; 68:964–977. [PubMed: 21145008]
141. Levine JD, et al. Altered circadian pacemaker functions and cyclic AMP rhythms in the drosophila learning mutant dunce. *Neuron*. 1994; 13:967–974. [PubMed: 7946340]
142. Wu MN, et al. The effects of caffeine on sleep in *Drosophila* require PKA activity, but not the adenosine receptor. *J Neurosci*. 2009; 29:11029–11037. [PubMed: 19726661]
143. Oike H, et al. Caffeine lengthens circadian rhythms in mice. *Biochem Biophys Res Commun*. 2011; 410:654–658. [PubMed: 21684260]
144. Lamia KA, et al. AMPK regulates the circadian clock by Cryptochrome phosphorylation and degradation. *Science*. 2009; 326:437–440. [PubMed: 19833968]
145. Lowrey, PL.; Takahashi, JS. Chapter 6 - Genetics of circadian rhythms in mammalian model organisms. In: Stuart, B., editor. *Adv Genet*. Academic Press; 2011. p. 175-230.
146. Um JH, et al. Activation of 5'-AMP-activated kinase with diabetes drug metformin induces casein kinase I ϵ (CKI ϵ)-dependent degradation of clock protein mPer2. *J Biol Chem*. 2007; 282:20794–20798. [PubMed: 17525164]

147. Zheng X, Sehgal A. AKT and TOR signaling set the pace of the circadian pacemaker. *Current Biology*. 2010; 20:1203–1208. [PubMed: 20619819]
148. Zhang HH, et al. S6K1 regulates GSK3 under conditions of mTOR-dependent feedback inhibition of Akt. *Mol Cell*. 2006; 24:185–197. [PubMed: 17052453]
149. Cross D, et al. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature*. 1995; 378:785–789. [PubMed: 8524413]
150. Ogawa S, et al. A seizure-prone phenotype is associated with altered free-running rhythm in Pten mutant mice. *Brain Research*. 2007; 1168:112–123. [PubMed: 17706614]
151. Kohsaka A, et al. High-fat diet disrupts behavioral and molecular circadian rhythms in mice. *Cell Metab*. 2007; 6:414–421. [PubMed: 17983587]
152. Akten B, et al. Ribosomal S6 Kinase cooperates with casein kinase 2 to modulate the *Drosophila* circadian molecular oscillator. *J Neurosci*. 2009; 29:466–475. [PubMed: 19144847]
153. Jones CR, et al. Familial advanced sleep-phase syndrome: A short-period circadian rhythm variant in humans. *Nat Med*. 1999; 5:1062–1065. [PubMed: 10470086]
154. Roenneberg T, et al. Epidemiology of the human circadian clock. *Sleep Med Rev*. 2007; 11:429–438. [PubMed: 17936039]
155. Dunlap JC, et al. A Circadian clock in *Neurospora*: how genes and proteins cooperate to produce a sustained, entrainable, and compensated biological oscillator with a period of about a day. *Cold Spring Harb Symp Quant Biol*. 2007; 72:57–68. [PubMed: 18522516]
156. Baker CL, et al. The circadian clock of *Neurospora crassa*. *FEMS Microbiol Rev*. 2012; 36:95–110. [PubMed: 21707668]
157. Baker CL, et al. Quantitative proteomics reveals a dynamic interactome and phase-specific phosphorylation in the *Neurospora* circadian clock. *Molecular Cell*. 2009; 34:354–363. [PubMed: 19450533]
158. Karatsoreos IN, et al. Disruption of circadian clocks has ramifications for metabolism, brain, and behavior. *Proc Natl Acad Sci USA*. 2011; 108:1657–1662. [PubMed: 21220317]
159. Scheer FAJL, et al. Adverse metabolic and cardiovascular consequences of circadian misalignment. *Proc Natl Acad Sci USA*. 2009; 106:4453–4458. [PubMed: 19255424]
160. Roenneberg T, et al. Social Jetlag and Obesity. *Current Biology*. 2012; 22:939–943. [PubMed: 22578422]

Box 1**Circadian period and health**

The adaptive value of having a ~24 h clock for life on earth is evident. An ideal ~24 h clock present in most organisms ensures that rhythmic processes occur at the most suitable time of day. An altered circadian period results in mistimed peaks and troughs of biological functions, and thus a mismatch with the environment, an extreme example of which is FASPS [153]. Variants of this allele and other genes may be present in quite large numbers in the general population.

Mismatch of circadian period with the environment, produced by such alleles, have adverse effects on health and lifestyle. For example, housing mice with an endogenous 24 h circadian period in 20 h light/dark cycles disrupts circadian rhythms and results in symptoms of metabolic syndrome, as well as changes in neural architecture (Figure I), cognitive flexibility and emotionality [158]. Human subjects exposed to a 28 h forced desynchronization protocol display impairment of cardio-metabolic functions [159]. Circadian disruptions in modern society are often created by shift work schedules and air travel. Although they are not due to altered period *per se*, the slow adaptation (or no adaptation in some cases) of the internal clock to the new environment or social/work schedules generates a phase difference that is similar to a mismatch of the internal circadian period with the environmental cycle. This type of social jetlag also exists in people who may have normal periods, but display strong preference for early or late schedules (ie., larks and owls, respectively)[154]. Importantly, even the standard weekly work /school schedule generates a chronic social jetlag that is strongly associated with increased body mass index (BMI)[160].

Figure 1. Simplified model of a transcriptional feedback oscillator in eukaryotic cells

(a) The activator promotes transcription of the repressor gene, and accumulating repressor proteins feedback to inhibit repressor gene transcription. A series of post-transcriptional and translational modifications are imposed to delay the accumulation of repressor proteins. In this simple model, a stronger activator, earlier feedback repression, or faster dissipation of the repression results in a short period, whereas a weaker activator, delayed or prolonged repression renders a long period. Post-translational modifications regulate the stability, cellular localization and activity of the repressor protein. The Activator also promotes transcription of other modulator genes, whose products positively and negatively feedback to regulate Activator gene transcription. (b) Such feedback regulation generates oscillations of clock molecules and of transcriptional activity. Cyclic activation and repression of clock gene transcription are interlocked (dark blue dotted line) so that the strength, timing and duration of one phase affects the other. We speculate that the overall duration of these processes forms the basis of period determination.

Figure 2. A basic molecular framework for circadian timing is conserved from fungi to flies and humans

(a) In *Drosophila*, CLK-CYC heterodimer activates transcription of *per*, *tim*. Accumulated PER and TIM proteins heterodimerize and feedback to repress CLK-CYC activity. CLK-CYC also activates transcription of *vri* and *Pdp1ε*, whose products feedback to repress and activate *Clk* transcription, respectively. (b) In mammals, the CYC homolog BMAL1 heterodimerizes with Clock to activate transcription of *Per*, *Cry*, *Ror* and *rev-erb*. PER forms a repressor complex with Cryptochrome (CRY), instead of TIM. There are three PERs (PER1, PER2 and PER3), of which PER2 may be most important, and two CRYs (CRY1 and CRY2), whose functions are not fully redundant. RORs and REV-ERBs feedback to regulate transcription of *Clock*, *Bmal1* and *Cry* [30, 32] (also see review in [145]). (c) In *Neurospora*, White collar (WC1/2) activates transcription of the repressor gene *frequency* (*frq*). Accumulated FRQ protein interacts with an RNA helicase, FRH, to repress WC1/2 activity. FRQ protein also positively regulates *wc1/2* transcription (see review in [155]). Similar negative and positive feedback regulation also exists in prokaryotes, although transcriptional feedback is not necessary to sustain the cyanobacteria circadian clock (see review in [4]).

Figure 3. Model for the core negative feedback loop of the *Drosophila* circadian clock

The circadian activator CLK-CYC promotes transcription of its target genes, the circadian repressors that include *per*, *tim* and *cwo*, during the day (1). mRNA expression of these genes peaks in the early evening, followed by accumulation of the proteins at night. DBT phosphorylates and promotes PER degradation, and this process is counterbalanced by PP2A (2). TIM mainly localizes to the cytoplasm in the early part of the night due to an active CRM/exportin mediated nuclear export mechanism (3). SGG phosphorylates and may also decrease TIM stability, while PP1 stabilizes TIM in the cytoplasm (4). Around midnight, accumulated PER and TIM proteins form heterodimers (5) and start to enter the nucleus. Nuclear translocation is facilitated by CK2 (perhaps also PP2A) and SGG phosphorylation of PER and TIM, respectively. The PER/TIM/DBT complex may dissociate briefly before nuclear translocation (6). In the late night, nuclear PER is progressively phosphorylated by DBT and reaches maximum repressor activity on the CLK-CYC heterodimer (7). CWO also binds to the E-box sequence of CLK-CYC target genes to help terminate their transcription. At daybreak, hyperphosphorylated PER is targeted by SLIMB for proteasomal degradation (8). TIM protein is degraded by light-dependent and independent mechanisms (question mark). The degradation of this PER-TIM repressor allows CLK-CYC to start a new cycle of transcription and feedback repression. Note that the only kinase for which a circadian time course of interaction with PER has been determined is DBT. Timing of clock protein association with other kinases and phosphatases is speculated (question mark) based upon cell culture and genetic assays. *Neurospora* and mammals have similar mechanisms (see review in [145, 156]).

Figure 4. Dual role of phosphorylation in regulating the stability and activity of circadian repressor proteins

(a) In *Drosophila*, newly synthesized PER is unstable, mainly due to DBT and CK2 mediated degradation [57, 68]. Progressive phosphorylation of the PER short domain by DBT creates a resistor that acts to slow down phosphorylation of PER at other sites, and hence stabilize PER. Inhibiting phosphorylation of this PER short domain by deletion or mutation facilitates phosphorylation of downstream and other sites, and hence faster PER degradation [66, 70]. Similar mechanisms may exist in mammals. (b) Human PER2 protein is phosphorylated at some unknown sites by CK1 and other kinases, which promote PER2 degradation. Phosphorylation of S662 promotes phosphorylation of neighboring sites by CK1, which generates a phosphor-cluster to inhibit phosphorylation of other sites responsible for PER2 degradation. However, current models regarding the FASPS mutation (S662G) are still unresolved [92-94]. It appears that the phosphorylation state of S662 also affects PER2 nuclear localization and *Per2* transcription. (c) In *Neurospora*, early phosphorylation of the C-terminus of the FREQUENCY (FRQ) protein inhibits phosphorylation of the PEST-1 domain and other sites, and thus slows down FRQ degradation. Deletion, or mutations that inhibit phosphorylation of the C-terminus, result in faster degradation and a shorter circadian period [157].

Figure 5. Hypothetical model for circadian period determination in *Drosophila*

We propose that the speed of a circadian oscillator is regulated by multiple temporally gated events: circadian transcription of repressor genes, posttranscriptional regulation of their mRNAs, translation and cytoplasmic accumulation of repressor proteins, nuclear accumulation and retention of repressors, and clearance of repressors from the nucleus. In a 24 h cycle, the repressor protein PER remains in the nucleus for 6-8 h after daybreak (time 0). Premature clearance of PER/TIM results in an earlier de-repression of CLK-CYC and thus a shorter circadian period, while extended presence of PER/TIM proteins slows it down. Progressive degradation of hyperphosphorylated PER/TIM enables gradual increase of CLK-CYC activity during the day, hence peak accumulation of *per/tim* mRNA around dusk. Newly translated PER protein is presumably targeted by DBT for degradation, thus it creates a delay between *per* mRNA and PER protein. Around midnight, accumulated PER and TIM start to translocate into the nucleus. Earlier nuclear accumulation of PER/TIM shortens circadian period, while delayed accumulation slows down the circadian clock. Nuclear PER is progressively phosphorylated by DBT, which maximizes its repressor activity in the late night and early morning (time 24). In the morning, hyperphosphorylated PER is gradually cleared from the nucleus by proteasome mediated degradation, thus allowing a new cycle to start (time 0). Some of these events maybe interlocked, while others are independent of each other. A dynamic assembly of these processes may form the basis of circadian period determination.

Figure I.

Mismatch of the endogenous 24 h circadian period with the environmental 20 h light:dark cycle results in morphological changes of medial prefrontal cortex (PFC) neurons in mice. (a) A layer III cell of prelimbic medial PFC neuron labelled with Lucifer yellow. Circadian disrupted mice showed shrunken apical dendrites in the PFC (b) but normal basal dendrites (c). Reproduced, with permission, from [158].