Circadian rhythm genetics: from flies to mice to humans

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A successful genetic dissection of the circadian regulation of behaviour has been achieved through phenotype-driven mutagenesis screens in flies and mice. Cloning and biochemical analysis of these evolutionarily conserved proteins has led to detailed molecular insight into the clock mechanism. Few behaviours enjoy the degree of understanding that exists for circadian rhythms at the genetic, cellular and anatomical levels. The circadian clock has so eagerly spilled her secrets that we may soon know the unbroken chain of events from gene to behaviour. It will likely be fruitful to wield this uncommon degree of knowledge to attack one of the most challenging problems in genetics: the basis of complex human behavioural disorders. We review here the genetic screens that provided the *entreé* into the heart of the circadian clock, the model of the clock mechanism that has resulted, and the prospects for using the homologues as candidate genes in studies of human circadian dysrhythmias.

Although there are 24-hour cycles in many biochemical and physiological processes, the regulation of the overall behaviour of an organism is the most overt and perhaps the most intriguing manifestation of circadian rhythmicity. The rhythm with which animals engage in the industry of everyday life extends from eating, drinking and spontaneous locomotion to the choice of when to go to sleep. To fully appreciate the genetic basis of the clock that underlies this rhythmicity, it is informative to first place the mechanism in its anatomical context.

A body of data has identified the suprachiasmatic nucleus (SCN) in the mammalian hypothalamus as the site controlling circadian behavioural rhythmicity[1]. Lesions of the SCN abolish locomotor rhythms and SCN transplants reinstate rhythmic behaviour with the circadian properties of the donor animal. The oscillatory mechanism of the clock is intracellular and can be monitored for weeks in individual SCN neurons in dispersed culture. The spontaneous firing frequency of these cultured cells mimics the behavioural profile of the animal from which they came, whether the animals have genetically fast, slow or arrhythmic circadian clocks[2]. The data placing SCN neurons in charge of regulating circadian behavioural rhythmicity are so compelling and multifaceted that the strength of this functional localization is unsurpassed by that of any other complex animal behaviour.

This master clock orchestrates the rhythms of peripheral oscillators (we use the terms clock, pacemaker, timekeeping mechanism and oscillator interchangeably) that reside within many other cells throughout the anatomy of the mammal[3]. Although not entirely self-sustaining without cues from the SCN, cycling of peripheral tissues can persist in culture for several days when organs are explanted[4] or when peripheral cells are immortalized[5,6].

Genetic screens

For 30 years, biologists have thrown the heft of forward genetics at the circadian pacemaker. This endeavour has been the most successful genetic dissection of any animal behaviour. First, it has resulted in the cloning of six genes that form the basis of the molecular oscillator. Second, the biochemical mode of action of each of these clock components has been studied and their individual functions have clustered into meaning with respect to how they endow a cell with the ability to tell time. Third, these genes are conserved from flies to mice to humans, and some of them even across kingdoms to plants[7].

The design of the screens, carried out mostly in the fruit fly *Drosophila melanogaster*, has resulted in the recovery of mutants that perturb timekeeping in a variety of ways[8]. Extensive coverage of both recessives[9–14] and dominants[10–16] has been achieved. Chemical mutagenesis[10–14,16,17] and P-element insertional mutagens[9,15] have been used. Eclosion, the emergence of adult flies from their pupal cases, was used to track clock function during metamorphosis[8,9,15,17]. The locomotion of the adult fly served as a gauge of rhythmicity in the mature brain[10–13,16]. Luminescence from a circadian promoter-driven luciferase transgene provided a measure of the oscillators of the body[14]. Some screens isolated mutants that fail to properly align their rhythm with the light/dark cycle[14,15,17], whereas others observed animals in constant darkness to reveal abnormalities in the free-running properties of the pacemaker[8–13,16].

The enterprise has led to the discovery of the central oscillator components designated *period* (*per*; ref. 17), *timeless* (*tim*; ref. 9), *Clock* (*Clk*; refs 11,18), *cycle* (*cyc*; ref. 12) and *double-time* (*dbt*; also known as *casein kinase 1e*; refs 10,19), as well as a gene, *cryptochrome* (*cry*; ref. 14), that allows the clock to perceive light, and one called *lark* that connects the clock to a physiological output[15]. Many of these genes were hit multiple times by independent means. *per* (ref. 8), *tim* (refs 9,10,16) and *dbt* (ref. 10) were each recovered many times in fly screens. *cyc* was discovered to be involved in circadian rhythms in a mutant screen with whole flies[12], and almost simultaneously in three biochemical studies by virtue of its physical interaction with the CLK protein using fly[20], rodent[21] and human[22] homologues. Also telling of evolutionary conservation in this system, *Clk* and *dbt* were obtained as circadian mutants in random/spontaneous mutageneses of both flies[10,11] and rodents[18,19] independently.

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Alleles were found that speed up the cadence of the clock, slow it down or cause rhythmicity to cease[9–13,16–18,23]. Splice mutants[2,24], small deletions[25], and missense[8,10,13,14,16] and nonsense[11,12,26] mutations were obtained that generate dominant-negative[2,11], semidominant[8,10,13,16], recessive[8,9,12,14] and homozygous lethal[10,15] alleles.

As thorough and unbiased as these studies were, some types of mutations were not picked up. No peptides or very small genes have yet been recovered in these screens, despite the fact that a reverse genetics approach determined that the neuropeptide pigment dispersing factor (PDF) is required for normal circadian rhythmicity[27]. All mutations from the screens affect amino acid sequence despite the existence of critical regulatory regions in and around these genes. Target size may be an important limitation in these cases.

Currently there are at least three new mouse mutagenesis screens being carried out for circadian rhythms. Progress in mouse genomics will soon reduce the time required to isolate the gene responsible for a given phenotype[28]. The mammalian genome may therefore be on its way to becoming as thoroughly pummelled for circadian mutants as is currently the case for flies.

Molecular machinery of the clock

Before describing the molecular details of the model, it is helpful to first consider the clock's overall design[29]. At its foundation, it consists of a self-sustaining, 24-hour rhythm in the expression of certain pacemaker genes, including the canonical members in *Drosophila*, *per* and *tim*. Their protein products act to repress transcription of their own genes in a negative feedback loop. Within the loop, they intermittently engage and disengage from transcriptional activators to form a dynamic multiprotein complex, or circadiasome. A key feature is that there is a lag between the transcriptional induction of *per* and *tim* on one hand and the nuclear translocation of the repressor proteins they encode on the other. This lag creates a temporal separation between phases of induction and repression, which is required to generate an oscillation. Without the separation, the transcriptional level would come to an equilibrium of the two forces. Another important feature is that the half-lives of the *per* and *tim* mRNAs and proteins are rather short, highly regulated[10,30] and precisely adapted to be part of the timekeeping mechanism.

If negative feedback regulation of these promoters is the heart of the circadian clock, the application of this rhythmic transcriptional apparatus to output genes is its soul. The genes outside the loop that are regulated by the circadiasome[31,32] are in turn believed to drive rhythmicity in physiology and behaviour. The feedback loop model has engendered widespread consensus among researchers, although it has not entirely escaped criticism[33].

Among species, the choreography of the *Drosophila* clock is best understood[29] (Fig. 1*a*). At about noon, the CLK protein with its partner, CYC, bind to E-box DNA elements and activate a slow transcriptional induction of the *per* and *tim* genes. *per* and *tim* RNA levels begin to rise, but phosphorylation by DBT prevents PER protein from accumulating[10]. Nightfall allows TIM, a light-labile protein, to rise to a level at which it can protect PER protein from degradation[10] and stable TIM:PER heterodimers begin to form. By midnight, heterodimerization has abrogated an apparatus that retains these proteins in the cytoplasm and the pair moves into the nucleus[7]. PER and TIM then physically associate with and inhibit the ability of the CLK:CYC protein complex to bind DNA (ref. 29) and transcription of these genes ceases. The half lives of *per* and *tim* mRNA dictate the pace of their decline throughout the night. Daybreak stimulates the photoreceptor, CRY, to sequester TIM protein and render it incapable of functioning as a transcriptional regulator. TIM ultimately becomes phosphorylated, ubiquitinated and degraded via the proteosomal pathway[29]. By noon the next day, PER and TIM proteins have waned to levels in the nucleus that can no longer inhibit CLK:CYC activity, and a new round of synthesis commences.

The transcriptional regulation of the *Drosophila* gene *clk* is the mirror image of that described for *per* and *tim* above[29]. In this case, CLK and CYC proteins

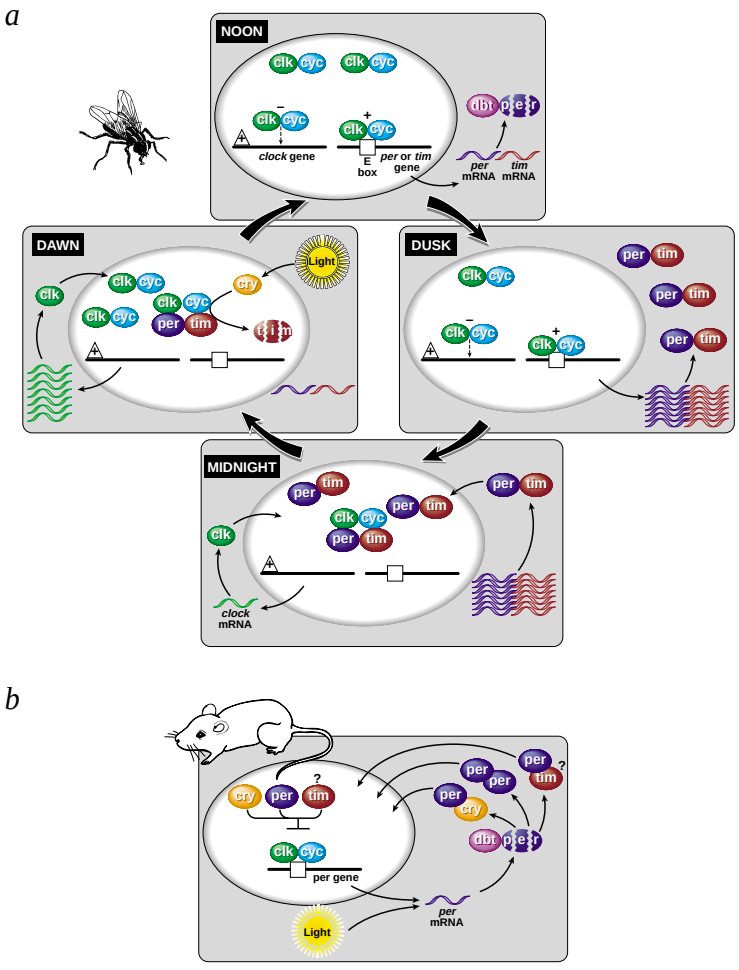

Fig. 1 Sequence of intracellular events at the core of the circadian clock. ***a***, In *Drosophila* at midday, CLK and CYC proteins (coloured circles) induce *per* and *tim* expression through E-Box elements in the promoters, but the PER protein is rendered unstable by DBT. At the same time, the CLK:CYC complex represses the *Clk* promoter, either directly or indirectly (denoted by the dotted arrow). Once night falls, TIM can accumulate and interfere with the action of DBT. In the middle of the night, PER:TIM dimers translocate into the nucleus (oval) and impede the functions of CLK:CYC. This brings about a cessation of *per* and *tim* mRNA production, and a simultaneous increase in *Clk* expression. The triangle with the plus sign indicates the presence of an unknown transcriptional activator for the *Clock* gene. The perception of light at dawn by CRY leads to degradation of TIM. These latter events cause a change in balance from an excess of PER:TIM in the nucleus to an excess of CLK:CYC, which frees the fly to take another trip around the clock. ***b***, The mouse clock is similar to that of the fly except that Cry has been conscripted into the feedback loop itself. Dbt phosphorylates and destabilizes Per. Per, Cry and possibly Timeless negatively regulate (denoted by the line ending in a bar) CLK:CYC-mediated expression of *Per*. Light resets the clock by stimulating production of *Per* mRNA.

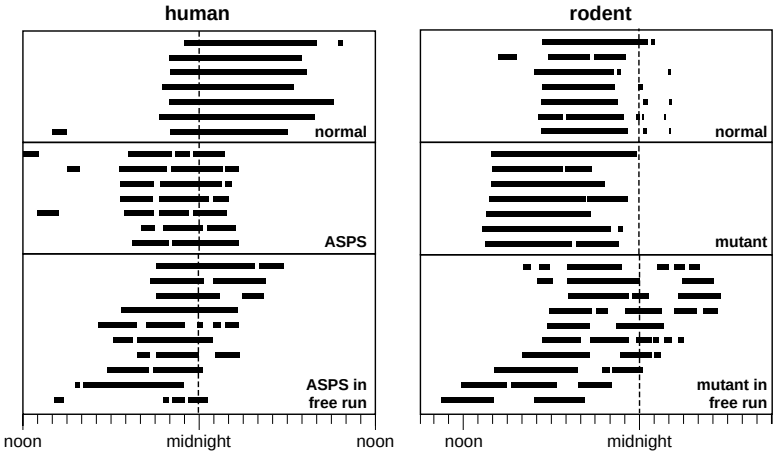

Fig. 2 Animal model of a human behavioural disorder. The phase of the daily rhythm in behaviour is assessed with sleep logs in human and by wheel running in rodent. The episodes of sleep or wheel running are depicted relative to midnight with black bars. Consecutive days are plotted from top to bottom of each subpanel. The abnormally early phase in advanced sleep phase syndrome is demonstrated by this 62-year male patient (redrawn from ref. 53) relative to a normal 69-year female subject JW-S from our study. The same deviation is seen in the *double-time* mutant hamster (known as *tau*) when compared with its normal littermate (redrawn from ref. 23). In the bottom subpanels, experimental subjects are put into an environment devoid of all temporal cues. A 69-year female proband of an advanced sleep phase kindred is shown here (redrawn from ref. 51) with a mouse mutant of *Per2* (redrawn from ref. 54) for comparison. Note the drift of the behavioural bout that indicates a shortened intrinsic circadian period length in both individuals. The normal intrinsic period in these species is nearly 24 hours[54,55].

repress the *clk* promoter, either directly or indirectly. PER and TIM block this repression, probably in the same manner that they block the other actions of CLK:CYC. The sign change in the action of the proteins on this promoter generates expression that is antiphasic to that of *per* and *tim*. These strategies, both *Clock*wise and counter*Clock*wise, probably apply to other promoters[29].

The architecture of the mammalian clock is largely shared with that of the fly[2] (Fig. 1*b*). Homologues of the entire bevy of *Drosophila* timekeeping genes exist in mammals. *Clock*, *Dbt* (MGI designation *Csnk1e*; ref. 19), *Cry1*, *Cry2* and *Per2* have been genetically altered in rodents and all were found to perturb behavioural and molecular circadian cycling[2]. Dbt phosphorylates and destabilizes Per in mammals as in flies[10,19,34]. The transcriptional activating and repressing capacities of mammalian *Clock*, *Cyc* (MGI designation *Arntl*), *Per* and *Timeless* have been demonstrated on E-boxes found in mouse *Per* and other circadianly regulated promoters[2].

Among these similarities between mice and flies, some differences have emerged[2]. Several clock genes that are unique in the fly have multiple homologous copies in the mouse genome. Some of the same physical interactions that occur among fly pacemaker proteins have been found for the mouse molecules, but some interactions are specific to one or the other species. Certain clock genes are rhythmically expressed in both species, whereas others oscillate only in one[2].

Some of the most important differences discovered so far are in the anatomical and molecular pathways by which light entrains the clock[35]. In mammals, light coming into the eyes is communicated transsynaptically to the SCN, where it leads to an induction of *Per* mRNA (refs 7,36). In *Drosophila*, light leads to a degradation of TIM all over that is independent of eyes[29,37,38]. Further differentiating the two species, the mouse Cry proteins have been shanghaied into the oscillator itself and have acquired many of the functions of *Drosophila* TIM. Mouse Cry proteins physically interact with Per protein and negatively effect transcription of the gene *Per*, as does *Drosophila* TIM (ref. 35). Many of the differences between these species may be explained by the evolutionary pressure that must have arisen when our ancestors became too large and opaque for light to penetrate appreciably to each clock-containing cell.

A number of major issues concerning the basis of circadian rhythmicity have been resolved, such as the logic of the oscillator, where it resides, and its main molecular components and their biochemical functions. Very important questions remain, however, such as the mechanism for coupling the body's oscillators together and uncertainties pertaining to input and output from the clock. A series of molecular events in which photoperception by cryptochrome leads to resetting has been outlined in flies, but the identity of the mammalian circadian photoreceptor is less clear[35]. Whether or not cryptochromes are involved in the light input pathway in mammals is controversial[35,39]. In both species, a poorly defined alternative pathway to cryptochromes exists for photoentrainment under certain circumstances[14,35].

The series of steps between the clock and behavioural rhythmicity has not been elucidated in any species, although some leads are being pursued. The clock's rhythmic machinery has been shown to directly regulate the transcription of certain output genes, including two related transcription factors, DBP in mammals[32] and Vrille in flies[40]. In addition, PDF is somehow circadianly regulated at the level of protein abundance in flies[41]. Mutations in the genes encoding these three proteins have been found to perturb locomotor rhythmicity, prompting some to speculate that they take part in the output pathway to behaviour[27,40,42,43]. However, each of them appears to input to the oscillator as well, making it difficult to determine whether the influence on behaviour is merely a consequence of their effects on the clock. On the other hand, a mutation altering the RNA-binding protein, LARK, affects the circadian rhythm of eclosion of adult flies from their pupal cases, but not the rhythm of locomotion[15]. This indicates that the oscillator itself is unaffected by this mutation and thereby places *lark* squarely in the output to eclosion. The mechanism by which circadian rhythm of LARK is regulated, which occurs at the protein level[44], is unknown, as are the means whereby this oscillating protein regulates eclosion.

Perspectives on the genetics of human circadian dysrhythmias

Several human disorders bear a striking resemblance to the phenotypes of experimental animals with mutations in their clock genes when these animals are monitored in normal light/dark cycles. The *dbt* mutation in some hamsters causes a syndrome consisting of fragmentation of the rest and active phases, early daily onset of activity, and a progressive decline in the level of locomotor behaviour in certain lighting conditions which remits in others[45]. These three symptoms are reminiscent of a clinical subtype of depression, perhaps even the seasonal form[46], and it will be of considerable interest to learn how closely this animal model mimics the clinical phenomena on further scrutiny. The rhythm and blues connection is buttressed by a recognized association between circadian alterations and psychiatric conditions in humans[47].

But the most compelling similarity of clock mutant phenotypes is to a family of sleep timing disorders. In one of these, advanced sleep phase syndrome (ASPS), the patient suffers intractable sleepiness in the early evening hours and, as a result,

Table 1 • Candidate genes for human circadian dysrhythmias

Gene	Behaviour of mutants in darkness	Behaviour of mutants in light/dark cycles	Molecular features	Biochemical function	Refs
period1, *-2*, *-3*	flies: short period	advanced activity peak	PAS domain	blocks CLK:CYC	2,17,20, 49,54,56
	long period	delayed activity peak			
	arrhythmic	behaviour responds to but doesn't anticipate dawn and dusk			
	mice: short period	advanced activity onset			
timeless	flies: arrhythmic	no abnormality reported	none	blocks CLK:CYC	9,2,16,20
	long period	not reported			
clock	flies: arrhythmic	irregular pattern of rest and activity	bHLH, PAS, glutamine-rich, triplet repeats	regulates transcription	2,11,18, 20–22*
	mice: long period	less sleep and more locomotion during rest phase with variable timing of activity onset			
cycle	flies: arrhythmic	irregular pattern of rest and activity	bHLH, PAS	regulates transcription	12,2,20–22
double-time	flies: short or long	no abnormality reported	casein kinase 1-ε	phosphorylates PER	10,19,23,34,45
	hamsters: short period	in some hamsters, early phase of activity; in others, depression-like syndrome, reduced sleep, or non-24-hour-like syndrome			
cryptochrome1, *-2*	flies: no behavioural abnormality reported	abnormal circadian responses to light	related to photolyases	photoreceptor and/or regulates transcription	14,35,57–59
	mice: short period	no abnormality reported			
	arrhythmic	behaviour responds simply to light and dark with no jet lag			
	long period	greater variance of phase			

Some examples are given of the free-running circadian phenotype of animals with different alleles of these genes along with the corresponding behaviour in normal light cycles. PAS is an acronym for a region of homology among proteins that functions as a protein:protein interaction domain. *E. Naylor, M.H. Vitaterna, J.S. Takahashi and F.W. Turek, pers. comm.

the habitual sleep episode is shifted unusually early in the 24-hour day[48], similar to certain 'short period' circadian mutant mammals[23] (Fig. 2). The advanced phase of rest and activity in these animals is caused by a pacemaker that runs too fast and yet is still able to entrain to the daily light/dark cycle. A circadian period length of substantially shorter than 24 hours is revealed when the animals are kept in constant darkness for several cycles[23]. In delayed sleep phase syndrome, the patient is persistently unable to initiate sleep until well after the conventional hour[48], as in long period mutant animals[49]. In non-24-hour sleep wake syndrome, the person does not have a stable sleep phase, but drifts around the clock[48]. This behavioural pathology is also seen in certain period length mutant animals who are not quite able to entrain to daily lighting cycles[23].

The link between mutant animals and these human disorders extends beyond phenotypic similarity. A polymorphism in the gene *CLOCK* is reported to be associated in humans with the person's early bird/night owl 'chronotype'[50], which can be considered the extension of sleep timing disorders into the normal range. Moreover, the proband of an advanced sleep phase family was found to have an intrinsic circadian period length that is about an hour shorter than normal using a lengthy temporal isolation procedure[51]. The human syndrome and the mutant animals, therefore, share both the abnormal phasing in light/dark cycles as well as the underlying cause for the aberrant phase, an altered period length (Fig. 2).

There are nine human homologues of the animal timekeeping genes (Table 1). To facilitate the use of these candidates in genetic analysis of the human circadian dysrhythmias, we have begun a study to catalogue sequence variation in subjects with familial circadian rhythm sleep disorders and to determine the consequences of these polymorphisms on biochemical function. In addition it would be worthwhile to find a biological, rather than a behavioural, phenotype that could quickly discriminate the subset of patients that have an abnormality in core pacemaker function. Perhaps cells could be removed from the patient, monitored in culture and assessed for circadian period length alterations *in vitro*. In our own preliminary experiments, however, we have been unable to find conditions that elicit reliable and robust rhythmicity in either primary or immortalized peripheral blood lymphocytes (K.W.-S., B. Schneirow, J.C. Gillin and S.A.K., unpublished data). Other cells such as punch biopsy fibroblasts or buccal scrape epithelial cells could be evaluated.

In making an inventory of various complex human behavioural disorders, the circadian dysrhythmias have several advantages for a genetic study. First, prior work in the field has produced a group of candidate genes whose candidacy is based not on speculation from the hypothesized pathogenesis, but on actual phenotypic similarity of mutant animals with the human disorder. The mutagenesis that led to the nomination of these candidates were largely unbiased and, based on the repeated recovery of most genes, nearly comprehensive. Second, our knowledge of the genetic determinants of circadian phenotypes is complimented by an understanding of how the environment influences the process, which is (at least for flies) at a high level of resolution. This gives human geneticists a molecular handle on the environmental variance that inevitably complicates behavioural genetic analysis. Third, the neuroanatomical substrate of circadian behavioural regulation is remarkable for its clarity and simplicity. Rather than being an emergent property of networks, the behavioural profile is contained within the firing pattern of single SCN neurons in dispersed culture. The discovery that the same living clock that generates the behavioural profile is intact and beating in immortalized mammalian cell cultures[5,6,52] makes this a behaviour that is amenable to *in vitro* experimental analysis. For example, determining the behavioural consequences of any human genetic variant will be aided by the ability to assess the effect of the polymorphism on timekeeping when the allele is transfected into clock-containing cell cultures.

The detailed genetic, molecular, cellular and anatomical understanding that exists for the circadian form of behavioural regulation is a precious commodity. This affords a unique opportunity

to tackle the extraordinarily difficult and important problem of complex human behavioural genetics from a rock-solid foundation. With the momentum that the field of circadian biology has gathered, the pace of further refining our understanding of the clock mechanism and its pathology is only likely to accelerate.

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