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REVIEW

Molecular chronobiology

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Abstract Recent years have seen exciting advances in the understanding of the mechanisms that underlie circadian rhythms in a variety of organisms, including mammals. Several key genes have been identified, whose products can be considered to represent bone fide clock molecules. Furthermore it appears that the same genes are important in generating rhythmic behaviour in both insects and man. There are some differences in the way these genes generate circadian output in the different taxa, but overall, the level of conservation of sequence and function is striking. The basic molecular oscillatory mechanism depends on a transcriptional/translational negative feedback loop, in which the PERIOD proteins play a cardinal role, together with other molecules, which interact to regulate circadian gene expression. In mammals, the brain oscillator resides in the suprachiasmatic nucleus, and its location in the hypothalamic region may have implications for understanding the rhythmic nature of some headache syndromes.

Key words Circadian • Clock • Gene • Molecule • Headache

Introduction

For several billion years, the Earth has rotated along its axis with a period close to 24 hours. It has been four billion years since the first replicating molecules arose in the sea, and almost every organism that has evolved since, simple or complex, has been touched by this relentless cycle of day followed by night. In turn, this has brought about the evolution of a mechanism which anticipates this oscillation, and which prepares the organism for the associated change in illumination and temperature. In today's extant taxa, we can observe this circadian oscillation from organisms as simple as the Cyanobacteria, through the fungi, to all higher organisms including insects and mammals. There is no need to tell anyone that they have a biological clock. The sleep-wake cycle makes this obvious, but what is not so obvious is that these rhythms are endogenous and are encoded genetically. Humans who are isolated from temporal cues will nevertheless slip into a circadian, 24-hour cycle of behaviour and physiology. This is not to say that environment cannot alter or modify that circadian clock: ask any trans-Atlantic traveller or shift worker. However, it was only about 30 years ago that the debate about whether circadian rhythms were determined by some cycling geophysical variable or by an internal clock was finally laid to rest. The argument was settled by a genetic experiment with fruitflies, performed by a young graduate student, Ronald Konopka at the California Institute of Technology in the late 1960s [1].

This study is arguably the most important in the field, and its findings have formed the focal point of an explosion in molecular circadian research which began about 15 years ago. So exciting have been these studies that the Christmas issues of *Science* magazine in 1997 and 1998 have placed molecular chronobiology within the top 10 scientific developments for those years. The molecular isolation of *per* in

the mid-1980s, and some of the subsequent work, form a substantial part of the book *Time, Love, Memory* [2], written by the Pulitzer-Prize winning author Jonathan Weiner. The book provides an inside view into how the work developed, and the personalities that led the charge from a genetical to a molecular description of the clock. As well as this book, which provides an introduction for the interested layman, a large number of more technical reviews of this field have been written. I will therefore refrain where possible from citing the primary literature, which numbers several hundred papers, and instead refer the reader to the more recent reviews of this field [3–9].

Konopka and Benzer's classic paper focused on a study of the fly's circadian pupal-adult eclosion rhythms [1]. When flies are ready to emerge from the pupa, they tend to do so at dawn, as this is the time when humidity is greatest. The word *'Drosophila'* means 'dew lover', and this taxonomical insight reflects the ancient adaptation which prevents the newly emerged adults from dessicating in the midday heat (these flies evolved in Africa), before they have had time to tan their cuticle and pump out their wings. Consequently, if a fly is ready to emerge in mid-afternoon, it waits, until the next morning. Thus a bottle of fruitflies containing a selection of mixed-aged pupae will show several cycles of morning eclosion until all the pupae have emerged as adults. This rhythm has a period of 24 hours in constant conditions of darkness and temperature. By feeding these flies a mutagen, Konopka and Benzer were able to identify three mutants in the next generation, which had abnormal eclosion cycles. These included a short 19-hour variant, a longer 29-hour fly, and an arrhythmic fly. These mutations all had corresponding effects on the individual fly's 'sleep-wake' rhythms, which can be measured as locomotor activity cycles (flies run around during the day, but 'sleep' at night). When these mutations were genetically mapped, they all appeared to be located to the same spot on the X-chromosome and defined a gene that Konopka called *'period'* or *'per'*. Normally the wild-type allele of *per* sits at this spot, and generates a 24-hour cycle, but Konopka's chemical mutagenesis had mutated this normal allele to a short, long or arrhythmic variant. One of the major take home messages from this study was that if a gene can be mutated to change the clock's rhythm, then clearly an external geophysical variable cannot be generating circadian timing.

The period feedback loop

The discovery of these clock mutants lay the foundations for the molecular analysis of circadian rhythms which was to occur some 15 years later. *per* was cloned, but translation of the primary amino acid sequence, which gave rise to a large putative protein of more than 1200 residues, gave few clues as to PER's function. Progress was made only after the subsequent discovery that levels of the PER protein and mRNA cycled in the fly's brain, with the transcript peaking early in the night phase (of a 12-h light – 12-h dark cycle, LD12:12), whereas the protein peaked late at night (Fig. 1). These molecular rhythms were also maintained in constant darkness

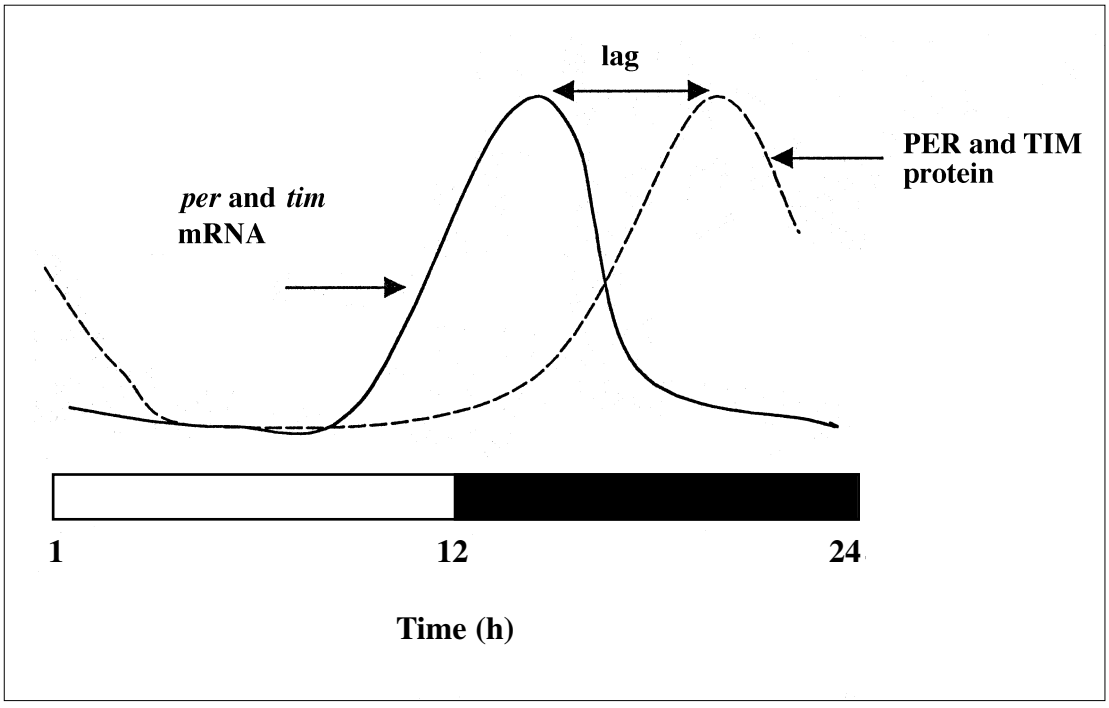

Fig. 1 *per* and *tim* mRNA and proteins cycle during a LD12:12 cycle or in constant darkness

with a period of 24 h (DD). This suggested that as the PER protein rises, it feeds back and shuts down its own mRNA production, giving rise to a negative feedback loop. Further evidence for this was provided by the *per* molecular cycles in the short 19-hour *per* mutant, which cycled with a corresponding 19-hour cycle. As the only difference between the normal wild-type PER protein and the mutant protein was a single amino acid change, this meant that the mutant protein had somehow fed back and influenced the mutant mRNA rhythm. The delay between mRNA and protein cycles would thus provide the necessary condition for the negative feedback to work, as without the delay, the protein would simply shut down transcription almost immediately, and cycling would damp.

Immunohistochemical studies using anti-PER antibodies also revealed that late at night, the PER protein was seen to move from the cytoplasm to the nucleus in the so-called lateral neurons in which PER was expressed. Experiments involving some sophisticated genetic trickery had shown that unless PER is expressed in these cells, the fly's sleep-wake cycle will be arrhythmic, so consequently, these neurons appear to represent the fly's behavioural pacemaker. The apparent nuclear role for PER in these neurons suggested that PER may be a transcription factor, which acts to influence its own mRNA expression by binding to its own promoter region. If this is how it worked, then the PER protein should carry a recognisable DNA-binding motif, which it does not.

In 1993 however, sequence analysis of PER and several other proteins, both in flies and mammals, revealed a marginal similarity in a 270 residue region that was termed PAS. In the past two years it has become clear that PAS is a recognisable motif found in many signalling molecules, including transcription factors. PAS acts as a dimerisation domain, and can mediate various types of protein-protein interactions, which perform a wide variety of functions, from light sensing in bacteria, to potassium channel deactivation in mammals [10]. The primary sequence similarity between PAS domains is very poor, yet the structural integrity of different PAS domains is remarkably conserved at the three-dimensional (3D) level [10]. The PER PAS domain would therefore be expected to physically interact with another molecule, and one of these binding partners is the product of the *timeless (tim)* gene. The first mutation identified in *tim* also gives arrhythmic behaviour, suggesting that *tim* is also an important clock gene. TIM protein and mRNA cycle in the same brain cells, and in approximately the same phase, as do the *per* products (Fig. 1). Thus not only is *tim* in a negative loop with itself, but it is also a cardinal component of the *per* loop. Without TIM, PER cannot get into the nucleus, and vice versa.

Late at night, as TIM and PER protein levels are on the rise, the two products dimerise via the PER PAS domain and translocate to the nucleus (Fig. 2). Inside the nucleus are the protein products of the genes *Clock* and *cycle (cyc),* both of which have bHLH (basic helix-loop-helix) and PAS regions. CLOCK and CYC can associate with each other via their PAS domains, and their bHLH motifs further allow them to bind to specific DNA sequences called E-boxes, which are found in the *per* and *tim* promoters. The CLOCK-CYC complex is therefore the positive element in the feedback loop and activates *per* and *tim* transcription during the day and early part of the night (Fig. 2). As the PER-TIM dimer enters the nucleus, it sequesters the CLOCK-CYC dimer, and represses *per* and *tim* transcription. The PER-TIM dimer therefore represents the negative element of the feedback loop. As PER and TIM degrade during the day phase, the CLOCK-CYC dimer is freed to reactivate *per* and *tim* transcription. In LD cycles, this process by which *per* and *tim* transcription is derepressed is also aided by another molecule, CRYPTOCHROME (CRY). CRY changes its conformation when stimulated by light, and binds to TIM, sequestering the PER-TIM dimer from the CLOCK-CYC complex, which then reactivates *per/tim* transcription (Fig. 2) [9]. CRY's light sensitivity thus appears to enhance the amplitude of the molecular oscillation in LD cycles.

The feedback loop therefore has positive (CLOCK and CYC) and negative (PER and TIM) components but relies on a delay between the translation of the proteins PER and TIM, and their negative feedback on their own promoters. This critical delay involves DOUBLETIME (DBT), a casein kinase, which phosphorylates PER monomers as they are produced in the cytoplasm in the early part of the night phase (Fig. 2). This earmarks PER monomers for degradation, and contributes to the delay between the observation of the peak levels of the *per* mRNA and the subsequent peak of the PER protein cycle (Fig 1). As TIM levels rise later in the evening, they somehow block the action of DBT on PER (Fig. 2), so PER monomers reach a level where they can dimerize with TIM, and so the PER-TIM complex moves into the nucleus [11, 12]. Mutations in *doubletime* which either shorten or lengthen the behavioural cycle have been isolated, but more severe mutations are lethal. This is not surprising as the kinase is likely to be involved in many other biological functions, and is not simply clock-specific. However, mutations in *Clock* and *cyc* are arrhythmic and non-lethal [13, 14], and a mutation in *cry* predictably leads to poorer circadian behavioural responses to light [15].

So much for fly rhythms, but can this molecular mechanism be extended to cover mammals? Circadian rhythms are found in almost all higher organisms, as well as some primitive single-celled bacteria. Furthermore the responses of circadian rhythms to different stimuli, for example heat or light pulses, are very similar, irrespective of taxa. Thus one might imagine that the underlying circadian mechanism might also be conserved. For years after *per* was cloned in

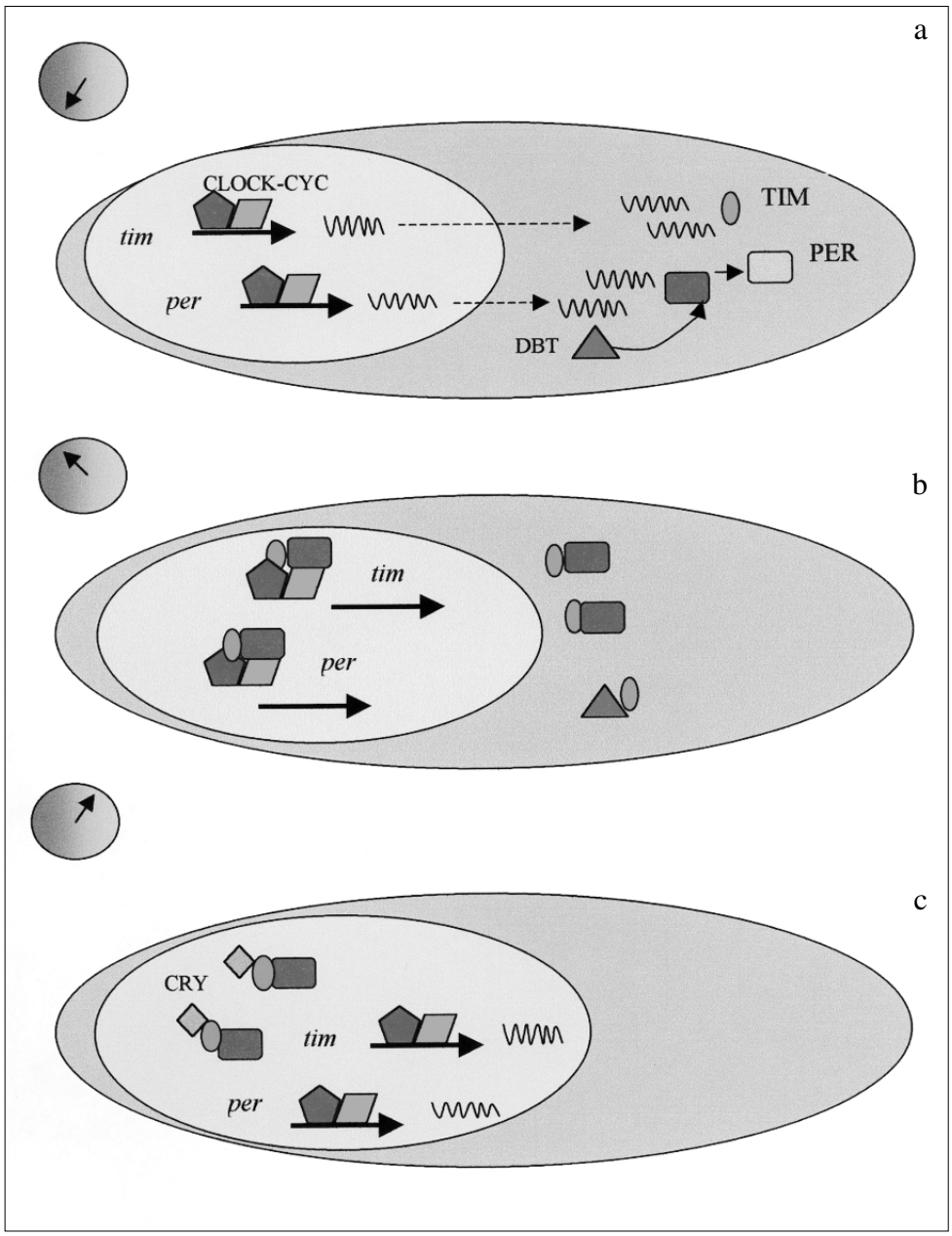

Fig. 2a–c Molecular/cellular basis of the *Drosophila* circadian clock. **a** Early in the evening the CLOCK-CYC heterodimer activates transcription of the *per* and *tim* genes (*thick arrows*). The mRNAs move out of the nucleus into the cytoplasm, where translation of PER and TIM monomers begins. The DBT kinase phosphorylates PER, leading to its degradation. **b** Late at night, and in the early hours of the morning, TIM blocks DBT's effects on PER, PER accumulates, and the PER-TIM dimer moves into the nucleus and sequesters the CLOCK-CYC heterodimer, so *per* and *tim* transcription is repressed. **c** At dawn, CRY sequesters TIM, and the degradation of TIM and PER allow the CLOCK-CYC heterodimer to reactivate *per* and *tim* transcription

the fly, workers tried to find a similar gene in the mouse, but all failed, and it was thought in many quarters that perhaps the fly clock mechanism would be specific to insects. One should never make up one's mind based on negative evidence, and this was underlined relatively recently, when mouse and human *per* genes were finally identified. In fact it turned out that the mouse has three *per* genes *(mper1, 2* and *3).* Many genes in mammals are duplicated, the most famous being the HOX gene complex, which has four copies, compared to *Drosophila*'s one [16]. These *mper* transcripts cycle in various parts of the mouse brain but in a different phase to that seen in the fly. Crucially, the *mper* genes are expresssed in the suprachiasmatic nucleus (SCN) of the hypothalamus, which has been known for many years to represent the circadian pacemaker of the mammal [6].

The mouse also has a *Clock* gene, which was defined by mutagenesis. The homozygous *Clock* mouse mutant has an arrhythmic circadian behavioural phenotype, and has a slightly longer period than normal in the heterozygote. There are also two murine *Cry* genes. Mutations in each produce slight changes in circadian behaviour, but a double mutant is arrhythmic, suggesting that mCRYs have a rather more direct function on the clock than *Drosophila* dCRY [17-19]. There is also a *cyc* homologue in the mouse (called

bmal1), a DBT homologue (casein kinase 1ε), and a *tim* homologue, *mTim.* Mutations in the latter three mammalian genes have not been reported, and the role of *mTim* particularly is unclear at the moment.

Thus some of the central components of the mammalian clock have been identified and not surprisingly, the duplication of clock genes in the mouse means that the different mPER or mCRY products will have slightly different functions from each other, providing a rather more complex regulatory network. These same clock components may generate circadian rhythmicity in the fly or the mouse, but the way in which these molecules are regulated will have altered through evolution. Even in insects, the regulation of PER and TIM in the brain of the giant silkmoth is quite dramatically different from that of the fruitfly [8]. No doubt, more clock components will be identified in the future, but my guess is that there will not be so many more involved in generating the feedback loop. There will certainly be many clock-controlled genes *(ccg's)* which connect the clock to the circadian phenotype, and some of these are already known in the mammal and the fly.

One example of the regulation of a *ccg,* concerns a recent study of the neuropeptide arginine vasopressin gene in the mouse, whose mRNA cycles with a circadian period in the mouse brain [20]. The gene's promoter has an E-box, to which CLOCK-BMAL1 heterodimers bind and activate *vasopressin* transcription. Addition of the mPER proteins or mTIM gives a modest repression of transcription, but the mCRY1 or mCRY2 products produce a far more powerful transcriptional inhibition, suggesting that mCRYs are part of the negative loop [19]. The mCRY proteins also dimerize with mPER proteins, revealing how mCRYs influence the negative regulation of the *mper* genes, and also explaining why mCRY double mutants are arrhythmic [18,19]. The role of the mCRYs thus contrasts with the role of dCRY in *Drosophila,* which seems to act predominantly as a circadian photoreceptor [9]. The regulation of the *vasopressin ccg* can therefore be directly controlled by the central clock components mPER, mTIM, mCLOCK, BMAL1 (CYC) and the mCRYs. However it is unlikely that all *ccg's* will be regulated in this way, because any mRNA which cycles with a different phase to *mper* genes may require other intermediates interfacing between the cycling actions of the core clock genes, and the transcription factors which ultimately control the *ccg*.

It is clear from this brief overview of molecular chronobiology that the basis of the clockworks in the mammal are beginning to be understood (thanks to the fly). This knowledge has broad implications for developing treatments for some of the clock problems that bedevil shift workers (>25% of the Western working population), insomniacs, and seasonal depressives, to name but a few.

Melatonin, headache, and the clock

Another output from the mammalian circadian clock is melatonin, which cycles with high levels at night, low levels during the day, and is produced predominantly from the pineal [21]. As there are a number of reports on the beneficial effects of melatonin administration for headache [22-24], perhaps examining the role of biological rhythms in headache may lead to new insights in the genesis of such disorders. There certainly are good reasons to suspect that the clock is involved in the circadian and seasonal patterns observed in cluster headache (CH) [23, 24], as well as a rare benign syndrome called hypnic or 'alarm clock' headache, which also shows monotonous temporal regularity [25]. In addition, positron emission tomography (PET) scans have implicated the hypothalamus in the onset of CH, and the possibility that CH is due to a neuro-vascular disorder has been discussed [26]. The region of the hypothalamus that seems to be the focus of CH lies uncomfortably close to the SCN.

Melatonin is a master hormone which controls the pituitary-hypothalamic-adrenal axis, and regulates genes which stimulate the immune system [27]. Melatonin stimulates T-helper 1 (Th1) cell cytokines, among them interferon-γ (INF-γ) and interleukin-2 (IL-2) [28–31]. IL-2 is reported to be at lower levels in CH sufferers [32], fitting with the observation that plasma melatonin levels are also too low during a cluster period [33, 34]. At night, a disruption of the Th1/melatonin signaling, caused by lower-than-normal melatonin levels, could signal the beginning of a CH attack. If this were the case however, then CH sufferers should show symptoms of a less-than-perfectly functioning circadian clock. In fact, there are reports of clock defects in such CH patients [22, 34], but disentangling cause from effect is not easy. Does a dysfunctional clock cause the disorder, or does the disorder cause the apparently defective clock? After all, a CH sufferer will be exhausted, sleep-deprived and distressed during the cluster period, leading to a number of correlated circadian changes. The simple procedure of waking up during a cluster episode and putting on the lights will itself reduce melatonin levels.

When melatonin is administered, as well as stimulating T-cells it will also stimulate the expression of many other genes, including those that are required to decrease body temperature. The hormone acts as a vasoconstrictor, so perhaps the beneficial effects of melatonin in CH and in other tension-type headaches which are associated with delayed sleep syndrome [35] are due to prevention of vasodilation. Melatonin's actions could therefore be mediated by stimulation of the immune system, or by melatonin's effects on any number of other targets, hormonal or circulatory. Whether there is a causal relationship between what is happening at the circadian level and headache remains to be demonstrated.

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