Cytosolic and Transcriptional Cycles Underlying Circadian Oscillations

1

Michael H. Hastings[1] and John S. O'Neill[2]

[1] *Division of Neurobiology, Medical Research Council Laboratory of Molecular Biology, Cambridge, UK*
[2] *Division of Cell Biology, Medical Research Council Laboratory of Molecular Biology, Cambridge, UK*

1.1 Introduction

Circadian (*circa-* approximately, *-dian* a day) clocks are the internal pacemakers that drive the daily rhythms in our physiology and behavior that adapt us to the 24-hour world (Duffy *et al*., 2011). They thereby maintain temporal coherence in our core metabolism, even when individuals are held in isolation, experimentally deprived of external timing cues such as light–dark (LD) cycles. As a result of this ability of our endogenous circadian system to define internal time and use it to drive daily rhythms, our brains and bodies can be viewed as 24-hour machines, alternating between states of wakefulness and sleep, catabolism and anabolism, growth/repair and physical activity. It is now widely recognized that disturbance of this daily program can carry significant costs for morbidity and even mortality (Hastings *et al*., 2003). Some personal insights into this can come from the subjective experiences of jet lag. More insidiously, however, the disturbance of nocturnal sleep, and consequent disaffected mood, loss of mental capacity and social disruption, is a common element of neurodegenerative and psychiatric conditions (Hatfield *et al*., 2004; Wulff *et al*., 2010; Bliwise *et al*., 2011) (This volume, several chapters). Moreover, epidemiological evidence now associates increased risk of cancer as well as cardiovascular and metabolic diseases with extensive experience of rotational shift-work (Knutsson, 1989; Viswanathan *et al*., 2007; Huang *et al*., 2011) (This volume, Chapter 13), a life-style that will inevitably compromise circadian coherence, and which represents a major and growing hazard to public health. Evolution has programmed us to live by a 24-hour day and where genetic, pathological, environmental or social factors drive us against this program, we pay a heavy price. Conversely, the recognition that our body is a 24-hour machine, with different metabolic and physiological states across day and night, provides a route into enhancing therapeutic efficacy by administering medicines on a schedule that maximizes their bioavailability and by targeting disease states at their most critical and vulnerable phases of the day (Levi and Schibler, 2007).

Key to appreciating the role of the circadian clock in both health and illness, and thereby identifying novel therapeutic strategies, is the unravelling of its molecular and cellular bases. Whilst the formal properties of circadian clocks have been understood for over 60 years, and the identification in 1972

Circadian Medicine, First Edition. Edited by Christopher S. Colwell.

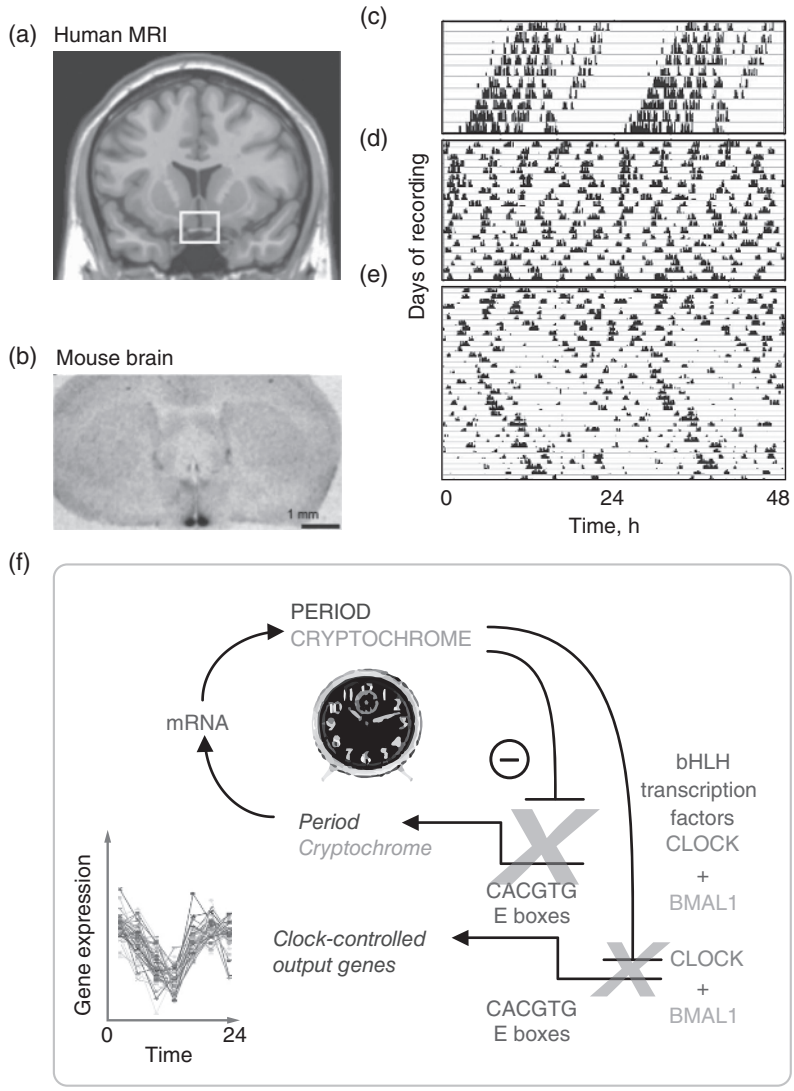

Fig. 1.1 The suprachiasmatic nucleus (SCN) as circadian pacemaker. (a) Frontal MRI view of human brain to identify location of SCN (boxed) in anterior hypothalamus at junction of third ventricle and optic chiasm (courtesy of Dr Adrian Owen, MRC CBU, Cambridge UK). (b) Comparable view of mouse brain labelled autoradiographically to reveal SCN in ventral hypothalamus. (c) Recording of wheel-running activity of mouse (double-plotted on 48-h time base) free-running in continuous dim red light, with a sustained circadian period of slightly less than 24 h (King *et al*., 2003). (d) Behavior of same mouse following ablation of SCN – note total loss of circadian organization in absence of SCN, but no change in overall activity level. (e) Behavior of same SCN-lesioned mouse following intracerebral graft of SCN from a Clock^{delta19} mouse. Note modest restoration of circadian patterning to behavior, but with a period longer than 24 h as determined by graft genotype. This genetic specification of circadian period proves that the rhythm is controlled by the grafted SCN and, thus, the SCN is the definitive pacemaker to circadian behavior. (f) Schematic representation of conventional TTFL at the heart of the SCN circadian pacemaker. (See text for details.)

of the suprachiasmatic nucleus (SCN) as the brain's principal pacemaker provided a neuroanatomical focus to circadian biology (Weaver, 1998; Chapter 3) (Fig. 1.1a–e), proper mechanistic understanding of the timing process proved to be elusive. This changed dramatically from the late 1970s onwards, when "circadian clock genes" and their mechanisms of action were identified: firstly in *Drosophila*, then in *Neurospora*, and more recently in mouse (Takahashi *et al*., 2008). The outcome of

these studies was to reveal that an autoregulatory negative feedback oscillator, based on sequential transcriptional and posttranslational processes, lies at the heart of the circadian timepieces of these divergent groups. Even though the molecular components may differ, the "logic" of the mechanism is conserved. But things move on, and there is growing realization that these transcriptionally based clocks do not operate in isolation; rather, they are mutually dependent upon intrinsically rhythmic cytosolic signals (cAMP, Ca^{2+}, kinases), such that the cell as a whole has a resonant structure, tuned to 24-hour operation (Hastings *et al*., 2008). Finally, the most recent development has been to show that even in cells lacking transcriptional apparatus (most notably mammalian erythrocytes), circadian cycles of metabolic state can be sustained (O'Neill and Reddy, 2011). The purpose of this chapter is to review the development of this molecular and cellular model of the circadian clockwork of mammals.

1.2 Assembling the transcriptional feedback loop

1.2.1 Discovering clock genes and their actions in lower species

The idea that a complex behavioral trait such as the circadian cycle of rest and activity could be understood from the viewpoint of single gene actions was, for some time, contentious in both the circadian field and also more widely. Nevertheless, the creation by Ron Konopka and Seymour Benzer of mutant *Drosophila* with atypically short or long periods to their circadian behavior, and the subsequent cloning of the *Period* gene as the molecular target of these mutations, initiated a revolution in clock biology (Konopka, 1987). Alongside the *Frq* (*Frq*) gene of *Neurospora*, cloned by Jay Dunlap and colleagues (Loros *et al*., 1989), *Period* (*Per*) provided an entry point into the molecular mechanisms of clocks: changes in the encoded proteins could make the clock run faster, or slower or not at all. They therefore MUST be an intrinsic part of the clockwork. Moreover, it became apparent that the key action of the encoded proteins was to inhibit the expression of their cognate genes. Given that there is an inevitable time lag between transcriptional activation and nuclear entry of the fully formed protein, an oscillation is bound to ensue, as in any other delayed negative feedback system (Hardin *et al*., 1990; Aronson *et al*. 1994). Indeed, autoregulation of this type is well recognized in molecular biology, with oscillations commonly occurring over a couple of hours. The critical property here, however, is that the dynamics of the contributory stages (gene activation, protein synthesis, intracellular transport, protein degradation) are extended such that the cycle runs for approximately 24 hours. Subsequent mutational and biochemical studies revealed that *Per* and *Frq* are components of dynamic, multiprotein complexes, the assembly of which is facilitated in part by their protein interaction domains (Hardin, 2005; Crosthwaite *et al*., 1997). Of particular note were the so-called PAS (Per–Arnt–Sim) interaction domains of *Per*. The positive drives to the feedback loops that stimulate expression of *Per* and *Frq*, comes from additional PAS-containing proteins: CLOCK and CYCLE in flies (Allada *et al*., 1998; Rutila *et al*., 1998), and WHITE COLLAR 1 and 2 in *Neurospora* (Crosthwaite *et al*., 1997; de Paula *et al*., 2007). After forming heteromers, these positive factors activate transcription via specific regulatory sequences in the enhancer regions of *Per* and *Frq*, respectively. Thus, positive factors drive the expression of negative factors, which in turn oppose the positive drive leading to a decline in negative factor abundance, which allows the cycle to start again approximately 24 hours after the previous point of initiation.

Although both systems are light sensitive – a prerequisite for synchronization with solar cycles and, thereby, environmental time, their molecular basis to entrainment is different. In flies, the stability of PER is dependent on association with another circadian protein, TIMELESS (Myers *et al*., 1996; Koh *et al*., 2006), which in turn is subject to degradation by CRY, a light-dependent factor with similarity to photolyase DNA repair proteins (Cashmore, 2003). Consequently, PER protein can only accumulate in the night, thereby stably entraining the entire molecular cycle to solar time. In contrast, the light-sensitive component in the *Neurospora* loop is the positive factor *White Collar-1*, which binds FAD (flavin adenine dinucleotide) as a chromophore (Crosthwaite *et al*., 1997; de Paula *et al*., 2007). Thus, expression of *Frq* is activated at the start of the day, again linking the phase of the molecular cycle to environmental (solar) time.

1.2.2 Discovering clock genes and their actions in mammals

Knowledge of the clock in flies and fungus played a large role in deciphering the mammalian clockwork (Fig. 1.1f). Homology cloning or sequence alignment of novel transcripts, based heavily on knowledge of the PAS domains of *Drosophila Period*, led ultimately to the discovery of three *Per* genes in mammals (Tei *et al*., 1997; Reppert and Weaver, 2002). Mammalian *Cryptochromes* (*Cry1* and *Cry2*) had previously been studied in the context of DNA repair, but findings from flies focused attention on their potential circadian role, which was confirmed by the demonstration that *Cry*-deficient mice cannot exhibit circadian behavior (van der Horst *et al*., 1999). Initial description of a mammalian *Timeless* gene was subsequently shown to be misleading, as the mammalian gene in question is, in fact, a homologue to *Timeout*, a different, noncircadian fly gene. A further difference, consistent with the absence of *Timeless*, is that mammalian CRY proteins are not the light-sensitive component of the cycle: resetting in mammals is mediated by the activation of *Per1* and *Per2* expression (see below) (Shigeyoshi *et al*., 1997; Albrecht *et al*., 1997), an echo of the light-dependent induction of *Frq* expression in *Neurospora*. Critically, both *Per* and *Cry* mRNA and proteins are expressed rhythmically in the SCN, with respective phases that are consistent with negative feedback action (Field *et al*., 2000). But what of the positive factors that would drive such a negative feedback system? The identification of mammalian *Clock*, by mutagenesis and subsequent transgenic rescue, was a landmark achievement by the laboratory of Joe Takahashi – it preceded the discovery of *Drosophila Clock*, and was dependent upon classical positional cloning, pre-dating the mouse genome era (King *et al*., 1997). As in flies, CLOCK forms heteromeric complexes to activate expression of *Per* and other circadian genes, including *Cry*. The partner to CLOCK is BMAL1, a homologue of *Drosophila Cycle*, which was initially identified by co-expression screens (Hogenesch *et al*., 1998).

Both CLOCK and BMAL1 contain PAS dimerization domains, but only CLOCK carries a poly-Q transactivation domain. Loss of BMAL1 leads to circadian incompetence at both molecular and behavioral levels, whereas loss of CLOCK has mixed effects that vary between tissues, depending upon whether or not NPAS2, a paralogue of CLOCK, can compensate (DeBruyne *et al*., 2007). Nevertheless, the original Clock^{delta19} mutation generated by Takahashi has compromised transactivation; consequently, circadian period is lengthened in the heterozygote and completely disorganized in homozygous mutants because of insufficient transcriptional drive to the *Per* and *Cry* genes. Finally, the negative feedback loop has been closed experimentally by the demonstration that CLOCK/BMAL1 heterodimers can acutely activate E-box mediated transcription and that this effect is suppressed by co-expression with PER and CRY (Kume *et al*., 1999). The details of this transcriptional repression are unclear, but both PER and CRY contribute (see below). In the established transcriptional model in mammals, therefore, the start of circadian day sees CLOCK/BMAL1 activation of *Per* and *Cry* expression via their E-box regulatory sequences (Fig. 1.1f). The accumulating mRNAs are translated into protein and by the end of circadian day SCN neurons have high levels of nuclear PER and CRY proteins. This is followed by a progressive decline in mRNA levels, reflecting the negative feedback action of the accumulated PER/CRY complexes. By late circadian night the existing PER/CRY complexes, no longer replenished in the absence of mRNA, are finally cleared from the nucleus such that CLOCK/BMAL1 activity is de-repressed and the cycle starts anew at circadian dawn. The application of this basic model to humans is described in Chapter 2.

1.2.3 Imaging the transcriptional clock in real time: a multitude of cellular oscillators appears

Circadian timing is an intrinsically dynamic process and major advances in analyzing circadian gene expression have come about with the development of real-time reporter genes in which circadian regulatory sequences are coupled to bioluminescent (firefly luciferase) or fluorescent proteins. Although recording of circadian rhythms of intrinsic bioluminescence in unicellular organisms has a long pedigree in clock research (Hastings, 2007), this approach found greater application when directed towards the newly discovered clock genes, firstly in plants and flies and more recently in mammals. Early examples are transgenic lines of mouse and rat in which upstream sequences of *Per1* (carrying five E-boxes) are used to drive luciferase. Organotypic slice cultures of SCN from such animals express robust, clearly defined bioluminescence rhythms arising from individual neurons (Fig. 1.2a–c), the phases of which are synchronized but exhibit a complex, wave-like progression across the SCN (Yamaguchi *et al*., 2003),

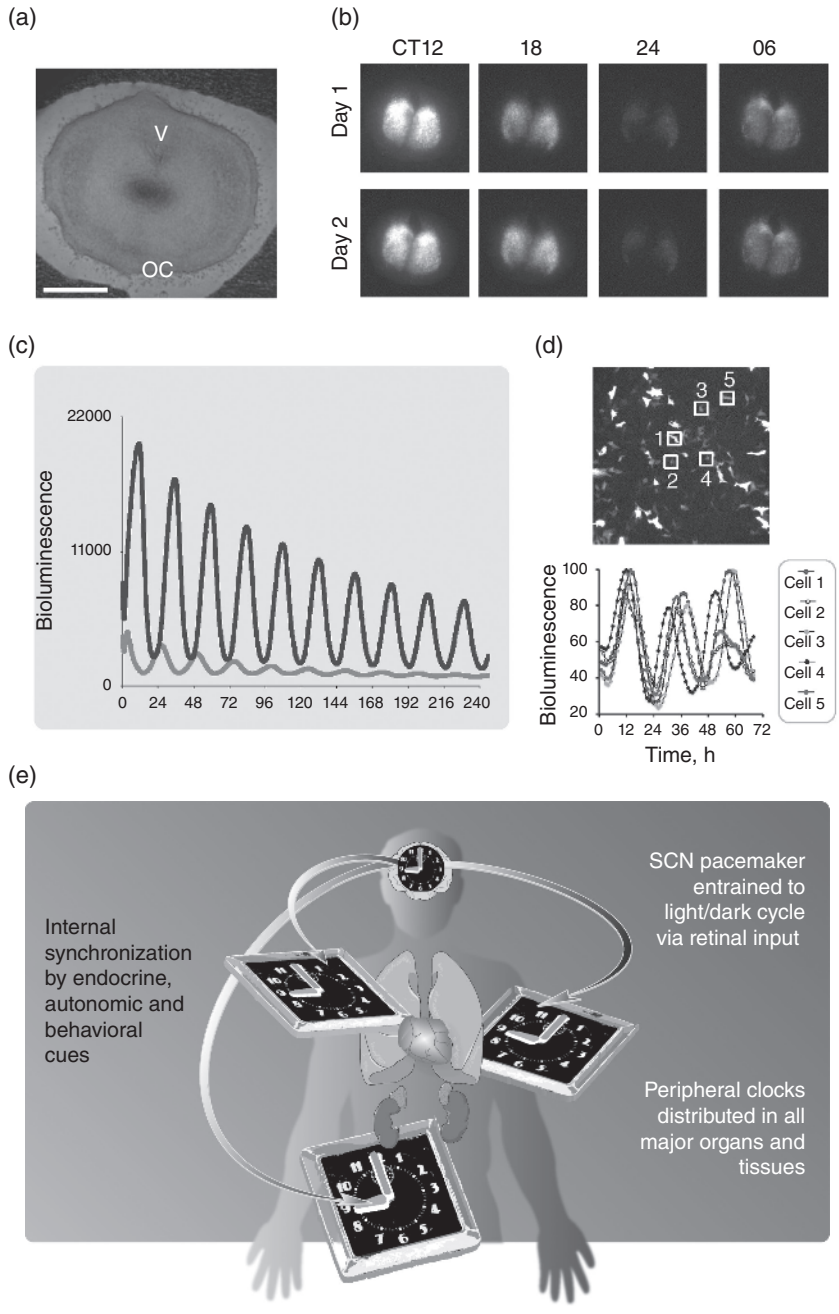

Fig. 1.2 Molecular pacemaking in SCN and other tissues and cells. (a) View of PER2::LUC SCN organotypic slice culture under phase illumination. V = third ventricle, oc = location of optic chiasm, scale bar = 500 um. (b) Serial bioluminescent images from same field of view as in (a), collected for 1 h every 6 h over 2 days in culture. Note stable and synchronized circadian oscillation in both SCN, with regionally specific phases of PER2 expression. CT = circadian time, CT12 = projected time of lights off. (c) Graphical plots of circadian bioluminescence from PER2::LUC organotypic SCN slices that are wild type (dark) or homozygote VIP-null (pale). Note stable molecular oscillation (with progressively smaller peaks due to luciferin substrate utilization) in wild type slices, but rapid loss of circadian organization in SCN lacking VIP. (d) Representative image of bioluminescent PER2::lUC fibroblasts (above) and plots of circadian rhythms of bioluminescence from individual cells in the culture (below). Note very stable molecular pacemaking, but no synchrony between cells. (e) Schematic representation of the internal circadian hierarchy in mammals, whereby local circadian clocks distributed across all major organs are governed by a variety of synchronizing cues ultimately derived from the SCN. In this way, daily rhythms across the body are synchronized to each other and also to solar time.

reflecting the transcriptional cycle of the clock. A second mutant mouse has in-frame luciferase coding sequences inserted into the endogenous *Per2* locus to generate an allele encoding a PERIOD::LUCIFERASE fusion protein (Yoo *et al*., 2004), and again individual SCN neurons express bioluminescence rhythms, this time in-phase with predicted native PER2 protein expression and, thereby, providing a posttranslational report of the clock mechanism.

Extensive studies using these and other reporter lines have revolutionized circadian biology because they, quite literally, provide a "window" on the SCN clock mechanism as it progresses through real time. They have, however, provided an even more profound understanding when applied to peripheral tissues and organs. A remarkable discovery based on Northern blot analyses of intermittent samples of cell culture extracts was that circadian genes are not only expressed in such cultures, but they are expressed with a circadian period: the transcriptional clock is active not only in the SCN but also in fibroblasts (Balsalobre *et al*., 1998) (Fig. 1.2d). Indeed, primary cultures of organs and tissues from circadian reporter animals could also exhibit self-sustained circadian transcriptional and posttranslational rhythms that can be imaged in real time by bioluminescent (Welsh *et al*., 2004; Yoo *et al*., 2004) or fluorescent reporters (Nagoshi *et al*., 2004). Importantly, these rhythms lack the "staying power" of the SCN, progressively damping out over a week or so. Nevertheless, circadian gene expression is sustained at the single cell level, but in the absence of any synchronising cues *in vitro*, the phases of individual cells within the culture dish or tissue gradually disperse and so the rhythm at the population level loses definition. The role of the SCN, therefore, is not to impose rhythms upon the rest of the brain and viscera. Rather, it is to coordinate the activity of the intrinsic transcriptional/posttranslational clocks distributed across innumerable cells in all of the major organs and tissues. The presence of such a complex spatio-temporal network underpinning metabolism and behavior has obvious relevance to health and disease. Moreover, it provides novel approaches for sophisticated diagnostic and therapeutic applications.

1.2.4 Elaborating the core transcriptional clockwork

Elucidation of the feedback actions mediated by PER/TIM in flies, FRQ in fungi and PER/CRY in mammals led to the idea of the "core" feedback loop, but developments in all three of the model organisms saw a gradual elaboration, adding additional rhythmic components and identifying rate-limiting enzymes. Importantly, in all three systems it became evident that some positive factors were rhythmically expressed due to the influence of their targets. In the case of mammals, this advance was facilitated by the tractable analysis of the transcriptional clockwork of peripheral tissues and cell lines, and the strongest early evidence came from identification of *Rev-Erb*α. This is a highly rhythmic circadian output gene driven by CLOCK/BMAL1 that encodes an orphan nuclear receptor that, in turn, inhibits *Bmal1* expression via its retinoic acid receptor-related orphan receptors response elements (RORE) regulatory sequences (Preitner *et al*., 2002). Thus, output of the "core" loop becomes its input. Further elaboration showed how a second circadian-controlled gene, *Rora*, acts as a positive factor to *Bmal1*, opposing the effect of *Rev-Erb*α at the RORE and thereby sculpting *Bmal1* expression. Whereas single-mutant mice show limited effects on the clock, mice lacking both *Rev-Erb*α and the closely related *Rev-Erb*β have major disruptions of metabolic and behavioral rhythms (Cho *et al*., 2012). Consequently, definition of the "core" clockwork progressively loses its focus as a network of transcriptional interactions develops. A further pair of basic helix–loop–helix transcription factors, DEC-1 and DEC-2, has also been implicated in the clock, insofar as they are expressed rhythmically in the SCN and also interfere with CLOCK/BMAL1 mediated transactivation. A final auxiliary loop consists of *Dbp* and *E4BP4*, which respectively activate and suppress transcription mediated by so-called D-boxes present in the *Per*, *Rev-Er*β and *Rora* genes. The clock-driven, rhythmic activities of DBP/E4BP4 will, therefore, feed back to influence the clock, generating a further autoregulatory group. The significance of this architecture of internested transcriptional loops is twofold (Ueda *et al*., 2005). First, it confers robustness to the overall behavior of the molecular oscillator and likely also boosts its amplitude. Second, because of the time constants of the various interlocking stages, the network establishes a phase map defined by serial episodes of activation and suppression of a number of genes, thereby providing more precise and definitive temporal resolution within the composite oscillation.

The discovery that cells and tissues contain transcriptional clocks very similar to those of the SCN was transformational for the experimental analysis of their regulatory mechanisms. The utility of cell cultures as a proxy for SCN pacemaking and the use of abundant tissues such as liver for biochemical analysis have made it possible to conduct studies that would be extremely difficult to perform on SCN. This has allowed a more comprehensive decoding of the molecular events associated with transcriptional activation. For example, with the description of the CLOCK/BMAL1 heterodimer to 2.3 Å it is now possible to define the roles of the basic helix–loop–helix and PAS domains in dimerization and DNA binding, and reveal key residues in the protein interfaces, mutations of which can alter transcriptional activity and the period of circadian pacemaking in fibroblasts (Huang *et al*., 2012). ChIP-seq and other biochemical analyses of liver have been able to track the various components of the CLOCK/BMAL1 complex (including RNA polymerase II, CRY, PER and associated factors) as it progresses through activated and suppressed states, associating with E-box-containing (and other) sequences (Koike *et al*., 2012). This cycle is accompanied by pronounced rhythms of histone modifications, including differentially phased cycles of methylation and acetylation as the oscillation progresses through times of transcriptional activation and suppression. Careful analysis of this molecular procession will likely provide important information regarding the general mechanisms of transcriptional coordination, with relevance well beyond the field of circadian clocks.

1.3 Keeping the transcriptional clockworks in tune

1.3.1 Entrainment of the SCN transcriptional clockwork

Retinal innervation of the SCN, carried via the retino-hypothalamic tract (RHT) is the means by which the transcriptional program of the SCN is synchronized to solar and seasonal time, as represented by the cycle of light and darkness (Reppert and Weaver, 2002). This pathway is described in detail in Chapter 3. The RHT consists of the axons of retinal ganglion cells (RGCs) and enters the ventro-lateral subdivision of the SCN, which contains neurons that express the neuropeptides vasoactive intestinal peptide (VIP) and gastrin-releasing peptide (GRP). The nonretinorecipient zone surrounding the core, termed the shell, is characterized by neurons expressing arginine vasopressin (AVP). Until recently it was assumed that conventional rods and cones are the circadian photoreceptors but a remarkable recent discovery was that a subclass of RGCs expresses a novel invertebrate-like opsin, melanopsin, that confers upon them intrinsic photoreceptivity (Rollag *et al*., 2003) (This volume, Chapter 3). These intrinsically photoreceptive RGCs (iPRGCs) are sufficient for circadian entrainment of the SCN, and they also mediate numerous other subliminal aspects of vision (Guler *et al*., 2008). They have broad receptive fields and act as luminance detectors rather than feature detectors: properties clearly adapted to their circadian role.

The principal neurotransmitter of RGCs is glutamate and the terminals of the RHT act upon the NMDA- and AMPA-type glutamate receptors expressed by retinorecipient SCN neurons. The subsequent influx of Ca^{2+} mediated by NMDAR increases the rate of firing of the neurons, which is otherwise low in circadian night (Kuhlman *et al*., 2003). It also activates a signaling cascade leading to increased gene expression mediated by the cAMP/Ca^{2+} response element (CRE) regulatory sequences in target genes (Obrietan *et al*., 1999; Schurov *et al*., 1999). Importantly, both *Per1* and *Per2* carry CREs, additional to their E-boxes (Travnickova-Bendova *et al*., 2002), so nocturnal light pulses acutely induce *Per* expression in the core SCN at a circadian time when it is otherwise very low (Albrecht *et al*., 1997; Shigeyoshi *et al*., 1997). This can be followed a few hours later by an increase in *Per* expression in the shell – likely triggered by the increased firing of action potentials by the core neurons and subsequent release of VIP, GRP and other transmitters onto the shell. These neuropeptides act via G-protein coupled receptors to activate cAMP and Ca^{2+} signaling, so will, in turn, increase *Per* expression via the CREs.

Thus, during circadian night, when spontaneous E-box-mediated expression of *Per* in the SCN is low, a light pulse will activate it and the additional pulse of PER protein will feed through the core loop and reset it to a new phase. If this occurs in the early subjective night when PER levels are falling, progression of the SCN molecular cycle is delayed, whereas light delivered in late circadian night when *Per* expression

is beginning to rise, will accelerate the rise and shift the molecular cycle forwards. During circadian daytime, when *Per* expression is already high, light has little impact on the molecular cycle. Thus, in the "real world," small phase adjustments to the molecular cycle at dusk (delay) and dawn (advance) will keep it synchronized to, and predictive of, the solar cycle, thereby ensuring appropriate phasing of the behavioral and physiological rhythms it controls. It is important to note that this entrainment by photic-induction of *Per* expression is equally applicable to both diurnal and nocturnal species because the cycle of *Per* expression in the SCN is the same in both: high in circadian day, low in circadian night, regardless of the animal's behavioral habits (Maywood and Mrosovsky, 2001). This transcriptional effect of light upon the SCN clock also enables the molecular cycle to encode season – the longer days of summer drive a broader peak of *Per* expression (Messager *et al*., 1999; Nuesslein-Hildesheim *et al*., 2000), which is ultimately decoded by brain and pituitary to engage adaptive seasonal changes such as altered appetite and nutrient utilization, reproductive status and migratory behavior (Dardente *et al*., 2010). The SCN can also be synchronized by cues other than light, principally behavioral arousal mediated by serotoninergic and neuropeptidergic cues from the brain stem (Hastings *et al*., 1997). In this case, the clock is most sensitive during circadian daytime, when *Per* expression is high and the cycle is acutely reset (advanced) by early suppression of *Per* – the mirror image of the effect of light. Indeed, because they have convergent but opposite molecular actions, light can block the resetting effect of arousal on the SCN and vice versa (Mead *et al*., 1992; Maywood *et al*., 2002).

1.3.2 Entrainment of transcriptional clocks in peripheral tissues

In contrast to the relatively limited number of mechanisms serving the SCN, the entrainment of the clocks within other brain regions, peripheral organs and cells is dependent upon a kaleidoscope of stimuli, some general and others specific to the cell types involved (Fig. 1.2e). The starting point is the SCN efferent innervation, which is distributed to a variety of target nuclei in the hypothalamus, brain stem and beyond, indirectly, to spinal cord and pituitary. The intrinsic clockwork of the SCN will enable it to convey time cues encoded as firing rate and very probably changes in the neurotransmitter types being released onto target cells. Although a small number of SCN neuropeptides have been implicated in transmitting circadian cues to the brain, we are far away from understanding how, at a systems-level such information is used to time fundamental neural processes, not least the alternation between states of sleep and wakefulness. In coordinating rhythms across the body, three general mechanisms are evident: behavior and the consequential cycle of feeding and fast; endocrine cues, especially the daily surge of corticosteroid hormones from the adrenal glands; and cues derived from the autonomic nervous system, such as the daily cycle of body temperature (Fig. 1.2e). Taking the liver clock as an example, under normal circumstances the SCN will determine the phases of feeding, core body temperature and corticosteroid secretion, which, in turn, will affect the local molecular pacemakers in the liver to ensure its various functions are appropriately timed to match the needs of the animal across day and night. This internal phasing can be altered experimentally by, for example, restricting the time of food availability or by injecting exogenous corticosteroids. Under such experimental conditions, the SCN remains phase-locked to solar time but the liver clockwork can be advanced or delayed by the new cues, thereby interfering with internal temporal coordination and, thus, metabolic efficiency.

The molecular basis of such entrainment is inevitably varied, although in the case of corticosteroid hormones (which are secreted at the start of the respective activity phase of nocturnal and diurnal species) the presence of glucocorticoid-response elements in *Per*, *Cry* and *Bmal1* genes provides a direct entry point to shifting the core clockwork. In contrast, entrainment by temperature cycles can involve transcriptional effects of heat shock factor (HSF 1), acting via response elements in the *Per2* gene, and posttranscriptionally via cold-inducible RNA-binding protein (CIRP), which appears to be necessary for normal expression and function of CLOCK protein. Indeed, the pivotal role of *Per2* as a sensor of entraining cues to the liver is demonstrated by the fact that in mice with a genetically compromised liver clock, *Per2* expression nevertheless continues to oscillate under the influence of systemic cues.

One clear demonstration of the functional importance of internal circadian tuning comes from the observation that mice lacking a liver clock but with otherwise normal circadian behavior are prone to

daily episodes of hypoglycemia because they are unable to perform the usual circadian up-regulation of hepatic gluconeogenesis to maintain glucose homeostasis during the fasting day. Notwithstanding these successes in identifying molecular pathways that entrain the local hepatic clock, the scale of the remaining problem remains enormous: the complexities of intercellular signaling between different tissues are already evident anatomically and biochemically, but now it must be taken to a new level by factoring in biological time. Solving this problem, however, will provide new therapeutic opportunities by the exploitation of circadian-based cues to regulate vital functions. One immediate example comes from the circadian morning surge in cardiovascular output (Fig. 1.3). Under normal circumstances this is adaptive, preparing the individual to engage with the world, but in those suffering from cardiovascular disease it represents a point of vulnerability – as reflected in the increased incidence of sudden cardiac death in hours immediately after awakening. Knowledge of the circadian signaling cascades from SCN to brain stem, to myocardium and to vascular endothelium that generate the morning surge could be used to develop time-based therapies to ameliorate the point of vulnerability without affecting baseline ongoing cardiovascular regulation (This volume, Chapters 8 & 9). This principle of circadian targeting applies to any number of systemic illnesses, not least metabolic syndrome and diabetes (This volume, Chapter 11).

1.3.3 Local tissue clocks direct local transcriptional and posttranscriptional programs

The relevance of transcription to circadian coordination extends far beyond the core feedback loops. A variety of DNA microarray studies and, more recently, RNA sequencing have shown that (depending on the algorithms used to detect significant rhythms) between 5 and 20% of the local transcriptome is subject to circadian modulation. Importantly in tissues such as the liver, this circadian modulation is most pronounced for transcripts involved in metabolic and signaling pathways (Akhtar *et al*., 2002; Koike *et al*., 2012; Menet *et al*., 2012), as well as cell cycle regulators. Characteristically, it is the enzymatic components of the cell that are clock-regulated rather than structural genes, such that the clock up- and down-regulates the "software" of the tissue, rather than its "hardware". The most immediate point of regulation of the circadian transcriptome is provided by the rhythmic activity of the proteins of the core oscillation, which periodically activates/suppresses the expression of target genes carrying E-boxes, D-boxes and ROREs. Recent ChIP-seq studies have established the genome-wide extent of such circadian control (and also highlighted the numerous targets of "clock" genes that are not circadian in their activity). Furthermore, some of the rhythmic targets of the core loop factors are themselves transcriptional regulators, for example, PPAR and HNF4a, so further tiers of circadian gene expression will be driven in a cascading effect. In addition, cues that entrain the core clock can also act upon clock-controlled genes directly, most obviously corticosteroids, which may act via glucocorticoid response elements (GREs) either independently or in concert with E-boxes, ROREs and other "circadian motifs." The transcriptome can, therefore, be viewed as a resonant network, enabling tissues to prepare to perform night- and day-specific metabolic and other functions in a timely manner, thereby supporting the individual's daily cycle of rest and activity.

The control of transcription is not, however, the only means to achieve temporal adaptation. Analysis of the cytosolic proteome of liver and SCN has revealed numerous proteins that are regulated at the level of protein abundance but not at steady-state transcript level. Furthermore, several isoforms of the same protein can be rhythmic but with contrasting phases of peak abundance, and recent RNA-sequencing studies suggest that only about 20% of rhythmically expressed genes in the liver are driven by *de novo* transcription. Clearly, posttranscriptional and posttranslational modifications are also important avenues for the clock to sculpt the functions of a tissue. Many RNA-binding proteins are circadian in their expression, and for example in liver and lung the clock and clock-related cues can influence the splicing of primary transcripts into different isoforms with contrasting temporal profiles. Posttranslational modifications, not least phosphorylation, are an additional circadian influence on the proteome, generating temporal diversity in cellular function. The prevailing view, however, remains one in which circadian cycles of gene expression drive rhythmic regulation of metabolism and signaling networks.

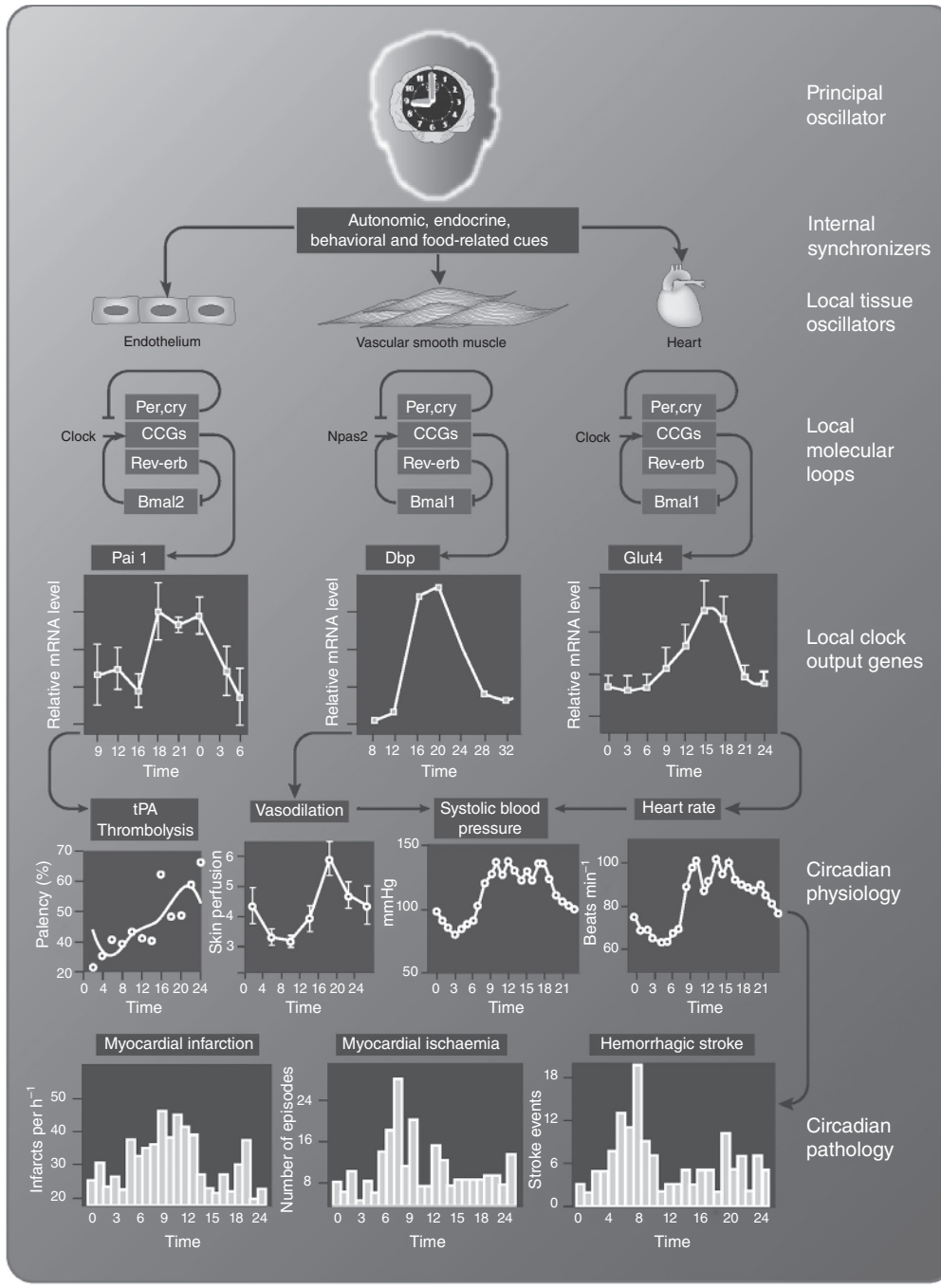

Fig. 1.3 Schematic view of systems-level circadian organization. Schematic view of circadian coordination across the individual in which the primary pacemaker of the SCN, entrained to solar time by retinal afferents, maintains and synchronises tissue-based clocks in the major organ systems by a blend of endocrine, autonomic and behavioral (feeding-related) cues. Disruption of these timing cues can result in pathology throughout the body. In this way, a robust circadian system contributes to our health and well-being while disrupting these rhythms as wide-ranging negative consequences. Redrawn with permission from Hastings *et al*. 2003.

1.4 Building posttranslational mechanisms into the circadian pacemaker

1.4.1 Posttranslational control of the clock: localization and stability of clock proteins

The obvious sophistication of posttranscriptional mechanisms in coordinating clock outputs raises the question of their potential role in the core pacemaking loop itself. For the nested transcriptional loops to oscillate effectively it is necessary for them to incorporate delays, which cannot be generated by the (inherently noisy) process of transcription itself (Suter *et al*., 2011). Rather, they can arise from regulation of the localization, activity and stability of the transcription factors that exert rhythmic transcriptional regulation (Hastings *et al*., 2007; Zheng and Sehgal, 2008; Asher and Schibler, 2011). These properties are themselves points of regulation by such mechanisms as phosphorylation and ubiquitinylation, and a conserved feature of the clock in fungi, flies and mammals is the role that dynamic protein phosphorylation plays in supporting rhythmicity *per se* and setting the clock's period (Hastings *et al*., 2008).

A good example is the ubiquitously expressed, highly conserved, and multifunctional serine/threonine-phosphorylating CASEIN KINASE 1 (CK1). In mammals both CK1e and CK1d (which are encoded by different genes) complex with and phosphorylate the PER proteins, directing their nuclear localization and stability. In the absence of both enzymes PER cannot be degraded, so the core transcriptional oscillation ceases (Etchegaray *et al*., 2010; Meng *et al*., 2010). Pharmacological inhibition of the enzymes slows down the rate of PER degradation, so progressively lengthens the period of the core loop and, thereby, slows down the behavioral activity rhythm. Conversely the gain-of-function *Tau* mutation in CK1e destabilizes PER protein and accelerates the pacemaker to 20 hours in homozygous mice and hamsters (Meng *et al*., 2008; Lowrey *et al*., 2000). In humans, mutations in both CK1d and the CK1 binding domain of PER2 are associated with a pronounced sleep disturbance, specifically advanced sleep phase, which is indicative of an accelerated circadian cycle consistent with the observations in rodents (Toh *et al*., 2001). The importance of CK1-dependent phosphorylation of PER is in its dual roles of licensing nuclear localization and yet also targeting the protein for ubiquitinylation by bTRCP and proteasomal degradation (Eide *et al*., 2005; Shirogane *et al*., 2005). In the case of CRY, stability is regulated in part by AMP kinase-mediated phosphorylation, which in turn licenses it for ubiquitinylation by the ligase FBXL3 (Godinho *et al*., 2007; Lamia *et al*., 2009). Various pharmacological and genetic manipulations of bTRCP and FBXL3 in mice, SCN and cell cultures can enhance PER and CRY stability, respectively, and thereby lengthen circadian period both *in vitro* and *in vivo* (Hirota *et al*., 2012).

Casein kinase 2 (CK2) and glycogen synthase kinase 3 (GSK3) are other well-known examples of ubiquitously expressed, multifunctional, highly conserved eukaryotic serine/threonine kinases that, in addition to their other established roles in the biology of the cell, have been shown to play critical roles in determining the cellular localization and/or stability of circadian transcription factors across a wide range of eukaryotes – even though the transcription factors themselves are not conserved (Yin *et al*., 2006; Hastings *et al*., 2008; Maier *et al*., 2009; O'Neill *et al*., 2011). As might be expected, the role of protein phosphatases, for example, PP1, is equally well conserved (Yang *et al*., 2004; Gallego *et al*., 2006; Fang *et al*., 2007; Schmutz *et al*., 2011). In the context of the transcriptional/translational feedback loop (TTFL) that has been proposed to account for cellular circadian rhythms, current data support a general model wherein a dynamic interplay between clock protein phosphorylation and de-phosphorylation by these ubiquitous enzymes acts as an interval timer to regulate the kinetics of complex formation, protein degradation and nuclear entry. Certain specific serine/threonine residues on each clock protein substrate are implicated in tipping the balance between degradation and nuclear localization (Reischl and Kramer, 2011).

1.4.2 Metabolic regulation of the transcriptional clockwork

An important question, therefore, is whether the posttranslational mechanisms discussed above are themselves circadian in nature or whether they are constitutively active and modify PER and CRY proteins as these proteins are generated. Although the expression level of most of these enzymes does not appear to be circadian, it remains possible that their activity is controlled in a rhythmic fashion and they act

coordinately, as in WNT signaling (Del Valle-Perez *et al*., 2011), to facilitate rhythmic intracellular localization and degradation of PER and CRY. In support of this possibility is the observation that the phosphorylation status, and therefore activity, of GSK3β is spontaneously rhythmic in cultured fibroblasts (Iitaka *et al*., 2005). Indeed the ubiquitin ligase FBXL21, which also targets CRY for degradation, is expressed in the SCN with high amplitude (Dardente *et al*., 2008). Even more intriguing is the fact that the kinase activity of AMPK is itself subject to the ratio of AMP and ADP:ATP in the cell (Oakhill *et al*., 2011). Thus, the ability of the cell to degrade CRY may vary as a function of the metabolic state of the cell, which in the case of the SCN, with its rhythm of electrical activity, is highly circadian. From this perspective a clock output (metabolic state) can be viewed as a clock input, and thus becomes part of the oscillator mechanism.

More generally it has been observed in several contexts that cellular metabolism is intrinsically rhythmic, for example, in mouse liver *in vivo* (Kaminsky *et al*., 1984; Eckel-Mahan *et al*., 2012; Fustin *et al*., 2012) and isolated mammalian cells *in vitro* (Radha *et al*., 1985; O'Neill and Reddy, 2011). This becomes of particular interest in light of observations that the cell's metabolic state can directly regulate transcription factor activity. For example, the DNA-binding activities of the CLOCK/BMAL1 and NPAS2/BMAL1 complexes is directly regulated by the redox state of nicotinamide adenine dinucleotide (NAD and NADP) cofactors, *in vitro* (Rutter *et al*., 2001).

In a still broader context, gene expression at loci bound by many clock gene transcription factors is associated with chromatin remodeling via recruitment of assorted histone methyl- and acetyl-transferases (Etchegaray *et al*., 2003; Ripperger and Schibler, 2006; Hosoda *et al*., 2009; Katada and Sassone-Corsi, 2010), all of which are ultimately reliant on the availability of their respective 1- and 2-carbon substrates (S-adenosylmethionine and Acetyl-CoA, respectively). These are generated by primary metabolism, and therefore also probably rhythmic, since intermediates in the pathways that generate them are (Eckel-Mahan *et al*., 2012). By the same token, the stability of PER2 and activity of BMAL1, are additionally regulated by dynamic lysine acetylation (Asher *et al*., 2008; Nakahata *et al*., 2008) and are deacetylated via specific recruitment of deacetylase SIRT1, which also targets histone H3, in an NAD^+-dependent manner. This facilitates the transition to transcriptionally repressive/inactive clock protein complexes later in the circadian cycle, again in a manner dependent on primary metabolism, in this case NAD^+ availability – which is also clock-regulated (Kaminsky *et al*., 1984; Ramsey *et al*., 2009). Finally, it is highly significant that several of the identified "clock gene" transcription factors are heme-binding proteins and exhibit reciprocal regulation between rhythmic heme metabolism and the heme protein's redox/ligand status, for example, heme-binding and, thus, activity of the nuclear receptor REV-ERBβ is governed by a redox-sensitive cysteine (Kaasik and Lee, 2004; Gupta and Ragsdale, 2011).

1.4.3 Cause versus effect in circadian transcriptional regulation

There are far more interactions between circadian timekeeping and metabolism than are discussed above; these are covered at length in some excellent reviews (Green *et al*., 2008; Asher and Schibler, 2011). In the latter, Asher and Schibler make the insight that "the discrimination between metabolic and circadian oscillations may be somewhat arbitrary." At the level of circadian timekeeping in cell culture or organotypic slice, therefore, there is an issue of cause and effect. The prevailing view is that circadian cycles of gene expression drive cellular rhythms of metabolism; however, as much evidence exists to support the contrary view that circadian cycles of metabolism drive rhythms of gene expression.

Certainly overexpression of clock gene transcription factors does not result in a major detriment to cellular timekeeping (Fan *et al*., 2007; Yamanaka *et al*., 2007; Asher and Schibler, 2011) and even gene knock-out does not completely abolish time-keeping in SCN slices (Liu *et al*., 2007; Ko *et al*., 2010; Maywood *et al*., 2011). Thus, whilst it is clear that circadian regulation of transcriptional circuits is essential for normal mammalian physiology and rhythmic behavior, at the cellular level rhythmic gene expression cannot be accounted for without delegating the majority of timekeeping function to rhythmic posttranslational regulation of clock protein localization, stability and activity. These are, in turn, determined by rhythmic enzyme activities and metabolic status – many of which are regulated at the level of transcription or translation: Catch 22 – or seemingly so.

1.5 Is the transcriptional clock paramount?

1.5.1 Cytosolic rhythms and the SCN pacemaker

To be effective as a central timekeeper, individual SCN neurons have to synchronize their molecular cycles, one to another and also to the light–dark cycle. When dispersed in cell culture, SCN neurons obviously lose synchrony as expected, but they are also less effective circadian pacemakers than when embedded in the usual SCN circuit – the transcriptional rhythms of individual cells lose amplitude and coherence. This dependence upon coupling is even more marked when the cells lack individual *Per* or *Cry* genes – single gene mutations that do not affect coherence at the level of the SCN ensemble. Clearly, intercellular signaling is a critical aspect not only in synchronizing the SCN cellular transcriptional clocks but also in maintaining them. Consistent with this, interference with electrophysiological signaling by tetrodotoxin (TTX) not only causes SCN neurons to become desynchronized but also to lose amplitude and definition to their transcriptional cycle (Yamaguchi *et al*., 2003; Maywood *et al*., 2007). There are several ways in which altering electrical communication across the circuit may affect the transcriptional clockwork. Suppression of action potential firing may alter intracellular Ca^{2+} signaling in both pre- and post-synaptic neurons, which in turn will alter transcriptional activation of *Per* and other genes via their CREs. In addition, the consequently reduced secretion of neuropeptides, including AVP, VIP and GRP, across the SCN will attenuate intracellular cues mediated via their G-protein coupled receptors, dis-regulating, *inter alia*, Ca^{2+}, cAMP and kinase cascades. Again this will compromise CRE-mediated transcription of *Per* and, thereby, undermine the core loops. A clear example of this is seen in mice lacking VIP or its cognate receptor, VPAC2. Not only are they behaviorally arrhythmic, but cellular transcriptional cycles in the SCN are also desynchronized and of low amplitude and coherence. These transcriptional cycles in the mutant SCN can be re-activated by paracrine cues, including AVP and VIP, derived from wild-type SCN grafted onto the mutant slice *in vitro* (Maywood *et al*., 2011). These peptides act via receptors that activate Gq and Gs signaling respectively, thereby driving Ca^{2+}, cAMP and kinase cascades to "rescue" the transcriptional loop. Under normal circumstances, the core loop drives the circadian rhythms of action potential firing, cAMP and Ca^{2+} levels, neuropeptide synthesis and secretion. Consequently, nontranscriptional outputs of the core loop within the SCN neuron are also its sustaining inputs, acting both within a neuron and between neurons. It can, therefore, be argued that this intercellular coupling is what makes the SCN special as a sustained pacemaker: the first amongst equals (Liu *et al*., 2007). Whereas bioluminescence recordings of other tissues (perhaps with the exception of the retina) progressively damp out as component, non-communicative cells lose phase coordination, that of the SCN slice will continue indefinitely (literally for many months, subject to an adequate supply of culture medium) with high amplitude and astonishing precision, as cells drive each other in reciprocal dependence. There is also a converse to this paramount competence – when dissociated from each other, SCN neurons may oscillate actually worse than individual fibroblasts do (Liu *et al*., 2007; Webb *et al*., 2009). The dependence upon coupling and intracellular signaling is, therefore, hardwired into the SCN as a condition of its pre-eminent role, and thus under contrived experimental conditions the SCN clockwork appears more vulnerable than that of a simple fibroblast.

The interdependence of intercellular cues, signaling cascades and the expression of clock genes blurs the distinction of a core loop. This distinction is further challenged by the observation that pharmacological manipulations of intracellular cAMP levels can change the canonical oscillatory properties of the transcriptional loop: its phase, amplitude and period (O'Neill *et al*., 2008). The case can be made, therefore, that the "real" pacemaker consists of both transcriptional and cytosolic components that are mutually dependent and act in concert. Some evidence for autonomous function of the cytosolic components comes from studies in *Cry*-null and *Bmal1*-null SCN, where the transcriptional loops are compromised but circadian cycles of *Per*-driven bioluminescence can still be observed in SCN neurons, albeit with shortened period and poor coherence (Ko *et al*., 2010; Maywood *et al*., 2011). This suggests that some capacity for cytosolic oscillation: a "cytoscillator," can exist independently of the transcriptional timer. Indeed, when transcription of nascent mRNA is compromised by treatment

with alpha-amanitin oleate (a potent transcriptional inhibitor), SCN slices can exhibit at least one and sometimes two further cycles of PER2::LUC- reported bioluminescence (O'Neill *et al*., 2013).

The possibility of a self-sustained cytosolic clock that normally couples with, but can run in the absence of, cycling clock genes has received further attention following recent observations made using undifferentiated embryonic stem cells (ESCs). ESCs were previously thought not to possess any intrinsic timekeeping because there is is no detectable cycling gene expression until differentiation. Paulose *et al*. report, however, a self-sustained rhythm in ESC glucose uptake prior to, and following, differentiation (Paulose *et al*., 2012). This again implies the existence of intrinsic timekeeping that is not reliant on any known transcriptional clock mechanism.

These observations reiterate earlier experiments in diverse model organisms that also addressed whether nascent transcription was necessary for cellular timekeeping, the earliest being in the alga *Acetabularia mediterranea*, where circadian rhythms of chloroplast movement persisted when the nucleus of the cell was removed (Sweeney and Haxo, 1961; Woolum, 1991). The landmark observations, however, were performed in the cyanobacteria *Synechococcus elongatus*, a prokaryote. Here it was shown that the approximately 24-hour rhythm of KaiA/B/C protein phosphorylation and complex formation that occurs in living cells, and which normally interacts reciprocally with genome-wide transcriptional regulation (Johnson *et al*., 2008), could be reconstituted *in vitro* using just the three recombinant proteins (KaiA, B and C) with ATP (Nakajima *et al*., 2005). Bacterial expression systems tend to work on a 1 protein = > 1 function principle, whilst mammalian proteins tend to possess multiple domains with multiple, context-dependent cellular functions. We therefore think it unlikely that a directly equivalent experiment can be performed for mammalian timekeeping. It does raise the possibility, however, that the smallest functional circadian timekeeping unit in mammals may not include the nucleus.

1.5.2 Totally transcription-free pacemaking

Recently the absolute requirement for nascent gene expression in mammalian cells was investigated *in vitro*. The ultimately cytotoxic effects of chronic inhibition of gene expression often confound pharmacological approaches to this question. To circumvent this, preparations of human red blood cells (which are naturally anucleate) were employed (O'Neill and Reddy, 2011). A rhythmic posttranslational modification of the peroxiredoxin (PRX) family of anti-oxidant proteins, first observed in mouse liver (Reddy *et al*., 2006), was used as a rhythmic marker. Briefly, the PRX family constitutes a major part of the cellular defense against reactive oxygen species (ROS), specifically H_2O_2, which are an unavoidable byproduct of aerobic metabolism. Erythrocytes express PRX at high levels (approximately 1% total protein), presumably due to the high ROS generation resulting from hemoglobin auto-oxidation. 2-Cys PRXs exist primarily as dimers that catalyze their own oxidation by H_2O_2 at conserved peroxidatic cysteine residues. The resultant sulfenic acid (Cys_p-SOH) may be reduced by a resolving cysteine on the opposing monomer (Cys_p-S-S-Cys_R), and ultimately reduced to the free thiol (SH) by the thioredoxin system. The kinetics of the resolving cysteine attack is quite slow, however, and in the presence of additional H_2O_2 overoxidation to the sulfenic (Cys_p-SO_2H) or even sulfonic (Cys_p-SO_3H) form, occurs (reversible through sulfiredoxin-catalyzed, ATP-dependent mechanisms). By performing anti-2-Cys PRX-$SO_{2/3}$ immunoblots on time-courses of erythrocytes, isolated in a minimal glucose/salt buffer under constant conditions, circadian rhythms of PRX oxidation were observed. These rhythms were temperature-compensated, entrainable by temperature cycles, and (predictably) robust to inhibitors of gene expression. In addition, the concentrations of several cellular metabolites ([ATP], [NADH], [NADPH]) appeared to be rhythmically modulated, as did an indirect fluorescence assay for hemoglobin multimeric state (O'Neill and Reddy, 2011).

As a marker for circadian timekeeping, the PRX oxidation rhythm appears to be highly conserved, being observable in representative organisms from across all three domains of life (Bacteria, Archeaea, Eukaryota), unlike any TTFL component (Edgar *et al*., 2012). Whilst PRX itself does not appear to play a critical timekeeping role, the redox rhythm it reports persists (albeit perturbed) in organisms that are deficient in "core" TTFL components. It is thus plausible that this remarkable conservation reflects either some underlying and ancient metabolic oscillation, which remains deeply embedded in the cellular machinery, or an evolutionary convergence upon rhythmic redox regulation to facilitate temporal segregation of mutually antagonistic metabolic processes.

1.5.3 A general model for mammalian cellular circadian timekeeping

Nascent transcription (cycling or otherwise) is not required for cellular circadian timekeeping (Tomita *et al*., 2005; O'Neill *et al*., 2011) but metabolism and signal transduction are required since they sustain life. In "normal" cells and organisms, however, circadian cycles of gene expression are observed and many of these cycling genes influence cell signaling and metabolism; ultimately facilitating rhythmic control of physiology. The activities of most known clock-relevant transcription factors are reliant upon metabolism and redox state, whereas their localization and stability, and in some cases acute induction, are determined posttranslationally and regulated by intracellular signaling systems. Furthermore, there are many established reciprocal pathways connecting redox balance and cellular metabolism with the activity of the various signaling mechanisms discussed above (Cheong and Virshup, 2011; Dickinson and Chang, 2011; Hardie, 2011; Sethi and Vidal-Puig, 2010; Metallo and Vander Heiden, 2010; Montenarh, 2010; Vander Heiden *et al*., 2009). In order to integrate these observations into a coherent framework (Fig. 1.4), therefore, we speculate that circadian rhythms in the cytoplasm persist through cyclical,

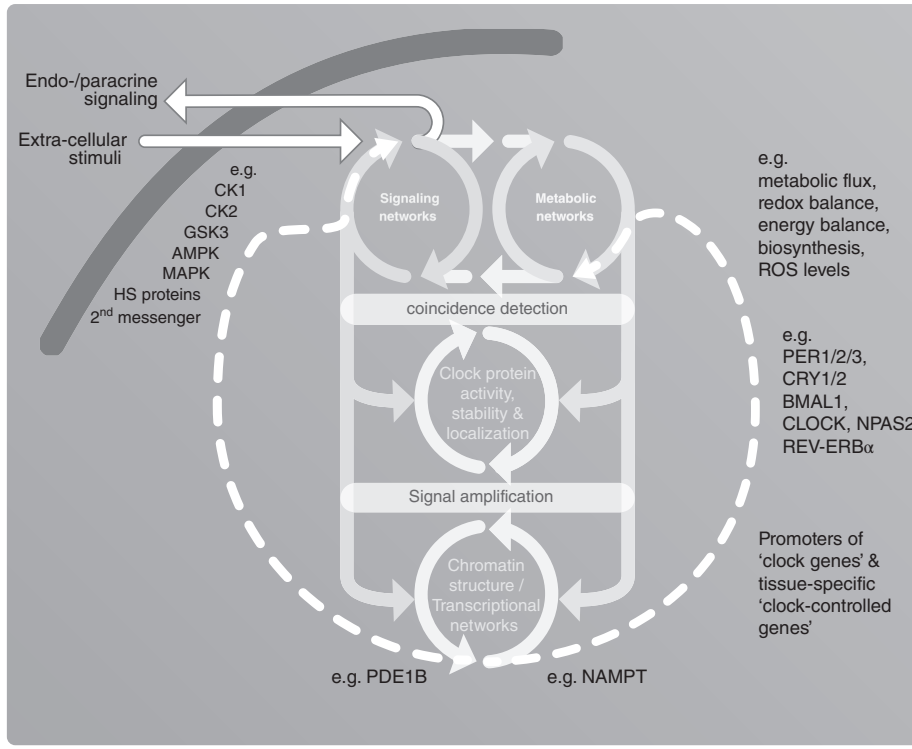

Fig. 1.4 A general model for mammalian cellular circadian timekeeping. Circadian timekeeping is functionally distributed within the cell's metabolic and signaling networks (top level) independently of nascent gene expression. In most (nucleated) cells, however, the integrated output from transcriptional networks (lower level) is manifest in the circadian cycles of protein activity/stability/localization (middle level) observed, for example, in canonical clock protein transcription factors which act as coincidence-detecting substrate effectors for network state. Rhythmically modulated chromatin structure facilitates coordinated temporal regulation of downstream networks of gene expression, including their own cognate clock gene circuitry, resulting in signal amplification. Rhythmic modulation of "clock-controlled genes" facilitates coordinated temporal regulation of physiology, and feeds forward into metabolic/signaling networks (right-hand flow), modulating expression of some component mechanisms, e.g., rhythmic NAMPT expression facilitates rhythmic activity of the NAD^+ salvage pathway (Ramsey *et al*., 2009), PDE1B degrades cAMP and affects rhythmic amplitude (Zhang *et al*., 2009). The circadian state of the signaling network modulates communication with local and distant targets, whilst selectively and temporally gating the capacity of relevant extracellular signals to affect circadian phase (left-hand flow).

distributed cross-talk between multiple metabolic and signaling networks, with transcriptional clock components acting as coincidence-detecting, substrate effectors. They thereby integrate the state of the network as a whole to coordinate genome-wide temporal, and cell type-specific, programs of gene expression. In this context, irrelevant network perturbations would be ignored but appropriate extracellular cues responded to in a phase-dependent fashion. Rhythmic licensing of transcription, with its slower kinetics, would impart robustness to the "cytoscillator" by rhythmic modulation of component protein/transcript levels. Critically, a rhythmic transcriptional contribution would not be required for oscillator competence but the additional repression of clock protein activity upon its cognate gene and CCGs would facilitate signal amplification. Similarly, rhythmic protein degradation is not required for cellular timekeeping (fibroblast rhythms are relatively insensitive to proteasomal inhibition) (Stratmann *et al*., 2012) but where present, it would increase the signal-to-noise ratio and amplify any transcriptional/translational contribution to the following cycle (Fig. 1.3). In essence, we suggest that, in contrast to the discreet clock mechanism in cyanobacteria, circadian timekeeping in mammalian (and by inference all eukaryotic) cells is functionally distributed amongst its component systems, which seem to have been coopted into the clock as soon as, or shortly after, they arose evolutionarily (Edgar *et al*., 2012).

1.6 Conclusion: cytoscillators, clocks and therapies

Since the acceptance that circadian rhythms are truly endogenous phenomena, driven by an internal timing mechanism rather than a response to undefined cyclical environmental cues, it has been obvious that an understanding of their nature and the mechanisms that govern them would provide a deeper insight into normal physiology and behavior, and thus identify new avenues for therapeutic intervention. There have been many surprises as the clock mechanisms have been unraveled; perhaps the greatest being that almost every cell has the potential to act as a circadian oscillator. This revelation brings complexity and opportunity in equal measure to biology and medicine. Because the paradigm of biology for the last decades has been genes and genomes, it is perhaps unsurprising that analysis of the clock mechanism focused on gene expression, leading to the development of the canonical model of TTFLs. Indeed, transcriptional processes are evident at all levels of the mammalian circadian system – from the core feedback loop, to entrainment by gene induction to orchestration of outputs and ultimate physiological rhythms by circadian transcriptomes. Nevertheless, the idea that the cellular environment within which the feedback loops are embedded influences their behavior has gained ground, leading to the view that transcriptional pacemaking and intrinsically rhythmic cytosolic oscillations are inescapably coupled, conferring precision and robustness. The latest revelations of transcription-free clocks in erythrocytes push this model further to show that cytosolic oscillations can exist independently of the nucleus: an echo of work on *Acetabularia* 50 years ago. Given the tractable "drugability" of cytosolic signaling in contrast to the dangers of meddling with transcription, it may well be that elucidation of the cytoscillator will provide the best entry point for future chronotherapies seeking to address diseases with a circadian dimension and, hence, circadian vulnerability (Fig. 1.3).

Acknowledgements

The work of the authors' laboratories is supported by the Medical Research Council (UK) (MC_UP_1201/4) and the Wellcome Trust (Grant 093734/Z/10/Z, JSO'N). The authors are indebted to Mr P. Margiotta, LMB Visual Aids Department, for expert assistance with the figures.

References

Akhtar RA, Reddy AB, Maywood ES, *et al*. 2002. Circadian cycling of the mouse liver transcriptome, as revealed by cDNA microarray, is driven by the suprachiasmatic nucleus. *Curr Biol* **12**: 540–50.

Albrecht U, Sun Z, Eichele G, Lee C. 1997. A differential response of two putative mammalian circadian regulators, mper1 and mper2 to light. *Cell* **91**: 1055–64.

Allada R, White NE, So WV, *et al*. 1998. A mutant Drosophila homolog of mammalian clock disrupts circadian rhythms and transcription of period and timeless. *Cell* **93**: 791–804.

Aronson BD, Johnson KA, Loros JJ, Dunlap JC. 1994. Negative feedback defining a circadian clock: autoregulation of the clock gene Frq. *Science* **263**: 1578–84.

Asher G, Schibler U. 2011. Crosstalk between components of circadian and metabolic cycles in mammals. *Cell Metab* **13**: 125–37.

Asher G, Gatfield D, Stratmann M, *et al*. 2008. SIRT1 regulates circadian clock gene expression through PER2 deacetylation. *Cell* **134**: 317–28.

Balsalobre A, Damiola F, Schibler U. 1998. A serum shock induces circadian gene expression in mammalian tissue culture cells. *Cell* **93**: 929–37.

Bliwise DL, Mercaldo ND, Avidan AY, *et al*. 2011. Sleep disturbance in dementia with Lewy bodies and Alzheimer's disease: a multicenter analysis. *Dement Geriatr Cogn Disord* **31**: 239–46.

Cashmore AR. 2003. Cryptochromes: enabling plants and animals to determine circadian time. *Cell* **114**: 537–43.

Cheong JK, Virshup DM. 2011. Casein kinase 1: Complexity in the family. *Int J Biochem Cell Biol* **43**: 465–9.

Cho H, Zhao X, Hatori M, *et al*. 2012. Regulation of circadian behavior and metabolism by REV-ERB-alpha and REV-ERB-beta. *Nature* **485**: 123–7.

Crosthwaite SK, Dunlap JC, Loros JJ. 1997. Neurospora wc-1 and wc-2: transcription, photoresponses, and the origins of circadian rhythmicity. *Science* **276**: 763–9.

Dardente H, Mendoza J, Fustin JM, *et al*. 2008. Implication of the F-Box Protein FBXL21 in circadian pacemaker function in mammals. *PLoS One* **3**: e3530.

Dardente H, Wyse CA, Birnie MJ, *et al*. 2010. A molecular switch for photoperiod responsiveness in mammals. *Curr Biol* **20**: 2193–8.

DeBruyne JP, Weaver DR, Reppert SM. 2007. CLOCK and NPAS2 have overlapping roles in the suprachiasmatic circadian clock. *Nat Neurosci* **10**: 543–5.

Del Valle-Perez B, Arques O, Vinyoles M, *et al*. 2011. Coordinated action of CK1 isoforms in canonical Wnt signaling. *Mol Cell Biol* **31**: 2877–88.

Dickinson BC, Chang CJ. 2011. Chemistry and biology of reactive oxygen species in signaling or stress responses. *Nat Chem Biol* **7**: 504–11.

Duffy JF, Cain SW, Chang AM, *et al*. 2011. Sex difference in the near-24-hour intrinsic period of the human circadian timing system. *Proc Natl Acad Sci USA* **108** (Suppl 3): 15602–8.

Eckel-Mahan KL, Patel VR, Mohney RP, *et al*. 2012. Coordination of the transcriptome and metabolome by the circadian clock. *Proc Natl Acad Sci USA* **109**: 5541–6.

Edgar RS, Green EW, Zhao Y, *et al*. 2012. Peroxiredoxins are conserved markers of circadian rhythms. *Nature* **485**: 459–64.

Eide EJ, Woolf MF, Kang H, *et al*. 2005. Control of mammalian circadian rhythm by CKIepsilon-regulated proteasome-mediated PER2 degradation. *Mol Cell Biol* **25**: 2795–807.

Etchegaray JP, Lee C, Wade PA, Reppert SM. 2003. Rhythmic histone acetylation underlies transcription in the mammalian circadian clock. *Nature* **421**: 177–82.

Etchegaray JP, Yu EA, Indic P, *et al*. 2010. Casein kinase 1 delta (CK1delta) regulates period length of the mouse suprachiasmatic circadian clock in vitro. *PLoS One* **5**: e10303.

Fan Y, Hida A, Anderson DA, *et al*. 2007. Cycling of CRYPTOCHROME proteins is not necessary for circadian-clock function in mammalian fibroblasts. *Curr Biol* **17**: 1091–100.

Fang Y, Sathyanarayanan S, Sehgal A. 2007. Posttranslational regulation of the Drosophila circadian clock requires protein phosphatase 1 (PP1). *Genes Dev* **21**: 1506–18.

Field MD, Maywood ES, O'Brien JA, *et al*. 2000. Analysis of clock proteins in mouse SCN demonstrates phylogenetic divergence of the circadian clockwork and resetting mechanisms. *Neuron* **25**: 437–47.

Fustin JM, Doi M, Yamada H, *et al*. 2012. Rhythmic nucleotide synthesis in the liver: temporal segregation of metabolites. *Cell Rep* **1**: 341–9.

Gallego M, Kang H, Virshup DM. 2006. Protein phosphatase 1 regulates the stability of the circadian protein PER2. *Biochem J* **399**: 169–75.

Godinho SI, Maywood ES, Shaw L, *et al*. 2007. The after-hours mutant reveals a role for Fbxl3 in determining mammalian circadian period. *Science* **316**: 897–900.

Green CB, Takahashi JS, Bass J. 2008. The meter of metabolism. *Cell* **134**: 728–42.

Guler AD, Ecker JL, Lall GS, *et al*. 2008. Melanopsin cells are the principal conduits for rod-cone input to non-image-forming vision. *Nature* **453**: 102–5.

Gupta N, Ragsdale SW. 2011. Thiol-disulfide redox dependence of heme binding and heme ligand switching in nuclear hormone receptor rev-erb{beta}. *J Biol Chem* **286**: 4392–403.

Hardie DG. 2011. AMP-activated protein kinase: an energy sensor that regulates all aspects of cell function. *Genes Dev* **25**: 1895–908.
Hardin PE. 2005. The circadian timekeeping system of Drosophila. *Curr Biol* **15**: R714–22.
Hardin PE, Hall JC, Rosbash M. 1990. Feedback of the *Drosophila* period gene product on circadian cycling of its messenger RNA levels. *Nature* **343**: 536–40.
Hastings JW. 2007. The Gonyaulax clock at 50: translational control of circadian expression. *Cold Spring Harb Symp Quant Biol* **72**: 141–4.
Hastings MH, Duffield GE, Ebling FJ, *et al*. 1997. Non-photic signalling in the suprachiasmatic nucleus. *Biol Cell* **89**: 495–503.
Hastings MH, Reddy AB, Maywood ES. 2003. A clockwork web: circadian timing in brain and periphery, in health and disease. *Nat Rev Neurosci* **4**: 649–61.
Hastings MH, O'Neill JS, Maywood ES. 2007. Circadian clocks: regulators of endocrine and metabolic rhythms. *J Endocrinol* **195**: 187–98.
Hastings MH, Maywood ES, O'Neill JS. 2008. Cellular circadian pacemaking and the role of cytosolic rhythms. *Curr Biol* **18**: R805–15.
Hatfield CF, Herbert J, Van Someren EJ, *et al*. 2004. Disrupted daily activity/rest cycles in relation to daily cortisol rhythms of home-dwelling patients with early Alzheimer's dementia. *Brain* **127**(5): 1061–74.
Hirota T, Lee JW, St John PC, *et al*. 2012. Identification of small molecule activators of cryptochrome. *Science* **337**: 1094–7.
Hogenesch JB, Gu YZ, Jain S, Bradfield CA. 1998. The basic-helix-loop-helix-PAS orphan MOP3 forms transcriptionally active complexes with circadian and hypoxia factors. *Proc Natl Acad Sci USA* **95**: 5474–9.
Hosoda H, Kato K, Asano H, *et al*. 2009. CBP/p300 is a cell type-specific modulator of CLOCK/BMAL1-mediated transcription. *Mol Brain* **2**: 34.
Huang N, Chelliah Y, Shan Y, *et al*. 2012. Crystal structure of the heterodimeric CLOCK:BMAL1 transcriptional activator complex. *Science* **337**: 189–94.
Huang W, Ramsey KM, Marcheva B, Bass J. 2011. Circadian rhythms, sleep, and metabolism. *J Clin Invest* **121**: 2133–41.
Iitaka C, Miyazaki K, Akaike T, Ishida N. 2005. A role for glycogen synthase kinase-3beta in the mammalian circadian clock. *J Biol Chem* **280**: 29397–402.
Johnson CH, Mori T, Xu Y. 2008. A cyanobacterial circadian clockwork. *Curr Biol* **18**: R816–R25.
Kaasik K, Lee CC. 2004. Reciprocal regulation of haem biosynthesis and the circadian clock in mammals. *Nature* **430**: 467–71.
Kaminsky YG, Kosenko EA, Kondrashova MN. 1984. Analysis of the circadian rhythm in energy metabolism of rat liver. *Int J Biochem* **16**: 629–39.
Katada S, Sassone-Corsi P. 2010. The histone methyltransferase MLL1 permits the oscillation of circadian gene expression. *Nat Struct Mol Biol* **17**: 1414–21.
King DP, Zhao Y, Sangoram AM, *et al*. 1997. Positional cloning of the mouse circadian Clock gene. *Cell* **89**: 641–53.
King VM, Chahad-Ehlers S, Shen S, *et al*. 2003. A hVIPR transgene as a novel tool for the analysis of circadian function in the mouse suprachiasmatic nucleus. *Eur J Neurosci* **17**: 822–32.
Knutsson A. 1989. Shift work and coronary heart disease. *Scand J Soc Med Suppl* **44**: 1–36.
Ko CH, Yamada YR, Welsh DK, *et al*. 2010. Emergence of noise-induced oscillations in the central circadian pacemaker. *PLoS Biol* **8**: e1000513.
Koh K, Zheng X, Sehgal A. 2006. JETLAG resets the Drosophila circadian clock by promoting light–induced degradation of TIMELESS. *Science* **312**: 1809–12.
Koike N, Yoo SH, Huang HC, *et al*. 2012. Transcriptional architecture and chromatin landscape of the core circadian clock in mammals. *Science* **338**: 349–54.
Konopka RJ. 1987. Genetics of biological rhythms in *Drosophila*. *Annu Rev Genet* **21**: 227–36.
Kuhlman SJ, Silver R, Le Sauter J, *et al*. 2003. Phase resetting light pulses induce Per1 and persistent spike activity in a subpopulation of biological clock neurons. *J Neurosci* **23**: 1441–50.
Kume K, Zylka MJ, Sriram S, *et al*. 1999. mCRY1 and mCRY2 are essential components of the negative limb of the circadian clock feedback loop. *Cell* **98**: 193–205.
Lamia KA, Sachdeva UM, DiTacchio L, *et al*. 2009. AMPK regulates the circadian clock by cryptochrome phosphorylation and degradation. *Science* **326**: 437–40.
Levi F, Schibler U. 2007. Circadian rhythms: mechanisms and therapeutic implications. *Annu Rev Pharmacol Toxicol* **47**: 593–628.
Liu AC, Welsh DK, Ko CH, *et al*. 2007. Intercellular coupling confers robustness against mutations in the SCN circadian clock network. *Cell* **129**: 605–16.

Loros JJ, Denome SA, Dunlap JC. 1989. Molecular cloning of genes under control of the circadian clock in Neurospora. *Science* **243**: 385–8.
Lowrey PL, Shimomura K, Antoch MP, *et al*. 2000. Positional syntenic cloning and functional characterization of the mammalian circadian mutation tau. *Science* **288**: 483–92.
Maier B, Wendt S, Vanselow JT, *et al*. 2009. A large-scale functional RNAi screen reveals a role for CK2 in the mammalian circadian clock. *Genes Dev* **23**: 708–18.
Maywood ES, Mrosovsky N. 2001. A molecular explanation of interactions between photic and non-photic circadian clock-resetting stimuli. *Brain Res Gene Expr Patterns* **1**(1): 27–31.
Maywood ES, Okamura H, Hastings MH. 2002. Opposing actions of neuropeptide Y and light on the expression of circadian clock genes in the mouse suprachiasmatic nuclei. *Eur J Neurosci* **15**: 216–20.
Maywood ES, O'Neill JS, Chesham JE, Hastings MH. 2007. Minireview: The circadian clockwork of the suprachiasmatic nuclei – analysis of a cellular oscillator that drives endocrine rhythms. *Endocrinology* **148**: 5624–34.
Maywood ES, Chesham JE, O'Brien JA, Hastings MH. 2011. A diversity of paracrine signals sustains molecular circadian cycling in suprachiasmatic nucleus circuits. *Proc Natl Acad Sci USA* **108**: 14306–11.
Mead S, Ebling FJ, Maywood ES, *et al*. 1992. A nonphotic stimulus causes instantaneous phase advances of the light-entrainable circadian oscillator of the Syrian hamster but does not induce the expression of c-fos in the suprachiasmatic nuclei. *J Neurosci* **12**: 2516–22.
Menet JS, Rodriguez J, Abruzzi KC, Rosbash M. 2012. Nascent-Seq reveals novel features of mouse circadian transcriptional regulation. *eLife* **1**: e00011.
Meng QJ, Logunova L, Maywood ES, *et al*. 2008. Setting clock speed in mammals: the CK1epsilontau mutation in mice accelerates circadian pacemakers by selectively destabilizing PERIOD proteins. *Neuron* **58**: 78–88.
Meng QJ, Maywood ES, Bechtold DA, *et al*. 2010. Entrainment of disrupted circadian behavior through inhibition of casein kinase 1 (CK1) enzymes. *Proc Natl Acad Sci USA* **107**: 15240–5.
Messager S, Ross AW, Barrett P, Morgan PJ. 1999. Decoding photoperiodic time through Per1 and ICER gene amplitude. *Proc Natl Acad Sci USA* **96**: 9938–43.
Metallo CM, Vander Heiden MG. 2010. Metabolism strikes back: metabolic flux regulates cell signaling. *Genes Dev* **24**: 2717–22.
Montenarh M. 2010. Cellular regulators of protein kinase CK2. *Cell Tissue Res* **342**: 139–46.
Myers MP, Wager-Smith K, Rothenfluh-Hifiker A, Young MW. 1996. Light-induced degradation of TIMELESS and entrainment of the Drosophila circadian clock. *Science* **85**: 1737–41.
Nagoshi E, Saini C, Bauer C, *et al*. 2004. Circadian gene expression in individual fibroblasts: cell-autonomous and self-sustained oscillators pass time to daughter cells. *Cell* **119**: 693–705.
Nakahata Y, Kaluzova M, Grimaldi B, *et al*. 2008. The NAD+-dependent deacetylase SIRT1 modulates CLOCK-mediated chromatin remodeling and circadian control. *Cell* **134**: 329–40.
Nakajima M, Imai K, Ito H, *et al*. 2005. Reconstitution of circadian oscillation of cyanobacterial KaiC phosphorylation *in vitro*. *Science* **308**: 414–5.
Nuesslein-Hildesheim B, O'Brien JA, Ebling FJ, *et al*. 2000. The circadian cycle of mPER clock gene products in the suprachiasmatic nucleus of the Siberian hamster encodes both daily and seasonal time. *Eur J Neurosci* **12**: 2856–64.
O'Neill JS, Reddy AB. 2011. Circadian clocks in human red blood cells. *Nature* **469**: 498–503.
O'Neill JS, Maywood ES, Chesham JE, *et al*. 2008. cAMP-dependent signaling as a core component of the mammalian circadian pacemaker. *Science* **320**: 949–53.
O'Neill JS, van Ooijen G, Dixon LE, *et al*. 2011. Circadian rhythms persist without transcription in a eukaryote. *Nature* **469**: 554–8.
O'Neill JS, Maywood ES, Hastings MH. 2013. Cellular mechanisms of circadian pacemaking: beyond transcriptional loops. In: *Handbook of Experimental Pharmacology: Circadian Clocks* (eds A Kramer, M Merrow). Springer, pp. 67–103.
Oakhill JS, Steel R, Chen ZP, *et al*. 2011. AMPK is a direct adenylate charge-regulated protein kinase. *Science* **332**: 1433–5.
Obrietan K, Impey S, Smith D, *et al*. 1999. Circadian regulation of cAMP response element-mediated gene expression in the suprachiasmatic nuclei. *J Biol Chem* **274**: 17748–56.
de Paula RM, Vitalini MW, Gomer RH, Bell-Pedersen D. 2007. Complexity of the Neurospora crassa circadian clock system: multiple loops and oscillators. *Cold Spring Harb Symp Quant Biol* **72**: 345–51.
Paulose JK, Rucker EB, 3rd, Cassone VM. 2012. Toward the beginning of time: circadian rhythms in metabolism precede rhythms in clock gene expression in mouse embryonic stem cells. *PLoS One* **7**: e49555.
Preitner N, Damiola F, Lopez-Molina L, *et al*. 2002. The orphan nuclear receptor REV-ERBalpha controls circadian transcription within the positive limb of the mammalian circadian oscillator. *Cell* **110**: 251–60.
Radha E, Hill TD, Rao GH, White JG. 1985. Glutathione levels in human platelets display a circadian rhythm *in vitro*. *Thromb Res* **40**: 823–31.

Ramsey KM, Yoshino J, Brace CS, *et al.* 2009. Circadian clock feedback cycle through NAMPT-mediated NAD+ biosynthesis. *Science* **324**: 651–4.

Reddy AB, Karp NA, Maywood ES, *et al.* 2006. Circadian orchestration of the hepatic proteome. *Curr Biol* **16**: 1107–15.

Reischl S, Kramer A. 2011. Kinases and phosphatases in the mammalian circadian clock. *FEBS Lett* **585**: 1393–9.

Reppert SM, Weaver DR. 2002. Coordination of circadian timing in mammals. *Nature* **418**: 935–41.

Ripperger JA, Schibler U. 2006. Rhythmic CLOCK-BMAL1 binding to multiple E-box motifs drives circadian Dbp transcription and chromatin transitions. *Nat Genet* **38**: 369–74.

Rollag MD, Berson DM, Provencio I. 2003. Melanopsin, ganglion-cell photoreceptors, and mammalian photoentrainment. *J Biol Rhythms* **18**: 227–34.

Rutila JE, Suri V, Le M, *et al.* 1998. CYCLE is a second bHLH-PAS clock protein essential for circadian rhythmicity and transcription of Drosophila period and timeless. *Cell* **93**: 805–14.

Rutter J, Reick M, Wu LC, McKnight SL. 2001. Regulation of clock and NPAS2 DNA binding by the redox state of NAD cofactors. *Science* **293**: 510–4.

Schmutz I, Wendt S, Schnell A, *et al.* 2011. Protein phosphatase 1 (PP1) is a posttranslational regulator of the mammalian circadian clock. *PLoS One* **6**: e21325.

Schurov IL, McNulty S, Best JD, *et al.* 1999. Glutamatergic induction of CREB phosphorylation and Fos expression in primary cultures of the suprachiasmatic hypothalamus *in vitro* is mediated by co-ordinate activity of NMDA and non-NMDA receptors. *J Neuroendocrinol* **11**: 43–51.

Sethi JK, Vidal-Puig A. 2010. Wnt signalling and the control of cellular metabolism. *Biochem J* **427**: 1–17.

Shigeyoshi Y, Taguchi K, Yamamoto S, *et al.* 1997. Light-induced resetting of a mammalian circadian clock is associated with rapid induction of the mPer1 transcript. *Cell* **91**: 1043–53.

Shirogane T, Jin J, Ang XL, Harper JW. 2005. SCFbeta-TRCP controls clock-dependent transcription via casein kinase 1-dependent degradation of the mammalian period-1 (Per1) protein. *J Biol Chem* **280**: 26863–72.

Stratmann M, Suter DM, Molina N, *et al.* 2012. Circadian Dbp transcription relies on highly dynamic BMAL1-CLOCK interaction with E boxes and requires the proteasome. *Mol Cell* **48**: 277–87.

Suter DM, Molina N, Gatfield D, *et al.* 2011. Mammalian genes are transcribed with widely different bursting kinetics. *Science* **332**: 472–4.

Sweeney BM, Haxo FT. 1961. Persistence of a photosynthetic rhythm in Enucleated Acetabularia. *Science* **134**: 1361–63.

Takahashi JS, Shimomura K, Kumar V. 2008. Searching for genes underlying behavior: lessons from circadian rhythms. *Science* **322**: 909–12.

Tei H, Okamura H, Shigeyoshi Y, *et al.* 1997. Circadian oscillation of a mammalian homologue of the Drosophila period gene. *Nature* **389**: 512–6.

Toh KL, Jones CR, He Y, *et al.* 2001. An hPer2 phosphorylation site mutation in familial advanced sleep phase syndrome. *Science* **291**: 1040–3.

Tomita J, Nakajima M, Kondo T, Iwasaki H. 2005. No transcription-translation feedback in circadian rhythm of KaiC phosphorylation. *Science* **307**: 251–4.

Travnickova-Bendova Z, Cermakian N, Reppert SM, Sassone-Corsi P. 2002. Bimodal regulation of mPeriod promoters by CREB-dependent signaling and CLOCK/BMAL1 activity. *Proc Natl Acad Sci USA* **99**: 7728–33.

Ueda HR, Hayashi S, Chen W, *et al.* 2005. System-level identification of transcriptional circuits underlying mammalian circadian clocks. *Nat Genet* **37**: 187–92.

Vander Heiden MG, Cantley LC, Thompson CB. 2009. Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science* **324**: 1029–33.

van der Horst GT, Muijtjens M, Kobayashi K, *et al.* 1999. Mammalian Cry1 and Cry2 are essential for maintenance of circadian rhythms. *Nature* **398**: 627–30.

Viswanathan AN, Hankinson SE, Schernhammer ES. 2007. Night shift work and the risk of endometrial cancer. *Cancer Res* **67**: 10618–22.

Weaver DR. 1998. The suprachiasmatic nucleus: a 25-year retrospective. *J Biol Rhythms* **13**: 100–12.

Webb AB, Angelo N, Huettner JE, Herzog ED. 2009. Intrinsic, nondeterministic circadian rhythm generation in identified mammalian neurons. *Proc Natl Acad Sci USA* **106**: 16493–8.

Welsh DK, Yoo SH, Liu AC, *et al.* 2004. Bioluminescence imaging of individual fibroblasts reveals persistent, independently phased circadian rhythms of clock gene expression. *Curr Biol* **14**: 2289–95.

Woolum JC. 1991. A re-examination of the role of the nucleus in generating the circadian rhythm in Acetabularia. *J Biol Rhythms* **6**: 129–36.

Wulff K, Gatti S, Wettstein JG, Foster RG. 2010. Sleep and circadian rhythm disruption in psychiatric and neurodegenerative disease. *Nat Rev Neurosci* **11**: 589–99.

Yamaguchi S, Isejima H, Matsuo T, *et al*. 2003. Synchronization of cellular clocks in the suprachiasmatic nucleus. *Science* **302**: 1408–12.

Yamanaka I, Koinuma S, Shigeyoshi Y, *et al*. 2007. Presence of robust circadian clock oscillation under constitutive over-expression of mCry1 in rat-1 fibroblasts. *FEBS Lett* **581**: 4098–102.

Yang Y, He Q, Cheng P, *et al*. 2004. Distinct roles for PP1 and PP2A in the Neurospora circadian clock. *Genes Dev* **18**: 255–60.

Yin L, Wang J, Klein PS, Lazar MA. 2006. Nuclear receptor Rev-erbalpha is a critical lithium-sensitive component of the circadian clock. *Science* **311**: 1002–5.

Yoo SH, Yamazaki S, Lowrey PL, *et al*. 2004. PERIOD2::LUCIFERASE real-time reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues. *Proc Natl Acad Sci USA* **101**: 5339–46.

Zhang EE, Liu AC, Hirota T, *et al*. 2009. A genome-wide RNAi screen for modifiers of the circadian clock in human cells. *Cell* **139**: 199–210.

Zheng X, Sehgal A. 2008. Probing the relative importance of molecular oscillations in the circadian clock. *Genetics* **178**: 1147–55.