Circadian rhythms coordinate our physiology at a fundamental level. Over the last 20 years, we have witnessed a paradigm shift in our perception of what the clocks driving such rhythms actually are, moving from ‘black boxes’ to talking about autoregulatory transcriptional/post-translational feedback loops with identified molecular components. We also now know that the pacemaker of the suprachiasmatic nuclei (SCN) is not our only clock but quite the opposite because circadian clocks abound in our bodies, driving local rhythms of cellular metabolism, and synchronised to each other and to solar time, by cues from the SCN. This discovery of dispersed local clocks has far-reaching implications for understanding our physiology and the pathological consequences of clock dysfunction, revealing that clocks are critical in a variety of metabolic and neurological conditions, all of which have long-term morbidity attributable to them. Without the currently available molecular framework, these insights would have not have been possible. In the circadian future, a growing appreciation of the systems-level functioning of these clocks and their various cerebral and visceral outputs, will likely stimulate the development of novel therapies for major illnesses.

Key words: sleep, period, metabolism, clock, corticosteroids, rhythm.

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principal circadian pacemaker was acknowledged (7) and circadian biology had its formalisms, endocrine profiles and neurobiology, but it lacked a mechanism. This debilitating shortcoming was resolved in 1997 with the identification of circadian clock genes in mammals (8–11), neatly dividing our two decades into work before and after this watershed. Whereas the genes encoding the negative regulatory factors Period (Per1, Per2, Per3) and Cryptochrome (Cry1, Cry2) were revealed via their homology to known factors in lower organisms (12), the *Clock* gene was discovered by forward genetics, screening for circadian behavioural mutants amongst the progeny of ENU-mutagenised mice (13). The ultimate cloning and functional rescue of the *Clock* mutation (characterised by a lengthened period and destabilised circadian behaviour) by transgenic manipulation in the pre-mouse-genome age was an inspiring intellectual and technical achievement (8, 9). With the subsequent identification of Bmal1 (Mop3) as a transcriptional partner of the Clock protein, and an essential component of the clockwork (14), a plausible model could be assembled of the SCN circadian oscillator that drew heavily on studies in fruit flies (15) and the fungus *Neurospora* (16).

This model pivots around an autoregulatory transcriptional/post-translational negative feedback loop (Fig. 1), in which heterodimers of Clock and Bmal1 proteins bind to E-box regulatory DNA sequences to activate the expression of *Per* and *Cry* genes at the start of circadian day. Approximately 12 h later, complexes of *Per* and *Cry* proteins are at peak levels in the nucleus, where they suppress E-box dependent gene activation, resulting in a fall in *Per* and *Cry* mRNA levels back to their nadir. The existing *Per* and *Cry* proteins are degraded by the proteasome and, in the absence of further translation, their levels decline until, by the end of circadian night, they have been cleared and Clock/Bmal1 heterodimers are now unopposed and free to start the cycle afresh.

The amplitude, precision and stability of this core oscillatory cycle are enhanced by an accessory loop involving two nuclear orphan receptor proteins, Rev-Erbα and RORA, encoded by genes that are also expressed rhythmically in phase with *Per* and *Cry* because they too carry E-box sequences. RORA and Rev-Erbα act through RORE regulatory sequences, respectively activating and suppressing *Bmal1* expression, with the result that *Bmal1* mRNA cycles in antiphase to that of *Per*, peaking late in circadian night and thus delivering a ‘positive surge’ of *Bmal1* coincident with the decline in negative feedback. Recently, heme, a cofactor in oxidative metabolism, has been shown to bind and regulate Rev-Erbα activity, thereby linking cellular metabolic cycles into the core clockwork (17).

The primary output of the oscillatory loops is in the form of rhythmic expression of clock-controlled genes (Fig. 1), the simplest of which carry E-box and RORE sequences, and thus are sensitive

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**Fig. 1.** Schematic representation of the molecular feedback loops that underpin circadian timing. Within neurons of the suprachiasmatic nuclei (SCN), temporally precise up- and down-regulation of the core clock genes is effected by complexes of proteins encoded by those genes in a series of autoregulatory, transcriptional/post-translational feedback loops (lower panel). In turn, this oscillatory expression of clock proteins directs the rhythmic activity of suites of downstream clock-controlled genes (upper panel), which are ultimately responsible for the circadian metabolic and electrical firing rate rhythms of the SCN. Key questions remain as to how clock proteins recruit and influence the activity of the basic transcriptional machinery and how their temporally regulated degradation sets circadian period, how rhythms in gene expression become manifest as electrical firing rate and neurosecretory rhythms, and how properties of the SCN as a circuit, via inter-neuronal signalling, sustain the intra-cellular molecular clockwork (for details, see text).
to intermittent repression by Per, Cry and Rev-Erbα. Amongst these are the E-box regulated PAR-domain basic leucine zipper transcription factor family, including albumin D-element binding protein (Dbp), that act via D-box sequences in their own target genes to control further downstream levels of transcriptional output. Consequently, between 5% and 10% of the SCN transcriptome is under circadian regulation (18), including genes encoding metabolic enzymes and ion channels. It remains an open question, however, as to how the pronounced circadian cycle of electrical firing rate in the SCN is maintained by these transcriptional programmes. Presumably up-regulation of cellular metabolic pathways is a precondition to support the increased energetic demands of electrical firing during circadian daytime, but what are the key conductance changes governing rhythmic membrane potential and how does the intracellular clockwork control them?

As for communication of circadian signals from the SCN to its neural targets, especially the sleep- and wake-regulatory centres of the pre-optic and dorsomedial hypothalamus (19), a number of clock-controlled neuropeptides and their receptors, including vasointestinal peptide (VIP), cardiotoxin-like cytokine and prokineticin 2, have been implicated. The relative contributions of electrical coding (changes in firing rates) and neurochemical coding (variation in which neuropeptides are released) to signalling circadian time are, however, yet to be determined. Indeed, unravelling the links from the molecular transcriptional oscillation, through rhythmic neuropeptide synthesis, firing rate and neurotransmitter secretion by the SCN and thence into the control of neuroendocrine circuits that choreograph behavioural, physiological and metabolic circadian rhythms, remains a major challenge to the field (Fig. 1). Thus, we have mapped out some of the parts, but have yet to establish a comprehensive view.

**Circadian times present: a new orthodoxy**

Identification of clock genes did more than propel circadian rhythms into molecular cell biology; it completely overturned the existing dogma that the SCN (alongside a specialised clock in the retina) were the only tissue capable of circadian pacemaking. The first indication that this was incorrect came from ex vivo expression studies showing that clock genes are expressed rhythmically in many (if not all) peripheral tissues. Moreover, the relative phasing of expression peaks and troughs of the different genes followed that observed in the SCN, as though the same feedback loops were operating peripherally, but with a phase delay relative to the SCN. Extension of these multi-sample gene expression studies then produced the remarkable result that immortalised fibroblasts contain an autonomous clockwork, expressing clock and clock-controlled genes with a circadian period in culture (20). The next step-change came with the creation of transgenic rats and mice in which fragments of the *Per1* promoter containing E-boxes or the entire *Per2* gene were used variously to drive green fluorescent protein or firefly luciferase optical reporters. This allowed the molecular pacemaker to be monitored continuously, and in real-time, in individual tissues isolated in culture (Fig. 2). This showed that not only the SCN, but also most major organ systems tested *in vitro* are able to exhibit self-sustaining rhythms of circadian gene expression. Not only is the body full of circadian clocks, but recent imaging studies also show that the molecular clockwork acts autonomously within individual cells (21).

Hence, the new orthodoxy is not that the SCN comprise the only clock and impose a circadian pattern onto a passive periphery; rather the SCN are the central coordinator of a plethora of tissue-based, autonomously active cellular clocks dispersed across the body. By virtue of their retinal innervation and effenter connections, the SCN have the unique role of maintaining appropriate synchrony between these local clocks and the local metabolic rhythms dependent upon them, to solar time, thereby ensuring that physiology across the entire organism is temporally integrated and

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**Fig. 2.** An intrinsic capacity for circadian timekeeping is widely distributed across the brain. Circadian cycles of bioluminescence emitted from organotypical slices of brain tissue cultured from mPer2::luciferase reporter mice (69). Note high amplitude and sustained circadian gene expression in suprachiasmatic nuclei (SCN), consistent with its autonomous pacemaker role. Note also that other brain regions have competent local molecular clocks that can continue to oscillate for several days when held in isolation. In vivo, these distributed local clockworks, which likely have the potential to regulate sleep-dependent cognitive and other neural functions, are synchronised and sustained by the SCN.
thus maximally adaptive (4). As with the SCN, approximately 5–10% of local transcriptomes are subject to circadian regulation, whereas an even higher proportion of local proteomes may vary with circadian time (22). Importantly, the local clockwork, synchronised by the SCN, directs the expression of rate-limiting and vital factors (e.g. the clock in the liver controls carbohydrate and nitrogen metabolism, xenobiotic detoxification and cell division).

The obvious question of how is internal synchrony achieved is rich territory for the neuroendocrinologist. Multi-synaptic pathways linking the SCN into the autonomic nervous system clearly play an important role; for example, by controlling the nocturnal secretion of melatonin to facilitate sleep (23), in regulating metabolic cycles dependent on the liver (24) and also mediating circadian and light-regulated gene expression in the adrenal (25). In turn, the daily cycle of corticosteroid secretion by the adrenal, ultimately governed by the SCN via both neuroendocrine and autonomic pathways, is a major coordinator of peripheral clocks. For example, a single treatment with the corticosteroid agonist dexamethasone acutely regulates approximately 60% of the circadian transcriptome of the liver (26). Furthermore, the important synchronising effect of nutrient availability arising from ingestion and digestion can be shown by subjecting animals to restricted feeding cycles that uncouple circadian gene expression rhythms in the liver and other peripheral tissues from control by the SCN clockwork (27). With so many potential synchronisers, it is not surprising to find redundancy and even antagonistic effects between cues, as seen with the inhibition by corticosteroids of the effects of restricted feeding (28).

Teasing apart the internal web of entrainment and the contribution of systemic and intrinsic factors in regulating local clocks is therefore extremely difficult, but one elegant approach involves conditional genetic suppression of the intrinsic clock of the liver (29). Although the bulk of the hepatic circadian transcriptome depends upon the hepatic clockwork, circadian rhythmicity of a minority of genes, including Per2, can be sustained by systemic signals even when the intrinsic clock of the liver is genetically inactivated. Indeed, just as in the SCN where retinal activation of Per expression synchronises the molecular loops of the SCN to environmental light, control of Per2 expression may be the critical feature in systemic entrainment of the liver and other peripheral clocks. Given that these entrainment pathways ultimately direct the expression of vital metabolic processes, characterisation of the responsible systemic factors and their intra-cellular mediators offers an opportunity to develop novel means of treating metabolic and other diseases (4, 30): can circadian regulation of cell division factors be used to suppress tumour growth, or can type 2 diabetes be managed via circadian influences on lipid and carbohydrate metabolism?

**Circadian futures**

**The core clockwork**

With a transcriptional feedback loop at the heart of circadian pacemaking, important advances are likely in understanding how the known complexes of Clock, Bmal1, Per, Cry and other circadian proteins assemble, how their activity is regulated and how they recruit and control the core transcriptional machinery. The dynamics of Clock/Bmal1 binding to E-boxes have been explored in relation to circadian gene expression (31), and Per- and Clock-interacting proteins with a circadian role have been identified (32, 33). These include the coactivator Nono that also binds to RNA polymerase II (34). Moreover, Clock has recently been shown to have intrinsic acetylase activity, not only for histones but also its binding partner Bmal1. Importantly, Clock-mediated acetylation of Bmal1 facilitates recruitment of Cry into the complex and appears to be necessary for circadian gene transcription (35).

A second central question is how intra-neuronal pacemaking in the SCN relates to inter-neuronal, circuit level activity. Suppression of electrical activity in the SCN slice by treatment with tetrodotoxin not only desynchronises the cells, but also compromises their molecular pacemaker, dampening rhythmic gene expression (36, 37). A comparable effect is observed when signalling via the VPAC2 receptor for VIP is compromised (38). This shows that circuit-level signals are essential to maintain SCN molecular pacemaking, challenging the concept of the SCN neuron as the ultimate self-sustained cell. By contrast, recent imaging studies have shown that fibroblasts continue, individually, to oscillate at high amplitude even though desynchrony between cells renders the aggregate culture apparently arrhythmic (21). A further demonstration of the particular reliance of the SCN pacemaker on circuit activity comes from single Cry and Per null mutants, which alone have no apparent effect on the molecular clock in the SCN slice with its neural architecture intact, but disable the clockwork of dispersed SCN cells lacking this circuitry (21).

**Setting clock speed and chronotypes**

Examination of these steady-state transcriptional mechanisms will not, however, explain the defining adaptive feature of the oscillator: its approximately 24-h period. One approach to identify factors that influence this is mutagenesis screening in mice, of the type that discovered the Clock gene, and this has revealed the F-box adaptor protein, Fbxl3, as a determinant of Cry protein stability (39, 40) (Fig. 3). Fbxl3 normally recruits Cry to the E3 ubiquitin ligase complex as a prelude to the proteasomal degradation of Cry. Mutations of the Cry-recognition motif of Fbxl3 compromise this recruitment and hence delay the clearance of Cry, selectively extending the phase of negative feedback and thereby lengthening the period of the circadian feedback loop. Although ubiquitinylation is usually preceded by phosphorylation, the relevant kinases and their sites of action on Cry are unknown, whereas, for Per, there is strong evidence that casein kinase 1 epsilon (Ck1ε) plays an important role in setting clock speed by targeting Per1 and Per2 for ubiquitinylation and then proteasomal degradation. In the homozygous tau mutant Syrian hamster, a point mutation in Ck1ε accelerates circadian behavioural and neuroendocrine rhythms to a period of 20 h (41). Recreation of the mutation in the mouse causes an equivalent phenotype (Fig. 3), accelerating the molecular feedback loop in the SCN and peripheral tissues by reducing the stability of Per (but not Cry) proteins (42).
approximately 20 h due to accelerated degradation of Per proteins (42), the mice were entrained to a 12 : 12 h light
Heterozygotes for the two alleles exhibit intermediate periods. For days 1–5 approximately 27 h because the rate of degradation of Cry is reduced (39).

Fig. 3. Specification of circadian period in mice by mutations that regulate the proteasomal degradation of Per or Cry proteins. Representative actograms of running-wheel behaviour in mice that are wild-type (+/+ or either heterozygous or homozygous for either the tau allele of Fbxl3 or the afh allele of Fbxl3. Tau homozygotes have a clock with a period of approximately 20 h due to accelerated degradation of Per proteins (42), whereas two copies of the afh allele slow the circadian pacemaker to approximately 27 h because the rate of degradation of Cry is reduced (39). Heterozygotes for the two alleles exhibit intermediate periods. For days 1–5 the mice were entrained to a 12 : 12 h light/dark cycle, and then were kept in continuous darkness to allow expression of their free-running period. Data are double-plotted to facilitate visualisation of free-run.

As the catalogue of factors influencing Per and Cry stability and their cellular translocation grows, then shall our understanding of how the clockwork operates within its cellular environment and is thereby tuned biochemically to solar time. This will in turn provide an appreciation of the genetic basis to the human trait of ‘chronotype’, the tendency of individuals to be ‘morning’ or ‘evening-types’ (6). An extreme morning-type condition is evident in familial advanced sleep phase syndrome where mutations in hPer2 or hCk1δ accelerate circadian period leading to a phase advanced sleep/wake cycle (43). In the general population, however, it is likely that more subtle allelic effects, acting at a variety of loci, determine individual pre-disposition. Nevertheless, the resulting phenotype may have a marked bearing on personality and health; for example, attuning individuals to a particular profession by virtue of its temporal demands, or making an individual more or less tolerant to shift work or sleep deprivation. Indeed, polymorphism in the hPer3 gene is associated both with chronotype and with altered homeostatic drive to sleep (44), such that individuals homozygous for a particular allele show greater cognitive deficits after 24 h of wakefulness.

Clocks and sleep

Sleep is a major health issue (3) and the search for effective treatments for various forms of dysomnia will benefit from approaches that exploit circadian mechanisms rather than simply depress neural activity by pharmacological means. Melatonin has long been regarded as a natural hypnotic and there is growing experimental evidence of its effectiveness in managing both the timing and the quality of sleep (23, 45, 46). Importantly, the question of sleep quality is now being understood at the level of cellular processes, and although sleep and circadian clocks have long been viewed as related processes, the new circadian orthodoxy provides a useful framework to consider the mechanisms of their interaction and its relationship with sleep quality. The function of sleep is to facilitate neural plasticity (47–49) be that developmental, as in the case of ocular dominance in the visual system, or synaptic, as in memory consolidation. Both processes require particular suites of genes to be expressed to support the necessary cellular rearrangements (50). Equally, during wakefulness, when sensory information is being acquired and motor patterns executed, such genes are down-regulated as neural circuits are primarily committed to ongoing cellular signalling and computational activities (51). Because the circadian clock of the SCN usually gates alternation between sleep and wakefulness, these changes can be viewed as local circadian transcriptomes equivalent to those of liver, heart or kidney. But at what level are they controlled? They may be a direct consequence of the states of sleep and wakefulness, imposed by altered electrophysiological activity. Alternatively, they may be driven in parallel to these state changes by efferent signals from the SCN. It is now clear, however, that principal brain regions, including the hippocampus, cerebral cortex and cerebellum, contain intrinsic circadian clockworks (Fig. 2). By analogy with circadian control in the periphery, therefore, it is likely that local transcriptomes in particular brain structures are driven by local clock mechanisms, which in turn are synchronised across the nervous system by cues from the SCN. These local clocks will thus establish global programmes in which appropriate suites of genes are up- and down-regulated in anticipation of daily sleep and wakefulness, thereby pre-adapting neurons to their alternating, brain region-specific functions across sleep and wakefulness. This model predicts that disruption of the local clocks will compromise cognitive functions (Fig. 4) and thus is supported by the evidence of sleep and cognitive disturbances in respect of polymorphisms in hPer3 (44).

Clocks and neurological disease

It is increasingly apparent that there is a complex interplay between the clock, normal neurophysiology and brain-specific disease processes. Recent work has demonstrated that circadian dysfunction is prevalent in various neurodegenerative conditions, most notably Alzheimer’s and Huntington’s diseases. In patients with dementia of
Clocks and metabolism

The liver is the ultimate metabolic regulator, and its biochemical, endocrine and autonomic inputs and outputs ebb and flow throughout the day. It is not surprising, therefore, that the clock and hepatic metabolism are entwined (Fig. 4). At both the transcriptome and proteome levels, there are clear circadian fluctuations in liver-specific metabolic pathways, often with rate-limiting enzymes exhibiting robust cycling (55, 56, 22). The regulation of these pathways by the clock is beginning to be unravelled and, in addition to Per2 (29), corticosteroid- and clock-regulated transcription factors, including DBP, HNF4α as well as heme-containing products, are amongst those that have received recent attention (57, 26, 17, 58, 59). When the clockwork goes awry, obesity and the metabolic syndrome arise (60–62), whereas loss of the clock-controlled output gene *nocturnin*, which encodes a deadenylase, confers resistance to hepatic steatosis and diet-induced obesity in mice (63). Conversely, inappropriate diets (e.g. too much fat) can disturb the clock; altering behavioural rhythms and circadian expression of genes that control fuel utilisation in the hypothalamus, liver and adipose tissue (64). Hence, understanding the interplay between the clock and metabolism has important and far-reaching consequences for dismantling the mechanics of energy balance and its control. Given the current epidemic of obesity and its attendant cardiovascular and other long-term morbidity, finding novel pathways for therapeutic intervention will be of great interest for the foreseeable future. Towards this goal, the transcriptional coactivator PGC-1α (a coregulator of the peroxisome proliferator-activated receptor (PPAR) family of nuclear hormone receptors) has been highlighted as a link between metabolic control and the core clockwork. Not only is this regulator of energy metabolism expressed rhythmically in liver and skeletal muscle, but it also controls expression of Bmal1 and Rev-Erbα (65). Moreover, members of the wider PPAR receptor family have been implicated in both regulating peripheral clocks (66) and the pathogenesis of type 2 diabetes, hyperlipidaemia and the metabolic syndrome. They have thus been identified as a promising target for modulating these conditions (67, 68), and their circadian regulation and activity may prove useful entry points for developing this therapeutic potential.

In summary, two decades have witnessed a great deal in circadian biology. It has grown up by solving the basic question as to how can a cell generate an internal representation of external time; a stimulus for which there is no material, chemical or electromagnetic manifestation. In this regard, the circadian axis is unique amongst sensory mechanisms. The past 20 years have also shown how tightly interleaved are our clocks and our metabolism and, at all stages of this growing understanding, neuroendocrine processes have been central to setting the biological context of circadian pacemaking. How this new framework is exploited will vary with society’s needs: the growing public health issue of long-term shiftwork, the morning prevalence of cardiovascular crises or the metabolic consequences of inappropriate diets, both in terms of content and when consumed. Clocks are not solely part of the problem: where certain times of day reveal an Achilles’ heel in pathological mechanisms, smart circadian targeting of therapies will enhance their efficacy while throwing the local circadian control switch may stop disease in its tracks. After all, it is not what you do, but when you do it that counts.

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