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Cellular Circadian Pacemaking and the Role of Cytosolic Rhythms

Review

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The daily rhythms that adapt organisms to the solar cycle are driven by internal circadian clocks. The hypothesis that the core pacemakers of these clocks consist of auto-regulatory transcriptional/post-translational feedback loops (TTFLs) was first developed in flies and fungi and has now been extended successfully to describe circadian timing mechanisms in mammals and plants. TTFL models revolve around the protein products of 'clock' genes that feedback periodically to regulate their own expression. From this simple beginning, the models have been expanded to encompass multiple, interlinked loops. However, experimental data now highlight the limitations of the TTFL model. Until recently, the focus on transcription caused rhythms in cytosolic signalling pathways to be viewed as outputs of the 'core' transcriptional clockwork, or else as a mechanism for its entrainment by extra-cellular stimuli. Recent work in *Arabidopsis thaliana*, *Drosophila melanogaster* and mammals now reveals that cytosolic rhythms in small signalling molecules have a central role within the circadian pacemaker. The logic is consistent across taxa: oscillatory cytoplasmic elements integrate with transcriptional feedback loops to sustain them and determine their rhythmic properties. Thus, clock outputs can constitute inputs to subsequent cycles and so become indistinguishable from a core mechanism. This emphasises the interdependence of nuclear and cytoplasmic processes in circadian pacemaking, such that the pacemakers of some species might encompass the entire cell and its intercellular environment.

Introduction

Circadian periodicity is a condition of eukaryotic life [1]. Endogenous circadian oscillators drive daily physiological and behavioural rhythms that anticipate and thereby prepare organisms for the regular and predictable demands and opportunities of the day, night and season [2]. Regardless of their particular cellular and biochemical constitutions, circadian systems of all species possess three canonical adaptive properties: First, they are autonomously capable of generating a period of approximately 24 hours, as revealed by the free-running rhythms of behaviour and physiology observed in subjects under experimental temporal isolation (Figure 1). Second, these autonomous oscillators can synchronise (entrain) to environmental signals that are reliable proxies for solar and seasonal time, such as light and temperature, and also social cues, such as feeding schedules. This entrainment makes the oscillators adaptive biological clocks and calendars. Third, circadian clocks are temperature-compensated at the level of both the whole organism and the single cell, with a temperature coefficient close to 1. This remarkable property runs counter to the general rule that cellular processes roughly double in rate for every 10°C increase in temperature.

Within cells, circadian mechanisms regulate and/or gate fundamental processes, including gene expression, replication, post-translational modifications of proteins, nutrient and xenobiotic metabolism and neural excitability [3,4]. Consequently, almost every aspect of physiology is regulated by circadian pacemakers, including neural function, manifest most obviously in the sleep–wake cycle [5]. Hence, morbidity and mortality in humans can exhibit a marked daily modulation, whilst disturbances of sleep and circadian timekeeping arising from environmental perturbation (rotational shift work, jetlag) or neurological conditions (e.g. Alzheimer's and Huntington's diseases) can impose a marked cost on both physical and mental well-being [6,7]. Thus, better understanding of how these circadian pacemakers work will not only enhance our appreciation of temporal regulation, and the relationship of temporal dysregulation to disease, it should also reveal novel and pervasive mechanisms of metabolic control within healthy cells and tissues, and thereby inform the management and treatment of disease.

The Search for Clock Mechanisms: The Rise of Transcriptional/Post-Translational Feedback Loops (TTFL)

So what is known of these remarkable endogenous timepieces? Notwithstanding their acceptance as *bona fide* biological phenomena in the late 1950s, for a long time the nature of circadian oscillators remained a 'black box', sandwiched between 'input to oscillator' and 'output from oscillator'. The anatomical localisation of pacemakers, such as to the retinal neurons of the molluscs *Bulla gouldiana* and *Aplysia californica*, and the hypothalamic suprachiasmatic nuclei (SCN) of mammals gave physical substance to the concept of endogenous clocks, whereas in higher plants and fungi pacemaking remained a more distributed property. Nevertheless, the ability of individual cells to act as circadian pacemakers was already evident from the diverse and extensive circadian rhythms of unicellular species [8]. Speculations on their biochemical mechanism were, however, hamstrung by the absence of genetic knowledge.

How might a single cell define 24 hours? Protein synthesis and post-translational modifications are important determinants of the cellular pacemaker because in the dinoflagellate *Gonyaulax* protein synthesis inhibitors phase-shift cellular bioluminescence and other rhythms, whilst inhibition of phosphorylation alters their circadian period [9]. Insight into the genetic basis of cellular pacemakers finally emerged, however, when the advent of molecular genetics facilitated the isolation of mutants with pronounced circadian phenotypes (most importantly alterations of free-running period) and subsequent identification of the mutated gene. By the early 1990s studies in *Drosophila melanogaster* [10] and

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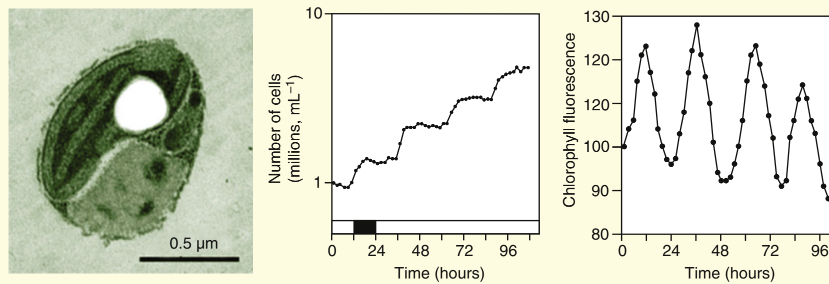
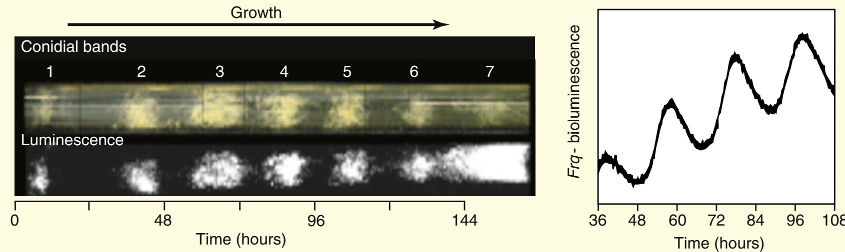
A Unicell: *Ostreococcus tauri* cell division and photosynthesis

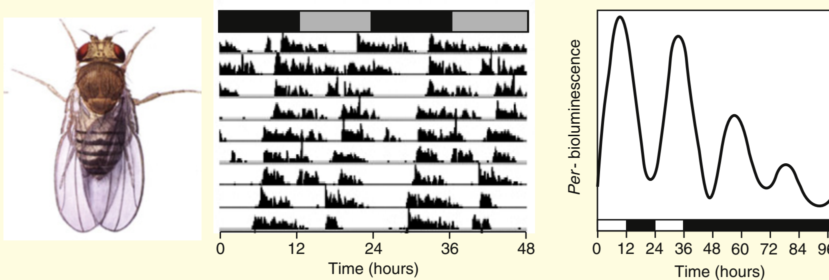


B Fungus: *Neurospora crassa* conidiation and *frq::luciferase* expression



C Higher plants: *Mimosa* leaf movements and *Arabidopsis* Ca2+ cycle

D Arthropod: *Drosophila* activity/rest cycle and *per::luciferase* in tissue explant



E Mammal: Mouse core body temperature and *Period2::luciferase* in SCN

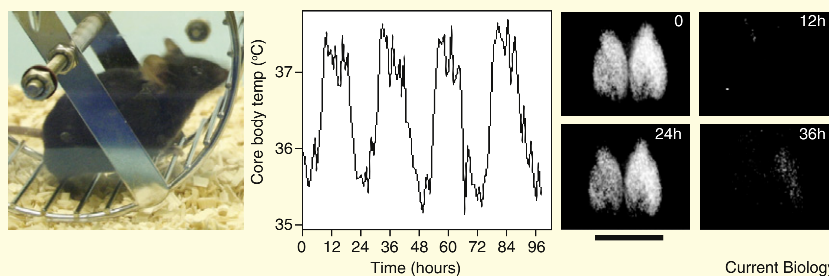


Figure 1. Circadian orchestration of gene expression, metabolism, physiology and behaviour amongst representative eukaryotes.

(A) The smallest free-living eukaryote, *Ostreococcus tauri* (left) exhibits circadian cycles of cell division (centre) and chlorophyll abundance (right) (redrawn with permission from [104]). (B) In *Neurospora*, conidiospore formation is clock-controlled. As the mould grows (left to right, upper track) it produces conidial bands (marked 1–7) for spore release at anticipated dawn. The clock gene *frequency* directs the conidial cycle and its circadian activation can be monitored (lower track), revealing circadian cycles of transcription (right) (redrawn with permission from [105]). (C) Circadian cycles of leaf movements in *Mimosa pudica* facilitate harvesting of light (leaves extended, left) and protection from dehydration (leaves retracted, centre) during anticipated day and night, respectively. Many clock-controlled rhythms of higher plants are driven by circadian cystosolic signals, including cycles of Ca^{2+}, monitored here in *Arabidopsis* (right) (redrawn with permission from [61]). (D) Circadian cycle of rest and activity (monitored by infra-red beams) of a *Drosophila melanogaster* fly held in continuous darkness, double-plotted on 48 hour time-base (centre). Activity occurs during anticipated day (grey bar), around dawn and dusk, with inactivity during anticipated night (black bar). This animal's intrinsic period is slightly less than 24 hours and so activity drifts to the left (redrawn with permission from [66]). In *period::luciferase* transgenic flies light-sensitive, autonomous local pacemakers can be monitored as cycles of bioluminescence, here recorded from an isolated wing, initially synchronised to 12 hours of light and 12 hours of darkness, and then in continuous darkness (redrawn with permission from [106]). (E) The circadian rhythm of activity in mice is accompanied by circadian variation in core body temperature (centre) (redrawn with permission from [107]). The molecular pacemaker of the mouse SCN in organotypic culture can be tracked by time-lapse imaging of Period2::luciferase bioluminescence (right), presented at peaks (Circadian time (CT) 0, 24 hours) and troughs (CT12, 36 hours) of expression over two circadian cycles. Scale bar = 1 mm.

Neurospora crassa [11] had contributed to a coherent model of the pacemaker as an autoregulatory TTFL, in which the protein products of 'clock genes', such as *period (per)* in the fly and *frequency (frq)* in the fungus, suppress the activity of their cognate genes. Importantly, transcriptional suppression occurs after an appreciable lag, reflecting the dynamics of translation, post-translational modification, intracellular trafficking and complex formation, and this prolonged delay enhances the oscillatory behaviour of the feedback loop. Only when any remaining, terminally processed proteins are cleared from the nucleus at the end of one cycle is negative feedback dissipated and the TTFL is able to proceed with a new wave of clock-gene activation. Such delayed negative feedback is commonly a cause of oscillations in transcriptional control [12]. The critical feature here is that

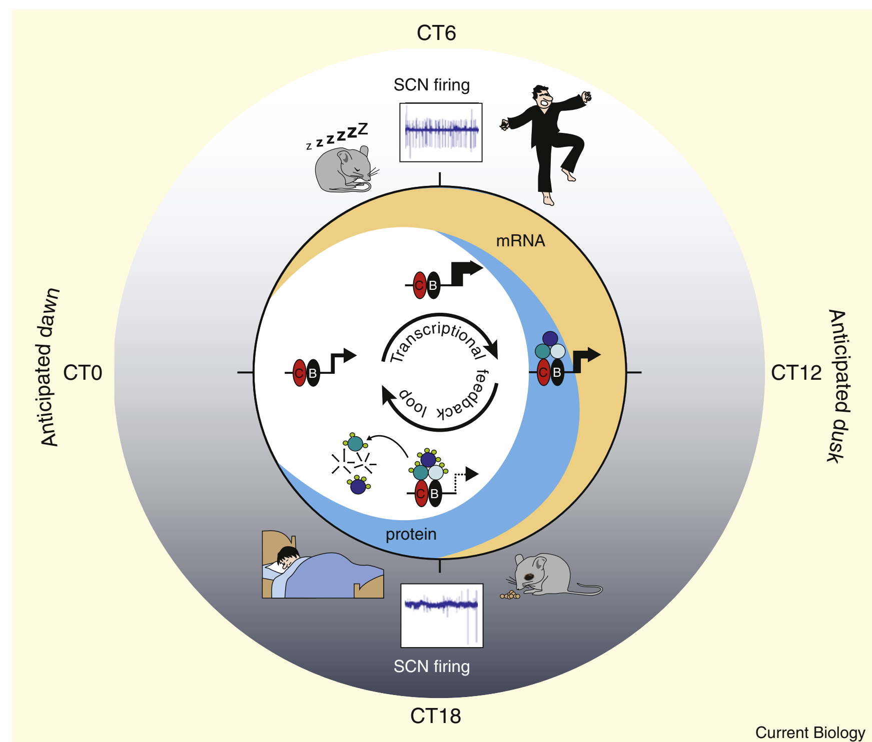


Figure 2. Schematic representation of the circadian transcriptional/post-translational feedback loop (TTFL) and its relationship to electrical activity of the SCN and behavioural rhythms in mammals.

Within the TTFL of the SCN (centre circle), heterodimers of Clock (C, red) and Bmal1 (B, black) start to drive transcription of *Per* and *Cry* after anticipated dawn. Consequently, levels of the respective mRNAs (orange shade) increase across circadian day (CT0 to CT12), followed by accumulation of Per and Cry proteins (blue shade). In the later circadian day Per, Cry and associated factors, including casein kinase (blue/green), form complexes with Clock and Bmal1, and start to suppress transcription. mRNA levels thus decline through circadian night. Phosphorylation of Cry/Per complexes during circadian night targets them for ubiquitinylation and proteasomal degradation, such that by the end of circadian night both mRNA and protein levels are minimal, and a new cycle of *Per* and *Cry* transcription is free to start. In parallel to this molecular cycle, the electrical firing rates of SCN neurons peak during circadian daytime at ca. 10 Hz (top, CT6) and decline at night to about 1 Hz (bottom, CT18). The molecular and electrical cycles of the SCN therefore code for anticipated solar time. Output signals from the SCN, both electrical and paracrine, determine the pattern of rest and wakefulness of the individual. These behavioural patterns are phased oppositely, relative to the electrical and molecular rhythms of the SCN, in nocturnal (e.g. mouse) and diurnal (e.g. human) species.

the time constants of the composite processes extend the loop to a period of approximately 24 hours. Moreover, by their ability to control the expression of clock-controlled genes not involved in the feedback loop, the clock proteins are able to impose daily metabolic rhythms on cells and organisms.

Following the cloning of various homologues of invertebrate clock genes [13] and discovery of the mouse *clock* gene [14], the TTFL model of cellular circadian pacemaking was extended to mammals. At the start of the circadian day, heteromeric complexes containing the basic helix-loop-helix transcriptional regulators Clock and Bmal1 facilitate chromatin remodelling to activate the expression of three *Per* and two *Cryptochrome* (*Cry*) genes via E-box DNA regulatory sequences [15] (Figure 2). Subsequently, Per and Cry protein complexes accumulate in the nucleus to suppress activation at E-boxes by Clock and Bmal. Reactivation of gene expression by Clock and Bmal1 can only occur some hours later, once Per and Cry have been degraded and cleared from the nucleus. *Bmal1* is apparently the only mammalian clock gene absolutely necessary for circadian competence *in vivo*, although combined null mutations of *Per1* and *Per2*, or *Cry1* and *Cry2* do compromise pacemaking, whilst a variety of single mutations either accelerate or lengthen circadian period and can affect the amplitude of overt rhythms [15].

This core negative feedback loop is accompanied by several accessory loops, the best characterised being the one mediated by the orphan nuclear receptors RORA and Rev-Erbα. The transcription of *RORA* and *Rev-Erbα* is activated during circadian day by Clock–Bmal complexes, and their respective proteins exert positive and negative transcriptional effects on Rev-Erbα/RORA response elements (RORE) in the *Bmal1* gene. This causes expression of *Bmal1* to cycle with a peak in circadian night, in anti-phase to the daytime peaks of the negative factors *Per* and *Cry*. These accessory loops thereby provide contrast enhancement within the cycle, such that circadian time is defined by a range of complementarily phased molecular markers. They also provide additional avenues for the transcriptional regulation of output genes containing, among others, RORE sequences [16], thereby increasing the repertoire of the circadian transcriptome. It is important to recognise, however, that all of these molecular cycles in the SCN code for solar time. Therefore, they hold the same phase, relative to dawn and to dusk, in species that are nocturnal as they do in diurnally active species [17]. What differs between such species is how this conserved molecular timer is interpreted, both by downstream genes in the SCN and also in its neural targets, as well as in peripheral tissues subject to neural and endocrine cues triggered by the SCN.

This logical structure of the oscillator, with a ‘core’ TTFL accompanied by accessory loops, is also evident in the cellular clockworks of other species, even if they consist of different biochemical components. In *Neurospora,* the positive factors White-collar1 (WC1) and WC2 drive the negative regulator Frq, which in turn feeds forward to activate WC1 whilst also repressing its own transcription [18]. In *Drosophila,* the positive factors clock and cycle (an orthologue of Bmal1) drive expression of the principal negative feedback regulators *per*, *timeless* (*Tim*) and *cry*, as well as the protein vrille, which in turn feeds-forward to sustain the rhythmic expression of clock [19]. In the higher plant *Arabidopsis,* at least three component loops can be identified, again involving nested negative and positive transcriptional regulation [2]. Within a few years, therefore, the concept of a ‘clock

loop' has become a 'clockwork web' across which the canonical properties of the pacemaker are distributed [20].

By using the regulatory sequences of 'clock' and 'clock-controlled' genes to drive the expression of genetically encoded bioluminescent or fluorescent reporters, real-time recordings have revealed the exquisite precision and astonishing persistence of these cellular oscillators. A pivotal discovery was that, in mammals, the molecular clockwork is active not only in the established neural pacemaker of the SCN, but also in the majority of peripheral organs as well as in primary cell cultures and transformed cell lines [21]. Indeed, autonomous cycles of circadian gene-expression can be monitored in cultures of human skin biopsy and blood [22]. Such distributed cellular pacemaking is also evident in *Drosophila* and zebrafish, but with the additional property that these local clocks are directly photo-responsive [19,23]. In mammals a sub-population of intrinsically photo-sensitive retinal ganglion cells, and their connections to the SCN, are the unique route for circadian entrainment by light [24].

The unanticipated revelation that autonomous circadian pacemaking is a general property of mammalian cells [25,26] has forced a radical re-appraisal of the mammalian circadian timing system. The SCN are no longer considered to be a driving pacemaker, imposing daily rhythms upon a passive body. Rather, they broadcast internal neural and endocrine cues that entrain the distributed network of cellular clocks to the solar cycle. In doing so, they impose an adaptive internal order to metabolic rhythms that are reflective of, and guide us through, our alternating states of wakefulness and sleep. Given that vital processes such as cell division, metabolic rate, synaptic remodelling, enzymatic and biosynthetic activities are actively and specifically up- and down-regulated across the day and night, it becomes easy to understand how disturbance of the normal circadian programme can be associated with cognitive impairment and chronic systemic illness [27,28].

Experimental Testing of the TTFL Model

The TTFL model has brought considerable advantages to the analysis of circadian pacemaking. First, as a self-sustained oscillator with very precise behaviour, the feedback loop readily lends itself to didactic mathematical simulation [26,29,30]. As such, it provides a novel exemplar for the formal modelling of several aspects of human behaviour [31]. Second, at an empirical level, the TTFL provides a conceptual framework within which to make strong, testable predictions, and this has supported the development of high-throughput mutational screens to identify functional domains within circadian factors and their interactions with relevant regulatory sequences that determine key attributes of the cellular pacemaker [16,32]. Lastly, the TTFL model has informed the phenotypic characterisation of the growing number of circadian mutants, and this has in turn facilitated further refinement of the model.

These points can be illustrated by considering how alterations in the rate of transcription of the core genes or changes in the stability and interactions of their encoded circadian proteins affect the pacemaker. For example, in *Neurospora* a mutation in the DNA-binding zinc-finger domain of the WC2 protein reduces transcriptional activation of *frq* and lengthens circadian period [33], whilst compromised transcriptional activity in the *Drosophila* $clock^{jrk}$ mutation and the mouse $Clock^{\Delta19/\Delta19}$ mutation leads to lengthened behavioural period or arrhythmia [14,34]. Clock is not, however, essential for circadian pacemaking in the SCN, where, in its absence, the closely related factor NPAS2 can partner Bmal1 [35]. Pacemaking in peripheral tissues does, however, require Clock protein [36], highlighting local variation within the general TTFL theme. Finally, for circadian pacemaking to be sustained, Clock and Bmal have to be able to bind Cry proteins and be sensitive to their negative feedback actions [32], the recruitment of Cry to the Clock/Bmal1 complex being facilitated by Clock-mediated acetylation of Bmal1 [37]. Overall, therefore, transcriptional efficacy within the TTFL is an important determinant of pacemaking.

The TTFL model also predicts that changes in protein stability will affect period length if they lead to a premature or delayed release of negative feedback. The circadian period of wheel-running and molecular pacemaking in tissues from homozygous *afterhours* and *overtime* mutant mice are slowed to about 27.5 hours [38,39]. These mice carry point mutations in the gene encoding Fbxl3, a protein that recruits phosphorylated substrates to the SCF–E3 ubiquitin-ligase complex, responsible for ubiquitination and proteasomal degradation (Figure 3). Cry1 and Cry2, but not Per proteins, are substrates for wild-type Fbxl3, but in the mutants the reduced ability of Fbxl3 to bind the Crys slows their rate of degradation, thereby lengthening the TTFL period. F-box-mediated degradation of phosphorylated clock proteins is also a feature of the TTFL in other taxa: for example, the slimb and jetlag proteins target per and tim, respectively, in flies; in *Arabidopsis,* Zeitlupe controls the degradation of the TOC1 protein, whilst in *Neurospora* Fwd-1 controls the degradation of phosphorylated Frq [40]. Mutations of these F-box proteins increase levels of their phosphorylated substrates and lengthen circadian period and/or cause arrhythmicity.

Conversely, accelerated degradation of clock proteins would be expected to shorten the period of the TTFL. The *tau* mutation of casein kinase 1 epsilon (CK1ε) in mice and hamsters is a gain-of-function mutation that destabilises Per, but not Cry, and thereby shortens the period of SCN and peripheral pacemakers [41]. Importantly, mutations of human CK1δ or of residues usually targeted for phosphorylation on human Per2 protein also shorten circadian period and lead to advanced sleep phase disturbance in patients [42]. The role of CK1 in setting clock speeds is strongly conserved. Various mutations of CK1 either accelerate or slow the *Drosophila* clock, whilst CK1 also plays a key role in *Neurospora* molecular rhythms [42]. Importantly, CK does not act in isolation — rather it acts in concert with other kinases, for example relying upon priming kinases before it can phosphorylate Frq protein in *Neurospora* and Per in mammals, whilst in *Neurospora* CK1-dependent degradation of Frq is opposed by a destabilising action of cAMP-dependent kinase [43]. Several other kinase and phosphatase families also appear to have important pacemaker roles. For example, GSK3, MAP kinases and Protein Phosphatase1 have all been implicated in the core mechanisms of the fly, mouse and *Neurospora* clocks [42], whilst genetic disruption of CK2 leads to some of the most pronounced period phenotypes in plant, fungal and animal clocks [44,45].

Thus, across taxa the circadian roles of several signal transduction elements appear to be as well conserved as those of transcription factors, and these observations have stimulated the idea that post-translational mechanisms are at least as important for circadian pacemaking as

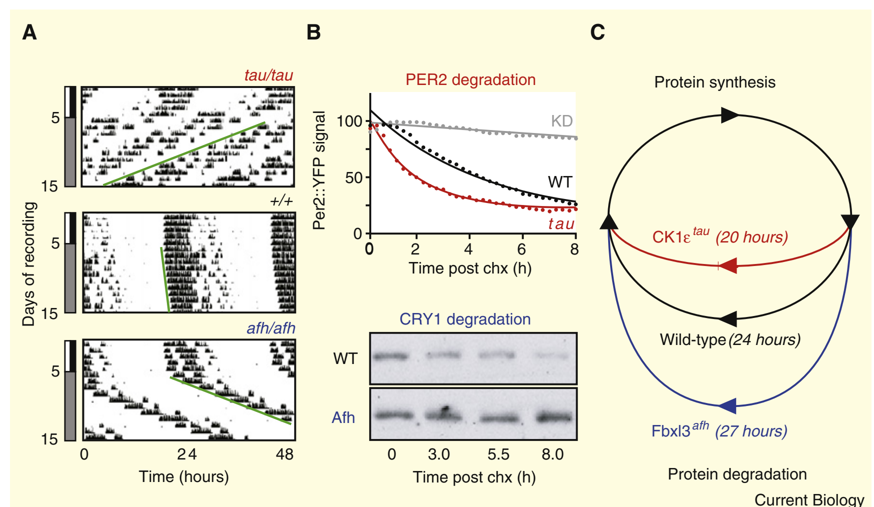


Figure 3. The stability of proteins within the TTFL determines circadian period: the effect of CK1ε^{tau} and Fbxl3^{afh} mutations in mice.

(A) Actogram plots of running wheel activity in mice initially held on a light–dark cycle (days 0–5) and then allowed to free-run in continuous dim light (day 6-) show accelerated (ca. 20 hour) and lengthened (ca. 27.5 hours) circadian periods in homozygous CK1ε^{tau} (top panel) and Fbxl3^{afh} (bottom panel) mutant mice, respectively, when compared to wild-type (middle panel, +/+, ca. 24 hours). Green lines indicate progressive activity onsets (Hastings *et al*. unpublished data). (B) Analysis of underlying biochemical defects in the TTFL of CK1ε^{tau} and Fbxl3^{afh} mice reveals altered protein stability. Upper panel: compared to kinase-dead enzyme (KD, grey trace) wild-type CK1ε (WT, black trace) accelerates the degradation of Per2 protein, monitored by a fluorescent tag in cycloheximide-treated COS cells. The CK1ε^{tau} mutation (*tau*, red trace) accelerates Per2 degradation even further (redrawn with permission from [41]). Lower panel: representative western blots for Cry1 protein in cycloheximide-treated lung tissue reveal slower degradation of Cry in Fbxl3^{afh} mutants ((A and B) redrawn with permission from [38]). (C) Schematic summary to demonstrate how altered stability of Per or Cry proteins in CK1 and Fbxl3 mutants can selectively change the duration of the interval of protein degradation within the TTFL pacemaker. This in turn shortens or prolongs the duration of protein-mediated negative feedback during circadian night and thereby determines circadian period length.

transcription. Indeed, by analogy with the cyclin-dependent kinases that drive eukaryotic cell division [46], it is tempting to speculate that the mechanisms that sustained a hypothetical ‘proto-clock’ were essentially (phospho-)enzymatic in nature [47], their complexity and the specific transcriptional targets they recruit diverging over evolutionary time to synchronise, first, cell division and, later, other cellular functions to the day–night cycle [1,8]. As this circadian network expanded it would have co-opted and incorporated additional feedback reactions governing cellular metabolism and biochemistry [30]. The question is, therefore, which came first: a clock based upon feedback transcription, fine-tuned by post-translational modifications, or one based upon serial modifications of proteins in the cytosol with transcription exploited as an output? Either way, having shown that both transcriptional efficacy and the post-translational modification of circadian proteins are determining features of pacemaking, it seems that the TTFL model incorporates the critical elements of the clockwork. But how comprehensive is it?

Can TTFLs Explain Everything about Circadian Pacemaking?

Observations that run counter to a simple interpretation of the TTFL are evident in both recent and historical work [48], not least because circadian photosynthetic rhythms persist in the green alga *Acetabularia crenulata* after enucleation and hence in the absence of nuclear transcription [49]. If pacemaking is solely based on the ordered transcription, complex formation and nuclear transport of clock proteins followed by orderly degradation of those complexes, the correct sequence and phasing of clock gene transcription and protein expression should be critical for oscillation. When *Drosophila per* and/or its partner *tim* are expressed constitutively in an arrhythmic null mutant background, however, their protein levels cycle and the flies are behaviourally rhythmic [50]. Furthermore, when the transcription of *clock* is temporally misaligned in transgenic flies by driving it with the *per* promoter, there is a minimal effect on the cycles of clock protein expression, and circadian behavioural cycles are maintained [51]. Similarly, circadian activity of an evening element promoter persists in plants over-expressing the CCA1 repressor [52], whilst in *Per1* transgenic rats, circadian gene expression in the SCN is dampened, but the animals nevertheless remain behaviourally rhythmic (albeit with lengthened period) in the face of constitutively elevated *Per1* transcription [53]. Finally, constitutive over-expression of the transcriptional activators Clock/Bmal1 [32] or repressor Cry1 [54] in mammalian fibroblasts does not substantially perturb oscillations, and circadian pacemaking is also maintained under continuous treatment with pre-expressed, cell permeable Cry protein [55].

The examples listed above therefore run counter to a simple reading of the TTFL model. Although rhythmic gene expression may be both a cause and a consequence of intermittent feedback suppression by circadian proteins, rhythmic transcription and rhythmic feedback are neither necessary nor sufficient for oscillation. It is, therefore, likely that post-transcriptional mechanisms, many of which are known to be clock-relevant [56,57], play a significant role in maintaining pacemakers. Equally, sustained protein expression is not a requirement for pacemaking because exposure of SCN slices to cycloheximide (a potent inhibitor of translation) for 18 hours beginning at the start of circadian daytime, has no significant effect on the phase of the oscillation [58]. The pacemaker in the eye of *Bulla* is similarly independent of ongoing protein synthesis; exposure to pulses of cycloheximide lasting 4–18 hours across the entire cycle having no effect on phase or periodicity, except when applied just prior to subjective dawn [59]. These data imply that in the absence of new protein expression, pre-existing cellular machinery is competent to sustain the oscillation for the majority of its cycle. This leads to the question: what sets the timing of these post-transcriptional mechanisms?

Cytosolic Oscillators as Partners of the TTFLs?

Might cytoplasmic oscillators, or at least interval timers, bridge the temporal gap between sequential daily episodes

of transcription and/or translation? A wide variety of cytosolic signalling factors — intracellular Ca^{2+} concentration, kinase activity, cyclic nucleotides and phospholipids — are under tight circadian regulation in a diversity of organisms [8]. For example, concentrations of free calcium in the cytoplasm and chloroplasts of *Arabidopsis* are highly circadian [60,61], as is that of cytoplasmic Ca^{2+} of SCN neurons [62] (Figure 4). In *Arabidopsis*, cyclic adenosine diphosphate ribose (cADPR) has been proposed as an upstream regulator of the Ca^{2+} oscillations [61] whilst in the SCN, ryanodine-sensitive stores provide the circadian Ca^{2+} cycles. What stimulates these stores on a rhythmic basis is, however, unclear. Circadian changes in electrical firing will certainly drive trans-membrane Ca^{2+} fluxes in the SCN, but the Ca^{2+} cycle persists when electrical signalling is silenced by tetrodotoxin (TTX) and blockade of L-type voltage sensitive Ca^{2+} channels. It is therefore likely that the Ca^{2+} cycle is controlled by the core TTFL, independently of electrical firing rhythms [62].

Given the fundamental role of Ca^{2+} signalling in biochemical integration and co-ordination, these intrinsic rhythms in plants and animals are ideally placed to impose circadian order across the many functions of a cell. Until recently, therefore, they and other circadian-relevant signalling pathways were viewed as outputs of a TTFL core pacemaker [63,64], but evidence is now accruing for a role of such pathways within the pacemaker. For example, cytosolic Ca^{2+} in the SCN is highest in advance of the peak in *Per* gene expression, suggestive of a role for Ca^{2+}-dependent signalling in driving transcription of these components of the TTFL, rather than being driven by it. Consistent with this, buffering of intracellular Ca^{2+} suppresses the circadian amplitude of *Per* gene expression in SCN slices [65]. In *Arabidopsis* pharmacological manipulation with nicotinamide has an even more telling effect, lengthening the period of both the Ca^{2+} rhythm and also the rhythms in leaf movement and clock-controlled gene expression [61] and it is proposed that cADPR is both an output and an input to the TTFL. A marked effect of altered cytoplasmic signalling on pacemaker period is also seen in the *Drosophila* brain, where expression of the Ca^{2+} buffer parvalbumin in pacemaker neurons lengthens the period of locomotor activity rhythms and the clock protein-dependent TTFL [66]. Furthermore, crosses between mutant fly lines (with ineffectually low levels of parvalbumin expression) and mild mutants of calmodulin (CaM) lead to synergistic lengthening of circadian period, revealing a role for signals that act via CaM-dependent protein kinase II (CaMKII) in driving the TTFL. How CaMKII-dependent phosphorylation may alter the TTFL awaits clarification, although it is notable that the fly clock protein is a substrate of a number of kinases, including CaMKII *in vitro* [67]. Whatever the effector pathway from cytosol to circadian transcription factor, electrical silencing of the pacemaker neurons, which would dysregulate Ca^{2+}-dependent signalling, not only impairs behavioural rhythms, it also compromises oscillation of the TTFL components within the silenced pacemaker cells [68]. Thus, circadian transcription and cytosolic signals depend on each other. Hence, in fly and *Arabidopsis*, small cytoplasmic signalling molecules that ostensibly are pacemaker outputs actually determine circadian period length, a core property of the TTFL pacemaker.

As noted above, cytoplasmic Ca^{2+} is also an important determinant of the amplitude of circadian gene expression in the SCN, but reduced cytoplasmic Ca^{2+} does not influence period. The reduced amplitude may, therefore, simply reflect a necessary role for a particular Ca^{2+} concentration to sustain gene expression. It does not prove that Ca^{2+} is a defining element of the SCN pacemaker. However, evidence that cAMP-dependent signalling may be just such a cytoplasmic component of the SCN pacemaker does exist [69]. In organotypic SCN slice-culture, the concentration of cAMP and transcriptional activity of the cAMP-response-element (CRE) regulatory sequences are both highly rhythmic, whereby cAMP levels peak in advance of the rhythm of *Per* gene expression (Figure 4). Irreversible pharmacological suppression of adenylate cyclase activity reduces cAMP concentration to basal levels, suppresses CRE rhythms and also severely attenuates rhythmic *Per* gene expression. The compromised circadian gene expression is, however, reversible and can be rescued by activation of an effector of cAMP, the guanine-nucleotide exchange protein activated by cAMP (Epac). Epac likely promotes downstream phosphorylation and activation of CRE-binding protein (CREB) a transcriptional activator of *Per* genes [70]. Thus, as with Ca^{2+}, a supposed circadian output, in this case cAMP synthesis is necessary for sustained circadian gene expression. Two further pieces of evidence place cAMP within the pacemaker: first, sustained pharmacological elevation of cAMP concentration disorganises SCN circadian gene expression, but on washout, as cAMP concentration returns to normal, the oscillation is restored. Importantly, the phase of the restored oscillation is set by the transition from elevated to normal cAMP concentration, independently of the phase of the cycle when the change occurred and for how long cAMP had been elevated beforehand. Hence, the phase of the TTFL pacemaker is determined by changes in cAMP concentration. Second, when antagonists at the catalytic p-site of adenylate cyclase are applied to reduce the rate of cAMP synthesis, the period of the SCN pacemaker lengthens dramatically, from ca. 24 to approaching 30 hours. Moreover, this effect is additive to the period lengthening associated with the $Clock^{\Delta 19/\Delta 19}$ mutation, indicating that the mechanism is independent of, and in parallel to, E-box mediated transcription [69]. Finally, inhibition of adenylate cyclase lengthens circadian period not only in the SCN but also in both mammalian cell lines and peripheral tissues [69].

Circadian Outputs Feed Back into the TTFLs

The re-entry of circadian cytosolic signals into the transcriptional core of the TTFL means that clock outputs also constitute inputs to current and subsequent cycles, and thus become indistinguishable from a core mechanism (Figure 5). Proposed mediators of this re-entry in mammals include canonical signalling networks such as cAMP and Ca^{2+}-dependent kinases, casein kinases [41], C-Jun amino-terminal kinases [71], ras-dependent (MAP) kinases [72] and their various downstream effectors and modulators (e.g. Epac, GSK3β, phosphodiesterases, protein phosphatases). Because of the well-established cross-talk between such networks [73] and their sequential, concerted actions towards target proteins, it may be helpful to consider the concept of a circadian 'signalosome' that is driven by the TTFL and which both sustains spontaneous cycling by the TTFL and mediates its acute resetting by extra-cellular cues. Many of the components of the signalosome converge on transcription factors such as CREB and its transcriptional co-activators CREB-binding protein (CBP) and p300 [74]. In flies, the availability of CBP limits clock/cycle-dependent

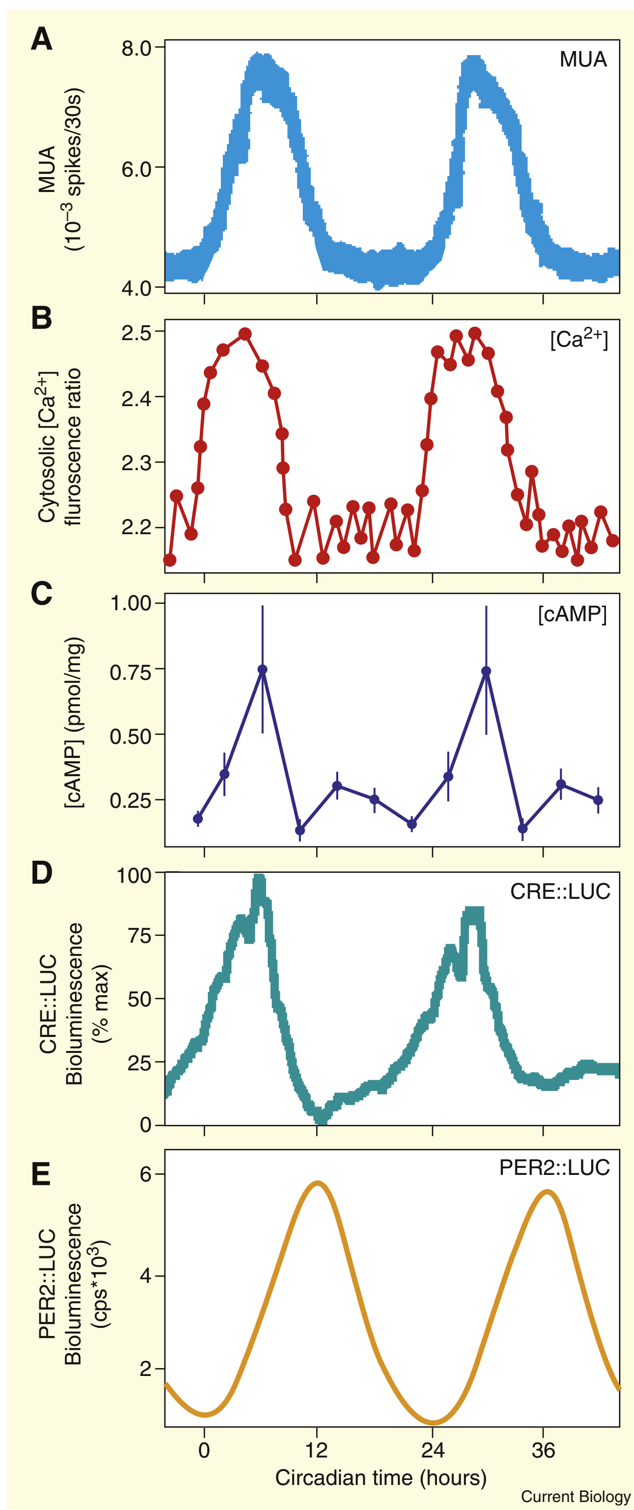


Figure 4. Autonomous circadian programming of electrophysiology, cytosolic signalling and gene expression in the suprachiasmatic nuclei held in organotypic slice culture.

When isolated in organotypical culture, the suprachiasmatic nuclei (SCN) sustain precise and stable rhythms in the activity of a series of inter-linked signalling cascades. (A) Multi-unit electrical firing rate of the SCN peaks in the middle of anticipated circadian daytime (CT06), and is preceded by (B) the peak elevation of cytosolic Ca^{2+} levels. The rhythms in Ca^{2+} and (C) cAMP concentration drive rhythmic transcriptional activity controlled by cAMP/Ca^{2+} response element (CRE) sequences (D). This rhythm in turn likely contributes to the rhythm in Per2::luciferase expression (E) which peaks at the end of anticipated circadian day (CT12). (A,B) Redrawn with permission from [62], (C–E) redrawn with permission from [69]. (C) and (E) are double plotted for clarity.

transcription and circadian behaviour [75] and CBP knockdown in pacemaker cells lengthens the period of behavioural rhythms and of rhythmic *per* and *tim* expression [76], whilst in mammals, CBP is reported to regulate trans-activation by Clock/Bmal1 [77]. Until recently, views on the role of CREB, its associated kinases and upstream regulators focussed on photic entrainment of the mammalian TTFL. This involved acute CRE-mediated induction of *Per* mRNA and protein leading to longer term re-phasing of the SCN feedback loops [70,78]. By facilitating re-entry from intra- and intercellular pathways, CREB/CRE-mediated signalling may now be viewed as a part of the pacemaker *per se*, facilitating both acute (entrainment) and circadian (Clock/Bmal1-dependent) activation of the TTFL [79].

Other routes for the re-entry of rhythmic cytosolic events into the TTFL are also being identified. For example, the porphyrin haem is an important co-factor for a number of metabolic enzymes and its synthesis is circadian. It is also a ligand for Rev-Erbα through which it controls the transcription of a number of metabolically relevant genes. Hence, a rhythmic cytosolic factor can again feedback into the TTFL [80]. GSK3, which regulates the *Drosophila* pacemaker by phosphorylating tim [46] prior to its degradation [81], is a regulator of Rev-Erbα in mammals [82] and itself displays spontaneous circadian oscillations in activity [83]. More broadly, redox changes secondary to cellular metabolic cycles may influence the TTFL by altering the formation of Clock/Bmal2 and Npas/Bmal1 complexes and their binding to DNA [84], whilst body temperature rhythms may sustain the TTFL [85] perhaps through heat-shock transcription factors. Finally, at the level of the organism, rhythmic behavioural arousal is an output of the SCN pacemaker, but changing states of sleep and arousal can feedback and reset SCN electrical activity and circadian gene expression [17,86].

Although the pathways under study vary between tissues and organisms, the logic of circadian outputs coming back into the TTFL is consistent across levels of biological organisation. This interlinking of the TTFLs and their various outputs has echoes of the concept of '*zeitnehmer*' developed by Roenneberg, Merrow and colleagues [30]. Literally meaning 'time-taker', a *zeitnehmer* is an input pathway that is itself rhythmically regulated by feedback from an oscillator and thus will create a rhythmic input to that oscillator even when the environment is held constant. When modelled, such a component can be shown to enhance oscillatory behaviour and offer reliable synchronization to environmental cycles [30]. The results in *Arabidopsis*, *Drosophila* and mammals now extend this concept to show that some cytosolic circadian signals should be considered as integral pacemaker components and not solely entraining agents. So, what might be the adaptive value of such an arrangement? Essentially, by integrating cytosolic and transcriptional processes, this dual-component model allows for both responsiveness to external cues and robustness of pacemaking. Responsiveness arises from the capacity of cytosolic networks to integrate diverse and noisy stimuli acting over differing time-courses and thereby facilitate coherent biological responses [73,87,88]. They are,

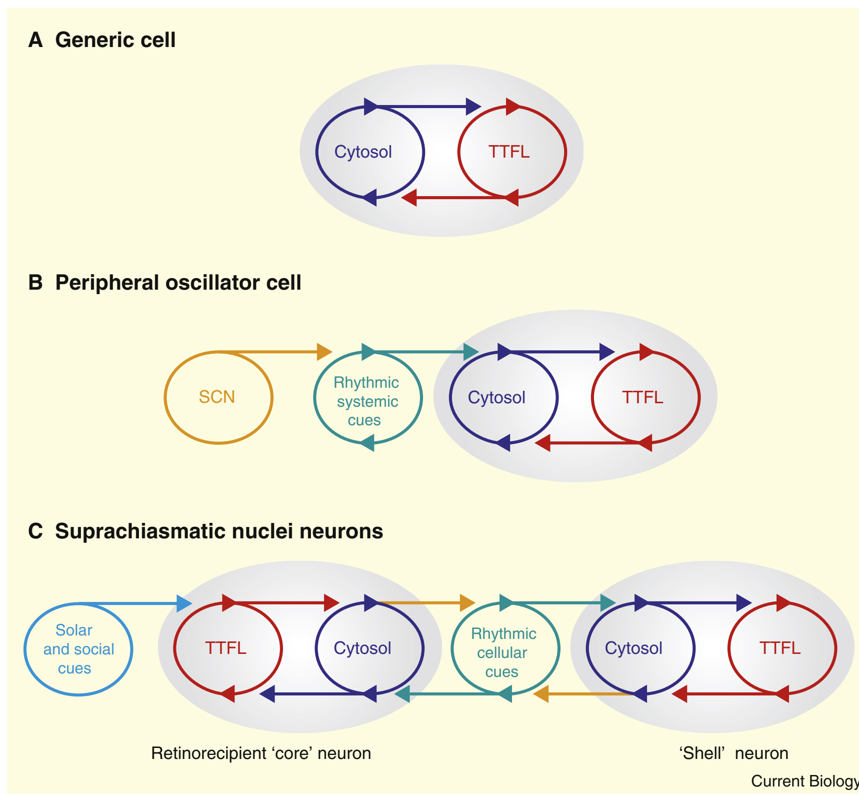


Figure 5. Schematic dual-component model of cellular circadian pacemaking incorporating inter-dependent, intra-cellular TTFL and cytosolic oscillations.

(A) A generic model of the cellular circadian oscillator in which the TTFL drives daily cycles in various intra-cellular signalling cascades (including cytosolic Ca^{2+}, cAMP, MAPK, metabolic signals) and these in turn feedback into the TTFL to sustain it. (B) In peripheral tissues of mammals, the cytosolic pathways provide an entry point for rhythmic, SCN-dependent systemic cues which entrain the cellular pacemaker to solar and social time. (C) In the SCN, the rhythmic extra-cellular cues that sustain and synchronise an individual neuron represent the output of other neurons in the circuit. In the absence of this inter-neuronal signalling, as in Vip2r-/- mutant tissue, the individual cellular pacemakers lose synchrony and amplitude, and in dispersed culture are less resilient in the face of genetic perturbation of the TTFL. In lower vertebrates and flies, in higher plants and *Neurospora*, intrinsically photoreceptive pacemakers extend throughout the organism. In mammals, however, synchronisation to external time is mediated by afferent innervation to the retinorecipient SCN core neurons, which in turn set the phase of the shell neurons and thence the peripheral pacemakers distributed throughout the individual.

therefore, well suited to having been co-opted into the protoclock to sustain cellular circadian rhythms and quickly integrate existing phase information with incoming environmental stimuli. In tandem, longer-term transcriptional processes will buffer the system from irrelevant perturbatory stimuli, thus stabilising phase and amplifying the signal (Figure 5).

Pacemaking in the SCN: Synchronising and Maintaining the TTFLs by Interneuronal Signals

So, how might this model of combined TTFL and cytosolic pacemaking work in a real biological clock? The SCN act through a variety of behavioural, autonomic and neuroendocrine pathways to co-ordinate circadian timing across the body's distributed clock network (Figure 5B). It is, therefore, essential that the ten thousand or so individual neurons of the SCN are coordinated [89]. Molecular pacemaking within individual cells is thus tightly synchronised across the population, albeit with phase variation: peak *Per* expression in the dorsomedial region of the SCN, characterised by neurons expressing the neuropeptide arginine vasopressin (AVP), is ahead in phase compared to the other areas — including the retinorecipient ventro-lateral SCN characterised by neurons expressing the neuropeptides vasoactive intestinal peptide (VIP) and gastrin-releasing peptide (GRP) [58]. The functional significance of this differential phasing is unknown: does it represent spatial separation of autonomous pacemaker cells and their driven slave oscillators, and/or is there a differential, phase-specific control of output pathways arising from particular sub-regions of the SCN that allows various locally appropriate time cues to be transmitted to specific target regions [89]? Importantly, the phase-distribution of the SCN clock cells is regulated *in vivo* by retinal innervation, which signals the light–dark cycle: The longer days of summer promote wider phase segregation of sub-populations of SCN cells than is seen in the short days of winter [90,91]. This photoperiodic dispersal of cellular phase underpins the role of the SCN as an 'internal calendar', ultimately co-ordinating seasonal cycles in physiology, behaviour and metabolism via changes in the nocturnal duration of melatonin secretion [92]. Retinally-driven plasticity within the inter-neuronal circuitry of the SCN is, therefore, central to its role. Illumination at dawn and dusk activates glutamatergic retinal afferents to the ventral SCN neurons, increasing their firing rate and inducing various CRE-regulated genes, including *Per1* and *Per2* [93]. Ultimate phase adjustment of behavioural and other rhythms is associated with subsequent induction of *Per* in the dorsomedial SCN, involving both neuropeptidergic (VIP, GRP) and GABA-ergic signalling from the retinorecipient cells.

The SCN can maintain precise circadian cycles indefinitely when isolated in organotypic slice culture. They therefore appear to be the pacemaker without equal. In contrast, circadian gene expression in peripheral tissues or fibroblast cultures damps rapidly, and is only restored by endocrine or metabolic stimulation [25,94]. Surprisingly, however, this damping is not a result of individual cells losing their pacemaking ability. Rather, it occurs because synchrony is lost across the culture, even though individual fibroblasts, for example, are very competent autonomous oscillators [95]. A defining difference between the SCN and peripheral cellular pacemakers, therefore, is the powerful inter-cellular synchronisation of the SCN [96] (Figure 5C), which is facilitated by various modes of communication, including conventional synaptic activity and gap junctions [97]. Importantly, electrical silencing with TTX not only desynchronises rhythms of circadian gene expression, but also damps its amplitude in

individual cells. On recovery from TTX the normal amplitude and phase-relationship of cellular pacemaking are restored. This highlights an unanticipated dependence of the TTFL in the SCN on afferent activation and inter-neuronal circuitry [98]. The neuropeptide VIP is a particularly important mediator of these circuit-level functions: loss of the *Vipr2* gene encoding the VIP receptor in the SCN, or the gene encoding VIP itself, both lead to disorganised circadian endocrine and behavioural rhythms *in vivo* and desynchronised cellular pacemaking *in vitro* [99,100]. As with TTX treatment, cellular desynchrony in the absence of neuropeptidergic signalling is accompanied by a dramatic reduction in the amplitude of circadian gene expression. In this regard, therefore, many of the SCN neurons appear to be less effective oscillators than the 'humble' fibroblast: synchrony and robust cellular pacemaking are two inter-dependent properties of SCN cells. In contrast, the TTFL of desynchronised fibroblasts is self-sustained over the long-term.

One interpretation of these results is that the SCN are so specialised for intercellular synchronisation that their molecular pacemaker has acquired dependence on synchronising signals in a way the fibroblast has not. Importantly, VIP signalling through its SCN receptor stimulates adenylate cyclase and thereby drives cAMP signalling. This interneuronal, extra-cellular signal, therefore, directly activates a cytosolic component of the circadian pacemaker. Similarly, the network of pacemaker cells in *Drosophila* brain that drives activity rhythms shows widespread receptivity to the neuropeptide pigment-dispersing factor which acts via a receptor closely related to Vip2r to stimulate cAMP synthesis [101]. Loss of pigment-dispersing factor not only compromises circadian patterning to behaviour, it also leads to the desynchronisation of molecular pacemaking across this cellular network [102], whilst mutations in cAMP signalling pathways are associated with entrainment or period defects, or behavioural arrhythmia [64]. Thus in both mouse and fly, interneuronal peptidergic cues that act via cAMP signalling synchronise and sustain neuronal pacemaking. This critical importance of intercellular signalling in the SCN is emphasised further by the impact of clock gene mutations on the robustness of pacemaking in isolated cells. In fibroblasts, *Cry1* and *Per1* knock-outs compromise cellular pacemaking, whereas SCN slice cultures maintain normal circadian oscillations in the face of such deficiencies. These genetic deficits do, however, compromise pacemaking once SCN cells are dispersed in culture and thus deprived of their circuit environment and appropriate intercellular signals [26]. In the absence of these cues, therefore, the TTFL of SCN cells is as sensitive to genetic disturbance as that of fibroblasts, or put another way, intercellular cues in the SCN circuitry are sufficiently powerful to overcome the loss of core genetic elements of the TTFL.

It remains to be seen whether, as in *Acetabularia*, biochemical oscillations persist in SCN and other mammalian cells in the complete absence of a nucleus-based TTFL. Nevertheless, in contrast to the cyanobacterial pacemaker, which can be reduced *in vitro* to a series of interactions within and between three proteins [103], the pacemakers of 'higher' species, involving both transcriptional and cytosolic oscillations, likely encompass the entire cell. Previously, when we were on the outside and looking in, the pacemaker was readily visible as a unitary phenomenon. Now that we have been able to move inside and are looking outwards, where the pacemaker starts and where it ends within the cell have become unclear. Indeed, given the many levels of biological scale over which circadian rhythms operate and the reciprocal communication between them, it may not even be helpful to reduce our model of the system to its simplest set of molecular components. Certainly we need a new vocabulary to replace 'input', 'oscillator' and 'output'. Perhaps talking of 'distributed function' between and within the TTFL and the cytoplasmic oscillator, or 'cytoscillator', may help.

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