Time after time: inputs to and outputs from the mammalian circadian oscillators

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Oscillating levels of clock gene transcripts in the suprachiasmatic nucleus (SCN) are essential components of the mammalian circadian pacemaker. Their synchronization with daily light cycles involves neural connections from light-sensitive photoreceptor-containing retinal ganglion cells. This clock orchestrates rhythmic expression for ~10% of the SCN gene transcripts, of which only 10% are also rhythmically expressed in other tissues. Many of the transcripts expressed rhythmically only in the SCN are involved in neurosecretion, and their secreted products could mediate SCN control over physiological rhythms by coordinating rhythmicity in other nuclei within the brain. The coordination of clock gene transcript oscillations in peripheral tissues could be controlled directly by specific signals or indirectly by rhythmic behavior such as feeding.

Circadian rhythms are the overt consequences of biological clocks – endogenous timers acting within cells. At the molecular level, circadian clocks are constructed from 'clock genes', some of which encode proteins able to feedback and inhibit their own transcription [1]. Cells experience daily variations in the transcript levels of clock genes and their protein products, and these changes thus reflect different phases of the daily cycle. The cells respond to these daily variations in clock components, at least in part, with changes in the patterns of genes they express which, in turn, affects their biochemistry and results in the observed rhythms.

The mammalian clock system is hierarchical, with a master clock located within the neurons of the suprachiasmatic nucleus (SCN) in the hypothalamus. The SCN receives signals from the environment and provides the principal timing cues for synchronizing the daily oscillations in peripheral tissues [2–5]. The past several years have seen considerable progress on three fronts. First, the clock mechanism in peripheral tissues and the nature of the synchronizing timing cues from the SCN are slowly being brought into focus. Second, the mechanism used to relay information from the environment to the master clock of the SCN has attracted much attention, including a debate on the nature of the photopigment involved in circadian photoreception. Lastly, high-density oligonucleotide array (microarray) technology has revealed the dramatic changes in gene transcription that occur over time in the SCN and in several different peripheral tissues.

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The same but different – the basic clockwork in the SCN and peripheral tissues

The components of the mammalian circadian oscillator have been studied primarily using mice carrying mutations in one or more of the clock genes. The components can be assembled into a basic wiring diagram (Fig. 1a) that involves a complex transcriptional feedback circuit of three *period* genes (*per1*, *per2* and *per3*), two *cryptochrome* genes (*cry1* and *cry2*), a *clock* gene (*clk*) and the gene encoding brain–muscle Arnt-like protein 1 (*bmal1*) [1,4,6,7]. Levels of all the transcripts (except those of *clk*) oscillate in the SCN, with *per* and *cry* transcripts peaking at roughly midday and those of *bmal1* peaking at around midnight (Fig. 1b). The complete circuit is formed from two interwoven negative-feedback transcription loops, with the gene products inside each loop acting negatively on their own transcription and positively on the transcription of genes in the other loop [8]. The stimulatory effect of the PER protein on *bmal1* transcription is the molecular equivalent of a grammatical double negative: expression of the nuclear orphan-receptor REV-ERBα is cyclic [9] and inhibits *Bmal1* transcription, yet is itself inhibited by PER [10].

When the patterns of gene expression are examined in detail, high PER levels correlate both with rising *bmal1* transcript levels, as expected considering the positive action of PER and CRY on *bmal1* transcription [8,11], and decreasing *per* transcript levels, as expected based on the negative feedback loop (Fig. 1b). The delay between the peaks of *per* and PER is believed to be an essential feature of the oscillatory mechanism [12]. However, some potential discrepancies remain, as times of maximum *per* accumulation do not correlate with peak BMAL1 levels. Clearly, the feedback circuit as drawn here is still incomplete. Some possible candidates for addition to the scheme include the leucine zipper transcription factor E4BP4 [13,14], which acts as an inhibitor of *per* transcription, and DBP (albumin gene D-site-binding protein) [15,16] whose role in the circuit is still unclear. Perhaps other PER-binding proteins, such as the PER-interacting protein of the SCN (PIPS), might also have a role to play [17]. It must also be noted that the SCN is not a uniform tissue with respect to clock gene expression [18–20].

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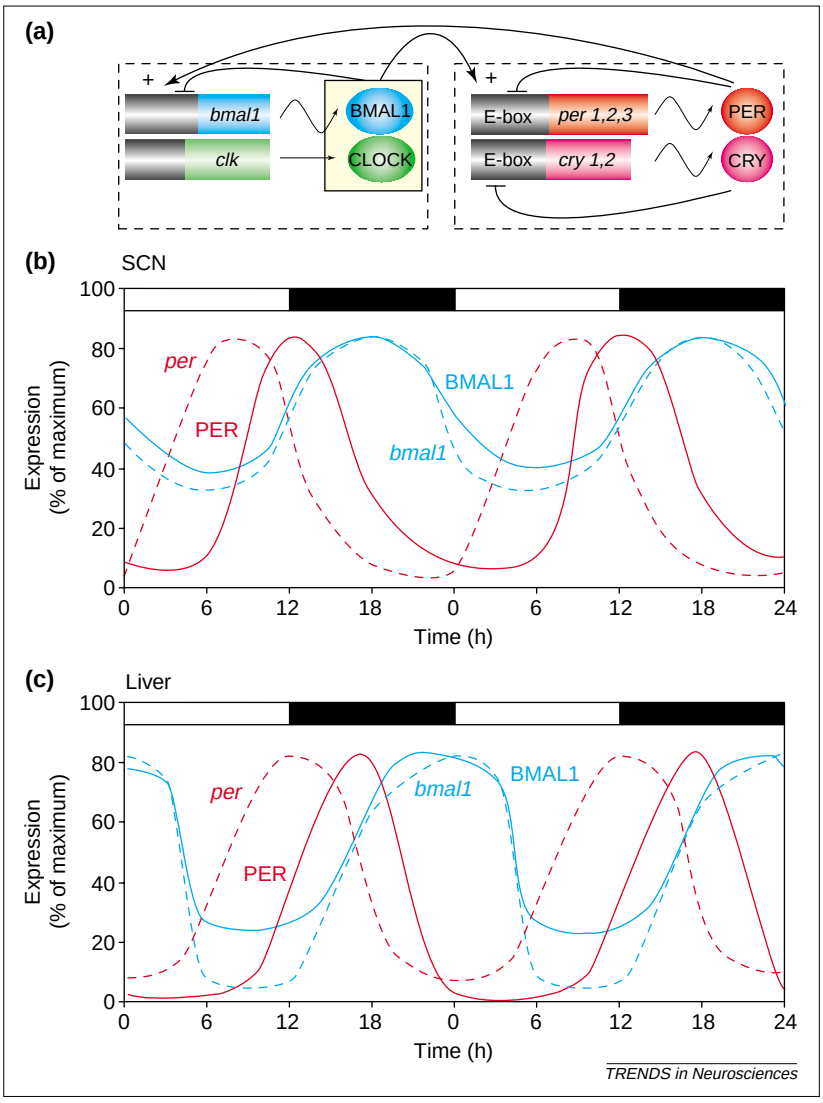

Fig. 1. Rhythmic oscillation of clock gene expression. (a) The mammalian circadian system has two negative feedback loops, one involving inhibition of *bmal1* transcription by a heterodimer of BMAL1 (brain–muscle Arnt-like protein 1) and CLOCK (left), the other involving inhibition of *period* (*per*) and *cryptochrome* (*cry*) transcription by PER and CRY (right). The two loops are linked by the positive action of PER on *bmal1* transcription and the positive action of the CLOCK–BMAL1 heterodimer (indicated by the yellow box) on E-box-containing promoters. Grey parts of the genes represent promoters; wavy lines indicate that levels of bmal1, per and cry transcripts oscillate. (b) In the mouse suprachiasmatic nucleus (SCN), *per* and *bmal1* transcripts oscillate almost 12 h out of phase, with *per* levels at a maximum around midday. PER and BMAL1 proteins also oscillate, but PER lags behind *per* RNA. (c) In peripheral tissues, such as the liver, the same clock gene components oscillate as in the SCN, but with peaks occurring later. The black and white bars above the graphs indicate night and day, respectively.

Clock gene transcripts also oscillate in peripheral tissues, with many properties similar to oscillations in the SCN [21]. However, the phase of the peripheral clock oscillations is delayed by 3–9 h (Fig. 1c), suggesting that the peripheral tissues might be receiving timing cues from the master oscillator in the SCN. Furthermore, oscillations in isolated peripheral tissues dampen rapidly, unlike the persistent rhythms in isolated SCN neurons [22,23]. Several examples of differences have now been uncovered. First, in the vasculature and regions of the forebrain, the gene encoding the CLOCK analog, MOP4 (a member of the PAS superfamily), is expressed at high levels, and MOP4 can activate *per* transcription in combination with BMAL1 [24,25]. In addition, *clock*$^{c/c}$ mutant mice (which are not null mutants but, rather, synthesize a CLOCK antimorph) display a rhythmic accumulation of *bmal1* and *per2* transcripts in heart and kidney but not in the SCN [26], suggesting that CLOCK might be functionally redundant in the periphery. Lastly, when fibroblasts from *per1*$^{-/-}$ mice are subjected to a serum shock, the period of the clock gene oscillations that are induced [27] is dramatically shorter than that of the activity rhythm of the animal, or of clock-gene oscillations in peripheral oscillators *in vivo* [28]. Because ablation of *per1* has a much more serious consequence for peripheral oscillators, PER1 might play a more important role there than in the SCN. Thus, these observations suggest that peripheral oscillators do indeed differ from those in the SCN, whether in the components used, the roles played by these components, or perhaps simply the relative amounts of the components.

Several studies underscore the role of phosphorylation in the clock mechanism. Almost all clock gene products are phosphorylated, some extensively [29], and casein kinase Iε (CKIε) can phosphorylate and destabilize PER *in vivo* and *in vitro* [30–32]. CKIε deficient in its ability to phosphorylate PER causes a short-period phenotype in the *tau* mutant hamster [33]. Similarly, a single base-pair mutation in the gene encoding human PER2, which eliminates the CKIε phosphorylation site, is associated with familial advanced sleep-phase syndrome (FASPS) [34]. Both these observations are consistent with accelerated degradation of phosphorylated PER compared with unphosphorylated PER: without phosphorylation, PER protein levels would build up more rapidly, causing the cycle to progress more rapidly (a short period could result in the advanced phase seen in FASPS if the clock were continually reset by light-dark cycles). However, as PER is phosphorylated normally in liver samples of the *tau* mutant hamster [29], it seems likely that the role of CKIε could be filled by additional kinases, perhaps including other casein kinase I isoforms, such as CKIδ [35].

Seeing the light – identifying the photoreceptor for light input to the clock

The identity of the photopigment involved in light signaling to the mammalian circadian system has long been a mystery [36]. Cryptochromes, which have been firmly implicated as circadian photopigments in *Drosophila* [37] and zebrafish [38], do not seem to be essential in mammals [39]. Another interesting candidate, melanopsin, was found to be expressed specifically in the ganglion cell layer of the retina [40,41]. This appeared important as a number of previous studies had localized the site of photic input to a subset of retinal ganglion cells [3] that connects to the SCN via the retinohypothalamic tract (RHT). When the subset of RGCs connecting to

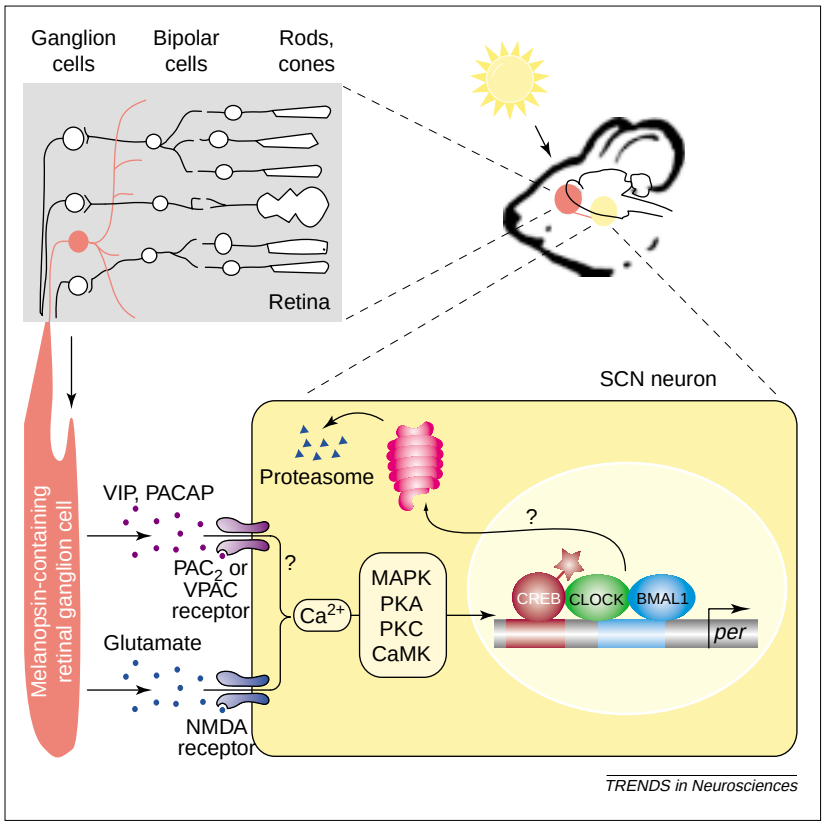

Fig. 2. Schematic view of retinal circuitry, showing light entering the circadian system through retinal ganglion cells that project to the suprachiasmatic nucleus (SCN) from the base of the retina. Melanopsin-containing cells also produce the pituitary adenylate-cyclase-activating polypeptide, PACAP (also known as vasoactive intestinal polypeptide or VIP) and glutamate. Signal transduction to the molecular oscillator inside individual SCN neurons could involve reception of glutamate and/or PACAP, through the NMDA receptor or the PAC_1 or $VPAC_2$ receptors, respectively (although the molecular action of the PAC_1 or $VPAC_2$ receptors is not yet clear). Changes in intracellular Ca^{2+} levels would lead to activation of MAPK (mitogen-activated peptide kinase), PKA (cAMP-activated protein kinase) PKC (protein kinase C) or CaMK (Ca^{2+}–calmodulin-dependent kinase) pathways. Ultimately, these signals could cause phosphorylation and activation of CREB (cAMP-response-element-binding protein; star indicates phosphate group), which can induce period gene (*per1* and *per2*) expression by binding to a cAMP-response-element in the promoter. Expression of *per* is also activated by heterodimers of CLOCK and BMAL1 (brain–muscle Arnt-like protein 1), and it is possible that light signaling accelerates proteasome-mediated degradation of BMAL1.

the SCN is identified, either by retrograde labeling or by staining for pituitary adenylate cyclase-activating polypeptide (PACAP), all of them contain melanopsin [42,43]. These RGCs connect up to the two lobes of the SCN and to other areas of the brain involved in different sorts of light responses [44]. Importantly, the melanopsin-containing RGCs are intrinsically photosensitive [44,45] and their action spectrum is in good agreement with that of photic entrainment [45]. Yet, without the genetic proof that only the phenotype of melanopsin-knockout mice will provide, all conclusions are arguable. Thus, the denouement of this fascinating conundrum is still unclear.

In part, the phase-shifting ability of light is believed to involve release of glutamate from the RGCs to NMDA receptors on the SCN neurons (Fig. 2). However, the PACAP found in melanopsin-containing RGCs can also produce phase shifts and induce *per1* and *per2* expression [46], whereas mice lacking the PACAP type-1 receptor show aberrant phase shifting and reduced induction of *per* by light [47]. The light signal could propagate through the SCN via release of vasoactive intestinal polypeptide (VIP) from SCN neurons, as VIP can induce phase shifts similar to light [48]. Even more interesting, mice lacking the $VPAC_2$ receptor, which mediates both VIP and PACAP signaling, display a striking loss of circadian rhythm [49].

Despite this evidence, it is still not clear how elevation of *per1* or *per2* levels alters the phase of the SCN molecular oscillator [50,51]. Perhaps induction of *per1* mRNA by light in the SCN will prove to be analogous to light induction of *frq* expression in *Neurospora* [1]. Alternatively, it is also possible that light-stimulated degradation of BMAL1 could constitute an important regulatory step [52], by a mechanism analogous to degradation of light-induced timeless protein (TIM) in *Drosophila* [53]. In any event, it is clear that light does affect transcription. This effect involves profound changes in chromatin architecture as well as activation of specific transcription factors. For example, a single light pulse induces phosphorylation at a specific serine residue in the N-terminal tail of histone H3, a modification that is linked to chromatin remodeling events and thought to increase accessibility of the locus to the transcriptional machinery [54]. Furthermore, activation of the *per1* promoter by induction of either the cAMP or mitogen-activated-protein kinase (MAPK) signaling pathways was shown to involve cAMP-response-element-binding protein (CREB) and to be independent of CLOCK and BMAL1 [55]. Although the classical phosphorylation site for activating CREB is Ser133 [56], light treatment of $CREB^{S142A}$-mutant mice produces smaller phase shifts in the locomotor rhythm and lower *per1* induction than it did in wild-type mice [57]. Phosphorylation of CREB at both Ser133 and Ser142 could, thus, be required to couple fully light reception to the phase shift of the molecular oscillator.

Getting the word out – synchronizing oscillations in the periphery

The role of the SCN as a master timer in the circadian system implies that timing cues are continually transmitted to the rest of the body. At least part of this timing information is transmitted to other areas of the brain via physical connections [58]. One recent study has traced an indirect link from the SCN through the dorsomedial hypothalamic nucleus to the noradrenergic nucleus locus coeruleus, which is involved in regulating arousal state [59]. The neurons of the SCN have a higher spontaneous discharge frequency during the day, which might help to transmit circadian output to other neurons. Recently, high frequency oscillations in Ca^{2+} have been observed in SCN neurons in brain slices [60]. These alter the membrane potential of the SCN neurons, and depolarization of the membrane facilitates

spontaneous firing. The mechanism for these oscillations is not yet known, although glutamate (the neurotransmitter of the RHT) can induce Ca^{2+} oscillations in astrocytes [61]; it will be interesting to see if this holds true for the SCN neurons too. It is also interesting that some kinases, notably Ca^{2+}–calmodulin-dependent kinase II (CaMKII) [62] can autophosphorylate to higher levels when the frequency of Ca^{2+} pulses increases. Perhaps the Ca^{2+} oscillations will have molecular consequences in addition to changes in membrane potential. Coincidentally, CaMKII can phosphorylate CREB at Ser142 [63], suggestive of an impact on *per1* transcription rates.

Timing cues are also given off in the form of diffusible signals [4,6,12]. For example, immortalized SCN cell lines can impose rhythmic metabolism on co-cultured fibroblasts, which are not by themselves rhythmic [64]. This is clearly independent of any neural connection. More recently, rhythmic secretion of transforming growth factor-α (TGF-α) from the SCN has been implicated in regulation of locomotor activity [65]. The ventricular infusion of TGF-α reversibly inhibited locomotor activity, and mice expressing a construct encoding EGF-R (a TGF-α receptor) with reduced ligand-stimulated kinase activity were more active during the day than were wild-type mice. The cysteine-rich protein prokineticin 2 (PK2) is also secreted rhythmically from the SCN; this suppresses wheel-running activity when infused into the ventricles and has receptors scattered throughout the brain – including in the SCN itself as well as in several SCN targets [66]. The *pk2* gene promoter contains several E-box elements, linking its transcription to oscillations in CLOCK–BMAL1 heterodimers. The relationship between TGF-α and PK-2 remains to be worked out, but already it seems clear they are not alone. Microarray analysis has demonstrated that the levels of transcripts encoding several prohormones and peptide hormones oscillate in the SCN [67], suggesting that many different chemical signals might be rhythmically produced by these neurons. Indeed, the largest group of coordinately cycling transcripts in the SCN involves genes implicated in protein synthesis, processing and secretion. Taken together, these results stress the importance of chemical signals that could potentially affect the behavior of other neurons or even of peripheral tissues.

Some of the timing information affecting the periphery could be provided by specific chemical signals. For example, retinoic acid can produce phase shifts in the vasculature [24]. This is likely to reflect an interaction between the nuclear retinoic acid receptors RARα or RXRα and the CLOCK–BMAL1 heterodimer, leading to a reduction in transcription from E-box-containing promoters. Other signals, such as glucocorticoids, transiently alter the phase of the peripheral clock oscillations [68] – an effect akin to masking. However, as restricted-feeding protocols can entrain peripheral oscillators without effect on the SCN clock [27,69,70], some entraining signals might be indirect effects of the SCN on behavior. Interestingly, the ability of tissues to respond to restricted-feeding protocols appears to be inhibited by glucocorticoids, which could, thus, provide a buffering effect against temporary changes in feeding schedules [71]. It has been suggested that food availability might contribute to changes in redox state in the cells, as binding of DNA by CLOCK–BMAL1 is exquisitely sensitive to changes in redox state *in vitro* [72]. However, the mammalian CRY is insensitive to redox state, unlike its *Drosophila* homolog [73]. The recent application of a transplantation procedure, in which mouse embryo fibroblasts (MEFs; a model of the peripheral clock) of a given genotype are implanted into a host with a different clock genotype, is likely to help greatly in study of the physiological connections between the SCN and peripheral clocks [27] (Fig. 3).

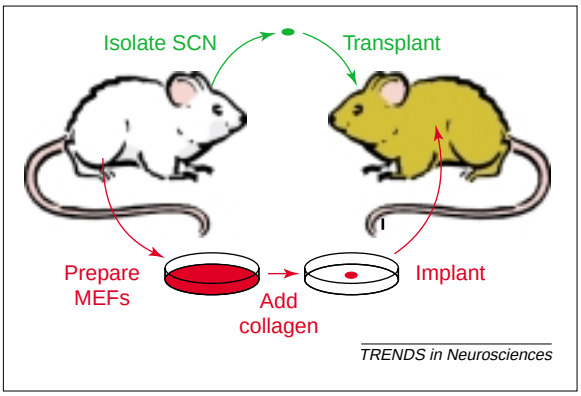

Fig. 3. The transplantation of cells from the suprachiasmatic nucleus (SCN) demonstrated the key role of this tissue in controlling circadian behavior (green; top). An analogous implant procedure, using surgical implantation of collagen-coated mouse embryo fibroblasts (MEFs), allows peripheral oscillators to be placed in an environment where they can receive and respond to the normal signals coordinating circadian oscillations in a host animal (red; bottom). This flexible procedure allows MEFs of any genotype to be implanted. As used in Ref. [27]

They've got rhythm – circadian gene expression in peripheral tissues

Independently of how timing signals arrive, to have any functional relevance the cells within various peripheral tissues must be able to do different things at different times of day. Several peripheral tissues have now been shown to regulate gene expression on a daily basis. Transcript profiling in serum-shocked synchronized fibroblasts [74,75], heart [76], liver [67,76–78], kidney [79] and the SCN itself [67] have all been reported. Several global conclusions can be drawn from this recent avalanche of data. First, although a substantial number of genes are rhythmic (2% of genes tested in fibroblasts and 10% in the SCN or peripheral tissues), the rhythmic genes are almost always different in the different tissues. For example, in comparisons of heart with liver [76] or SCN with liver [67], only 10% of the cycling transcripts are found in both tissues (Fig. 4a). Although some of these

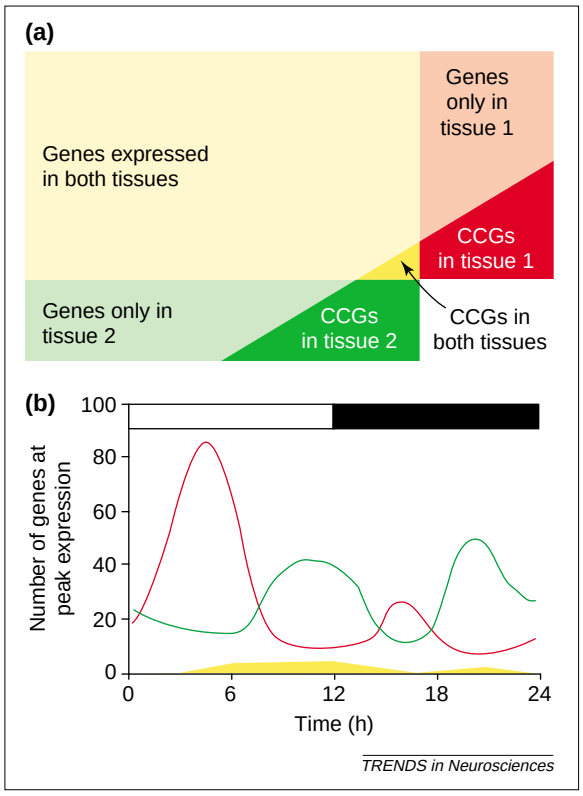

Fig. 4. Differences in circadian-regulated gene expression. (a) Venn diagram of gene expression in two different tissues (red and green), showing that roughly 80% of the genes expressed in one tissue will also be expressed in the other (yellow overlap). Circadian-controlled genes (CCGs; darker hues for each color) constitute only a small percentage of the genes expressed in both tissues, although ~10 times this number of genes could be under circadian control in any one tissue. (b) Different circadian-controlled genes might peak at different times during the circadian cycle. Analysis is based on data comparing gene expression in two different tissues [67,76].

common transcripts are clock genes, the roles of the others are not clear. Some might be other core components of the oscillatory mechanism.

Alternatively, they could correspond to transcripts directly responsive to circadian humoral signals. Second, in a given tissue, peaks of gene expression can be found at almost all different phases (Fig. 4b), suggesting that a single transcription factor (e.g. the CLOCK–BMAL1 heterodimer) is unlikely to regulate all genes. Third, different groups of genes are regulated in different tissues, despite similarities in the basic clock mechanism. This suggests that either oscillating clock gene products interact with tissue-specific transcription factors or co-activators, or that humoral signals drive the rhythmic transcriptional response of the different tissues. Lastly, sorting of the cycling transcripts into functional groups has revealed that the major classes of regulated genes are implicated in processes specific to the tissue in which they are found. For example, many cycling transcripts in the liver are involved in nutrient metabolism and intermediate metabolism [67]. It is also of interest that many of the regulated transcripts correspond to rate-limiting steps in their respective pathways, indicating that control is selective and efficient.

Of course, cycling transcripts are just the beginning in the search for understanding of the impact the body clock has on cell physiology. It will be important to verify that the proteins encoded by these transcripts also cycle (as there are cases in which cycling transcripts do not result in cycling proteins), and to evaluate whether the cycling proteins produce changes in metabolic activity (as metabolic flux through a given biochemical pathway will be sensitive only to changes in the levels of an enzyme catalyzing the rate-limiting step). It will also be important to assess whether proteins act together to regulate a given step, as their effects could multiply rather than add. Finally, a caveat: where the protein is could be more important than its amount [80]. Clearly, many more exciting results will come with time.

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