Environmental stimulus perception and control of circadian clocks

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Circadian rhythms are regulated by clocks located in specific structures of the central nervous system, such as the suprachiasmatic nucleus (SCN) in mammals, and by peripheral oscillators present in various other tissues. Recent discoveries have elucidated the control of central and peripheral clocks by environmental signals. The major synchroniser in animals is light. In mammals, a subset of retinal ganglion cells receive light signals that are transmitted to the SCN via the retinohypothalamic tract. Photoreception is probably elicited by a novel opsin, melanopsin, although cryptochromes may also play a role. These signals feed directly to the SCN master clock, which then provides timing cues to peripheral clocks. In contrast to mammals, peripheral tissues in the fly and in the fish are directly photoreceptive. However, alternative routes exist. Some peripheral clocks in mammals can be specifically entrained in an SCN-independent manner by restricting food during the light period.

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Abbreviations

BMAL1	brain and muscle ARNT-like protein 1
CREB	cAMP-responsive element binding protein
CRY	CRYPTOCHROME
LD	light:dark
LNvs	ventral group of lateral neurons
MAPKs	mitogen-activated protein kinases
PER	PERIOD
RGCs	retinal ganglion cells
RHT	retinohypothalamic tract
SCN	suprachiasmatic nucleus
TIM	TIMELESS

Introduction

Most organisms adapt the timing of their physiology to the cyclic changes of their environment using intrinsic time-keeping systems called circadian clocks. In the absence of external cues, circadian clocks can sustain rhythms of about 24 hours — hence the name circadian, meaning 'about a day' — for extended periods of time [1–3]. In mammals, the main circadian clock is located in the suprachiasmatic nucleus (SCN), a group of several thousand cells located in the anterior hypothalamus. Neurons of the SCN exhibit circadian rhythms even when isolated in culture. The cell-autonomous nature of these oscillators prompted the search for clock genes [1–3]. Clock genes and their protein products interact in a complex series of negative and positive regulatory feedback loops, which result in oscillation of specific transcripts and proteins, and ultimately, circadian rhythmicity and behaviour.

Environmental cues can reset daily the phase of molecular internal rhythms, ensuring that the organism's behaviour remains tied to the rhythms in its environment. The main resetting cue for animals is light, provided by the day-night cycles [1–3]. Light signals are perceived by the retina and information is conveyed to the SCN through the retino-hypothalamic tract (RHT) [4], and induces in retinorecipient neurons of the SCN a cascade of events including the activation of mitogen-activated protein kinases (MAPKs) [5] and cAMP-responsive element binding protein (CREB) [6•,7•], upregulation of several genes including clock genes [1,2] and specific chromatin modification [8] (Figure 1a). Here, we focus on recent reports that deal with the reception of environmental cues by circadian clocks in animals, including photoreception by the retina and by different anatomical clock structures, as well as responses to other environmental signals.

Photoreceptors for the central clock

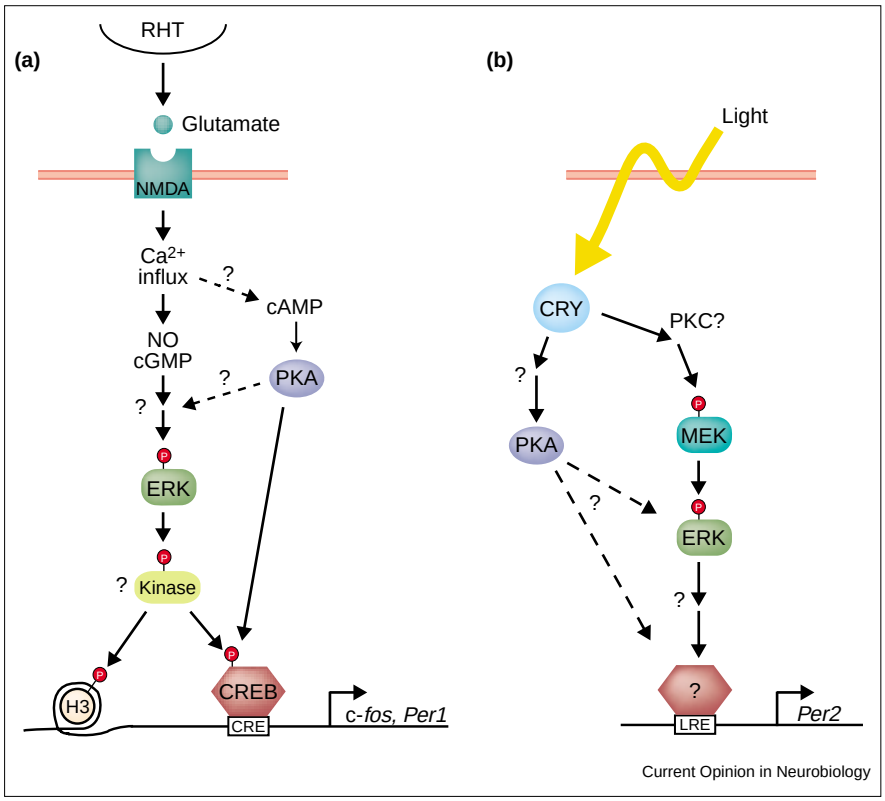
Opsins and cryptochromes in mammals

Circadian photoreception involves pathways that are distinct from those used for image formation. This is clearly shown by the resetting of activity rhythms and the suppression of melatonin in response to light in rodless, coneless mice [9]. Photoreceptive cells for these processes must therefore be located elsewhere within the retina (Figure 2). Retrograde tracing experiments were used to identify retinal cells targeting the SCN through the RHT. These cells constitute a small subset of retinal ganglion cells (RGCs), formed of type III or type W cells [4]. Recently, these cells were shown to display intrinsic phototransduction, with photic properties matching those of clock entrainment [10••].

What is the photopigment involved in the reception of photic cues for the circadian clock? The main candidates attracting attention in the last few years are melanopsin [11] and the cryptochromes [12].

Several recent papers build a strong case for melanopsin. This protein is a novel opsin found in the eye and in other photoreceptive structures in amphibians, and exclusively in the eye in primates and rodents [11]. Its distribution is restricted to a subset of RGCs, an observation that initially suggested a role in circadian photoentrainment. This idea was supported by the demonstration that the cells projecting to the SCN and mediating entrainment are the same as the ones expressing melanopsin [13,14,15••]. Moreover, all photoreceptive RGCs contain melanopsin, whereas RGCs

Figure 1



Light signalling in SCN neurons and zebrafish peripheral cells. **(a)** Light signalling in SCN neurons in response to signals from the retina. RHT neurons release glutamate in synapses of the ventrolateral SCN. Activation of the *N*-methyl-D-aspartate (NMDA) receptor initiates a cascade of events leading ultimately to induction of immediate-early genes (such as c-*fos*) and clock genes (such as *Per1*), specific chromatin remodelling (such as histone H3 phosphorylation [8]) and phase resetting of SCN-controlled rhythms. *Per1* and c-*fos* gene induction is dependent upon the activation of mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK) and the transcription factor CREB. A role for the cAMP-protein kinase A (PKA), nitric oxide (NO) and cyclic GMP (cGMP) pathways has also been suggested. **(b)** Signaling in zebrafish cells (Z3 line) in response to light. CRY perceives light and induces signal transduction pathways involving MAPK kinase (MEK), PKA and protein kinase C (PKC). This leads to the induction of clock genes (such as *Per2*) and subsequent phase resetting of the molecular rhythms of the cells. NO, nitric oxide; CRE, cAMP response element; LRE, light response element; H3, histone H3.

lacking light response are devoid of this protein. The ultimate confirmation of melanopsin as the primary circadian photoreceptor in mammals should be obtained using loss-of-function approaches by homologous recombination in the mouse and/or by targeted ablation of melanopsin-containing cells.

The photoreceptive RGCs and the photopigment melanopsin may be also used for several other non-visual tasks, which are independent of the presence of rods and cones. For example, the pupillary light reflex — a quick pupillary constriction in response to light — appears to rely on an opsin/vitamin A-based photopigment [16]. Moreover, the peak sensitivity of this reflex occurs at a wavelength similar to that of circadian rhythm entrainment and SCN-targeting RGCs [10••]. In addition, acute light suppression of melatonin levels presents similar action spectrum characteristics [17]. Finally, masking, a suppressive effect of light on wheel-running activity that happens independently of the SCN [18], may also involve the same RGCs [19].

Besides melanopsin, the products of the two *Cryptochrome* (*Cry*) genes are candidate circadian photopigments. Initially, mutation of these genes in the mouse indicated a key role for these molecules in the core clock mechanism [1–3,12]. Molecular studies confirmed this view. Heterodimers of the CLOCK and BMAL1 (brain and muscle ARNT-like protein 1) factors, which are intimately linked to circadian activity, activate the expression of *Period* (*Per*) and *Cry* genes [1–3]. The products of *Per* and *Cry* form multimeric protein complexes and translocate to the nucleus, where CRY proteins inhibit CLOCK–BMAL1 activity. This inhibition closes the negative feedback loop essential for maintenance of molecular circadian rhythms [1–3,20,21]. CRYs were also shown to control PER2 protein stability [21].

Given this role for CRYs as components of the central oscillator, a key question is whether they also have photoreceptive properties in mammals. The amino acids critical for intramolecular redox reactions involved in light perception in *Drosophila melanogaster* CRY (see below) [22] can be mutated in mouse CRYs without affecting their transcriptional repression activity [23], although this is not observed in *Xenopus laevis* [24]. Furthermore, *Cry*-deficient mice present some defects in light dependent *Per* gene induction in the SCN [25]. Finally, mice devoid of rods, cones and CRYs are nearly arrhythmic in light:dark (LD) conditions and display severely blunted c-*fos* gene response to light in the SCN [26]. Taken together, these results suggest a role for CRYs in photoreception. However, some of these effects could be indirect, due to CRYs modifying characteristics of melanopsin-containing RGCs. More compelling evidence for CRY involvement in photoreception comes from the recent report that vitamin A-lacking mice exhibit *Per* gene induction in the SCN in

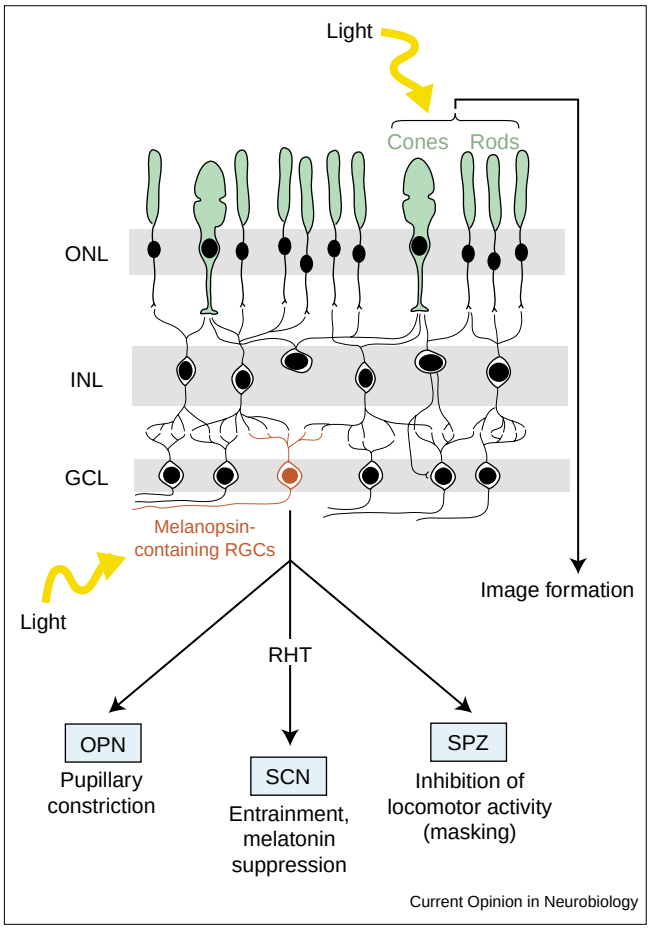
response to light [27]. Thus, the identity of circadian photopigments may be more complicated than originally thought: despite the strong evidence for melanopsin as a key player in circadian photoreception, a role for CRYs should not be excluded.

A reversed situation in the fruit fly

Mammals are unique in that their central clock is not photoreceptive; thus, specialised structures have evolved to perceive light. Other vertebrates such as amphibians, birds and fish harbour extra-ocular photoreceptive structures. This is also the case for *Drosophila*, in which a small group of pacemaker neurons responsible for controlling activity rhythms — the ventral group of lateral neurons (LNvs) — are directly photosensitive [28]. In contrast to the situation in mammals, where CRYs seem to function primarily as clock components, albeit with putative roles in photoreception, the *Drosophila* homologue, dCRY, is a major photoreceptor for the circadian clock [29,30]. A mutation in the *cry* gene — the cry^b mutation — abolishes clock protein oscillations in the eye and the response of the clock to brief light pulses [30]. In contrast to wild-type flies, cry^b flies are rhythmic in constant light, acting as if they were blind to light [31]. This suggests that CRY does not form an essential part of the molecular oscillator in flies, in contrast to its role in mammals. Interestingly, light response is recovered in *Drosophila* when CRY is overexpressed specifically in the LNvs of cry^b mutant flies [28]. This implies that, within a single cell, the molecular component of the clock can be directly affected by CRY in response to light. However, CRY is not the only photoreceptor in the fly, because cry^b mutants can be entrained to LD cycles [30]. Combined impairment of *cry* and non-LNv photoreceptive structures — the compound eye, the ocelli and the Hofbauer-Buchner eyelet — abolishes all circadian photoresponses, and the fly rhythms then free-run completely (i.e. activity rhythms can occur but they are independent of LD cycles) [32•]. Thus, other photoreceptors, probably opsins, may also contribute to entrainment of the clock in *Drosophila*. LD entrainment studies in different mutant flies suggest that CRY may contribute mainly to adjusting the evening circadian activity peak, whereas other pathways are involved in setting the morning peak [28].

As in mammals, the clock in *Drosophila* relies on regulatory feedback loops [3]. However, in the fly clock, PER and TIMELESS (TIM) proteins act in the negative limb of the feedback loop, repressing their own expression by inhibiting the activity of the CLOCK–CYCLE heterodimer (CYCLE is the fly homologue of BMAL1). CRY can bind both PER and TIM in a light dependent fashion and stop them from inhibiting the activity of CLOCK–CYCLE [3,33]. Light-induced changes in redox potential appear to cause conformational changes in CRY [22], which ultimately result in ubiquitination and degradation of TIM [22]. These events are not observed with the mutant CRY^b protein [22,34•]. These observations outline the initial events in the response of the *Drosophila* clock to light, and explain how photoreception and clock function can be linked in the same cells. In this mechanism, CRY is a molecule sensitive to light that transmits its message directly to clock components, but it is not part of the clockwork mechanism itself. However, this is not likely to be the whole story, and other roles for CRY in clock mechanisms were uncovered when comparing oscillations in LNvs and in other tissues (see below).

Figure 2



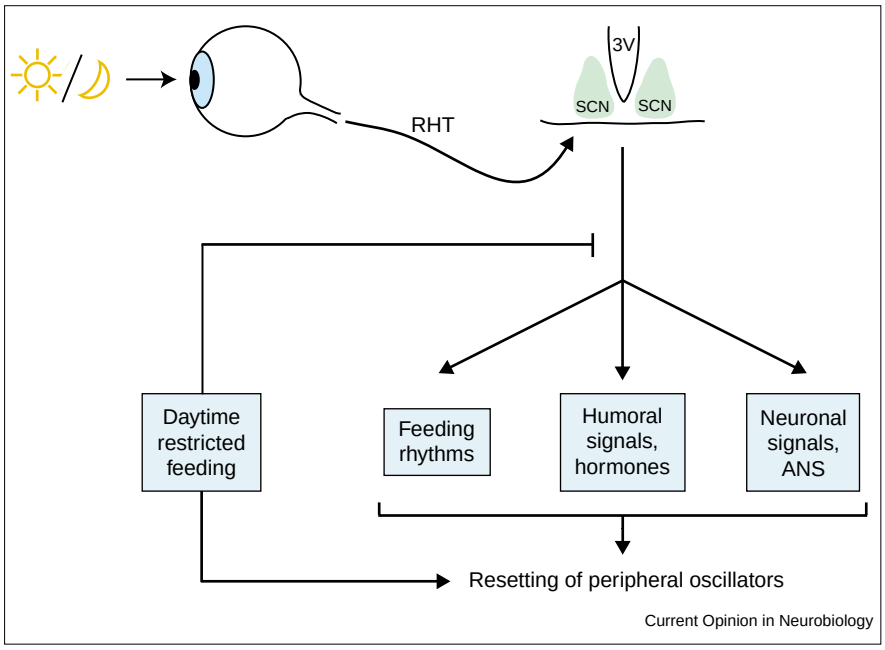
Distinct photoreceptors in the mammalian retina for vision and non image-forming tasks. Cones and rods mediate light perception for image formation. Other light-regulated processes depend on different photoreceptors within the retina. These non image-forming processes are thought to involve a small subset of RGCs that express melanopsin and are light sensitive (in contrast to other RGCs). Melanopsin-containing RGCs innervate the SCN through the RHT, thus allowing entrainment of the pacemaker. Pineal melatonin suppression may also depend on this RHT/SCN-dependent pathway. Melanopsin cells also project to the olivary pretectal nucleus (OPN), allowing pupillary reflex, and to the subparaventricular zone (SPZ), thus possibly mediating the light-dependent inhibition of locomotor activity. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer.

So many clocks to synchronise…

Non-neuronal clocks that see the light

The simple view of a single or a few centralised clock structures has been reevaluated over the past few years.

Figure 3



The regulation of peripheral oscillators in mammals. Environmental LD cycles reset the SCN pacemaker through the retina and the RHT. The SCN in turn synchronises the oscillators in peripheral tissues. This control may be exerted by a combination of neuronal signals, humoral factors and restriction of feeding to the times of the day in which the animals are awake. The tight control of the SCN over peripheral oscillators can be circumvented by restricting feeding to the daytime (for nocturnal animals). This resets rhythms in several organs (e.g. liver, kidney) by a still unknown mechanism, but does not reset SCN rhythms. This peripheral tissue-specific entrainment by feeding schedule appears to dominate over other possible routes of entrainment by the SCN. ANS, autonomic nervous system; 3V, third ventricle.

Drosophila [35,36], zebrafish [37] and mammals [38] were all shown to possess circadian oscillators in various tissues, including non-neuronal tissues. For example, cultures of *Drosophila* wings and antennae [36], or of zebrafish hearts and kidneys [37], display circadian oscillations of clock genes in constant conditions. In the fly and in the fish, there is a relative independence of these peripheral clocks from the central clock. Indeed, clock gene oscillations exhibit distinct patterns of expression from tissue to tissue in the zebrafish [39]. In *Drosophila*, excretory tubules taken from one fly maintain their phase of oscillations even when grafted to another fly that is entrained on a reversed LD cycle [35].

Peripheral clocks in *Drosophila* and zebrafish display an even more striking feature: they are directly light responsive. No need for an eye or other specialised structures: clock gene circadian expression in cultured *Drosophila* tissues [36] and zebrafish organs [40] can be reset by LD cycles. In the case of zebrafish, light-responsiveness has even been demonstrated for cultured cells [40,41]. We recently established a zebrafish embryonic cell line, named Z3, which recapitulates the light response characteristics of a clock. In Z3 cells, clock gene oscillations can be entrained to new LD cycles and clock gene expression can respond acutely to light [41]. The action spectrum of *Per2* induction in Z3 cells revealed that a subset of the six zebrafish CRYs [42] are likely to be involved in this response to light (Figure 1b), whereas the function of other CRYs in these cells may be restricted to the clockwork itself [43•]. Circadian photoreception could employ distinct photopigments in the retina and in peripheral tissues. It is not known at the moment what mechanism is used in the eye of the zebrafish.

In *Drosophila*, although CRY acts as a photoreceptor in peripheral tissues, it probably plays an additional role in clock function in these locations. This notion is supported by studies on olfactory responses [44•] and on *tim* gene expression in excretory tubules and lateral neurons [34•] in cry^b mutant flies. Thus, both in vertebrates and in insects, CRYs cannot be restricted to either photoreception or clock function.

How mammalian peripheral oscillators deal with the environment

No peripheral photoreception has been shown to occur in mammals. Light has an effect on mammalian peripheral oscillators, but this effect is indirect: the SCN integrates photic cues from the retina and the RHT, and then synchronises peripheral oscillators through output pathways [38,45] (Figure 3). In the absence of SCN signals, oscillations (in clock gene transcripts or in expression of a reporter in *Per1*-luciferase transgenic animals) rapidly dampen in peripheral oscillators [38,45,46]. The signals from the central clock must thus entrain these dampened oscillators. These signals could follow neuronal pathways, either to various areas of the brain [45,47] or to tissues via the autonomic nervous system [48]. The SCN was also proposed to reset peripheral clocks through humoral signals. This is supported by the observation that a serum shock can induce oscillations in cultured fibroblasts [46], and that forskolin, an adenylate cyclase activator, can restart oscillations in dampened tissues *in vitro* [38,45]. Moreover, by coculturing SCN neurons with 3T3 fibroblasts, the former can induce oscillations in the latter cells, via a signalling molecule that can pass through a semi-permeable membrane [49]. Using a novel approach that

involves mouse embryo fibroblasts/collagen implants, we have recently extended these observations *in vivo*. We found that the SCN imposes rhythmicity onto peripheral tissues through a diffusible signal, which, evidently, is able to access the implanted fibroblasts. Importantly, this study demonstrates that the central clock can phenotypically rescue an important genetic defect intrinsic to a peripheral oscillator [50•].

What is the nature of this diffusible signal? Various substances have been proposed as candidates. Glucocorticoids seem to play a role, because dexamethasone induced the same circadian gene expression in fibroblasts as did serum shock. Dexamethasone can also provoke transient changes in the phase of clock gene oscillations in peripheral tissues when injected into mice [51]. Another possible synchroniser is retinoic acid, which can delay the *Per2* rhythm in vascular smooth muscle cells both in culture and *in vivo*, possibly due to an interaction of retinoic acid receptors with CLOCK or its homologue MOP4 [52•]. Thus, different nuclear receptors may be able to mediate distinct responses in peripheral clocks.

Food may also result in diffusible signals affecting the phase of peripheral clocks. For example, when the feeding of nocturnal animals such as mice is restricted to daytime instead of being available *ad libitum*, the phase of peripheral oscillators, for example in the liver and in the kidney, but not that of the SCN, is shifted by 12 hours [53••,54••] (Figure 3). The mechanism underlying this entrainment is still unknown, but one intriguing observation has been made recently: on shifting from food *ad libitum* to restricted food accessibility, peripheral clocks take some time to entrain to their new phase, and this slow phase resetting is due to glucocorticoids (GCs) [55]. Mice that do not make GCs have peripheral clocks that entrain much faster, as do organs lacking GC receptors. Thus, GCs apparently have opposite roles: first they cause a phase shift when injected in mice; second, they oppose the phase shift caused by food-induced entrainment. These experiments, in addition to giving insights on how the environment impinges on peripheral clock function, provide another possible mechanism by which the central clock could synchronise peripheral oscillators: the SCN indirectly controls the time of feeding by regulating activity rhythms and arousal. In nocturnal animals, most of the food is absorbed during the dark period [53••]. Accordingly, food restriction to daytime would bypass the normal communication between the SCN and the peripheral oscillators. Consequently, the SCN pathway must be dominant over the neuronal and humoral pathways discussed above, because, during daytime feeding, the SCN and peripheral tissue rhythms appear completely uncoupled.

Conclusions and future directions

The abundance of information accumulated over the last year or two raises numerous issues regarding circadian photoreception and illustrates the complexity of the links between the environment and the clock. In the future, it will be important to understand the exact contribution of different input pathways to the entrainment of clocks, in particular when considering the actions of different synchronisers. For example, the SCN can be entrained by photic signals, but also by physiological non-photic cues [56]. Peripheral clocks can receive signals from the central clock, but also other signals from the environment, for example food restriction. How do these cues — sometimes opposite in effect — impact on the oscillators? Other crucial questions will address the identification of signals involved in peripheral clock resetting, how food availability can entrain specifically peripheral clocks, what links can be found between clocks and metabolism, and the existence of other unexpected environmental synchronisers. Many more surprises await us in the near future.

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