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The regulation of circadian clocks by light in fruitflies and mice

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A circadian clock has no survival value unless biological time is adjusted (entrained) to local time and, for most organisms, the profound changes in the light environment provide the local time signal (*zeitgeber*). Over 24 h, the amount of light, its spectral composition and its direction change in a systematic way. In theory, all of these features could be used for entrainment, but each would be subject to considerable variation or 'noise'. Despite this high degree of environmental noise, entrained organisms show remarkable precision in their daily activities. Thus, the photosensory task of entrainment is likely to be very complex, but fundamentally similar for all organisms. To test this hypothesis we compare the photoreceptors that mediate entrainment in both flies and mice, and assess their degree of convergence. Although superficially different, both organisms use specialized (employing novel photopigments) and complex (using multiple photopigments) photoreceptor mechanisms. We conclude that this multiplicity of photic inputs, in highly divergent organisms, must relate to the complex sensory task of using light as a *zeitgeber*.

Keywords: circadian; cryptochrome; *Drosophila*; mice; opsin; photoreceptors

1. LIGHT, TIME AND LIFE

The role of the circadian system in any organism is to coordinate the phase of a biological event to a specific feature or phase of the environment, and to ensure that the phases of multiple rhythmic events within an organism are appropriately coupled. Thus, circadian clocks provide the endogenous timetable for development, behaviour, physiology and biochemistry, as well as photoperiodic events in most organisms. This appropriate phasing can only occur when biological time is adjusted or entrained to local time. The entrainment of a biological clock requires an input pathway for the detection of specific environmental signals (*zeitgebers*) that provide time-of-day information. Depending on the species, biological clocks respond to a variety of different *zeitgebers*. For example, many micro-organisms, plants and heterothermic animals can be entrained by rhythmic changes in environmental temperature (Edmunds 1988), while social signals in birds can act as *zeitgeber* (Gwinner 1966). However, the stable daily change in the light environment provides the most reliable indicator of the time of day. As a result, most organisms use changes in the quantity and quality of light around dawn and dusk as their primary *zeitgeber* to effect what has become known as 'photoentrainment' (Roenneberg & Foster 1997).

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Until recently, photoentrainment has been regarded as yet another type of image detection, essentially no different from other visual tasks. Circadian physiologists have appreciated, however, that the circadian system must have evolved specializations that enable it to extract time information from the light environment, and that these specializations have nothing to do with classical vision (Roenneberg & Foster 1997). In a general sense, the sensory task of photoentrainment is complex, but can be considered fundamentally the same for all organisms. We should predict therefore, that the photoentrainment pathway will show convergent features in highly divergent organisms. One of the central aims of this review is to test this hypothesis, and so we compare the photoreceptors that mediate photoentrainment in both flies and mice, and assess their degree of convergence.

Most multicellular animals have evolved a master clock in the central brain that regulates behavioural rhythmicity and that coordinates the rhythms of peripheral tissues. Furthermore, these master clocks have specialized photoreceptor organs for photoentrainment. In recent years, however, some multicellular organisms have been shown to have autonomous clocks in peripheral tissues, which can be locally entrained by light, or more accurately radiant energy (Emery *et al*. 1997; Giebultowicz & Hege 1997; Giebultowicz *et al*. 2000; Plautz *et al*. 1997; Whitmore *et al*. 2000; Giebultowicz 2001). Our understanding of these autonomous peripheral clocks, and their entrainment mechanisms, is only just emerging. As a result, we have restricted this review to the photoentrainment of master clocks by specialized photoreceptor organs.

2. *DROSOPHILA*

In *Drosophila melanogaster*, the master clock or 'circadian pacemaker centre' resides within a few neurons in the lateral central brain called the 'lateral neurons' (LNs); for review, see Kaneko (1998). A ventral group of the LNs contains both the molecular clockwork necessary to generate circadian rhythms (Kaneko 1998) and a circadian output factor—the neuropeptide pigment-dispersing factor (Helfrich-Förster 1995; Park *et al*. 2000; Renn *et al*. 1999). Furthermore, mutants that lack the LNs are behaviourally arrhythmic (Helfrich-Förster 1998), although molecular rhythms can still be detected in peripheral cells of flies with no LNs (Zerr *et al*. 1990).

The best-studied behavioural rhythms in *Drosophila* have been pupal eclosion and the locomotor activity of individual adults. Pittendrigh and co-workers (Chandrashekaran & Loher 1969; Konopka *et al*. 1989) studied the rhythm of eclosion in detail in *Drosophila pseudoobscura*. More recent studies have monitored the activity rhythms of individual *D. melanogaster*, the model organism for geneticists. The overall rhythmicity of both species is similar, and the same genes appear to be involved in rhythm generation. Eclosion and activity rhythms of *Drosophila* are very sensitive to light. They can be entrained to light–dark (L:D) cycles of very low irradiance (less than 0.1 lux for activity) and phase-shifted by short light pulses (15 min of 0.1 lux for eclosion). Furthermore, continuous light lengthens the periods of free-running rhythms and suppresses rhythmicity when light intensity exceeds a certain threshold.

Describing the spectral sensitivity or action spectrum of a light-dependent response is a crucial step in characterizing the photopigment on which the response is based. The light-sensitive photopigments that mediate all photoreceptive pathways have discrete absorbance spectra that describe the probability of photons being absorbed as a function of wavelength (table 1). In an attempt to define the photopigments involved in circadian photoreception, the phase-shifting effects of monochromatic light treatments on *Drosophila* circadian rhythms have been studied. The first action spectrum examined phase delays and advances in eclosion rhythms, and showed a very broad spectral response between 420 and 480 nm. Wavelengths longer than 540 nm were ineffective in shifting the eclosion rhythm (Frank & Zimmermann 1969). A more detailed action spectrum for phase delays of the eclosion rhythm showed that 'blue' light of 457 nm was the most effective wavelength, but there were additional peaks of sensitivity around 473, 435 and 375 nm (Klemm & Ninnemann 1976). These multiple peaks more closely resemble the absorbance characteristics of a flavoprotein rather than that of an opsin–vitamin A (retinaldehyde–carotenoid)-based photopigment (Wolken 1995). Some support for a non-opsin-based photopigment comes from studies in which flies were fed on a diet depleted of carotenoids (Zimmerman & Goldsmith 1971). In these flies, the sensitivity of the visual receptors (compound eyes) was decreased by three orders of magnitude, whereas the photosensitivity of the circadian eclosion rhythm was not affected. The unambiguous interpretation of these experiments is that gross changes in visual sensitivity do not affect the entrainment of eclosion, and suggests that non-ocular photoreceptors might be important for this process. This conclusion was first made by Engelmann & Honegger (1966), who showed that flies lacking the compound eyes were still able to synchronize their eclosion rhythm to the L:D cycle.

Action spectra for phase-shifting or entraining the activity rhythms of adult flies, from several different laboratories, have demonstrated peak spectral responses close to 500 nm with some additional photosensitivity in the red part of the spectrum (table 1) (Blaschke *et al*. 1996; Ohata *et al*. 1998; Suri *et al*. 1998; for a review, see Helfrich-Förster & Engelmann 2001). These action spectra show a greater degree of similarity to the absorption spectra of opsin–vitamin A photopigments than the flavoproteins. As with eclosion, the compound eyes are not required for entrainment of locomotor activity at normal light intensities (Dushay *et al*. 1989; Helfrich 1986; Helfrich & Engelmann 1983; Wheeler *et al*. 1993). Although the compound eyes are not required for entrainment, they do appear to contribute to the overall photosensitivity of the circadian system under dim light conditions (below 1 lux). For example, eyeless flies show a decrease in sensitivity of about 2 log units compared with wild-type flies (Ohata *et al*. 1998). Significantly, the action spectrum of eyeless flies is much narrower than that of wild-type flies, with a maximum spectral response near 460 nm, and no sensitivity beyond wavelengths of 525 nm (table 1) (Ohata *et al*. 1998). Collectively, these data suggest that under normal circumstances both the eyes and extraocular photoreceptors contribute to the photoentrainment of activity rhythms. It seems likely that this is also true for the entrainment of pupal eclosion. The larval eyes, which use opsin–vitamin A photopigments, project directly to the larval LNs (Kaneko *et al*. 1997), and functional larval eyes are necessary to entrain the molecular rhythms in the larval LNs in mutants that lack extraocular photoreceptors (Kaneko *et al*. 2000).

Thus, *Drosophila* appears to use both ocular and extraocular photoreceptors for the entrainment of activity and eclosion rhythms. But what is the nature of these extraocular photoreceptors? In 1989, Hofbauer & Buchner discovered a pair of putative extraocular photoreceptors in adult flies that were later named the H–B (Hofbauer–Buchner) eyelets (Hofbauer & Buchner 1989). Significantly, these structures were still present in eyeless *sine oculis*^{1} mutants. Recent electron microscopic studies have shown that each of these H–B eyelets is composed of four photoreceptor-like cells with numerous microvilli arranged into coherent rhabdomeres (Yasuyama & Meinertzhagen 1999). These rhabdomeres are immunolabelled by antibodies raised against *Drosophila* rhodopsin (Rh6), and arrestin (a molecule of the phototransduction cascade) (Swinderen & Hall 1995). The H–B eyelets, like the compound eyes, are strongly labelled by antibodies against the period protein PER (Hall 1998*a*), and seem to use both histamine (Pollock & Hofbauer 1991) and acetylcholine (Yasuyama & Meinertzhagen 1999) as neurotransmitters. Significantly, the H–B eyelets project directly into the brain region where the LNs are located (for reviews, see Hall 1998*b*; Helfrich-Förster 1996). Analogous H–B photoreceptive-like structures have also been reported for other insects (for a review, see Yasuyama & Meinertzhagen 1999).

Table 1. A comparison of action and absorption spectra in *Drosophila* spp. (*a*) Action spectra. The maximum sensitivity (λ_{max}) for a variety of action spectra associated with the circadian system of *Drosophila* spp. (*b*) Absorbance spectra. Absorbance spectra for the opsin-based photopigment and CRY.

(In flies, the opsin-based photopigments or rhodopsins (R-form) are converted to metarhodopsins (M-form) upon illumination. The latter are then reconverted into the R-form by absorption of another quantum of light. The λ_{max} of the R- and M-form differ significantly. In the table, only λ_{max} for the R-form is given as it reflects a fairly accurate estimate of the spectral sensitivity measured physiologically (Feiler *et al*. 1988, 1992; Salcedo *et al*. 1999). It is important to note that this table compares only the λ_{max} of action and absorbance spectra. We have not attempted to compare the other important feature of absorbance spectra and action spectra—their spectral profiles. TIM, timeless protein.)

(*a*) action spectra

assay	λ_{max} (nm)	species	reference
eclosion phase-shift, wild-type	*ca*. 460	*D. pseudoobscura*	Frank & Zimmermann (1969), Klemm & Ninnemann (1976)
activity phase-shift, wild-type	*ca*. 500	*D. melanogaster*	Suri *et al*. (1998)
activity entrainment, wild-type	420 and *ca*. 480, *ca*. 500	*D. melanogaster*	Blaschke *et al*. (1996), Ohata *et al*. (1998)
activity entrainment, *sine oculis*^{1}	420 and *ca*. 460, *ca*. 480	*D. melanogaster*	Blaschke *et al*. (1996), Ohata *et al*. (1998)
activity entrainment, *sine oculis*^{1}*;glass*^{60j} double mutant	420	*D. melanogaster*	Helfrich-Förster & Hofbauer (2001)
TIM degradation phase-shift	*ca*. 450	*D. melanogaster*	Suri *et al*. (1998)

(*b*) absorbance spectra

rhodopsins	λ_{max} (nm)	location	reference
Rh1	486	R1–6	reviewed in Salcedo *et al*. (1999)
Rh2	418	ocelli	reviewed in Salcedo *et al*. (1999)
Rh3	331	R7	reviewed in Salcedo *et al*. (1999)
Rh4	355	R7	reviewed in Salcedo *et al*. (1999)
Rh5	442	R8	Salcedo *et al*. (1999)
Rh6	515	R8, H–B eyelet	Salcedo *et al*. (1999), Yasuyama & Meinertzhagen (1999)
CRY	420[a]	LN, retina[b]	Selby & Sancar (1999)

[a] Although the absorbance spectra of the opsin-based photopigments is well resolved (Lythgoe 1979), cryptochrome (CRY) absorbance spectra are much more variable (Ahmad & Cashmore 1996). Thus, it is difficult to empirically predict the shape of action spectra of CRY-mediated events for comparison with experimental observations (see text (§ 2) for details).
[b] The presence of CRY in the retina is only inferred. We assume that CRY is expressed in the retina because the absence of CRY in the *cry*^{b} mutant is associated with a loss of PER–TIM cycling in the eyes (Stanewsky *et al*. 1998).

Action spectra in two mutants of *Drosophila* suggest that the H–B eyelets are responsible for entrainment photosensitivities around 480 nm. This was concluded from the comparison of the action spectra of eyeless *sine oculis*^{1} mutants with that of *sine oculis*^{1}*;glass*^{60j} double mutants that lack both the H–B eyelets and compound eyes. In *sine oculis*^{1} mutants, two sensitivity peaks were found in the action spectrum, one at 420 nm and the second around 480 nm (Helfrich-Förster & Hofbauer 2001). The 480 nm peak was absent in *sine oculis*^{1}*;glass*^{60j} double mutants, suggesting that the H–B eyelets are the source for this sensitivity peak. Significantly, the absorption spectrum of the *Drosophila* Rh6 photopigment (which appears to be expressed in H–B eyelets) has a spectral maximum at 515 nm (table 1) (Salcedo *et al*. 1999; Yasuyama & Meinertzhagen 1999). However, the H–B eyelets cannot be the only extraocular circadian photoreceptors in *Drosophila*. We know this because the *glass*^{60j} mutants, (lacking both compound eyes and the H–B eyelets) as well as *sine oculis*^{1}*;glass*^{60j} double mutants, which lack all compound eyes and the H–B eyelets (also lacking both compound eyes and the H–B eyelets), are still capable of entraining and phase-shifting circadian rhythms of locomotor behaviour (Hall 1998*a*; Helfrich-Förster & Hofbauer 2001; Helfrich-Förster *et al*. 2001).

The best candidate we have for this additional extraocular photopigment is the recently discovered blue-light-absorbing protein cryptochrome (CRY) (Emery *et al*. 1998; Stanewsky *et al*. 1998). CRY is expressed in the LN (Egan *et al*. 1999; Emery *et al*. 2000*a*) and the CRY absorption spectrum (Selby & Sancar 1999) has some resemblance to the behavioural action spectra of *sine oculis*^{1}*;glass*^{60j} double mutants (C. Helfrich-Förster, unpublished data). Unfortunately, implicating CRY-type photoreceptors using action spectrum approaches is not straightforward. The main problem is that the absorbance profiles of photolyase–CRY proteins are potentially very variable (Ahmad & Cashmore 1996). This variability is partly caused by the presence of two independent cofactors (a pterin or 5-deazoflavin and a flavin (FAD)) either or both of which may act as the primary chromophore, and partly by the change in spectral sensitivity of the FAD cofactor according to its redox state (Ahmad & Cashmore 1996; Galland & Senger 1991; Lin *et al*. 1995;

Wolken 1995). When oxidized, FAD absorbs blue–ultraviolet (UV) light. However, FAD can also exist in reduced (FADH) and intermediate (flavosemiquinone) states. FADH absorbs near UV light and is relatively insensitive to longer wavelengths (Galland & Senger 1991). Flavosemiquinone also shows maximum sensitivity to near UV, but additionally exhibits significant absorption of blue–green (400–500 nm) and to a lesser extent longer wavelengths of light (Galland & Senger 1991; Lin *et al*. 1995). This potential for variation makes the fitting of a standard absorbance template problematical. Consequently, it is difficult to empirically predict the shape of action spectra of CRY-mediated events for comparison with experimental observations. This problem prevents us from distinguishing in the discussion below between CRY acting as a photopigment, or as an element of a phototransduction cascade (also see discussion in § 3).

Genetic manipulation of *Cry* gene dose will modify the photoentrainment of the activity rhythms. A lower dose of the *Cry* gene reduces the magnitude of phase-shifts (Egan *et al*. 1999), whereas overexpression of *Cry* leads to larger phase-shifts (Emery *et al*. 1998). Furthermore, the level of CRY in head extracts of *Drosophila* is profoundly affected by light exposure (Emery *et al*. 1998). Under a 12 L:12 D cycle CRY levels decrease upon illumination reaching a low point during the second half of the light phase. By contrast, after lights-off CRY levels increase and reach a peak towards the end of the night.

Further evidence that CRY is involved in circadian photoreception was gained by isolation of a mutation in the *Cry* gene that was called cry^{baby} (cry^{b}) (Stanewsky *et al*. 1998). The cry^{b} mutation affects a highly conserved amino acid probably involved in binding FAD, one of the two cofactors necessary for CRY functioning. cry^{b} mutants are unable to reset their clock to short light pulses (Stanewsky *et al*. 1998) and cry^{b} mutants do not become arrhythmic under continuous light conditions (Emery *et al*. 2000*b*). The presence of the compound eyes and H–B eyelets in cry^{b} mutants allows these animals to entrain their activity rhythms to L:D cycles rather normally, and also PER and TIM oscillations in the small LNs can be entrained by L:D cycles (Stanewsky *et al*. 1998). It is only when cry^{b} mutants are in genetic backgrounds that eliminate the function of the compound eyes that photoentrainment is clearly disrupted. *Drosophila* that carry the *no receptor potential* ($norpA^{P41}$) mutation have a disrupted phototransduction cascade in both larval and adult photoreceptor cells.

In $norpA^{P41};cry^{b}$ double-mutant larvae, the molecular rhythms in the LNs cannot be entrained to L:D cycles (Kaneko *et al*. 2000) and, in adults, entrainment of molecular rhythms is restricted to a subgroup of the LNs (Helfrich-Förster *et al*. 2001). Behaviourally, $norpA^{P41};cry^{b}$ double mutants take many cycles to entrain to the new phase of an adjusted L:D cycle (Emery *et al*. 2000*a*; Stanewsky *et al*. 1998). Indeed, some flies never re-entrain. The residual capability of $norpA^{P41};cry^{b}$ adults to entrain could be due to parallel phototransduction pathways that remain unaffected by the $norpA^{P41}$ mutation, and/or the H–B cells, which may use a different phototransduction cascade from the compound eyes.

Support for the latter comes from recent experiments using $glass^{60J}cry^{b}$ double mutants. These lack all ocular and extraocular photoreceptor structures and functional CRY. As might be expected, such mutants are totally blind to circadian time-cues. Both molecular rhythms in the LNs and behavioural rhythms failed to become entrained to L:D cycles (Helfrich-Förster *et. al*. 2001). The location of CRY in the LNs suggests that this is its site of action—within the master clock. Indeed, the entrainment deficiency of the $norpA^{P41};cry^{b}$ double mutants could be rescued by expressing the *Cry* gene exclusively in the LN (Emery *et al*. 2000*a*). This indicates that CRY directly interacts with components of the molecular feedback loop that generate rhythmicity in the LNs (for a review, see Scully & Kay 2000).

Entrainment of the molecular feedback loop to L:D cycles relies on the degradation of the timeless protein TIM in response to light. As TIM is not directly light sensitive, the light signal has to be transduced via photopigments to TIM, and there is growing evidence that CRY is involved in this process. In cry^{b} mutants, TIM is not degraded in the presence of light but stays at a constant high level in the photoreceptor cells of the compound eye (Stanewsky *et al*. 1998). Furthermore, in *in vitro* cell-based assays (S2 and yeast cells), CRY is able to interact directly with TIM (Ceriani *et al*. 1999). This interaction can be induced *in vitro* upon intense illumination and renders the PER–TIM complex inactive and unable to participate in the negative feedback loop. The degradation of TIM in the proteasome (Naidoo *et al*. 1999) may be an immediate consequence of the PER–TIM blockage by CRY. This CRY model of circadian photoreception in the LNs of *Drosophila* offers a simple explanation for the observation that CRY is capable of rendering PER–TIM activity light sensitive. The fact that transfection with *cry* alone is sufficient to render PER–TIM light sensitive suggests that CRY acts alone and is capable of both absorbing light and transmitting that information directly to the oscillator. However, a note of caution should be introduced. The *in vitro* assays of Ceriani *et al*. (1999) relied on high light exposure to induce alterations in PER–TIM activity. We know that the *Drosophila* clock is capable of responding to short-duration low-intensity light pulses and it would be appropriate to examine the effects of light exposures within the physiological realm to control for any non-specific thermal effects of light–radiant energy on CRY activity (Lucas & Foster 1999*a*).

In *Drosophila*, the latest evidence places CRY firmly in the photoentrainment pathway and raises the intriguing possibility that it acts as a photopigment, capable both of absorbing light and transducing that information directly to the oscillator. This appears to be in contrast to the recent results in mammals, which have shown that the CRYs are at the heart of the rhythm generating process (see § 3). Interestingly, CRY appears to have a similar clock role in *Drosophila*—not in the master clock but in the compound eyes. The cry^{b} mutation was found due to its elimination of clock functions (cycling of PER and TIM) in the compound eyes and in peripheral body tissues (Stanewsky *et al*. 1998): cry^{b} mutants lacked the typical cycling in *per* and *tim* when monitored in a luciferase-reporter assay. Similarly, TIM and PER levels on Western blots of head extracts did not show any daily cycling in abundance in cry^{b} mutants. The overall arrhythmicity in PER and TIM levels in the compound eye

appears to be caused by an interruption of clock function in individual photoreceptor cells (Hall 2000). Thus, CRY appears to have at least two possible roles in the fruitfly's circadian system: as a clock component in the photoreceptor cells of the compound eyes, and as a photopigment in the fly's master clock.

Although CRY might act as a photopigment in entrainment of *Drosophila* circadian rhythms it is not the only photopigment. Opsin-based photopigments in the compound eyes and the H–B eyelet are important in entraining the circadian system to L:D cycles. Photoentrainment is only abolished when all known photoreceptors are eliminated as in $glass^{60J}cry^{b}$ double mutants (Helfrich-Förster *et al*. 2001). Thus, *Drosophila* uses multiple photoreceptors for photoentrainment, and future studies will be necessary to define the specific roles of these different photic inputs into the circadian system.

3. MICE

In mammals, light information from the eye reaches the suprachiasmatic nucleus (SCN) via a distinct neural projection called the retino-hypothalamic tract (RHT) (Moore & Lenn 1972). This tract arises from a small subset of retinal ganglion cells (RGCs) and forms a relatively small percentage of the fibres of the optic nerve. For example, in the mouse, rat and cat retina, *ca*. 0.1% of the RGCs form the RHT projection to the SCN (Provencio *et al*. 1998). Although the RGCs that form the RHT have been to some degree characterized, the photoreceptors that are connected to these cells have not. Disentangling which of the retinal cells mediate photoentrainment from the mass of neurons dedicated to image detection has been a major problem.

The natural assumption was that the rods and cones, and their well-characterized photopigments, were responsible for collecting information for the SCN as well as for the image-forming visual system. However, work on mice with naturally occurring genetic disorders of the eye cast doubt on this assumption in the early 1990s. Mice that are homozygous for *retinal degeneration* (*rd*/*rd*) experience a progressive and ultimately massive degeneration of the rods and cones. By 60 days of age all rod cells have degenerated, and between 90 and 150 days of age even the crudest electrophysiological and behavioural responses to bright light have disappeared (Provencio *et al*. 1994).

Although all rods are lost in the *rd*/*rd* retina, a few cone cells survive. These cones lack outer segments and constitute only 2–5% of the cone cells found within the normal (wild-type, +/+) retina. Despite this loss of photoreceptors, *rd*/*rd* mice show circadian responses to light that are indistinguishable from those of congenic mice with phenotypically normal retinas (*rd*/+, +/+). The light intensity required to produce both saturating and half-saturating responses was found to be the same for all groups. It is important to stress that not only does some photosensitivity remain in mice with degenerate retinas, but that the circadian photosensitivity shown by these animals is not different from that of wild-type mice (Foster *et al*. 1991).

It should be emphasized that the site of circadian photoreception must reside within the eye of *rd*/*rd* mice because enucleation of these animals abolishes all circadian responses to light (Foster *et al*. 1991). The studies on the *rd*/*rd* mouse reported above contradict an earlier study that suggested that this mutation attenuates circadian photosensitivity. Ebihara & Tsuji (1980) determined the threshold for entrainment in C57 wild-type mice to be *ca*. 2 log units lower than the threshold for entrainment in C3H *rd*/*rd* mice (table 2). However, these authors failed to address the potential effects of genetic background on the *rd*/*rd* mutation by comparing C57 +/+ mice with C3H *rd*/*rd* mice. When this is taken into account, by comparing congenic C3H +/+ with C3H *rd*/*rd* mice, no reduction in circadian photosensitivity is observed (table 2).

More recent studies by the same laboratory have compared the circadian photosensitivity of CBA/N (+/+) and CBA/J (*rd*/*rd*) mice (Yoshimura & Ebihara 1998; Yoshimura *et al*. 1994). CBA/J (*rd*/*rd*) mice were reported to show a decrease in sensitivity of *ca*. 2 log units. Once again, the interpretation of these results is complicated by the failure to compare mice of the same genetic background. CBA/N mice were obtained from an inbred colony in Japan (Hamamatsu) and CBA/J mice were obtained from a separate inbred colony from the USA (The Jackson Laboratory). Inbred lines of mice of the same strain designation can have highly differing genotypes, and hence phenotypes, and every effort must be made to compare the impact of a genetic defect with wild-type animals of a congenic background (Mellor 1992; Sigmund 2000; Simpson *et al*. 1997).

The most recent experiments to address the impact of rod and cone photoreceptor loss on the circadian system have compared mouse models that lacked all rods and cones with congenic wild-type controls. These rodless + coneless mice were produced by crossing coneless transgenic (*cl*) mice with either *rd*/*rd* mice (Foster *et al*. 1991), or transgenic mice (*rdta*) (McCall *et al*. 1996) in which the rods had been ablated. In these two lines of rodless + coneless mice, photoentrainment and pineal melatonin suppression were intact (Freedman *et al*. 1999; Lucas *et al*. 1999). Again, removal of the eyes abolished all circadian responses to light, demonstrating that the eyes must house these novel photoreceptors.

Support for functionally distinct visual and circadian photoreceptors in our own species comes from several recent studies. These show that a significant subset of individuals who have eyes but have lost conscious light perception, due to retinal disease, retained the ability to suppress melatonin (Czeisler *et al*. 1995), as well as the ability to shift their circadian rhythms (Lockley *et al*. 1997).

Collectively, these results in mice and humans lead to the striking conclusion that mammals must use some unidentified photoreceptor outside the rod and cone receptors and, in the absence of an outer nuclear layer, the search for these photopigments is directed to the inner layers of the retina. The hypothesis that the mammalian inner retina contains novel photoreceptors is given indirect support by the discovery of an entirely new opsin-based photopigment (VA opsin) within the inner retina of fishes (Soni *et al*. 1998). To date, no VA opsin homologues have been identified in mammals, but a number of other opsin-like candidates exist, the strongest

Table 2. Three separate studies that compare the percentage of mice entrained to L:D cycles of varying irradiance. In these experiments the impact of mouse strain on the threshold for entrainment has been determined.

((*a*) Results reprinted from Ebihara & Tsuji (1980) showing the percentage entrainment of C57 +/+ and C3H *rd*/*rd* mice to 12 L:12 D of varying irradiances (lux). (*b*) Extension of the study by Ebihara & Tsuji (1980) by Argamaso-Hernan (1996). In this study the threshold for entrainment of C57 +/+, C3H *rd*/*rd*, and C3H +/+ mice to 12 L:12 D was determined. Note that C57 +/+ mice can entrain to light of a lower irradiance than C3H +/+ mice, and that the thresholds for entrainment in C3H *rd*/*rd* and C3H +/+ mice are similar. (*c*) In this study, the thresholds for entrainment of C3H +/+ and C3H *rd*/*rd* mice to 16 L:8 D were determined. Again, the thresholds for entrainment in C3H *rd*/*rd* and C3H +/+ mice are similar. Numbers in parentheses denote numbers of animals.)

	lux				
strain	100	10.0	1.00	0.10	0.01
(*a*) Percentage C57 +/+ and C3H *rd*/*rd* mice entrained to 12 L:12 D					
C57 +/+	100 (9)	100 (9)	88 (8)	86 (7)	83 (6)
C3H *rd*/*rd*	100 (12)	100 (18)	32 (19)	0 (17)	—
(*b*) Percentage C57 +/+, C3H *rd*/*rd* and C3H +/+ mice entrained to 12 L:12 D					
C57 +/+	100 (12)	100 (14)	100 (14)	100 (10)	75 (8)
C3H *rd*/*rd*	100 (16)	100 (11)	94 (16)	24 (17)	6 (18)
C3H +/+	100 (4)	100 (2)	32 (2)	0 (4)	0 (4)
(*c*) Percentage C3H +/+ and C3H *rd*/*rd* mice entrained to 16 L:8 D					
C3H +/+	100 (28)	100 (8)	50 (10)	13 (8)	0 (8)
C3H *rd*/*rd*	100 (27)	100 (7)	100 (8)	13 (8)	0 (10)

of which is a mammalian homologue of *Xenopus* melanopsin (Provencio *et al*. 2000). Significantly, melanopsin is expressed in a small number of cells within the ganglion and amacrine cell layers in the inner retina of both rodents and primates. Unfortunately, functional expression studies are lacking, and we do not know whether melanopsin is capable of forming a photopigment.

It is worth noting that in addition to circadian physiology, many other aspects of mammalian biology are influenced by gross changes in environmental light, including pupil size, blood pressure, mood and attention (Wetterberg 1993). It is possible, therefore, that an inner retinal photoreceptor might form the basis of a general 'irradiance detection' pathway mediating many, if not all, non-image responses to light. Preliminary support for this hypothesis comes from very recent work using rodless +coneless mice. In addition to circadian responses to light, these animals also show a partially intact pupillary light reflex (Lucas *et al*. 2000). The action spectrum for this response demonstrates the involvement of an opsin–vitamin-A-based photopigment with a wavelength of maximum sensitivity that is very different from the known mouse photopigments (Lucas *et al*. 2001). Until we have matched the action spectra for pupillary and circadian responses to light, it remains possible that these aspects of physiology are driven by different novel photoreceptors. However, the principle of parsimony would argue against this.

Several research groups studying *Arabidopsis* (plant), *Drosophila* and mice have suggested that CRYs might act as photopigments and mediate photoentrainment in these phylogenetically diverse organisms (Devlin & Kay 1999; Miyamoto & Sancar 1998; Thresher *et al*. 1998). In mammals at least, the evidence for this hypothesis was always weak (for reviews, see Lucas & Foster 1999*a*,*b*,*c*) and has recently faltered as a result of detailed studies by Griffin *et al*. (1999) who failed to uncover any effect of light on the activity of these proteins. Furthermore, the disruption of the CRY genes (*Cry1* and *Cry2*) in $Cry1^{-/-}Cry2^{-/-}$ mice does not block the light-induced expression of the two clock genes *mPer1* and *mPer2* in the SCN (Okamura *et al*. 1999). Note that this finding differs from a similar study by Vitaterna *et al*. (1999). These researchers showed that although *mPer2* could still be light induced in $Cry1^{-/-}Cry2^{-/-}$ mice (cf. Griffin *et al*. 1999), *mPer1* was constitutively elevated.

Why the two studies differ is unclear, but may relate to the small number of experimental animals used by Vitaterna *et al*. ($n = 2$). Rather than photopigments, the mammalian CRYs appear to be essential components of the murine clock. The strongest evidence for this comes from the work of Van der Horst *et al*. (1999) who showed that $Cry1^{-/-}Cry2^{-/-}$ mice have a completely arrhythmic phenotype, with no indication of a functional circadian clock under conditions of continuous darkness, and no indication that they can anticipate L:D transitions under experimental photoperiods. An important indication of the function of *Cry1* and *Cry2* was provided by the observation that although the double-knockout mice were totally arrhythmic, functional circadian rhythms were retained following the ablation of either *Cry* gene alone. Thus, it was clear that these genes perform overlapping functions in the maintenance of circadian rhythms. Since then, *in vitro* reporter gene experiments conducted by two independent research groups (Griffin *et al*. 1999; Kume *et al*. 1999) have indicated that both CRY1 and CRY2 are extremely potent repressors of Clock–Bmall complex-induced gene transcription. In fact, they are more effective in this role than any of the mPER or mTIM proteins either singly or in combination. These results, along with the arrhythmicity of the double-knockout mice, strongly

suggest a role in the negative limb of the circadian feedback loop.

Although there is considerable positive evidence that CRY1 and CRY2 have a fundamental role in the generation of mammalian circadian rhythms (Shearman *et al*. 2000), some groups have argued that the mammalian CRYs have a dual function both as components of the oscillator and as photopigments (Vitaterna *et al*. 1999). This view may have much to do with the original proposed function of the mammalian CRYs. CRY1 and CRY2 were designated as photopigments largely on the basis of their sequence similarity to the photoreceptive plant CRYs (Cashmore *et al*. 1999; Miyamoto & Sancar 1998; Thresher *et al*. 1998). However, assigning function on the basis of sequence similarity alone can be very misleading. For example, the opsin-like proteins share many features that are common to the superfamily of G-coupled receptors. The original assignment of gene function profoundly influences the interpretation of subsequent experimental results. It is therefore crucial that the criteria used to assign a role to a gene are appropriate. Photopigment identification has traditionally been based on a number of criteria. The candidate pigment should (i) be expressed in cells known to be photoreceptive; (ii) be capable of forming a functional photopigment; and (iii) have an absorbance spectrum that matches the action spectrum of the response in question. Only when these criteria have been met can gene-ablation studies be taken into account. Ablation studies on their own can associate a gene with a light-dependent process, but cannot demonstrate a photopigment function. When the candidate photopigment is ablated, the response to light should be either lost or attenuated and, if attenuated, show an altered action spectrum that would be predicted on the basis of the absorbance spectrum of the photopigment. On the basis of the criteria listed above there is no positive evidence to support a photopigment function for the mammalian *Cry* genes.

The role of CRY in the master clock of *Drosophila* and mice appears to differ very markedly. In *Drosophila*, CRY is clearly part of the photoentrainment pathway and may even act as a photopigment (see discussion of the criteria above), whereas in mice the CRYs have a crucial role in the negative limb of the circadian feedback loop. We speculate that during the course of animal evolution the CRYs may have shifted from a role within the light-input pathway to become central clock components, losing their photosensitivity in the process. The fact that they have retained both FAD and pterin 'chromophore' binding sites suggests that these cofactors are functionally important, but the function of the CRY cofactors remains to be resolved. In the photolyase proteins, these cofactors are thought to act both as light-absorbing pigments and as electron donors–acceptors in the repair of DNA dimerization. Perhaps similar redox reactions will prove to be an important aspect of the biology of the *Drosophila* and mammalian CRYs (Lucas & Foster 1999*a*).

There is now overwhelming evidence that unidentified (non-rod, non-cone) photoreceptors within the mammalian eye mediate photoentrainment. However, this does not mean that the classical rod and cone photoreceptors have no role in this process. The experiments on rodless + coneless mice outlined above merely suggest that these receptors are not required. Indeed, indirect evidence for a contribution from cone photoreceptors in photoentrainment comes from studies on an extraordinary animal called the 'blind mole-rat' (*Spalax ehrenbergi*). *Spalax* is a subterranean rodent with subcutaneous atrophied eyes and shows a massive reduction (87–97%) of those regions of the brain associated with the image-forming visual system (Cooper *et al*. 1993).

Although visually blind (Haim *et al*. 1983; Rado *et al*. 1992), the minute eyes (little more than 0.5 mm in diameter) can perceive light and are used to entrain circadian rhythms (David-Gray *et al*. 1998; Goldman *et al*. 1997). Photoentrainment is thought to occur in the wild when *Spalax* removes debris from its tunnel complex and is exposed to brief periods of natural light (Rado & Terkel 1989). Over the past 30 million years, evolutionary processes appear to have disentangled and eliminated the image-forming visual system of this animal while retaining those components of the eye that regulate the biological clock.

Remarkably, a cone opsin has been isolated from the eye of *Spalax*, and this opsin has been shown to form a fully functional photopigment (David-Gray *et al*. 1998, 1999). These results provide strong, although indirect, evidence that cone photopigments contribute (at some level) to photoentrainment in *Spalax* and, by implication, other mammals. This conclusion would appear to contradict the findings that the loss of both rod and cone photoreceptors has no effect on rodent photoentrainment, and that the retina contains novel circadian photoreceptors (Freedman *et al*. 1999; Lucas *et al*. 1999). But we should not be forced into an either/or answer. Indeed, there is a clear precedent for the involvement of multiple photopigments in the regulation of temporal physiology in many groups of vertebrates (Roenneberg & Foster 1997). For example, in non-mammalian vertebrates the pineal organ is often an important part of the circadian timing system (in some ways analogous to the mammalian SCN) and is itself directly light sensitive (unlike in mammals) (Gwinner & Brandstätter 2001). This photosensitivity is attained using multiple photopigments, with rod- and cone-like opsins, as well as novel opsins, coexisting within the same organ (Philp *et al*. 2000*a*,*b*; Shand & Foster 1999).

4. MULTIPLE PHOTOPIGMENTS AND TWILIGHT DETECTION

Our discussion of *Drosophila* and mice has emphasized that multiple photopigments seem to mediate the effects of light on temporal physiology. Why this should be, and how these photopigments might interact, remain a mystery but must surely relate to the common task of extracting time-of-day information from dawn and dusk (Roenneberg & Foster 1997). During twilight, the quality of light changes in three important respects: (i) the amount of light, (ii) the spectral composition of light, and (iii) the source of light (i.e. the position of the sun). These photic parameters all change in a systematic way, and in theory could be used by the circadian system to detect the phase of twilight (Roenneberg & Foster 1997). However, each is subject to considerable sensory 'noise' (table 3), and the impact of this noise will depend on the

Table 3. The major sources of 'noise' associated with the photic regulation of temporal physiology.

(Like other sensory systems (Dusenbery 1992), the two main sources of noise for twilight detection are associated with variation in the light stimulus and variation in exposure to the light stimulus. In each case, the impact of this noise will depend on the organism and the environment that it inhabits. Some examples of the type of noise that might be expected to complicate photoentrainment are listed.)

variation in the stimulus	
channel–signal noise	fluctuations in the light signal; e.g. cloud cover or daylength
environmental noise	extraneous light signals; e.g. starlight, moonlight and lightning
receptor noise	molecular noise of the receptor pathway; e.g. variation in external temperature
variation in exposure to the stimulus	
sensory adaptation	changing receptor thresholds; e.g. receptor habituation, changes in pupil size and ocular pigment migration
behavioural noise	behavioural state; e.g. emergence from burrow, place of rest, feeding, courtship and migration
developmental noise	stage of development; e.g. feeding niche, body pigmentation, neural connections and developmental niche (in egg, pupae or *in utero*)

organism and the environment that it inhabits. One can also make the general point that in all sensory systems, much of the complexity observed is associated with noise reduction. A classic example of this is the visual system in colour vision. Colour vision is a mechanism for increasing the signal-to-noise ratio of an object against its background by exploiting the fact that different objects do not equally reflect the same wavelengths of light.

We know that the circadian system of the mouse (Provencio & Foster 1995) and the hamster (Von Schantz *et al*. 1997) are sensitive to both green light and near-UV irradiation. However, we do not know how these signals might be used. Perhaps a form of wavelength discrimination is important not only for contrast perception but also for the detection of twilight? At twilight there are very precise spectral changes, primarily an enrichment of the shorter wavelengths (< 500 nm) relative to the mid–long wavelengths (500–650 nm). If the circadian system was capable of using multiple photopigments to ratio changes in the relative amounts of short and long wavelength radiation, and of coupling this information with irradiance levels, then the phase of twilight could be determined very accurately.

5. CONCLUSION

Until recently, circadian biologists have tended to use light merely as a 'hammer' to shift the clock, but of course twilight detection is not a straightforward stimulus. It is highly dynamic and subject to considerable noise. Yet despite this high degree of environmental noise, entrained organisms show remarkable precision in their daily activities. Thus, the photosensory task of entrainment is likely to be very complex. On the basis of what we know about other sensory systems we should predict that the photic inputs regulating temporal physiology will be both specialized and complex. Indeed, we have discussed the evidence for both novel (specialized) and multiple (complex) inputs regulating temporal physiology in two highly divergent organisms—the fruitfly and the mouse. Furthermore, multiple photopigments appear to contribute to photoentrainment in unicellular organisms such as *Gonyaulax* (Roenneberg & Deng 1997). As outlined in this review, considerable progress has been made in identifying the different photoreceptor organs and photopigments of animal circadian systems. The time is now right for circadian biologists to think about photoentrainment in a different way, to stop asking 'what is the circadian photopigment?' and ask the more sophisticated question of 'how do multiple photic channels interact to reduce the noise problem inherent in twilight detection?'

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