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The role of retinal photoreceptors in the regulation of circadian rhythms

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Abstract The circadian clock is an evolutionarily, highly conserved feature of most organisms. This internal timing mechanism coordinates biochemical, physiological and behavioral processes to maintain synchrony with the environmental cycles of light, temperature and nutrients. Several studies have shown that light is the most potent cue used by most organisms (humans included) to synchronize daily activities. In mammals, light perception occurs only in the retina; three different types of photoreceptors are present within this tissue: cones, rods and the newly discovered intrinsically photosensitive retinal ganglion cells (*ip*RGCs). Researchers believe that the classical photoreceptors (e.g., the rods and the cones) are responsible for the image-forming vision, whereas the *ip*RGCs play a key role in the non-image forming vision. This non-image-forming photoreceptive system communicates not only with the master circadian pacemaker located in the suprachiasmatic nuclei of the hypothalamus, but also with many other brain areas that are known to be involved in the regulation of several functions; thus, this non-image forming system may also affect several aspects of mammalian health independently from the circadian system.

Keywords Circadian · Melanopsin · Retina

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1 Introduction

The circadian clock is an evolutionarily, highly conserved feature of bacteria, plants and animals that allows organisms to adapt their physiological processes to the time of day in an anticipatory fashion [1, 2]. This internal timing mechanism coordinates biochemical, physiological and behavioral processes to maintain synchrony with the environmental cycles of light, temperature and nutrients. Circadian rhythms reflect extensive programming of biological activities that meet and exploit the challenges and opportunities offered by the periodic nature of the environment [2].

In mammals, circadian rhythms are driven by a timing system comprised of a master pacemaker in the suprachiasmatic nuclei (SCN) of the hypothalamus, and peripheral oscillators located throughout the organism. Independent circadian oscillators exist within each cell of almost every tissue and/or organ investigated, including the liver and heart [3, 4]. The mammalian circadian system appears to be arranged in a hierarchical manner, with the SCN acting as the "master pacemaker." The SCN drives and coordinates peripheral clocks through as yet poorly defined humoral and neural signals, as well as indirectly by modulating activity and feeding.

Several studies have shown that light is the most potent Zeitgeber to entrain the mammalian circadian system. In mammals, light perception occurs only in the retina [5], where three different types of photoreceptors are present: cones, rods and the newly discovered intrinsically photosensitive retinal ganglion cells (*ip*RGCs). Researchers believe that the classical photoreceptors (e.g., the rods and the cones) are responsible for the image-forming vision, whereas the *ip*RGCs play a key role in the non-image-forming vision.

In the present review, we will summarize the experimental evidence collected thus far on the role that the

different photoreceptors play on the photic entrainment of the mammalian circadian rhythms and investigate how the *ip*RGC may contribute to the regulation of other behavior.

2 Circadian photoreception

The study of the photoreceptors that mediate non-image-forming vision in mammals is emerging as a new and promising aspect of retinal neurobiology. The current view is that, while all effects of light on the circadian visual system are accounted for by the three types of photoreceptors, no single photoreceptor is necessary for entrainment. For example, mice that lack rod photoreceptors (*rd)* or rods and cones *(rdcl)* have a normal phase response to light [6, 7], and the action spectrum for phase shifts peaks at 480 nm [8]. These findings indicate that an undiscovered photoreceptor/photopigment in the mammalian retina is responsible for the photo-entrainment of circadian rhythms.

Data from many laboratories have provided compelling evidence that a mammalian homologue of *Xenopus* melanopsin (also known as *Opn4*) is a new photo-pigment responsible for a key role in the photic entrainment of the circadian system [9]. Studies have also shown that most vertebrates express two melanopsin genes, termed O*pn4m* (mammal) and O*pn4x* (*Xenopus*). In non-mammalian vertebrates, O*pn4m* and O*pn4x* are expressed in many tissue types, e.g., retina, brain, and skin [10–12], whereas mammals express only O*pn4m* [13].

In mammals, melanopsin mRNA and protein are present in only a small population (about 2%) of the retinal ganglion cells (RGCs) [14]. These RGCs express pituitary adenylate cyclase-activating polypeptide (PACAP) [15] and form the retinohypothalamic tract [16, 17].

A series of elegant studies has demonstrated that these cells, named "intrinsically photosensitive RGCs" (*ip*RGCs), are directly photosensitive and have an absorption peak around 470–480 nm [18, 19]. These *ip*RGCs were no longer intrinsically photosensitive in melanopsin knock-out (KO) mice, although their number, morphology, and projections were unchanged [20].

Studies have also reported that, since light response from these melanopsin cells can be obtained before the rods and cones become functional, *ip*RGCs are the first photoreceptive system to develop in mammalian retina [21, 22]. The role played by these photoreceptors at this early stage is not yet known. Some investigations have also reported that the regulation of melanopsin levels (at least in the rat) is dependent on the light and the length of the photoperiod [23–25].

Although these experimental data provided compelling evidence to suggest that melanopsin is a functional photopigment, a direct demonstration was obtained in 2005 when three groups published concurrent and mutually supportive evidence of the full functionality of expressed melanopsin in human, mouse and *Xenopus* cell lines [26–28].

Several studies using behavioral assay have also reported that removal of the melanopsin gene in the mouse (melanopsin knock-out, KO) does not prevent the entrainment of the circadian system and does not affect the free-running period in constant dark conditions. However, phase response to light is attenuated in the KO animals since the magnitude of the phase-shift is about half (40%) that of wild-type mice at each of three non-saturating irradiance levels [29]. A saturating white light pulse also produced a diminished phase shift in the KO animals [30]. The length of the free-running period that follows exposure to constant light is reduced to about 55–65% of that of controls in melanopsin KO [29, 30].

Melanospin levels in the Royal College of Surgeons (RCS) rat homozygous for retinal dystrophy (*rdy*) are dramatically down-regulated once the classical photoreceptors have degenerated [31]; however, despite this massive reduction in the photoreceptive apparatus, their capability to phase shift in response to a pulse of light is not affected [32].

Melanopsin has also been implicated in the regulation of pupillary light reflex (PLR). Transgenic mice that lack both rod and cone photoreceptors (*rdcl*) retain a PLR, a response driven by a photopigment with peak sensitivity around 479 nm [33]. Melanopsin KO animals showed a PLR indistinguishable from that of the wild-type mice at low irradiances, but at high irradiances the reflex was incomplete. This result suggests that the melanopsin-associated system and the classical rod/cone system are complementary in function [34]. Melanopsin KO mice and mice that carry the *rd* (or *rdcl*) mutation, show normal light-induced suppression of pineal melatonin [35], whereas melatonin in mice lacking rods, cones and melanopsin cannot be suppressed by light [36], and the circadian rhythms of locomotor activity cannot be entrained by light [20, 36].

Accumulated experimental data indicate that melanopsin also plays an important role in mediating the photic entrainment of human circadian rhythms. Brainard et al. [37] reported that, in humans, the action spectra for melatonin suppression has a lambda max around 460 nm, suggesting that melanopsin is a key player in the photic regulation of melatonin levels and of the circadian rhythms and sleep in humans. Studies have also shown that blue light in the range of 440–480 nm is highly effective in phase-shifting the human circadian clock [38].

A few studies have reported that light can modulate sleep. In the rat, light exposure during the night (the normal active period for a nocturnal animal) induced sleep, and exposure to darkness promoted wakefulness [39], while neuroanatomical studies have described a retinal pathway in the mouse that originates in *ip*RGCs and innervates the

ventrolateral preoptic nucleus (VLPO) [20, 40]. Two recent studies investigated the contribution of the image-forming and the non-image-forming system to the regulation of sleep. In the first study, Lupi et al., [41] investigated whether the removal of melanopsin or of the rods and cones affected light-induced sleep. As was the case in rats, light exposure during the night induced sleep in normal mice and in mice that lacked the classical photoreceptor, whereas the photic regulation of sleep was almost completely abolished in melanopsin KO mice, light exposure did not induce c-fos expression in the brain areas that are believed to mediate this response (VLPO and superior colliculus).

In the second study, Altimus et al. [42] reported the effects that light exposure during night, or dark exposure during the day, have on sleep in mutant mice that lack the *ip*RGCs or lack functional rods and cones, or melanopsin. The data presented in the study indicated that the acute regulation of sleep and wake by light and dark requires both rod-cone and melanopsin-signaling through *ip*RGCs and that such regulation is independent of image formation. The differences between these two studies are probably due to the different experimental designs used by the two research teams. Another study reported that only classical photoreceptors are involved in the modulation of the cardiovascular system by acute exposure to light [43].

Studies have also shown that blue light in the range of 440–480 nm is highly effective in phase-shifting the human circadian clock, can increase alertness, and may be used to treat seasonal affective disorders [44–46].

Because of these widespread effects on many different regulatory systems, several studies have focused their attention on identifying the central projections of these new photoreceptors. Most of these projections were initially identified by retrograde labeling [16–18] and then by using a transgenic mouse line in which the melanopsin gene was replaced with tau-lacZ gene [17, 40]. These studies identified the SCN as one of the main recipients of the *ip*RGCs [16, 18, 40]. Another brain area that receives projections from these cells has been identified in the intergeniculate leaflet, a brain area known to be involved in the regulation of the photic entrainment of the circadian system [40]. In addition, the *ip*RGC cells project to the olivary pretectal nucleus (a key center in the control of PLR) [17, 47], the VLPO (a well established center in the regulation of sleep), the lateral habenula, and the superior colliculus [40–47].

Although the number of *ip*RGCs in the mouse that project to the visual center seems to be negligible or even absent, in primates it has been reported that an anatomically distinct population of "giant" melanopsin-expressing ganglion cells projects to the lateral geniculate nucleus, which is the thalamic relay to the primary visual cortex [48]. Hence, at least in the primate, the non-image-forming and the image-forming pathways may merge, so the melanopsin-based photoreception may contribute to conscious visual perception. A similar situation may be present in humans, since new experimental evidence has suggested that visual awareness may be present in humans with no conscious light perception [49].

Finally, newly obtained data have shown that there are at least two types of *ip*RGCs: the melanopsin *ip*RGC type 1 (M1) cells, which mostly project to the SCN, and the melanopsin *ip*RGC type 2 (M2) cells, which mostly project to the olivary pretectal nucleus (OPN) responsible for the pupillary light reflex ([40, 50]; see Fig. 1 for more details).

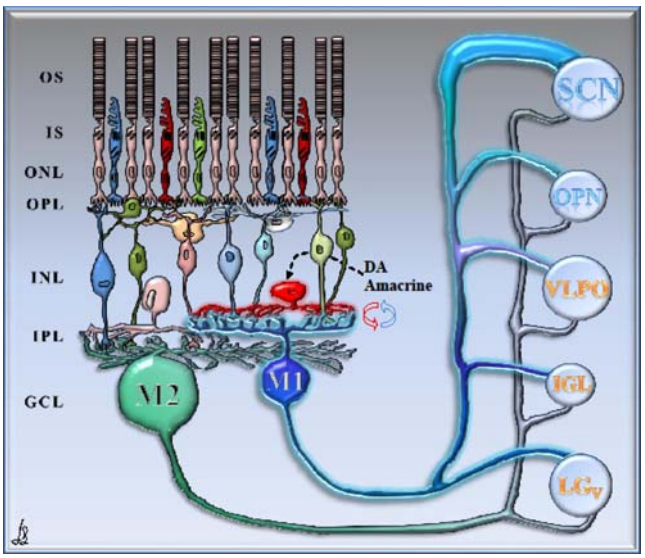

Fig. 1 Two types of melanopsin containing retinal ganglion cells (mRGCs) M1 and M2 are present in the retina. Earlier research highlighted type III mRGC'scells and type II mRGC'smore recent studies refer to these as M1 cells (type III) and M2 cells (type II) based upon morphologic and physiologic comparisons. The width of the projection from the M1 cell to the suprachiasmatic nucleus (SCN) of the hypothalamus represents the proportion of innervation, 80%, versus 20% from the M2 cells. The innervation to the olivary pretectal nucleus (OPN) from M1 cells is 55% versus 45% from M2 cells. A recent study shows that the dendrites of M1 mRGC'sare localized to sublamina A of the innerplexiformlayer (off), while M2 dendrites localize to sublamina B (on) as depicted by the difference in stratification. The dendrites of the M2 mRGC'sare considerably more complex and span a larger diameter than the M1 mRGC's. The M1 cells are considerably smaller but respond with significantly larger depolarizations and light-induced currents than do the M2 cells. The red and blue arrows represent the exclusive dendrodentdriticplexus between M1 mRGC'sand the dopaminergicamacrine cells of the inner nuclear layer. The other neural targets of mRGCs not shown in the figure include the pre-optic area, sub paraventricular zone, anterior hypothalamic nucleus, lateral hypothalamus, medial amygdaloid-nucleus, lateral habenula, lateral geniculate nucleus (dorsal division), bed nucleus of the stria terminalis, periaqueductal gray, and superior colliculus. (*OS* outer segments; *IS* inner segments; *ONL* outer nuclear layer; *OPL* outer plexiform layer; *INL* inner nuclear layer; *IPL* innerplexiform layer; *GCL* ganglion cell layer)

3 Circadian photoreception within the retina

Previous work has demonstrated that the mammalian retina contains its own autonomous circadian clocks that control several retinal functions [51–53]. One of the most intensely investigated of these functions is melatonin biosynthesis, which displays a clear circadian rhythm *in vivo* and *in vitro* [51–53].

Cultured mammalian retinas show a clear circadian rhythm in melatonin release [54], and *Arylalkylamine N-acetyltransferase* (an important regulatory component in the synthesis of melatonin) mRNA is rhythmic in animals in which the suprachiasmatic nuclei of the hypothalamus have been lesioned [55].These findings indicate that the retinal clock can generate circadian rhythmicity independent of the circadian clock located in the SCN.

Melatonin and dopamine (DA) play opposing roles in the regulation of retinal adaptive physiology (reviewed in: [51–53]). Dopamine functions as a humoral signal for light and produces light-adaptive physiology, while melatonin has dark-adaptive effects. In many species, the synthesis and release of both melatonin and dopamine are under circadian control, with melatonin released at night and dopamine during the daytime. Melatonin inhibits the release of dopamine through an action on melatonin receptors, and dopamine inhibits the synthesis of melatonin from photoreceptor cells by acting on dopamine receptors [51–53]. Thus, the melatonin-secreting photoreceptors and dopamine-secreting amacrine/IP cells form a cellular feedback loop that functions to regulate circadian retinal physiology.

More recently, it has been reported that, in the RCS rat (*rdy*), light-dependent regulation of the dopaminergic system within the retina is not affected by the loss in the photoreceptive system [56], and it has been proposed that dopamine and its metabolites may provide an important signal for entrainment of retinal circadian rhythms [57]. A recent study using long-term monitoring of PER2::LUC mice retina *in vitro* has also shown that, among the major retinal neurotransmitters, dopamine, acting through D1 receptors, is responsible for the resetting (entraining) of the phase of the retinal circadian clock [58].

Studies have also investigated whether melanopsin-based photoreception is involved in the modulation of the retinal circadian rhythms. Hartwick et al. [59] investigated the light responses evoked in DA neurons in the presence of L-AP4, a blocker of retinal ON-bipolar cells, and found that the light responses observed were similar to those observed in ipRGCs. Moreover, these light responses persisted in mice with degenerated retinas, suggesting the presence of centrifugal outflow signals within the retina.

A second study that measured DA release reported an increase in the DOPAC:DA ratio in wild-type mice and in mice lacking melanopsin or lacking rod phototransduction, whereas the light response was not observed in mice that lacked both rods and cones. These data suggest that light regulation of DA depends on the presence of rods and cones and that melanopsin is not involved in the regulation of DA release [60]. The reasons for the discrepancy between the results obtained in these two studies are unclear, and further studies will be needed to address the role of melanopsin-based photoreception in the regulation of the retinal circadian clock.

Finally, the loss of the melanopsin gene abolishes circadian control in some parameters of cone electroretinogram, causing significant attenuation of the diurnal variation in cone vision [61]. These new data suggest a melanopsin-dependent regulation of visual processing within the retina and reveal an important function for inner retinal photoreceptors in optimizing classical visual pathways according to time of day. In humans, it has also been reported that this new photoreceptive system may actually modulate the entire visual system [45].

4 Role of the photoreceptors in regulation of circadian rhythms

Retinal photoreceptors play a key role in the circadian organization of the whole organism since they are the only source of photic input to the SCN and, hence, to the rest of the body. Some investigations have reported changes in several parameters of the circadian rhythm in locomotor activity after photoreceptor degeneration or bilateral enucleation [32, 62, 63]. Bilaterally enucleated hamsters showed a wider range of free-running periods than did intact hamsters held for the same length of time in constant darkness; this effect seems to be independent from the age at which the animals were enucleated (postnatal days 1, 7, or 28); on the other hand the average free-running period of intact animals maintained in DD from days 7 or 28 was longer than that of intact animals kept in DD from day 1 or that of any of the enucleated groups, suggesting that the exposure to light-dark cycles in the early days of postnatal life affect the free-running period [63].

Lupi et al. [62] showed that, in *rdta* mice (a transgenic mouse in which the rod photoreceptors degenerate very rapidly), the free-running period is significantly shorter the magnitude of light-induced phase shift is significantly increased, and the irradiance required to produce a saturating phase shift is also significantly increased with respect to the wild-type. In RCS rats, the free-running period of the rat with dystrophic retina was significantly shorter, and the magnitude of the light-induced phase shifts tended to be larger in rats with dystrophic retinas than in the control group [32]. However, in *rd* mice and in mice lacking of all the photopigments (i.e., rhodopsin, cone

opsin, and melanopsin), the free-running period was not different from that of the wild-type [6, 36].

A few authors have proposed that the changes in the free-running period observed after photoreceptor degeneration or enucleation are due to alteration in the relationships between the retinal and the SCN clocks [62–64]. However, previous studies have shown that, in RCS rats with retinal dystrophy, the retinal circadian clock still functions [56, 65], so it is unlikely that the changes we have observed in the free-running period of *rdy* rats are a consequence of the presence or absence of the retinal circadian clock. The same explanation is probably valid also for the mouse since the loss of photoreceptors in the mouse also does not necessarily destroy the retinal clock [66].

We believe that the changes in the circadian parameters observed in animals with retinal degeneration are likely to be due to the inappropriate signals transmitted from the retina to the SCN, either *via* the retinal hypothalamic tract or *via* accessory pathways, or that the changes in the free-running periods observed are the result of a secondary effect of photoreceptor degeneration on the developing SCN.

Recent studies have reported that retinal degeneration in mice may change the phase at which the animals are active (nocturnal vs. diurnal). Under normal conditions, mice are nocturnal; however, mice that lack rods and functional *ip*RGCs [67] and mice that lack melanopsin and RPE65 (a key protein used in retinal chromophore recycling) became diurnal [68], suggesting that changes in retinal input can affect the temporal niche at which the animals are active.

Overall, these data indicate that the influence of the retinal photoreceptors on the regulation of circadian rhythm of locomotor activity may be difficult to predict (Table 1). It is not clear whether these changes are the result of interactions between the two clock systems or to other unknown factors that may be altered by photoreceptor degeneration or removal of the eyes.

5 The SCN plays a role in light transduction

Finally, there is evidence that light has separate and discrete effects on the circadian system. When light is presented at night, glutamate release will phase-shift the circadian pacemaker located in the SCN through its actions on NMDA and non-NMDA excitatory amino acid (EAA) receptors [69–71]. In addition to this effect on the circadian clock, light exposure at night induces a rapid decrease in the melatonin levels (within 5 min), which is due to the action of proteasomal proteolysis on the arylalkylamine N- acetyltransferase protein [72]. Since melatonin is not stored within the pineal but freely diffuses as soon as it has been synthesized, the rapid destruction of the AA-NAT protein has, as a consequence, an almost immediate decrease in pineal, and then plasma, melatonin levels.

A series of recent studies has investigated the converging mechanisms that underlie these two different responses to light. First, it was shown that injections of either NMDA or non-NMDA excitatory amino acid (EAA) receptor antagonists into the SCN inhibit the ability of light to increase *Per1* mRNA levels but do not disrupt the ability of light to suppress pineal melatonin levels [73]. Then it was demonstrated that sodium-dependent action potentials in the SCN region are necessary for the retina to signal the pineal gland that light is present [74]. Although tetrodotoxin (TTX) was unable to block the effects of NMDA on *Per1* and *Per2* mRNA levels and phase-shifting, TTX did block the ability of NMDA to suppress pineal melatonin, suggesting that the SCN cells that mediate the suppression of pineal melatonin in response to light are separate from and independent of

Table 1 Summary of the effects that retinal photoreceptor removal and enucleation produces on the period length and phase in nocturnal rodents (KO=knock out)

Species	Phenotype	Effect	References
Mouse	rodless (*rd*)	no change	[6, 36, 62]
Mouse	rodless/coneless (*rdcl*)	no change	[7]
Mouse	rodless (*rdta*)	shorter (0.3 h)	[62]
Mouse	*Opn4* KO	no change	[30, 31, 36]
Mouse	*Opn4-Rpe65* KO	phase-change	[68]
Mouse	Only cones	phase-change	[67]
Mouse	*Opn/rd* KO	no change	[36]
Mouse	*Opn4/Gnat1/Cnga3* KO	no change	[20]
Mouse	enucleation	no change	[64]
Hamster	enucleation	no change	[64]
Hamster	enucleation	broader free-running	[63]
Rat	enucleation	no change	[32]
Rat	*rdy* (retinal degeneration)	shorter (1 h)	[32]

those that phase-shift circadian rhythms. The simplest explanation for these observations is that TTX blocks the electrochemical activity of SCN cells that are essential for light to suppress melatonin but that action potentials in these cells are not involved in regulating *Per1* and *Per2* mRNA levels or phase-shifting [74]. Another study has also shown that NPY-receptor activation can inhibit the light-induced increase in *Per1* mRNA but not the light-induced suppression of melatonin [75]. Therefore, it is possible to modulate pineal melatonin levels via the SCN without modulating *Per1* mRNA levels in the SCN, and vice versa. Taken together, these results suggest that the photic transduction pathway within the SCN that results in suppression of pineal melatonin during the night is independent of the photic transduction pathway that upregulates *Per1* mRNA.

An important implication of these studies is that SCN cells have a discrete phototransductory role in which they function as a neural relay between retinal ganglion cells and the sympathetic nervous system. It may not seem economical for SCN cells to develop a separate mechanism to communicate light signals to extra-circadian processes, but the SCN exert control over the pineal through a sympathetic pathway that includes the paraventricular nucleus, the intermediolateral cell column of the spinal cord, and the superior cervical ganglion [76–78]. In rodents whose SCN are intact, light acutely influences an array of autonomic functions that include stimulatory and inhibitory effects (depending on the time of day) on heart rate [43, 79, 80] and blood pressure [80]. Light also acutely reduces core body temperature [81] and acutely increases glucocorticoid secretion from the adrenal glands [82]. These studies further demonstrated that, in addition to abolishing circadian rhythms, SCN ablation eliminates the acute effects of light on each of these processes. If the SCN serve as both the primary circadian pacemaker and a relay point for sympathetic structures downstream of the SCN, and both functions have to be responsive to light, then it makes sense for SCN cells to have developed separate mechanisms to communicate light information. An extension of this view is that, under certain circumstances, SCN cells can discriminate between the two roles and convey light signals to one pathway at the expense of the other. Such a mechanism would be effective for enhancing sympathetic activity without disturbing the pacemaker or, conversely, for entraining the pacemaker without influencing sympathetic activity.

6 Conclusions

Studies in the last 10 years have demonstrated that a new type of photoreceptor is present in the mammalian retina and that this new photoreceptive system plays a key role in the entrainment of the circadian rhythms. This non-image-forming photoreceptive system projects not only to the SCN, but also to several brain areas that are involved in the regulation of several functions (e.g., sleep). Therefore, this non-image-forming system may also affect several aspects of the mammalian health independently from the circadian system.

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