Multiple Photoreceptors Contribute to Nonimage-forming Visual Functions Predominantly through Melanopsin-containing Retinal Ganglion Cells

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In the absence of functional rod and cone photoreceptors, mammals retain the ability to detect light for a variety of physiological functions such as circadian photoentrainment and pupillary light reflex. This is attributed to a third class of photoreceptors, the intrinsically photosensitive retinal ganglion cells that express the photopigment melanopsin. Even though in the absence of rods and cones, mammals retain the ability to detect light for various nonimage-forming visual functions, rods and cones can compensate for the absence of the melanopsin protein in nonvisual light-dependent physiological behaviors. Several studies have addressed the relative contribution of each photoreceptor type to nonimage-forming visual functions; however, a comprehensive model for these interactions is far from complete. Under conditions where melanopsin-containing retinal ganglion cells were genetically ablated, image formation is maintained, whereas circadian photoentrainment and pupillary light reflex are severely impaired. The findings indicate that multiple photoreceptors contribute to nonimage-forming visual functions through signaling via melanopsin-containing retinal ganglion cells. Future studies will aim to determine more quantitatively the relative contributions of each retinal photoreceptor in signaling light for nonimage-forming visual functions.

INTRODUCTION

Daily and seasonal changes in the light/dark cycles of the earth due to the tilted rotation around its axis provide all organisms with temporal information that allows them to better adapt to their environment. Leaf senescence, which is preceded by the remarkable autumn foliage colors such as that seen at the Cold Spring Harbor Laboratory, results from the shortened day length during fall to allow plants to cope with pressures of the winter months (Thomas and Stoddart 1980). The ability of organisms to anticipate changes to the solar day with their internal biological clock permits them to coordinate their physiology for optimal performance throughout the day/night cycle. For the internal clock to be relevant, a synchrony between the light/dark cycle and circadian rhythms has been established. The synchrony between the light/dark cycle and circadian rhythms has been dubbed circadian photoentrainment.

For nearly a century, the retina, particularly the classical photoreceptors (rods and cones), has been extensively studied using a variety of mutant or lesioned animals to understand phototransduction and image formation. In vertebrates, rods and cones are the photoreceptors for image formation under dim- and bright-light conditions, respectively. Although studies on nonmammalian vertebrates during the 1960s revealed that extraocular organs, such as the pineal gland, were photoreceptive for circadian photoentrainment (Menaker 1968; Menaker et al. 1997), research on mammals blinded by eye removal demonstrated that all photoreception for photoentrainment and vision occurs exclusively in the mammalian retina (Nelson and Zucker 1981). Later, mice with outer retinal degeneration that lacked functional rods and cones were shown to photoentrain but not form visual images (Freedman et al. 1999; Lucas et al. 1999). Studies on blind humans also demonstrated that photoentrainment without the ability to form conscious vision is possible (Czeisler et al. 1995; Klerman et al. 2002). The ability of an animal to photoentrain in the absence of intact classical photoreceptors puzzled researchers and pointed to the presence of a distinct photoreceptor located beyond the outer retina that is responsible for photoentrainment.

The recent identification of the photopigment melanopsin (Opn4) in a unique subpopulation of the retinal ganglion cells resolved this long-standing conundrum (Provencio et al. 1998, 2000). Electrophysiological recordings from melanopsin-expressing ganglion cells (also known as intrinsically photosensitive retinal ganglion cells [ipRGCs]), and cells expressing melanopsin protein ec-topically, clearly demonstrated that melanopsin is capable of responding to light and that it functions as a true photopigment (Rollag et al. 2000; Berson et al. 2002; Newman et al. 2003; Melyan et al. 2005; Panda et al. 2005; Qiu et al. 2005). The ipRGC response to light is less sensitive and more “sluggish” than the classical photoreceptors rods and cones (Berson et al. 2002). The ipRGCs tile the entire retina and project to nonimage-forming visual centers, including the suprachiasmatic nucleus (SCN) (Hattar et al. 2002), making them the premier candidate for the specialized cells of irradiance detection and signaling. The ipRGCs located in the inner retina slowly
depolarize and fire action potentials during extended light stimulation, which allows them to signal directly (monosynaptically) to the SCN. This is in contrast to the rapidly activating and desensitizing responses observed in elsewhere (Berson 2003).

To the classical photoreceptors has been reviewed in detail brain (Fig. 1). The initial physiological and morphological cascade to signal light information to the classical photoreceptors of the outer retina, which require above the threshold for rod light detection, but that the optimal photosensitivity of rods, these authors noted not only that the threshold for this response is 6 log units above the threshold for rod light detection, but that the phototransmission cascade is also capable of integrating light information for extended periods of time. Because this is atypical of classical photoreceptors, the authors implicated a novel photoreceptive system mediating the entrainment of mammalian circadian rhythms. Since then, researchers have shown that melanopsin is a part of this novel photoreceptive system (Hattar et al. 2002; Panda et al. 2002; Ruby et al. 2002). Although animals that lack the melanopsin protein showed attenuated phase-shifting responses, the maximal sensitivity of melanopsin cells is 480 nm (Yoshimura and Ebihara 1996; Hattar et al. 2003), which does not match the maximal spectral sensitivity of phase shifts in hamsters. This indicates that multiple photoreceptors contribute to phase shifting the circadian oscillator. Determining the relative contribution of each photoreceptor to phototransmission will allow the creation of better models to understand how light interacts with the circadian pacemaker.

**CURRENT MODELS ARE NOT SUFFICIENT TO EXPLAIN THE RELATIVE CONTRIBUTION OF EACH PHOTORECEPTOR TO CIRCADIAN PHOTOTRANSITION**

Although the animals that lack the rod, cone, and melanopsin photoreceptive functions (gnat1<sup>–/–</sup>; cnga3<sup>–/–</sup>; opn4<sup>–/–</sup>) (Hattar et al. 2003) or that lack melanopsin and have outer retinal degeneration (rd/rd; opn4<sup>–/–</sup>) (Panda et al. 2003) demonstrated that the three photoreceptor types are sufficient for the proper operation of nonimage func-
tions, there are still unexplained observations that are worth mentioning. In mice that lack the rod, cone, and melanopsin photoreceptive functions, a variable transient 5% response to light was reported in their pupil light reflex, indicating that these animals are not completely blind (Hattar et al. 2003). A possible source of this signal is transient membrane hyperpolarization of the receptor potential of rods and cones caused by charge movements associated with conformational changes of the visual pigments after photoisomerization (Cone 1967; Murakami and Pak 1970). Alternatively, a redundant inefficient signaling cascade in one of the mutant photoreceptor cell types might account for this response. For instance, the Cngβ3 subunit is known to be expressed in cone cells and might be able to substitute for the eliminated Cnga3 and form a homomorphic form of the cyclic nucleotide channel, hence leading to minuscule light responses. The other animal model, which utilized the melanopsin knockout in conjunction with the rd/rd mutation, did not show the residual transient light responses in PLR (Panda et al. 2003). This could indicate that the photoisomerization-dependent hyperpolarization in the classical photoreceptors is the cause for the residual 5% transient response. The rd/rd animals have a mutation in the rod phosphodiesterase (Pde6b) (Bowes et al. 1990), which causes the initial ablation of rods leading to a secondary yet incomplete degeneration of cones giving rise to opsin-positive perikarya (Foster et al. 1995). These functional aberrant cone photoreceptors are capable of surviving in the rd/rd animals for many months (Garcia-Fernandez et al. 1995). Provencio and Foster (1995) demonstrated that rd/rd animals can be phase-shifted by monochromatic light at 515 nm as well as 375 nm (around the maximal spectral sensitivity of MW-cones and S-cones, respectively) implicating that both S- and MW-cones participate in photoentrainment. Moreover, Dkhissi-Benyahya et al. (2007) recently demonstrated that the mw-cones are important in signaling light information for circadian photoentrainment. In light of these findings, it is surprising that the rd/rd; opn4–/– animals do not show any light responses in non-image-forming functions, and further investigation is warranted to settle the discrepancy between these animal models with respect to cone function. It is crucial to understand how it is possible that the remaining cone cells are incapable of signaling any light information in the rd/rd; opn4–/– animals. It is possible that rd/rd; opn4–/– animals have a retina that is rewired and incapable of signaling properly for nonimage-forming functions due to degeneration and the absence of the melanopsin protein. Alternatively, as demonstrated by Yoshimura and Ebihara (1996), the maximum light response in rd/rd animals is 480 nm, which matches the spectral sensitivity of melanopsin and implies that cone photoreceptors in rd/rd animals are not involved in phase shifting the circadian oscillator.

The role of cone cells in signaling light information for circadian photoentrainment becomes even more complicated when considering mice containing only functional cone photoreceptors. Mrosovsky and Hattar (2005) achieved this by knocking-out essential components of rod (gnat1–/–) and melanopsin (opn4–/–) phototransduction machinery, leaving the cone system intact. These animals, despite having a nonfunctional rod-signaling cascade, do not have rod degeneration. Therefore, cone cells do not suffer secondary degeneration as in rd/rd mutants. Albeit the sample size in these experiments was low, some of these animals demonstrated attenuated or possibly a complete lack of circadian photoentrainment. Furthermore, another subset of animals with only functional cones (gnat1–/–; opn4–/–) showed a preference for activity in the light portion of the cycle, i.e., became diurnal. These results indicate that cone input independent of rods is not sufficient to compensate for circadian photoentrainment in the absence of melanopsin protein. Dkhissi-Benyahya et al. (2007) studied mice mutated in the thyroid hormone receptor (TRβ–/–), which leads to selective elimination of MW cones. Animals without the MW cones have defects in detecting light for circadian responses, especially at longer wavelength and shorter duration light pulses. Taken together, the observations from the Mrosovsky and Dkhissi-Benyahya studies beg the question: How could cone photoreceptors have a major role in circadian phase-shifting yet fail to photoentrain the animals? To resolve this issue, phase-shifting studies in animals with only functional cones should be undertaken. In addition, because several mouse lines that have specific elimination of cone function (cnga3–/– or cl animals) are available, the results obtained with TRβ–/– mice should be confirmed with the same light parameters that the Dkhissi-Benyahya study utilized. The contribution of cone input to circadian phase shifting is also shown in human studies. Lockley et al. (2003) showed that a 555-nm light that preferentially activates cone photoreceptors in humans is twofold less efficient in phase shifting the oscillator than a 460-nm light that preferentially activates the melanopsin photoreceptors. In conclusion, despite the fact that the role and relative contribution of cone photoreceptors in circadian photoentrainment are still not fully resolved, animals that lack rod function and melanopsin protein have weaker circadian photoentrainment than animals lacking melanopsin protein only. Therefore, do rod photoreceptors contribute to circadian photoentrainment?

One way to determine the role of rod photoreceptors in circadian photoentrainment is to use animals that have only functional rod cells intact (cnga3–/–; opn4–/–). However, the only available data in the literature about rod function comes from animals lacking the Rpe65 protein (Redmond et al. 1998). Rpe65 is the retinoid isomerase that is required for regeneration of chromophore for classical photoreceptors but is not essential for melanopsin function (Jin et al. 2005; Moiseyev et al. 2005; Redmond et al. 2005). Animals that lack this isomerase have rods as the only remaining functional classical photoreceptor, albeit with reduced photosensitivity (Fan et al. 2005). Doyle et al. (2006) demonstrated that animals that are null for melanopsin protein in addition to the Rpe65 gene (opn4–/–; rpe65–/–), despite having no cone function and reduced rod sensitivity, can photoentrain; moreover, some of these animals showed a preference for day activity and become diurnal. Their ability to photoentrain indicates that
rods may be important for signaling light for nonimage-forming functions. This is surprising given that the threshold for the circadian light response is well above the threshold for rod light signaling.

So far, the current research in this area does not conclusively clarify the role of each photoreceptor to circadian light functions. These reports demonstrate that all three photoreceptor cell types work together in order to signal for irradiance-dependent functions. However, how can we determine the specific contribution of each photoreceptor for nonimage-forming functions? To answer this question, future studies should utilize multiple animal models that preserve photoreceptive systems individually and models that manipulate specific aspects of the retinal circuitry without affecting photoreception.

AN AMAZING FEAT: DECODING LIGHT INFORMATION FOR BOTH CONTRAST AND IRRADIANCE DETECTION

The intricate architecture of the mammalian retina provides precise pathways for light signaling for both image- and nonimage-forming functions. Although both functions require detection of light, they are distinct in the nature of light information attained. Although image formation involves the ability of the retina to differentiate between two juxtaposing points in the visual field, the nonimage-forming functions require the knowledge of the absolute environmental luminosity.

The light information that is detected by rods and cones is signaled via the inner nuclear layer of the retina to RGCs, the only output neurons of the retina (see Fig. 1). Two classes of RGCs, ON and OFF, either depolarize and activate by a light or a dark signal from the outer retina, respectively. Because the ipRGCs receive very few OFF-light “signals” (Brown and Silva 2004; Dacey et al. 2005; Wong et al. 2007), primarily ON-light responses will be discussed.

To form images, the retina absorbs light from the environment and deciphers this overwhelming amount of information into differences of contrast between objects and signals this information through retinotopic maps to the visual cortex. For this visual function, the total intensity of the light is not utilized as much as the relative contrasts, which allows us to be able to read not only outside under bright sunlight (50,000 lux), but also inside a dimly lit room (100 lux). The adaptation of cones to a wide range of light intensities (up to 9 log units) allows this remarkable ability of reading under such vastly different light conditions (Knox and Solesso 2006). Although very advantageous for reading a book, light adaptation with such vigor might cause uncertainty about the intensity of light from the perspective of the tissues receiving this information for phototainment, such as the SCN. In contrast to cone photoreceptors, rod photoreceptors can decode differences between even a few photons but only at low light intensity (scotopic conditions). Rods bleach at medium (mesopic) to high (photopic) light intensities and hence cannot detect further increases in light irradiances (Burns and Baylor 2001; Stockman and Sharpe 2006). Although the cone pathway under mesopic and photopic conditions is adapted and might not supply adequate irradiance information, the continuous bleached signal of rods could be used to indicate the presence or absence of light in the environment. Therefore, the signals from rods and cones could be decoded to measure irradiances even in the absence of melanopsin protein.

MELANOPSIN-CONTAINING RGCs ARE REQUIRED FOR NONIMAGE-FORMING FUNCTIONS

The role of melanopsin-expressing ganglion cells as photoreceptors has been well-studied, but their role as RGCs, responsible for mediating rod/cone-dependent signaling to the brain, has not been established independently of their photosensitivity. The ability of melanopsin knockout animals to photoentrain and constrict their pupil to light has been attributed to the light detected by rods and cones; however, the route used to signal to nonimage-forming centers by the classical photoreceptors for these functions has not been elucidated. Morphological studies indicate that although the majority of the projections to SCN are ipRGCs, some of these projections are of classical RGC origin (Gooley et al. 2001; Morin et al. 2003; Sollars et al. 2003). Furthermore, the olivary pretectal nucleus is highly innervated by the nonmelanopsin cells (Hattar et al. 2006). What is the role of the contrast-detecting classical RGCs in nonimage-forming functions? Do melanopsin expressing RGCs integrate all light input from the three types of photoreceptors and relay this information to nonimage-forming centers as decoders of irradiance?

To appreciate all of the functions carried out by ipRGCs, the elimination of not only the melanopsin photopigment, but the whole cell is necessary. Because only RGCs that do not express melanopsin survive, this ablation also indirectly determines the relative contribution of the classical RGCs to nonimage-forming functions. We accomplished the specific ablation of ipRGCs by expressing the attenuated form of diphtheria toxin subunit A (aDTA), a translational inhibitor, at the melanopsin locus (Güler et al. 2008). As expected, animals carrying a single copy of aDTA (opn4aDTA/aDTA) demonstrate nearly complete loss of melanopsin-expressing cells with approximately 17% of the cells surviving at 6 months of age. As expected, the projections from these cells to their targets (i.e., SCN) are reduced in the aDTA animals.

Bilateral ocular injections of the cholera toxin B subunit revealed that the projections by classical RGCs remained intact in both nonimage- and image-forming centers, leaving only few fibers projecting to the SCN. When the ipRGCs were ablated to a greater extent by the generation of aDTA homozygotes (opn4aDTA/aDTA), we observed a further elimination of RGC projections to the SCN, leaving few fibers at 1 year of age. This indicates that there are minimal classical RGC innervations to the SCN. These morphological observations indicate that light signaling for nonimage functions may be studied independent of image formation. In fact, we showed that the aDTA animals are capable of forming images quite normally using several classical vision tests.
THE SIGNAL FOR PLR CONVERGES AT THE LEVEL OF MELANOPSIN-EXPRESSING RGCs

Light-dependent pupil constriction regulates the amount of light that enters the eye based on overall illumination. When light enters the retina, light intensity information is sent to the olivary pretectal nucleus via RGCs, whose firing rate to the Edinger Westphal nuclei is modulated by irradiance. The oculomotor nerve originating from Edinger Westphal nuclei synapses to the ciliary ganglion, which controls pupil size (Zhang et al. 1996). Because irradiance detectors within the retina control the PLR, it was thought that the melanopsin-containing RGCs would be involved; however, Lucas et al. (2001) established that rods and cones are the main input for pupil constriction at low light intensities, whereas rods and cones are not able to execute full pupil constriction at higher light intensities in the absence of melanopsin protein. Therefore, the ability to fully constrict the pupil at higher light intensities depends on the intrinsic photosensitivity of the melanopsin-containing RGCs (Lucas et al. 2003). By utilizing the DTA mouse, we were able to determine whether rod- and cone-dependent pupil constriction acts through melanopsin-expressing cells or through the classical RGCs. In mice expressing aDTA from the melanopsin locus, pupil constriction at low light intensities was significantly weakened compared to that in wild-type mice. This suggested that the rod and cone signal necessary for pupil constriction is routed through the melanopsin cells even at low light intensities. In animals with nearly complete ablation of ipRGCs, the ability to constrict the pupil is attenuated to a greater extent at high light intensities than in animals that lack the melanopsin photoreceptor. These results indicate that melanopsin-containing RGCs are necessary for PLR at high and low light intensities by acting both as the primary light detectors and as conduits for rod/cone light information to the olivary pretectal nucleus.

IN THE ABSENCE OF MELANOPSIN-CONTAINING RGCs, CIRCADIAN PHOTOENTRAINMENT IS ABOLISHED

To fully assess the ability of ipRGC-ablated animals to photoentrain, we exposed them to a “jet-lag” paradigm (three 2-week 12:12 light/dark cycles advanced and delayed by 6 hours consecutively). Although wild-type animals had a close phase relationship with each light/dark cycle, the animals expressing aDTA segregated into two distinct phenotypic groups, neither of which photoentrain. The first group, group A, showed no light responses and completely free-ran similar to genetically engineered mice that lack all three photoreceptor types or bilaterally enucleated animals. Although the second group of animals, group B, was unable to photoentrain, they exhibited weak light responses with unstable and large phase angles to the light/dark cycles. The differences in light responses between these two animal groups may be attributed to the variability among mutants in the number of remaining melanopsin-expressing cells. Because phase-shifting experiments are a more quantitative indication of an animal’s ability for circadian light response, we tested whether either group A or B mutant animals can be phase-shifted by a light stimulus that elicits approximately 2-hour phase delays at circadian time (CT) 16 in wild-type mice. Interestingly, neither mutant group showed any phase delays to this light stimulus. This reveals that the photoentrainment capability of animals that lack ipRGCs is severely diminished. The rod/cone-dependent light signal for photoentrainment observed in wild-type and melanopsin knockout animals is routed through the ipRGCs. Unlike rod/cone or melanopsin photoreceptive functions, signaling of light input to the SCN via the ipRGCs is required for photoentrainment.

THE CIRCADIAN OSCILLATOR IS NOT COMPROMISED IN aDTA ANIMALS

In constant darkness, wild-type mice are able to maintain a regular, approximately 23.6-hour rhythm in locomotor activity reflecting their endogenous clock. Animals lacking an endogenous circadian oscillator will not maintain a regular rhythm under constant conditions and will be arrhythmic. Because both groups A and B of the aDTA-expressing mice have intact circadian rhythms in constant darkness, we concluded that the endogenous clock in these animals is not affected by the attenuated light input to their SCN. Aschoff’s rule states that generally in nocturnal species, tau is positively correlated with light intensity in constant light, i.e., as the light intensity is increased, the period length of the rhythm will lengthen. Diurnal animals respond oppositely and will shorten their period as light intensity increases in constant light (Aschoff 1961; Daan 2000). As expected, wild-type mice in our experiments lengthen their free-running periods from 23.3 hours in constant dark to 25.5 hours in constant light. Although the group B mice do not show a significant difference in their period length between constant dark and light conditions, group A mice shorten their periods from 23.8 hours in constant dark to 23.4 hours in constant light. This trend toward a shortened period in constant light in animals that have only conventional RGCs remaining is characteristic of diurnal animals. Interestingly, mice with only functional cone photoreceptors (gnat<sup>1+/-</sup>; opn4<sup>-/-</sup>) and rpe65<sup>1+/-</sup>; opn4<sup>-/-</sup> show diurnal tendencies (Mrosovsky and Hattar 2005; Doyle et al. 2006). In light of these findings, it would be interesting to determine whether these animal models with diurnal tendencies also have shortened periods under constant light conditions.

MASKING DEMONSTRATES THAT THE MELANOPSIN PROTEIN MAINTAINS PROLONGED RESPONSES TO LIGHT

Organisms are able to coordinate their activity to the light/dark cycles by either synchronizing their internal clock so that they are able to anticipate the light cycle (i.e., photoentrainment) or responding directly to light changes (masking). It is thought that these two responses work together to control daily cycles. Photoentrainment is mediated by light input from the retina to the SCN; however, the light pathway and brain regions mediating masking are unknown. The most-prominent defect observed in mice
lacking melanopsin protein is their masking responses to light. To measure the ability of the melanopsin protein to detect light for prolonged periods in vivo, Mrosovsky and Hattar (2003) presented 3-hour light pulses that inhibit locomotor activity, 2 hours after the onset of activity in the dark. In animals that lack melanopsin protein, the initial rod/cone-mediated inhibitory effect on locomotor activity is not maintained compared to wild-type animals that sustain this masking response for the duration of the light pulse (Mrosovsky and Hattar 2003). This confirms that the melanopsin protein is an irradiance detector that can measure photons for a prolonged period of time.

Using a 7-hour light/dark cycle (3.5 hour:3.5 hour light/dark cycle) to which mice cannot readily entrain, melanopsin knockout mice confine approximately 75% of their activity to the dark portion compared to the wild-type mice at a light intensity that confines 98% of their activity to the dark phase (Mrosovsky and Hattar 2003). In this paradigm, animals that are unable to mask should distribute their activity equally between the light and the dark phases and have approximately 50% of their activity in the dark. At a light intensity where the wild-type animals confined 84% of their activity to the dark portion of the ultradian cycle, the opn4mDTA/aDTA mice that free-run through all light paradigms (group A) confined 62% of their activity to the dark portion of the light/dark cycle. The mutant mice with weak light responses (group B) had slightly higher masking ability with 67% of their activity confined to the dark phase of the light/dark cycle. Because there exists a possibility that group A animals contain fewer melanopsin-expressing cells than group B animals, the positive correlation between the strength of masking and the melanopsin protein is an irradiance detector that can measure photons for a prolonged period of time.

Melanopsin-expressing ganglion cells in the mouse retina are responsible for masking. The mutant mice with weak light responses (group B) had slightly higher masking ability with 67% of their activity confined to the dark phase of the light/dark cycle. Because there exists a possibility that group A animals contain fewer melanopsin-expressing cells than group B animals, the positive correlation between the strength of masking and the melanopsin protein is an irradiance detector that can measure photons for a prolonged period of time.

CONCLUSIONS

The selective ablation of ipRGCs reveals a clear distinction between the function of the melanopsin protein and the melanopsin-containing RGCs. The ipRGCs are the main conduit for light information to nonimage-forming functions combining light responses from all three types of photoreceptors. Rods/cones contribute to nonimage functions mainly at low light intensities, whereas the melanopsin protein is important at high light intensities and especially for prolonged measurement of light. A delicate balance between all three photoreceptor systems coordinates physiologically important functions to light detection.

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REFERENCES


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