

Melanopsin and inner retinal photoreception

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Abstract Over the last ten years there has been growing acceptance that retinal photoreception among mammals extends beyond rods and cones to include a small number of intrinsically photosensitive retinal ganglion cells (ipRGCs). These ipRGCs are capable of responding to light in the absence of rod/cone input thanks to expression of an opsin photopigment called melanopsin. They are specialised for measuring ambient levels of light (irradiance) for a wide variety of so-called non-image-forming light responses. These include synchronisation of circadian clocks to light:dark cycles and the regulation of pupil size, sleep propensity and pineal melatonin production. Here, we provide a review of some of the landmark discoveries in this fast developing field, paying particular emphasis to recent findings and key areas for future investigation.

Keywords Retina · Ganglion cells · Photoreception · Circadian rhythms · Opsin · Photosensitivity

Introduction

The discovery of melanopsin and inner retinal photoreceptors has its origin in the study of circadian clocks. These endogenous timing mechanisms perform the important task of fine-tuning behaviour and physiology according to the varying demands of the astronomical day, and are a near ubiquitous feature of life on earth. In mammals, circadian oscillators can be found in multiple cell types and tissues,

but these are subservient to a dominant circadian clock located in the hypothalamic suprachiasmatic nuclei (SCN) within the brain. A cardinal feature of circadian clocks is that they are self-sustaining, capable of running with a period close to 24 h without any external input. However, in order to be of benefit to the organism, the ‘internal’ representation of time of day must provide an accurate estimate of external time. Circadian clocks achieve this goal by being regularly reset (or entrained) to rhythmic cues in the physical environment. The most reliable external indicator of time of day is the light:dark cycle and, as a result, light is generally regarded as the most powerful entraining signal for the clock.

The sensory requirements of circadian photoentrainment are fundamentally different from those of image-forming vision. Time of day is correlated with ambient light intensity (irradiance), and it is exactly this parameter (integrated over long timescales) that defines the magnitude of circadian clock responses to experimental light stimuli [1–3]. By contrast, classical visual pathways are optimised for spatial and temporal contrast acuity, with rather limited requirement to accurately encode irradiance. Since at least the beginning of the twentieth century, it has been clear that non-mammalian vertebrates respond to this fundamental difference in sensory requirements by having separate photoreceptors for form vision and for irradiance detection. Karl von Frisch first suggested such extra-ocular photoreceptors in his study of skin pigmentation control in minnows [4], and this has been followed by a great body of work describing photoreceptors extrinsic to the retina. These include receptors associated with circadian photoentrainment and other physiological and behavioural responses to environmental irradiance in the central nervous system, skin and peripheral organs of fish, amphibia, reptiles and birds [5–8].

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In contrast to the prevalence of extra-ocular photoreceptors in other vertebrate classes, enucleation of the eye in rodents results in a loss of all light detection. This finding implicated retinal photoreceptors as the origin of both image-forming vision and circadian entrainment in mammals [9, 10]. The obvious implication, that the same rod and cone photoreceptors support both tasks, used to be widely accepted but is now seen as outdated. Early indications that this was not the case came from reports that laboratory rodents suffering extensive degeneration of rods and cones retain circadian photoentrainment [11–13]. While it was thought that residual cone photoreceptors in these mice may account for their ability to entrain [14–16], the possibility of an unknown retinal photoreceptor dedicated to circadian entrainment was also raised [11, 12, 15, 17–19]. Case reports of human subjects lacking conscious light perception and yet retaining circadian light responses provided support for this latter possibility [20]. A direct test of this hypothesis came with the generation of transgenic mice lacking detectable rod and cone photoreceptors. These mice retained a variety of irradiance responses including circadian photoentrainment, light-induced suppression of pineal melatonin, and a pupillary light reflex [21–23]. The clear implication that the mammalian eye contains a non-rod non-cone photoreceptor dedicated to measuring ambient light intensity was further supported by descriptions in mice and humans of irradiance responses whose spectral sensitivity did not match that of any known retinal photoreceptor class [23–26].

The first direct description of these non-rod non-cone photoreceptors came with the publication in 2002 of two papers describing a sub-set of retinal ganglion cells with the extraordinary ability of responding to light even in the absence of synaptic inputs [27, 28]. These so-called ‘intrinsically photosensitive retinal ganglion cells’ (ipRGCs) project to the SCN and express melanopsin [27], a member of the opsin family of G-protein-coupled receptors that was initially discovered in the photosensitive dermal melanophores of *Xenopus laevis* [29]. The subsequent generation of melanopsin knockout mice (*Opn4^{-/-}*) confirmed that this protein is critical for the intrinsic light response of ipRGCs [30]. Confirmation that this reflects its function as a light absorbing photopigment came from experiments showing light-dependent G-protein activation by melanopsin in vitro [31] or under heterologous expression in a variety of vertebrate cell types [32–34].

The significance of melanopsin photoreception for irradiance responses was initially investigated in melanopsin knockout mice. These animals retain circadian photoentrainment and a pupillary light reflex, but show significant alterations in stimulus:response relationships [30, 35, 36]. It was not until melanopsin knockout was

combined with lesions of rod and cone phototransduction that mice lacking photo responsiveness were obtained [37, 38]. This latter result provided the final confirmation that melanopsin retinal ganglion cells represent the non-rod non-cone photoreceptor whose existence was implied by the earlier rodent and human studies.

Although the study of ipRGCs has its origins in attempts to explain circadian photoentrainment, it was clear from a relatively early stage that their influence was not restricted to the clock. Mice lacking rods and cones retain a variety of responses to environmental irradiance including pineal melatonin suppression, a pupillary light reflex, inhibition of locomotor activity, and regulation of sleep propensity. In humans, there is evidence that this system regulates body temperature, mood and aspects of arousal and concentration. It seems, therefore, that melanopsin-expressing ipRGCs represent a distinct sensory modality encoding environmental irradiance for a wide variety of physiological and behavioural systems (Fig. 1). Their input to such an array of important biological processes places an imperative to achieve a deeper understanding of their anatomy and physiology, as well as their contribution to our sensory capabilities. Here, we review some of the progress made over the last 5–6 years to address these important questions.

Anatomy of ipRGCs

Since the correlation between expression of melanopsin and intrinsic photosensitivity was first established [27], immunohistochemistry using melanopsin antibodies has been the primary approach to exploring ipRGC morphology. Antibodies against melanopsins from a wide variety of species and recognising both N- and C-terminal epitopes are now available [27, 39–43]. There have been reports of melanopsin immunoreactivity in a subset of cones and in the retinal pigment epithelium [44, 45]. However, at least in mammals, by far the most consistent staining is observed in a small number of retinal ganglion cells whose cell bodies may be found in the ganglion cell or inner nuclear layers [42, 43, 46]. The proportion of ganglion cells immunoreactive for melanopsin varies according to the species under investigation and, to some extent, the antibody used, but never exceeds a few percent. Compared to other ganglion cell classes, they are distinguished by their sparse dendritic arborisation but wide receptive fields [39].

It is becoming increasingly clear that melanopsin ganglion cells do not, in fact, form a single homogenous group. In the mouse, at least three anatomically distinct subtypes have been reported. Termed M1–3, these differ mainly in their dendritic stratification (Fig. 2). Ganglion cells in the vertebrate retina receive synaptic input in the

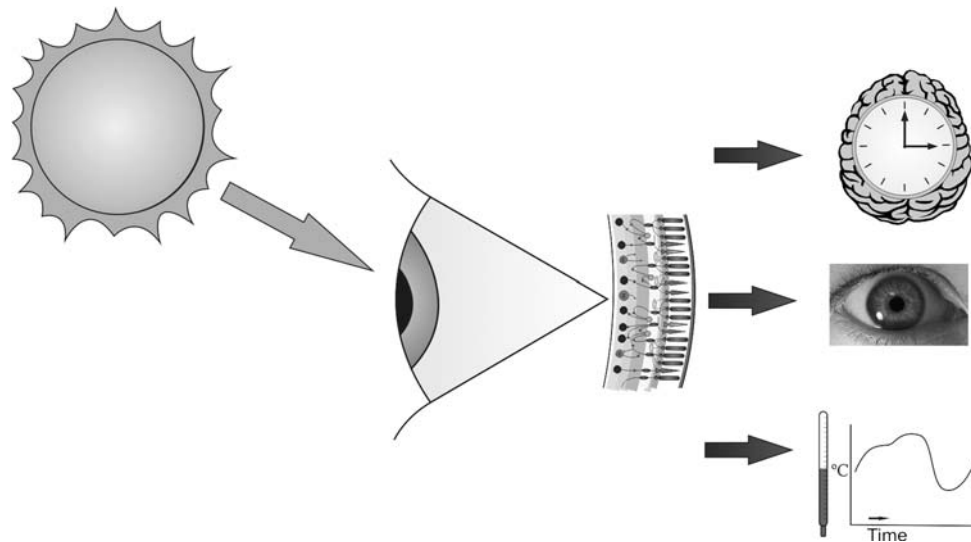


Fig. 1 A schematic figure of the non-image-forming (NIF) visual system in mammals involving ipRGCs. Light falling on the retina is detected by rod and cone photoreceptors and by the melanopsin phototransduction cascade in ipRGCs. The resultant signals are combined by ipRGCs to send an integral signal of irradiance to the brain. In turn, light information is passed to the brain down the optic

nerve, triggering a diverse range of non image-forming responses in addition to normal form vision. NIF responses comprise the entrainment of circadian rhythms including sleep propensity (shown here by an image of a clock superimposed on a brain), the pupil light reflex, regulation of pineal melatonin levels and, in humans, regulation of body temperature and mood

inner plexiform layer (IPL), which can be subdivided into anatomically distinct sublaminae. The major distinction is between sublamina a, closest to the rods and cones, in which they make connections with so-called OFF bipolar cells that are excited by decrements in light intensity, and sublamina b, proximal to the major concentration of ganglion cell bodies, characterised by synaptic input from ON bipolars excited by light increments. Dendrites from the so-called M1 cells stratify in sublamina a, those of M2 in sublamina b, while M3 ganglion cells are bistratified with dendrites in both a and b sublaminae [53, 55–57]. One might expect M1 cells therefore to receive mainly OFF signals from the outer retina, but as this appears not to be the case [48–52], the functional significance of this difference in dendritic stratification remains unknown. There are, moreover, other morphological differences between classes. M2 cells have larger soma, larger dendritic fields, more branched/complex arborisation, and greater overall dendritic length than M1 cells [58]. Circumstantial evidence suggests that these classes also differ in their degree of melanopsin expression. Thus, a lac-Z reporter knocked into the melanopsin gene reaches detectable levels of expression only in the M1 class [55, 56], and the intrinsic light response of M2 cells is also smaller and less sensitive than that of M1 [58]. In addition to murines, primates also have separate populations of melanopsin ganglion cells with dendrites either in the inner or outer border of the IPL [43, 59]. However, the degree to which these are correlates of the rodent M1/2 cells remains unclear.

All species examined thus far show melanopsin-positive cells distributed across the retina (with the exception of the primate fovea) with only relatively minor regional differences in concentration [27, 43, 55, 60]. Taken together with their wide dendritic arborisation this means that they form what has been termed a ‘photoreceptive net’ across the retina [39], capturing light from almost all parts of the visual scene. It now also appears that the dendritic fields of neighbouring M1 and M2 cells show significant overlap when filled and traced with neurobiotin, suggesting an overlap of the receptive fields of the two subtypes [58].

Intra-retinal connectivity

Physiological and anatomical studies indicate that excitatory ON and OFF bipolar cells (signalling light increments and decrements respectively), as well as inhibitory amacrine cells, are presynaptic to ipRGCs [47, 51, 57, 59, 61]. Thus, despite their unique ability to respond directly to light, ipRGCs share much of the intra-retinal connectivity of classical retinal ganglion cell classes. However, a number of unusual aspects of ipRGC connectivity have been suggested. Firstly, M1 cells receive strong ON signals from the outer retina, despite having dendrites in the OFF sublamina of the IPL [43, 48]. This has recently been explained by description of a class of ON bipolar which contacts ipRGC dendrites in the OFF sublamina [50]. Secondly, there is anatomical evidence that rod ON bipolars, which normally do not make direct contacts with

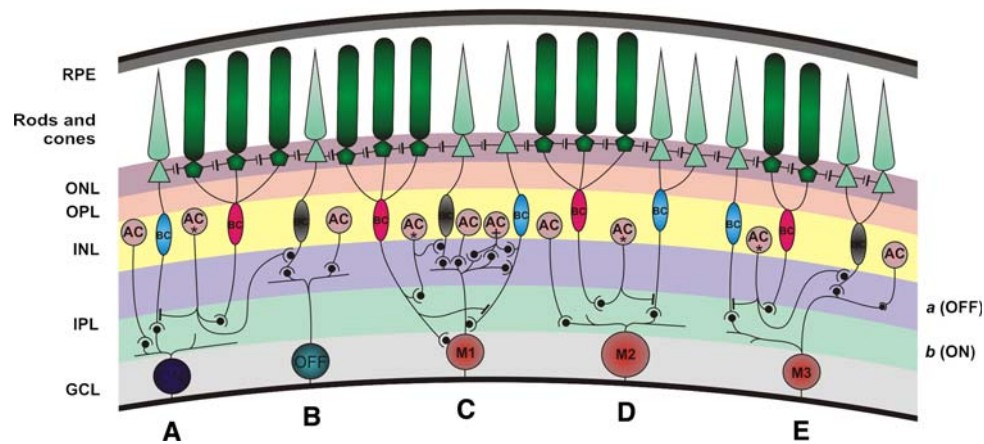


Fig. 2 A schematic diagram of retinal connections relevant to ipRGCs in the rodent retina. *A* and *B* show examples of classical wiring through ON (triggered by increments of light) and OFF (triggered by decrements of light)-cell pathways of the retina, respectively. The typical pathway of vertical transmission flows from photoreceptor cell to bipolar cell to ganglion cell. The inner plexiform layer (*IPL*) can be subdivided into sublaminae *a* and *b*. Bipolar and ganglion cells stratifying in sublamina *a* are OFF cells, while those stratifying in sublamina *b* are ON. A cone ON bipolar cell synapses directly onto an ON ganglion cell in sublamina *b*. Rod bipolar cells are all ON cells but do not contact ganglion cells directly in this classical pathway, but rather synapse onto AII amacrine cells in sublamina *b*. AII amacrine cells in turn contact cone ON bipolar cell dendrites through sign-conserving gap junctions, and OFF cone bipolar cells via sign-inverting synapses (*B*). Cone OFF bipolar cells directly synapse onto OFF ganglion cells. Diverse amacrine cell types modulate ON and OFF pathways mainly through inhibitory inputs. ipRGCs can be subdivided into three morphological types (M1–M3). M1 cell dendrites stratify in sublamina *a* (*C*), M2 in sublamina *b* (*B*), while M3 cells are bistratified in both sublaminae (*D*). In contrast to

RGCs, do form synapses with ipRGCs [47] (Fig. 2). Finally, ipRGCs seem capable of exporting information not only onto the brain but also back out to the rest of the retina. Thus, in both humans and mice, there is evidence that melanopsin activity contributes to diurnal/circadian control of cone-based visual pathways [62, 63]. The route by which they exert this influence is currently unknown. There is physiological evidence that ipRGCs make gap junction connections with neighbouring cells in the ganglion cell layer [64]. As the mouse ganglion cell layer contains many amacrine cell bodies, this could allow ipRGCs to influence the major neuromodulator systems of the retina. Alternatively, ipRGCs may provide a more direct regulation of a particular class of amacrine cell that produces dopamine. Dopamine is released from these cells in response to light [65], and modulates many aspects of retinal function [66–70]. A subset of ipRGCs form a discrete plexus with dopaminergic amacrine cells [46, 50, 53, 54], and receive synaptic input from them. Information may also flow in the opposite direction. Thus, Zhang et al. [54] recently presented evidence that, in the mouse, sustained light-dependent firing of dopaminergic amacrine cells is

normal retinal cell stratification patterns, however, M1 cells stratifying in sublamina *a* receive strong ON signals [43, 47, 48]. This reflects direct input from cone ON bipolar cells at the M1 cell body and, anomalously at dendrites in sublamina *a* [49, 50], as well as direct synaptic contacts with rod bipolar cells [47]. Rod bipolar cells may also influence their activity via the classical AII amacrine cell route. Physiological results predict additional input from cone OFF bipolar cells and inhibitory amacrine cells [51, 52]. Finally, there are direct connections between dopaminergic amacrine cells and presumed M1 cell dendrites in sublamina *a*, possibly going in both directions [46, 47, 50, 53, 54]. The connections of M2 and M3 cells are currently unknown. Those shown here are predictions based on connectivity of conventional ganglion cells with similar dendritic stratification patterns. Not shown are horizontal cells which modulate information transfer at the OPL. *ONL* outer nuclear layer, *OPL* outer plexiform layer, *INL* inner nuclear layer, *IPL* inner plexiform layer, *GCL* ganglion cell layer, *HC* horizontal cell, *AC* amacrine cell, *AC** aII amacrine cell, *AC+* dopaminergic amacrine cell, *BC*, bipolar cell. *Blue* bipolar cell body, *cone* ON bipolar; *red* bipolar cell body, *rod* bipolar (ON); *grey* bipolar cell body, *cone* OFF bipolar

driven by melanopsin photoreceptors. Consequently, this pathway would be well placed to translate melanopsin activation into modifications in retinal physiology. It should be noted, however, that melanopsin phototransduction does not appear either necessary or sufficient for large-scale dopamine release in the retina [71].

Projections and functions of ipRGCs

Currently, the most comprehensive analysis of central projections for ipRGCs has been from Hattar et al. [55]. That work employed mice in which a tau-lacZ transgene was knocked into the melanopsin locus. The resultant expression of β -galactosidase throughout ipRGC axons enables standard histochemical techniques to be used to trace their passage through the mouse brain. The projection pattern observed was surprisingly widespread and the reader is referred to that paper for a detailed description. In brief, the most intense innervation was in the SCN, with a bilateral projection extending throughout the nucleus. Elsewhere in the hypothalamus, the ventral subparaventricular zone, the

lateral hypothalamus and the supraoptic nucleus (SON) all receive ipRGC input, providing a potential route for the direct effects of light on gross physiology and the neuroendocrine axis. ipRGC axons were also found in the ventrolateral preoptic nucleus, consistent with evidence that melanopsin provides photic regulation of sleep propensity [72–74]. Other notable projections were traced to the olivary pretectal nucleus (OPN; the origin of the pupil light reflex) and the intergeniculate leaflet (a known component of the circadian entrainment pathway; [75]).

The major drawback of the tau-lacZ approach to tracing ipRGC projections is that β -galactosidase expression seems to be restricted to the M1 subtype [76]. Thus, the projection pattern described by Hattar et al. [55] is probably incomplete. There are indications that M2 and M3 cells might have quite different projection patterns. Viral tracing techniques suggest that while M1 cells comprise the primary projection to the mouse SCN (80% M1/20% M2), equal numbers of M1 and M2 cells project to the OPN (45% M1/55% M2; [76]). In fact, the relative projection pattern of these two classes suggests a new anatomical subdivision of this nucleus, with M1 axons terminating in a hollow ovoid ‘shell’ surrounding the OPN, and M2 cells innervating the central core.

Another brain region in which the work of Hattar et al. [55] probably underestimates the ipRGC projection is the lateral geniculate. β -galactosidase staining reveals strong innervation of the intergeniculate leaflet but not the neighbouring dorsal lateral geniculate (the major thalamic relay centre for visual information). However, Dacey et al. [43] successfully labelled ipRGCs in the primate using injections of retrograde tracers from the lateral geniculate. Moreover, in our own unpublished work we have observed widespread responses to melanopsin activation in neurons of the lateral geniculate (Brown and Lucas, unpublished observations). It seems reasonable, therefore, to speculate that this brain region receives input from M2/M3 cells. An important area for future study will be to determine what this signal contributes to visual processing.

Recently, fMRI techniques have been applied to exploring irradiance-dependent activity in the human brain. The results reveal fairly widespread changes in activity in sub-cortical and limbic areas [77]. At present, these have not been unambiguously assigned to melanopsin photoreception. However, they are consistent with evidence that this system has widespread effects on attention, mood and perhaps cognitive function.

Physiology of ipRGCs

Historically, a major barrier to a physiological characterisation of the ipRGC light response has been their

comparative scarcity within the retinal ganglion cell layer. Data on the number of ipRGCs in the retina depends on the method used to identify them, but is around 1,200–1,600 cells, or $\sim 1.5\%$ of the total ganglion cell population in mice and hamsters [30, 55, 76, 78], and up to 3,000 cells in human and macaque retinæ (0.2% of total population; [43]). As a result, their contribution to unbiased samples of physiological activity in the ganglion cell population would be very slight. A number of strategies have been used to overcome this problem.

Retrograde tract tracing of retinal ganglion cells

The original discovery of ipRGCs was made by recording from cells retrogradely traced from the SCN [79–82]. The success of this approach relies upon the fact that the SCN receives predominantly ipRGC innervation [83]. Although it has been used very successfully in a number of studies, this strategy is labour intensive. Moreover, as selective targeting of other ipRGC-recipient nuclei is not always possible, its use has so far been mostly limited to studying those ipRGCs afferent to the SCN.

Identification of light responses in the absence of signals from the outer retina

One of the early confirmations that cells in the mouse ganglion cell layer could be intrinsically photosensitive came from calcium imaging of the ganglion cell layer in rodless + coneless mice [64]. In that work, the ability to survey activity over relatively large areas of the retina, combined with genetic ablation of rod/cone activity, enabled a small number of intrinsically light responsive cells to be identified and studied. Similar approaches employing multi-electrode arrays, and replacing genetic with pharmacological lesions of outer retinal signalling have since been successfully employed in the same way [51, 84, 85].

Viral tracing

An alpha (Bartha) strain of the pseudorabies virus (PRV-Ba), incapable of anterograde transport [86–88], labels retinal ganglion cells afferent to the SCN/OPN when injected into the contralateral eye. This is a fifth order event, following infection of autonomic ganglia innervating the uvea and subsequent retrograde infection of the ipsilateral Edinger-Westphal nucleus (EW), then four bilateral retinorecipient structures (the OPN followed by the IGL, the lateral terminal nucleus—LTN, of the accessory optic system and the SCN; [89]). PRV-Ba has been used by successive authors to investigate aspects of the SCN [89, 90] as well as to identify RGCs of the

mammalian retinohypothalamic tract. PRV-Ba constructs have been engineered to express enhanced fluorescent proteins (EGFP/RFP) [53, 78, 91, 92], allowing infected RGCs to be targeted with recording electrodes [49].

Immunopanning

Two groups have successfully purified ipRGCs from rodent retinal homogenates by immunopanning with a melanopsin antibody [93, 94]. These purified ipRGC populations can then be easily isolated for physiological responses.

Genetic

Several mouse lines now exist in which fluorescent proteins are expressed under control of the melanopsin promoter [57, 95, 96]. These fall into two main categories. The first carries a BAC transgene including a fluorescent protein downstream of the melanopsin promoter that has been randomly integrated into the mouse genome [57, 96]. In the second, a Cre recombinase coding sequence has been knocked into the melanopsin locus [95]. Crossing these *Opn4^{Cre}* mice with transgenics carrying a ‘floxed’ reporter (a reporter coding sequence driven by a strong promoter upon Cre-mediated excision of a STOP cassette flanked by loxP sites) allows specific expression of the reporter protein in melanopsin-expressing cells. Each type of reporter mouse has its own advantages. Both strategies appear to label not only M1 but also other ipRGC classes, and as they may use fluorescent proteins to do this can be adapted to physiological as well as anatomical analyses. The Cre/loxP system dissociates the strength of reporter expression from the degree of melanopsin promoter activity, as once the STOP cassette has been excised the reporter is driven by its own, much stronger promoter. This could provide a more extensive coverage of the ipRGC population, but is also more liable to yield false positives as a small degree of ‘leaky’ Cre expression could result in strong reporter expression [97], perhaps due to early developmental activity of the promoter in a cells ontogeny. In both cases, it is theoretically possible that the introduction of FP/Cre coding sequences disrupts promoter/enhancer elements sufficiently to produce significant numbers of false positives and/or negatives. In fact, so far these potential limitations appear not to be a big consideration for the melanopsin reporter mice [57, 95, 96], and given their advantages over other methods for identifying ipRGCs, their use looks set to increase.

Light response characteristics in ipRGCs

The basic characteristics of the ipRGC light response have been accepted from the earliest days of their discovery, and

fit well with their role as irradiance detectors. Unlike rods and cones, which hyperpolarise in response to light, ipRGCs depolarise, triggering action potentials which propagate to the brain [28, 43, 58, 96, 98]. Although the light intensities required to elicit a change in ipRGC firing rate are well within the physiological range [57], their intrinsic light reaction is significantly less sensitive than either rods or cones [28, 43]. The ipRGC response can also be extremely sluggish, taking several seconds to reach peak under dim light intensities [28]. Brighter stimuli elicit more rapid responses [96, 99], however, but the response latency for the pupillary light reflex in rodless+coneless mice suggests that even at the highest irradiances the melanopsin-mediated response is delayed by ~ 300 ms compared to that of the conventional photoreceptors [23].

Recently, the origins of these sensory characteristics were addressed in a study describing the response of ipRGCs to absorption of a single photon of light [96], reviewed in [100]. This confirmed the low relative sensitivity of ipRGCs (the photon flux required to elicit a half saturating response is $\sim 10^4\times$ that of cones [101]), and presented evidence that this is not a reflection of inefficient phototransduction, but rather of very poor photon catch associated with low pigment density. In fact, the membrane current induced by single photon absorption was greater than that reported in rod photoreceptors, and long lasting. The single photon response also recapitulated the sluggish response kinetics reported for ipRGCs, with a time to peak some ~ 20 times slower than rods and ~ 100 times slower than cones [101, 102]. As elements of the melanopsin phototransduction cascade become identified it will be important to determine the origin of these response characteristics, e.g. does the relatively large sustained depolarisation of the single photon response reflect high biochemical gain in the phototransduction cascade and/or persistent channel opening?

Diversity in the intrinsic light response has been described in studies of ipRGCs using calcium imaging [64], multi electrode array [85, 103], whole-cell [28, 104] and perforated patch recordings [96]. Only very recently, however, have these varying physiological characteristics been assigned to anatomical subtypes of ipRGCs [58]. Schmidt and Kofuji [58] described $10\times$ higher sensitivity and greater maximal responses in the intrinsic light response of M1 compared to M2 cells, consistent with the suggestion that they express more melanopsin. Differences in intrinsic membrane properties of M1 and M2 cells were also reported, with M2 cells having lower input resistance, a more hyperpolarised resting membrane potential and higher peak and average firing rates. It is tempting to interpret these data in terms of the relative significance of extrinsic (rod/cone) versus intrinsic (melanopsin) signals in defining the firing pattern of M1 and M2 cells, and thus the

sensory characteristics of downstream responses. However, this remains unproven.

Phototransduction

Melanopsin is a member of the opsin sub group of G-protein-coupled receptors. In general, opsins bind 11-*cis* retinaldehyde (or related compounds) in the dark, which acts as an inverse agonist inhibiting G-protein interaction. Light is absorbed by this cofactor, which is isomerised to the all-*trans* isoform in the process. All-*trans* retinaldehyde is a receptor agonist and the photoactivated opsin therefore binds its cognate g-protein, setting in train second messenger signalling cascades. There is currently no reason to suspect that melanopsin's mechanism of action deviates from this basic model.

Across the animal kingdom, there are examples of opsins coupling to G-proteins from Gt, Gq-, Go- and Gs classes (see [105] for review and [106]). Vertebrate rod and cone opsins couple to a Gt-protein (transducin) and trigger a phosphodiesterase-dependent cascade resulting in cyclic nucleotide gated channel closure. The downstream effectors of melanopsin have yet to be proven conclusively. However, most available evidence supports the hypothesis that its cognate G-protein is of the Gq/11 family activating the IP3/DAG pathway and ultimately TRP channels, similar to many invertebrate phototransduction cascades [33, 34, 107–114]. Light triggers an increase in intracellular calcium in ipRGC somata, and calcium responses are highly correlated with action potential firing [64, 93, 115, 116]. In vivo data implicate canonical transient receptor potential (TRP) [110, 111] and/or voltage gated calcium channels [93] as the origin of this calcium increase. Direct evidence for melanopsin interactions with Gq/11 G-proteins is currently limited to heterologous cell expression studies [34]. Melanopsin can also bind other G-proteins under these conditions [31, 32], but there is always the concern of promiscuous G-protein interactions outside of a native cellular environment [117]. Nonetheless, ipRGCs express Gq and G11 G-proteins as well as several phospholipase C isozymes [99] and TRPC channels (TRPC6 and TRPC7; [110, 111]). Moreover, the ipRGC intrinsic photoresponse has been inhibited using pharmacological blockers of phospholipase C [99] and TRPC channels [110, 111].

Signal integration: the triplex retina

Over the last year, three independent studies have confirmed that, at least in mice, cytotoxic lesions of ipRGCs abolish all irradiance responses [95, 118, 119]. These findings indicate that ipRGCs provide a unique sensory

modality that cannot be provided by other ganglion cell classes. Nonetheless, while rodless+coneless mice retain a variety of irradiance responses [21–23], so do animals lacking melanopsin [30, 35, 36]. The implication that ipRGCs are capable of supporting irradiance responses either through their own intrinsic photosensitivity or by integrating signals from rods and/or cones is supported by the finding that animals lacking all three photoreceptor classes are truly photo-insensitive and by anatomical and physiological studies showing synaptic input to ipRGCs from the outer retina [28, 43, 47, 48, 51, 52, 57, 59, 61, 118, 119].

This raises the question of what benefit the irradiance response system gains from receiving input from the three different photoreceptor classes. A definitive and comprehensive answer to this question will take some time to arrive. However, the evidence to date is that each receptor contributes to irradiance responses according to its own sensory capabilities and that the inclusion of all three extends the conditions under which irradiance can be accurately encoded. Studies of pupillary responses in rodless+coneless and *Opn4*^{-/-} mice are consistent with a variety of physiological studies in indicating that melanopsin phototransduction lacks the sensitivity and temporal fidelity of rod and cone pathways [23, 30, 38]. Thus, a simple view in which melanopsin encodes very bright stimuli while rods and cones provide additional sensitivity and increase the temporal bandpass of irradiance detection has gained currency. This model, however, does not readily explain data from other irradiance responses, especially those which may integrate irradiance signals over many tens of minutes or hours [12, 36].

Ontogeny

ipRGCs provide the earliest light detection in mammals. Melanopsin expression is first detected in the mouse retina at embryonic day (E)10.5 [120], following the appearance of retinal ganglion cells at E9. The rodent RHT is present at postnatal day (P)0 [121, 122], and ipRGCs are functional and capable of inducing *c-fos* within the SCN at least as early as that [115, 121, 123]. By contrast, rod opsin expression is not detectable until P5 [124] and rod/cone responses until P10–12 [125–127]. Rod/cone input to ipRGCs appears also to begin at P11 [57]. Thus, melanopsin photoreception precedes that of the classical visual system. Interestingly, an analogous situation has been noted in chickens, where multi-electrode array recordings measured action potentials from ipRGCs in response to blue light as early as E13 [103]. Pineal photoreception in lampreys [5], fish [128] and amphibians [129] also develops in advance of rods or cones. Such examples of the early development of irradiance detection systems across diverse vertebrate

classes suggests that measuring irradiance has far-reaching ecological advantages in early vertebrate life, perhaps in phototaxis as a predator avoidance strategy.

In mice, ipRGCs undergo substantial postnatal changes in cell number, morphology and physiology [115]. Just like the general ganglion cell population [130], ipRGCs are overproduced at birth and subsequently reduced back to their adult numbers [115]. Interestingly, this pruning fails to occur in a mouse model that undergoes photoreceptor degeneration at an early stage of development (complete by the first month of life; CBA/J), indicating that ipRGC pruning may be under photoreceptor regulation [131]. The molecular mechanisms responsible for ipRGC differentiation and for determining their unusual projection pattern are largely unknown. However, recent work suggests that the transcription factor *Bmn3b* is essential for correct axonal structure and path finding in ipRGCs [132].

Melanopsin spectral sensitivity and photobiology

A photoreceptor's spectral sensitivity function describes its relative responsiveness to light of different wavelengths. It is determined by the absorbance spectrum of the light-absorbing photopigment (which lies at the origin of the photoreceptor light response), adjusted according to any wavelength dependent pre-receptor filtering by screening pigments/absorptive structures [133]. The first estimates of the spectral sensitivity of the non-rod non-cone photoreceptors that turned out to be ipRGCs came from descriptions of the wavelength dependence of behavioural responses in retinally degenerate mice. Working in rodless (*rd/rd*) mice Yoshimura and Ebihara [24] described peak sensitivity for circadian entrainment around 480 nm, which was distinct from that of any mouse photoreceptor known at the time. The significance of this finding was not widely appreciated, partly because it was not supported by other work in this genotype [134] and because the possibility that it reflected a polymorphism in cone spectral sensitivity could not be discounted. In 2001, a more detailed action spectrum was described for pupillomotor responses in rodless+coneless (*rd/rd cl*) mice. It matched the predicted absorbance spectrum of an opsin: vitamin A-based photopigment and again showed peak sensitivity (λ_{\max}) around 480 nm [23]. Subsequently, a 480 nm λ_{\max} was also reported for rat ipRGCs in *in vitro* recordings [28], and this is now accepted as an accurate representation of melanopsin's spectral sensitivity in a variety of mammalian species [43, 135]. There was initially some doubt over whether this extended to humans, as action spectra for suppression of pineal melatonin reported shorter wavelength sensitivity [25, 136], but more recent studies support a 480 nm λ_{\max} [135, 137], and it seems likely that, given

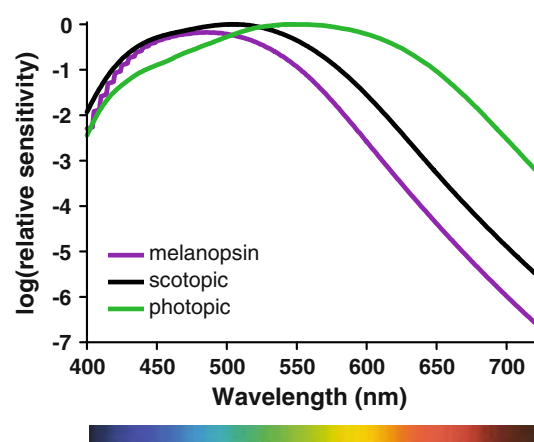


Fig. 3 Melanopsin is maximally sensitive to short wavelength 'blue' light. The spectral sensitivity profile of melanopsin photoreception in a standard human observer is approximated by the nomogram for an opsin: vitamin A based photopigment with peak sensitivity at 480 nm corrected for likely wavelength-dependent lens absorption. The spectral sensitivity of scotopic (rod-based) and photopic (cone-based) vision (Commission Internationale de l'Eclairage, CIE, luminosity functions) are presented for comparison. A very rough approximation of perceived colour is shown below. Note that although melanopsin sensitivity peaks around 480 nm, it shows good responsiveness well into the green/yellow portion of the spectrum

the challenges of human experimentation, these are all in agreement (Fig. 3).

The most satisfactory way of confirming these estimates of melanopsin spectral sensitivity would be to directly measure its absorbance properties. This has been achieved for rod and cone opsins *in vivo* by microspectrophotometry and by spectroscopic analysis of *in vitro* purified opsin protein (see for example [138, 139], and [140] for review). Unfortunately, the low concentration of melanopsin in the retina impacts both of these approaches. As an alternative, heterologous expression methods have been developed to produce melanopsin in cell culture. Success with this approach has been somewhat limited, and the small number of published studies reported only modest yields of light absorbing protein compared to that routinely obtained for e.g. rod opsins [141]. Purified mouse melanopsin also had a λ_{\max} (424 nm) quite divergent from the 480 nm pigment from behavioural studies [31]. More success has been reported from non-mammalian melanopsins, with Koyanagi and colleagues reporting λ_{\max} of 485 nm for a melanopsin-related pigment from amphioxus [107], and Torii et al. [142] peaks at 476 nm and 484 nm for two chicken melanopsins. These papers notwithstanding, there is still a need for reliable methods for melanopsin production and purification that would allow *in vitro* spectroscopic, biochemical and perhaps direct structural analysis.

A question that needs to be addressed by more detailed spectroscopic analysis of mammalian melanopsins is that of

chromophore recycling. Rod and cone opsins bind only *cis* isoforms of retinaldehyde, and following light absorption, release all-*trans* retinaldehyde for regeneration in neighbouring retinal pigment epithelium and, probably, Müller glial cells. An alternative mechanism of bleach recovery is used by invertebrate opsin photopigments. These retain all-*trans* retinal and use a second photon to regenerate the *cis*-isoform. Phylogenetic analyses show that melanopsins are more closely related to invertebrate opsins than other vertebrate opsins, and there have subsequently been suggestions that melanopsin also employs such an intrinsic bleach recovery mechanism (see [143] for review). The most comprehensive analysis of this issue has been undertaken for *Amphioxus* melanopsin which, when reconstituted with 11-*cis* retinal, was shown to form a stable photoproduct whose spectral sensitivity was shifted to longer (redder) wavelengths. Further light exposure could regenerate a pigment whose spectral sensitivity matched that of the original 11-*cis* reconstituted melanopsin, supporting the hypothesis that these data reflect light dependent transitions of melanopsin from *cis* to *all-trans* retinaldehyde binding states. There is indirect evidence from in vivo [108, 137, 144] (although see also [145]) and in vitro [32, 34] studies that mammalian melanopsins may behave in the same way, although the field awaits more direct confirmation of this hypothesis. Moreover, chromophore extraction from mouse ipRGCs in dark adapted retinas only showed evidence of 11-*cis* but not all-*trans* retinaldehyde. This indicates that irrespective of any intrinsic photocycle, melanopsin employs some independent source of 11-*cis* retinaldehyde for bleach recovery in vivo [94].

Comparative considerations

Since the initial discovery of melanopsin in *Xenopus* [29], melanopsin genes have now been described in a variety of vertebrates, including species of fish, reptiles and birds [146–149]. Until recently, it was assumed that these were all orthologues of a single gene. However, the discovery of two melanopsin genes in the chicken turned this thinking on its head [150]. One of these, designated *Opn4m*, is the true orthologue of mammalian melanopsin, while the other (*Opn4x*) was found to be orthologous to the original *Xenopus* melanopsin gene [150]. A re-examination of other species revealed that these two branches of the melanopsin family are widespread in non-mammalian vertebrates, but that *Opn4x* had been lost due to substantial chromosomal reorganisation early in mammalian evolution [150, 151]. A similar loss of cone opsin genes also occurred in mammalian evolution, and it is tempting to conclude that both events reflect a retrenchment of photosensory capability during the nocturnal phase of our evolution [152]. The

consequences for mammalian sensory biology of only having one melanopsin gene remain unknown. There is significant sequence divergence between *Opn4m* and *Opn4x* branches of the melanopsin family, and although early data suggest that they do not differ in spectral sensitivity [142], there is scope for them to couple to different signalling cascades or differ in other aspects of their activity.

Perspectives

The decade since the demonstration that mammalian photosensitivity could not be entirely explained by the activity of rods and cones, and since the discovery of melanopsin, has witnessed the birth of a new branch of sensory biology. Few questions regarding melanopsin and ipRGC photoreceptors remain entirely unaddressed (although the factors regulating melanopsin gene expression and ipRGC fate determination are notable exceptions). However, even fewer have received a definitive answer. The coming years should see a more quantitative and analytical understanding of the basic physiology of ipRGCs emerge, alongside full elucidation of its signalling cascade. There is also work to do on melanopsin photobiology: is its spectral sensitivity really invariant between species, and does it have an intrinsic bleach recovery mechanism? A great contribution to this latter effort would be development of heterologous expression and protein purification strategies capable of reliably producing large amounts of melanopsin for spectroscopic, biochemical and ultimately structural investigation. Further insight into the diversity of ipRGCs will come from new reporter mice in which melanopsin-expressing cells can be identified for anatomical and physiological experiments. Thanks to this diversity it seems likely that melanopsin influence will be detected in a wider range of light responses [77], including perhaps in the functioning of the classical image-forming visual system. As challenging as any of these endeavours will be the development of new models of how the ‘triplex’ (rod, cone, melanopsin) retina encodes the light environment. Studies of cell types presynaptic to ipRGCs will be critical to this effort, as will quantitative descriptions of stimulus:response relationships for diverse irradiance dependent behaviours under a variety of lighting conditions.

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