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Intrinsically photosensitive retinal ganglion cells: many subtypes, diverse functions

Tiffany M. Schmidt[1,*], **Shih-Kuo Chen**[1], and **Samer Hattar**[1,2,*]

[1]Department of Biology, Johns Hopkins University, Baltimore, MD 21218, USA

[2]Department of Neuroscience, Johns Hopkins University-School of Medicine, Baltimore, MD 21218, USA

Abstract

For decades, rods and cones were thought to be the only photoreceptors in the mammalian retina. However, a population of atypical photoreceptive retinal ganglion cells (RGCs) expresses the photopigment melanopsin and is intrinsically photosensitive (ipRGCs). These ipRGCs are critical for relaying light information from the retina to the brain to control circadian photoentrainment, pupillary light reflex, and sleep. ipRGCs were initially described as a uniform population involved solely in signaling irradiance for non-image forming functions. Recent work, however, has uncovered that ipRGCs are unexpectedly diverse at the molecular, cellular and functional levels, and may even be involved in image formation. This review summarizes our current understanding of the diversity of ipRGCs and their various roles in modulating behavior.

Introduction

Light is an important regulator of physiology and behavior in animals, influencing a variety of non-image forming functions such as melatonin synthesis, daily activity rhythms, and sleep [1]. In mammals, eyes are absolutely required for photoreception [2, 3] and in humans light has additional effects on mood, concentration, and mental health [4]. For decades, rods and cones were thought to be the only photoreceptors in the retina. However, recently discovered retinal ganglion cells (RGCs) that express the photopigment melanopsin are themselves atypical photoreceptors [5, 6]. These intrinsically photosensitive RGCs (commonly known as ipRGCs) project to several brain nuclei that regulate non-image forming functions, such as the suprachiasmatic nucleus (SCN) to photoentrain circadian rhythms and the olivary pretectal nucleus (OPN) to control the pupillary light reflex (PLR) [6–9].

Initially, ipRGCs were thought to be a relatively uniform population of RGCs that can detect light levels (irradiance detectors) [5, 6]. Accumulating evidence indicates, however, that ipRGCs consist of several subtypes that are morphologically and physiologically distinct. These ipRGC subtypes contribute differently to non-image and image-forming behaviors. The purpose of this review is to discuss recent advances in our understanding of the

*To whom correspondence should be addressed. shattar@jhu.edu, TMSchmidt@jhu.edu. Editorial correspondence: Dr. Samer Hattar, Assistant Professor, Johns Hopkins University, Department of Biology, 3400 N. Charles Street/Mudd 227, Baltimore, MD 21218, Tel: 410-516-4231, fax: 410-516-5213, shattar@jhu.edu.

morphological and molecular diversity of ipRGCs and their functional roles in light-evoked behaviors.

Discovery and function of ipRGCs

The retina is a highly organized structure, where the cell bodies of distinct neuronal types reside in well-defined nuclear layers and make synaptic connections in two distinct plexiform layers (Figure 1). The classical photoreceptors, rods and cones, transform light energy into an electrical signal and convey information for both image- and non-image-forming visual functions through RGCs, the only neurons in the retina that send axonal projections to the brain. The classical view that rods and cones are the only photosensitive cells in the retina was challenged when it was discovered that blind individuals with degenerated rod and cone photoreceptors still show decreased melatonin production in response to light, despite having no conscious visual perception [2, 10]. Similarly, mice that lack rod and cone photoreceptors maintain photoentrainment and the pupillary light reflex [11–14]. The fact that humans and mice retain light-responsive behaviors when rods and cones are lost implies that additional non-rod/non-cone photoreceptors must exist in the mammalian retina [11, 13, 15]. The idea of other retinal cell types acting as photoreceptors was bolstered when the photopigment melanopsin (encoded by the gene *Opn4*), first identified from the dermal melanophores of *Xenopus laevis* [16], was subsequently localized to a small population of RGCs in the ganglion cell layer of the mammalian retina [15, 17].

Seminal work by Berson and colleagues in 2002 conclusively identified the elusive third class of retinal photoreceptors [5]. By retrogradely labeling SCN-projecting RGCs in the rat, they demonstrated that these cells are *bona fide* photoreceptors, with electrophysiological and spectral characteristics that match those observed behaviorally in measures of circadian photoentrainment [5]. Simultaneously, a study [6], which utilized a genetic approach to incorporate the *tau-LacZ* reporter gene into the mouse melanopsin locus ($Opn4^{tau\text{-}LacZ}$), led to the expression of the tau-β-galactosidase (β-gal) fusion protein in melanopsin-expressing cells [6]. β-gal, as revealed by X-gal (5-bromo-4-chloro-3-indolyl- beta-D-galactopyranoside) staining, was observed in approximately 700 out of 30,000 RGCs (~1–2% of RGC population). Double labeling with a melanopsin antibody revealed that this subset of RGCs had dendrites arborizing in the outer sublamina of the inner plexiform layer (IPL) of the retina. The tau signal peptide results in axonal localization of the associated β-gal enzyme, which upon X-gal staining allows the tracing of projections from these outer stratifying ipRGCs to the brain [6]. X-gal positive ipRGCs were found to project to a variety of brain regions involved in non-image-forming visual functions, such as the SCN and the OPN (Figure 1) [6, 9]. These initial studies led to the prevailing view that ipRGCs comprise a uniform population of neurons with outer stratifying dendrites that are involved mainly in circadian photoentrainment and the PLR. Recent studies indicate, however, that ipRGCs are composed of different subtypes, each with unique morphological and physiological properties and potentially distinct functional roles. The originally described cells are now known to correspond only to the M1 subtype [9].

Diversity of ipRGCs

The initial hint that ipRGCs are not a uniform population came from morphological studies using a highly sensitive melanopsin antibody. Immunolabeling revealed melanopsin-positive RGC dendrites in not only the outer sublamina but also in the inner sublamina of the IPL [15]. Arguably the most influential approach in advancing the study of ipRGC structure and function has been the use of genetic mouse models to label or ablate ipRGCs selectively. Detailed anatomical studies utilizing genetic mouse models have since distinguished several different morphological subtypes of ipRGCs (Figure 1 and Table 1). The three most well-

characterized subtypes are the M1 ipRGCs which stratify in the outermost sublamina of the IPL, the M2 ipRGCs which stratify in the innermost sublamina of the IPL, and the bistratified M3 ipRGCs, with dendrites in both inner and outer sublaminae [18–23]. In addition to differences in dendritic stratification, M2 cells have larger soma sizes and larger, more highly branched dendritic arbors than M1 cells [19, 20, 24]. Detailed study of the M3 ipRGCs has revealed that these bistratified cells, in contrast to other bistratified RGCs, show variability in the proportion of dendritic stratification in the inner and outer sublaminae [25]. The M3 ipRGCs are otherwise similar to M2 cells in the size and complexity of their dendritic arbors [25].

Further morphological diversity in the ipRGC population was discovered using the Cre/loxP system [24]. Specifically, crosses between a mouse line in which the melanopsin gene was replaced by Cre recombinase (*i.e.*, $Opn4^{Cre}$) and a Cre-dependent green fluorescent protein (GFP) reporter line (Z/EG) revealed two additional subtypes of ipRGCs, M4 and M5 (Table 1). Both of these cell types stratify in the inner sublamina of the IPL (Figure 1), but each has a unique morphology and can be differentiated from M2 cells using several quantitative parameters including soma size, total dendritic length and number of dendritic branchpoints [24]. M4 cells have the largest soma of any described ipRGC subtype, as well as larger and even more complex dendritic arbors than M2 cells [24]. In contrast, M5 ipRGCs have small, highly branched arbors arrayed uniformly around the soma (referred to as bushy dendritic arbors) [24]. These two new subtypes cannot be stained with even the most sensitive melanopsin antibody, although they do display a consistent yet weak intrinsic light response [24]. This suggests that the M4 and M5 ipRGCs do express functional photopigment.

The morphological diversity within ipRGCs raises the intriguing possibility that these subtypes are functionally distinct from one another. Extracellular recording from multi-electrode arrays (MEA) and calcium imaging were the first approaches to uncover physiological diversity in ipRGC light-evoked responses [26, 27]. However, in these initial studies, physiological properties were not directly correlated with specific cellular morphologies. This correlation of structure and function was accomplished when researchers recombineered a bacterial artificial chromosome (BAC) to label ipRGCs with enhanced GFP (eGFP) and directly demonstrated that distinct ipRGC morphological subtypes have different physiological properties [20, 21]. M1 cells have larger, more sensitive intrinsic light-evoked responses, whereas M2 cells have significantly smaller light responses that are at least one log unit less sensitive to light [20]. The dissimilar physiological properties of M1 and M2 cells are not limited to the intrinsic light response since M1 cells have higher input resistance, a more depolarized resting membrane potential, and spike at lower frequencies than M2 cells [20] (Table 1). In-depth electrophysiological analyses of the rare M3 cells demonstrated that these cells are remarkably invariable, which is surprising given the dendritic variability within this subtype [25] (Table 1). Use of the Cre/LoxP system to identify and record from M4 and M5 cells showed that these subtypes have smaller and even less sensitive intrinsic light responses than M2 cells [24]. Collectively, these findings reveal the diversity within ipRGCs in their level of dendritic stratification, dendritic morphology, membrane properties, and melanopsin expression levels.

Atypical ipRGC photoreceptors can also act as conventional RGCs by receiving extrinsic (synaptic) rod/cone input via bipolar cells [22, 28–30]. A well-established paradigm in the retinal field is that RGCs with dendrites stratifying in the outer sublamina of the IPL receive excitatory synaptic inputs from bipolar cells that respond to light decrements (OFF), whereas RGCs with dendrites stratifying in the inner sublamina of the IPL receive synaptic inputs from bipolar cells that respond to light increments (ON). Based on the stratification patterns of the ipRGC subtypes (M1, OFF; M2, M4 and M5, ON; M3, ON/OFF), the prediction is that M1 cells would receive synaptic inputs from the OFF pathway, M2, M4,

and M5 cells would receive input from the ON pathway, and M3 cells would receive input from both the ON and OFF pathways. Contrary to this expectation, both ON (i.e. M2), OFF (i.e. M1), and ON-OFF-stratifying (i.e. M3) ipRGC subtypes receive predominantly ON-input [31, 32], although, a very weak OFF input to M1 cells has been reported, but only under pharmacological blockade of amacrine cell inputs [30]. Anatomical studies have revealed the source of this unusual synaptic input: ON bipolar cells make *en passant* synapses with M1 ipRGC dendrites that stratify within the OFF sublamina of the IPL [33–35]. M1 ipRGC dendrites also colocalize with dopaminergic amacrine cells [33, 36]. Dopaminergic amacrine cells were recently implicated in guiding M1 cell dendrites to the OFF layer of the IPL [37]. Previous observations have also suggested that ipRGCs signal back to dopaminergic amacrine cells in the opposite direction of classical retinal circuits [38]. The function of this unusual reverse signaling between M1 ipRGCs and dopaminergic amacrine cells is unknown. The unexpected findings concerning M1 connectivity indicate that ipRGC circuitry in the retina is far more complex than previously appreciated.

Although all ipRGCs predominantly receive ON input, the degree of this input varies across ipRGC subtypes. In *Opn4*$^{-/-}$ mice, which lack intrinsic but not synaptic light input, the light responses of M2 and M3 cells are similar to those of wild-type animals [25, 32]. This suggests that the activity of M2 and M3 cells is shaped largely by synaptic input from the outer retina triggered by conventional photoreceptors. In contrast, intrinsic melanopsin-mediated signaling is predominately responsible for the light responses of M1 cells as the light responses of this subtype are severely attenuated in *Opn4*$^{-/-}$ animals [25, 32]. The experiments on Opn4 mutants were conducted on isolated retinas under light intensities that saturated the rod response, rendering rods non-functional during the course of the assay. This suggests that, at light intensities that saturate the rod response, cone input is critical for M2 and M3 responses, but is less important for M1 function. The contribution of cones to different ipRGC-dependent behaviors is therefore constrained by which subtypes integrate and relay cone signals to the relevant brain regions. Anatomical studies demonstrate a direct input from rod bipolar cells onto M1 ipRGCs [39] (Figure 1). Future studies using light intensities that do not saturate rods may reveal whether this direct rod bipolar input onto M1 cells provides a stronger rod input to M1 ipRGCs compared to non-M1 ipRGCs.

Phototransduction and chromophore regeneration in ipRGCs

In the absence of the melanopsin protein, ipRGCs lose the intrinsic light response [40]. Ectopic expression of melanopsin in heterologous cell systems further demonstrates its sufficiency to act as a functional photopigment [41–45]. Sequence analysis shows that the melanopsin photopigment closely resembles invertebrate rhodopsins, whose phototransduction pathway leads to the depolarization of photoreceptors through a Gq-mediated signaling cascade and opening of a transient receptor potential (TRP) channel [46]. This scheme differs from that of vertebrate opsins, whose phototransduction pathway involves a signaling cascade through the Gi/o family G protein transducin (Gt) that results in the closure of a non-selective cyclic nucleotide gated channel. This leads to a hyperpolarization of rods and cones [46]. Current evidence suggests that melanopsin couples to a Gq protein and signals through a membrane-bound component of the phospholipase C (PLC) signaling pathway to open a TRP channel [47–51]. However, the diversity of ipRGCs raises the possibility that different subtypes utilize unique phototransduction pathways. The mammalian genome contains several Gq proteins and TRP channels [52, 53] that could couple to melanopsin, which may account for the difficulty the field has experienced in identifying a specific phototransduction pathway using mouse mutants.

Another difference between mammals and *Drosophila* is how the chromophore regenerates after light stimulation. Mammalian rod and cone opsins are bound to 11-*cis* retinal (11-

CRAL). Light induces a conformational change of the chromophore from 11-CRAL to all-*trans* retinal (ATRAL), thereby activating the photopigment to initiate the phototransduction cascade. Subsequently, ATRAL dissociates from the photopigment and is converted back to 11-CRAL in the retinal pigmented epithelium (RPE) through an isomerase known as RPE65 [54]. 11-CRAL is then supplied back to the photoreceptor to regenerate the functional photopigment. In addition to the RPE, ATRAL is also regenerated into 11-cis retinol (11-CROL) in Müller glial cells and then transferred to cone photoreceptors where it is converted into 11-CRAL (Figure 2; reviewed in [54]). In *Drosophila* photoreceptors, the rhodopsin photopigment is bistable, meaning that after the chromophore has been converted from 3-hydroxy-11-*cis*-retinal (3-OH-11-CRAL) to 3-hydroxy-all-*trans*-retinal (3-OH-ATRAL) by blue light (short wavelength), subsequent exposure to orange light (long wavelength) restores sensitivity by converting the chromophore back to 3-OH-11-CRAL (Figure 2; reviewed in [55]).

Similar to *Drosophila* opsins, the melanopsin photopigment has been shown to be bistable in heterologous systems, as melanopsin renders cells photosensitive in the presence of ATRAL [42, 44]. Furthermore, in agreement with melanopsin bistability, the light-evoked responses of SCN neurons *in vivo* and the PLR in humans are both potentiated by exposure to long wavelength light [56, 57]. These findings indicate that chromophore regeneration is an intrinsic property of ipRGCs and does not require additional RPE or Müller cells as do the rods and cones of vertebrates.

Other data suggest that chromophore regeneration in ipRGCs may be more complicated. For example, in mammals, contradictory results about melanopsin bistability were obtained using MEAs [58]. Even in invertebrates recent work has challenged the sufficiency of photoreceptor bistability to regenerate chromophore, in that *Drosophila* photoreceptors also require pigment cells for chromophore regeneration [59] (Figure 2). Furthermore, it has been shown that in dark-adapted retinas, only 11-CRAL is isolated from ipRGCs. However, if bistability of melanopsin is solely responsible for chromophore regeneration in ipRGCs, then a mixture of both ATRAL and 11-CRAL should be present under dark-adapted conditions [60]. Thus, an additional light-independent mechanism for chromophore regeneration likely exists in ipRGCs. *RPE65*$^{-/-}$ mice that lack a key component of the light independent pathway to regenerate 11-CRAL from ATRAL [61] can be used to determine whether ipRGCs are dependent on this chromophore cycle to regenerate functional photopigment. *RPE65*$^{-/-}$ mice have no cone function, but retain weak rod function. Intriguingly, RPE65 mutant mice fail to phase shift [62], suggesting that chromophore regeneration is compromised in ipRGCs [62–64]. Introduction of a second mutation that ablates rods (*rdta)* [65] into RPE65 homozygous mutants enhances the sensitivity of circadian light responses [62], possibly due to an increased availability of chromophore to ipRGCs in the absence of rods. Thus, the message from these results is that melanopsin may utilize the retinoid cycle as well as bistability to maintain chromophore bound to the photopigment (Figure 2).

Melanopsin is detected in M1 and M2 subtypes in wild-type mouse retinas stained with an antibody against the protein [15, 62]. However, only M1 cells are labeled in retinas from *Rpe65*$^{-/-}$ mice [62]. One explanation for this difference in immunodetection is that melanopsin localization at the cell membrane may vary between ipRGC subtypes depending on their access to chromophore. In *Drosophila*, the chromophore is essential for localizing opsins to the photoreceptor membrane [66, 67]. The reduction of melanopsin in the M2 cells of *Rpe65*$^{-/-}$ mutants mice may indicate a similar dependence on the chromophore cycle to maintain membrane-localized photopigment in mammals. Alternatively, the lower expression level of melanopsin in M2 cells could render them more susceptible to decreased chromophore availability compared to M1 cells [9, 18–20]. The RPE, which provides

chromophore to both rods and cones, is far-removed from the ganglion cell layer, where ipRGCs reside, and therefore is an unlikely source of chromophore. However, as discussed above, Müller glia also supply cones with chromophore [68, 69] (Figure 2) and, given their close proximity to ipRGCs, are the most likely source of chromophore to these neurons [22]. Future studies should directly test whether Müller glia contribute to chromophore regeneration in ipRGCs.

Axonal projections of ipRGC subtypes

Using the *Opn4*$^{tau\text{-}LacZ}$ line, M1 ipRGCs were shown to project to the SCN and the shell of the OPN for mediation of circadian photoentrainment and the PLR, respectively [6, 8, 9]. In addition, M1 ipRGCs project to other brain regions involved in circadian behaviors such as the intergeniculate leaflet (IGL) and the ventral lateral geniculate nucleus (vLGN) [8, 9], and to other structures such as the supraoptic nucleus, ventral subparaventricular zone, medial amygdala, and lateral habenula [8, 9]. Non-M1 ipRGC subtypes innervate additional targets in the brain [18]. For example, the M1 ipRGCs innervate only the shell of the OPN, the relay station for the PLR [70]. However, crossing the *Opn4*^{Cre} line with a Cre-dependent alkaline phosphatase (AP) reporter line (Z/AP) that allows for the axonal tracing of all ipRGCs, revealed additional innervation of the core of the OPN by non-M1 cells [24] (Figure 1). The functional significance of non-M1 cell projections to the OPN core is unclear. Non-M1 cells also contribute substantial synaptic input to the dorsal lateral geniculate nucleus (dLGN), a structure involved in image-forming vision, and the superior colliculus (SC), the mammalian equivalent to the optic tectum [24] (Figure 1). In comparison, M1 cells only send a few fibers to these regions [9, 24]. Targeting of non-M1 ipRGCs to the SC and dLGN suggests that they play an unexpected role in vision. Accordingly, avoidance of light by mouse pups attributed to the SC is melanopsin-dependent [71, 72] and most likely mediated by non-M1 ipRGCs (Figure 3), because at the same developmental stage, only non-M1 ipRGCs target the SC [73].

Behavioral outputs of ipRGCs

The melanopsin field has expanded dramatically in the last few years, providing a deeper understanding of the molecular, cellular, and connectivity features of ipRGCs. Furthermore, behavioral studies have highlighted the diverse and important role of ipRGCs in various light-driven behaviors (Figure 3). Mechanistic insights from this atypical photoreceptive system may also apply more broadly toward understanding the complexity of circuits and modulation of behavior in the central nervous system.

Animals lacking either the intrinsic light response of ipRGCs or lacking rods and cones are still able to photoentrain and constrict their pupils to light [13, 40], indicating that conventional photoreceptors and ipRGCs both contribute to circadian photoentrainment and PLR (Figure 3). In fact, rod/cone and melanopsin driven light responses have complementary roles in non-image forming visual functions. In the absence of rods and cones, melanopsin-mediated light responses contribute to the pupillary reflex only at high light intensities [13]. Conversely, in melanopsin mutants, rod and cone mediated pupil constriction is normal at low light intensities but is attenuated at high light intensities [13, 40, 74]. Similar findings were observed for circadian photoentrainment, where rods are necessary for entrainment at low light levels, but melanopsin-mediated light responses are the major contributor to entrainment at high light levels [74, 75].

To understand the role of ipRGCs in non-image forming functions, it is necessary to eliminate the cells themselves, thus removing both melanopsin-mediated responses and rod/cone responses conveyed to brain targets via ipRGCs. This was achieved in three independent studies, using Saporin-conjugated melanopsin antibody [76], melanopsin-driven

diphtheria toxin [77], or Cre-activated diphtheria toxin receptor [78] to ablate ipRGCs, while leaving other RGCs and vision intact. Despite the diversity of methods, the results consistently showed severe deficits in circadian photoentrainment and PLR, demonstrating that ipRGCs are essential for the mediation of non-image forming behaviors and are the conduit for both melanopsin-mediated and rod/cone-mediated light signaling for non-image forming behaviors [76–78].

The ability of rod/cone photoreceptors to signal through ipRGCs to influence non-image forming visual functions even in the absence of the melanopsin protein raises the question of whether there is a unique contribution of melanopsin phototransduction to non-image forming visual functions? The contribution of the intrinsic light response can be quantified for the PLR by comparing animals with ipRGCs partially ablated to mutants just lacking the melanopsin protein. Wild type animals fully constrict their pupils under high light intensity, whereas in the absence of the melanopsin protein the pupil does not achieve full constriction [40]. Surprisingly, even with the loss of more than 80% of ipRGCs, full pupil constriction under high light intensity could be observed provided the melanopsin protein was still present in the remaining ipRGCs [77]. Experiments such as these show that a fraction of ipRGCs that retain the melanopsin photopigment can drive pupil constriction better than a complete repertoire of ipRGCs that lack melanopsin and solely depend on rod and cone input. These data illustrate the importance of melanopsin-based photoreception to behavior at high light levels.

An additional role for melanopsin phototransduction has been discovered in the induction and regulation of sleep (Figure 3). Sleep is controlled by homeostatic and circadian factors [79]. The homeostatic factor accumulates during wakefulness and then dissipates during sleep, hence determining the quality and amount of sleep. The circadian clock determines the time at which sleep should occur during the daily cycle. One long held assumption is that light effects on sleep are mediated only via changes in the circadian clock. However, several research groups have shown that light given at night induces sleep directly [80, 81] through ipRGCs [82]. Light presented to melanopsin null animals fails to induce sleep, which indicates that the contribution of rods and cones to sleep regulation is minimal although it may be gated by the circadian clock [81]. In addition to the direct light effects on sleep, the amount of sleep (homeostat) is also affected when the intrinsic light response of ipRGCs is eliminated [81]. This raises the intriguing idea that the melanopsin-mediated light response plays a central role in not only sleep induction, but also sleep modulation [83].

Mice are nocturnal animals and light induces sleep, whereas in humans, darkness promotes sleep. To simulate the effect of light on alertness in humans, a recent study investigated the effect of darkness on mouse behavior during the day [82]. Lights were turned off for 3 hours during the day on mice placed under a 12:12 hour light:dark cycle. The absence of light in the day induced alertness in wild type mice corresponding to a time when they were usually asleep, but did not alter the behavior of mutants that lacked ipRGCs [82]. Involvement of these cells in alertness was surprising, considering that ipRGCs respond strongly to light ON but not light OFF signals. The important conclusion is that increased alertness is due to repression of ipRGC signaling, rather than a *bona fide* OFF signal from rods and cones [82]. The results of this work further suggest that ipRGCs are continuously signaling the presence of light to the brain and could explain why shorter day length (i.e., presumably weaker melanopsin signaling) often leads to seasonal affective disorder (SAD), a seasonal form of depression. Consistent with this idea, a melanopsin gene variant has been associated with an increased risk of SAD [84].

While the major function of ipRGCs is to subserve non-image forming behaviors, the innervation pattern of non-M1 cells to the dLGN and SC implicates ipRGCs in image-

forming vision [24] and light avoidance behavior [71] (Figure 3). Consistent with these innervation patterns, when rod/cone phototransduction is eliminated, mice that retain melanopsin-expressing ipRGCs still possess a rudimentary form of pattern vision [24]. Specifically, these mice show induction of the immediate early gene, *c-fos*, in the visual cortex following presentation of patterned, but not diffuse, light stimuli [24]. Furthermore, melanopsin-mediated responses have been detected in the dLGN and visual cortex by MEA electrophysiological recording and intrinsic optical imaging of the dLGN and visual cortex [85]. Given earlier reports that ipRGCs receive color information from cones and project to the LGN in monkeys, ipRGCs could also play a role in pattern and color vision in primates [29]. Consistent with this possibility, a single case of a human patient who lacks rods and cones showed a rudimentary visual awareness to the wavelength of light most effective at activating melanopsin [86].

It has become increasingly clear that ipRGCs relay light information to influence a myriad of neural processes and behavioral responses. For example, the segregation and refinement of retinogeniculate afferents in the dLGN was thought to be only dependent on spontaneous retinal waves and to be independent of visual experience [87]. However, a recent study has shown that light input through melanopsin-mediated phototransduction modulates the spiking of the retinal waves and enhances the segregation of retinogeniculate afferents [87]. Another recent example is a study on humans demonstrating a thalamocortical function for ipRGCs in light regulation of migraine headache pain [88]. How the melanopsin-expressing cells of the retina accomplish such diverse physiological and behavioral tasks will require considerably more information about the specialized roles individual ipRGC subtypes play.

Future directions

Although M1 ipRGCs have been well studied for irradiance detection, the function of non-M1 cells in light-dependent behaviors and their importance in image forming vision are poorly understood. One pressing question is whether the intrinsic light response of ipRGCs contributes to image formation when functional rods and cones are present. Melanopsin intrinsic light responses convey environmental light levels to the visual cortex even in animals with intact rod/cone function [85]. Determining whether vision is impaired in melanopsin mutant mice is the next logical step. Furthermore, to develop a model of how light detection by ipRGCs is integrated with the output from rods and cones to modulate vision, it will be essential to determine the ipRGC subtypes and the circuitry of how ipRGCs signal to the visual cortex. M1 cells do not directly project to image centers in the brain, but one possibility is that they may indirectly modify cortical activity and visual processing by modulating dopaminergic amacrine cells.

The discovery of the morphological diversity of ipRGCs demonstrates that understanding a seemingly complex neuronal system requires parallel molecular, physiological, genetic, circuitry and behavioral approaches. By selectively eliminating specific cells, one can assess aspects of physiology and behavior that are influenced by a single subtype. To do this, however, identifying distinct markers for individual subtypes of ipRGCs will be a critical next step. For example, a recent study determined that subpopulations of ipRGCs can be molecularly defined by their expression of the Brn3b transcription factor [86]. Brn3b is expressed in all non-M1 ipRGCs but only in a fraction of M1 cells, suggesting that the M1 subtype actually consists of two distinct sub-populations (Figure 1). Additionally, Brn3b-positive M1 ipRGCs do not innervate the SCN, although they project to all other M1 ipRGC brain targets (Figure 1). Consistent with this innervation pattern, ablation of Brn3b-positive ipRGCs severely impairs the PLR, but does not affect circadian photoentrainment [89]. Which transcription factor(s) are responsible for specifying Brn3b-negative ipRGCs and how M1 sub-populations are differentially targeted to regions of the brain are issues that

remain to be resolved. However, similar genetic strategies using cell-type specific tools will eventually uncover the complete circuitry and specialized functions of ipRGC subtypes.

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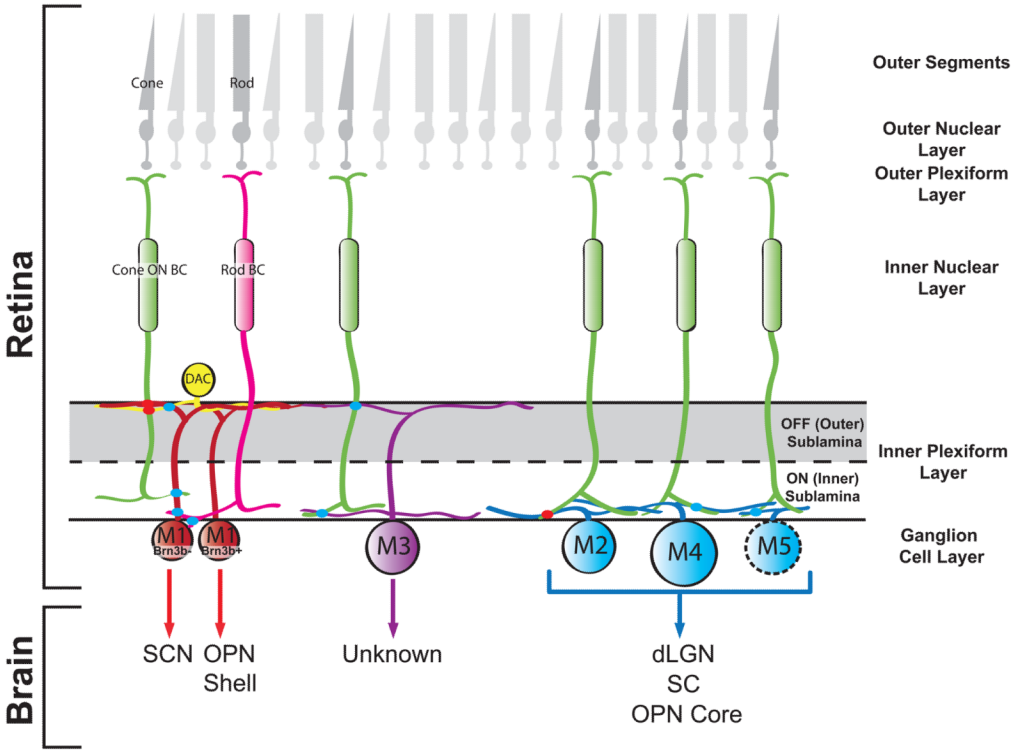

Figure 1.

Schematic diagram illustrating the connectivity and location of the five distinct morphological subtypes (M1–M5) of ipRGCs and projections to their predominant targets in the brain. The mammalian retina contains three nuclear layers (outer, inner and ganglion) and two plexiform layers (outer and inner). Synaptic connections between photoreceptors (rods and cones), horizontal and bipolar cells (BC) occur in the outer plexiform layer, whereas synaptic connections between bipolar, amacrine and ganglion cells occur in the inner plexiform layer (IPL). The outer nuclear layer contains the classical photoreceptors rods and cones (shown in gray). The inner nuclear layer (INL) contains horizontal cells (not shown), bipolar, and amacrine cells, of which only dopaminergic amacrine cells (DAC) are shown. The ganglion cell layer contains conventional ganglion cells (not shown) and the ipRGCs. For simplicity, M1 ipRGCs displaced to the INL [6] are not depicted in this diagram. M1 ipRGCs stratify in the OFF sublamina (red); M2, M4, M5, stratify in the ON sublamina (blue); and M3, stratify in the ON and OFF sublamina (purple) of the IPL of the retina. M4 ipRGCs have the largest cell body size, and M1 cells have smaller body size than M2–M4 cells [20, 24, 25]. The cell body size of M5 is not known (dotted line). The proportion of ON and OFF stratification in M3 ipRGCs varies considerably between cells [25]. Recent findings suggest that the M1 subtype consists of two distinct subpopulations that are molecularly defined by the expression of the Brn3b transcription factor [89]. Red dots indicate synaptic connections for which both functional and anatomical evidence exists [22, 28, 32, 33, 35]. Blue dots indicate synaptic connections for which either functional or anatomical evidence exists [24, 25, 38, 39]. ipRGC subtypes project to distinct non-image and image-forming nuclei in the brain [9, 24]. M1cells predominantly project to non-image forming centers such as the suprachiasmatic nucleus (SCN) to control circadian photoentrainment and the shell of the olivary pretectal nucleus (OPN) to control the pupillary light reflex. M1 innervation of brain targets could be further divided by Brn3b expression with Brn3b-negative (Brn3b-) M1 ipRGCs predominantly projecting to the SCN [89]. M3 brain targets are completely unknown at this time. M2, M4 and M5 are included

together since no specific genetic marker exists for a single subtype. Collectively, they project to image forming areas in the brain such as the lateral geniculate nucleus (LGN) and the superior colliculus (SC), but also to the core of the OPN of which no specific function is assigned to this brain region [24, 85]. Retrograde analysis confirms that M2 cells project minimally to the SCN and strongly to the OPN [18].

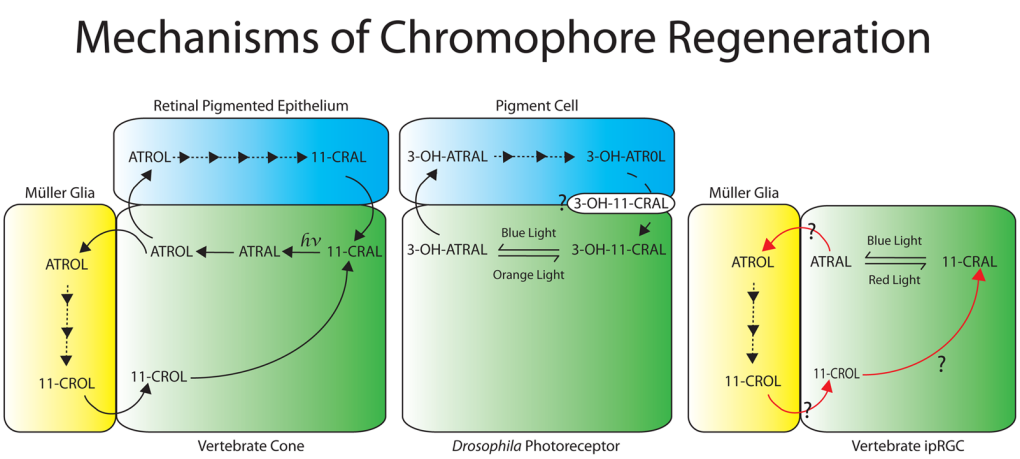

Figure 2.

A proposed pathway for chromophore regeneration in ipRGCs. This figure provides a simplified summary of chromophore regeneration (the visual cycle) for vertebrate cones [rods use only the retinal pigmented epithelium (RPE) pathway], *Drosophila* photoreceptors, and a proposed pathway for ipRGCs. Photopigments of rods and cones composed of the opsin and the chromophore (11-cis retinal; 11-CRAL) respond to light through the absorption of photon energy by the 11-CRAL which is then converted to all trans retinal (ATRAL) leading to the activation of the opsin's G-protein pathway. ATRAL dissociates from the opsin and is first converted to the alcohol form (ATROL) in the photoreceptors and then transported to the RPE. In the RPE, the ATROL is converted back through an RPE65 dependent multistep process to 11-CRAL. Recent studies showed that cones also use Müller glia to convert ATROL to 11-CROL, which is then converted to 11-CRAL within cones. Dotted line indicates multiple steps that are not depicted in this figure for simplicity. Note that only in *Drosophila* and ipRGCs, the *trans* form of the chromophore (3-OH-ATRAL and ATRAL, respectively) can be converted back to the *cis* form (3-OH-11-CRAL and 11-CRAL, respectively) in the photoreceptors by exposure to long wavelength light (orange light for *Drosophila* photoreceptors and red light for ipRGCs). Recent discovery in *Drosophila* revealed an alternative pathway that depends on pigment cells for chromophore regeneration, where similar to vertebrate rods and cones, all trans form is converted to the cis form outside the photoreceptor itself. The site of conversion from the alcohol to the cis form is currently unknown, hence depicted by a question mark. We propose that ipRGCs also use a chromophore regeneration pathway requiring the Müller glia, similar to cones. Red lines indicate proposed steps for chromophore regeneration in ipRGCs and question marks indicate that these pathways have not been demonstrated, although the dependence of ipRGCs on RPE65 is well documented [62].

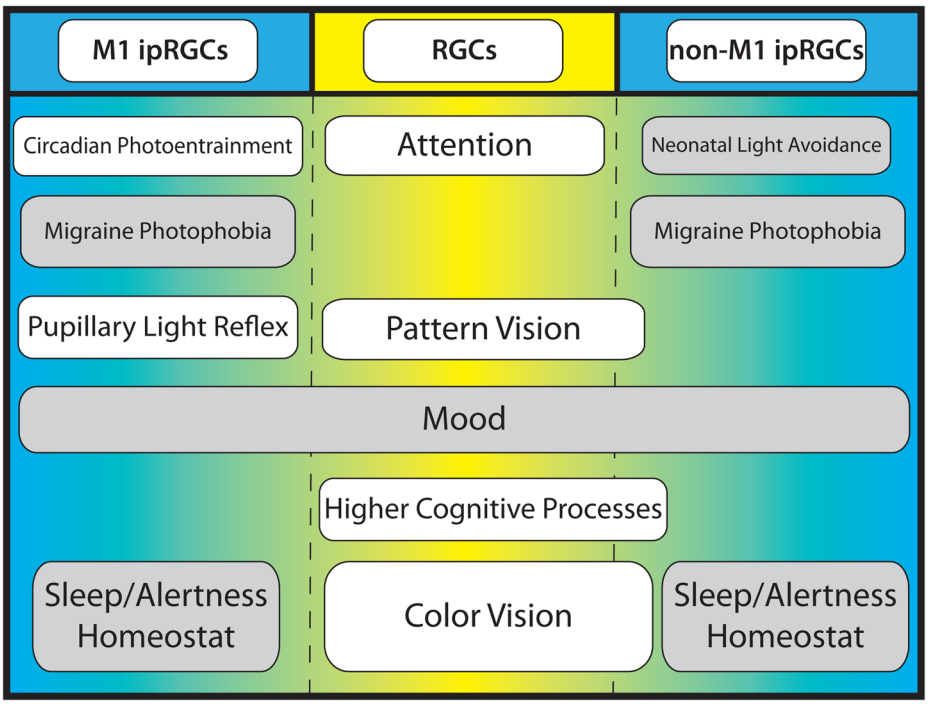

Figure 3.

Schematic diagram illustrating the behavioral functions attributed to ipRGCs (M1 and non-M1) and the relative contribution of conventional RGCs to various behaviors. White boxes indicate functions that have been demonstrated to depend on a specific class of ipRGC and/or RGC. Gray boxes indicate functions that are ipRGC dependent, but the relative contribution of individual ipRGC subtypes has not been fully resolved. The role of M1-Brn3b-negative ipRGCs for circadian photoentrainment and the M1-Brn3b-positive ipRGCs for pupillary light reflex is the best documented [89]. The individual contribution of M2–M5 ipRGCs to specific functions is currently unknown and these subtypes are hence grouped together as non-M1 ipRGCs. Conventional RGCs, secondarily through rods and cones, influence higher cognitive functions, attention, pattern and color vision. Based on retinal recordings and brain innervation patterns, non-M1 cells may contribute to higher cognitive functions, pattern and color vision [24, 29, 85, 86]. Mood is known to be affected by light (as in seasonal affective disorder [SAD]). However, the contributions of ipRGCs and conventional RGCs to mood are not yet resolved, although an association between a mutant variant of melanopsin and SAD was recently described [84]. Melanopsin phototransduction is capable of relaying light signals to influence migraine photophobia in the absence of classical vision, but the contribution of each subtype is currently unknown [88]. For both sleep and alertness, melanopsin phototransduction is absolutely required for the light and dark effects on both functions, but again the individual contribution of ipRGC subtypes is unknown [82]. Finally, neonatal light avoidance requires melanopsin-based photoreception [71]. Based on morphological analysis during development [73], the neonatal avoidance task is most likely mediated by non-M1 ipRGCs, although this has not yet been shown directly.

Table 1

Morphological and physiological properties of ipRGC subtypes

	Morphology				Physiology		
	Molecular Identity	**Dendritic Stratification**	**Dendritic Field**	**Soma Size**	**Intrinsic Light Response**	**Synaptic Light Response(cones)**	**Membrane Properties**
M1	Brn3b + and Brn3b − Strongly Opn4+ $Opn4^{tau\text{-}lacZ}$ + $Opn4^{Cre}$ +	Outer	<M2, M4	<M2, M4	Large and most sensitive	• ON- predominant, small and sustained. • Very weak OFF under pharmacological manipulations	High input resistance, depolarized resting membrane potential, spike at low frequencies
M2	Brn3b + Weakly Opn4 + $Opn4^{tau\text{-}lacZ}$ – $Opn4^{Cre}$ +	Inner	>M1, <M4	<M4	Small and low sensitivity	ON-predominant, large and sustained	Low input resistance, hyperpolarized resting membrane potential, spike at high frequencies
M3	Brn3b + Weakly Opn4+ $Opn4^{tau\text{-}lacZ}$ + (?) $Opn4^{Cre}$ +	Inner-Outer	=M2	=M2	Small and intermediate sensitivity	ON-predominant, large and sustained	Low input resistance, hyperpolarized resting membrane potential
M4	Brn3b + Opn4 − $Opn4^{tau\text{-}lacZ}$ – $Opn4^{Cre}$ +	Inner	Largest	Largest	Small and insensitive	Unknown	Unknown
M5	Brn3b + Opn4 − $Opn4^{tau\text{-}lacZ}$ – $Opn4^{Cre}$ +	Inner	Small and symmetrical	Unknown	Small and insensitive	Unknown	Unknown

Brn3b + and Brn3b -: Brn3b positive and Brn3b negative, respectively

Opn4 +: immunostains with melanopsin antibody

Opn4 −: no staining with melanopsin antibody

$Opn4^{tau\text{-}lacZ}$ + and $Opn4^{tau\text{-}lacZ}$ –: labeling or lack of labeling with X-gal, respectively $Opn4^{Cre}$ +: labeling by melanopsin Cre line