

REVIEW



Diversity of intrinsically photosensitive retinal ganglion cells: circuits and functions

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Abstract

The melanopsin-expressing, intrinsically photosensitive retinal ganglion cells (ipRGCs) are a relatively recently discovered class of atypical ganglion cell photoreceptor. These ipRGCs are a morphologically and physiologically heterogeneous population that project widely throughout the brain and mediate a wide array of visual functions ranging from photoentrainment of our circadian rhythms, to driving the pupillary light reflex to improve visual function, to modulating our mood, alertness, learning, sleep/wakefulness, regulation of body temperature, and even our visual perception. The presence of melanopsin as a unique molecular signature of ipRGCs has allowed for the development of a vast array of molecular and genetic tools to study ipRGC circuits. Given the emerging complexity of this system, this review will provide an overview of the genetic tools and methods used to study ipRGCs, how these tools have been used to dissect their role in a variety of visual circuits and behaviors in mice, and identify important directions for future study.

Keywords Intrinsically photosensitive retinal ganglion cells · Melanopsin · Non-image-forming visual pathway · Pattern vision · Circadian · Retina

Abbreviations

AAV	Adeno-associated virus	iPLR	Intrinsic pupillary light reflex
aDTA	Attenuated version of the diphtheria toxin	ipRGC	Intrinsically photosensitive retinal ganglion cell
AH	Anterior hypothalamus	LH	Lateral hypothalamic area
AP	Alkaline phosphatase	LHb	Lateral habenula
BST	Bed nucleus of the stria terminalis	MA	Medial amygdaloid nucleus
CNO	Clozapine N-oxide	PACAP	Pituitary adenylate cyclase-activating polypeptide
cOPN	Olivary pretectal nucleus core	PAG	Periaqueductal gray
CTB	Cholera toxin B subunit	PDE	Phosphodiesterase
dLGN	Dorsal lateral geniculate nucleus	PHb	Perihabenular nucleus
DREADDs	Designer receptors exclusively activated by designer drugs	PLC	Phospholipase C
DTA	Diphtheria toxin	PLR	Pupillary light reflex
GABA	Gamma-aminobutyric acid	pSON	Peri-supraoptic nucleus
GFP	Green fluorescent protein	R26	Rosa26
iDTR	Inducible diphtheria toxin receptor	RGC	Retinal ganglion cell
IGL	Intergeniculate leaflet	SC	Superior colliculus
IPL	Inner plexiform layer	SCN	Suprachiasmatic nucleus
		sOPN	Olivary pretectal nucleus shell
		SPZ	Subparaventricular zone
		TRPC	Transient receptor potential channels
		vLGN	Ventral lateral geniculate nucleus
		VLPO	Ventral lateral preoptic area
		X-gal	5-Bromo- 4-chloro-3-indolyl-b-D-galactopyranoside

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Introduction

The day/night cycle is one of the most predictable changes in our daily existence, and has resulted in the evolution of robust systems to anticipate and respond to changes in environmental light. Light is required for the ability to navigate our environments, find prey, and perceive the environment around us. Beyond its role in forming image vision, light has numerous subconscious effects on our physiology and behavior ranging from photoentrainment of our circadian rhythms, to driving the pupillary light reflex (PLR) to improve visual function, to modulating our mood, alertness, learning, sleep, and thermoregulation. Despite this stunning diversity in light-evoked functions, little is understood about the specific circuits from the eye to the brain underlying them. This deficit stems in part from a lack of genetic tools to label, manipulate, and ablate single types of the retinal projections neurons: the retinal ganglion cells (RGCs).

RGCs receive synaptic inputs relayed from cones and rods and send visual information through their axons to approximately 50 brain regions, each of which processes different information about the visual environment. The melanopsin-expressing RGCs represent a specialized class of RGC that is capable of responding to light in the absence of rod and cone input (Fig. 1). These intrinsically photosensitive (ip)RGCs are a morphologically and physiologically heterogeneous population that project widely throughout the brain and mediate a wide array of

visual functions from circadian photoentrainment to contrast sensitivity [1–5]. The presence of melanopsin as a unique molecular signature of ipRGCs has allowed for the development of a vast array of molecular and genetic tools to study ipRGC circuits. Given the emerging complexity of this system and the nuances of the tools that have been developed to study them, the goal of this review is to highlight the diversity of ipRGCs, their roles in behavior, the tools commonly used to manipulate, label, and ablate specific ipRGC subtypes, and identify important directions for future study.

ipRGC diversity

ipRGCs, like all RGCs, receive inputs from the outer retina through bipolar cells and amacrine cells (Fig. 1). The variability of the photoresponses from different ipRGC subpopulations is the result of the integration of the intrinsic melanopsin responses, and extrinsic synaptic inputs which are influenced by the location of the dendritic stratification and the degree of integration with the rod and cone circuitry. There are currently six known subtypes of ipRGC (M1–M6) (Fig. 2a). These subtypes are identified primarily by their morphological characteristics including level of dendritic stratification within the retinal inner plexiform layer (IPL) as well as their dendritic arbor size and complexity. M1 ipRGCs stratify in the OFF sublamina of the IPL, have the smallest soma size of any ipRGC subtype, and have relatively small and simple dendritic arbors (Fig. 2a). M2

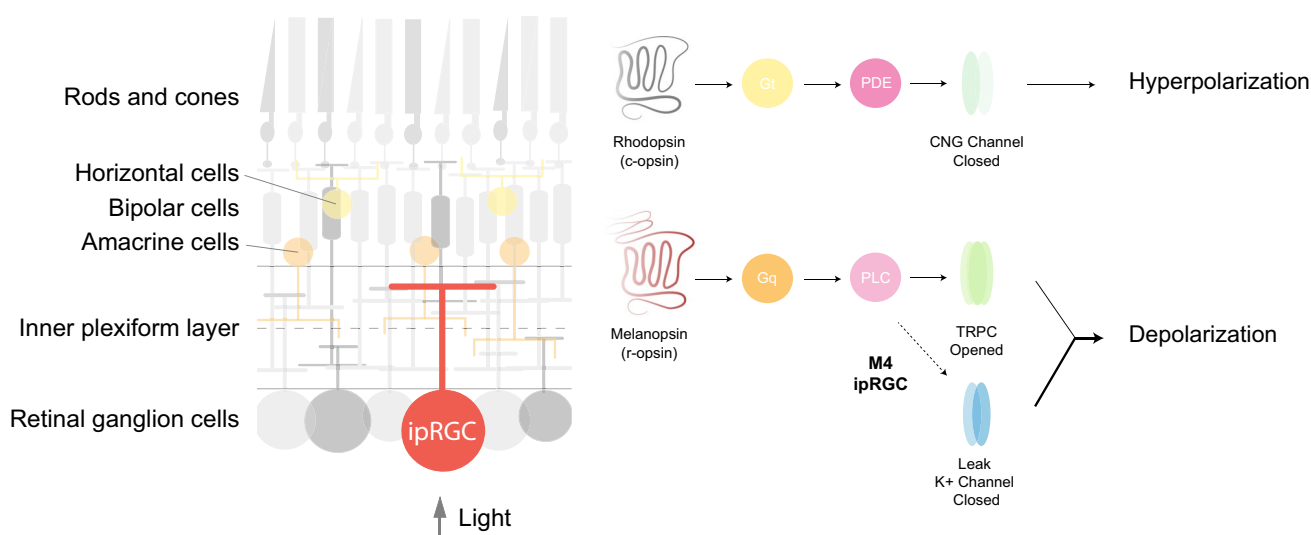


Fig. 1 Simplified schematic representation of a cross-retinal section (left). Signaling cascade of rhodopsin and melanopsin molecules (right). When light activates rhodopsin, Gt (transducin) triggers an activation of phosphodiesterase (PDE) that closes Na^+ channels and induces hyperpolarization. In M1 ipRGCs, melanopsin activates a Gq

protein that triggers the activation of phospholipase C (PLC) followed by opening of transient receptor potential channels (TRPC) which give rise to membrane depolarization [24, 92, 101, 136, 137]. In addition to TRPC cascade, in M4 ipRGCs, melanopsin increases cellular excitability via closure of potassium leak channels [55]

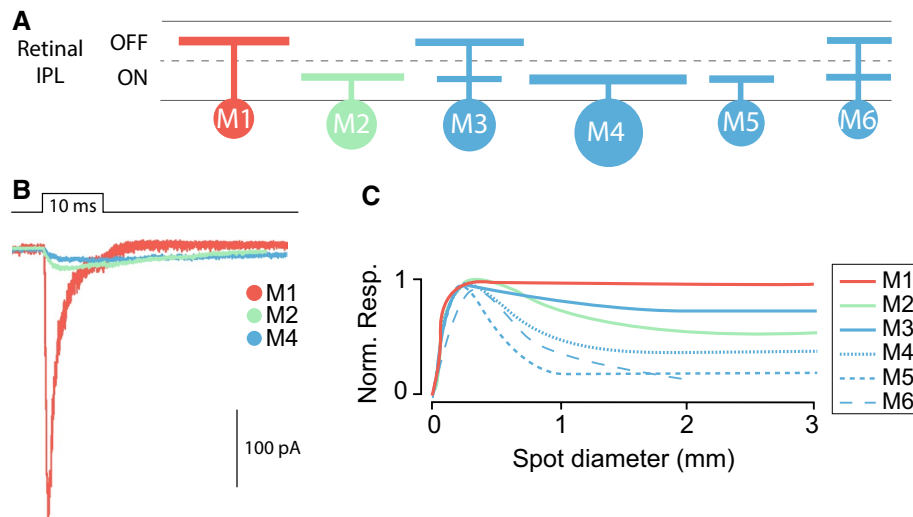


Fig. 2 ipRGC diversity. **a** Schematic representation of M1–M6 ipRGC stratification in the ON and/or OFF sublaminae of the retinal inner plexiform layer (IPL). **b** Schematic representation of melanosin-driven intrinsic electrophysiological responses of M1, M2, and M4 ipRGCs (drawn based on [20]). **c** Schematic representation of

the M1–M6 ipRGCs receptive fields. M1 ipRGC receptive field lacks surround antagonism of the inhibitory input nullifying the surround of the excitatory input. M2–M6 ipRGCs have center–surround-organized receptive fields (drawn based on [9, 10])

ipRGCs have dendrites that stratify in the ON sublamina, and have larger somata and larger, more complex dendritic arbors than M1 cells [6]. M3 ipRGCs are similar to M2 cells in terms of soma size and dendritic arbor size and complexity, but have dendrites that bistratify in both the ON and OFF sublaminae of the IPL. M4 ipRGCs have the largest soma size and largest dendritic arbors of all ipRGCs, ramify in the ON sublamina of the IPL, and have the lowest levels of melanosin expression [5, 7, 8]. M4 ipRGCs are synonymous with ON sustained alpha RGCs and are also the only ipRGC subtype that is labeled with an antibody to a non-phosphorylated form of the neurofilament heavy chain protein (SMI-32). ON-stratifying M5 cells have small and highly branched dendritic arbors [7], while M6 cells have the smallest soma size of any ipRGC subtype, and small, highly branched, bistratified dendritic arbors [9].

In addition to their diverse morphological properties, each ipRGC subtype exhibits distinct responses to light. This diversity in output response between M1 and M6 ipRGCs stems from the differential ratio of melanosin versus rod and cone contributions, and from the morphological differences between each subtype. The magnitude, latency, and duration of the light response varies across M1–M6 ipRGCs in an intensity dependent manner. The intrinsic photoreponses of M1 cells have lower threshold, higher amplitude, and faster responses than those of non-M1 ipRGCs (Fig. 2b). The receptive field of M1 cells also lacks surround antagonism of synaptic inputs [10]. M2–M6 ipRGCs, however, have center–surround-organized receptive fields which suggests a capacity to detect spatial contrast (Fig. 2c).

Indeed, the response of M4 ipRGCs (i.e., ON alpha RGCs) to spatial contrast has been well studied over many decades because these cells have been identified in multiple species [5, 7, 11–15].

Beyond their diverse signaling properties, ipRGC subtypes also project to distinct downstream targets in the brain. M1 cells project primarily to non-image-forming targets such as the suprachiasmatic nucleus (SCN) (to drive circadian photoentrainment), and the olivary pretectal nucleus (OPN) (to drive the pupillary light reflex), among other targets [16, 17]. M4–M6 cells project to brain nuclei involved in image-forming vision such as dorsal geniculate nucleus (dLGN) and superior colliculus (SC). Interestingly, M2 cells project to both image forming (SC and dLGN) and non-image-forming brain areas (SCN and OPN) [7, 17–20]. While M3 ipRGCs have been reported to project to the SC, no other targets have been identified, likely because this subtype has not been as extensively studied [10]. Advances in the development of genetic tools, in combination with viral neuronal tracers, have allowed researchers to map ipRGC–brain projections and determine their role in shaping image forming and non-image-forming behaviors.

Though not the focus of this review, ipRGC subtypes have been noted in other mammalian species. M1–M5 ipRGCs have been identified in rat retinas, and seem to exhibit similar morphological variations to the parallel subtype in mouse [2, 20–29]. Similarly, ON- and OFF-stratifying ipRGCs have been identified in the macaque, marmoset, and human retina [30, 31], with some work, suggesting that there are M1, M2, M3, and M4 ipRGCs in the human retina [32].

Moreover, functional data from multielectrode recordings in human donor retinas have identified three classes of ipRGC responses that may correspond to specific ipRGC subtypes [33]. Thus, though much remains to be done regarding the properties and behavioral functions of ipRGC subtypes in species other than mouse, data thus far points to conservation of ipRGC subtypes from mice to humans, and potential parallels in behavioral roles [30, 34–37].

A diverse toolkit for studying ipRGC circuits

In ipRGCs, the photopigment melanopsin is encoded by the gene *Opn4*. Researchers have capitalized on this specificity to generate a host of immunohistochemical and genetic tools in mice to label, manipulate, and ablate ipRGCs. This arsenal of approaches has allowed researchers to study visual circuits with higher resolution than has been possible for many RGC types. Each of the available tools to label and/or manipulate ipRGCs has its own benefits and drawbacks, and many label or ablate specific subsets of ipRGC subtypes. In the following section, we will review many of the major tools used in ipRGC research, and provide an overview of the nuances, benefits, and drawbacks of each.

ipRGC labeling

All ipRGCs express the melanopsin protein. However, M1 ipRGCs have the highest levels of melanopsin, followed by M3, then M2, and, finally, M4–M6 ipRGCs [6, 9, 38, 39] (Fig. 3). Immunohistochemistry for the melanopsin protein was one of the first methods used to identify ipRGCs [2, 40] and is still a major method used to label ipRGC distributions across the retina and stratification within the IPL. Though this method is straightforward, it is limited by the inability of the antibody to label ipRGCs that express low levels of melanopsin. Currently, the most sensitive melanopsin antibody detects the N-terminus of the melanopsin protein, and only labels M1–M3 ipRGCs. It labels very few-to-no M4–M6 ipRGCs [7, 38, 40] (Fig. 3). Given that M1 cells express the highest levels of melanopsin, they show the brightest immunolabeling and their dendrites in the OFF sublamina are readily visible. The M2 and M3 ipRGCs show detectable, but less robust, labeling of the cell body, and the dendrites of some M2 and M3 cells can be traced to the ON (or ON and OFF in the case of M3 ipRGCs) sublamina of the IPL. It is possible to detect melanopsin immunolabeling in M4–M6 ipRGCs when used in conjunction with the TSA amplification technique. Using this amplification approach, M4–M6 ipRGCs show detectable levels of melanopsin in the soma, but the labeling tapers off in the proximal dendrites [9, 20, 41].

There are now multiple genetic tools to label ipRGCs. Depending on the sensitivity of each tool, different ipRGC subtypes are labeled. Two knock-in lines, *Opn4^{LacZ}* and *Opn4^{Cre}*, and three BAC transgenic lines, two *Opn4-GFP* lines and one *Opn4-tdTomato* line, have been developed (Fig. 3). In the *Opn4^{LacZ}* line, the melanopsin gene has been replaced with the gene for tau-lacZ. Tau-lacZ codes for a protein containing the beta-galactosidase enzyme fused to a signal sequence from tau that preferentially targets the protein to the axon (Fig. 3). This allows for visualization of ipRGC axons at their downstream brain targets using immunolabeling for B-gal or X-gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside) labeling. In this mouse line, the amount of protein expression is under control of the melanopsin promoter, and only M1 ipRGCs (and potentially M3 ipRGCs, [42]) are visible using either antibodies (immunolabeling) or X-gal labeling [2].

There are two different *Opn4-GFP* BAC transgenic lines currently available. The first of these was developed by the Kofuji lab. In this line, a BAC transgene contains 29 kb of upstream and 155 kb of downstream regulatory regions of the melanopsin locus, but the melanopsin gene itself has been replaced by GFP [43]. In this mouse line, M1–M3 ipRGCs are labeled, but M4 ipRGCs (and likely M5–M6 ipRGCs) are not labeled in the adult retina [44]. However, early in development, M4 ipRGCs also show detectable GFP expression, likely because melanopsin levels in M4 ipRGCs are higher during development [44, 45]. A major benefit to the BAC transgenic approach is that it can be used to label ipRGCs, but leaves both copies of the melanopsin locus itself intact for further genetic manipulation (i.e., knockout of melanopsin or use with a Cre or LacZ allele). A second *Opn4-GFP* BAC transgenic line was generated as part of the GENSAT project [46]. This mouse line was also generated using a BAC containing the melanopsin gene, but GFP was inserted upstream of the melanopsin gene on the BAC, leaving a copy of the melanopsin gene intact on the transgene. Thus, in the GENSAT *Opn4-GFP* line, it is impossible to generate a melanopsin null animal that also contains this transgene. However, the GFP signal in this mouse line is such that some ipRGC axons are detectable in the brain with immunolabeling, allowing for mapping some projections of M1–M3 ipRGCs. Thus, it is critical that the *Opn4-GFP* line is chosen with care depending on experimental needs. The *Opn4-tdTomato* line was developed in the Yau lab [47]. This line primarily labels M1 and M2 (and presumably M3) ipRGCs, but has been reported to label some M4 ipRGCs, as well [47, 48]. While *Opn4-GFP* lines have the disadvantage that GFP excitation and melanopsin excitation share a preferred wavelength and thus strongly activate the melanopsin phototransduction cascade if epifluorescence is used, the *Opn4-tdTomato* line has the advantage of allowing

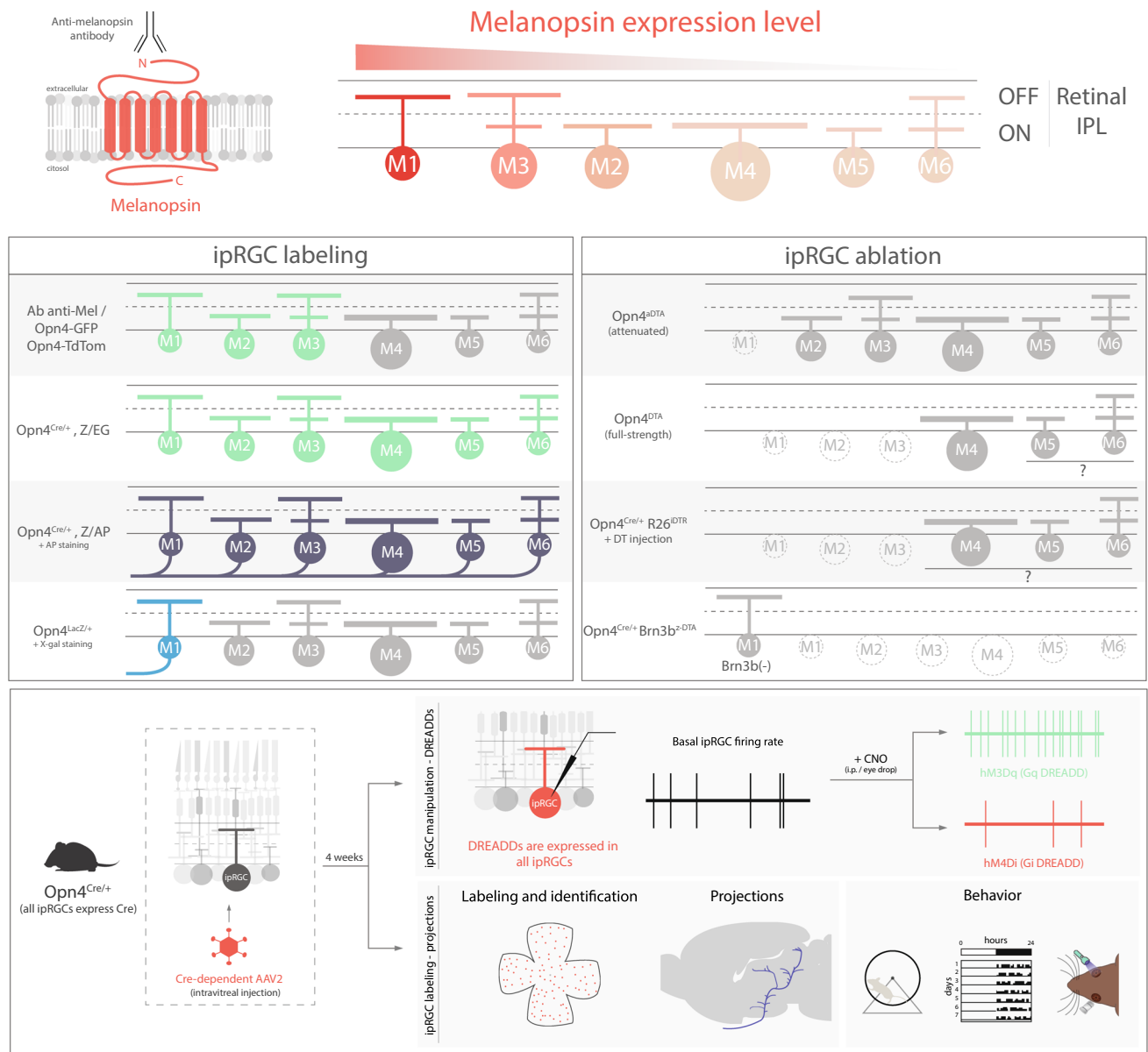


Fig. 3 Diverse toolkit for studying ipRGCs circuits. Top, differential melanopsin expression in M1–M6 ipRGCs. Anti-melanopsin antibody binds to the N-terminus extreme, labeling M1–M3 ipRGC subtypes. ipRGC labeling box: *Opn4-GFP* line labels M1–M3 ipRGCs in the adult mouse retina and also M4 cells prior to P14 [43]. The *Opn4-tdTomato* line labels similar subtypes to *Opn4-GFP*. *Opn4^{Cre/+}; Z/EG* labels M1–M6 ipRGCs [7]. *Opn4^{Cre/+}; Z/AP* line labels somas and axons of M1–M6 cells after AP staining [7]. *Opn4^{LacZ/+}* line labels

M1 ipRGCs somas and axons after X-gal staining [2]. ipRGC ablation box: in the *Opn4^{aDTA}* line M1 ipRGCs are ablated [19]; in *Opn4^{DTA}*, M1–M3 cells are likely ablated and *Opn4^{Cre/+}; R26^{DTR}* mice potentially all ipRGCs are ablated [49, 53]. In *Opn4^{Cre/+}; Brn3b^{Z-DTA}*, all ipRGCs are ablated except Brn3b-negative M1 ipRGCs [99]. Bottom, use of adeno-associated viruses (AAV) to manipulate ipRGC activity through DREADDs (top) or to label somata and axons of ipRGCs (bottom) [50–52, 55–60]

for the use of longer wavelength light to excite the tdTomato fluorophore.

The *Opn4^{Cre}* line is by far the most sensitive mouse line for ipRGC identification and manipulation that has been developed and has allowed for the discovery of new ipRGC subtypes [7, 49]. In this mouse line, Cre recombinase is knocked into the melanopsin locus. The best characterized

of the two developed Cre lines was generated by the Hattar Lab [7] and is described in detail here. When crossed with either the *Z/EG* or *Z/AP* reporters, this mouse line labels all currently known ipRGC subtypes (Fig. 3). There is evidence of wider Cre expression in the retina when this mouse line is crossed to a stronger, Rosa-based reporter such as tdTomato (Ai9), AP (R26iAP), and synaptophysin–tdTomato

[50, 51]. The identity of these additional retinal neurons has not been reported and it is unclear if these are additional M1–M6 ipRGCs, new ipRGC subtypes, or ‘ectopic’ labeling of other retinal neurons (amacrine and/or cone cells). The *Opn4^{Cre}* line can also be combined with intravitreal injection of Cre-dependent AAV reporters and used to label somata and projections. This strategy is very effective for driving reporter expression in ipRGCs [52] (Fig. 3). Although the high sensitivity of this Cre-loxP-based genetic approach of *Opn4^{Cre}* line has allowed the identification of different subtypes of ipRGCs, using *Z/EG* or *Z/AP* reporters, it also labels some cone photoreceptors [7]. Furthermore, the *Opn4^{Cre}*; *Z/EG* lines show extraretinal expression in areas that are not thought to express melanopsin like brainstem, diencephalon, and cortex [7]. This evidence suggests either leaky expression of Cre recombinase or that these non-retinal cells transiently express melanopsin during some stage of development [7]. These issues can be partially circumvented by intravitreal injection of AAV into adult animals to restrict expression to retinal neurons that express melanopsin in adulthood. Intravitreal AAV also avoids transgene expression in cones. Regardless of these caveats, the *Opn4^{Cre}* line has greatly expanded our understanding of how ipRGCs function within their specific circuits.

ipRGC ablation

Given the ability to successfully introduce transgenes into the endogenous melanopsin locus, researchers have been able to develop multiple lines for ipRGC ablation. These models have been used to great effect in understanding the functions of ipRGCs in light-evoked behavior. These advances will be discussed in detail in a subsequent section, but, here, we will review the tools for ipRGC ablation that have been developed thus far. In 2008, Güler et al. developed the first mouse line designed to ablate ipRGCs, the *Opn4^{aDTA}* line [19]. To generate this mouse line, researchers replaced the melanopsin locus with an attenuated version of the diphtheria toxin (aDTA). Using this strategy in combination with the *Opn4^{LacZ}* line, researchers determined that *Opn4^{aDTA/+}* eliminated mostly the M1 ipRGCs at adult ages (Fig. 3). ipRGC loss in *Opn4^{aDTA/aDTA}* mice cannot be further tested without crossing to a BAC transgenic line, because the two copies of aDTA mean a loss of melanopsin protein expression, making antibody immunolabeling impossible. *Opn4^{aDTA/aDTA}* animals also preclude crossing with other knock-in lines to assess the extent of ipRGC loss. However, behavioral analyses (described below) suggest that the loss of ipRGCs in aDTA animals is mostly due to loss of M1 ipRGCs. The Hattar lab has since generated an *Opn4^{DTA}* line that expresses the full-strength diphtheria toxin in the melanopsin locus [53]. The *Opn4^{DTA}* mouse line shows greater loss of ipRGCs and more severe behavioral phenotypes, though

as with the *Opn4^{aDTA}* line, only *Opn4^{DTA/DTA}* homozygous mice show free running circadian rhythms [53]. Moreover, SMI-32 labeling is normal in *Opn4^{DTA/DTA}* mice, suggesting that at least M4 ipRGCs survive [53]. Based on comparably low melanopsin expression levels between M4 and M5/6 ipRGCs, it is possible, even likely, that M5 and M6 ipRGCs also escape ablation in the *Opn4^{DTA/DTA}* line, though this has not been explicitly tested. Importantly, the *Opn4^{aDTA}* animals show ipRGC loss only after 3–6 months of age, while *Opn4^{DTA}* animals show ipRGC loss by the first postnatal week [19, 53] (Fig. 3).

Researchers have also taken the strategy of crossing the *Opn4^{Cre}* line with other ablation lines. For example, the *Opn4^{Cre}* line has been crossed with a *Brn3b^{Z-DTA}* line. This knock-in line expresses a Cre-dependent, full-strength DTA under the control of the *Brn3b* promoter. Therefore, when crossed with the *Opn4^{Cre}* line to generate *Opn4^{Cre/+}*; *Brn3b^{Z-DTA/+}* mice, this mouse line ablates all RGCs that express both *Brn3b* and melanopsin. This mouse line results in ablation of all M2–M6 ipRGCs (all of which express *Brn3b*) and a subset of M1 ipRGCs that are *Brn3b*-positive (Fig. 3). Researchers also crossed the *Opn4^{Cre}* line with a mouse strain in which the inducible diphtheria toxin receptor (iDTR) is knocked-in to the *ROSA26* locus (*R26^{iDTR}*) [49]. This knock-in mouse line expresses a Cre-dependent diphtheria toxin receptor under the *Rosa26* promoter. Therefore, when crossed to the *Opn4^{Cre}* line, this mouse line results in ablation of M1, M2, and M3 ipRGC subtypes when mice are injected with diphtheria toxin (Fig. 3). Göz et al. also took the strategy of using saporin-conjugated melanopsin antibodies to ablate ipRGCs and study their role in behavior [54].

ipRGC manipulation

The development of the *Opn4^{Cre}* line has made all ipRGC subtypes amenable to cell-type specific manipulations to either activate or silence ipRGCs themselves or to manipulate ipRGC gene expression. One widely used tool to manipulate the activity of neurons are the Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) activated in the presence of artificially designed, specific ligands [e.g., clozapine N-oxide (CNO)]. Particularly, for ipRGC manipulation, Cre-dependent DREADDs sequences are packaged in adenovirus vectors (AAV2) and injected intravitreally into the eyes of *Opn4^{Cre}* mice (Fig. 3). Gq-DREADDs (hM3Dq) have been used to mimic melanopsin phototransduction in M4 ipRGCs to assess the role of melanopsin in ipRGC physiology [55] or to activate all ipRGCs and assess behavioral outcomes [56–59]. On the other hand, it can also be leveraged to chemogenetically silence ipRGCs with inhibitory Gi-DREADDs (hM4Di) [60]. AAV can also be used to selectively express channelrhodopsins in

ipRGCs to map their postsynaptic targets and assess their neurotransmitter content in specific brain regions [61–63] or to study neurite outgrowth of postnatal retinal explants [64]. Sulfonamide compounds been also used as antagonists of melanopsin [65]. These opsins are able to inhibit melanopsin-mediated photoresponses and behaviors such as PLR and phototaxis in neonatal mice [65].

The *Opn4^{Cre}* line has also been combined to knock out specific genes in ipRGCs, most of them related to neurotransmitters glutamate, pituitary adenylate cyclase-activating polypeptide (PACAP), and gamma-aminobutyric acid (GABA) to study their role in specific behaviors such as PLR and photoentrainment [63, 66]. These conditional knockout tools have also been used to study the importance of ipRGC apoptosis during development [67] and as well as to rescue melanopsin expression in ipRGCs to study the role of human melanopsin polymorphisms associated with abnormal behaviors [68]. Researchers have used AAVs to rescue melanopsin expression in melanopsin knockout mice (*Opn4^{Cre/Cre}*) with a full or modified version of the melanopsin sequence (lacking combinations of C-terminal phosphorylation sites) using AAV2 vectors to study the role of signal cascade on the intrinsic light response, the PLR, and photoentrainment [69, 70]. The possibilities for using these strategies are vast and allow for molecular level insights into ipRGC function. As the ability to specifically manipulate subsets of ipRGC subtypes is expanded, these approaches will provide important insight into the contribution of single RGC subtype to visual behavior.

Knock-in lines that target the melanopsin locus also provide the opportunity to knockout the melanopsin gene using homozygous mice such as *Opn4^{LacZ/LacZ}* and *Opn4^{Cre/Cre}*. These lines allow for a determination of the role of melanopsin in various behaviors and cellular functions [3, 7, 69–71]. One concern with these manipulations is the global knockout of melanopsin from early developmental stages. In studies that have addressed this question, behavioral or cellular deficits that are attributed to melanopsin can be rescued by acute activation of the melanopsin phototransduction cascade in ipRGCs [55] or by expressing melanopsin in adult ipRGCs in melanopsin null animals [70]. The ability to acutely rescue behavioral deficits argues that at least in the behaviors tested thus far, deficits are not likely to arise from circuit level rewiring due to melanopsin knockout.

ipRGC projections and behavior

Non-image-forming visual system

The non-image-forming system is a visual circuit that operates in parallel to conscious visual perception. This non-image-forming visual system regulates numerous

subconscious effects on our physiology and behaviors such as photoentrainment of our circadian rhythms, the regulation of light-dependent pupillary constriction, sleep–wake cycles, body temperature, and improvement of learning and visual function. Almost a century ago, Clyde Keeler observed that blind mice were still able to constrict their pupils in response to light [72]. In 1999, the Foster lab demonstrated in two different papers that mice lacking rods and cones were able to adjust their circadian rhythms to light and maintain a robust PLR [73, 74]. In clinics, it was observed that some blind patients maintained circadian entrainment and suppressed melatonin secretion despite an apparently complete loss of visual function [75]. These observations suggested that there must exist a third class of non-rod, non-cone photoreceptor. Taking a cue from the continued photoentrainment of blind individuals, the Berson lab tested whether the retinal ganglion cells that project to the central circadian pacemaker, the SCN, were in fact directly light sensitive. To do this, they recorded from SCN-projecting RGCs and found that these responded to light in the absence of rod/cone input [1]. Concurrently, the Yau lab used *Opn4^{LacZ}* animals to show that melanopsin-positive RGCs project axons to a variety of non-image brain targets including the SCN. Researchers initially concluded that ipRGCs comprised a uniform population of RGC that primarily influenced non-image-forming behaviors. As mentioned earlier in this review, however, we now know that there are multiple ipRGC subtypes that mediate a variety of behavioral functions. Subsequent research showed that the ipRGC input to the non-image-forming visual system primarily arises from M1 ipRGCs which project to brain areas involved in circadian photoentrainment (SCN, intergeniculate leaflet (IGL)), ventral lateral geniculate nucleus (vLGN), the pupillary light reflex (OPN), mood (perihabenular nucleus (pHb)), sleep (ventrolateral preoptic area (VLPO) and lateral hypothalamic area (LH)), and multiple other hypothalamic regions such as peri-supraoptic nucleus (pSON), IGL, anterior hypothalamus (AH), subparaventricular zone (SPZ) among others [16, 17] (Fig. 4). Below, we outline what is known about ipRGC contributions to non-image-forming behaviors.

Circadian photoentrainment, sleep, and alertness

Originally, it was assumed that ipRGCs played a major role in circadian photoentrainment, because they were found to project to the SCN. However, when the melanopsin gene was removed, abolishing ipRGC intrinsic sensitivity to light, circadian photoentrainment remained largely intact with mild impacts on phase shifting [76, 77] and period lengthening in constant light [76, 77]. This indicated that rod/cone signaling was sufficient for circadian photoentrainment [78]. Rods, cones, and melanopsin are the only photoreceptors that contribute to circadian photoentrainment, because a triple

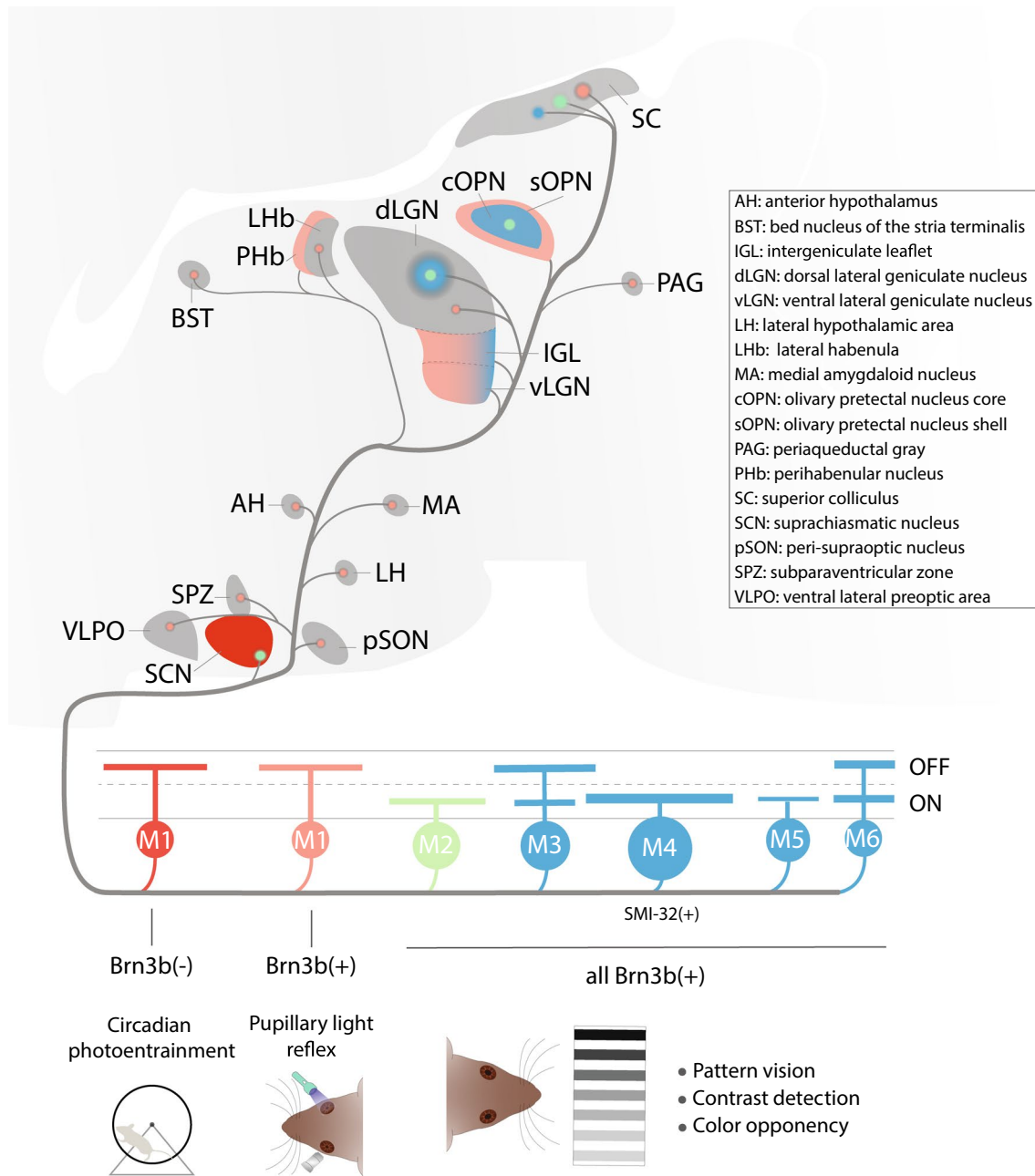


Fig. 4 Schematic representation of the main brain targets of ipRGCs. Brn3b-negative M1 ipRGCs are sufficient to drive circadian photoentrainment and project mainly to the SCN, while Brn3b-positive M1 ipRGCs project to sOPN and are necessary for the PLR [99]. Brn3b-positive M1 ipRGCs constitute the majority of sparse M1 ipRGC innervation of the thalamus, hypothalamus, and midbrain (VLPO, SPZ, pSON, AH, MA, vLGN, IGL, dLGN, PAG, BST, PHb,

and Lhb) [16, 17]. M2 cells project to both image forming (SC and dLGN) and non-image forming (SCN and OPN) visual areas and M4–M6 cells project to brain nuclei involved in image-forming vision such as dorsal geniculate nucleus (dLGN) and superior colliculus (SC) [7, 17–20]. While M3 ipRGCs have been reported to project to the SC [10], no other targets have yet been identified

knockout of phototransduction in rods, cones, and ipRGCs results in a complete loss of this behavior.

The sufficiency of both rod/cone signaling and melanopsin phototransduction for photoentrainment led to a critical question: is all light information routed through ipRGCs or are melanopsin versus rod/cone signals routed through

separate types of RGCs? This question was tackled directly by ablation of ipRGCs. Using the *Opn4^{cre}* line, researchers demonstrated that ablation of ipRGCs resulted in a complete loss of circadian photoentrainment, demonstrating conclusively that ipRGCs are the principal conduit for light to drive this behavior. These conclusions were supported

by studies in the same year using *Opn4^{Cre}*; *Rosa^{iDTR}* mice, or saporin-conjugated melanopsin antibodies to ablate ipRGCs, and later by removing synaptic transmission from ipRGCs or using the full-strength *Opn4^{DTA}* line [49, 53, 54, 79]. Subsequent dissection of the specific rod versus cone contributions to circadian photoentrainment revealed that rod photoreceptors play a critical role in photoentrainment. Rods are required for circadian photoentrainment from low-light intensities of at least 0.1 lux to surprisingly bright light intensities (10 lux) where rod signaling was not thought to influence behavior [78, 80]. When rod phototransduction is abolished, animals show deficits in photoentrainment below 10 lux. At high light intensities, melanopsin signaling is required for proper circadian photoentrainment. Mice lacking cone phototransduction show no deficits in photoentrainment, while mice where cones are the only functional photoreceptor fail to photoentrain [78]. Moreover, spectral analysis of circadian photoentrainment also indicates a major role for rod, but not cone, photoreceptors [80].

ipRGCs also play a major role in sleep. Mice are nocturnal rodents; they are active during subjective night and sleep during the subjective day. A light pulse presented during the early part of the subjective night (i.e., ZT 14–17) results in increased sleep. The circadian photoentrainment of sleep and the induction of sleep by light are both absent when ipRGCs are ablated using *Opn4^{DTA}* mice [3]. Furthermore, the induction of sleep is attenuated in melanopsin null (*Opn4^{LacZ/LacZ}* or *Opn4^{Cre/Cre}*) animals or animals lacking rods, though photoentrainment of sleep rhythms remained intact, indicating that rod/cone inputs can compensate for the loss of melanopsin to photoentrain sleep rhythms [3, 71]. Moreover, melanopsin null animals showed a reduction in c-Fos expression in neurons from sleep promoting areas such as VLPO and SC [71]. Sleep and circadian brain regions are heavily innervated by M1 ipRGCs, which are likely to be the primary ipRGC subtype lost in *Opn4^{DTA}* line. Because photoentrainment of sleep rhythms and circadian rhythms along with sleep induction by light is lost in the *Opn4^{DTA}* line, the available evidence suggests that M1 ipRGCs are necessary for these behaviors.

Pupillary light reflex

The PLR is a non-image-forming behavior that is critical to regulate the amount of light entering the eye for proper vision. The OPN is a midbrain nucleus, the shell of which plays a central role in PLR pathway. Neurons in the OPN shell project to the Edinger–Westphal nucleus which, in turn, sends projections to the ciliary ganglion. The ciliary ganglion neurons innervate the iris muscle to induce contraction [35–37, 81–84]. For many decades, it was believed that PLR was driven only by rod and cone signaling. However, in 2001, Lucas et al. showed that animals lacking rods and

cones continue to show a PLR, albeit with greatly reduced sensitivity [85]. Loss of rod, cone, and melanopsin phototransduction results in a complete loss of the PLR [86], indicating that the PLR in rodless/coneless animals arises from ipRGCs. In agreement with the reduced sensitivity of the PLR in rodless/coneless animals, animals that lack only melanopsin signaling show a normal PLR except at the brightest light levels, but cannot maintain pupil diameter for long periods in constant light [66, 80, 85, 86].

Multiple papers revealed that the OPN receives innervation from multiple ipRGCs subtypes. Studies using *Opn4^{LacZ}* mice showed that M1 ipRGCs heavily innervate the OPN shell but avoid the core [2, 17, 18]. However, ipRGC labeling using a *Z/AP* reporter line combined with the *Opn4^{Cre}* line (*Opn4^{Cre/+}*; *Z/AP* mice) (Fig. 3) revealed that the OPN core region is heavily innervated by non-M1 ipRGCs [7, 18], and a retrograde tracing study has indicated that M2 ipRGCs may contribute to the OPN core innervation [18]. The role of ipRGC innervation of the OPN core in behavior is currently unknown. In addition to the conventional PLR pathway, multiple studies in fish, amphibians, birds, and some mammals suggest that a PLR can be generated by intrinsic phototransduction within the iris muscle even when the eye is physically separated from the brain [87–91]. This intrinsic (i) PLR is very insensitive to light and is dependent upon both melanopsin phototransduction and cholinergic neurotransmission [92, 93]. ipRGCs from the ciliary marginal zone, mainly from the nasal retina, project directly to the ciliary body to induce pupillary constriction [93].

Mood and learning

M1 ipRGCs directly project to limbic regions such as the lateral habenula (LHb), the medial amygdala (MA) [2, 17], and different brain region involved in mood and behavioral state such as bed nucleus of the stria terminalis (BST), paraventricular nucleus (PVN), and periaqueductal gray (PAG) [52] (Fig. 4). Moreover, circadian rhythms and sleep can be disrupted by manipulation of the light environment and lead to mood and learning defects in mice and humans [94–96]. These two lines of evidence prompted the hypothesis that ipRGCs may drive direct effects of aberrant light on mood and learning. To model aberrant light, researchers used long-term exposure to an ultradian cycle consisting of 3.5-h light and 3.5-h dark (T7). This paradigm provides light input at multiple times in the circadian cycle where light would not normally be encountered but does so while leaving the endogenous circadian and sleep rhythms intact [97]. Exposing mice to this T7 cycle induces depression-like behaviors and hippocampal-dependent learning deficits. *Opn4^{DTA/aDTA}* mice where M1 ipRGCs are ablated, fail to show these deficits when exposed to the T7 light/dark cycle, indicating that ipRGCs relay this aberrant light information. These direct

effects on mood are driven by a direct projection of (likely M1) ipRGCs to the PHb and LHb [61, 97] (Fig. 4). There is also evidence that a disynaptic connection between M4 ipRGCs and GABAergic neurons in the vLGN and IGL, which in turn project to the LHb, may decrease depression-like behavior in mice and provide a potential mechanistic explanation for the treatment of depression by light [98].

Direct effects of ipRGCs on behaviors related to anxiety and alertness have also been studied using molecular genetic strategies (Fig. 3). Milosavljevic et al. selectively activated ipRGCs by intravitreally injecting AAVs containing a Cre-dependent hM3Dq (Gq-DREADD) into the eyes of *Opn4^{Cre}* animals [56] (Fig. 3). Induction of ipRGC activity through injection of the specific Gq-DREADD agonist CNO resulted in alertness and anxiety-like behaviors in mice. Moreover, ipRGC activation induced downstream neuronal c-Fos induction in multiple brain regions in the hypothalamus, thalamus, and limbic system that are commonly associated with aspects of autonomic and neuroendocrine activity. While the specific pathways underlying these behaviors are yet unknown, this evidence indicates that ipRGCs could drive a light-dependent switch in behavioral motivation toward a more alert, risk-averse state [56].

Molecular subtypes of ipRGC: differential expression of *Brn3b*

Until 2002, M1 ipRGCs were considered a homogenous population with uniform molecular, morphological, and functional properties. However, this homogeneity was questioned in 2011 when Chen et al. demonstrated that M1 ipRGCs differentially express the transcription factor *Brn3b* (encoded by *Pou4f2* gene), while all non-M1 ipRGCs express *Brn3b*. Taking advantage of this molecular distinction, researchers generated mice to either selectively label (*Opn4^{Cre}; Brn3b^{cKOAP/+}*) or selectively ablate (*Opn4^{Cre}; Brn3b^{Z-DTA/+}*) *Brn3b*-positive ipRGCs. Researchers found that *Brn3b*-positive M1 ipRGCs innervate the shell of the OPN, while *Brn3b*-negative M1 ipRGCs innervate the SCN. Ablation of *Brn3b*-positive ipRGCs resulted in a loss of the PLR but retention of nearly normal circadian light responses [99]. Thus, *Brn3b*-positive ipRGCs are necessary for the PLR, while *Brn3b*-negative ipRGCs are sufficient to drive circadian photoentrainment.

Li and Schmidt studied the projection patterns of M1 ipRGC subtypes beyond regions associated with circadian photoentrainment and the PLR [16]. They found that *Brn3b*-positive M1 ipRGCs constitute the majority of sparse M1 ipRGC innervation of the thalamus, hypothalamus, and mid-brain (Fig. 4). This work demonstrated that *Brn3b*-positive and *Brn3b*-negative M1 ipRGCs have very little overlap in their projection patterns. In agreement with non-SCN projections arising largely from *Brn3b*-positive M1 ipRGCs,

Fernandez et al. demonstrated that a *Brn3b*-positive M1 ipRGC projection to the PHb is primarily responsible for the effects of aberrant light exposure on mood [61]. Moreover, Rupp et al. examined the role of *Brn3b*-positive and *Brn3b*-negative ipRGCs in driving the circadian and acute effects of light on body temperature and sleep [59]. They found that *Brn3b*-positive ipRGCs are necessary for the acute light induction of sleep and acute light induction of body temperature decrease. However, *Brn3b*-negative M1 ipRGCs are sufficient for the photoentrainment of both sleep/wake cycles and body temperature rhythms. Collectively, these data point to distinct and diverse functions for these molecularly sub-divisible *Brn3b*-positive and *Brn3b*-negative ipRGCs.

Image forming visual system

While M1 ipRGCs contribute primarily to non-image-forming vision, non-M1 ipRGCs project to brain regions involved in conscious visual perception such as the dLGN and SC, and these regions receive only very sparse M1 ipRGC inputs [7] (Fig. 4). Ecker et al. were the first to demonstrate that non-M1 ipRGCs projected to image-forming brain regions. As a first test of whether melanopsin signaling was sufficient to drive rudimentary pattern vision, they demonstrated that mice lacking classical rod-cone photoreception and, therefore, entirely dependent on melanopsin for light detection were able to discriminate coarse grating stimuli from equiluminant gray [7]. Therefore, animals lacking rod/cone phototransduction had measurable, albeit very low, visual acuity, indicating that ipRGCs could, in principle, play a role in visual perception in the intact animal. Soon after this study, the M4 ipRGCs arose as a candidate to influence visual perception. Indeed, melanopsin null (*Opn4^{LacZ/LacZ}* and *Opn4^{Cre/Cre}*) mice showed a reduction in two measures of behavioral contrast sensitivity but normal visual acuity. These results indicated that melanopsin phototransduction in non-M1 ipRGCs was necessary for normal contrast sensitivity, even in the presence of functional rod/cone signaling. Ablation of the non-M1 ipRGCs using *Opn4^{Cre/+}; Brn3b^{Z-DTA/+}* animals showed a further reduction in contrast sensitivity, consistent with non-M1 ipRGCs relaying rod, cone, and melanopsin signals to influence contrast sensitivity [5].

Our recent work demonstrated that melanopsin phototransduction is also necessary for normal contrast sensitivity within M4 cells themselves [55]. Melanopsin phototransduction in ipRGCs had been well characterized as slow in onset and offset, decaying on the order of tens of seconds or even minutes, as well as very sustained [6, 7, 10, 20, 100]. Moreover, in light-adapted tissue, melanopsin phototransduction had been demonstrated to be active only at very bright, photopic, light levels. Each of these three features (slow kinetics, sustained signaling, and insensitivity

to light) seemed, at face value, to be inconsistent with a potential role for melanopsin in enhancing visual perception, which requires spatial and temporal precision that melanopsin phototransduction lacks. Sonoda et al. found that melanopsin phototransduction acts across a range of physiological light intensities from photopic to scotopic to enhance the contrast sensitivity of M4 ipRGCs [55] (Fig. 5). Melanopsin phototransduction does this through the use of a

novel phototransduction cascade in M4 ipRGCs that serves to both depolarize the resting membrane potential of these cells as well as increase their excitability through the closure of potassium channels. Combined, these influences serve to boost the response of M4 ipRGCs to the incoming temporally and spatially precise rod/cone signals, in effect enhancing the contrast sensitivity of M4 ipRGCs through modulation of its cellular properties [55]. This mechanism is distinct

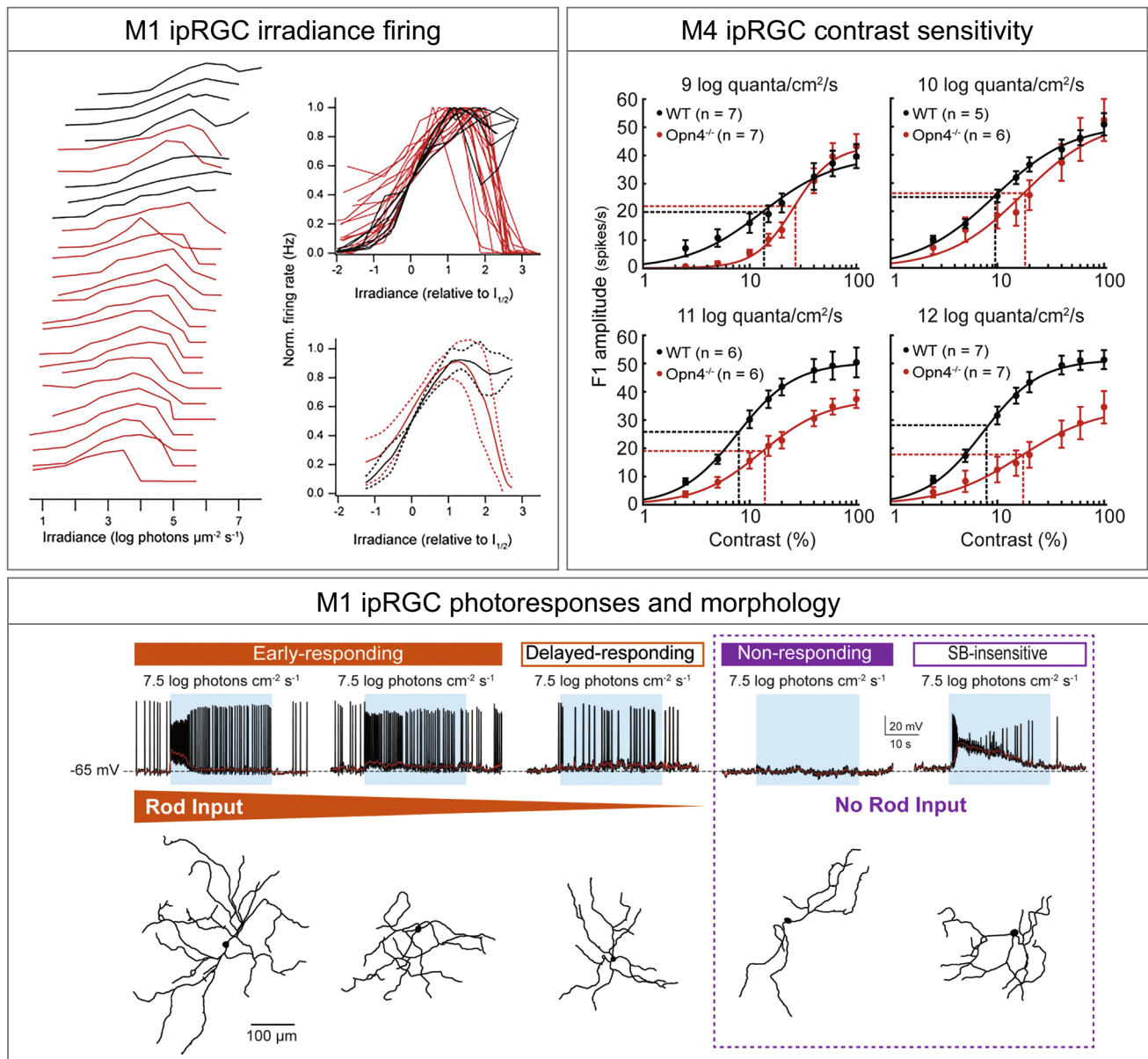


Fig. 5 Re-evaluating the roles of ipRGCs. M1 ipRGCs irradiance firing box: every individual M1 cell operates over a narrow range and the population covers irradiances from moonlight to full daylight (left); relations overlaid and aligned by $I_{1/2}$ for comparison of shape (right) [109]. Melanopsin signaling in both M1 and M4 is active into the scotopic range. M4 ipRGC contrast sensitivity box: contrast response functions of M4 cells in WT (black) and *Opn4*^{-/-} (red)

retinas recorded at background light levels from 9 to 12 log quanta/cm²/s. Vertical dotted lines indicate C_{50} and horizontal dotted lines indicate half-maximal response [55]. M1 ipRGC photoresponses and morphology box: top, representative recording examples, each from various types of M1 response arranged based on degree of rod input (orange gradient). Blue rectangle indicates start and end of light stimulus. Bottom, dendrite tracing image for each recorded cell [111]

from that reported in M1 ipRGCs [48, 92, 101] (Fig. 1), and indicates that different ipRGC subtypes utilize distinct melanopsin phototransduction cascades that properly tune each cell for its unique behavioral functions. Moreover, this influence of melanopsin was detectable even at dim, scotopic light intensities where only rods were thought to influence RGC responses. These data demonstrate that melanopsin phototransduction is active and influencing cellular function at much lower light intensities than previously appreciated (Fig. 5). The role of melanopsin, rod, and cone inputs to shaping the M4 ipRGC response is still an active area of research, with much yet to be resolved [5, 20, 48, 102].

Recently, new evidence suggests that M5 ipRGCs, like M4 ipRGCs cells, contribute to ‘image-forming’ or spatial vision. In 2018, Stabio et al. reported M5 ipRGCs to exhibit opponent responses to different wavelengths of light (color opponency) [39]. They showed that M5 ipRGCs had sustained ON responses, receptive-field centers driven by balanced input from UV and mid-wavelength cone (M-cone) opsins, and a strong suppressive surround dominated by input from M-cones. M5 ipRGCs project axons to the image-forming visual thalamic nuclei, and it could provide chromatic signal to the primary visual cortex of mice and could contribute to color vision [103–105]. These cells can be distinguished from the temporal M4 ipRGCs (which are also bushy and stratify in the ON sublamina) by their lack of SMI-32 labeling and a higher number of branch points and smaller somata [39, 106]. These morphological and molecular distinctions are important as it has been reported that strong light adaptation of the ex-vivo retina can lead to color-opponent responses even in M4 ipRGCs [106]. Future work will be needed to elucidate the mechanisms behind ipRGC influences at a cellular and circuit level and, ultimately, on image-forming visual behaviors.

Re-evaluating the roles of ipRGCs

As our understanding of ipRGCs has evolved, the field has made exciting discoveries about ipRGC diversity and their functions. In this section, we will touch on some of the recent advances that are reshaping our understanding of ipRGCs.

A spectrum of M1 ipRGC properties

As mentioned before, there are important interactions between melanopsin phototransduction or rod and cone inputs to ipRGCs that are required to modulate the visual responses that will ultimately be relayed to the brain. Many studies have shown that the ipRGCs (M1 ipRGCs in particular) can individually track light intensity over a wide range [1, 30, 107, 108]. However, instead of all M1 ipRGCs

each encoding a wide range of irradiance, the recent evidence from the Do lab suggests that every individual M1 cell operates over a relatively narrow intensity range (Fig. 5). However, the M1 ipRGC population covers irradiances from moonlight to full daylight [109] (Fig. 5). In parallel, the same group also demonstrated that M1 ipRGCs vary widely across other morphological and biophysical properties [110]. In addition to the intrinsic photoresponse of M1 ipRGCs, rod pathway input is necessary for normal pupil constriction and normal circadian photoentrainment across a relatively broad range of light intensities [66, 78]. Recent work from our lab indicates that M1 ipRGCs also exhibit variability in their processing of signals arising from the rod pathway, and that the degree of rod pathway input correlates with certain morphological features of M1 ipRGCs [111] (Fig. 5). As with M4 cells, we also find that M1 ipRGCs show melanopsin-evoked responses well into the scotopic regime, again indicating that melanopsin phototransduction at the cellular level is much more sensitive than previously appreciated (Fig. 5), and may only be detectable when tissue is dark adapted and cells are identified with 2-photon excitation of GFP [55, 111]. Collectively, these recent data and the molecular evidence for variability in Brn3b expression in M1 ipRGCs suggest that these are not a uniform population of cell, but perhaps exist on a wide continuum of properties.

A subset of ipRGCs release GABA

All RGCs, including ipRGCs, were thought to influence behavior through the release of the excitatory neurotransmitter glutamate at downstream targets in the brain. M1 ipRGCs, and perhaps other ipRGC subtypes, also release the neuropeptide PACAP, which is critical for sustained pupil constriction in constant light [66]. However, our recent work has demonstrated that a subset of ipRGCs (likely of the M1 subtype) release the inhibitory neurotransmitter GABA [63]. We found that ipRGC GABA release serves to dampen the sensitivity of both circadian photoentrainment and the pupillary light reflex in dim light. These new findings explain, at least in part, why ipRGCs in the retina are, in fact, quite sensitive to light, but their associated non-image-forming behaviors are so insensitive to light.

ipRGCs play critical roles in visual system development

In addition to their role in visual circuits in the adult retina, increasing amounts of evidence link ipRGCs to early retinal and visual circuit development. ipRGCs are photosensitive from embryonic stages, and are the only functional photoreceptors before eye opening, at which point the rod/cone circuitry is functionally connected to the RGCs [43, 112–117] (Fig. 6). Moreover, ipRGCs connect with the SCN

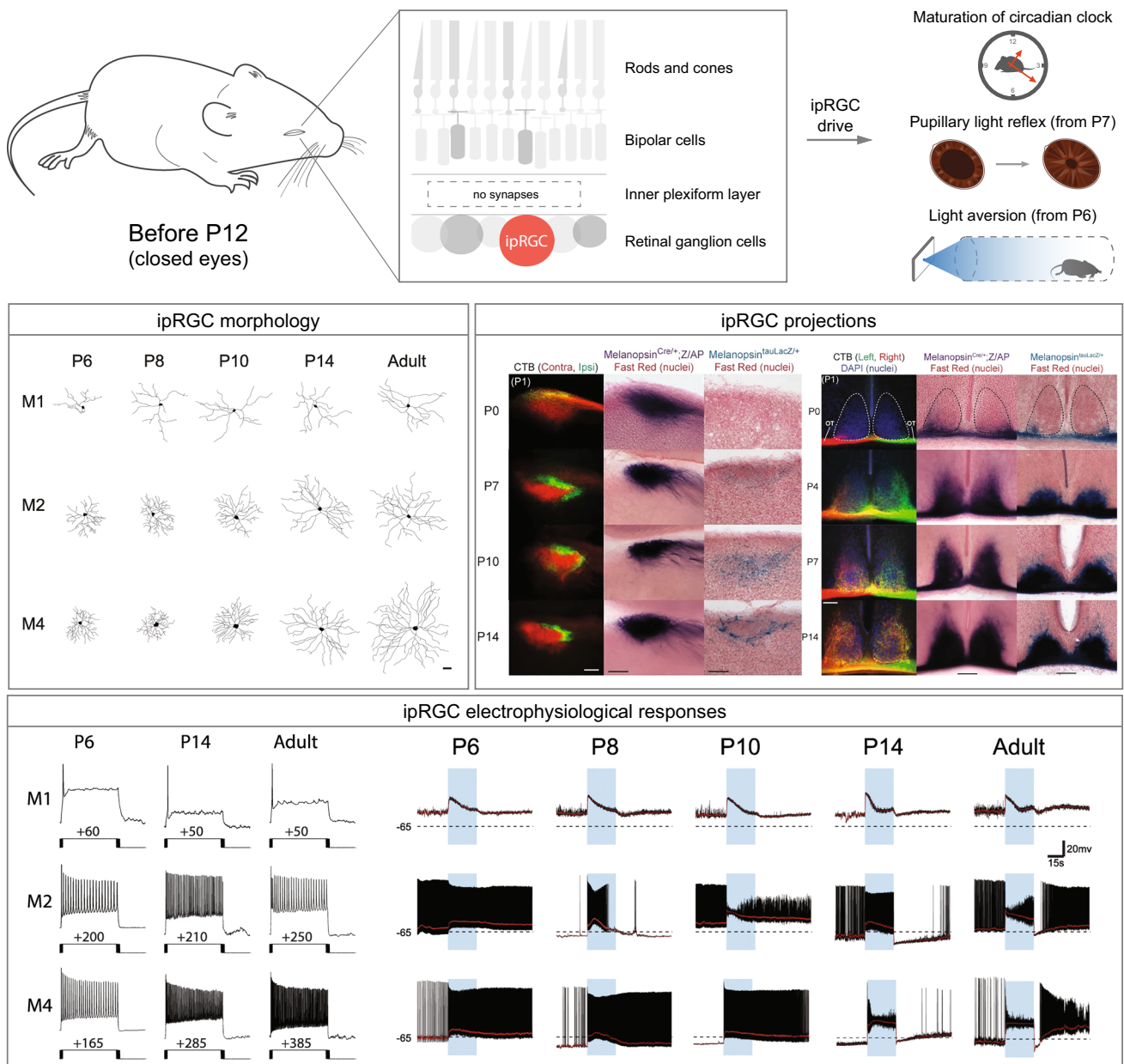


Fig. 6 ipRGC morphology, projections, and behavior during development. Top, ipRGCs are the only photosensitive cells up to ~P12, prior to the opening of the eyes and before the connection between bipolar cells and RGCs [115]. ipRGCs set the length of the circadian period [53], and drive rudimentary PLR at P7 [114] and light aversion in mice as young as P6 [138]. ipRGC morphology box: representative cell tracings of M1, M2, and M4 subtypes for P6, P8, P10, P14, and adult time points [44]. ipRGC projections box: left, representative images of olivary pretectal nucleus (OPN) innervation through contralateral (red) and ipsilateral (green) eye injection with fluorescent cholera toxin B subunit (CTB) at P0; *Opn4^{Cre}; Z/AP*-labeled axons show a similar innervation pattern from P0. In contrast, *Opn4^{LacZ/+}*-

labeled axons are not detected until P7 and show adult-like innervation of the OPN shell (sOPN) by P14 [114]. Right: representative images of suprachiasmatic nucleus (SCN) innervation using CTB. Retinal fibers fill the SCN by P7 and innervation becomes bilateral by P14. Similar innervation pattern was observed in both *Opn4^{Cre}; Z/AP* and *Opn4^{LacZ/+}* lines [114]. ipRGCs electrophysiological responses box: left, representative traces from depolarizing current injection that elicited the maximum spike output for M1, M2, and M4 subtypes at P6, P14, and adult time points [44]. Right, representative light response traces from M1, M2, and M4 cells at P6, P8, P10, P14, and adult time points. Blue rectangle indicates start and end of light stimulus. Black dotted line indicates -65 mV [44]

and dLGN as early as P0, positioning ipRGCs to relay light information to the brain from late early postnatal stages [114, 118] (Fig. 6). Light signaling by ipRGCs has been linked to

multiple developmental effects on visual system development. For example, ipRGCs modulate the development and branching of the retinal vasculature, influence spontaneous

activity during retinal development, send retinofugal axons to the outer retina, couple widely to amacrine and RGCs, and even regulate refinement of retinofugal projections [53, 119–125]. Researchers have begun to dissect the properties of ipRGC development and how they signal within the retina and to the brain. Recently, the Hattar group has proposed a new role for ipRGCs during development controlling non-photic entrainment [126]. Innervation of ipRGCs to IGL in early postnatal stages tunes the SCN to allow entrainment to time-restricted feeding [126]. Currently, the mechanisms behind these influences during retinal and visual system development, and the ipRGC subtypes involved are unknown. ipRGC subtypes exhibit many of their distinguishing features relatively early in development, making a study of ipRGC signaling at a subtype specific level feasible [44]. Moreover, because melanopsin expression occurs early in retinal development, ipRGCs are amenable to genetic manipulation throughout most of retinal development, making an understanding of their role in visual system development an achievable future goal.

Concluding remarks

In this review, we synthesized what is known about the contribution of ipRGCs to diverse visual circuits. We highlighted the complex diversity of ipRGC subtypes and discussed the different molecular, histological, genetic, circuitry, and behavioral approaches that researchers have used to classify them. Functionally, the ipRGCs can be classified into those that influence non-image-forming behaviors (e.g., photoentrainment and the PLR; largely driven by M1 ipRGCs), and into those that influence image-forming vision (largely driven by non-M1 ipRGCs). Interestingly, the M2 ipRGCs project to nuclei involved in both forming and non-forming image visual pathways, indicating that they may influence both classes of visual behavior. While this simplified functional classification can be useful in a practical sense, each subtype has unique morphological features (dendritic field and soma size, as well as level of dendritic stratification), melanopsin expression level, electrophysiological characteristics, and axonal projection patterns, which suggest possible specific functional roles for each subtype within these broad categories.

Much has yet to be uncovered regarding how ipRGCs function within their associated visual circuits. For example, how does melanopsin function within each ipRGC subtype and how does that integrate with rod/cone input at the cellular level? How do each of these individual subtypes modulate their associated light-evoked effects on physiology and behavior? What are the downstream cellular synaptic targets of each ipRGC within the ~20 brain regions innervated by

ipRGCs? How does this feed into more complex downstream circuits to influence physiology?

A final remaining major question is how this system functions within humans. ipRGCs have been identified in non-human primate and human retina. ON and OFF subtypes of ipRGC have been identified, and they seem to target both image and non-image-forming brain regions in the primate and influence the PLR, circadian system, and even conscious visual perception in humans [33, 34, 127–132]. Work has also indicated that ipRGCs play a role in photophobia in migraines, sleep disorders, anxiety, and depression in humans [94, 133, 134]. A recent molecular analysis of RGCs from both primate retina and mouse retina indicated that the ipRGCs are one of the few cell types that appear well conserved across these species [135], bolstering the rationale for continued in-depth study of these cells in the rodent, making use of the suite of genetic tools that have been (and likely will be) developed.

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