# REVIEW

# Seeing More Clearly: Recent Advances in Understanding Retinal Circuitry

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Among 10 breakthroughs that *Science* announced at the end of 2002 was the discovery of a photosensing (melanopsin-containing) retinal ganglion cell (RGC) and its role in entraining the circadian clock. This breakthrough exemplifies the ultimate goal of neuroscience: to understand the nervous system from molecules to behavior. Light-sensing RGCs constitute one of a dozen discrete RGC populations coding various aspects of visual scenes by virtue of their unique morphology, physiology, and coverage of the retina. Interestingly, the function of the melanopsin-containing RGCs in entraining the circadian clock need not involve much retinal processing, making it the simplest form of processing in the retina. This review focuses on recent advances in our understanding of retinal circuitry, visual processing, and retinal development demonstrated by innovative experimental techniques. It also discusses the advantages of using the retina as a model system to address some of the key questions in neuroscience.

The dream of many neuroscientists is to track a behavior right down to the activities of particular molecules. A small part of the dream came true in 2002. A series of elegant studies firmly established that a photopigment-like molecule, melanopsin, located in a specific population of the RGCs, is responsible for resetting the biological clock.

## From Molecules to Behavior

It had been clearly established that the entraining signal comes from the eyes (*1*, *2*), but that photoreceptors (rods and cones) are not needed (*3*, *4*). When Provencio and colleagues looked for a photopigment in frog skin using an antibody against bovine opsin, they identified a molecule in the dermal melanophores and named it melanopsin (*5*). Melanopsin has also been localized in the eye of the mouse, monkey, and human and, more precisely, in the ganglion cell layer of the mouse and monkey (*5*–*7*). These results indicated that some RGCs might contain a potential photopigment and therefore could directly sense light. In situ hybridization localized the melanopsin message to the RGCs projecting to the suprachiasmatic nucleus (SCN), a center related to the biological clock, further linking the melanopsin RGCs with a role in regulating circadian rhythm (*8*). The melanopsin messenger RNA has also been found in the PACAP (pituitary adenylate cyclase-activating polypeptide)-containing RGCs (*9*) that were previously shown to project to the SCN, indicating that PACAP might be used as a transmitter or modulator in this pathway.

Electrophysiological recordings from the SCN-projecting and melanopsin-containing RGCs (mcRGCs) revealed that they respond to light when synaptic transmission within the retina is blocked and even when they are isolated from the retina, directly demonstrating that the SCN-projecting mcRGCs are intrinsically light sensitive (*10*, *11*). The light responses of these RGCs show very long latency and little adaptation, properties inappropriate for coding dynamic images of vision. The spectral sensitivity of the mcRGCs is similar to the effective light spectrum of photo-entrainment (*11*). Construction of a transgenic mouse with labeled mcRGCs showed that they form a complete coverage of the retina, suggestive of a discrete population (Fig. 1). In addition to the SCN, these cells project to several non–image forming brain centers related to pupillary responses and circadian rhythm (*10*). All of the evidence mentioned above suggested that this newly characterized RGC type participates in entraining the circadian clock.

Researchers next questioned whether the entrainment is affected when melanopsin is depleted. Two groups independently constructed melanopsin-null mice and showed that, in the knockout mice, the rhythm and period of the clock were unaffected, but the circadian phase shift (a brief exposure of light during an activity phase delays the onset of the next activity phase in a continuous darkness regime) was significantly less delayed (*12*, *13*). In an independently constructed melanopsin-deficient mouse, the SCN-projecting RGCs lost intrinsic light responses. Furthermore, the pupillary response to intense light was abolished (*14*). These data show that melanopsin is necessary for generating intrinsic light responses, regulating pupillary responses and entraining the circadian rhythm. Most recent studies showed that depletion of melanopsin in the mice lacking rods and cones completely abolished photoentrainment, pupillary responses, and other non–image forming responses (*15*, *16*), demonstrating that photoreceptors and mcRGCs are the only inputs to the non–image forming systems.

The function of melanopsin in the non–image forming system has now been firmly established, and the activity of an individual molecule has been directly linked to certain aspects of a behavior.

## Complex Circuitry: Computation for Motion Direction

Electrophysiological recordings and electron microscopic investigation showed that only about 30% of mcRGCs receive inputs from rods and cones (*17*, *18*). It is sufficient for some of the non–image forming functions that the mcRGCs directly sense the light change and transmit the signal to the SCN and other related brain centers (*11*, *14*); little retinal processing is necessary. The direction-selective ganglion cell (DSGC) represents an example at the other end of the spectrum. A particular DSGC responds preferentially to movement of visual stimuli in one of the four cardinal directions (the preferred direction) and generates virtually no response to the opposite movement (the null direction). It is amazing that a simple structure as the retina (containing two steps of serial transmission and two levels of lateral interactions) can generate such a highly selective response. The mechanisms involved in computing the direction of motion have been much discussed over the past four decades (*19*).

With the help of experimental techniques ranging from gene manipulation to two-photon microscopy, major advances in understanding this complex retinal processing have been made in the last few years. One of the critical questions in computing motion direction is "where does the computation take place?" There are three obvious possibilities: it takes place on the dendrites of DSGCs (the postsynaptic model), in the neurons presynaptic to the DSGCs (the presynaptic model),

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or by a combination of the two. A key observation to be explained is the absence of DSGC response to motion in the null direction, indicating the involvement of an inhibitory mechanism. The starburst amacrine cell, a retinal interneuron, has many attractive properties and has long been the prime suspect for computing direction (*20*, *21*). Laser ablation of patches of starburst cells in combination with the application of various receptor blockers revealed connectivity between starburst cells and DSGCs. The starburst cells are symmetrically connected with the DSGCs for excitatory signals. There is no evidence that starburst cells supply the DSGCs the asymmetrical inhibition needed for direction selectivity (*19*, *22*, *23*). However, a transgenic mouse in which over 90% of starburst cells can be ablated by immunotoxin showed that both directionality and optokinetic nystagmus (OKN, a type of eye movement) were diminished (*24*). Therefore, the remaining starburst cells in the laser ablation experiment might be sufficient for direction selectivity, or the γ-aminobutyric acid (GABA)–releasing mechanism may have survived the ablation.

Patch clamp recordings from DSGCs showed conflicting results; some claimed a postsynaptic computation (*25*), whereas others found a presynaptic computation (*26*, *27*) or different computation sites for directional ON- and OFF-responses (*28*). A two-photon imaging study showed that the $Ca^{2+}$ signal in the tips of starburst cell processes was much stronger for the centrifugal movement than for the centripetal movement, suggesting that the starburst cells may underlie the fundamental computation of the direction (*29*). Consistent with this interpretation, simultaneous patch clamp recording from a DSGC and an overlapping starburst cell revealed that only starburst cells on one side supply inhibitory inputs to the DSGCs and that the direction of this asymmetrical inhibitory connection coincides with the DSGC's null direction (*27*). These studies established the importance of the starburst cells in the retinal direction selectivity and further highlighted the remaining issues: how do starburst cells generate asymmetrical signals and how do they form selective connection with the DSGCs?

## Functional Implication of DS Outputs

Very early in the investigation of retinal direction selectivity (DS), attempts have been made to link the retinal DS with eye movement. Two populations of the RGCs code motion direction in the retina: the ON DSGCs (responding to the onset of a flashing stimulus) and the ON-OFF DSGCs (responding to both the onset and offset of a flashing stimulus). The distribution of the preferred directions of the ON-OFF DSGCs coincides with the lines of action of the four extraocular recti muscles, leading to the proposal that the outputs of the ON-OFF DSGCs drive the extraocular recti muscles (*30*). It was later shown that ON DSGCs may be more important for the OKN: They project to the accessory optic system (AOS), and there are similarities in response dynamics and in the distribution of the preferred directions between the ON DSGCs and the AOS neurons (*31*). In mice lacking the metabotropic glutamate receptor subunit (mGluR6), OKN is greatly impaired (*32*). The mGluR6 mediates the ON signal; therefore, the effect of deletion of the subunit is consistent with a contribution of ON DSGCs in the OKN. Ablating starburst amacrine cells resulted in abolition of directional responses of ON-OFF DSGCs and blockade of the OKN (*24*, *33*). Although it is not clear whether the ON DSGCs were directly affected by the treatment, the observation raises a possibility that the ON-OFF DSGCs participate in the OKN. Here lies an opportunity to identify the RGCs responsible for OKN, to dissect the receptors identities on the DSGCs, and to monitor the system output (OKN) while manipulating the activity of a distinct RGC population.

## Wiring Up the Circuitry

The vertebrate retina is a simple and orderly structure with three neuronal layers and two synaptic layers. The processes of different RGC populations stratify in different sublaminae, where they receive inputs from different bipolar and amacrine cells (Fig. 1).

The dendritic fields of the retinal ganglion cells undergo a great deal of remodeling during development (*34*, *35*). The dendrites of the DSGCs begin to contact the starburst cell plexus very early during development, and the extent of contact increases during development (*36*), reflecting the maturation of the circuitry.

Factors affecting dendritic remodeling include intrinsic genetic programs, extrinsic factors, and neuronal activity. Application of brain-derived neurotrophic factor (BDNF) to

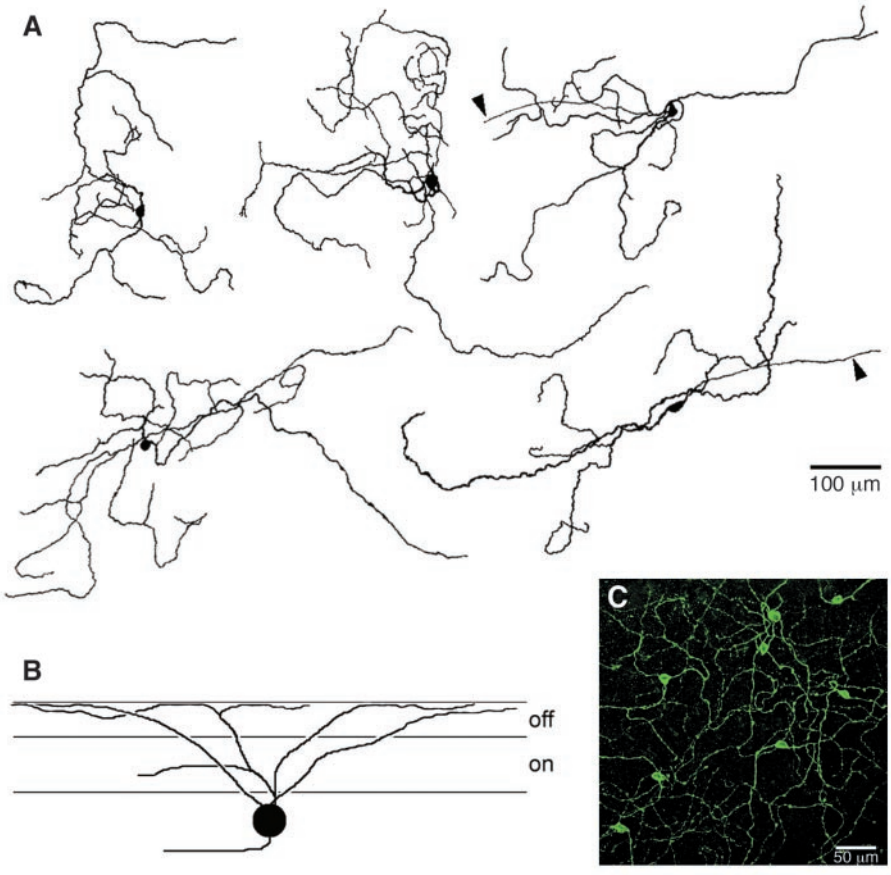


**Fig. 1.** The morphology of photosensitive RGCs. (**A**) Camera lucida drawings of cells stained with lucifer yellow in retinal flat mounts. Arrowheads indicate axons. (**B**) Schematic summary of the location of dendrites of these cells, predominantly in the sublayer of the inner plexiform layer responsible for the OFF response. (**C**) Stacked confocal image of immunocytochemical staining of RGCs with melanopsin $NH_2$-terminal specific antibody, in which all focal planes containing labeled processes are combined. Because the stacking increased background, the sensitivity of the camera was reduced, making some faint processes not clearly visible. [(A) and (B) Adapted from (*11*); (C) adapted from (*10*)]

the developing tectum promotes dendritic growth of RGCs, whereas BDNF applied to the eye inhibits dendritic growth, indicating that BDNF may serve as a retrograde signal to regulate retinal wiring of RGCs after their axons reach the tectal targets (*37*). Contact with amacrine cells inhibits axonal growth and promotes dendritic growth, suggesting that signals from retinal neurons also play a role in controlling RGC dendritic development (*38*).

In addition to trophic factors and surface signals, neuronal activity is also important for dendritic remodeling. Blocking neurotransmission greatly reduces dendritic motility (*34*). Neurotransmitters involved in promoting dendritic motility vary at different developmental stages, correlating with neurotransmitters responsible for the retinal waves (*39*). $Ca^{2+}$ release from internal stores, triggered by local $Ca^{2+}$ entry induced by synaptic activity, is critical in stabilizing dendritic branches, whereas the global $Ca^{2+}$ activity induced by spiking is ineffective in dendritic remodeling (*40*). Thus, while waves of transmitter release promote dendritic motility, local $Ca^{2+}$ concentration (indicative of synaptic contact) stabilizes dendritic terminals. However, disruption of acetylcholine (ACh)-mediated spontaneous activity by depleting β2 subunits of nicotinic ACh receptors delays the gross stratification of the RGCs but does not seem to affect their gross morphology (*41*).

In order to regulate dendritic motility, different signals such as trophic factors, surface signals, and neurotransmitters must eventually converge on the regulation of cytoskeletons. The Rho family of small guanosine triphosphatases (GTPases) appear to be important components of intracellular pathways, with Rac and Rho in opposing roles in regulating dendritic remodeling (*34*).

The RGC could be a useful model for studying dendritic development and interaction because of its largely two-dimensional structure (Fig. 1). Among various model organisms, zebrafish may lead the way, due to the clear genetic background, short duration to reach maturity for the retina (~100 hours), and relative ease of observing single RGCs in vivo from birth to maturity (*42*, *43*).

## Coupled Networks

Many types of retinal neurons form gap junctions with neurons of the same type (homologous coupling) or of different types (heterologous coupling). Gap junctions are composed of connexins (Cxs); the first neuronal connexin successfully identified was Cx35 in fish retina (*44*). Subsequent efforts cloned more connexins in fish retina and tested their properties in a functional expression system (*45*). Intracellular injection of tracers linked with biotin revealed three types of gap junctions in the mammalian retina (*46*).

A series of elegant studies established the importance of Cx36, the murine ortholog of fish Cx35, in the retinal circuitry coding rod signals. Rod photoreceptors contact rod bipolar cells, and rod bipolar cells form synapses with a retinal interneuron called AII amacrine cell that forms gap junctions with ON cone bipolar cells and glycinergic (inhibitory) synapses with OFF cone bipolar cells. Therefore, rod signals are "piggybacked" onto cone pathways. Immunocytochemical staining in mouse and rat retinas using a polyclonal antibody against Cx36 showed the puncta of immunoreactivity concentrated in the area where AII amacrine cells form gap junctions with neighboring AII amacrine cells (*47*). No Cx36 positive immunoreactivity was detected on ON cone bipolar cells (*47*). In the rabbit retina, Cx36 has been localized predominantly to the crossings of AII amacrine cell processes, with staining also observed between bipolar axon terminal and single AII process (*48*). Replacing Cx36 gene with reporter genes revealed that Cx36 is expressed not only in AII amacrine cells but also in a few types of bipolar cells, some of which form gap junctions with AII amacrine cells (*49*). This finding supports previous reports that the gap junctions between AII amacrine cells and ON cone bipolar cells are bidirectional, both in passing tracers (*50*) and in electric current (*51*).

Recording field potentials, electroretinogram (ERG) and visual evoked potential (VEP), in Cx36 null mice revealed that (i) a-wave was not affected both under dark-adapted and light-adapted conditions, indicating photoreceptors are not affected by the genetic manipulation; (ii) the b-wave amplitude was much reduced under dark-adapted condition and increment threshold was much increased under light-adapted condition; (iii) at lower intensity, mutant animals showed a significant increase in VEP time-to-peak (*52*). These findings suggest that the ON pathway is impaired in Cx36 null mice, within the limits of interpretation of field potentials. Extracellular recordings from RGCs showed that only cone signals get through to RGCs in Cx36 knockout mice, demonstrating that Cx36 is essential for transmitting rod signals (*49*). This result indicates that other pathways communicating rod signals to RGCs, for example, gap junctions between rods and cones, utilize Cx36 as well.

Much remains to be clarified. The most prominent gap junctional coupling takes place between horizontal cells, and the identity of the connexin underlying this gap junction is still unknown. Even for the AII-ON cone bipolar cell coupling, the cause of the ultrastructral differences on two sides of the gap junction and the difference in tracer permeability remain to be learned.

## Nature's Brain Slice

The easy accessibility of the retina makes it an ideal model for addressing many important questions in the central nervous system. The fact that all connections are contained in a tissue about 100 μm thick provides a good opportunity to manipulate an input neuron while observing the activities of the receiving neurons in a normal neural environment. This advantage is nicely illustrated by experiments that employed multiple patch clamp recordings to illustrate the asymmetrical inhibitory connections between the starburst cells and the DSGCs (*27*). In addition, DeVries carried out an experiment in which he was able to simultaneously monitor the activities in two bipolar cells while driving a single photoreceptor. He found that the parallel temporal processing streams of the retina begin at the very first synapse by differential expression of AMPA or kainate receptors on different bipolar cells (*53*).

The two-dimensional layout of the RGCs is advantageous for application of multielectrode arrays, which were first used to reveal spontaneous waves in the developing retina (*54*) as well as the mosaic arrangement of RGC receptive fields (*55*). Such recordings also allowed extraction of information coded by neighboring cells in forms of synchronized activity (*56*–*59*). The discovery that there might be more signals than conventionally thought in the information transmitted to the higher brain centers by the RGCs prompted a reconsideration of the ways in which retinal outputs might be read.

The retina is also an attractive preparation for imaging experiments, because of its transparency and the laminar distribution of the dendritic arbors. In addition to the findings discussed on dendritic field remodeling, imaging technology expands the horizon of our knowledge of retinal development and processing. Imaging technology is essential in revealing the dynamic properties of the retinal waves during development (*60*). With multiphoton microscopy that avoids bleaching photo pigment, it is possible to peek into dendrites that would otherwise be out of reach (*29*, *61*).

## Outlook

Much remains to be clarified in the retinal circuitry and the functional connections of the RGCs and their targets. Combining various approaches and technologies to tackle problems in a relatively simple model of the central nervous system has been successful in elucidating the function of mcRGCs, the computation of motion direction, and the formation of connections of retinal circuitry. Because of these successes, we expect more breakthroughs to emerge in aspects of elucidating functions of other types of RGCs, revealing rules of connection during develop-

ment and uncovering mechanisms of neural computation. Knowledge and principles learned from the retina should be applicable in approaching similar problems in the other parts of the brain. As a result, we will see more clearly not only how we see but also how we think.

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