Chapter 2
Fundamental Retinal Circuitry for Circadian Rhythms

Enrica Strettoi and Vincenzo Parisi

Abstract A remarkable piece of tissue, the retina is a true outpost of the brain, peripheral only for its location on the back of the eye. Downstream of the photoreceptors, the specialized cells which transduce light energy into electric signals then conveyed to the brain by the optic nerve, approximately 60 types of neurons belonging to five classes are arranged in a sophisticated architecture and provide the substrate for extracting information pertinent to contrast, position in space, intensity, chromatic content, and movement. Light reaching photoreceptors and other photosensitive retinal neurons is also coded as temporal information pertinent to the alternation of night and day and to seasonal changes. This information is transmitted to a central clock located in the brain, which tunes biological rhythms to environmental light–dark cycles. Thus, a single sensory organ, the retina, informs the brain of light changes functional to vision, as well as to variations of light occurring in time, providing the core information for the existence of circadian rhythms. Correspondingly, this chapter summarizes fundamental features of retinal organization providing an overview of the main principles according to which the mammalian retina is built and operates as an organ of the visual system. The focus is, however, on retinal neuronal types and circuits forming the substrate for the establishment and function of circadian rhythms. Indications are given for appreciating the elaborate architecture of the whole retinal neurome and the likely existence of retinal channels deputed to code features of the visual scene of so far unsuspected complexity.

E. Strettoi, Ph.D. (✉)
Department of Pharmacology, Neuroscience Institute, Italian National Research Council (CNR), Area della Ricerca CNR, 56124 Pisa, Italy
e-mail: enrica.strettoi@in.cnr.it

V. Parisi, M.D.
GB Bietti Foundation for Ophthalmology, Via Livenza 3, 00198 Rome, Italy
e-mail: vmparisi@gmail.com

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2.1 Introduction

The retina is essentially a piece of the brain with a peripheral location. For its embryological origin (the neuroepithelium of the optic cup, an evagination of the diencephalon) and structural organization, comprising neurons and glia, the retina is part of the CNS. Functionally, it is much more complex than a plain relay station or than the simple photographic camera to which it is often compared. Rather, photoreceptors, the specialized sensors, transmit light-initiated signals to a cohort of about 60 types of neurons, arranged in an exquisitely ordered architecture, altogether extracting from light information pertinent to intensity, chromatic content, contrast, movement, and position. This bulk of data is then conveyed to the visual centers of the brain by the optic nerve using a complex (and partially undeciphered) code. Noticeably, the light reaching photoreceptors and other photosensitive retinal neurons is also coded as temporal information pertinent to the alternation of night and day and to seasonal changes. This information is transmitted to a central clock located in the brain that tunes various, and even distant, biological rhythms (body temperature, heart rate, fertility, etc.) to environmental light–dark cycles.

This book deals principally with circadian rhythms, a direct consequence of the ability of the retina to inform the brain about changes of light in time [1]; hence, this chapter will focus on retinal neuronal types directly involved in such a functional task. Also, it will illustrate fundamental principles of organization typical of the retina of mammals only and provide a necessarily simplified information, which is instrumental to the following chapters. Accordingly, most of the references quoted consist of review articles meant to address the reader toward fundamental literature, condensing decades of studies of many laboratories in the field of retinal organization.

2.2 Classes and Types of Retinal Neurons

The mammalian retina is only about 200 μm thick: light has to traverse it completely and with minimal distortions to reach the photosensitive elements placed on the opposite side of the leaflet. Within this minimum width, stereotyped networks of neurons (functional units) are repeated in an orderly fashion to ensure total coverage of the retinal surface and, correspondingly, adequate sampling of the outside world (Fig. 2.1).

Retinal neurons belong to five classes only: photoreceptors (rods and cones); horizontal cells (occurring in two main types); bipolar cells (further divided into rod

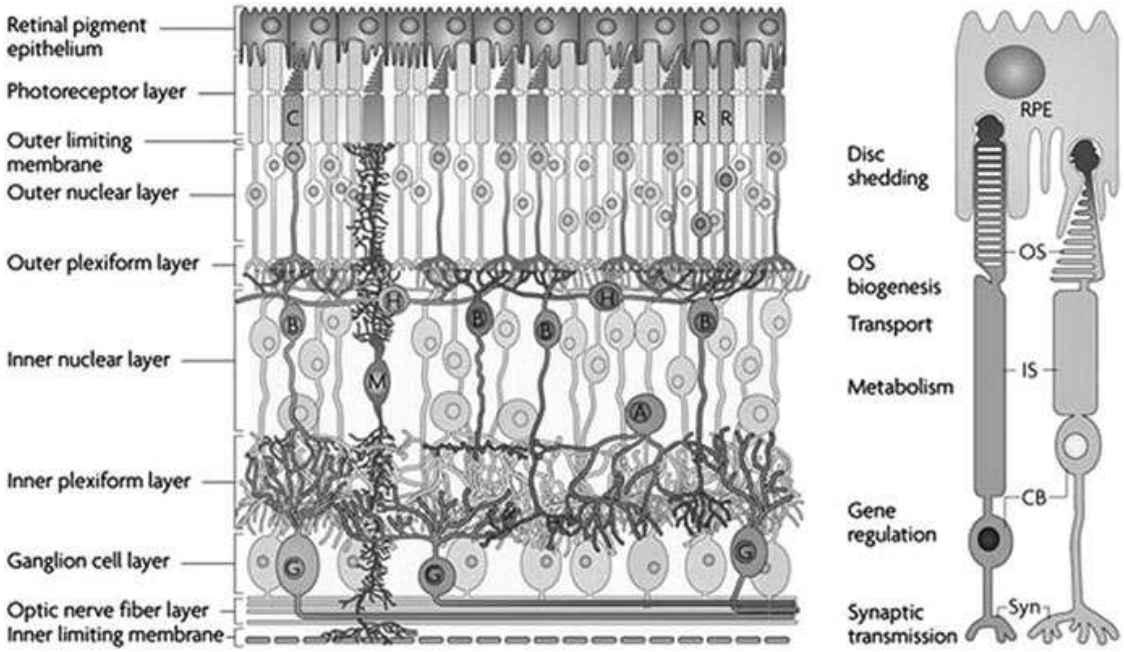

Fig. 2.1 Retinal organization. *Left side*: Rod (R) and cone (C) photoreceptors have cell bodies in the outer nuclear layer. Rod and cone inner segments (IS), rich in mitochondria and other organelles, and outer segments (OS) lay beyond the outer limiting membrane. Outer segments penetrate the microvilli of the retinal pigment epithelium (RPE). Photoreceptor synaptic terminals reside in the outer plexiform layer where synapses with horizontal (H) and bipolar (B) cells are established. The inner nuclear layer contains the cell bodies of bipolar, horizontal, and amacrine (A) cells, as well as those of Müller glial (M) cells. In the inner plexiform layer, bipolar cells establish synapses with amacrine and ganglion (G) cells: these send their axons to the optic nerve that carries signals to the brain. Processes of Müller cells form the outer limiting membrane, while their end-feet form the inner limiting membrane. A representative cone pathway is shown in *blue* and a representative rod pathway is shown in *orange. Right side*: Illustrations of rod and cone morphologies that include subcellular locations of functions (Modified with permission from [52])

bipolars, existing as a single type, and cone bipolars, occurring in a dozen different types); amacrine cells (approximately 30 types); and, finally, ganglion cells, which are believed to occur in some 20 distinct varieties [2]. Different classes of retinal neurons subserve globally different functions according to a hierarchical arrangement: upon light absorption and subsequent excitation of rods and cones, the visual signal has to travel through a cascade of other neurons (first bipolar and horizontal cells, then amacrine cells), to reach the final retinal station, represented by ganglion cells, which, with their axons, form the optic nerve and transport the signal to the visual centers of the brain. Traditionally, photoreceptors and bipolar and ganglion cells are assigned to the vertical, retinofugal pathway, while horizontal and amacrine cells are considered as modulatory interneurons with a horizontal arrangement. Exceptions apply to this rule: amacrine cells occur in two broad categories, i.e., wide-field and small-field neurons. While the wide-field amacrine cells have a large lateral spread and perform what is called "lateral integration," some small-field amacrines are true components of the vertical retinal pathway, as explained later for the case of AII cells.

Each stage of the retinal pathway (i.e., from photoreceptors to bipolar cells) does not simply implicate conduction and transfer of information but rather introduces integration, elaboration, and processing operated by neuronal networks. Elaboration relies both upon classes of cells performing globally different tasks in series (i.e., photoreceptors and bipolar cells) and on the existence of morphologically and functionally different types of neurons within a class. A retinal cell type is defined by a set of specific features, among which morphology (shape), size, level of stratification in the inner plexiform layer (IPL), density over the retinal surface, and molecular signature are highly distinctive. Different cell types perform different functional tasks, typically in parallel. Thus, a given light signal is decomposed in retinal parallel streams, each of them devoted to a particular property (chromatic composition, brightness, contrast, etc.) of the visual stimulus.

Remarkably, the identity of the neuronal types participating in the retinal networks (the so-called retinal neurome) is largely known [2]. That is to say that, unlike the case of other CNS areas, i.e., the cerebral cortex, the virtually complete panel of neuronal types existing in the retina has been deciphered, defining each of them in terms of stringent morphological and topographic properties as well as according to broad functional characteristics. However, data about exact physiological roles of known morphological types are still missing. The disparity of available morphological and physiological information is especially evident for amacrine and ganglion cells, which occur in many types. Even the code used to deliver information toward the brain through the optic nerve is partially obscure and the variety of visual properties embedded in a scene and encoded in ganglion cell signals more complex than previously believed [3].

2.3 Photoreceptors: Cones and Rods

Most mammalian retinas possess two or three types of cones and one type of rods distinguishable by their slightly different size and shape but equally perfectly aligned as it can be appreciated in a retinal vertical section (Figs. 2.2 and 2.3).

Cones are larger than rods, display an elliptical structure, and have their cell bodies (containing the nucleus and a small rim of cytoplasm) aligned in a row immediately below the outer limiting membrane (OLM). Their outermost portion is composed of an inner segment, mostly comprised of endoplasmic reticulum, Golgi apparatus, and a rich complement of mitochondria, in addition to a tapered outer segment. This is a modified cilium consisting of regularly tiled and tightly packed membranes containing the visual pigments (cone opsins). Inner and outer segments of cones project into the subretinal space toward the retinal pigment epithelium. Cones are specialized for vision in bright light (photopic vision), have low sensitivity, and also carry chromatic information. Their response to light is rapid.

Rods are more slender than cones and have a cylindrical (rod-like) shape. They also have thin inner and outer segments located beyond the OLM and the tips of their outer segments are equally surrounded by the apical processes of the retinal

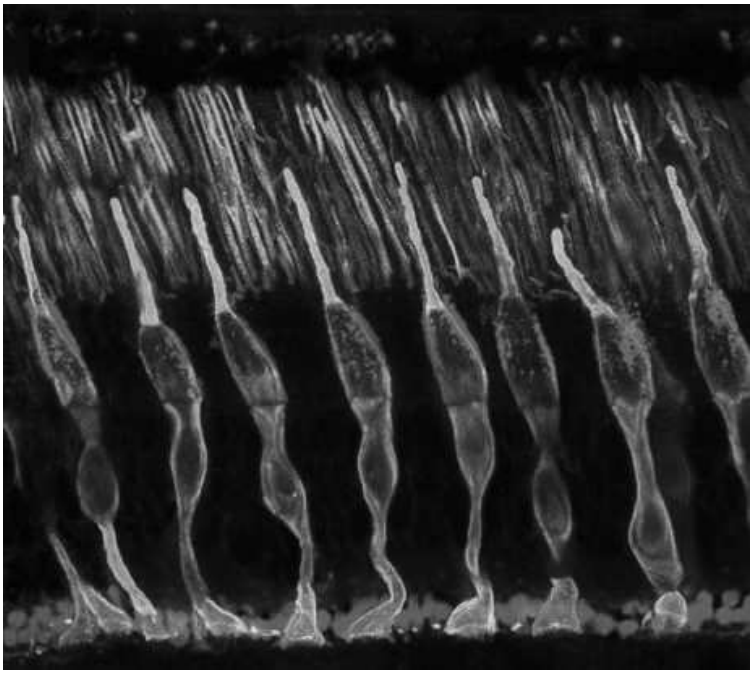

Fig. 2.2 Vertical section from a monkey retina stained with photoreceptor-specific antibodies revealing the detailed morphology of these cells. Cones (*green*, elongated cells) have *red*, punctate inner segments. Slender rods are visible in the background as *vertical, green lines*, corresponding to their outer segments. Synaptic terminals of rods appear as *bright red spherules* (Picture by Nicholas Cuenca, reproduced with permission from http://www.vision-research.eu/index.php?id=471&no_cache=1&sword_list[]=cuenca)

pigment epithelial cells. While cone cell bodies (mostly occupied by their nuclei) form a single row in the outer nuclear layer (ONL), rod nuclei are distributed in multiple layers, whose number changes in different species and is typically quite high (10–12 rows) in the small eye of a mouse. Rods have extremely high sensitivity and are specialized for vision at low light levels (scotopic vision); they actually detect single quanta of light, with an efficiency similar to the most sensitive of physical instruments. However, rods respond to light stimulation much more slowly than cones.

The morphological similarity of rods and cones is maintained at the ultrastructural level, which allows appreciation of the extremely specialized organization of these small neurons in spatial terms. Outer segments are comprised of stacks of regularly arranged membranes containing the highest density of proteins found in mammals. Besides photosensitive elements (rhodopsin for rods and cone opsins for cones), the various proteins constituting and controlling the phototransduction cascade are located here.

A noticeable difference between rods and cones is that in rods, the outer segments are separated from the cell membrane (they are called disks) (Fig. 2.4). In cones, they are rather invaginations of the plasma membrane itself. Inner segments of both rods and cones are rich in organelles and are very similar. Nuclei are different as the chromatin of rods is typically more condensed; this feature is often used to distinguish rods and cones by simple DNA staining methods. Another important

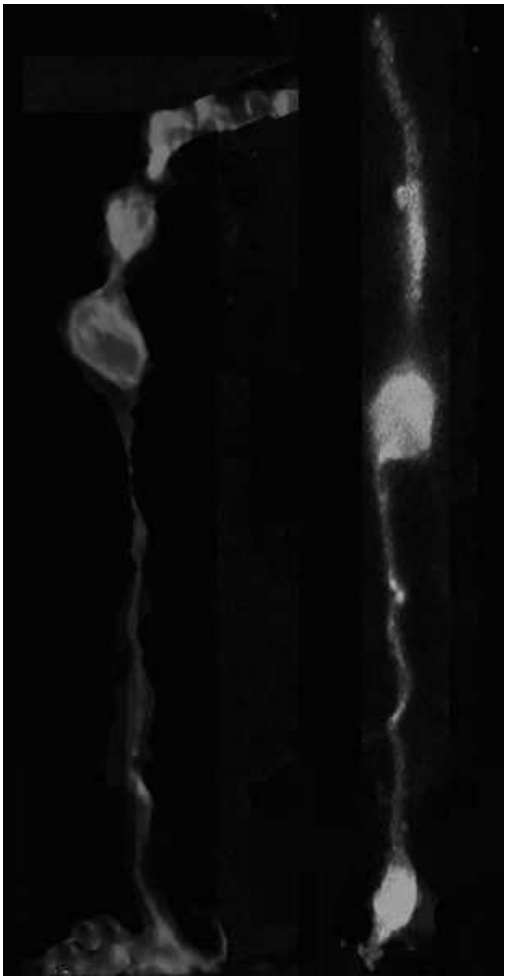

Fig. 2.3 Montage of photomicrographs of cone (*red*) and rod (*green*) photoreceptors from the mouse retina, stained by gun delivery of fluorescent nanoparticles [53]. The different size of their synaptic endings, at the bottom of the picture, is clearly visible

element of distinction is given by the size and shape of synaptic endings: these are simple ball-shaped terminals, called spherules, in rods, and larger, more complex, pyramid-shaped endings (pedicles) in cones (Fig. 2.5). In both cases, the main constituents of the ending are synaptic vesicles, filled with glutamate. However, there is dissimilarity in synaptic connectivity: a rod spherule typically establishes one or two synaptic connections with the dendrites of its dedicated second order neurons (rod bipolar and horizontal cells), while a cone makes numerous connections with second order neurons (cone bipolar and horizontal cells).

The cone ending is considered one of the most elaborate synaptic complexes of the whole CNS: in the human retina it can engage as many as 500 synaptic connections [4]. The reason of this diversity will be explained later.

Rods and cones are joined by tiny gap junctions made out of connexin 36. These contribute to the transfer of visual signals from the scotopic to the photopic channels and might be also implicated in what is known as the bystander effect. This is the complex of non-cell-autonomous events occurring in a cell type due to the effect of factors deriving from nearby cells. In the case of photoreceptors, this effect is believed to play a role when a mutation occurring in the rods, and consequently causing their death, leads to the secondary degeneration of nearby cones, such as happens in retinitis pigmentosa, a severe disease leading to near blindness [5, 6].

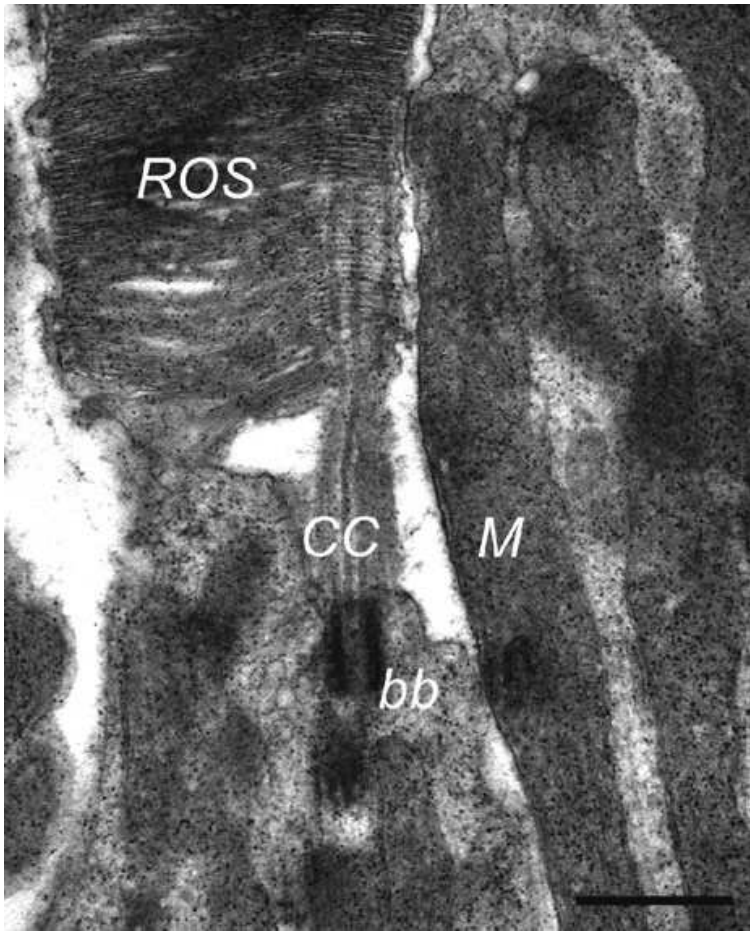

Fig. 2.4 Electron micrograph of photoreceptor outer and inner segments. The regularly stacked disks of the rod outer segment (ROS) and the inner segment, rich in mitochondria (M), are shown. *CC* connecting cilium, *bb* basal body, Bar is 0.5 μm

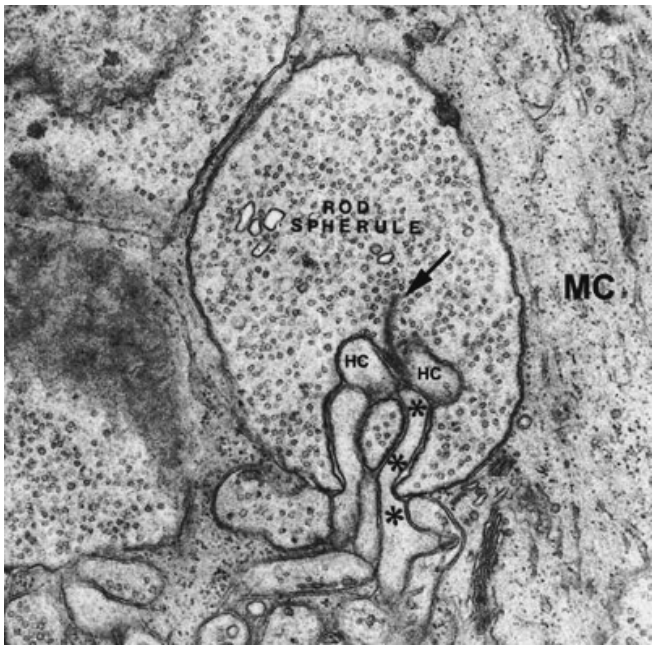

Fig. 2.5 Electron micrograph of the outer plexiform layer of the rabbit retina, showing the ultrastructure of a rod spherule, filled with synaptic vesicles. The presynaptic site is marked by a characteristic ribbon (arrow); postsynaptic processes are arranged in a triad, where the central element is the terminal dendrite of a rod bipolar cell (*asterisks*) and the two lateral elements belong to horizontal cells (HC). The large process of a Müller cell (MC) is also visible (Modified with permission from [54])

2.4 Outer Segment Renewal

Outer segments of rods and cones, containing the visual pigments and thus constituting the photosensitive part of the cell, are membranous protrusions evaginating from the base, near the photoreceptor cilium. Here, the opsin protein, synthesized in the inner segments and modified in the Golgi apparatus, becomes incorporated in the outer membrane by means of specialized areas and mechanisms of fusion [7]. The protein is synthesized by the photoreceptor itself, and the vitamin moiety of the photopigment is provided by the pigment epithelium. Binding of the two components takes place at the base of the outer segments. While the base is actively growing, the tip of the outer segment is constantly detached and eliminated: the pigment epithelium phagocytoses the apical portions of the outer segments that are being continuously renewed. Molecules of newly synthesized opsins can be followed while they move gradually from the base to the tip of an outer segment until the latter is "shed" [8]. Remnants of outer segments phagocytosed by the pigment epithelium are degraded by lysis. Shedding takes place regularly, so that a whole outer segment is completely renewed in a time interval of approximately 10 days. Moreover, the process of shedding is deeply influenced by external factors and by light in first instance. In amphibians, disk shedding in rods is activated by melatonin, which is produced by photoreceptors at night. Melatonin production is inhibited by light and dopamine. Conversely, dopamine (that is synthesized in neurons of the inner retina) is stimulated by light and inhibited by dark and the presence of melatonin. Because of the existence of rhythms and of opposite actions, rod outer segment disks are shed at light onset (in the morning), while cone outer segments are eliminated at the onset of darkness (at dusk) [9, 10]. The mechanisms regulating circadian disk shedding in mammal have yet to be clarified. For more details about disk shedding rhythms and their regulation by dopamine and melatonin, the reader is invited to refer to the Chap. "Role of Melatonin and Dopamine in the Regulation of Retinal Circadian Rhythms".

2.5 Horizontal Cells

Horizontal cells (HCs) are laterally interconnecting neurons with large cell bodies located in the outermost region of the inner nuclear layer (INL) and processes restricted to the outer plexiform layer (OPL). These neurons occupy a strategic position as they control signal processing in the outer retina and are themselves under neuromodulatory control from the retina and from the brain [11].

Most mammalian retinas have two types of horizontal cells, which can be named A- and B-types. Remarkably, the mouse retina has only one type of HC, morphologically similar to the B-type HC of other species. A-type HCs (Fig. 2.6) are large neurons with stout primary dendrites emerging directly from the cell body with a radial orientation giving rise to small, vertical terminals reaching the synaptic base of the photoreceptors. The B-type HC exhibits a smaller, bushy arborization with

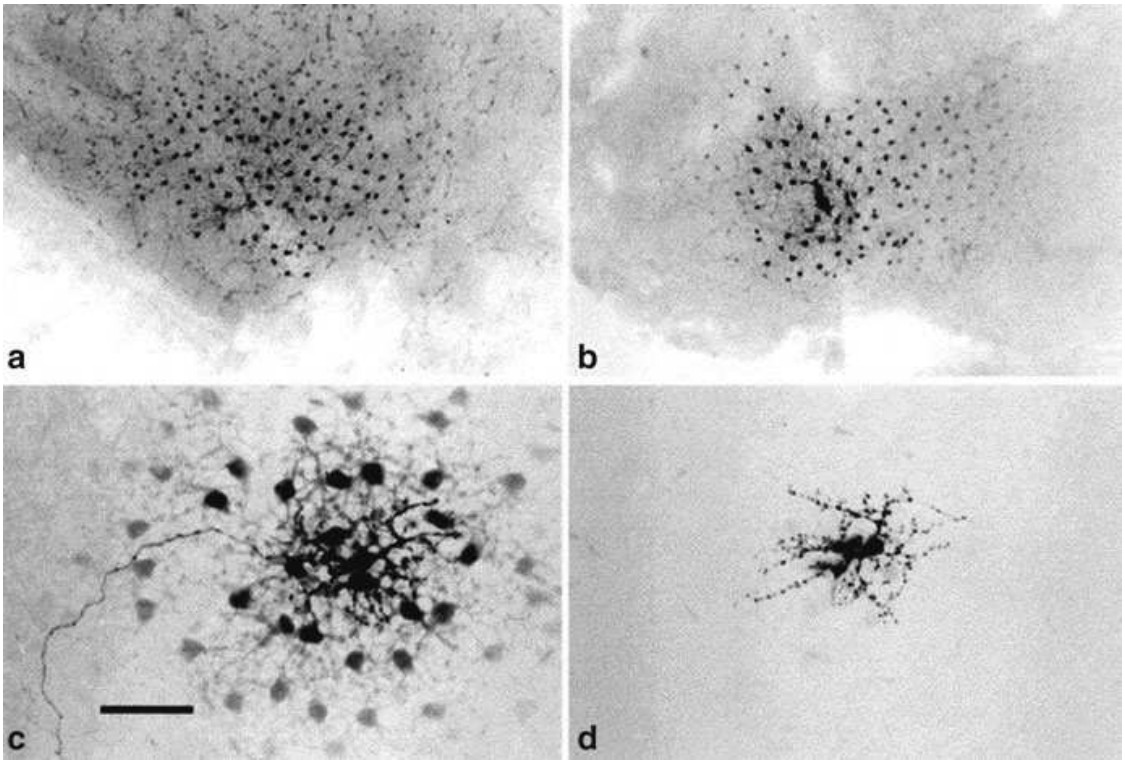

Fig. 2.6 Gap junctional coupling between horizontal cells in the mouse retina and regulation by Dopamine. (**a**) Neurobiotin tracer coupling in basal conditions. (**b**) Application of dopamine (100 μM) greatly reduces coupling. (**c**) Application of a pharmacological D_1 antagonist increases coupling. (**d**) Conversely, application of analogs of cAMP reduces the extent of coupling, as expected for a D_1 mechanism. Scale bar is 50 μm (Reproduced with permission from [55])

radial orientation, as well as a long, thin axon with horizontal course in the OPL. This atypical axon gives rise to a wide axonal ending, usually larger than the dendritic tree, with numerous terminals in the OPL. Because such axons are very long and thin, passive electrotonic spread of signals from one side of the cell to the other is negligible, and the two parts of the same cell behave independently. The dendrites of both A-type and B-type HCs are postsynaptic to cone pedicles, where they occupy the lateral elements of ribbon synapses. The axon terminals of the B-type HCs end in rod spherules, also constituting the lateral elements of ribbon synapses (Fig. 2.5).

Horizontal cells receive excitatory input directly from photoreceptors via chemical synapses mediated by glutamate acting on AMPA receptors and respond to light with graded hyperpolarization. They use GABA as a neurotransmitter but are considered unconventional GABAergic neurons for their unusual morphology and mode of transmitter release, which is not vesicular for the major part. An important feature of horizontal cells is that cells of the same type are connected by large gap junctions, the largest in the retina, through which they become excited by neighboring (homologous) neurons. For this reason, their receptive field is very wide, well beyond the extent of direct synaptic contact with photoreceptors.

The extent of HC coupling (as for other gap junction-connected neurons) can be visualized by intracellular injections of dyes that cross the gap junctions, thus revealing their morphological and functional syncytium across the whole OPL

(Fig. 2.6) [12]. Gap junctions might be constituted by connexin 57 or by connexin 50, where the diversity in their molecular composition contributes to the different properties of the various HC types.

A specific function of HCs is to send visual information back to cones by means of feedback connections established by dendrites penetrating cone pedicles. The functional effects of HC feedback onto cones are measurable even though conventional chemical synapses from HCs onto photoreceptors are observed only in a few species [13]. Responses of cones to light are antagonized from opposite responses due to the HC feedback effect. An important consequence of HC antagonistic feedback is "spatial opponency," documented for cones only. Horizontal cells have extremely large receptive fields, and their influences on photoreceptors and bipolar cells, whose receptive fields are much narrower, can be observed in the far periphery of these narrow-field cells. Wide light stimuli exert a depolarizing (antagonistic) effect on cones as an effect of horizontal cell feedback; such a depolarization opposes the direct hyperpolarization produced by the direct excitation of light falling onto cones. This spatial opponency is retained in the transmission from cones to bipolar cells to ganglion cells and contributes to the fundamental center-surround organization of the receptive fields of these neurons.

Horizontal cells also exert a direct, opposing action onto bipolar cells, reinforcing the effect of feedback onto cones. This feedforward action is particularly clear in OFF cone bipolars, which undergo delayed depolarization as an effect of wide-field stimuli affecting HCs. Thus, the "center-surround" spatial organization of bipolar cells is shaped due to the contribution of HCs.

The functional properties of horizontal cells can be modified as an effect of various substances released by retinal neurons as a function of changes in conditions of illumination. These substances (neuromodulators) have the important role of matching retinal physiology to ambient conditions. Dopamine is the best known neuromodulator and is released by dopaminergic amacrines (see below), which exerts multiple effects on horizontal cells acting on D1 and D2 receptors. The most relevant of these effects is a decrease in the extent of gap junction coupling and a consequent reduction of the receptive field size of HCs. This, in turn, is reflected in their changing outcome onto bipolar cells and onto cones through feedback action. In summary, horizontal cells contribute to the organization of spatially opponent receptive fields of bipolar cells and modulate the photoreceptor signal with different lighting conditions also adjusting what is known as the synaptic gain in the outer retina. Further, the horizontal cell itself is under the control of dopamine, which deeply affects its receptive field properties according to light conditions [11].

2.6 Bipolar Cells and ON and OFF Channels

In mammals, cones and rods converge upon separate sets of bipolar cells, correspondingly named cone and rod bipolars [2] (Fig. 2.7). All bipolar cells, like photoreceptors, use glutamate as a neurotransmitter and possess the adequate machinery for transport and release of this amino acid.

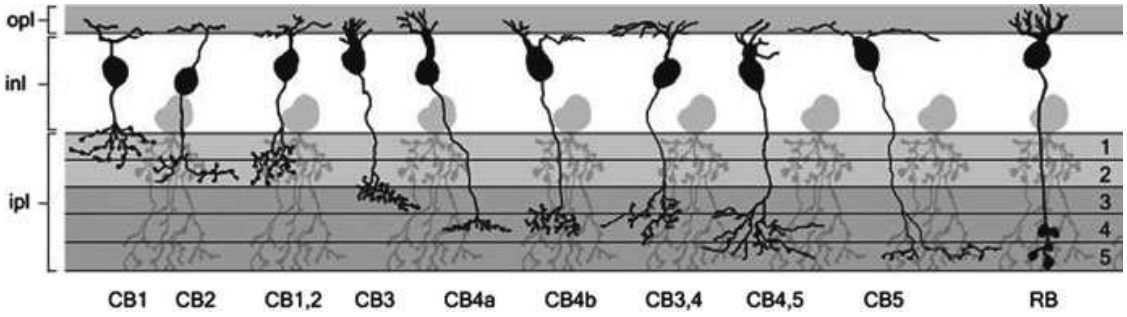

Fig. 2.7 Drawing of the types of mouse bipolar cells identified by means of gun delivery of fluorescent nanoparticles. One single type of rod bipolar cell (RBC) and 9 types of cone bipolar cells are illustrated (Reproduced with permission from [17])

Cone bipolar cells are now known to occur in a dozen different types [14, 15]. Each cone pedicle establishes multiple contacts in the OPL, making at least one synapse with each type of cone bipolar cell and all the bipolars spanning its territory. In the primate retina, cone pedicles have a highly complex structure and are engaged in up to 500 synaptic connections [4]. This arrangement splits signals generated in cone-mediated illumination conditions (the photopic range) into parallel streams, each extracting a specific parameter from the stimulus and, in turn, informing ganglion cells about this parameter.

The first known set of parallel channels generated at the cone-to-cone bipolar synapse is the dichotomous separation of the signal into ON and OFF channels. Actually, roughly one half of the existing types of cone bipolar cells belong to the so-called ON-center type, for they respond with a graded depolarization to light falling in the center of their receptive field. Since cones respond to light with graded hyperpolarizations, the synapse between cones and ON-center cone bipolars is called sign inverting. Inversion is achieved by the interaction of glutamate released by cones with mGluR6, a distinct type of metabotropic glutamate receptor, almost exclusively located in the retina on the dendritic tips of ON bipolar cells [16]. Although ON cone bipolars occur in different types (5–6 according to various classifications) [14, 17], with peculiar morphological and molecular signatures, all their terminal axonal arborizations remain confined to the innermost two thirds of the IPL, near the ganglion cell bodies. This is the so-called sublamina ON or, in anatomical terms, sublamina b. The other half of the cone bipolar cell population belongs instead to the OFF-center variety. These cells carry ionotropic glutamate receptors that maintain the cone-to-cone bipolar synapse sign conserving. Thus, as photoreceptors, these cells respond to light falling in the center of their receptive fields with graded hyperpolarizations; accordingly, their axonal arbors end in the outer part of the IPL, also called sublamina OFF or sublamina a. Fundamental rules of retinal organization are that (a) cone bipolars are presynaptic to ganglion cell dendrites in the corresponding (and spatially restricted) sublamina of the IPL and (b) cone bipolars establish sign-conserving synapses with ganglion cell dendrites. Ganglion cells also occur in ON and OFF functional types and their dendrites follow the rule of being stratified within the innermost and outermost portions of the IPL, respectively. This holds true for dendrites of amacrine cells as well; when cells with ON–OFF functional properties are found (i.e., amacrines and ganglion cells

responding both at light increments and decrements), these are multistratified through both IPL sublaminae. Because of the exquisite association of morphology and function in the retina, prediction on the physiology of newly discovered cell types can be made on the basis of their anatomical properties and vice versa [18].

Noticeably, ON-center cells display OFF responses when light falls in the periphery of their receptive field and vice versa, so that each cell is maximally sensitive to contrast. An obvious functional consequence of the ON–OFF dichotomy is that one retinal channel is specialized in informing the brain about stimuli brighter than the background (the ON-center channel), while the second is better tuned for stimuli darker than the background (the OFF-center channel). This property contributes to the high sensitivity of the retina (and more generally of the visual system) to stimulus contrast, as first demonstrated by Kuffler from cat ganglion cells recordings [19].

ON and OFF functional differences among cone bipolar cells are established due to molecular diversity in glutamate receptors (i.e., metabotropic versus ionotropic). Glutamate receptors expressed by different types of cone bipolar cells vary in their inactivation kinetics, which might be rapid or slow. Corresponding cone bipolar types are transient or sustained, respectively [20, 21], and are tuned with phasic or tonic temporal properties of light stimuli. Also, certain types of cone bipolars are dedicated to process precise chromatic features of light signals [22]; in particular, an ancient retinal pathway has been described dedicated to blue light where a "blue cone bipolar" selectively contacts the short wavelength sensitive cones, thus ensuring that the chromatic information is not degraded while moving along the vertical retinal pathway [23]. In this particular channel, light is coded in terms of chromaticity, while other channels respond better to luminosity content. Additional elements of variety among bipolar cell types are found in the molecular composition of gap junctions in the IPL, which in turn determine their electrical properties and response to external control factors including calcium, pH, and dopamine [24, 25].

Our understanding of the functional abilities specific to each cone bipolar cell types is still limited. Bringing to light the full panel of molecular differences subserving specific functional prerogatives of these neurons represents one of the challenges of modern retinal research.

2.7 The Rod Pathway and the Piggyback Arrangement

Rod bipolar cells belong to a single morphological and functional type. Characterized by cells with cell bodies located in the outer part of the INL with a profuse dendritic arborization terminating in small tips, each of them receives a synaptic contact by a single rod terminal in the OPL. Although numerical variations in different species occur, the principle holds true that convergence in the rod pathway is high and that a large number of rods (i.e., from about 20 in a mouse to up to 80 in a rabbit) make connections with one postsynaptic bipolar cell. Thanks to this arrangement, summation of inputs at a postsynaptic level is achieved and sensitivity of the rod system

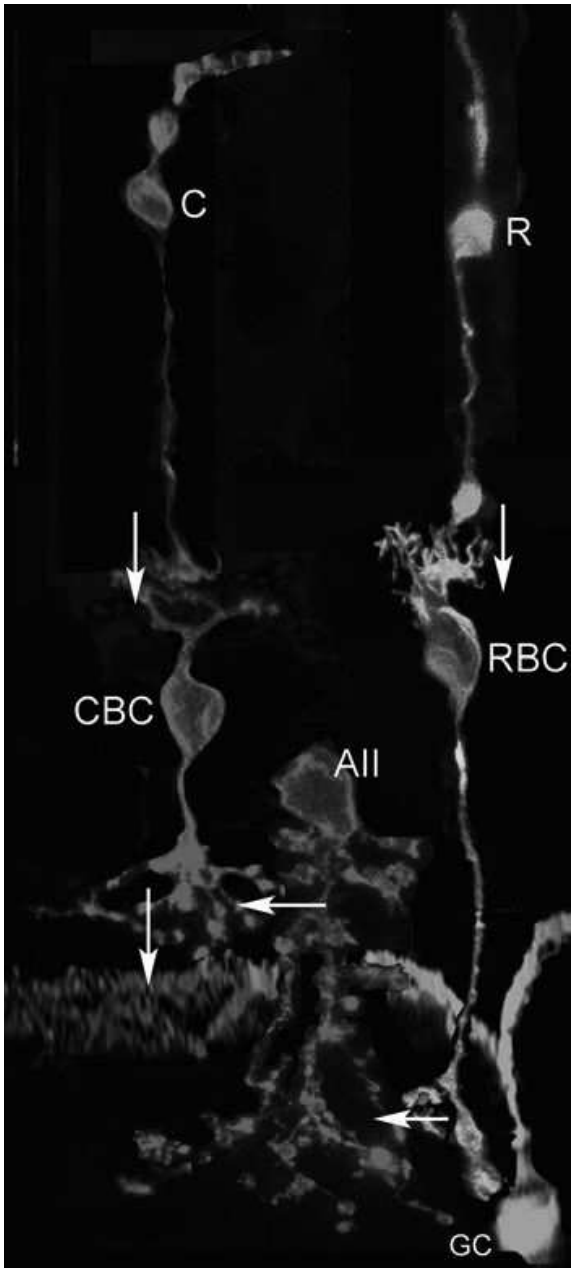

Fig. 2.8 Semi-schematic representation of the rod pathway. Images of neurons have been obtained from mouse retinal sections stained by delivery of lipophilic fluorescent dies, in the mouse retina. Rods (R) and cones (C) converge upon separate sets of bipolar cells. Rod bipolar (RB) cells are presynaptic s to AII amacrine cells, which, in turn, send the information to cone bipolar (CB) cells. Retinofugal connections with ganglion cells (GCs) are established by axonal endings of CBs making synapses onto GC dendrites (Reproduced with permission from [56])

increased. Extreme amplification in rods by the phototransduction cascade [26] and high convergence along all the steps of the rod pathway [27] result in enormous sensitivity of the retina to dim light: a change in cat ganglion cell electrophysiological response can be recorded even when only one of the thousands of rods within its receptive field captures a single photon [18]. In turn, each rod bipolar engages connections with all the rods within its reach. From the inner part of the rod bipolar cell body, a stout axon emerges, whose bulbous endings terminate in the deepest part of the IPL, near the cell bodies of ganglion cells. Here, synapses to and from third order neurons are established (Fig. 2.8). Functionally, rod bipolar cells have a center-surround organization of their receptive fields like cone bipolars. Their membrane potential undergoes a graded depolarization in response to stimulation of photoreceptors located in the central area of their receptive field, while a graded

hyperpolarization is evoked by peripheral (annular) light stimulation. Hence, the synapses between rods and rod bipolars are sign inverting, exactly like ON-center cone bipolars, where inversion is achieved by the interaction of glutamate released by rods with mGluR6 receptors: rod bipolars are "ON-center" neurons showing a typical "OFF response" when the periphery of their circular receptive field is stimulated [4].

2.8 AII Amacrine Cells

The specialized chain of neurons carrying signals generated in rods across the retina to the ganglion cells is called the rod pathway and represents one of the most studied and best characterized networks of the mammalian retina. Here, a summary of this fundamental module of neuronal architecture is schematized in Fig. 2.8.

Axonal arborizations of rod bipolar cells occupy the deepest part of the ON sublamina of the IPL. Here, they establish multiple synaptic contacts with the dendrites of "dedicated" narrow-field amacrine cells called AII. Only exceptionally do the axonal arbors of rod bipolars make direct connections with ganglion cells. AII amacrines were first described in the cat [28] and are true hallmarks of the mammalian retina. These cells exhibit a typical bistratified morphology (Fig. 2.8). The outer dendritic arbor, restricted to the OFF sublamina of the IPL, consists of round, synaptic endings, known as lobular appendages, while the innermost arborization, spreading in the ON part of the layer, is comprised of thin, elongated dendrites, with tangentially oriented tips called "tufted processes." Such processes (a) receive multiple synapses from the axonal endings of rod bipolar cells, for which AII amacrines are the principal postsynaptic target; (b) engage homologous gap junctions with similar dendrites of nearby AIIs; and (c) form large, heterologous gap junctions with the axonal endings of most types of cone bipolar cells that ramify in the deepest layer of the IPL (ON cone bipolars) [29, 30]. AII amacrines respond to light with a graded depolarization similar (but more transient) to that generated in rod bipolar cells and, strictly speaking, they belong to the ON channel. The ON–OFF dichotomy generated in the cone pathway at the first cone-to-bipolar synaptic station becomes available to the rod pathway thanks to the bistratified morphology of AII amacrines. In fact, in the outermost part of the IPL, the lobular appendages of AII cells form sign-inverting, glycinergic synapses with the axonal arborizations of OFF cone bipolars, thus "feeding" the rod-generated signal in the OFF channel. Electrophysiological studies have shown that the heterologous gap junctions established in sublamina ON allow sign-conserving transfer of information between AII amacrines and cone bipolars terminating there. Transmission in the opposite direction occurs but it is less effective [31]. By means of this peculiar 5-neuron chain, the depolarizing response generated by light excitation of the central receptive field of a rod bipolar cell is transferred to AII amacrine cells, then split into the ON and the OFF retinal sublaminae and fed into the homologous channels of the cone pathway; output synapses of cone bipolar axonal arborizations onto ON and OFF ganglion

cells dendrites in the corresponding sublaminae of the IPL are the gate through which the scotopic signal gains access to the retinal exit.

Although few direct synapses exist linking AII amacrine cells and dendrites of ganglion cells directly, physiology indicates that rarely ganglion cells are purely rod driven. Through AII amacrines, the bulk of the scotopic signal is injected directly into the cone pathway, in what has been named a "piggyback" array. AII amacrine cells, therefore, can be regarded as true elements of the "vertical" retinal pathway, even though usually cells of their class are considered as modulatory interneurons with a lateral arrangement.

More than simple conduits of signals, AII amacrine cells constitute nodal points of intersection of both the rod and cone systems and the ON and OFF channels. In the scotopic range, light excites rods, which, in turn, evokes a depolarization in rod bipolar cells, causing the release of glutamate from their axonal endings. Glutamate excites AII amacrines, causing simultaneously (1) the release of glycine onto OFF cone bipolar cells, translated into inhibition of OFF ganglion cells; (2) the depolarization of ON cone bipolar cells via gap junctions, coded as excitation of ON ganglion cells; and (3) the diffusion of the signal through the network of AIIs, through their homologous gap junctions. In summary, rod-generated signals produce inhibition of the OFF pathway and excitation of the ON pathway.

In photopic conditions, AII amacrine cells receive inputs from both ON (through gap junctions) and OFF cone bipolars (by means of chemical synapses), which also independently feed the corresponding ganglion cells. ON-center responses cause strong glycine release from AII lobular appendages, which, accordingly, inhibits OFF cone bipolar cells. Hence, in bright light the OFF channel is inhibited by the ON channel [24].

2.9 The Rod Pathway and Dopaminergic Amacrine Cells: The Power of Being Few

The piggyback arrangement does not implicate unidirectional information flow: first of all, cone bipolar cells make feedback synapses onto the lobular appendages of AII amacrines, thus creating the ground for transfer of data from the photopic to the scotopic channel. In addition, each AII cell receives a profuse innervation from dopaminergic amacrine (DA) cells. These are wide-field neurons, first described as A18 amacrines, occurring at low density (only 600 of them in a mouse retina, or 0.1 % of the total amacrine cell population in this species). However, these cells have very wide dendritic arbors with extensive ramifications ensuring efficient coverage of the retinal surface. Their dendrites are largely restricted in the outermost portion of the IPL (the sublamina 1), in close proximity to the amacrine cell bodies, and entangle a dense network of processes forming characteristic rings. These are essentially synaptic varicosities encircling the main dendrites of AII amacrines that therefore receive multiple contacts from dopaminergic endings [32]. In addition,

A18 amacrines give rise to long axon-like processes running in different strata of the IPL and forming a plexus in the OPL, for which they are also named interplexiform cells. A second type of dopaminergic cell (type 2) has been described using genetic labeling for catecholamines [33]. Electrophysiological evidence suggests that the two types respond to light in sustained and transient manners, respectively.

By means of their endings as well as their distal processes in the outer retina, dopaminergic amacrines can control their targets both through synaptic release of dopamine and via a paracrine discharge of the neurotransmitter [34]. Dopamine is a very important global regulator of retinal sensitivity to light and a powerful modulator of gap junction permeability. This transmitter controls multiple elements of the retinal circuitry; it alters the gap-junctional conductance between photoreceptors [35], horizontal cells, and amacrine cells; increases the responses of ionotropic glutamate receptors in bipolar cells; and ultimately affects the center-surround balance of ganglion cells. In scotopic and photopic conditions, and with the contribution of the control exerted by dopaminergic innervation, the AII amacrine cell operates as a switch from one input pathway to another, with high efficiency [36]. Because of a weaker coupling of gap junctions in photopic conditions, the dissipation of cone-initiated signals through the homologous network of AII amacrines is limited, so that this can reach ganglion cells more effectively. Dopaminergic control of gap junction permeability is, among others, an important component of the retinal circadian clock, as extensively described in the following chapters.

2.10 Possible Explanation and Advantages of the Piggyback Arrangement

Additional pathways exist in the retina through which light signals generated in rods can reach ganglion cells [37]. First of all, rods are linked by very small gap junctions, mainly established by telodendria, thin processes at the base of rod spherules and consisting of few connexons each. One of the advantages of these gap junctions is that noise arising in single rods (because of quantal fluctuation of neurotransmitter) is reduced by electrical coupling. Moreover, in some species rods are coupled electrically to cones, and in theory scotopic information can enter the cone system and bypass the rod bipolars. Actually, the functional effect of rod–cone gap junctions does not seem very high on ganglion cells, and their precise role is still debated. In several species, rods (albeit only a fraction) make direct connections with cone bipolar cells of the OFF pathway that are therefore called mixed bipolars. The effect on ganglion cell physiology varies among mammals.

Noticeably, all these pathways originate from rods but directly, or later along the retinal pathway, converge upon cone bipolars to access ganglion cells [2]. A possibility is that all the rod systems, and particularly the piggyback arrangement, originate evolutionarily from a common, ancestral pathway dedicated to photopic vision. It is known that rods and vision in dim light appear only after the evolution of the

jawed vertebrates [38]. Hence, retinal network profiles were initially shaped by cones. Because of the creation of parallel channels built at the cone-to-bipolar synapses, it is possible to imagine an antique vertebrate retina comprised of cones, various types of cone bipolars, and cone-driven amacrine cells ultimately driving ganglion cells.

Thanks to the evolution of rhodopsin and its segregation into a new type of photoreceptor, the prototype of a rod, the retina became duplex. However, needless duplication of the whole inner circuitry was avoided by making preexisting pathways accessible to the rod system by means of rod bipolars and AII amacrine cells. High convergence of rods upon dedicated bipolars made the latter highly sensitive. AII amacrines ensured appropriate connectivity to the ON and OFF channels also thereby injecting the rod-generated information into the diversity (and processing abilities) of the various types of cone bipolars. The shared inner retinal circuitry was made more efficient by a dedicated network of dopaminergic amacrines controlling sensitivity by affecting gap junction permeability

A reflection of the fact that the original retinal circuitry was shaped by cones can be appreciated considering that, although rods are usually one order of magnitude more numerous than cones in most mammalian species, cone bipolars are instead much more numerous than rod bipolar cells [15], a fact only partially explained by the high convergence of the rod pathway mentioned above. Cone bipolars are numerous because they come in different types, each of them dedicated to a specific function and each cone has to contact them all to ensure parallel processing of the signal. However, the piggyback arrangement guarantees the access of the scotopic signal to the whole retinal processing originally evolved in the cone system.

2.11 Diverse and Complex Retinal Neurons: Amacrine and Ganglion Cells

The variety of cell types within a class increases from the outer to the inner retina and reaches its maximum for amacrines and ganglion cells [2]. Giving an account of such a complexity is well beyond the scope of this chapter, which is primarily focused upon those retinal neurons more directly involved in circadian rhythms. However, some general and important rules underlying the main properties of these cell classes should be mentioned.

Amacrine cells are by definition neurons without an axon, although some of them break this (quite atypical) rule. They occur in a large variety probably not yet completely exploited, and this, together with the relatively inaccessible position they occupy in the wiring diagram of the retina, makes their physiology still largely unexplored. As already mentioned, amacrine cells occur in two broad varieties, i.e., small-field and large-field cells. In general, small-field cells (like AII amacrines) have a radial arrangement in the IPL, might span across the ON and OFF sublaminae, and use glycine as primary neurotransmitter. Conversely, large-field amacrines

(such as dopaminergic amacrines, or cholinergic, starburst, amacrine cells) have a radial, starlike morphology, with a tangential spread in the IPL and dendrites which are usually restricted in specific sublaminae. They use GABA as primary neurotransmitter.

Amacrines in general receive their synaptic inputs from bipolar cells, on which they might return feedback synapses; amacrine cells are also pre- and postsynaptic to each other and might be joined by gap junctions. Finally, they are presynaptic to ganglion cells. Connections involving amacrine cells can occur laterally as well as vertically across the ON and OFF sublaminae of the IPL. Since amacrine cells are inhibitory interneurons, the synaptic arrangements described above mediate feedback inhibition (i.e., from amacrines back to bipolar cells), lateral inhibition (i.e., between amacrine cells arranged horizontally), crossover inhibition (i.e., involving amacrines spanning radially across the two sublaminae of the IPL), and feedforward inhibition (i.e., from amacrines to other amacrines or to postsynaptic bipolar cells). While synapses established by bipolar cells in the IPL can be distinguished ultrastructurally for the presence of characteristic ribbons, amacrine cells make conventional chemical synapses by means of varicosities filled with synaptic vesicles. Such varicosities are quite similar for most amacrine cell types, making it hard to attribute a given contact to a specific parent amacrine cell. Hence, their circuitry in the IPL has yet to be explored. A connectome approach (i.e., the reconstruction of a complete map of the retinal neural connections), in which neuronal networks are traced by large-scale electron microscopy and extensive computation, is going to contribute sensibly to an advancement in this arduous task [39]. Functionally, amacrine cells refine the output of bipolar cells, performing computations that, ultimately, shape the physiological properties of ganglion cells and create task-specific types among them. Recognized examples of tasks to which the computational capabilities of amacrine cells are known to contribute are center and surround effects and directional selectivity [40] (i.e., the capability of certain ganglion cells to become excited by a stimulus moving in one direction and inhibited by a stimulus moving in the opposite direction). The list of newly discovered properties of amacrine cells continues to grow [41].

2.12 Ganglion Cells

Ganglion cells have been hard to classify so far, although many different types have been labeled by antibody staining, intracellular injections, genetic targeting, etc. (Fig. 2.9). Recent data suggest that the retina of common laboratory mammals has approximately 20 ganglion cell types [2]. This number reflects the fact that besides an input from the 12 types of bipolar cells described above, ganglion cells are shaped also by the contribution of amacrine cells, so that additional functional channels are created at the retinal exit [42]. Thus, moving from the outer to the inner retina, the number of visual channels expands, increasing the computational power

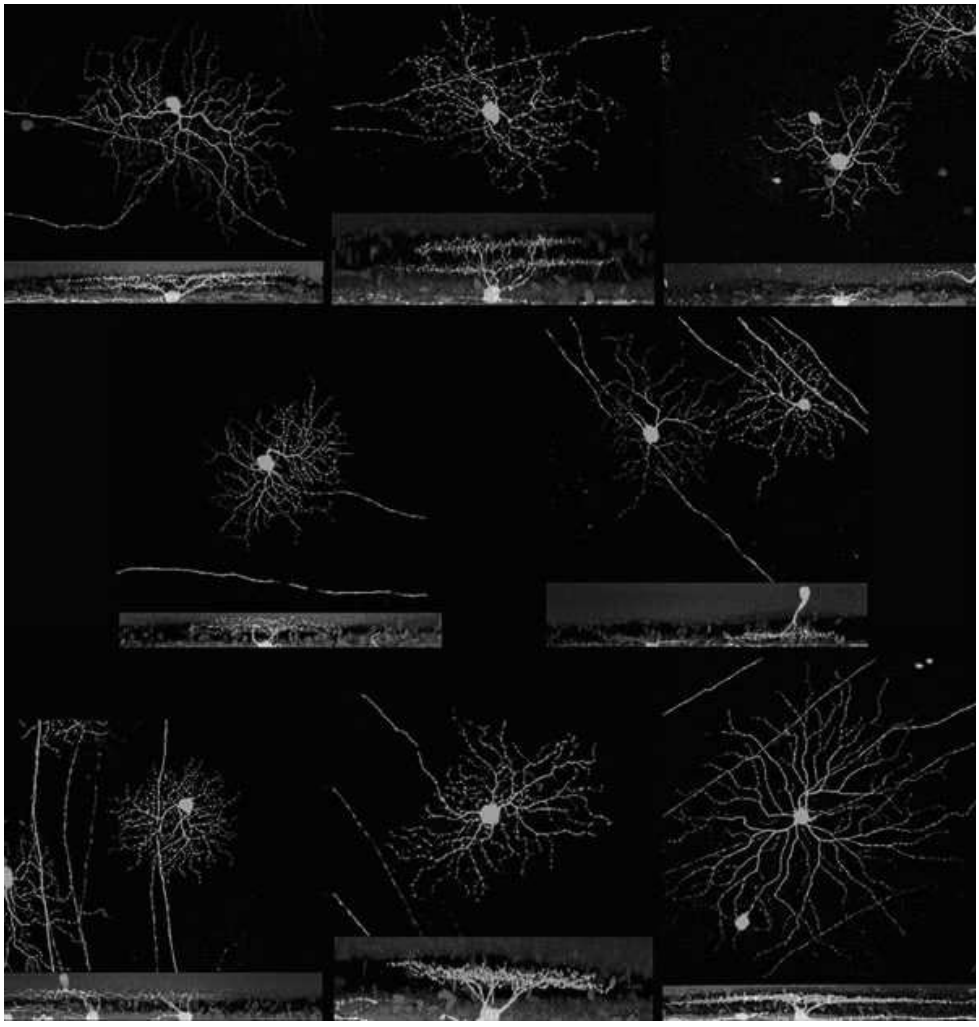

Fig. 2.9 Composite of different types of ganglion cells from the mouse retina. Transgenic expression of GFP in ganglion cells (shown in *green*) reveals their detailed morphology in retinal whole mounts. Computer rotations in the *vertical plane* show the different levels of stratification of their dendrites in the IPL, whose boundaries are marked by the nuclei of cells located in the inner nuclear and ganglion cells layers, labeled by a *red* DNA-binding molecule (see [47])

and feature selectivity operated on the signal. The visual scene is then presented to the brain by means of trains and patterns of action potentials traveling along the axons of ganglion cells constituting the optic nerve fibers and the optic nerve. As stated before, the functional properties of ganglion cells (best known from electrophysiological studies conducted in the rabbit retina) are actively explored and still partially obscure. Especially intriguing is the task of deciphering the code through which ganglion cells provide the results of the computation performed in the IPL to downstream brain areas through the optic nerve [3]. This code appears to have (previously unsuspected) multiple levels of complexity.

Morphological and physiological correlates of ganglion cell properties have allowed the identification of various types comprising (among others) ON-tonic and OFF-tonic cells; blue-ON and blue-OFF ganglion cells; an ON direction selective

cell, which projects to the accessory optic system and forms the basis of optokinetic nystagmus; and ON–OFF directionally selective cells of various types. A recently discovered type, the most common in the retina of the mouse, was demonstrated to detect small moving objects down to the receptive field size of bipolar cells, but only on a featureless or stationary background. These cells may serve as "alarm neurons" for overhead predators [2]. A type of its own is represented by the intrinsically photosensitive (melanopsin) ganglion cells described below.

2.13 Melanopsin Ganglion Cells

A second role for the eye has been described besides sight: independently of vision of forms, built-in sensors paired to canonical photoreceptors have the function of measuring ambient light. These dedicated sensors are intrinsically light-sensitive ganglion cells, whose discovery represents a breakthrough in the field of retinal organization [43, 44].

These neurons (wide and sparse, see Fig. 2.10) contain a photopigment called melanopsin (with an absorption peak of the light at a ~480 nm) and are involved in various reflexive responses of the brain and body to the presence of (day)light, such as the regulation of circadian rhythms, pupillary reflex, and other nonvisual responses to light. Melanopsin ganglion cells project to various brain targets including the olivary pretectal nucleus (responsible for controlling the pupil of the eye), the lateral geniculate nucleus (LGN), and the suprachiasmatic nucleus of the hypothalamus (the master clock of circadian rhythms). Intrinsically photosensitive retinal ganglion cells (ipRGC) comprise only ~1–3 % of all the retinal ganglion cells and can be visualized selectively with melanopsin-specific antibodies (Fig. 2.10) [45].

Studies using profoundly blind humans lacking functional rods and cones showed that, similarly to other mammals, the human retina contains some type of non-rod, non-cone photoreceptor, whose identity was eventually found to be a ganglion cell. These studies were performed examining patients with rare diseases leading to rod and cone degeneration but preserving ganglion cells. Similarly to mice with inherited photoreceptor diseases leading to progressive death of rods and cones, these patients continued to exhibit circadian photoentrainment, circadian behavioral patterns, and pupil reactions, with peak spectral sensitivities compatible to that for melanopsin photopigment [46]. In both humans and mice, melanopsin ganglion cells appear particularly robust to secondary effects of degeneration affecting the survival of photoreceptors, such as retinitis pigmentosa [47, 48], as well as in pathologies primarily affecting ganglion cells, like mitochondrial optic neuropathies (i.e., Leber hereditary optic neuropathy and dominant optic atrophy). The latter selectively involve ganglion cells and cause major visual loss with a relatively preserved pupillary light reflex. Recent studies show melanopsin retinal ganglion cells are resistant to neurodegeneration caused by dysfunction of mitochondria as

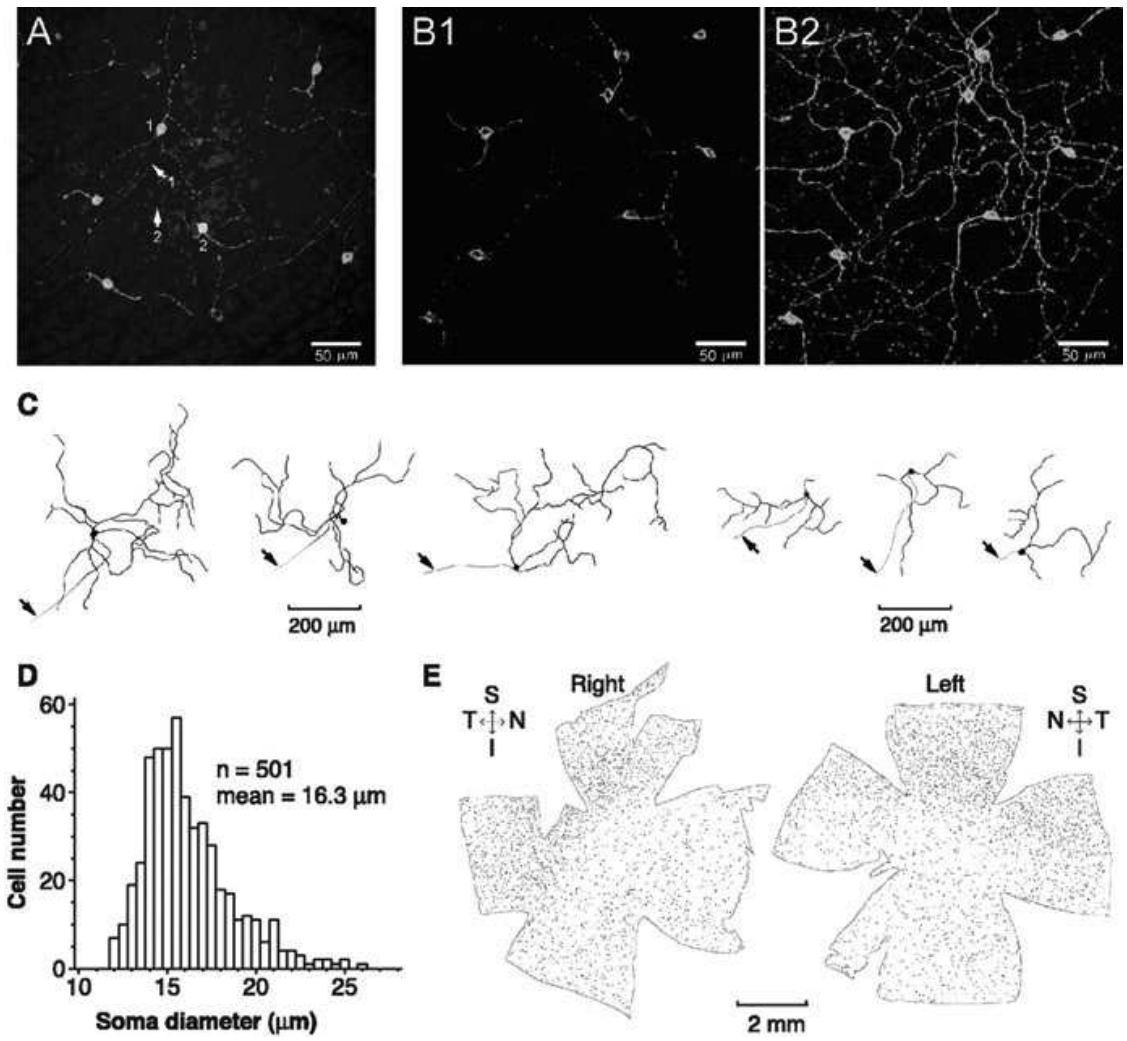

Fig. 2.10 Ganglion cells in the flat-mounted rat retina after melanopsin-antibody staining (*green* signal in **A**, **B1** and **B2**). (**C**) Drawings of examples of melanopsin GCs, where *arrows* indicate axons. (**D**) Soma-size distribution of melanopsin GCs. (**E**) Distribution of melanopsin-positive GCs on whole-mount retinas, showing the higher cell density in the superior (S) and temporal (T) quadrants (Reproduced with permission from [45])

shown by retention of non-image-forming functions in visually impaired patients [48]. Intrinsic resistance of melanopsin ganglion cells to degeneration might open new avenues for vision restoration based on these cells; their robustness to metabolic insults might be explored in search of intrinsic protective mechanisms that might be applied to glaucoma or similar disorders [49]. Indeed, the existence of intrinsically photosensitive ganglion cells has inspired experiments of vision restoration based on inner retinal intervention: transgenic technology can target the expression of melanopsin in inner retinal neurons (bipolar or ganglion cells) of individuals in which primary photoreceptors have been irretrievably lost by degenerative diseases [50].

2.14 Conclusions

In summary, there are two parallel pathways for vision: one arising in the outer retina and based upon rod and cone photoreceptors and a second channel detecting visual brightness arising from the inner retina. The outer and inner retinal channels are not entirely separate: rods and cones themselves also feed into intrinsically photosensitive ganglion cells, which are more complex than initially assumed, at least in some species, and send projections to multiple brain targets, including those deputed to image formation [51]. Intrinsically photosensitive ganglion cells thus might contribute to non-image-forming functions like circadian rhythms, behavior, and pupil reactions, as well as to conscious sight, with a proposed role in mesopic vision. Knowledge of the exact contribution of melanopsin ganglion cells in vision and in non-image-forming functions may have an impact on basic science and on human health in general: the consequences of altered circadian rhythms, when these cells are defective, are already of obvious relevance to clinical medicine, while the possibility of targeting these neurons for vision repair strategies is actively investigated.

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