

Unsolved retinal questions

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ABSTRACT

The era of modern retinal studies began over 60 years ago when it became possible to record and stain intracellularly individual neurons in each of the five retinal cell classes (MacNichol and Svaetichin, 1958; Tomita et al., 1967; Werblin and Dowling, 1969; Kaneko, 1970). Other technological advances over the past half century have also greatly facilitated research on molecular and cellular mechanisms underlying retinal function and synaptic circuitry. At the anatomical level, cellular mechanisms and classification has advanced with antibody labeling (Ghosh et al., 2004) and the use of genetic markers (Sanes and Masland, 2015). The ultrastructure of retinal synapses and their arrangements has been revealed with electron microscopic analysis (Dowling and Boycott, 1966), and more recently, connectomic methods have been used to study the ultrastructural cellular and synaptic connectivity in a given retinal volume (Marc et al., 2013; Kim et al., 2026). At the physiological level, light responses from all classes of retinal neurons were first recorded with sharp micropipettes, and then with patch clamping techniques (Werblin and Dowling, 1969; Wu et al., 2000). Further, living retinal slices and flat-mounted retinal preparations were developed that allow for the recording of pharmacologically identifiable excitatory and inhibitory currents in single or multiple retinal cells under visual control (Werblin, 1978; Gao et al., 2000; Pang et al., 2024). Finally, the use of mutant animals to uncover aspects of visual function and behavior has come to the fore as well (Emran et al., 2007). Three senior scientists, either retired or close to retirement, whose laboratories participated in a number of these studies review this progress and suggest directions for future investigations.

1. Introduction

The retina is a true piece of the brain, having been pushed out into the eye during development. Studies on the retina have contributed in many ways to an understanding of brain mechanisms. It has been suggested that the retina may be the best understood part of the central nervous system (CNS), but many questions remain. Here we point out a number of unresolved issues with the hope that studies will be undertaken by the next generation of investigators to solve them. For each of the unresolved issues, we provide a brief summary on what is presently known as background, and state what is unresolved.

This essay clearly does not include every remaining unknown but focuses on areas of research that the authors have primarily focused upon. For example, many questions remain on the development of the retina, the genes involved in retinal function, and gene mutations that affect retinal function, subjects the authors have worked on to some extent, but are not included here.

We begin with the photoreceptors and then move on to the other retinal cells that process the visual information sensed by the photoreceptors. Most retinas contain two basic types of photoreceptors – rods and cones. Rods are concerned mainly with dim light vision and visual

adaptation, whereas cones operate at higher light intensities, are responsible for color vision and high acuity vision.

2. Photoreceptors

A number of interesting unanswered questions regarding the photoreceptors relate to how they operate, their sensitivity to light and the synapses they make. For example, it is well known that the photoreceptors are maintained in the dark in a partially depolarized state, due to Na^+ influx through cGMP-gated channels in the outer segment. An advantage of maintaining photoreceptor's dark potential in a partially depolarized state is to allow the photoreceptors to generate larger dim light postsynaptic responses near their dark potential, because the input-output relations of the photoreceptors are non-linear with the highest gain near the depolarized photoreceptor dark potential (Attwell et al., 1987). Light closes the outer segment Na^+ channels, hyperpolarizing the cell, which suppresses a dark release of its transmitter, L-glutamate. The glutamate release is calcium dependent, and the calcium activation curve in photoreceptors has its greatest slope near the dark potential, resulting in the highest synaptic gain in that voltage region. Thus, the photoreceptors release their transmitter in the dark, and the light

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evoked signal to second order cells is via a decrease in transmitter (Dowling and Ripps, 1973).

Why do vertebrate photoreceptors work this way, by decreasing transmitters in the light? Elsewhere in the CNS, neurons release transmitter when they are depolarized. Thus, vertebrate photoreceptors act as if they are excited by the dark and that light turns them off. Is there an advantage for doing it in this way? Clearly being on continuously in the dark is a substantial energy drain.

2.1. Visual adaptation

As one reduces the light-sensitive visual pigment levels in rod photoreceptors by raising rats, for example, on a vitamin A deficient diet, or by bleaching away visual pigment by light, the sensitivity of the retina decreases as one would expect. However, this happens not as a linear relationship but as an approximate log/linear relationship. That is, if half the visual pigment is lost, light sensitivity does not decrease by 2-fold (0.3 log units), but by two to three thousand-fold (2-3 log units) (Dowling and Wald, 1958; Dowling, 1960, 1963).

We now know why bleached pigment causes significant sensitivity loss in the photoreceptors (Cornwall and Fain, 1994; Matthews et al., 1996). The bleached pigment protein (opsin) weakly activates the G-protein transducin and the transduction cascade and so acts as if a dim light is falling on the retina, as suggested many years ago by Stiles and Crawford (1932). Presumably the same holds for vitamin A deficiency in which opsin without its chromophore, retinal, stimulates weakly the transduction cascade, raising visual threshold (Dowling, 1963).

On the other hand, when a background light is applied to a rat or human eye, the light sensitivity decreases linearly with intensity, the well-known Weber-Fechner incremental threshold relationship as shown in Fig. 1A (Dowling, 1963). However, as shown in Fig. 1A, the loss of visual pigment has little effect on the increment threshold level. Further, with dim background lights that do not bleach a significant amount of visual pigment, the increment threshold can be raised as

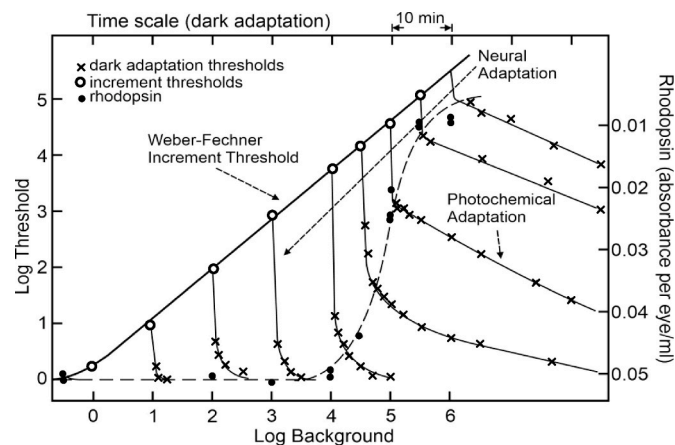


Fig. 1A. The effects of 5-min of background light adaptation over a range of intensities on rhodopsin content (filled circles, dashed line), increment thresholds (open circles, thick line, showing Weber's increment law relationship), and dark adaptation (crosses, thin lines). The increment threshold and dark adaptation data were obtained by recording the ERG b-wave from the eyes of the anesthetized rats. The rhodopsin content was not significantly reduced until the eye was exposed to background light intensities greater than $\log I = 4$. The log increment threshold, on the other hand, rose linearly with intensities greater than $\log I = 0.5$. Dark adaptation (crosses) was rapid until the eye was adapted to light that bleached a significant fraction of the rhodopsin (that is, greater than $\log I = 4$). Thus, dark adaptation consists of two components, an initial rapid phase (neural adaptation), followed by a slower component (photochemical adaptation). The extent of the slow component of dark adaptation depends on the amount of rhodopsin bleached. (From Dowling, 1963; 2012).

much as 3 log units or more. With bright background lights that bleach significant amounts of visual pigment (>10%), the bleached pigment affects the rate and extent of dark adaptation, and that relationship between bleached pigment and visual threshold during dark adaptation is identical to the effect of loss of visual pigment on the visual threshold in vitamin A deficiency.

The data shown in Fig. 1A were obtained by measuring the b-wave of the ERG which derives from the second order ON-bipolar cells in the retina. However, measurements from single cones from the mudpuppy retina (Fig. 1B) show that incremental sensitivity changes in background light originate in the photoreceptors (Normann and Werblin, 1974). What causes the substantial loss of visual sensitivity in photoreceptors with dim background lights that do not bleach significant amounts of visual pigment is not known and needs study.

With non-bleaching background illumination, and also immediately following lights that bleach significant amounts of visual pigment, visual threshold levels drop rapidly, within just a few minutes to dark-adapted levels or to a level appropriate to the amount of bleached pigment. In the latter case, dark adaptation then proceeds as visual pigment is regenerated, and after a strong bleach it can take up to 2 h or so in a rat (Dowling, 1963), or 30 min in humans (Rushton, 1961), for the rod pigment, rhodopsin, to fully regenerate. The same holds for cones and cone visual pigments, although cone visual pigments regenerate much faster than does rhodopsin. The slow phase of dark-adaptation depending on visual pigment levels and pace of regeneration, is called photochemical dark-adaptation, and the initial rapid phase of dark-adaptation following the cessation of an adapting light, neural adaptation (Fig. 1A). The mechanisms underlying neural dark adaptation in photoreceptors are unknown and clearly need investigation (Dowling, 1963; Normann and Werblin, 1974).

2.2. Rod saturation and network adaptation

It has long been known that when rods are exposed to a bright background light, they saturate and become totally unresponsive to light

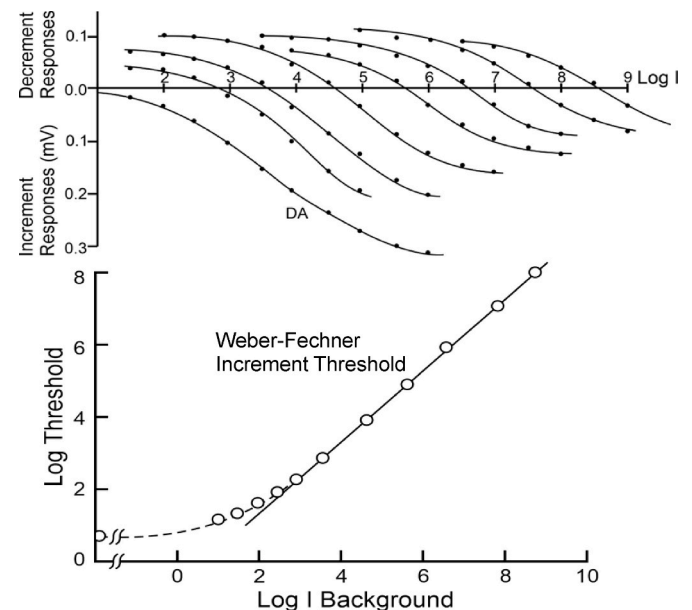


Fig. 1B. Upper panel: operating curves of mudpuppy cone light responses during light adaptation. Light-adapted curves at 8 background levels. Responses to test flashes brighter and dimmer than each background level are plotted below and above the abscissa, respectively. Background illumination caused a right-shift in the operating curve along the log intensity axis. Lower panel: Increment threshold curve for the cones derived from the operating curves in the upper panel, showing the Weber-Fechner increment threshold relationship. (From Normann and Werblin, 1974).

flashes, regardless of intensity (Aguilar and Stiles, 1954). However, in the all-rod retina of the skate, it was found that following rod saturation, if one waits for several minutes (~10–15 min) in the saturating background light, the rods begin to respond again, regaining some light sensitivity and response amplitude over time (Dowling and Ripps, 1970, 1972). Long thought to be an exception, Tikidji-Hamburn et al. (Tikidji-Hamburn et al., 2017) showed that mouse rods do the same thing. With time in a saturating background light, mouse rods escape saturation and become light sensitive again. Do rods in all species do this? We do not know at present.

The mechanism underlying this escape has been suggested to relate to the movement of the G-protein transducin from the outer to the inner segments in the light, a movement known to occur but whose significance has not been understood (Frederiksen et al., 2021). Also in bright light, there is photoregeneration of rhodopsin which likely contributes to the recovery (Dowling and Hubbard, 1963). Whether recovery from saturating background lights in skate relates to the movement of transducin and photoregeneration of rhodopsin is not known.

As noted earlier, significant changes in retinal sensitivity to light occur when very dim background lights are shone on the retina. Whereas with brighter background lights, the rise of increment thresholds reflects the decrease in light sensitivity of the photoreceptors (Fig. 1), with very dim background lights is this the case? Experiments in skate suggest this is not so. For example, with very dim backgrounds, no change in ERG rod sensitivity is observed, and the same holds true for horizontal cell responses (Dowling and Ripps, 1971). However, at the same time, both the ERG b-wave (reflecting bipolar cell activity) and on-ganglion cell discharges show a significant decrease in light sensitivity, suggesting there is a network adaptation mechanism in the inner retina not seen in the outer retina (Green et al., 1975). Further, the kinetics of the fast (neural) dark adaptation in the outer retinal cell responses is faster than in the proximal retinal cells. At present, we know little if anything, about the mechanisms underlying this network adaptation or the cells/synapses involved, although there is some suggestion that K^+ accumulation could be involved (Dowling and Ripps, 1976). There is evidence of similar findings in the mouse retina, suggesting that the skate findings are more general (Dunn et al., 2006). In mouse it has been proposed that the site of retinal network adaptation is the synapse between the rod bipolars and the All amacrine cells, and that Ca^{2+} may be involved (Griffis et al., 2023). But again the mechanisms underlying network adaptation are not clear and require further investigation.

2.3. Photoreceptor synapses

There are also some interesting and unusual features of the synapses made by the photoreceptors. For example, the excitatory synapses made by the photoreceptors, both rods and cones, are found within invaginations along the basal surfaces of the photoreceptor terminals (Fig. 2) (Dowling and Boycott, 1966; Dowling, 1968). Electron-dense synaptic ribbons sit just above the invaginations and neurotransmitter (L-glutamate) is released from the terminals at the presynaptic membrane just below the ribbons (Kolb, 1994). Dendrites from the second order ON-bipolar cells and processes from the horizontal cells enter into the invaginations to receive input from the photoreceptors. Thus, the central elements in the invaginations are depolarizing ON-bipolar cell dendrites whereas the lateral elements are horizontal cell processes (Dowling, 1970). It has long been supposed that invaginations promote interactions among the photoreceptors, On-bipolars and horizontal cells, but what is the nature of these interactions? There are also synapses made by the photoreceptors other than the ribbon synapses, the so-called flat or basal synapses. These are synapses on the dendrites of OFF-bipolar cells that hyperpolarize when activated. There is no aggregation of presynaptic synaptic vesicles at these junctions nor synaptic ribbons, but usually only some electron microscopic-visualized membranous filaments or densities associated with the junctions that identify them.

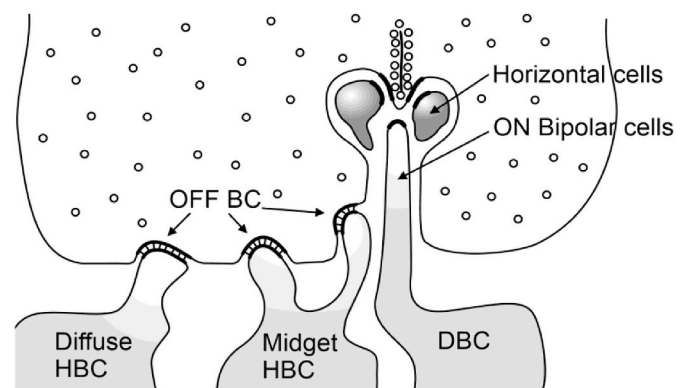


Fig. 2. Schematic drawing showing the arrangements and kinds of junctions made by bipolar cell dendrites and horizontal cell processes with the primate cone pedicle. Horizontal cell processes always end laterally and deeper in the invaginations. The invaginating depolarizing ON- midget bipolar cell and diffuse ON-bipolar cell dendrites (DBC) end as the central element in the cone terminal invaginations. The flat midget hyperpolarizing OFF- bipolar dendrites (MHBC) terminate at basal junctions that are in close proximity to an invagination, whereas the dendrites of the flat diffuse bipolar cells (DHBC) usually contact the basal surface of the cone terminal away from the invaginations. (modified from Dowling, 2012).

What activates the postsynaptic processes at these basal synapses? Is it from transmitter released into the invaginations from the synaptic ribbons above the invaginations that somehow finds its way to the basal junctions, by what has been called a spill-over mechanism (DeVries et al., 2006). This has been suggested, but evidence supporting this conjecture is still uncertain. The ON-bipolar cell dendrites and horizontal cell processes are within 20–40 nm of the presynaptic membrane where the transmitter is released, but some of the basal junctions can be as much as 1000 nm from where the transmitter is released. Some OFF-bipolar-cell dendrites, especially from small (midget) bipolar cells, hug closely to the central ON-bipolar cell dendrites as they enter the invaginations, in a semi-invaginating fashion, and these dendrites could easily be sensing the ribbon-related transmitter, but this seems unlikely for the distant basal junctions from larger, diffuse OFF-bipolar cells.

Is there another mechanism operating to release transmitters from distal basal junctions? In the zebrafish *nrc* (no optokinetic response c) mutant, there are no typical invaginations nor ribbon synapses made, but basal junctions are present and OFF-bipolar cells (and subsequently OFF-ganglion cells) are activated, suggesting this might be the case (Allwardt et al., 2001; Emran et al., 2007). In apparent confirmation of this finding, when ON-responses of zebrafish retinas are blocked pharmacologically, OFF responses can be recorded from the retinas both at the ERG level (d-waves which come from OFF bipolars (Wong et al., 2004) and from OFF-ganglion cells, but no ERG b-waves (from ON-bipolars) nor pure ON- ganglion cell responses (Emran et al., 2007).

If there is another mechanism causing distal basal synapses to release transmitter, there is another question to be answered, because aggregation of synaptic vesicles is not seen presynaptically at these junctions. Is transmitter not released via vesicles in this case as is typically so at CNS synapses? A suggestion in this regard is that transmitter might be released by a reverse transporter mechanism (Schwartz, 1982), but again this is conjecture and not proven.

Finally, why are ribbon synapses invaginated? One possible reason for the geometrically tortuous invaginating synapses is that it slows glutamate diffusion in the synaptic cleft and thus shapes synaptic responses, and there is some evidence for this (DeVries et al., 2006). A second reason is that the invaginations isolate one release site from another, thus avoiding mixtures of synchronized vesicular release at adjacent ribbons. A careful connectomic reconstruction along with patch clamp and calcium imaging analysis of the postsynaptic responses could perhaps shed light on these issues.

3. Retinal cells and their synapses/pharmacology

Four classes of neurons are found in the vertebrate retina, and they form two synaptic layers, one in the outer retina, the other in the inner retina. Thus, the retina can be considered as the equivalent of two brain nuclei; the outer one (outer plexiform layer), concerned primarily with the spatial and color aspects of a visual image falling on the retina, while the inner one (inner plexiform layer) is concerned primarily with temporal and dynamic aspects of an image (Dowling, 2012). The mammalian retina also contains three types of glial cells, the most prominent one being the Muller cells that extend radially across the retina, from the photoreceptors to the inner retinal surface where they form the inner limiting membrane. The other glial cells are astrocytes, found along the inner edge of the retina among the ganglion cells and their axons, and microglia which serve as retinal phagocytes, but are also involved in pruning neuronal processes especially in the developing retina.

Whereas each retinal cell class can be clearly differentiated anatomically and physiologically from cells of another class (with one or two exceptions), it has been shown that there are numerous subtypes in most retinal classes, with horizontal cells having the least (2-4), and amacrine cells the most with 50 -70. Various methods from anatomical to genetic are continually increasing the number of subtypes of cells present in a retina (McNeil and Masland, 1998; Martersteck et al., 2017), so some authors suggest there may be a total of over 130 subtypes of neurons in mammalian retinas. Thus, the number of cell subtypes claimed as being present in retinas is still uncertain, and defining what a particular subtype means and signifies is as yet unclear.

How are subtypes of retinal cells determined? Classically, subtypes of retinal cells were determined by morphology with the use of Golgi staining or similar methods. For example, amacrine and ganglion cells were classified as being stratified or diffuse, large or small field, etc. But even here difficulties are encountered (Cajal, 1893; Polyak, 1941; Boycott and Dowling, 1969). As shown by Boycott and Wässle (1974) neurons of the same general morphology in the cat's retina can look quite different in the center of the retina as compared with the retinal periphery. Are these the same or different subtypes of cells? Physiological criteria for classifying cells also have issues. Midget ganglion cells in the center of the primate retina receive input from a single cone photoreceptor via one midget bipolar cell, whereas in the periphery, they receive input from several photoreceptors via several midget bipolar cells (Boycott and Dowling, 1969). The peripheral midget ganglion cells thus differ physiologically from those in the retinal center, so should they be considered as a separate cell subtype?

Today, one favored classification scheme is based on RNA expression (scRNA-seq) that claims very large numbers of subtypes of amacrine and ganglion cells (Yan et al., 2020), but this technique raises other issues with regard to subtyping of cells. So, for example, in the inner plexiform layer (IPL) where these cell processes are located, there are many neuroactive substances found and presumably released from neurons at synapses and elsewhere. Some of these substances, such as retinoic acid, are capable of not only directly modulating synapses, but also the expression of RNA, thus altering other aspects of neuronal/cellular function as a result, for example, of circadian rhythms, dendritic and synaptic growth, etc. (McMahon and Dowling, 2023). Certainly, ganglion cells innervating different brain regions are also likely to show different RNA expression patterns, even if the ganglion cells are basically of the same subtype. So, should these cells be considered the same or different subtypes?

But even if some of the techniques used to classify retinal subtypes of cells have questions, it is still clearly the case that there remain numerous subtypes of amacrine and ganglion cells that participate in the complex processing of visual information in the IPL, that we only partially understand today.

4. Horizontal cells

Horizontal cells are second-order neurons that receive input directly from the photoreceptors as the lateral elements in the photoreceptor invaginations (Dowling and Boycott, 1966). They have channels in their membranes that open to cations; thus, they are depolarized in the dark and generally hyperpolarize in the light. They make three types of synapses: first and most importantly, feedback synapses onto cone photoreceptors (and to rods in some species), electrical (gap junction) synapses with adjacent horizontal cells, and, perhaps, feed-forward synapses onto bipolar cells.

4.1. Horizontal cell feedback synapses

The evidence for inhibitory feedback from horizontal cells (HC) to photoreceptors goes back more than 50 years (Baylor et al., 1971), but no anatomical evidence for such a junction has been seen. Rather the evidence is all physiological in nature but unequivocal. Two unconventional mechanisms have been proposed to explain the feedback, and there is some evidence for both, but which is correct, if either or both, is not yet proven. One mechanism suggests that a channel in horizontal cells opens when horizontal cells hyperpolarize in the light, allowing for an outward flow of positive current from the horizontal cell processes into the photoreceptor invaginations (Kamermans and Fahrenfort, 2004). This current then depolarizes the photoreceptor presynaptic membrane, enhancing Ca^{2+} dependent glutamate release from the terminal and antagonizing the effect of light on decreasing glutamate release from the photoreceptors. The second mechanism suggests that horizontal cell hyperpolarization in the light increases pH (negativity) in the invaginations, leading to an increase in the pH dependent Ca^{2+} current flow into the photoreceptor terminals, thus increasing glutamate release and again opposing the light-induced decrease of glutamate from the terminals (Barnes and Bui, 1991; Hirasawa and Kaneko, 2003). A recent study showed that the sodium-bicarbonate co-transporter (Slc4a5) is involved in the HC-cone feedback signal, as knockout or knockdown of the co-transporter abolishes the feedback, a finding consistent with the pH mechanism (Morikawa et al., 2024). Both mechanisms also suggest another reason for the invaginations found in photoreceptor terminals. That is in both cases it would be important to contain the current flows proposed, and the invaginations could provide for that. But again, this is conjecture and not proven.

A third mechanism proposes that the horizontal cell induced depolarization in cones can be mediated by chloride currents activated by GABA, which is found in some horizontal cells, but not all (Zhang and Wu, 2021). Another complication is that horizontal cells in fish and salamanders appear to be involved in feedback and feed-forward interactions with photoreceptors that result in some horizontal cells (the C-type) displaying depolarizing or hyperpolarizing responses depending on the color of the light they are exposed to (MacNichol and Svaetichin, 1958; Kaneko and Yamada, 1972; Stell et al., 1975; Tomita et al., 1967).

All three proposed mechanisms described above have merits and limitations, and it may be that different animals use one or more of these mechanisms. Perhaps systematic, cross-species studies are needed to resolve this issue. The horizontal to cone feedback synapse is the first negative feedback system in the visual pathway and it is important to understand not only because it is crucial in image coding and synaptic modulation in the outer retina, but also in how negative synapses can work in the CNS.

4.2. Electrical (gap junctional) synapses

Horizontal cells make large gap junctional synapses with each other, which serves to expand significantly the receptive field size of a contacted horizontal cell. These junctions are modified by neuromodulatory agents such as dopamine, nitric oxide and even retinoic acid (McMahon and Dowling, 2023). But elsewhere in both the outer and inner retina,

there are also numerous gap junctions that participate in neuronal processing.

For example, rods and cones are electrically coupled, and this coupling is regulated by a circadian rhythm via dopamine. Coupling increases in bright light and weakens in dim light or darkness (Yang and Wu, 1989; Werblin, 1978). And as described below, the feed-forward synapses from horizontal cells to ON-bipolar cells, employ a gap junctional synapse.

Another prominent gap junction is found between one type of amacrine cell (A2 cell) and the terminals of ON-bipolar cells. Rods in mammals do not contact ganglion cells directly, but through A2 cells (Kolb and Famiglietti, 1974). Rod bipolar cell terminals synapse deep in the inner plexiform layer (IPL) onto A2 amacrine cell processes, which more proximally in the IPL contact the terminals of ON- and OFF-bipolar cells. The ON-bipolar cell contact is a sign-conserving electrical gap junction (Veruki and Hartveit, 2002), whereas the OFF-bipolar cell contact is a sign-reversing chemical synapse (Chun et al., 1993). Gap junctions have now been seen on all classes of retinal neurons, it is likely they are involved in many circuits other than those mentioned above (see below), and it seems likely that they are relatively common throughout the CNS.

4.3. Horizontal cell feed-forward synapses

Properties of the horizontal-bipolar cell feedforward synapses are even less well understood. Although anatomical evidence has suggested chemical synapses made by horizontal cells onto bipolar cells in the cat and mudpuppy retina (Dowling et al., 1966; Dowling and Werblin, 1969), in most retinas, such synapses have not been seen. If they are present, it is unclear how functional they are. Application of GABA onto bipolar cell dendrites in Co^{2+} Ringer's does not elicit a response in tiger salamander retinas (Maple and Wu, 1996), indicating that there are no GABA receptors directly on bipolar cell dendrites. Therefore if HC feed-forward synapses are chemical, the neurotransmitter is not GABA. Histochemical evidence suggests that not all horizontal cells in retinas are GABAergic, but the identity of other neurotransmitters in horizontal cells is unknown (but unlikely to be glycine because no glycinergic neurons have been found in the outer retina (Menger et al., 1998). Further work is needed to clarify this issue.

One study in salamander retina does suggest that a horizontal (HC) to ON-bipolar cell feedforward synapse is functional. Application of L-AP4, which blocks the cone to ON-bipolar cell (DBC) synapse and thus blocks the HC-cone-DBC feedback pathway, converts the DBC depolarizing response to a sustained hyperpolarizing response, without affecting the HC and OFF-bipolar cell responses. This DBC sustained hyperpolarizing response is likely to be mediated by HCs via a sign-preserving synapse, because in L-AP4 the HC response is sustained, whereas the response of the amacrine retinal cell that sends signals to the DBCs is transient at light offset (Yang and Wu, 1991). It also has been shown that the HC somas and axon terminals are dye-coupled with some DBCs (whose axons ramify in the inner half of the IPL) (Zhang and Wu, 2009). Therefore, the sustained hyperpolarizing response in the DBC in the presence of L-AP4 is likely to be mediated by a HC-DBC feedforward electrical synapse, a notion that is supported by a report showing that such responses can be blocked by the electrical synapse blocker MFA (meclofenamic acid) (Zhang and Wu, 2009). If the feedforward synapses are mainly electrical, this could explain why classic feedforward chemical synapses are not seen in most retinas. It is worth noting that it is not possible to perform similar experiments on OFF-BCs (HBCs), because these cells and HCs use the same glutamate receptors, and thus blocking the cone-HBC synapses with AMPA receptor antagonists will also suppress HC light responses. Clearly, further study of possible feed-forward horizontal to bipolar cell synapses is needed.

5. Bipolar cells

Bipolar cells carry the visual message from the outer to inner retina and they synaptically act as excitatory neurons for both the ON- and OFF-ganglion cells, releasing L-glutamate onto ganglion cell dendrites (Ehinger et al., 1988). However, a significant number of bipolar cells (~10%) contain GABA, an inhibitory transmitter (Yang and Yazulla, 1994), and a few others glycine, the other principal inhibitory transmitter in the retina and central nervous system. However, it has been proposed that glycine may come from glycinergic amacrine cells to which some bipolar cells are electrically coupled (Marc and Liu, 1985; Hendrickson et al., 1988). However, the role of GABA in those bipolar cells that contain it and presumably release it, is not at all understood.

Bipolar cells, like photoreceptors and horizontal cells respond to light with graded potentials for the most part (Werblin and Dowling, 1969). At low light levels, bipolar cells may generate some regenerative responses (spikes) that may act as an amplifying mechanism to ensure that the cells postsynaptic to bipolars are sufficiently activated (Pan et al., 2001; Ichinose et al., 2005), but this conjecture has not been proven.

Bipolar cells group into two major subtypes – ON bipolar cells that depolarize in the light, and OFF cells that hyperpolarize in the light (Werblin and Dowling, 1969). The terminals of the bipolar cells generally end in one of two major laminae in the inner plexiform layer IPL (Pang et al., 2024) – ON cells in the lower half lamina, and OFF cells in the upper half lamina (Famiglietti and Kolb, 1976; Nelson et al., 1978; Roska and Werblin, 2001). In mammals, there are generally 10-12 cone bipolar cell subtypes which are recognized based on anatomical differences and level in the IPL lamina into which they extend their terminals. Rod bipolar cells, on the other hand, all terminate in the lowermost part of the ON lamina where they contact A2 amacrine cells as described earlier (Kolb and Nelson, 1983). There is then something like 10-20 or so sublaminae in the IPL where the various subtypes of ganglion cells receive their input from bipolar and amacrine cells (Ghosh et al., 2004). It is important to note that the exact number of bipolar cell types is perhaps less relevant than the fact that different functional types have axon terminals ramified at different sublayers of the IPL. A major task before us is to work out the wiring in each of the sublaminae to begin to understand the response properties of the various types of ganglion cells (see below).

The various types of bipolar cells also show a variety of waveforms when recorded in-situ in response to light. But since bipolar cells receive substantial input from amacrine cells, how much of the waveform variety is intrinsic to a bipolar cell subtype or to the input from amacrine cells, which also have a variety of response waveforms, is as yet unclear. It has been shown that the light-evoked voltage responses of most bipolar cells correlate with the waveform and spectral sensitivity of the light-evoked cation current (ΔI_C , mediated by photoreceptor inputs), rather than the light-evoked chloride current (ΔI_{Cl} , mediated by amacrine cell inputs), partially because that the dark membrane potential of bipolar cells is near the chloride equilibrium potential (Gao et al., 2000; Wu et al., 2000; Roska and Werblin, 2001; Pang et al., 2004; Zhang and Wu, 2009). There is some suggestion that all bipolars when isolated from amacrine and horizontal cell input, for example, in the presence of a GABA_A receptor blocker I4AA (Gao et al., 2000), respond basically the same way – with a graded and sustained response, either depolarizing (ON cells) or hyperpolarizing (OFF cells), but this conjecture needs to be shown (See also Euler et al., 2014).

Segregation of ON and OFF neurons in the visual system starts at the photoreceptor-bipolar cell synapses in the outer plexiform layer. All rod and cone photoreceptors cells release glutamate from their terminals in darkness (Dowling and Ripps, 1973), but the postsynaptic glutamate receptors on ON- and OFF-bipolar cells mediate opposite actions: glutamate closes TRPM 1 cation channels in ON-bipolar cells via a mGluR6 receptor cascade and opens cation channels in OFF-bipolar cells via AMPA receptors (Brandstatter et al., 1998; Nawy, 1999; Thoreson

and Witkovsky, 1999). Light suppresses glutamate release, which opens cation channels resulting in membrane depolarization in ON-bipolar cells and closes cation channels resulting in membrane hyperpolarization in OFF-bipolar cells. These segregated ON and OFF neurons persist in downstream visual neurons, including retinal ganglion cells and neurons in higher visual centers through sign-preserving synapses.

In cone depolarizing ON-bipolar cells in fish, glutamate has been found to activate a glutamate transporter-mediated chloride current (I_{glu}) associated with opening chloride channels (Grant and Dowling, 1996). Since the DBC resting potential is between the cation and chloride reversal potentials, an elicited decrease of glutamate results in depolarizing responses in the DBC. It is unclear, however, whether this glutamate transporter-mediated chloride current exists in DBCs of other vertebrates, and what would be the advantage of having two mechanisms associated with two opposite conductance changes (but see below).

5.1. Center-surround organization

All vertebrates have ON- and OFF-bipolar cells with distinct center-surround antagonist receptive fields (Werblin and Dowling, 1969; Kaneko, 1970); depolarizing cone bipolar cells (DBC) depolarize to central illumination and hyperpolarize to surround illumination (ON-center/OFF-surround cells) and hyperpolarizing cone bipolar cells (HBC) hyperpolarize to central illumination and depolarize to surround illumination (OFF-center/ON-surround) (Fig. 3). It is generally agreed that the surround responses in bipolar cells result from horizontal cells antagonizing the center response, and they are the first cells in the visual system to show substantial center-surround antagonism. One curious observation is that the antagonistic responses elicited by surround illumination in bipolar cells cannot be seen in darkness, but only in the presence of concomitant center light. This is surprising because synaptic transmission is strongest in the dark. Such surround responses in the absence of concomitant center light have not been observed in cones, bipolar cells or ganglion cells (Werblin and Dowling, 1969; Wunk and Werblin, 1979; Skrzypek and Werblin, 1983; Zhang and Wu, 2009, 2010, 2021). The mechanism underlying this phenomenon is unknown and warrants systematic investigation.

6. Amacrine cells

In terms of neuronal subtypes, amacrine cells are the most numerous, diverse, and heterogeneous of all retinal cells. In mammals, more than 60 subtypes have been suggested, and in non-mammalian vertebrate retinas, as many as 70 subtypes have been claimed (McNeil and Masland, 1998; Yan et al., 2020). Why so many amacrine cells? Because it is clear amacrine cells are involved in complex processing of visual information, and we are finding out more and more examples of this occurring in the retina all the time. And why might there be more amacrine cells in non-mammalian retinas? One possible answer is that much of the more

complex processing of visual information occurs in the cortex, and mammals have much more cortex than non-mammals who need to process complex information upstream in retinal plexiform layers.

Most amacrine cells respond to light with depolarizing potentials, either transient or sustained, but some sustained hyperpolarizing amacrine responses are observed as well. Many of the depolarizing amacrine cells that respond transiently show ON and OFF responses, and they can generate action potentials both at ON and OFF. Other amacrine cells only respond transiently at the onset of light, but they too can generate action potential, as can the sustained responding amacrine cells.

Amacrine cells also vary widely in process spread, ranging from about 25 μm in diameter up to 3 mm in diameter. The widest amacrine cells are referred to as polyaxonal amacrine cells in that they have numerous processes clustered around the cell body with spread of about 400 μm and then fewer but longer processes extending out several mm. in all directions. The long processes generate action potentials, but exactly how these cells function, and their roles remain unclear. Amacrine cells may also extend their processes only within a narrow sublamina in the IPL (stratified cells) or diffuse throughout the IPL (diffuse cells).

Amacrine cells are very diverse anatomically, making classic feedback synapses onto bipolar cells, feed-forward synapses onto other amacrine cells and ganglion cells as well as electrical synapses with other amacrine, bipolar and ganglion cells (Dowling, 2012). Reciprocal and serial amacrine synapses also occur, so sorting out the wiring of amacrine cells in the various sublaminae of the IPL is a gigantic challenge before us but may be the key in understanding retinal wiring and the generation of the various physiological types of ganglion cells. We believe that understanding how feedback, feedforward, reciprocal and serial amacrine cell synapses mediate visual signaling in the inner retina is critical for an understanding of retinal function (Dowling, 1968; Diamond, 2017).

Bipolar, amacrine, and ganglion cells interact in between 10 and 20 laminae in the inner plexiform layer that are defined by the ramification of dendrites from specific ganglion cell subtypes (Fig. 4). Each subtype of cell receives distinct synaptic inputs from bipolar and amacrine cells and generates a unique response pattern. Ganglion cells with dendrites near the midline of the IPL tend to generate more transient responses than ganglion cells with dendrites at the inner and outer boundaries of

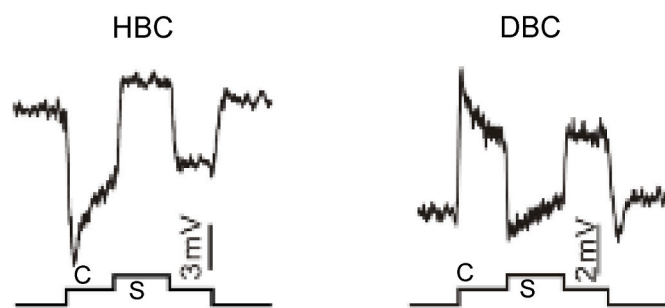


Fig. 3. Voltage responses of the hyperpolarizing bipolar cell (HBC) and depolarizing bipolar cell (DBC) elicited by a center light spot (C) and a surround light annulus (S). (modified from Zhang and Wu, 2009).

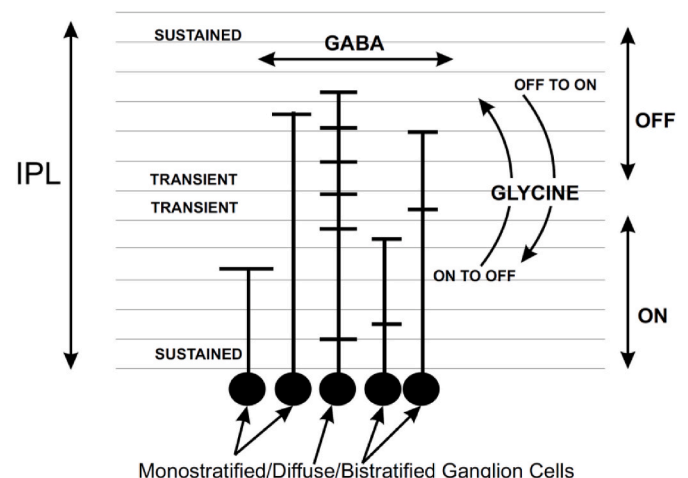


Fig. 4. Schematic drawings illustrating dendritic distribution and interactions of various types of retinal ganglion cells (GCs) in the IPL of the rabbit retina. GCs with transient light responses have dendrites ramifying in the central sublayer of the IPL whereas those with sustained responses ramify close to the two margins of the IPL. GCs receive inhibitory inputs from amacrine cells (ACs): GABAergic inhibition spreads laterally as GABAergic ACs are wide or medium-field cells, whereas glycinergic inhibition moves vertically as glycinergic ACs are narrow-field cells (modified from Werblin, 2011).

the IPL. GABAergic amacrine cells ramify laterally across the IPL and also may cross layers, generating center-surround activity in the ganglion cells they subserve (Werblin, 2011). But, there are also vertically oriented amacrine cells that release glycine and mediate a linearizing crossover inhibition as described below.

6.1. Specific amacrine cell functions

Synapses are by nature nonlinear, releasing more transmitters for voltage increments than for voltage decrements. Retinal bipolar cells operate around a mid-level of activity. They release incrementally more transmitter when they depolarize than when they hyperpolarize. For ON-bipolar cells, a light increment elicits a larger incremental release than a dark light increment. OFF-bipolar cells generate the opposite - a larger incremental release for a dark light increment than for a light increment. If ganglion cells received input from only ON-or OFF-bipolar cells their voltage responses to light and dark increments would be rectified. But the ON and OFF signals from bipolar cells are synaptically combined so that this distorting rectification is eliminated. ON-ganglion cells receive inhibition from the OFF pathway, and OFF-cells receive inhibition from the ON pathway. These so-called crossover signals are mediated by glycinergic amacrine cells. One class of these glycinergic amacrine cells receives synaptic input from ON-bipolar cells and inhibits OFF-ganglion cells; the other class receives input from OFF-bipolar cells and inhibits ON-ganglion cells. These crossover signals allow more linear voltage responses to light increments in both ON- and OFF-ganglion cells (Molnar et al., 2009). It is important to determine whether these crossover signals occur in all ganglion cells across species and whether it is a general mechanism for synaptic linearization in the nervous system.

A few amacrine cell types with unique morphologies have been identified and have been shown to be involved in specific visual functions (Kolb, 1994). A radial symmetric amacrine cell, the “starburst cell” is a key component of a complex of cells that generate movement and directional selectivity in certain ganglion cells (Wei, 2018). These starburst cells release both GABA and acetylcholine (ACH) in response to outward movement from their cell bodies, both exciting (ACH) and inhibiting (GABA) the dendrites of such cells (Lee et al., 2010).

Another class of amacrine cell, broadly distributed across the retina, responds transiently at both ON and OFF of light with a burst of activity in response to broad field changes in illumination. This class of amacrine cell transiently suppresses ganglion cell responses when there is a global change in illumination and may be responsible for the transient suppression of ganglion cell activity during an eye movement saccade (Roska and Werblin, 2003).

Another example of a specific type of amacrine cell playing a significant role for a subtype of ganglion cell is with regard to the detection of an approaching (looming) object (Munch et al., 2009). The analysis of this circuit is informative in another way in that an important component of the circuit is the A2 amacrine cell that plays a critical role in the transmission from rod bipolar terminals to cone bipolar terminals discussed earlier. But rather than information flow mediating rod responses from A2 amacrine cell processes to cone ON- and OFF-bipolar cell terminals, the flow of information here is from the cone ON-bipolar cell terminal to the A2 amacrine cell. Thus, in the former case, the A2 amacrine cell is mediating scotopic signals, whereas in the case of this ganglion cell circuitry the A2 cell is mediating a photopic signal. Thus, the same amacrine cell can be used in different circuits that mediate different functions.

The above-mentioned amacrine cells exemplify how these cells mediate specific visual functions in the inner retina. Such studies need to be extended to more and perhaps all “types” of amacrine cells (Thoreson and Mangel, 2012; Diamond, 2017). There will not be, until we achieve such a goal, a true meaningful classification scheme of amacrine cells.

6.2. Neuromodulation

Most amacrine cells (80%) contain and release either GABA or glycine, the classic CNS inhibitory transmitters, but a variety of other neuroactive substances are also found in GABAergic amacrine cells. Most of these other neuroactive substances presumably act as neuromodulators, not initiating neural activity but modulating it in various ways and via different mechanisms. Understanding neuromodulation is perhaps the greatest unknown we have regarding retinal and brain function.

Two major classes of neuromodulatory substances have been identified in the retina (and CNS); monoamines and neuropeptides. Most insights so far have been from studies on monoamines, especially dopamine, at least in the retina (Dowling and Ehinger, 1975; Hedden and Dowling, 1978). In the retina dopamine acts via receptors on postsynaptic neurons that activate adenylate cyclase which produces the second messenger, cyclic AMP, that promotes PKA to phosphorylate (or dephosphorylate) proteins. In the retina, dopamine is known to alter the conductance of the electrical, gap junction channels (Lasater and Dowling, 1985), but it can also modify the sensitivity of glutamate channels on horizontal cells (Knapp and Dowling, 1987). Neuromodulation by dopamine (and perhaps in conjunction with other neuromodulatory substances) underlies many of the circadian rhythms that occur in the retina, including switching from rod to cone vision in the light and vice versa, altering overall retinal sensitivity in dark and light, modulating center-surround strengths, and so forth (Mangel and Dowling, 1987; Yang et al., 1988; McMahon et al., 1989; Li and Dowling, 2000). In young larval zebrafish, a circadian rhythm appears to shut off almost completely retinal activity in the dark, presumably to save energy for the animal at this critical time in its life (Emran and Dowling, 2010). How other monoamines present in the retina (serotonin and norepinephrine) function is not known but there are other substances in the retina that have been shown to act as neuromodulators such as nitric oxide, cannabinoids, zinc, adenosine and even retinoic acid (RA). RA is particularly interesting in that it appears to act directly on gap junction proteins to modify junctional conductance without activating a second messenger system (Dowling, 2012; McMahon and Dowling, 2023). How various monoamines modulate retinal synapses and whether and why they may be co-released with GABA warrants investigation.

6.3. Neuropeptides

By far the most mysterious neuroactive substances found in the retina are the neuropeptides. What their role is in retinal and brain function is almost entirely unknown. The neuropeptides are the largest group of neuroactive substance found in the retina. At least 15 different neuropeptides have been identified in the retina (Dowling, 2012). They are found almost exclusively in amacrine cells, and usually in amacrine cells that also contain GABA. It is presumed that these amacrine cells release both the neuropeptide and GABA. Instances of two different peptides in the same amacrine cell, or even three, have been reported. Again, it is thought that most peptides are neuromodulatory in nature. The peptide-containing amacrine cells are quite rare, usually accounting for only one or a few percent of the amacrine cells, but those amacrine cells do spread their processes quite widely in the IPL.

Observations so far do suggest that the peptides act as modulators by involving second-messenger systems, but they can also act as classic excitatory neurotransmitters by directly depolarizing postsynaptic cells. VIP in the retina does both in fish; it increases cAMP in horizontal cells but also directly depolarizes the cells (Lasater et al., 1983). What is the significance of this? How the neuropeptides are released from retinal cells and where they exert their effects is also variable. Ca^{2+} -dependent release has been demonstrated in several cases, but it has also been shown that neuropeptides can have effects on neurons many micrometers away from the releasing neuron, and so the release may not be confined to an adjacent postsynaptic site. Why are the neuropeptides

usually found in amacrine cells that also contain GABA or glycine. Much work needs to be done in this area.

7. Ganglion cells

Ganglion cells are the output neurons of the retina, carrying the visual information sensed and processed in the retina to higher visual centers, and thus our discussion on ganglion cell function in the retina will be minimal. A substantial number of subtypes of ganglion cells are present in all retinas (Sanes and Masland, 2015), but within the retina, the ganglion cells appear not to be involved in the sensing and processing of visual information. They do not usually make synapses in the IPL with other neurons. The exception is the intrinsically photosensitive ganglion cells (ipRGCs) that perform many important functions such as contraction of the pupil, entrainment of circadian rhythms, etc., but they play little role in analyzing visual images. They do provide some feedback to retinal cells containing dopamine, and thus the release of dopamine whether in light or dark (Do, 2019). In other words, most ganglion cells are read-out cells that depend on the wiring and interactions of the photoreceptors, horizontal, bipolar, and amacrine cells, and so our focus in this essay has been on how the retina senses light and then processes visual information.

The different ganglion cell types carry all of the visual information that an animal can access. In mammalian retinas, as many as 20-30 subtypes of cells may exist, differing in their anatomy and physiology. All generate action potentials that carry the visual information from eye to brain via the optic nerve and usually each ganglion cell sends its axons to different parts of the brain. Embedded in these representations is a complete code that forms the basis for an animal's visual world. It speaks a rich neural language that we do not yet fully understand.

With present day connectomics techniques, it is possible to work out the precise wiring of excitation and inhibition in each of the strata in the IPL (Marc et al., 2013; Kim et al., 2026). However, at present neuromodulatory synapses, for the most part, cannot be identified in connectomic studies, and in some cases, neuromodulatory substances are released some distance from the releasing neuron, so no obvious synaptic structure is present. Thus, connectomic studies can give us a good notion of the potential basic wiring in a bit of neural tissue, but not complete information about various synaptic interactions that may be occurring.

8. Conclusions

Whereas we have learned an enormous amount about the retina and how it functions, there is much more to learn. We hope this essay encourages researchers in the field. The retina is a very rich area of research that for us has been very satisfying. The questions we are able to address today are worlds apart and much more sophisticated compared to the naïve questions we addressed beginning 50-60 years ago due to the many technological advances made along the way that were noted earlier. And, of course, it will be the case that in the future new methods will be developed to measure, for example, the intracellular activity of many neurons simultaneously, the analysis by AI methods of complex data, and the formulation of realistic computer models of how retinas function when stimulated with natural scenes. We are confident it will be possible to understand much better how this small but very sophisticated bit of the brain works.

CRediT authorship contribution statement

John E. Dowling: Writing – review & editing, Writing – original draft. **Frank S. Werblin:** Writing – review & editing, Writing – original draft. **Samuel M. Wu:** Writing – review & editing, Writing – original draft.

Unsolved questions

1. Why do vertebrate photoreceptors decrease transmitter release in the light? Vertebrate photoreceptors act as if they are excited (depolarized) by the dark and that light turns them off. Being continuously in the dark is a substantial energy drain. Does this suggest vertebrates are in the light more than in the dark to minimize energy drain?
2. We know that the slow phase of dark adaptation in photoreceptors depends on visual pigment levels and the pace of regeneration. The mechanisms underlying the rapid phase of dark adaptation in photoreceptors are unknown and clearly need investigation.
3. Is escape from rod saturation during light adaptation, such as shown in skate and mouse, a general phenomenon in all rods, and is the migration of transducin from outer to inner segment the underlying mechanism?
4. What cells/mechanisms underly network light adaptation that occurs in the inner retina?
5.
 - (i) It has been proposed that the invaginations of ribbon synapses promote interactions among the photoreceptors, ON-BCs and HC s, but what is the nature of these interactions?
 - (ii) Why are ribbon synapses invaginated? Is it because the geometrically tortuous invaginations slow glutamate diffusion in the synaptic cleft and thus shape synaptic responses? Or do the invaginations isolate one release site from another, thus avoiding mixtures of synchronized vesicular release?
 - (iii) What activates the postsynaptic processes at the basal synapses? Is it from transmitter released at the synaptic ribbons above the invaginations that somehow finds its way to the basal junctions or another mechanism?
6. Classification of retinal neuronal subtypes remains an important question. The existing classification categories are cell morphology, response polarity and waveform, transmitter content and genetic markers, but can any one of these categories define the function of a specific subtype of retinal neuron? A challenging problem is to design a comprehensive classification scheme which may include the above categories as well as specific input and output connections.
7.
 - (i) All three proposed mechanisms for the HC feedback synapses onto photoreceptors have merit but limitations. It is unclear whether different animals use one or more of these mechanisms, and needs study.
 - (ii) How does adaptation, circadian rhythms and various modulatory agents modify coupling parameters and HC receptive fields? HC-HC coupling varies in different retinas; systematic studies on the strengths and HC coupling patterns warrant investigation.
 - (iii) Are the electrical synapse-mediated HC-ON bipolar cell feedforward signals found in other species? If feedforward synapses are electrical, this could explain why classic feedforward chemical synapses (with vesicles) are not seen in most retinas.
8.
 - (i) What are the functions of GABAergic BCs? Is GABA co-released with glutamate? Is glycine in BCs simply caused by leakage from ACs via gap junctions or does it mediate a functional inhibitory synapse?
 - (ii) Does the glutamate transporter-mediated chloride current exist in ON bipolar cells of other vertebrates, and what would be the advantage of having two mechanisms associated with two opposite conductance changes.

- (iii) Why is the surround response only seen in the presence of concomitant center light? This mechanism warrants further systematic investigation.
9. As amacrine cell subtypes are the most numerous of all retinal cells and they serve different specific visual functions as exemplified by the A2 ACs and starburst ACs, not until we extend such studies on the specific circuitries and functions to more and perhaps all “types” of amacrine cells, can a true meaningful classification scheme for amacrine cells emerge.
- 10
- (i) How do other monoamines in the retina, besides dopamine, (i.e. serotonin and norepinephrine) function? Also, how do other substances in the retina that have been shown to act as neuromodulators such as nitric oxide, cannabinoids, zinc, adenosine and retinoic acid function? How do various neuromodulators modify retinal synapses and whether and why they may be co-released with GABA?
- (ii) Why are the neuropeptides usually found in amacrine cells that also contain GABA and what is the significance of this colocalization? VIP in the retina increases cAMP in horizontal cells but also directly depolarizes the cells. Is this typical of neuropeptides?
11. Will it be possible to understand ganglion cell function well enough to generate a complete functional basis for an animal's visual world which we do not yet fully understand. The different GC types carry all of the visual information that an animal can access. For a meaningful GC classification scheme, the synaptic inputs in the inner retina (perhaps by connectomic methods) as well as their output targets in the brain have to be systematically characterized. This is one of the ultimate research goals for vision researchers to achieve.

Authors' statement

All authors have seen and have approved the final version of the revised and resubmitted manuscript. The article is the authors' original work, has not been published elsewhere and is not under consideration for publication elsewhere.

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