Cell Communication Mechanisms in the Vertebrate Retina
The Proctor Lecture

Robert F. Miller

The vertebrate retina has a unique position within the panoply of the nervous system networks: Our understanding of its complex circuitry of interacting neurons and glia has become the gold standard of our current knowledge of network operations. This presentation is about work from my laboratory that contributed to some of the concepts that support our contemporary views of the functional retina. Early in the pursuit of retinal function, a vital issue was that of understanding the synaptic mechanisms and neurotransmitters required for information to flow from the photoreceptors to the ganglion cells. My research contributions to this effort include the discovery of inhibition and the GABA and glycine modes of inhibitory mechanisms. Our work on inhibition was followed by the discovery of the APB (mGluR6) receptor of On bipolars, the first metabotropic glutamate receptor described in the nervous system. This finding was followed by a body of work carried out in salamander and rabbit retinas on the pathways of glutamatergic excitation revealed through the use of agonists and antagonists of increasing selectivity. We separated sign-conserving from sign-inverting responses in the outer retina and provided compelling evidence that bipolars, like photoreceptors, had a glutamatergic mode of neurotransmission. We identified NMDA (N-methyl-D-aspartate) and KA (kainic acid)/AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolpropionic acid) receptors in amacrine and ganglion cells and revealed that both receptor classes are activated by light. Additional studies on neuropeptides illustrated how many of these, including substance P, somatostatin, and neurotensin have actions such that receptor classes are activated by light. Additional studies on neuropeptides illustrated how many of these, including substance P, somatostatin, and neurotensin have actions such that they should be considered major neuromodulators in the retina. My laboratory also made significant contributions to structure-function relationships and mechanisms of glial–neuronal interactions. (Invest Ophthal Vis Sci. 2008;49:5184 –5198) DOI:10.1167/iovs.08-2456

Modern neuroscience was born on the thrust of Ramon y Cajal’s prodigious anatomic studies of the nervous system and his insistence that single cells form the fundamental composition of its organization. During the course of his career, Cajal revealed a rich array of cell morphologies whose diversity of form reached dizzying heights. Among the preferred sites for his studies of the nervous system, the vertebrate retina stood out as the CNS region from which he generated many of his most important insights about the relationship between the structure and function of nerve cells.1,2 Cajal’s extensive studies evolved into a transgenerational challenge to determine whether the variance in morphology revealed through his Golgi staining methods was matched by an equal diversity of function. This challenge became a Holy Grail of cellular neuroscience in the latter half of the 20th century. As a young medical student in the 1960s, I was fascinated by my first glimpse of Cajal’s camera lucida drawing of a Purkinje cell of the cerebellar cortex, with its impressive dendritic structure. When I learned that little was known at the time about the physiology of these magnificent cells, my career interests gained a new focus. Research experience in medical school encouraged me to believe, as Cajal did throughout his career, that the vertebrate retina is one of the few places in the central nervous system where one can begin to tractably address Cajal’s challenge of relating the structure of a nerve cell to its function.

I arrived at John Dowling’s laboratory in 1967, just as the Werblin and Dowling work3,4 was reaching its creative heights. With John’s encouragement, I eagerly took up the new technique of intracellular recording and obtained the first intracellular recordings from Müller cells.5 These results stimulated additional studies of Müller cells, which in turn led directly into studies of the retinal network and structure-function correlations of single nerve cells. More recently I have returned to examining the role of glial cells in the retina and their relationship to ω-serine synthesis and release and how they participate in the regulation of NMDA receptors and ganglion cell excitability.

The upper panel of Figure 1 illustrates the fundamental connections which underlie the physiological polarity of the three classes of ganglion cells in the retina, including On, Off, and On-Off subtypes. The color coding reflects the polarity of the light response, with blue as depolarizing or On, red as hyperpolarizing or Off, and purple as On-Off, reflecting cells that are depolarized at the onset and termination of a light stimulus. Rods and cones all hyperpolarize to light and the segregation of On and Off pathways is provided by the two opposite polarity bipolar cells which feed directly into ganglion cells through excitatory, glutamatergic connections. This cartoon illustration is based on our knowledge of the amphibian retina, which lacks the specialized rod bipolars found in the mammalian retina that feed indirectly into ganglion cells through amacrine-mediated pathways.

The lower panel of Figure 1 illustrates a more integrated cartoon of the retina, retaining the same color coding as that of the upper panel. The left partition reflects the neuronal architecture of the amphibian retina, with the Müller cells (green) in the background, while the right partition conveys a more realistic structure of the retina, in which Müller cells form intimate relationships with the cell bodies and synaptic processes of retinal neurons. We now know that Müller cells participate very broadly in retinal control mechanisms: These cells not only generate light-evoked responses, but they also...
generate slow calcium waves⁶ and participate in regulating the excitability of retinal neurons.⁷⁻⁹

A Doorway through Chloride

From Müller Cells to the Neural Network of the Retina

While expanding my pursuit of Müller cell physiology using the perfused isolated retina of the frog,¹⁰ I attempted to simplify the experimental approach by removing external chloride ions, replacing them with an impermeant anion (sulfate/methylsulfate). With this strategy, I hoped to avoid complications of Cl⁻ and water movements that can cause tissue swelling when elevating external K⁺.¹¹ To my surprise, the replacement of Cl⁻ in the bathing medium promptly abolished the b-wave of the ERG, but did so in such a way that its actions reflected a Cl⁻ dependency of the retinal network, rather than one that could be attributed to Müller cells.¹²,¹³ For this reason I transitioned my attention from Müller cells to the neuronal network of the retina.

Insights into Retinal Connections through the Chloride Door

Figure 2A summarizes how a chloride-free (C-F) environment changed the retinal network by illustrating extracellular ganglion cell recordings from On, Off, and On-Off cells in the
The discharge pattern observed under C-F conditions with a large-diameter stimulus that covered the entire receptive field. The discharge pattern evoked by rabbit retina. For each cell, the response to a focal (center) light stimulus was contrasted with the discharge pattern evoked by a large-diameter stimulus that covered the entire receptive field. The discharge pattern observed under C-F conditions showed elimination of all On ganglion cell activity with enhanced Off discharge.

Figure 2B summarizes the C-F effects on the major types of retinal neurons, based on a comprehensive study of the mudpuppy retina: only two cell types had their light responses eliminated in a C-F environment, including the horizontal cell and the depolarizing bipolar. The hyperpolarizing bipolar cell light responses persisted under these conditions, although these cells lost their antagonistic surround, presumably because of horizontal cell response elimination. This work on the mudpuppy retina was published in a series of papers in 1976. Because only one of the two types of bipolar cells was eliminated under C-F conditions, these results allowed us to make conclusions about the polarity of connections between the bipolars and the amacrine and ganglion cells. Since both the depolarizing bipolar and On ganglion cells were silenced under C-F conditions, it meant that they must connect through an excitatory synapse. Conversely, the hyperpolarizing bipolars, whose light response persisted under C-F conditions, must be connected to Off ganglion cells through an excitatory synapse. Thus, both bipolar cell types that connect with excitatory synapses to neurons of the inner retina. This was the first clear indication of the bipolar connectivity arrangement. The following year, Naka confirmed this conclusion in his study of the catfish retina.

On-Off Ganglion Cells Are a Unique Class of Neuron

A second impact from the C-F studies related to the On-Off ganglion cells. Although Hartline had originally separated ganglion cells of the frog retina into three types, including On, Off, and On-Off, it was never clear whether the On-Off cells could be justified as a separate class. But, the C-F studies revealed that On-Off ganglion cells were unique: They received excitatory inputs from both bipolar types, a connectivity pattern that justified their inclusion into the trinity of On, Off, and On-Off ganglion cells. Thus, it is this group of three ganglion cell types that represents the fundamental wiring diagram of the retina. Additional synaptic interactions superimposed on this fundamental set of connections is responsible for encoding the more elaborate trigger features evident within the ganglion cell population. We generally assign the On cells as positive contrast neurons, the Off cells as negative contrast cells and the On-Off cells as contrast insensitive, meaning they respond equally well to either positive or negative contrast.

The Discovery of Inhibition

Perhaps the single most important contribution of our C-F studies was the discovery of inhibition in the retina. The fact that this inhibition turned out to be Cl-dependent helped us to think about the neurotransmitters that might mediate the inhibitory responses we observed. Until this work, inhibition had not been identified and characterized. We now know that ganglion cells hyperpolarize to light by two very different mechanisms, including inhibition from amacrine cells and disinhibition as a means by which Off bipolars generate the light-evoked hyperpolarization seen primarily in Off ganglion and amacrine cells. Figure 3A illustrates an intracellular recording of an On-Off ganglion cell in the mudpuppy retina and shows how the initial On and Off EPSPs are followed by On and Off hyperpolarizing responses that can be isolated by stimulating with an annulus (middle recording). These hyperpolarizing responses are associated with a significant conductance increase; Figure 3C illustrates how hyperpolarizing, On-Off IPSPs rapidly converted to depolarizing responses during an initial exposure to a Cl-free Ringer. This reversal occurred because the...
chloride reversal potential rapidly changed to a more positive value by the explosive reduction of external Cl\(^{-}\). This early response inversion unequivocally established the Cl\(^{-}\) dependency of the responses. Figure 3B shows a similar C-F exposure to an On-Off amacrine cell, demonstrating relatively minor effects on this recording, except that the repolarization response at light On and Off is slowed due to reversal of “shunting” type of IPSP that normally makes the response briefer in duration. (C) Ganglion cell IPSPs revealed when ganglion cell is depolarized which masks the EPSPs. Brief exposure to a Cl\(^{-}\)-free Ringer quickly reversed hyperpolarizing responses to depolarizations due to rapid reduction of Cl\(^{-}\) reversal potential. (A, C) Miller and Dacheux, 1976. Originally published in *The Journal of General Physiology* 67:661–678. (B) Miller and Dacheux, 1976. Originally published in *The Journal of General Physiology*. 67:639–659.

Inhibition in Directionally Selective Ganglion Cells in the Rabbit Retina

Early in the course of our studies, we obtained intracellular recordings in the rabbit retina and demonstrated that directionally selective ganglion cells generated a large IPSP for stimuli moving in the null direction and net excitation for targets moving in the preferred direction. Feedforward inhibition onto the ganglion cells has now been confirmed as a major component of the directionally selective mechanism.

The Counterintuitive C-F Mechanism of Action

Throughout the studies of inhibition, we continued to examine the cellular mechanisms by which chloride modulates the retinal network in a selective way. We developed ion-selective electrodes for Cl\(^{-}\) and applied Cl\(^{-}\)-imaging methods and patch-recording techniques, all of which eventually converged on the idea that the network-selective actions of a C-F environment result from a Cl\(^{-}\)-sensitivity of L-type Ca\(^{2+}\) channels, which mediate synaptic transmission from photoreceptors. The full dimensions of this work are beyond the scope of this article, but the topic remains a lively area of development and interest.

INHIBITION IN THE RETINA

Neurotransmitters of Inhibition

When we first discovered Cl\(^{-}\)-dependent inhibition in the retina, it was generally accepted that GABA was the inhibitory transmitter of the brain, while glycine served the same function in the spinal cord. We discovered that, in the retina, both GABA and glycine inhibition are present, serving in parallel, though with slightly different targets. Our first paper on the dual nature of inhibition in the retina appeared in *Science* in 1977. Ignited by these preliminary findings, we wanted to know why both GABA and glycine appear to function as inhibitory neurotransmitters in the retina, and how pervasive was their influence. We determined that GABA and glycine target powerful actions in the inner retina (Fig. 4) but seem to play a minor role in outer retinal processing. GABA and glycine inhibition had three modes of action: (1) feedforward inhibition onto ganglion cells (Fig. 4B); (2) feedback inhibition onto bipolar cells (Fig. 4D); and (3) lateral inhibition between amacrine cells mediated by GABA (Fig. 4F). Every ganglion cell had some degree of inhibition mediated by GABA and/or glycine, which was direct, hyperpolarizing inhibition (Fig. 4A). However, the sensitivity to exogenous GABA and glycine varied from one cell to another, with GABA showing a higher preference for On ganglion cells and glycine showing a higher preference for Off ganglion cells, while many ganglion cells had coequal sensitivity, including most On-Off ganglion cells. All amacrine cells were affected by GABA and glycine, whose action was shunting rather than hyperpolarizing (Fig. 4E). Bipolars showed a differential sensitivity, with On bipolars more sensitive to GABA (Fig. 4C, top two traces) and Off bipolars more sensitive to glycine (4C, lower trace). Consistent with this differential sensitivity, we found that On bipolar light responses were enhanced by GABA antagonists (Fig. 4D, right column), while Off bipolars were enhanced by the glycine antagonist strychnine (Fig. 4D, left column). It seemed likely to us at the time that bipolar cells receive tonic inhibitory input with GABA more prominently involved with the On bipolars and glycine more so with the Off bipolars. We argued that inhibitory interactions between On-Off amacrine cells were mediated by GABA, since picrotoxin enhanced the light response while slowing the repolarization (Fig. 4F, top traces, arrows). In contrast, strychnine enhanced the light response without significantly changing its waveform (Fig. 4F, bottom traces). So, by having the GABergic On-Off amacrine cells contributing to a more rapid repolarization of the On and Off responses of all amacrine cells, blocking that input should slow the recovery of the light response. This phenomenon was also evident in exposing On-Off amacrine cells to a C-F environment (Fig. 3C). Interestingly, Burkhardt observed the same phenomenon when comparing strychnine and picrotoxin in his recordings of the proximal negative response (PNR), an
extracellular recording of the inner retina that reflects amacrine and ganglion cell activity.

We had determined that amacrine cells are the source of the feedforward inhibition onto ganglion cells, and so we argued that they had to be the source of the GABA and glycine inhibition directed to bipolars and other amacrine cells. However, because chloride was distributed passively in amacrine cells, generating a shunting type of inhibition when GABA or glycine was applied, the nature of this inhibitory signal was more subtle in its presentation. Figure 4G illustrates one way of demonstrating a surround inhibitory input into amacrine cells. In this example, the voltage traces of an Off-On amacr ine cell show that a focal light stimulus evoked a larger response than the large-diameter stimulus. Simultaneous recording of the impedance change was made by using a lock-in amplifier, whose detection signal was based on the application of a small 100-Hz current pulse, balancing out the in-phase circuitry to permit a resistance change to be measured in one of the output channels. Note that the large-diameter stimulus, though it resulted in a smaller amplitude voltage response, generated a larger conductance change detected by the lock-in amplifier. The latter observation makes sense, since the surround inhibition evoked by the larger light stimulus generated an additional conductance increase that added to the conductance change associated with the relatively pure excitatory response generated by the small spot. In other words, the inhibitory and excitatory inputs cancel at the voltage levels, but add when conductance changes are determined because both types of signals open ion channels, though of opposite polarity. The lack of any polarization change associated with the inhibitory signal is consistent with the shunting type of inhibition present in these cells.

Neither GABA nor Glycine Mediates the Antagonistic Surround of Bipolars

We looked carefully to see if GABA or glycine might be involved in mediating the surround response of bipolars or the feedback response observed in cone recordings, both of which have been attributed to horizontal cells. In our studies, neither picrotoxin, bicuculline, nor strychnine blocked the surround responses of bipolar cells (Figs. 4H, 4I) or the feedback component of cones (not shown), an idea that is now widely accepted.

A Model of Inhibitory and Excitatory Connections

Our work on inhibition led to the publication of three papers, in which we identified the kinds of cells that are affected by glycine and GABA agonists and antagonists. We then deduced the nature of the inhibitory and excitatory connections that would be required to replicate the light-evoked responses that we observed and analyzed. We added the power of studying inhibition by observing the transient actions of a C-F perfusion, a method that was highly complementary to our pharmacologic studies because it separated the process of hyperpolarizing by disfacilitation from Off bipolars versus hyperpolarizing inhibition from amacrine cells.

We proposed a model in the third paper of the GAB/GLY series that attempted to summarize the ways in which feedforward inhibition onto ganglion cells plays a variable role in shaping the patterns of light-evoked postsynaptic responses. The background set of connections, on top of which our models for inhibitory inputs were superimposed, consisted of the basic photoreceptor and bipolar interactions that formed the On, Off, and On-Off ganglion cell polarities that were identified by our C-F experiments. An additional element to the background network included the connections and presumed actions of the horizontal cells that form the surround organization of bipolars and whose mechanism of action proved immutable to the GABA/glycine antagonists we used in our studies. Although focal and wide-field light stimulation were used in our studies, the classification scheme presented in this work was based on the polarity of the inhibitory inputs (On, Off, and On-Off) not on their spatial organization (small or large-field inhibition). We purposely avoided studying the spatial properties of inhibition, because of earlier studies of amacrine cells suggesting that a single cell could behave like a small- or large-field neuron, depending on the degree to which dendritic versus somatic impulse activity was activated.

More recent analysis in which a modeling strategy was combined with physiological recordings has strongly endorsed that concept.

Figure 4. (A) GABA and glycine applied to a ganglion cell before and after blocking synaptic transmission with Co2+ generated hyperpolarization of the cell associated with an increase in conductance. (B) Ganglion cell IPSP (Ba) partially blocked by strychnine, picrotoxin and bicuculline (top two traces) compared to glycine (middle trace); glycine has greater sensitivity on Off bipolar compared to GABA (bottom trace). (C) Left column: strychnine increased light response of Off bipolar; right column: bicuculline (BCC) increased On bipolar light response. (D) On-Off amacrine shows large increase in conductance associated with exogenous GABA with larger Off response; glycine application suppressed both On and Off components with a large increase in conductance (positive current pulse applied to balanced bridge). (E) Off-On amacrine cells exposed to picro (top two traces) which enhanced On and Off responses and markedly slowed the time course of repolarization for both On and Off transients (arrows); strychnine application (bottom two traces) enhanced On and Off response amplitudes without significantly changing transient response repolarization (arrows). (F) Top traces show voltage recordings of light response to focal and large diameter light stimulus while the bottom traces show the resistance change of each response recorded simultaneously through a lock-in amplifier. The focal light stimulus evoked a larger voltage change, while the large field stimulus evoked a larger conductance increase, illustrating a type of surround inhibition that shunted the light response: the conductances for inhibition and excitation add, while the voltages for the two events are subtractive. (G) Picro applied to Bipolar increased response amplitude, but did not block antagonist surround generated by continuous focal stimulus with two flashes of an annulus which evoked hyperpolarizing response demonstrating preservation of antagonistic surround. (H) Strych application to Off bipolar stimulated with alternate focal and annulus stimulus shows gradual enhancement of response amplitude associated with progressive depolarization of cell; during strychnine exposure focal plus annulus stimulation evoked depolarizing responses, indicating persistence of antagonistic surround response. Strychnine, strychnine; picro, picrotoxin; BCC, bicuculline. (A, E) Reprinted, with permission, from Miller RF, Fromkes TE, Slaughter M, Dacheux RF. Physiological and pharmacological basis of GABA and glycine action on neurons of mudpuppy retina. II. Amacrine and ganglion cells. J Neurophysiol. 1981;45:764–782. © 1981 The American Physiological Society. (B, F) Reprinted, with permission, from Miller RF, Dacheux RF, Fromkes TE. Amacrine cells in Necturus retina: evidence for independent y-amino butyric acid- and glycine-releasing neurons. Science. 1977;198:748–750. © 1977 by American Association for the Advancement of Science. (C, D, G, H) Reprinted, with permission, from Miller RF, Fromkes TE, Slaughter M, Dacheux RF. Physiological and pharmacological basis of GABA and glycine action on neurons of mudpuppy retina. I. Receptors, horizontal cells, bipolars, and G-cells. J Neurophysiol. 1981;45:745–763. © 1981 The American Physiological Society.
feedback onto On bipolars and an Off glycinergic amacrine, that seemed to interact more with the On ganglion cells and amacrine cells (Fig. 5B), including an On GABAergic amacrine that was more sustained and attributed it to two sustained On-Off inhibition. We also proposed another form of inhibition cells (On, Off, and On-Off) to form a broad representation of these amacrine cells interacted with all three types of ganglion inner retina to account for observations of GABA and glycine actions.

All ganglion cells receive direct excitation from bipolars and inhibition from amacrine cells. On-Off amacrine cells consist of GABA and glycineric cells which provide feedforward inhibition onto On, Off, and On-Off ganglion cells. (B) Two sustained amacrine cells provide feedforward inhibition onto ganglion cells and feedback inhibition onto bipolar terminals. On amacrine is GABAergic and Off amacrine is glycineric; both converge onto some On-Off ganglion cells to provide “hybrid” On-Off inhibition where On inhibition is GABA-mediated and Off inhibition is glycine-mediated. Reprinted, with permission, from Frumkes TE, Miller RF, Slaughter M, Dacheux RF. Physiological and pharmacological basis of GABA and glycine action on neurons of mudpuppy retina. III. Amacrine-mediated inhibitory influences on ganglion cell receptive-field organization: a model. J Neurophysiol. 1981; 45:783–804. © 1981 The American Physiological Society.

Figure 5 summarizes our GABA/glycine findings in the form of two models. To account for our observations, it was necessary to propose two types of On-Off amacrine cells (Fig. 5A), one of which released glycine, while the other released GABA. These amacrine cells interacted with all three types of ganglion cells (On, Off, and On-Off) to form a broad representation of On-Off inhibition. We also proposed another form of inhibition that was more sustained and attributed it to two sustained amacrine cells (Fig. 5B), including an On GABAergic amacrine that seemed to interact more with the On ganglion cells and feedback onto On bipolars and an Off glycineric amacrine, which targeted feedforward inhibition onto Off ganglion cells and feedback inhibition onto Off bipolars.

The Special Character of Inhibition in Retinal Ganglion Cells

Once we identified inhibition in prominent partnership with excitation as the two dominant light-evoked synaptic inputs into retinal ganglion cells, we were immediately confronted by the unique character of inhibition in these cells. At the time of our initial discovery, the primary model for inhibition in the central nervous system was that of the spinal cord motoneuron, which was generally regarded as a soma-dominant form of inhibition. It seemed reasonable that the soma-dominant form of inhibition in motoneurons might be very important in preventing impulse firing in motoneurons for actions related to movements. But we recognized, because of the anatomical work done by Dowling and Boycott through his elucidation of amacrine-to-ganglion cell dendrodendritic synapses, that inhibition in the retina was largely a phenomenon that took place within the dendrites of ganglion cells and, for that reason, it was far more subtle and perhaps more surgical in its mode of action. Hence, to understand the significance of inhibition in the retina, we had to appreciate that it was not an all or none phenomenon as it might be if inhibition was restricted to the soma. The selective placement of inhibitory inputs in different regions of the dendritic tree meant that any single inhibitory connection could surgically eliminate excitation from the distal branches, but not necessarily affect those inputs into other dendritic regions of the cell. It is the presence of dendritic inhibition that probably contributes important tuning and precision to those receptive field characteristics, such as directional selectivity, that depend on inhibitory inputs into retinal ganglion cells. We speculated on how these kinds of interactions may be integrated to achieve those objectives. Thus, viewing inhibition in retinal ganglion cells as in any way similar to its role in motoneurons almost surely denies inhibition its proper place setting at many different tables. In retinal ganglion cells the role of inhibition is not necessarily to prevent impulse activity from taking place, but to shape the cell’s ability to respond in a very specific way to a specific form or movement and it is dendritic inhibition that enables this surgical procedure to achieve success in the mission.

Our work on GABA/glycine inhibition was published many years before newer methods, including those of molecular biology, revealed the diversity of GABA and glycine receptors that we now know more extensively enrich the mechanisms of inhibition. The presence of GABAA, GABAB, and GABAc receptors, in addition to the diversity of glycine receptors, provides a greatly expanded repertoire of inhibition and inhibitory options, as differences in receptor sensitivity, rate of desensitization, and receptor kinetics have been responsible for further expansion of the synaptic repertoire subserved by inhibition.

Moving on to Excitation

When we discovered chloride-dependent inhibition in the retina, it was something of a no-brainer to immediately postulate the likely transmitters for this action, since they had been identified and studied in several regions of the brain and spinal cord. But, in contrast, no readily available transmitters had been identified for excitation. Indeed, this was one of the great challenges in the last quarter of the 20th century, a more modern Holy Grail.
How Do Photoreceptors Communicate?

In the 1960s, Trifonov and Byzov\textsuperscript{40} suggested that photoreceptors release a transmitter maximally in the dark and that the light-evoked hyperpolarization reduces the rate of transmitter release. This concept was supported by Dowling and Ripps,\textsuperscript{41} who blocked synaptic transmission with elevated Mg\textsuperscript{2+} and demonstrated that horizontal cells are hyperpolarized as synaptic transmission from photoreceptors is blocked. Early in the formation of this concept, the physiological studies were all done on horizontal cells. We decided to extend these experiments to see if bipolar cells conformed to this idea. Our results confirmed and extended the concept that photoreceptors release a transmitter maximally in the dark and minimally in the light.\textsuperscript{32} But what was the identity of that neurotransmitter and what kinds of synaptic receptors did it activate? Of equal curiosity was a related issue raised by our findings. It was easy to understand how hyperpolarizing Off bipolars and horizontal cell light responses are generated by a dark-released photoreceptor transmitter. One only had to imagine an excitatory transmitter that opened cationic channels in the dark, which would then be opened in the light. Alternatively, one could imagine an arrangement where intracellular chloride was elevated in its concentration so that chloride served as a depolarizing response. In this way, the photoreceptor transmitter could close chloride channels in the dark, allowing a depolarization to occur during light. Whatever the outcome, every retinal physiologist understood that the ionic and transmitter mechanism accounting for the On bipolar light response would be different from any other known synaptic mechanism.

The Discovery of APB


We discovered that APB had unique, profound effects on the retinal network, though its sole action was to rapidly block the On bipolar light response (Fig. 6). This action eliminated all On responses throughout the retina, but not those mediated by the Off bipolar.\textsuperscript{44} At the level of the ganglion cell, it cleanly separated On from Off activity.

A critical component of our analysis with APB was to show that it acts as an agonist and blocks the light response by interacting with the same synaptic receptors as those activated by the endogenous photoreceptor neurotransmitter. The alternative explanation was that APB shunts the cell by opening nonsynaptic receptors. We differentiated between the "specific" and "nonspecific" modes of action in the following way. First, we demonstrated that the actions of APB were direct and evident after synaptic transmission was blocked with Co\textsuperscript{2+}. Second, we showed that APB increases the input resistance of the cell and third, when synaptic transmission was blocked with Co\textsuperscript{2+} APB polarized the bipolar in the same direction as that of the endogenous transmitter. The alternative "shunting" hypothesis was eliminated because APB increased the input resistance of the cell. If the increased input resistance by APB was through nonsynaptic ion channels, the light response would be increased, not eliminated. This analysis unequivocally established that APB acts on the same synaptic receptors as those of the endogenous photoreceptor neurotransmitter. As APB is clearly a glutamate analogue, we felt that our analysis strongly pointed to an excitatory amino acid as the photoreceptor neurotransmitter and favored glutamate over aspartate.

The APB receptor we discovered is now known as the mGlur6 receptor, based on the molecular biology studies of Nakajima et al.\textsuperscript{45} It was the first metabotropic receptor discovered in the nervous system and preceded by many years similar discoveries in the brain and spinal cord. Today, APB, or L-AP4 as it is now officially named, has been available for more than 25 years and is arguably the most commonly used drug for evaluating issues of retinal circuitry. Peter Schiller\textsuperscript{46} used APB to explore the segregation of On and Off inputs into cells of the
visual cortex and visually guided behavior. His use of APB in the primate provided evidence that the On system subserves eye movements for positive contrast images, whereas the Off system subserves a similar function for negative contrast images.

Today, we know that metabotropic glutamate receptors form a large family of slow glutamate receptors that function at both presynaptic and postsynaptic sites. Although these receptors have different modes of action, they are all coupled to G-proteins which convey their actions. Despite the diversity of metabotropic receptors, the APB or mGluR6 receptor we discovered in 1981 is found exclusively in the retina and is restricted in its role to generating the light responses of rod bipolars and cone On bipolars. Because the light response of On bipolars was eliminated in a C-F medium, we initially thought that it might be generated by chloride, but additional studies in my laboratory with Wally Thoreson demonstrated unequivocally that the action of APB was to close nonspecific cation channels, with a reversal potential near 0 mV.

The paper on APB marked the first in a series of studies and discoveries in which we progressively elucidated the sites of synaptic transmission in the retina that used excitatory amino acid receptors, including (1) as a complement to APB, we reported that cis-2,3-piperidinedicarboxylic acid (PDA) suppresses the light response of Off bipolars and horizontal cells without having a significant effect on On bipolars (Fig. 7A); (2) later that year we demonstrated that amacrine and ganglion cell light responses are antagonized by PDA and that Off ganglion cell light responses are significantly blocked by PDA at concentrations of 1 mM which had little effect on outer retinal neurons. (A) Reprinted, with permission, from Slaughter MM, Miller RF. An excitatory amino acid antagonist blocks cone input to signconserving second-order retinal neurons. Science. 1983; 219:1230–1232. © 1983 by American Association for the Advancement of Science. (B) Reprinted, with permission, from Slaughter MM, Miller RF. Bipolar cells in the mudpuppy retina use an excitatory amino acid neurotransmitter. Nature. 1983;303:537–538. © 1983 Nature Publishing Group.

KA Interacts with Light-Activated Glutamate Receptors in the Inner Retina

One of the major problems with PDA was its nonselective mode of antagonism. While it suppressed light responses of Off bipolar and horizontal cells and all inputs into amacrine and ganglion cells, it did not discriminate between the agonists NMDA, quisqualate, and KA. We found that D-3-O-phosphoserine

FIGURE 7. (A) Application of cis-2,3-piperidinedicarboxylic acid (PDA) on outer retinal neurons with virtually no effect on photoreceptors (Aa) or On bipolars (Ab) (except the antagonistic surround is hyperpolarizing in control, but more depolarizing in presence of PDA), but significant depression of horizontal cell responses and Off bipolar responses. (B) PDA actions on inner retinal neurons. (Ba) Off ganglion cell light response was suppressed by PDA with virtually no change in the input resistance of the cell (Bb). (Bc) On-Off amacrine shows significant PDA suppression of On and Off response by PDA; (Bd) Off ganglion cell light responses depressed PDA and (Be) Off ganglion cell light response significantly blocked by PDA at concentration of 1 mM which had little effect on outer retinal neurons. (A) Reprinted, with permission, from Slaughter MM, Miller RF. An excitatory amino acid antagonist blocks cone input to signconserving second-order retinal neurons. Science. 1983; 219:1230–1232. © 1983 by American Association for the Advancement of Science. (B) Reprinted, with permission, from Slaughter MM, Miller RF. Bipolar cells in the mudpuppy retina use an excitatory amino acid neurotransmitter. Nature. 1983;303:537–538. © 1983 Nature Publishing Group.
was more selective in attenuating the light response, while it also selectively suppressed the response to exogenous KA, but had very little action on NMDA or quisqualate (another glutamate agonist). These experiments indicated that KA interacts with a population of receptors that are also activated by the neurotransmitter released from bipolars (Fig. 8).52,53 In addition, we carried out an extensive analysis of many different antagonists and agonists to evaluate their selectivity properties against the primary agonists that defined different glutamate receptors at the time (Table 1). A summary of our early work on excitatory amino acid receptors appeared in 1986.56

**KA Receptors are AMPA Receptors**

The techniques of molecular biology, cloning, and expression eventually determined that KA responses are mediated by AMPA receptors, not KA receptors. In binding studies, KA binds to KA receptors, but physiologically opens them only transiently. On the other hand, KA opens AMPA receptors tonically by preventing their rapid desensitization mechanism, which results in very large currents evoked by this agonist. Although our own work on the synaptic physiology and pharmacology of glutamate receptors in the retina pointed to glutamate rather than aspartate as the neurotransmitter, studies of agonists and antagonists cannot be used to identify the endogenous neurotransmitter. Ayoub et al.57 provided direct measurements of glutamate release from cone photoreceptor terminals of the lizard and, to this day, we look at glutamate as the major transmitter of bipolar cells and photoreceptors.

**Rabbit Retina Uses Glutamate Receptors**

Our work on glutamate receptors was carried out in both the amphibian and rabbit retina. There were many advantages in working with the rabbit because cellular identification was more secure, in part based on our own studies using horseradish peroxidase as a cell staining and identification technique. This work was headed by Stewart Bloomfield. Initially, our work in the rabbit retina was done by Steve Massey. His studies revealed that the rabbit retina, like that of the salamander, uses excitatory amino acid receptors for neurotransmission from photoreceptors and bipolar cells.58–60

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**Table 1. Comparison of Various EAAR Antagonists at Concentrations which Suppress Light-Evoked Activity**

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>Concentration</th>
<th>KA</th>
<th>NMDA</th>
<th>QQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>γ-D-Glutamyltaurine (D-GT)</td>
<td>5–6 mM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>γ-D-Glutamylaminomethylsulphonate (GAMS)</td>
<td>5–6 mM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-O-Phosphoserine (DOS)</td>
<td>5–6 mM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cis-2,3-piperidine dicarboxylic acid (PDA)</td>
<td>5–6 mM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kynurenic acid (KYN)</td>
<td>3–5 mM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>γ-D-Glutamylglycine (D-GG)</td>
<td>5–10 mM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-(β-Chlorobenzoyl)piperazine-2,3-dicarboxylic acid (cPZA)</td>
<td>3–5 mM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-(β-Bromobenzoyl)piperazine-2,3-dicarboxylic acid (bPZA)</td>
<td>3–5 mM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6-Cyano-7-nitroquinoxaline-2,3-dione (CNOX/FB 9065)</td>
<td>25 μM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6,7-Dinitroquinoxaline-2,3-dione (DNQX/FG 9041)</td>
<td>25 μM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6,7-Dichloro-3-hydroxy-2-quinoxalinecarboxylic acid (DHQA)</td>
<td>100 μM</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Segregation of NMDA and AMPA Receptor Pathways in the Inner Retina

When we introduced the highly selective AMPA receptor antagonist, NBQX61 (a quinoxaline compound: 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo-f-quinoxaline), we demonstrated its unique properties on some specialized ganglion cells, such as the directionally selective ganglion cells of the rabbit retina. NBQX blocks the directionally selective encoding of the cell, while preserving the vigorous On and Off light-evoked impulse activity. In the presence of NBQX, DS cells respond to moving targets in both the preferred and null directions, rather than in the preferred direction only, which is characteristic of DS ganglion cells. Although NBQX blocks the AMPA/KA receptor inputs into ganglion and amacrine cells, the remaining NMDA receptors provide strong light-evoked excitation, presumably because NBQX leads to enhanced release of glutamate from bipolar cells, but also antagonizes glycine actions on ganglion and amacrine cells.62,63 However, it was clear that a KA/AMPA receptor pathway is critical for encoding the DS mechanism. Dixon and Copenhagen64 showed that sustained amacrine cells are activated by AMPA receptors, whereas transient amacrine cells (On-Off) are activated by both AMPA and NMDA receptors. This arrangement means that blocking AMPA receptors can eliminate sustained amacrine cells, which may account for the NBQX-induced loss of directional selectivity that we observed in these ganglion cells.

Peptide Actions in the Retina

During the 1980s, we witnessed an explosion in the number of new transmitter candidates that were almost instantaneously elevated to neurotransmitter status through immunostaining methods. In this respect, the retina turned out to be one of the most provocative regions of the nervous system, as a large number of peptides were localized to different cell types of the retina.65 As these new revelations surfaced, we felt compelled to examine the actions of peptides on retinal neurons and the retinal network. We found that virtually all peptides localized to the retina by immunostaining have actions on retinal neurons. Peptides were applied using iontophoretic application or by adding them to the bathing medium in a perfused retina–eyecup preparation of the rabbit.67–69 We provided convincing evidence that substance P contributes to the tonic (dark) impulse firing of retinal ganglion cells and that somatostatin has unique actions on the retinal network that give it special status as a potential contributor to adaptive mechanisms in retina network operations. The full spectrum of peptide actions in the retina is a topic still worthy of pursuit, as our understanding of the physiological actions of peptides and the full spectrum of their functional contributions remains at a very preliminary level. The work that has already been done however, suggests that peptides may provide a new rich layer of functional diversity uniting communication mechanisms of many different systems, including vascular, metabolic, glial, and neuronal in ways we have yet to appreciate or imagine.

\(\alpha\)-Serine in the Vertebrate Retina

The Road Back to Glia

This brings me to the final section of my lecture. It relates to the special control mechanisms of NMDA receptors, the recept-

\(\alpha\)-Serine and serine racemase are present in the vertebrate retina and contribute to the physiological activation of NMDA receptors. Proc Natl Acad Sci U S A. 2005;100:6789–6794. Copyright © 2003 by the National Academy of Sciences.

FIGURE 9. (A) Whole-cell recording from rat retinal ganglion cell with inward currents evoked by puff application of NMDA from a pipette placed over the cell body of the ganglion cell. \(\alpha\)-Serine reversibly enhanced the response. (B) Rat ganglion cell WCR with inward currents evoked by puff of NMDA. Addition of the enzyme D-AAO reversibly depressed the NMDA response. (C) WCR from retinal ganglion cell showing noise level of recording increased with \(\alpha\)-serine application, followed by a decrease in noise and lack of \(\alpha\)-serine effect in presence of DL-AP7, with recovery to right. (D) Graphs of \(\alpha\)-serine and D-AAO application showing significant actions of \(\alpha\)-serine and D-AAO: (E) WCR from salamander ganglion cell with light evoked inward current increased in amplitude by \(\alpha\)-serine application. (F) Recording of PNR in salamander in presence of picrotoxinin and NBQX to block inhibitory input and AMPA receptors. The addition of AP7 to the cocktail decreases response demonstrating high degree of NMDA receptor input to response. (G) PNR enhanced by picrotoxinin and NBQX. The addition of D-AAO to the bathing medium reversibly depressed the response to approximately that illustrated in (F). (H) Summary of WCR and PNR experiments showing significant \(\alpha\)-serine enhancement, decrease in PNR response with AP7 and D-AAO exposure. Reprinted, with permission, from Stevens ER, Esguerra M, Kim PM. et al. \(\alpha\)-Serine...
tors we discovered in ganglion and amacrine cells some 25 years ago (1983), when we demonstrated that virtually every ganglion cell could be depolarized in response to both KA and NMDA and that both receptors participate in mediating light-evoked activity. But, the state of knowledge in the field at that time was such that activation of NMDA receptors was assumed to indicate aspartate as the endogenous neurotransmitter, while the activation of KA receptors was assumed to indicate glutamate as the neurotransmitter. But a year after our discovery of separate NMDA and KA receptors in retinal ganglion cells, binding studies revealed that glutamate is more effective than aspartate as an agonist for NMDA receptors and since then, glutamate has generally been regarded as the only excitatory neurotransmitter in the retina and all other areas of the central nervous system.

NMDA Receptors Require Two Agonists for Activation

NMDA receptors play important roles in events such as memory and learning, but their role in retinal function has not been clearly established and there is no uniform agreement on the extent to which NMDA receptors are involved in mediating light-evoked synaptic activity. Shortly after NMDA receptors were identified as a distinct subpopulation of glutamate receptors, Johnson and Ascher reported that, in order for glutamate to open the NMDA receptor ion channel, a cofactor has to be present in the environment, and they initially identified glycine as the critical cofactor. Additional work demonstrated that many amino acids can substitute for glycine as a coagonist and NMDA and D-serine is among them. Hashimoto et al. pursued the possibility that D-serine may be present in the brain and, to everyone's surprise, he found substantial D-serine levels that varied in different regions of the brain, as determined by microdialysis experiments. A few years later, Wolosker et al. discovered the enzyme serine racemase, which synthesizes D-serine from L-serine, and localized it to astrocytes.

As these initial reports were emerging, I decided to determine whether D-serine had functional significance in the retina. Using antibodies initially obtained from Sol Snyder, we discovered that D-serine and the enzyme serine racemase are readily detected in the retina and that their distribution is consistent with localization into Müller cells and astrocytes. Furthermore, we demonstrated that, in the intact isolated retina of salamanders and rats, NMDA receptors were not saturated at their coagonist sites, such that the exogenous application of D-serine further enhanced the postsynaptic currents of NMDA receptors (Figs. 9A–F).

Encouraged by these initial studies, we decided to pursue more detailed physiological questions about the role of D-serine in retinal function. Previous studies, including our own, had suggested that the coagonist binding site of NMDA receptors was tonically saturated and hence, an unlikely site of any excitatory neurotransmitter in the retina and all other areas of the central nervous system. NMDA receptor coagonist sites are saturated and cannot be enhanced by adding exogenous D-serine (E. R. Stevens, preliminary observation, 2007).

Is D-Serine the Only NMDA Receptor Coagonist in the Retina?

While glycine levels are under tight regulatory control through the high-affinity GlyT1 transporter, we determined that the uptake of D-serine in the salamander retina is mediated by a low-affinity, Na+-dependent ASCT2 amino acid transporter. To determine the extent to which D-serine serves as the coagonist when the glycine transporter was functionally present, we used two different D-serine-degrading enzymes, including the fast-acting D-serine deaminase (DsdA) and the slower, endogenous enzyme, D-amino acid oxidase (DAAO). These enzymes were added independently to the extracellular bathing environment. To our surprise, the NMDA receptor currents were reduced to virtually the same level as those observed when NMDA receptors were blocked with antagonists, suggesting that D-serine may serve as the only endogenous coagonist for NMDA receptors in the retina, at least under the conditions of our experiments.

More recently, we have started to address issues of D-serine function in the retina by using knockout or mutant mice with a defect in one or more areas related to coagonist function. One mutant mouse which we obtained from Ryuichi Konno lacks functional DAAO, an enzyme essential for regulation of D-serine. The retinas of these animals have a normal adult layering pattern, but may have an excessive branching of Müller cell processes in the inner retina, with heavy labeling for serine racemase (Catherine Morgans, personal observation, 2007). We have also been studying D-serine release from these animals and have detected a light-evoked release using the isolated retina. A second mouse line that we have studied is a GlyT1 knockout mouse. In this mouse line, homozygotes die at birth, but the heterozygote reaches maturity. We have studied heterozygote mice, which show a high degree of saturation of the NMDA receptor coagonist sites when compared to their wild-type litter mates.

Further description of these additional studies on D-serine synthesis, storage, and release in the retina is beyond the scope of this report. However, our preliminary observations suggest that D-serine is itself under the regulatory control of glutamate release from retinal cells, which in turn may activate AMPA receptors on Müller cells. D-Serine release may also take place through transport exchange, as demonstrated by Dun et al. Clearly, many elements critical to our understanding about D-serine regulation and its control of NMDA receptors and ganglion cell excitability remain to be worked out. But who can deny that fascinating new insights await these additional studies simply because they seem to indicate that glial cells have a far greater impact on controlling ganglion cells than we have previously imagined.

Acknowledgments

I have many people to acknowledge and thank. First, I want to thank Thomas E. Ogden, who, when I was a medical student at the University of Utah, opened my eyes not only to the fascination of research, but also pointed me toward the very tissue that has fascinated me for nearly 50 years and has been the delightful focus of my entire research career. Tom convinced me that my early intrigue with Purkinje cell structure–function relationships, based on seeing one of Cajal's Golgi stains as a first-year medical student, could be better served by pursuing studies of the vertebrate retina. I also have to thank Carlton C. Hunt who was chairman of the Physiology Department at the University of Utah for his mentorship and for allowing me to pursue some of my own research interests.
Utah when I was a medical student. He assembled a truly remarkable faculty whose research energy and accessibility stimulated me to think about a career as a neurophysiologist. I am flattered that Carlton recognized how important his faculty and physiology course were in shaping my career.86 But my career probably would have not prospered as it did without the experience I had in John Dowling’s laboratory. I have always looked at John as the gold standard for mentoring. It was John and the stimulating people who were in his laboratory at the time, including Dwight Burkhardt, Frank Werblin, Mark Dubin, Robert Frank, Dick Chappell, and Helga Kolb, combined with stimulating, intermittent visits from Bryan Boycott, and lasting conversations with Harris Rips that broadened my research interests and knowledge. It was in John’s laboratory that I began to appreciate that science had a social component and cohesiveness, not just a bunch of oases in the desert of a disinterested public. The experience in John’s laboratory provided a solid research direction and strategy that I still carry with me today. I am grateful too that John and I have remained good friends and associates. And, it was in John’s laboratory that I met Dwight Burkhardt who has been a good friend and great collaborator over many years. I am grateful for his introduction at the Proctor Medal talk at ARVO this past spring and his contribution to this journal. Werner Noell gave me my first assistant professor position in Buffalo in 1971, and introduced me to new ways of thinking about biochemical and physiological mechanisms. He had a wonderfully penetrating, intuitive grasp of issues and mechanisms that I have rarely seen in others.

I can never adequately express the gratitude I have for those who came through my own laboratory as students or postdocs: Those who have remained in research continue to work in the retina and all have established strong research programs and visibility of their own. I have had academic appointments in three different institutions, and in each place I have been blessed with colleagues of prodigious talent and work capacity; and of course, they did all the work that I have described. Ramon Dacheux started as my research assistant in Buffalo and eventually earned his PhD in my laboratory. He went on to establish an excellent research career of his own, through a long collaboration and friendship with Elio Raviola, followed by establishing his own laboratory at the University of Alabama, Birmingham, until his untimely death in 2006. The many others who came through my laboratory include Malcolm Slaughter, Stewart Bloomfield, Steve Massey, Stuart Mangel, Tom Frumkes, Wally Thoreson, Ethan Cohen, Connie Bove, Janet Olsen, Evan Dick, Robert Cunningham, Robert Zalutsky, Marty Arkin, Ron Nitzan, Toby Velte, Jon Gottesman, Mike Sikora, Jurgen Fohldrester, Manuel Esquerra, Carol Toris, Dori Henderson, Eric Stevens, Kylie O’Brien, Mary Fagerson, Eric Gustafson, Pratip Mitra, and Steve Sullivan. All of my colleagues who came through my laboratory have been both students and teachers for me. I had outstanding technical help from Judy Dodge, Bob Wolfe, Jody Eisland, and Terry Doerr. I currently enjoy excellent graphics, rhetorical, and editorial support from Derek Miller.

Most of all, I want to thank my family: My wife Rosemary patiently shaping my career.86 But my career probably would have not prospered as it did without the experience I had in John Dowling’s laboratory. I have always looked at John as the gold standard for mentoring. It was John and the stimulating people who were in his laboratory at the time, including Dwight Burkhardt, Frank Werblin, Mark Dubin, Robert Frank, Dick Chappell, and Helga Kolb, combined with stimulating, intermittent visits from Bryan Boycott, and lasting conversations with Harris Rips that broadened my research interests and knowledge. It was in John’s laboratory that I began to appreciate that science had a social component and cohesiveness, not just a bunch of oases in the desert of a disinterested public. The experience in John’s laboratory provided a solid research direction and strategy that I still carry with me today. I am grateful too that John and I have remained good friends and associates. And, it was in John’s laboratory that I met Dwight Burkhardt who has been a good friend and great collaborator over many years. I am grateful for his introduction at the Proctor Medal talk at ARVO this past spring and his contribution to this journal. Werner Noell gave me my first assistant professor position in Buffalo in 1971, and introduced me to new ways of thinking about biochemical and physiological mechanisms. He had a wonderfully penetrating, intuitive grasp of issues and mechanisms that I have rarely seen in others.

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