0270-6474/83/0308-1701$02.00/0

The Journal of Neuroscience
Vol. 3, No. 8, pp. 1701-1711
August 1983

THE ROLE OF EXCITATORY AMINO ACID TRANSMITTERS IN THE MUDPUPPY RETINA: AN ANALYSIS WITH KAINIC ACID AND *N*-METHYL ASPARTATE[1]

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Received December 20, 1982; Revised March 24, 1983; Accepted March 25, 1983

Abstract

A variety of glutamate and aspartate analogues were used to characterize the excitatory amino acid receptors in the mudpuppy retina. This approach revealed two general classes of receptors which were represented by the agonists kainic acid and *N*-methyl aspartic acid. Kainic acid was found to be a potent photoreceptor transmitter agonist on all three types of second-order neurons, and it was a powerful excitant of amacrine and ganglion cells. *N*-Methyl aspartate had little effect in the outer retina, but it had potent stimulatory effects on inner retinal neurons. *N*-Methyl aspartate antagonists selectively blocked light responses in some sustained OFF ganglion cells. These results suggest that both photoreceptors and bipolar neurons may use glutamate or an analogue, whereas aspartate may be utilized by a class of sustained ON amacrine cells.

The direct afferent pathway in the vertebrate retina is a bisynaptic connection from photoreceptors, through bipolar neurons, to the ganglion cells. Horizontal cells mediate lateral interactions at the first synaptic region, and amacrine cells provide lateral interactions at the second synaptic location (Ramon y Cajal, 1972). Aspartate and glutamate may play a role in neurotransmission at synaptic sites in both plexiform layers (Murakami et al., 1972, 1975; Ikeda and Sheardown, 1982; Slaughter and Miller, 1983). Therefore, we wished to characterize the excitatory amino acid receptors in the retina. In general, the identification of acidic amino acid synapses has been hampered by a paucity of site-specific histochemical and pharmacological techniques. In particular, the limited effectiveness of many presumed antagonists has prevented a clear identification of the transmitter and the properties of the synaptic receptors. As an alternative approach, several agonists have been developed which show selective actions and thus disclose discrete receptor populations. At present, this approach has distinguished three types of excitatory amino acid receptors: (*1*) a glutamate/quisqualate receptor, (*2*) an aspartate/*N*-methyl aspartate receptor, and (*3*) a kainic acid receptor (Watkins, 1982). We have employed this method in an attempt to identify the excitatory amino acid receptors among retinal neurons in the mudpuppy. We have found the responses to kainic acid and quisqualic acid to be similar, the former being slightly more potent. However, the actions of *N*-methyl aspartate were distinctly different. Therefore, in this paper we have contrasted the effects of kainic acid and *N*-methyl aspartate. We have found that the former was a potent agonist on all second- and third-order neurons, whereas *N*-methyl aspartate activity was principally restricted to the proximal retina. Our evidence suggests that glutamate or an analogue is used by photoreceptors and bipolars, whereas aspartate may be the transmitter of a group of sustained ON amacrine cells.

Materials and Methods

Experiments were conducted in the superfused retina-eyecup of the mudpuppy, *Necturus maculosus*, using standard intracellular recording procedures (Miller and Dacheux, 1976). The animal was beheaded, the lower jaw was removed, and the remaining portion of the head was sagittally hemisected. The eyecup and the surrounding tissue were dissected from the skull and placed on Ringer's soaked cotton. The eyelids, cornea, iris, and lens were removed, and the vitreous was absorbed with a Kimwipe tissue. When the retina was fully exposed, another tissue, containing a hole slightly smaller than the eyecup, was placed over the eye leaving only the retina exposed. This preparation was placed on a Ring-

[1] We wish to thank Dr. J. C. Watkins, Dr. P. Krogsgaard-Larsen, and Dr. S. C. Massey for generously donating several compounds used in these experiments. We thank Ms. Rita Drochelman for typing this manuscript. This work was supported by National Eye Institute Grants R23 EY 03868 and R01 EY 03014.

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er's soaked cork which was in contact, through a salt bridge, with a silver-chloride ground electrode.

A two-channel light bench equipped with quartz iodide lamps was used to project a small spot (200 μm), an annulus (inner diameter = 400 μm, outer diameter > retinal diameter), or a full field light stimulus onto the retina. Neutral density filters were used to regulate the light intensity (irradiance = 5×10^{-9} W/cm^2 at retina), and photocells recorded the opening and closing of the shutters. Studies with monochromatic lights indicated that we were monitoring cone-dominated activity under our experimental conditions.

Electrodes were made from 1.2 mm outer diameter, 0.6 mm inner diameter omega dot glass tubing (Glass Company of America), fabricated in a Narishige vertical electrode puller, and were filled with 3 M K^+ acetate. The electrodes were advanced using a hydraulic microdrive, and capacitative oscillations were used to facilitate cell impalement. The recording apparatus consisted of a WPI 707 high impedance amplifier connected through a Tektronix AM 502 amplifier (used single-ended) to a Tektronix 5113 storage oscilloscope and a Brush 260 penwriter. A DC current source was used to inject 0.1 nA through the stimulus input of the WPI for resistance measurements.

Pharmacological agents were dissolved in amphibian Ringer's (111 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 10 mM dextrose, and 5 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)) buffered to pH 7.80. The solutions were contained in aspiration bottles, bubbled with oxygen, and connected through Teflon-lined valves (Hamilton) and individual Teflon tubes to a mixing chamber. The mixing chamber could accept up to 10 lines and had a single output which ran through a short length of polyethylene tubing to a glass pipette. The pipette was positioned at the rim of the eyecup, and the flow rate over the retina was maintained at 1 to 2 ml/min. The perfusion bottles were positioned about 3 feet above the level of the eyecup so that the flow was maintained by gravity.

Kainic acid, *N*-methyl aspartate (DL form), D-α-amino adipate, and quisqualate were obtained from Sigma Chemical Co. AMPA (amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) was a gift from Dr. P. Krogsgaard-Larsen, α-amino suberate was a gift from Dr. J. C. Watkins, and *N*-methyl D-aspartate was a gift from Dr. J. C. Watkins or from Dr. S. C. Massey.

Results

Outer retina, horizontal cells. In the mudpuppy retina, the photoreceptor neurotransmitter depolarizes horizontal cells and OFF bipolars, whereas it hyperpolarizes ON bipolars (Dacheux and Miller, 1976). In the outer retina kainic acid mimicked the action of the photoreceptor transmitter on all three types of second-order neurons, whereas *N*-methyl aspartate had little or no effect. Neither agent attenuated the light response of photoreceptors. Figure 1 illustrates the action of kainic acid and *N*-methyl aspartate on mudpuppy horizontal cells. The figure shows the response of horizontal cells to small spot and annular stimulation. The application of 50 μM kainic acid produced a depolarization of the cell and a loss of the light response within 20 sec. In contrast, 1 mM *N*-methyl DL-aspartate had no effect during a 100-sec application period. To ascertain that kainic acid was acting on horizontal cell receptors, as opposed to an indirect excitation involving synaptic input from other neurons, we repeated these experiments in the presence of cobalt. Cobalt application caused a hyperpolarization of the cell and a loss of the light response, consistent with the elimination of synaptic input from photoreceptors. In the continued presence of cobalt, 25 μM kainic acid caused a depolarization which reached the level of the original dark membrane potential, whereas 500 μM *N*-methyl DL-aspartate had no effect on the cell's potential.

Bipolar cells. The ON bipolar is also very sensitive to kainic acid treatment. The *upper trace* of Figure 2 shows the response of an ON bipolar cell to full illumination. One micromolar kainic acid caused a diminution of the light-evoked depolarization, compatible with the action of a photoreceptor transmitter agonist. The application of 100 μM *N*-methyl DL-aspartate had no effect on the light response of this neuron. Fifty micromolar kainic acid caused a similar but more pronounced suppression of the light response by driving the cell toward its dark membrane potential. The *middle trace* of Figure 2 serves to demonstrate that even at a concentration of 1 mM, *N*-methyl DL-aspartate does not affect the light responsiveness of ON bipolars. The experiment depicted in the *lower trace* of Figure 2 indicates that kainic acid is acting on receptors endogenous to the ON bipolar. Cobalt application, by blocking synaptic input from photoreceptors, caused the ON bipolar to depolarize. Adding 50 μM kainic acid in conjunction with 3 mM cobalt resulted in a hyperpolarization of the cell to approximately the dark resting level. The far right side of the trace shows the beginning of recovery from this combined treatment with kainic acid and cobalt.

The OFF bipolar cells display less sensitivity to kainic acid than do horizontal cells or ON bipolars and also show a slight *N*-methyl aspartate sensitivity. The response of an OFF bipolar to small spot and annular illumination is shown in Figure 3. At the far left of the *top trace* the response to a small spot and an annulus are shown. Then the small spot is left on continuously while the annulus (*arrow*) is flashed twice to elicit the antagonistic surround (Werblin and Dowling, 1969). Under these conditions the surround caused a depolarization believed to be mediated by horizontal cell feedback. The adapting center spot was then removed, and intermittent small spot and annular stimuli were again presented. One millimolar *N*-methyl DL-aspartate produced a slight decrease in the light response evoked by both the small spot and the annulus. When an adapting center spot was reapplied, the surround response appeared unchanged. Thus *N*-methyl DL-aspartate seemed to have a slight ability to drive the OFF bipolar toward the dark membrane potential, and it thus acted as a weak photoreceptor transmitter agonist. The *lower trace* of Figure 3 demonstrates that kainic acid is more potent than *N*-methyl aspartate on this cell but less potent than it was on the two other second-order neurons. When 100 μM kainic acid was administered, the light response de-

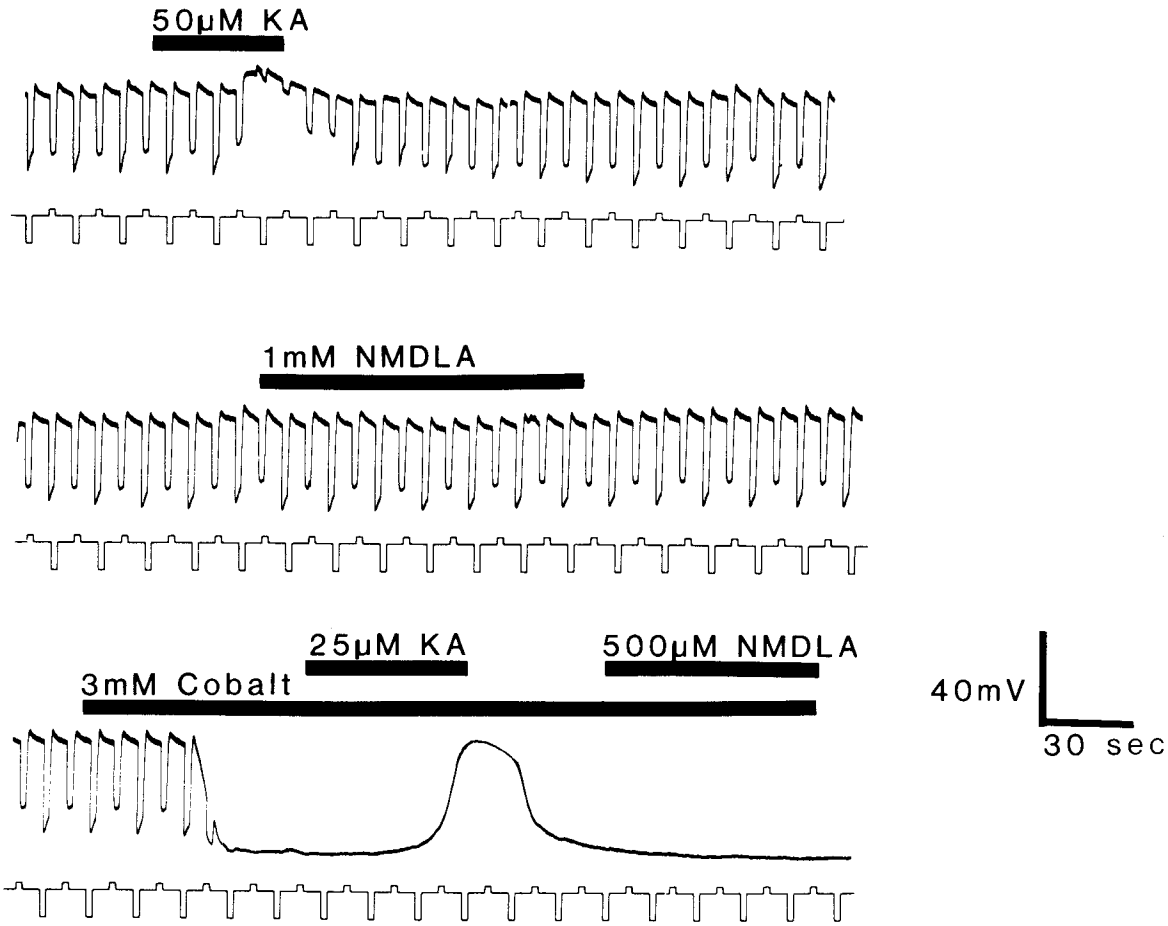

Figure 1. This shows the potent effect of kainic acid (*KA*), but not *N*-methyl DL-aspartate (*NMDLA*), on a horizontal cell before and after the application of cobalt, which blocks synaptic transmission. The figure shows a continuous recording from a single horizontal cell. The format for this and all following figures is that the *bar* above each voltage trace represents the duration of application of the agent indicated, and the *square wave pulses* below the voltage trace indicate the timing of light stimuli. When square waves in both directions are shown, the upward deflection signals a small spot (200 μm) stimulation and the downward deflection represents annular (400 μm outer diameter, inner diameter greater than retinal diameter) stimulation. When a unidirectional square wave pulse is shown, a diffuse light stimulus covering the entire retina was applied.

creased and the cell depolarized beyond the dark membrane potential level. The surround response could no longer be elicited, as would be expected from kainic acid's effect on horizontal cells. This loss of the antagonistic surround is also reflected in the similarity of the response waveform to the small spot and the annulus. At the very end of the recovery period, a better seal was obtained between the cell and the microelectrode, resulting in an enhanced response to both small spot and annulus.

N-Methyl aspartate effects in the outer retina. In the course of our experiments we found that both horizontal cells and ON bipolars were sometimes affected by 1 mM *N*-methyl aspartate when it was applied for the first time in a particular eyecup. After noticing this phenomenon, we studied it by withholding application of *N*-methyl aspartate until a suitable cell was obtained. We found that approximately two-thirds of the horizontal cells and ON bipolars tested in this manner responded to the first *N*-methyl aspartate application. Subsequent applications of *N*-methyl aspartate were without effect. In several horizontal cells we attempted to determine whether *N*-methyl aspartate was acting directly by applying cobalt and then *N*-methyl aspartate. In these instances *N*-methyl aspartate had no effect. However, we could not be sure if these cells would not have been among the group that would not have shown *N*-methyl aspartate sensitivity in any event. We naturally were concerned that this effect was masking a relevant synaptic receptor. To evaluate this possibility we used D-α-amino adipate, an *N*-methyl aspartate antagonist. As described below, we have found that D-α-amino adipate is an effective *N*-methyl aspartate antagonist in the mudpuppy retina. When D-α-amino adipate was applied while recording from a horizontal cell (Fig. 4), no hyperpolarization or attenuation of the light response was observed. Thus, the horizontal cell response is not mediated through an *N*-methyl aspartate receptor. We also wanted to test whether the "desensitization" resulting from *N*-methyl aspartate application would alter the cell's response to kainic acid. Therefore, we compared *N*-methyl aspartate and kainic acid activity during repetitive drug applications, as exemplified in Figure 4. The first application of *N*-methyl DL-aspartate to this retina (*upper trace*) caused depolarization and attenuation of the light re-

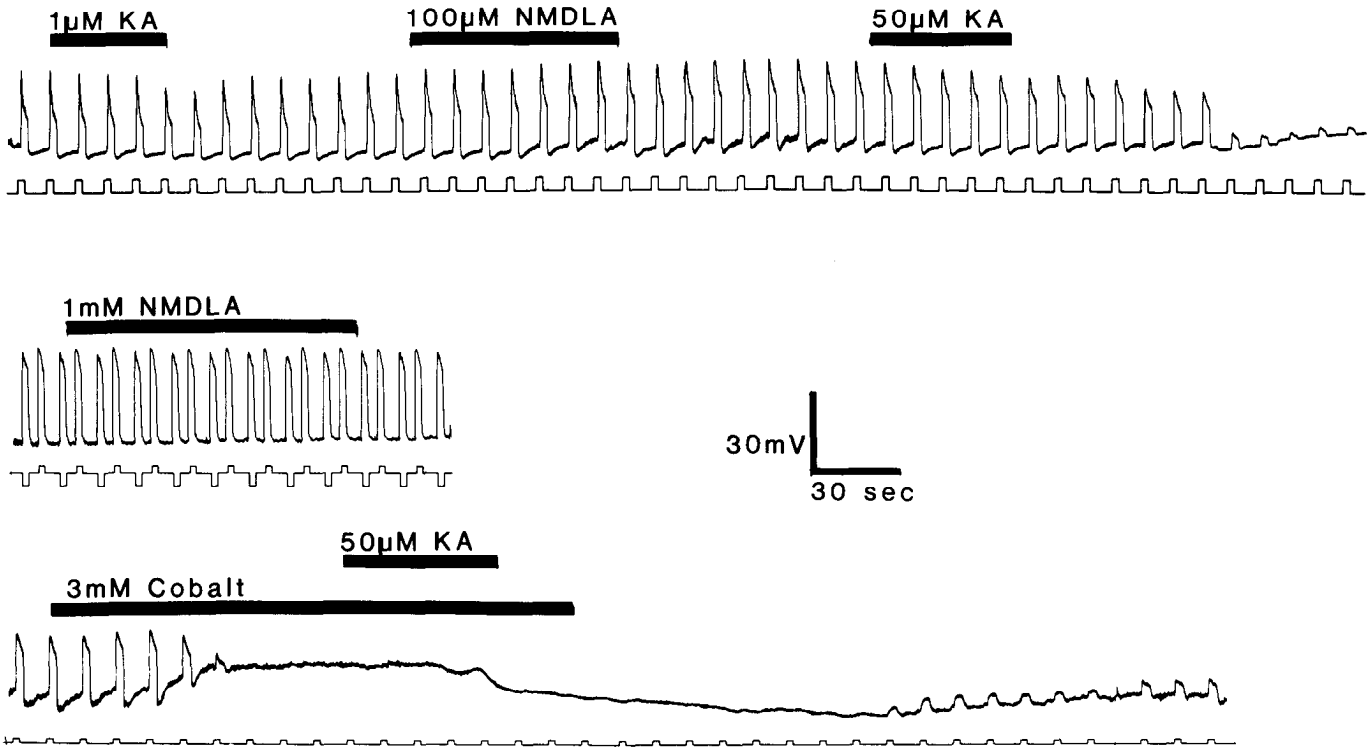

Figure 2. Recordings from several ON bipolars contrasting the effects of differing concentrations of kainic acid and *N*-methyl DL-aspartate on the light response and on the membrane potential after cobalt application.

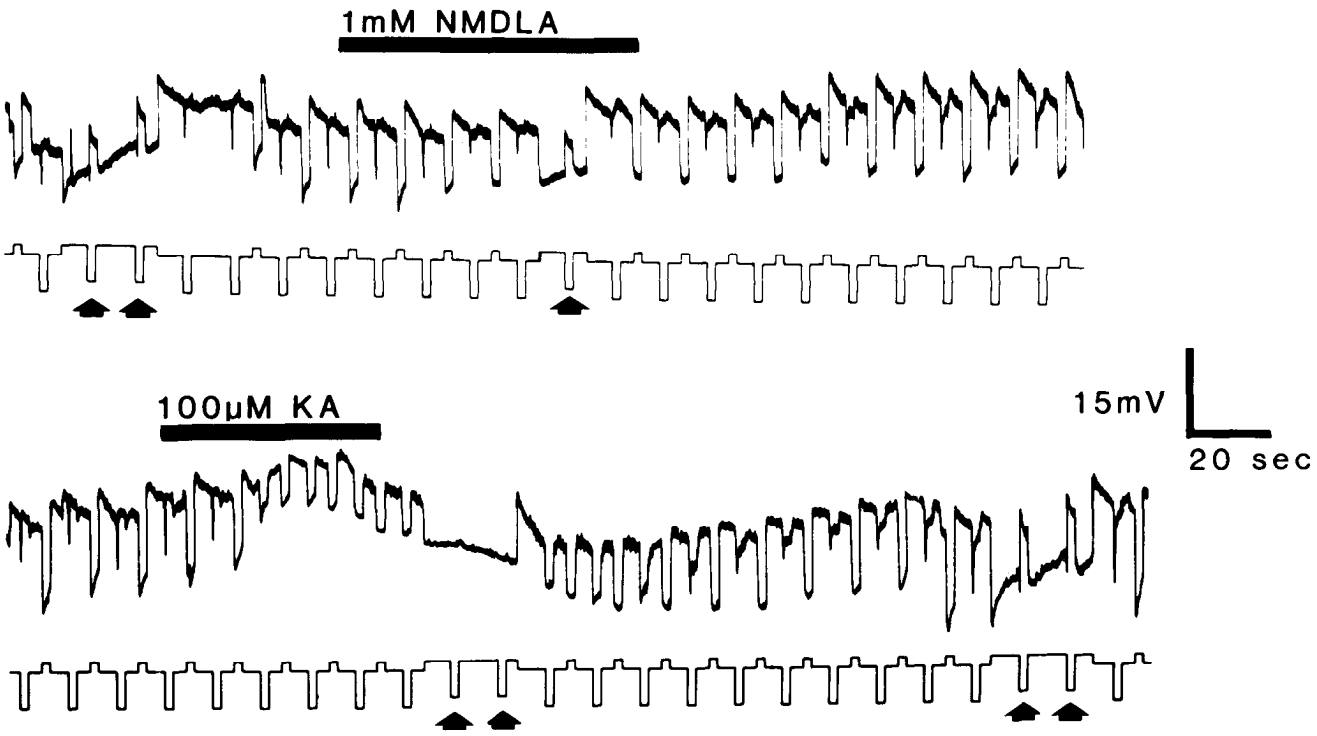

Figure 3. The effect of kainic acid and *N*-methyl DL-aspartate on the center, annulus, and antagonistic surround (*arrows*) responses of an OFF bipolar cell. Comparing this to the previous figures of the other second-order neurons, *N*-methyl DL-aspartate has a slight effect, whereas kainic acid is not as effective an agonist. Note that *N*-methyl DL-aspartate does not eliminate the antagonistic surround but kainic acid does.

sponse of the horizontal cell. However, the second application of 1 mM *N*-methyl aspartate had no effect. Furthermore, the third application of *N*-methyl aspartate (*middle trace*) had no effect on either the synaptically driven light response or the kainic acid-induced depolarization of the horizontal cell. A similar phenomenon was found in the responses of ON bipolar neurons. The origin of this effect is unclear. However, it apparently does not affect synaptic or kainic acid receptors.

Inner retina, amacrine cells. In the inner retina kainic

acid is still a potent excitatory agent, but now *N*-methyl aspartate also shows a prominent stimulatory action. This is epitomized in the ON-OFF amacrine cells of Figure 5. The *upper two rows* depict the responses of one neuron. In this cell 500 μM *N*-methyl DL-aspartate caused a rapid and large depolarization which induced high frequency spiking and the loss of the light response. When cobalt was applied and all the light responses were blocked, 500 μM *N*-methyl aspartate still resulted in a depolarization and an increase in spike activity. The left side of the *middle row* simply shows the partial recovery of this cell following the removal of *N*-methyl aspartate and cobalt. The ability of D-α-amino adipate to antagonize the action of *N*-methyl aspartate in this cell is illustrated at the right of the *second row*. When 500 μM D-α-amino adipate was applied, it had no effect on the cell's response to either small spot or annular stimulation. However, in the presence of D-α-amino adipate, *N*-methyl aspartate produced only a slight depolarization and did not eliminate the light response, as it had previously. The conductance change associated with the action of *N*-methyl aspartate is shown in the *lower trace* of Figure 5. We used 2 mM *N*-methyl aspartate to accentuate the conductance change, although similar results were seen with lower doses. A fast sweep at the left shows the ON and OFF EPSPs and the spike activity elicited by diffuse light. Negative current pulses (0.1 nA) were applied between light flashes, and the bridge of the WPI 707 amplifier was balanced. A short application of *N*-methyl DL-aspartate produced a rapid depolarization with a burst of spiking. At the same time the application of the negative current pulses resulted in a positive deflection of the voltage trace, indicating a large decrease in the input resistance of the cell.

Similar effects on amacrine cells were seen with kainic acid, although the ligand-receptor interactions were not identical since D-α-amino adipate did not block the action of kainic acid. The effect of kainic acid is illustrated in the ON-OFF amacrine cells of Figure 6. The cell in the *upper trace* showed transient ON and OFF depolarizations to annular stimulation and both transient and sustained depolarizing components to small spot stimulation. Addition of 25 μM kainic acid produced a depolarization, a few spikes, and a loss of the light response. A similar depolarization was seen after synaptic transmission was blocked with cobalt, indicating the presence of kainic acid receptors on this neuron. The hyperpolarizing notch seen after the peak kainic acid-induced depolarization was commonly observed. Its origin has not been determined, although it may represent a phase of sodium channel inactivation or increased potassium or chloride conductance. Since it is seen in the presence of cobalt, it is apparently neither synaptically mediated nor due to a calcium conductance. The *lower trace* of Figure 6 depicts

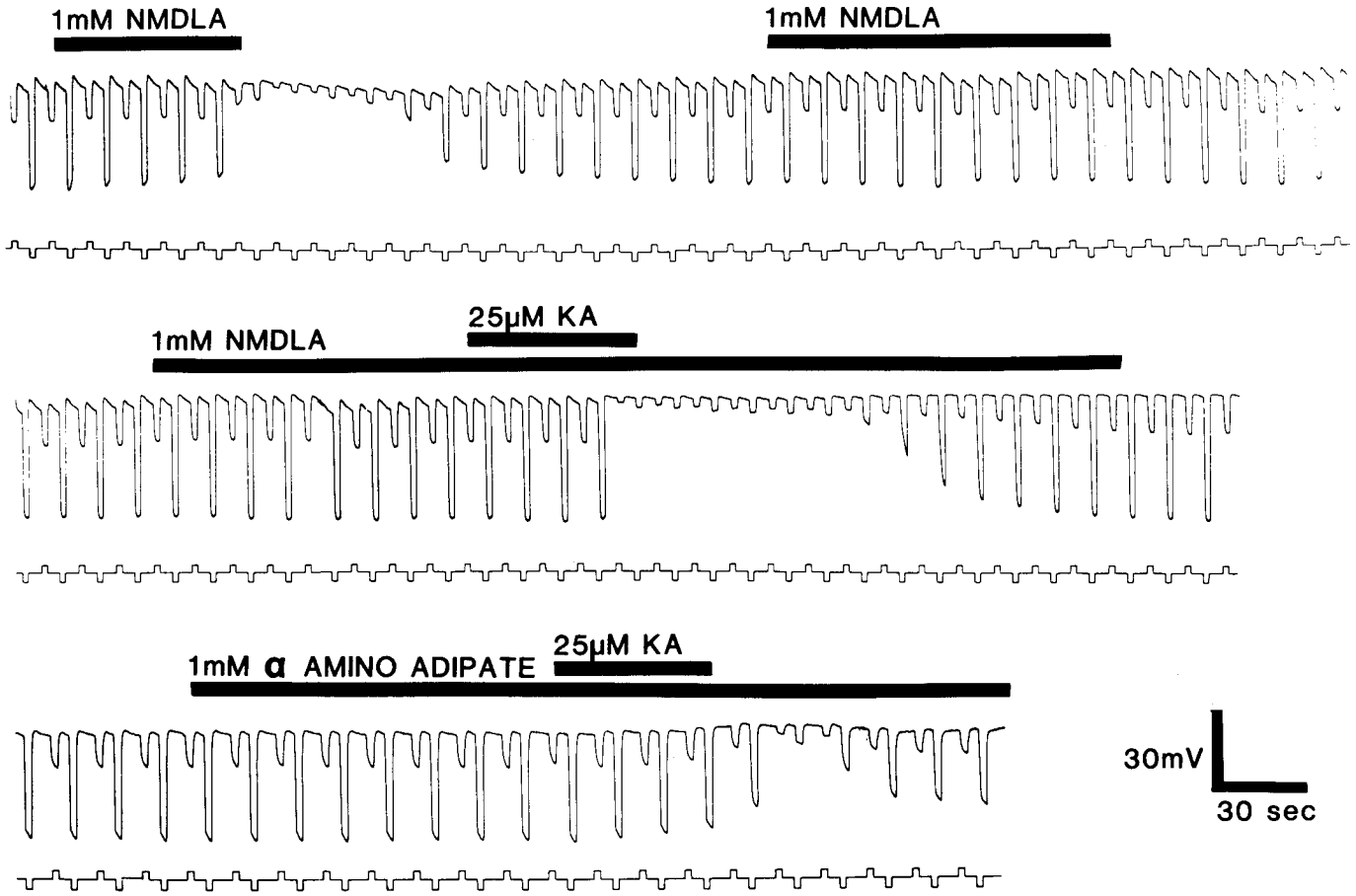

Figure 4. This continuous recording from a horizontal cell shows that *N*-methyl DL-aspartate, when applied for the first time to this retina, drove the cell close to the dark resting membrane potential. But subsequent applications of *N*-methyl DL-aspartate had no effect on the light response or on the normal action of kainic acid. Also, D-α-amino adipate, an *N*-methyl aspartate antagonist, did not alter the light response, nor did it block the kainic acid-mediated depolarization of this neuron.

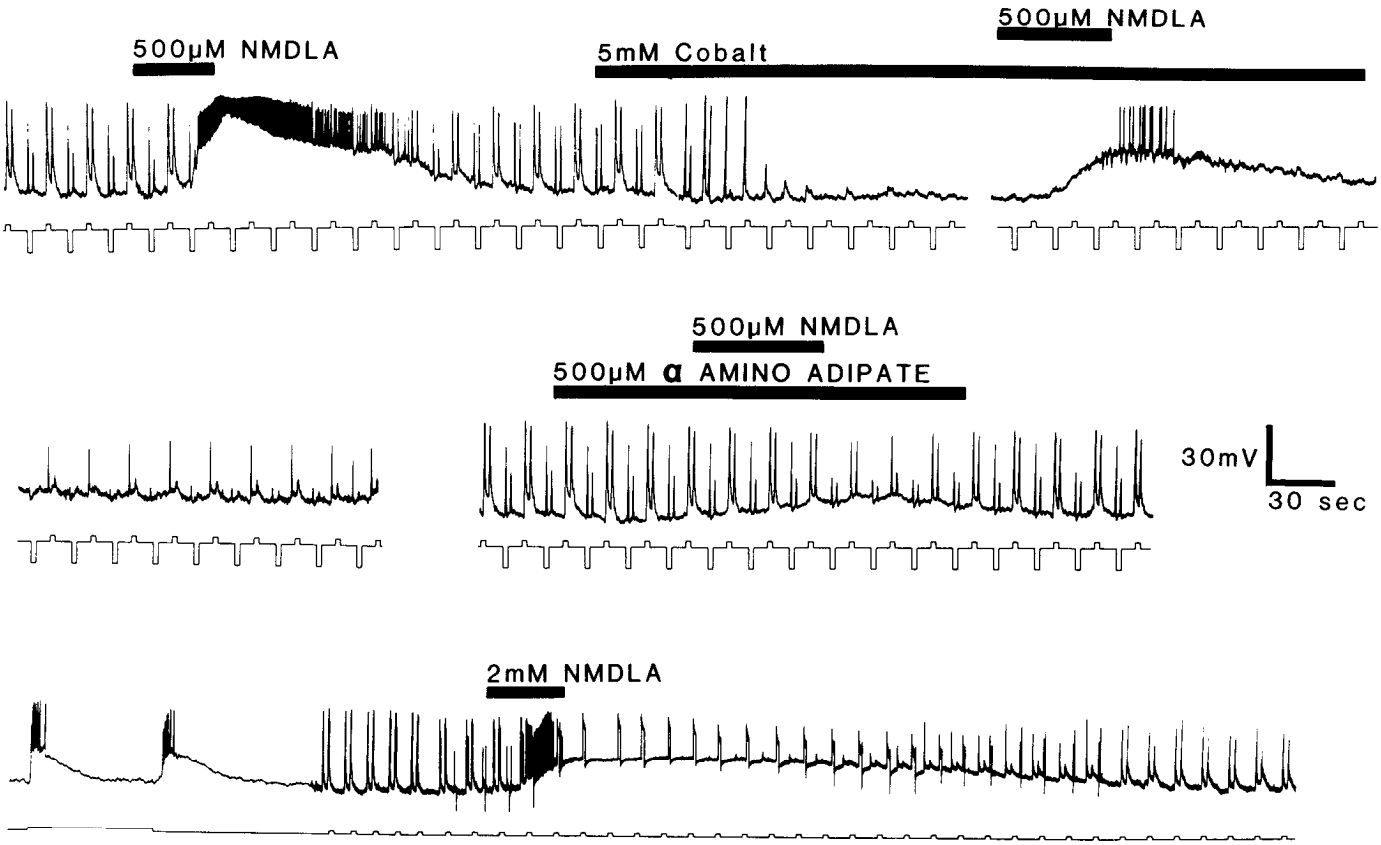

Figure 5. The *top* and *middle rows* illustrate responses from a single ON-OFF amacrine cell. In this neuron, *N*-methyl DL-aspartate caused a depolarization before and in the presence of cobalt. D-α-Amino adipate was able to block this effect. In the ON-OFF amacrine in the *lower trace*, *N*-methyl DL-aspartate caused a large depolarization which eliminated the light response and decreased the input resistance of the cell. In this and several following figures, a 0.1-nA negative current pulse was applied, and the bridge circuit was balanced prior to drug application. After drug application, a positive voltage deflection during the current pulse indicated a decrease in the input resistance of the cell. The time interval between the two parts of the *top trace* was 45 sec, the *left middle trace* was taken 5 min later, and the *right middle trace* was after another 3 min.

another ON-OFF amacrine cell that responds equally to small spot and annular illumination. Negative current pulses (0.1 nA) were applied in the dark, and the bridge was balanced. The positive deflection of the voltage trace during current application indicates that the kainic acid-induced depolarization is associated with a large decrease in the input resistance of the amacrine cell.

Ganglion cells. Ganglion cells are also sensitive to both *N*-methyl aspartate and kainic acid. The effect of *N*-methyl aspartate on several types of ganglion cells is illustrated in Figure 7. In general all but one type of spiking ganglion cell were depolarized by *N*-methyl aspartate. The ganglion cell at the *top* of Figure 7 shows a sustained ON response to small spot illumination but a transient ON response followed by a sustained ON hyperpolarization to an annulus. Five hundred micromolar *N*-methyl DL-aspartate caused a large depolarization with an almost total suppression of the light response. The neuron on the left side of the *middle trace* showed ON and OFF spikes and a slight sustained hyperpolarization to a small spot but a large sustained ON hyperpolarization with superimposed ON and OFF transient IPSPs to annular stimulation. In this cell also, *N*-methyl DL-aspartate caused a large depolarization, an initial burst of spiking, and a loss of the light response.

The cell shown at the right of the *second row* is a sustained OFF ganglion cell which produced similar voltage responses to both small spot and annular illumination. We have found several varieties of sustained OFF ganglion cells in the mudpuppy. The sustained ON hyperpolarization may be due to inhibition (Belgum et al., 1982) or disfacilitation (Dacheux et al., 1979) or a combination of these two mechanisms. The inhibitory synaptic input is derived from the ON channel and can be blocked by 2-amino-4-phosphonobutyrate (Slaughter and Miller, 1981a), whereas the disfacilitation is from the OFF channel and is insensitive to this agent. When both types of synaptic input converge on one sustained OFF ganglion cell, the use of 2-amino-4-phosphonobutyrate isolates the disfacilitation, which can now be inverted by positive current. As seen in Figure 7, the sustained OFF ganglion cells are the only type of spiking ganglion cell which is hyperpolarized by *N*-methyl DL-aspartate. The hyperpolarization is accompanied by a suppression of the light response. This action is associated with sustained OFF ganglion cells which receive inhibitory ON input. It has not yet been determined if this is true for those sustained OFF ganglion cells in which the ON hyperpolarization is purely disfacilitation.

The responses of the ON-OFF neuron at the *bottom* of

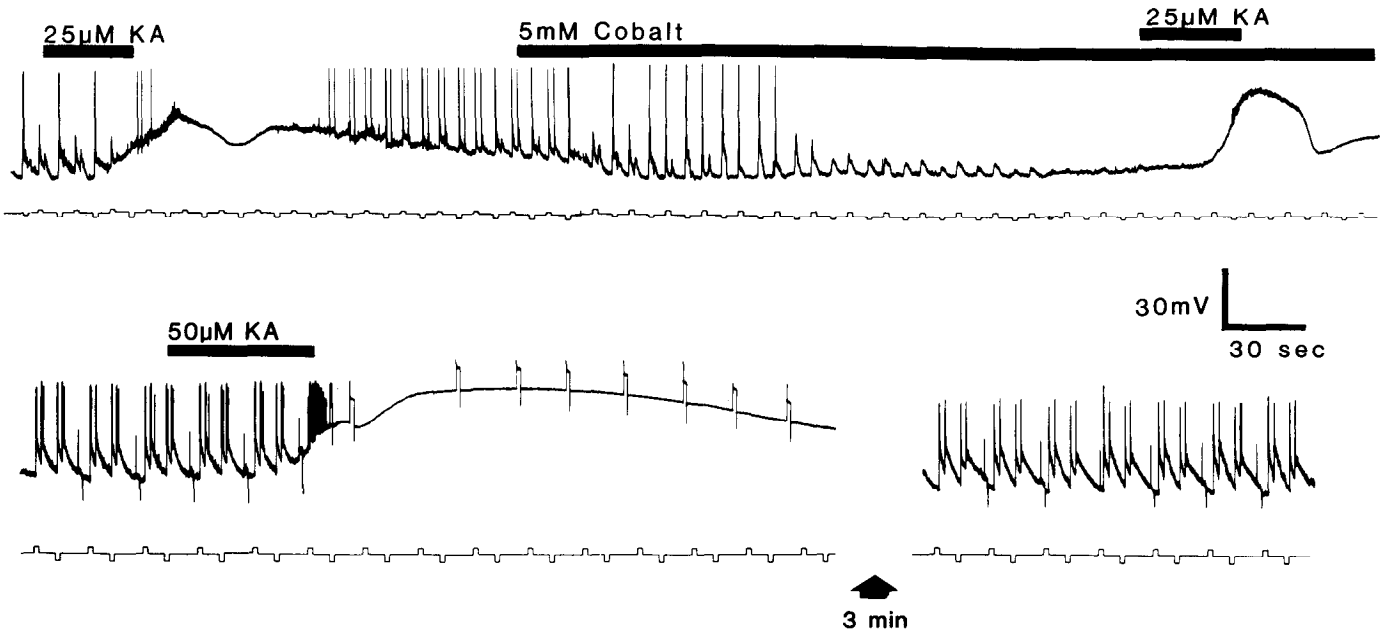

Figure 6. This illustrates that kainic acid causes a depolarization of amacrine cells which is associated with a resistance decrease and occurs when synaptic transmission is blocked by cobalt.

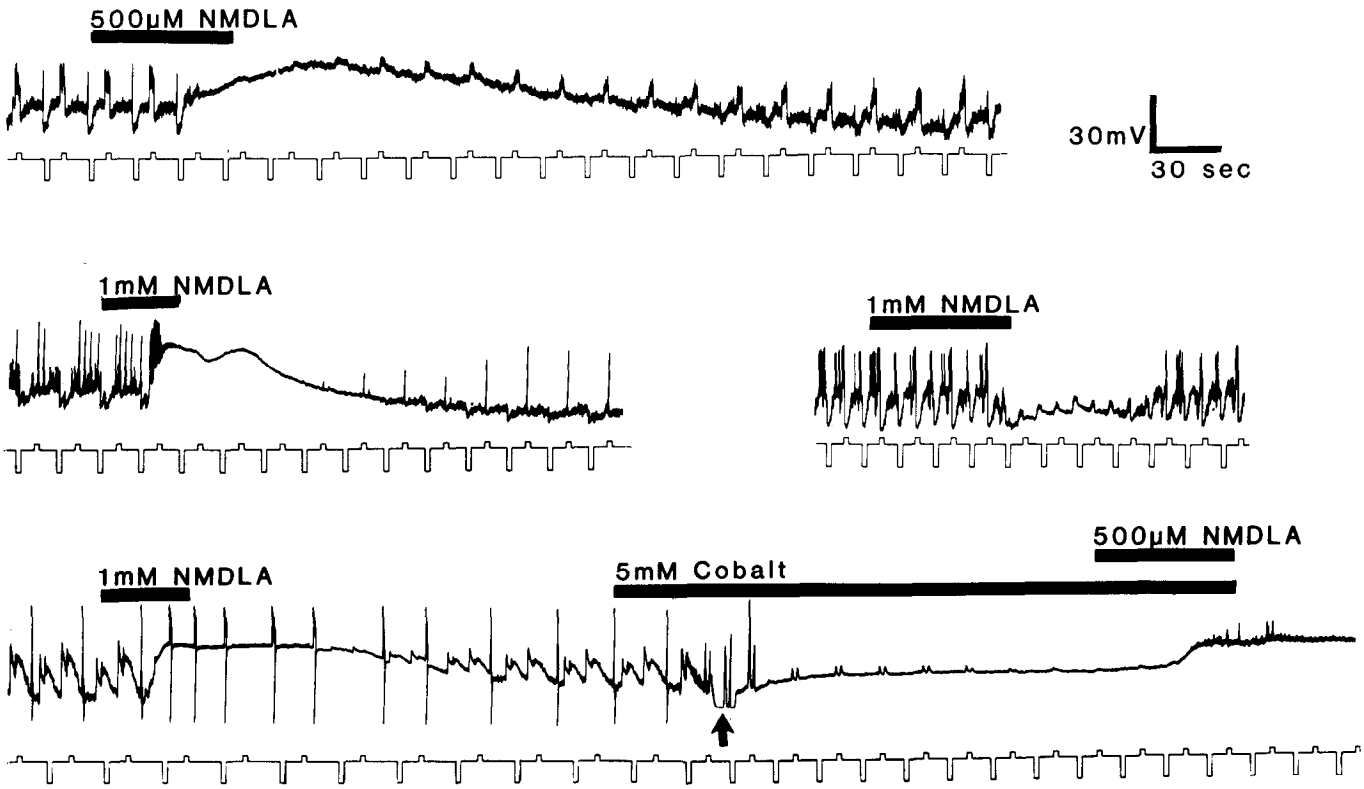

Figure 7. Responses of several types of ganglion cells to *N*-methyl DL-aspartate (detailed in the text), illustrating the effects on membrane voltage and light response. The *lower trace* demonstrates that the action of *N*-methyl DL-aspartate is accompanied by a resistance decrease and occurs in the presence of cobalt.

Figure 7 typify the conductance changes and the direct effect of *N*-methyl aspartate on ganglion cells. Initially, negative current pulses (0.1 nA) were applied in the dark, and the bridge was balanced. The administration of 1 mM *N*-methyl DL-aspartate caused a large depolarization and a decrease in the input resistance of the cell as shown by the positive voltage deflections during the current application. After removal of the *N*-methyl aspartate the cell recovered to the original dark membrane potential, the light response returned, and the current pulses indicated that the input resistance of the cell had returned to control levels. The current pulse was turned off, and

cobalt was added to block synaptic transmission. This resulted in a large hyperpolarization (the trace went off scale and had to be manually adjusted at the point indicated in the figure). After cobalt had blocked all synaptic input, 500 μM *N*-methyl DL-aspartate still produced a depolarization.

Kainic acid is also a potent excitor of ganglion cells. In the transient ganglion cell at the *top* of Figure 8, kainic acid caused a depolarization and a loss of the light response. After cobalt was applied to block synaptic transmission, the same dose of kainic acid produced an even larger depolarization, indicating the presence of endogenous kainic acid receptors on this neuron. This neuron appears to receive both excitatory and inhibitory input so that kainic acid not only excites this cell directly but it also excites inhibitory neurons which have synaptic input to this cell. When synaptic input is blocked by cobalt, the input resistance of the cell increases, and now kainic acid only activates the direct, excitatory receptors on this cell. These factors may account for the larger depolarization seen in the presence of cobalt. As with many amacrine cells, there is a hyperpolarizing notch near the peak of the kainic acid-induced depolarization of this ganglion cell, and it persists in the presence of cobalt.

The *middle* and *lower traces* of Figure 8 illustrate that the kainic acid depolarization of ganglion cells is accompanied by a decrease in the cell's input resistance. The neuron in the *middle trace* shows a transient ON spike followed by a sustained ON hyperpolarization and a sustained OFF spiking. Kainic acid caused a depolarization and a decrease in the input resistance as indicated by the positive deflection of a 0.1-nA negative current pulse. In the sustained ON ganglion cell in the *lower trace*, kainic acid produced a depolarization that exceeded the potential of the light-driven EPSP, and again this was associated with a decrease in input resistance. There is also a prominent hyperpolarizing notch at the peak of the kainic acid depolarization.

Action of N-methyl aspartate antagonists. Several antagonists are available that block the action of *N*-methyl aspartate. Three of these are α-amino adipate, α-amino suberate, and 2-amino-5-phosphonovalerate (Watkins et al., 1981). The latter also mimics the effect of 2-amino-4-phosphonobutyrate (although less potently) on the ON bipolar (Slaughter and Miller, 1981b); therefore, we used only the first two agents. As mentioned in the description of Figure 5, D-α-amino adipate was found to be an effective *N*-methyl aspartate antagonist. We found that α-amino suberate had a similar antagonist activity in the retina. Because of the widespread effectiveness of *N*-methyl aspartate in the inner retina, we tested the effect of α-amino adipate and α-amino suberate on light-evoked activity. Of the cells that we studied, these antagonists were only effective on the light responses of sustained OFF ganglion cells. For example, in the sustained OFF

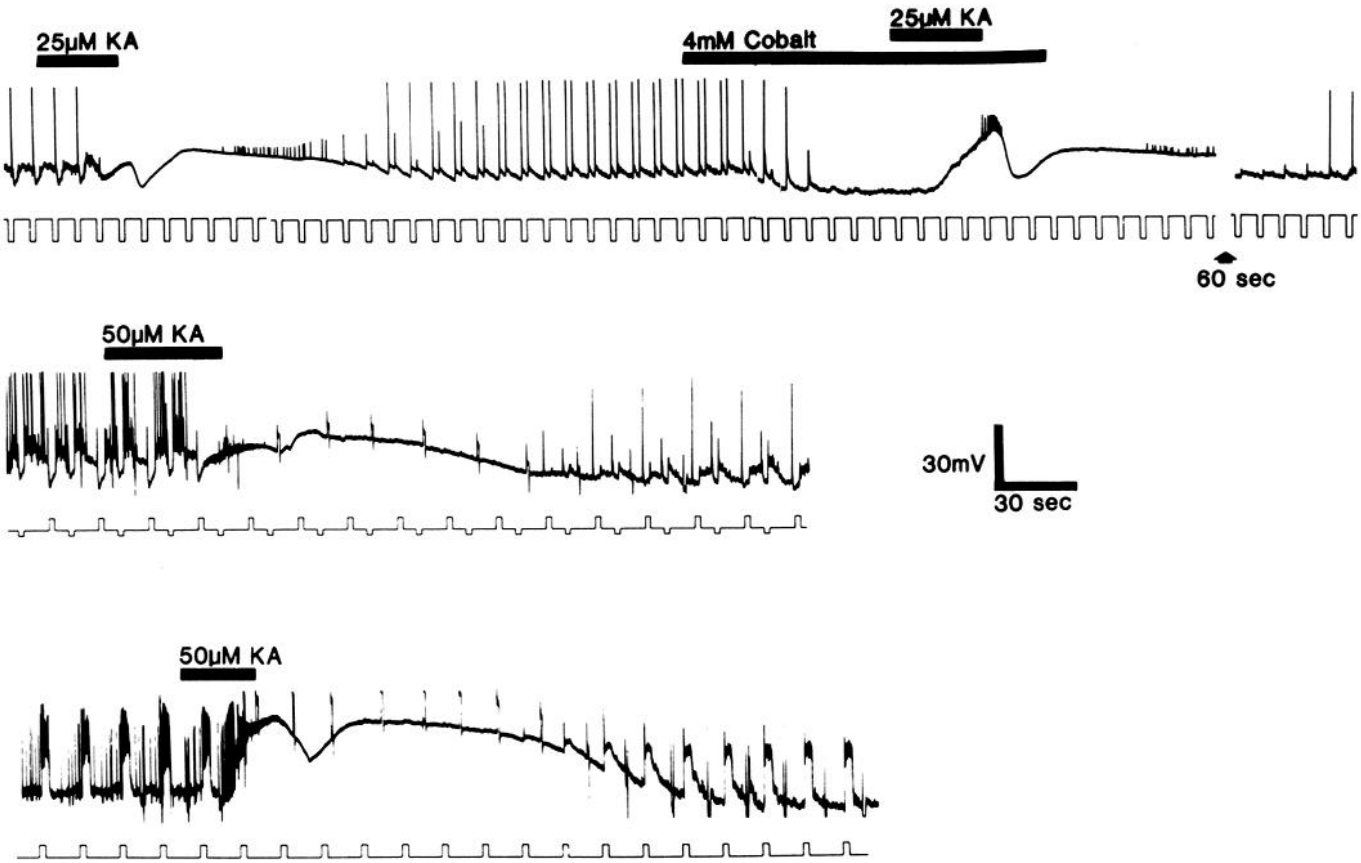

Figure 8. Kainic acid is shown to depolarize and block the light responses of several types of ganglion cells. The *top trace* shows that kainic acid is effective in this cell when synaptic transmission is blocked, whereas the *two lower traces* show that this depolarization is associated with a resistance decrease.

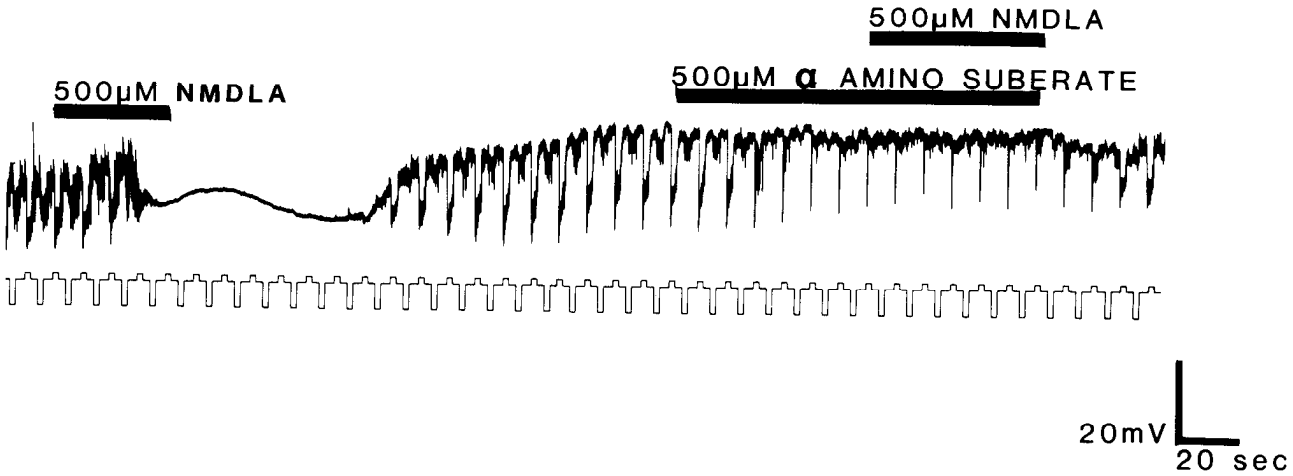

Figure 9. In this sustained OFF ganglion cell, α-amino suberate blocks the sustained hyperpolarizing component of the light response and it also blocks the hyperpolarization evoked by *N*-methyl DL-aspartate.

ganglion cell illustrated in Figure 9, *N*-methyl aspartate caused a hyperpolarization and a loss of the light response. After recovery, 500 μM α-amino suberate was applied, resulting in an almost complete suppression of the sustained portion of the light-induced hyperpolarization, leaving a transient ON component. When *N*-methyl aspartate was added in the presence of α-amino suberate, it had only a very slight hyperpolarizing action. Thus α-amino suberate blocked both the sustained light-evoked hyperpolarization and a similar hyperpolarization produced by *N*-methyl aspartate.

Other excitatory amino acids. A number of other glutamate analogues were tested, such as AMPA, quisqualate, and homocysteine sulfonate. They all produced effects that were similar to those of kainic acid on all classes of retinal neurons. AMPA and quisqualate were approximately as potent as kainic acid. We have not studied these agents as extensively as kainic acid and there may be subtle differences, but, in the context of this paper, they appear to be activating kainic acid-sensitive neurons as opposed to *N*-methyl aspartate-sensitive neurons. We have also tried dihydrokainic acid, which we found to be an ineffective agonist.

Using small amounts of *N*-methyl D-aspartate that we had available, we compared its effect with *N*-methyl DL-aspartate. We found no qualitative differences in the action of the racemic mixture and the D enantiomer. Magnesium ion has been reported to block the effect of *N*-methyl aspartate in some cases (Evans et al., 1977). However, Mg^{2+}-free Ringer's did not enhance the effect of *N*-methyl aspartate in our preparation. In the figures shown in this paper, *N*-methyl DL-aspartate was applied in concentrations of 500 μM to 2 mM, which was the range we most often used. However, we found that many neurons in the inner retina responded to 100 μM *N*-methyl DL-aspartate, and the figures reflect a sampling bias in this respect.

Discussion

In the mudpuppy retina it appears that most, if not all, postphotoreceptor neurons possess excitatory amino acid receptors. This evidence supports the notion that dicarboxylic amino acids play a predominant role in excitatory transmission in both plexiform layers. Despite doubts arising from the widespread action of these agents in the central nervous system, other evidence indicates that these amino acids are indeed likely transmitters used by photoreceptors, bipolars, and amacrine cells.

Support for aspartate and/or glutamate as the photoreceptor neurotransmitter has come from histological studies (Marc and Lam, 1981; Altschuler et al., 1982), biochemical release experiments (Miller et al., 1982; Miller and Schwartz, 1983), and physiological evidence (Murakami et al., 1972, 1975; Wu and Dowling, 1978; Ishida and Fain, 1981; Lasater and Dowling, 1982; Rowe and Ruddock, 1982). In the mudpuppy retina, we have found that one excitatory amino acid analogue, 2-amino-4-phosphonobutyrate, binds to the receptors of the ON bipolar (Slaughter and Miller, 1981a), whereas another analogue, *cis*-2, 3-piperidine dicarboxylic acid, acts at the horizontal cell and OFF bipolar receptors (Slaughter and Miller, 1983). These agents block the light responses of these neurons, indicating that they bind at the synaptic receptors normally activated by the photoreceptor transmitter. One object of our recent experiments was to determine whether aspartate or glutamate was the more likely transmitter candidate. Results at several sites in the nervous system indicate that *N*-methyl aspartate and its antagonists can be used to identify synapses at which aspartate is the likely transmitter (McCulloch et al., 1974; Watkins, 1982). This contention is supported by studies in the fish (Wu and Dowling, 1978) and the cat (Ikeda and Sheardown, 1982) retinas that have demonstrated the effectiveness of *N*-methyl aspartate antagonists at synapses that show a much greater sensitivity to aspartate than to glutamate. Thus, the ineffectiveness of *N*-methyl aspartate and its antagonists in the outer retina implies that aspartate is not the transmitter used by mudpuppy cones. Another possible role for aspartate in photoreceptor neurotransmission is as a co-transmitter which potentiates the effectiveness of the simultaneously released glutamate. Dual release has been proposed at the lobster neuromuscular junction where glutamate is thought to be the transmitter. At this synapse

both aspartate and glutamate are released (Shank and Freeman, 1975), and noise analysis indicates that aspartate potentiates the activation of the glutamate receptor (Crawford and McBurney, 1977). We have examined this possibility in the mudpuppy retina by using the same ratio of aspartate to glutamate (3:1) proposed at the lobster neuromuscular junction, but we did not find a potentiation of the glutamate response.

In contrast, the potent action of kainic acid on all three second-order neurons suggests that glutamate may be the photoreceptor transmitter. In support of this is our finding that 2-amino-4-phosphonobutyrate, a glutamate analogue, but not 2-amino-3-phosphonopropionate, an aspartate analogue, is a potent agonist at the ON bipolar synapse (Slaughter and Miller, 1981a). Also, while *N*-methyl aspartate antagonists are ineffective in the outer retina, *cis*-2,3-piperidine dicarboxylic acid blocks the synaptic input to the horizontal cells and the OFF bipolars and it also blocks the kainic acid-induced depolarization of these neurons (Slaughter and Miller, 1983). The evidence that both the photoreceptor transmitter and kainic acid were blocked by *cis*-2,3-piperidine dicarboxylic acid suggests that kainic acid acts at synaptic receptors in the outer retina. Thus, the action of both agonists and antagonists indicates that glutamate or its analogue is the cone transmitter in the mudpuppy retina. Physiological studies in the fish retina (Rowe and Ruddock, 1982) also indicate that glutamate is the neurotransmitter and that kainic acid acts synaptically (Shiells et al., 1981) and mimics the action of glutamate.

The observation that all second-order neurons do not have identical synaptic receptors for the photoreceptor transmitter (the possibility that photoreceptors release two or more dicarboxylic acids that interact at discrete receptors as opposed to one amino acid that has several active conformations does not alter this analysis of postsynaptic receptors) was first apparent when Wu and Dowling (1978) demonstrated that α-amino adipate selectively blocked the light response of L-type horizontal cells in carp. This concept has been extended by the use of 2-amino-4-phosphonobutyrate (Slaughter and Miller, 1981a) and *cis*-2,3-piperidine dicarboxylic acid (Slaughter and Miller, 1983) to demonstrate that the ON bipolar receptor is distinct from that on the horizontal cell or the OFF bipolar. In the present paper, the difference in sensitivity of the horizontal cell and the OFF bipolar to kainic acid and *N*-methyl aspartate hints that the receptors on these two neurons may also differ. McLennan (1981) has proposed that excitatory amino acid receptors can be characterized by evaluating the distance between the amino group and the terminal carboxyl group of active ligands. For glutamate this distance can range between approximately 2 and 5 Å, whereas for *N*-methyl aspartate the range is 2.5 to 4 Å. We have found that the synaptic receptors of the ON bipolar preferentially bind to agonists which match the extended conformation of glutamate, indicating that this receptor preferentially binds ligands with an α to γ carboxyl distance ≥ 4 Å (M. M. Slaughter and R. F. Miller, manuscript in preparation). The actions of *N*-methyl aspartate and kainic acid on the OFF bipolar raise the possibility that receptors on this neuron bind to glutamate when it is in a folded configuration. In this scheme, the horizontal cell would possess receptors that preferentially bind to a partially folded glutamate molecule.

In the inner retina the dichotomy between the actions of kainic acid and *N*-methyl aspartate is more distinct. Based on studies using cobalt to block synaptic transmission, many amacrine and ganglion cells have endogenous receptors for kainic acid and *N*-methyl aspartate. The *N*-methyl aspartate receptors can be distinguished pharmacologically, using α-amino adipate or α-amino suberate, from the kainic acid-sensitive receptors. *N*-Methyl aspartate has a potent action on amacrine and ganglion cells, but kainic acid is still more potent. This suggests that glutamate may be an important excitatory amino acid transmitter in the inner retina, perhaps the bipolar cell transmitter. We have found that ON bipolars may use an excitatory amino acid transmitter which can be blocked by *cis*-2,3-piperidine dicarboxylic acid (Slaughter and Miller, 1982). Kainic acid mimics the depolarizing action and the conductance increase produced by ON bipolar input to third-order neurons and to some extent mimics the pharmacology of this transmitter inasmuch as it is also blocked by *cis*-2,3-piperidine dicarboxylic acid. However, to date there has been no histochemical support for the presence of a dicarboxylic amino acid transmitter contained in bipolar cells. Radiolabeled uptake of D-aspartate has been detected in photoreceptors and some cells at the inner margin of the inner plexiform layer (presumably amacrine cells), but not among bipolar cells (Marc and Lam, 1981). Immunohistochemical labeling of aspartate aminotransferase shows a similar distribution (Altschuler et al., 1982). Nor does labeled D-glutamate show selective uptake into bipolars, although it does appear in photoreceptors (Marc and Lam, 1981). This negative evidence may be due to technical considerations, or it may signify that another excitatory amino acid with a similar pharmacology but a different uptake pathway and separate enzymatic machinery may be the actual transmitter. Cysteic and homocysteic acids are often mentioned as alternative acidic amino acid transmitters (Iwata et al., 1982), and we have found that both of these sulfonic amino acids are potent excitatory agents in the inner retina.

As mentioned above, an aspartate-releasing amacrine cell group has been proposed based on uptake and immunohistochemical studies. The action of *N*-methyl aspartate in the inner retina supports the idea that third-order neurons are rich in aspartate receptors. However, the selective action of α-amino adipate and α-amino suberate on the sustained OFF ganglion cells indicates a more restricted role for aspartate in the inner retina of the mudpuppy. The data suggest that the output of a sustained ON cell is blocked by these antagonists. Our results, combined with the histochemical localization of aspartate to some amacrine cells in the retina of other species, points to the existence of a sustained ON amacrine cell that utilizes aspartate as a neurotransmitter. The presumed aspartate amacrine may be postsynaptic to ON bipolars and have a direct inhibitory action on OFF ganglion cells. Alternatively, the putative aspartate amacrine may excite a conventional inhibitory amacrine (GABAergic or glycinergic) which then synapses on the OFF ganglion cell.

In summary, our present findings indicate that in the

mudpuppy retina: (*1*) cone photoreceptors release glutamate, and second-order neurons have distinct receptors that are related to the functional class of the neurons; (*2*) bipolar cells release an excitatory amino acid which may be glutamate or an analogue; and (*3*) there is a group of sustained ON amacrine cells which releases aspartate.

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