Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 1127–1131, February 1995
Neurobiology

Quinoxalines block the mechanism of directional selectivity in ganglion cells of the rabbit retina

(starburst/2,3-dihydroxy-6-nitro-7-sulfamoylbenzo-*f*-quinoxaline/2-amino-4-phosphonobutyric acid/excitatory amino acids/α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor)

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Communicated by Leo M. Hurvich, University of Pennsylvania, Philadelphia, PA, October 7, 1994

ABSTRACT **Direction selectivity is a receptive field property displayed by neurons throughout the visual system. Previous experiments have concentrated on the role of lateral connections that use γ-aminobutyric acid and acetylcholine. We have examined the role of excitatory amino acid receptors on direction-selective ganglion cell function in the rabbit retina. Application of the quinoxalines, a group of kainate/α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor antagonists, selectively blocked the directional-selectivity mechanism, leaving cells responsive to both directions of movement. In contrast, direction selectivity was unaffected by *N*-methyl-D-aspartate receptor antagonists or L-2-amino-4-phosphonobutyric acid. Large reductions in acetylcholine release by starburst amacrine cells appear to parallel losses of direction selectivity observed in the quinoxalines. These results shed additional insights into the mechanism of direction selectivity.**

Direction-selective ganglion cells (DSGCs) fire a series of action potentials to motion of a bar stimulus moved across their receptive field in one direction, termed the "preferred" direction, while movement in the opposite direction, or "null" direction, evokes little or no response. The rabbit retina contains two classes of DSGCs: a common On–Off center and a rarer On-center variety (1–3). Each class of direction-selective cell has a unique set of angles of directional preference across the retinal surface (3) and sends its axonal input to different areas in the thalamus (4–6). The direct physiological and anatomical bases for the direction-selective mechanism are not presently known.

Several studies have suggested that starburst amacrine cells are involved in direction selectivity (DS). The common On–Off class of DSGC in the rabbit retina has bistratified dendrites that arborize in both the On and Off sublaminae of the inner plexiform layer (IPL) (7) and is thought to receive extensive synaptic input from starburst amacrine cells (8–10). The starburst amacrine cells are a specialized group of cholinergic- and γ-aminobutyric acid (GABA)-containing neurons, which consist of two mirror symmetric cell populations whose dendrites arborize in either the On or Off sublamina of the IPL (11–14). While the dendrites of starburst amacrine cells appear to closely appose the dendrites of DSGCs, there is currently little evidence of any direct synaptic contact (15–17).

Both excitatory and inhibitory processes are thought to play a role in the DS mechanism (17–19), using either GABA or acetylcholine (20–22), while excitatory amino acids (EAAs) have been viewed as being only indirectly involved in the direction-selective process. Thus, the prevailing view is that the direction-selective mechanism probably resides in the inner retina and requires only EAA input from bipolar cells in order to operate. However, we have reported an unexpected finding: EAA receptors of the kainate/α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) class appear to be critical to the function of the direction-selective mechanism (23).

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EXPERIMENTAL PROCEDURES

DSGCs were recorded with tungsten-in-glass electrodes (24) and an ac amplifier. The extracellular spike time events and bar positions were recorded by a computer for offline peristimulus time histogram (PSTH) generation and analysis. DSGCs were isolated on a mesopic background of either 5.6 or 0.2 cd/m^2. Moving bars, 100 or 500 μm wide (typically 1–1.2 log units above background), were projected onto the receptive fields of DSGCs using a Maxwellian view optical system. Bar stimuli were controlled by stimulus generators driving electromagnetic shutters and moved with pen motors modulated by a function generator. Rabbit retina eyecups were prepared as described (25). Rabbits were anesthetized with either urethane and/or ketamine-xylazine by a university-approved protocol. The eye was removed and hemisected, and the posterior half containing the retina was placed over a Teflon dome and superfused with a heated (36–37°C) and oxygenated (95% O_2/5% CO_2) bicarbonate-buffered Ringer's solution containing the following salts: 120 mM NaCl, 3.1 mM KCl, 0.5 mM KH_2PO_4, 23.0 mM $NaHCO_3$, 1.2 mM Mg_2SO_4, 1.15 mM $CaCl_2$, 0.5% equine serum, and 26 vitamins and amino acids (26) at a rate of 5.5 ml/min. The chamber volume was 250 μl. Drugs were held in gassed wells and bath applied. The antagonists 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo-*f*-quinoxaline (NBQX) and 6,7-dinitroquinoxaline-2,3-dione (DNQX) were obtained as gifts from T. Honoré (Inst. Ferrosan A/S, Soeborg, Denmark); *N*-methyl-D-aspartate (NMDA), L-2-amino-4-phosphonobutyric acid (APB) and D-2-amino-7-phosphonoheptanoic acid (DAP7) were obtained from Tocris Neuramin (Langford, U.K.).

RESULTS

We studied the action of bath-applied EAA antagonists and agonists on the receptive fields of 38 DSGCs (mainly On–Off cells) by using a superfused rabbit retina eyecup preparation and extracellular recording (24). Fig. 1*A* shows the continuous raw spike record of an extracellularly recorded On–Off DSGC

Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; NMDA, *N*-methyl-D-aspartate; NBQX, 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo-*f*-quinoxaline; APB, L-2-amino-4-phosphonobutyric acid; DNQX, 6,7-dinitroquinoxaline-2,3-dione; DAP7, D-2-amino-4-phosphonoheptanoic acid; DS, direction selectivity; DSGC, direction-selective ganglion cell; LE, leading edge; TE, trailing edge; DHBE, dihydro-β-erythroidine; PSTH, peristimulus time histogram; IPL, inner plexiform layer; GABA, γ-aminobutyric acid; EAA, excitatory amino acid.

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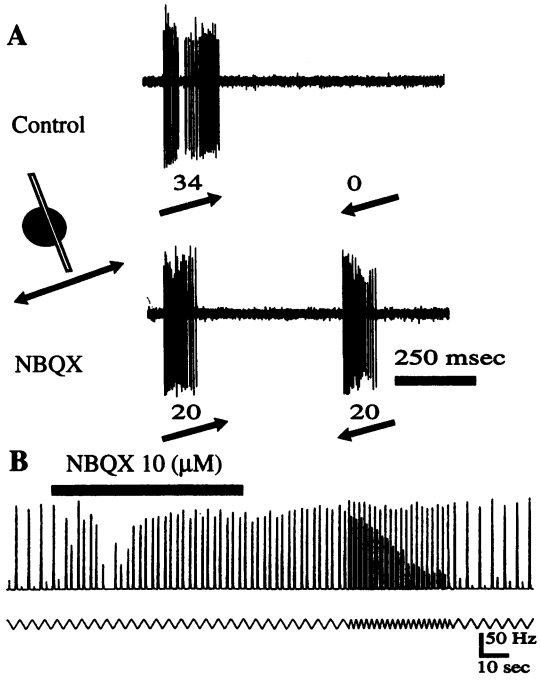

FIG. 1. Effect of the EAA antagonist NBQX on DS. (*A*) Raw extracellular spike discharge of an On–Off DSGC to an oscillating bar stimulus moving across the receptive field (bar velocity, 18°/sec.) (*Upper*) Control response. Cell responds in the preferred direction of bar motion (right arrows) but not when movement reverses (null direction, left arrows). (*Lower*) In 10 μM NBQX, DSGC now responds to bar movement in both directions. (*B*) Rate meter record (0.1-sec time constant) of another DSGC in the presence of 10 μM NBQX. Lower trace indicates bar position across DSGS's receptive field. DS is rapidly lost, and a loss–recovery sequence is seen for bar responses (27). Upon NBQX washout, DS recovers (recovery time base compressed).

in response to a thin (100 μm wide) bar of light moved back and forth across the receptive field. In the control condition (Fig. 1*A Upper*), the DSGC responded with a burst of spikes to bar movement in the preferred direction (right arrows) but elicited no response to bar movement in the null direction (left arrows). When the quinoxaline antagonist NBQX (10 μM) was added to the bath, DS was abolished (Fig. 1*A Lower*), as indicated by spiking in both directions (27). Fig. 1*B* shows a rate meter record of the discharge pattern of another On–Off DSGC to 10 μM NBQX. Note that NBQX caused a loss of DS, as seen by impulse doubling for preferred and null movement, followed by a transient loss of light-evoked activity, after which a steady state condition was maintained in which DS was eliminated (28). Similar results were seen on 34/35 cells tested with NBQX.

Unlike other agents reported to block DS, such as picrotoxin or physostigmine, NBQX did not increase the spontaneous discharge rate of DSGCs (33/35 cells) (20–22). As little as 1 μM NBQX was capable of blocking DS (12/13 cells), although in some cells the loss of DS was incomplete (see Figs. 2 and 3*A*). This dose had little effect in the outer retina.§ Examination of 3 On-center type DSGCs revealed their DS was also blocked by 10 μM NBQX.

§Intracellular recordings from horizontal cells revealed that 1 μM NBQX had little effect on their light-evoked responses or dark membrane potential, while 10 μM NBQX or more was capable of blocking their light-evoked responses. Similar results were seen with combinations of NBQX and 200 μM DAP7 on the light-evoked responses of three ganglion cells tested (unpublished observations). A dose of 10 μM NBQX was chosen for most studies in order to try to completely block retinal kainate/AMPA EAA receptor function.

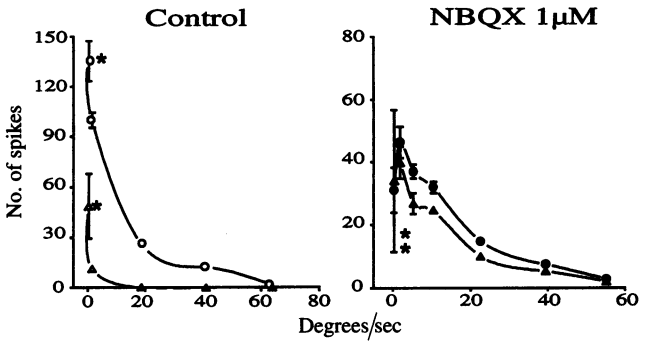

FIG. 2. Examination of the loss of DS on bar stimulus velocity. Circles, number of spikes per bar passage in the preferred direction; triangles, number of spikes per bar passage in the null direction. Each point is the average ± SD of four presentations, except the lowest stimulus velocity (*) (two presentations). In the presence of NBQX, responses in the null direction increased nearly to control levels for all stimulus velocities tested.

Some agents that block DS, such as the GABA antagonist picrotoxin, can dramatically increase the maximum stimulus velocities to which DS cells respond (21, 22). The effects of NBQX (1 μM) were examined at different bar stimulus velocities (n = 3 On–Off cells; Fig. 2). In the presence of NBQX, responses were nearly equal for both preferred and

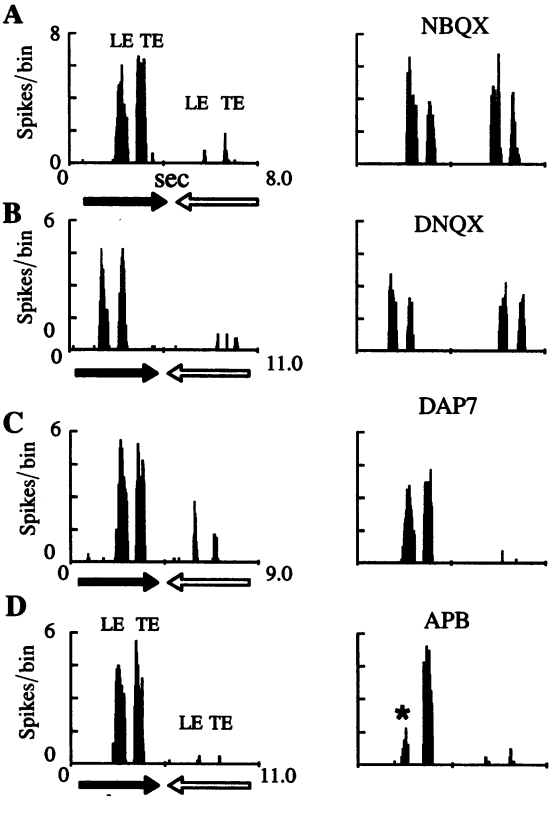

FIG. 3. PSTHs of the responses of On–Off DSGCs to EAA agonists and antagonists (20-msec bin width, five-presentation average). Solid arrows, preferred direction bar movement, open arrows, null direction movement. (*Left*) Control responses. (*Right*) Effect of drugs. (*A*) NBQX (1 μM) abolishes DS, and both the LE and TE responses of the bar stimulus persist. (*B*) DNQX also caused a loss of DS, but the LE and TE light-evoked responses are more suppressed. (*C*) The NMDA antagonist DAP7 had no effect on DS, although bar responses were depressed. (*D*) Application of APB (50–100 μM) reduced the LE response (*), but DS persisted. All cells showed recovery from drug application.

null directions over virtually the entire range of stimulus velocities tested (0.3–55°/sec). However, at the slowest stimulus velocity tested, the total number of impulses per stimulus was reduced when compared to the control (Fig. 2 *Right*). Thus, NBQX and picrotoxin have different effects on DSGCs. This was also true when the drugs were directly compared on the same DSGC (n = 3 cells) (10 μM NBQX; 20 μM picrotoxin).

We studied the action of three different EAA receptor antagonists on the DS of On–Off DSGCs (Table 1). The ratio of the null to preferred direction responses and the total number of spikes were compared in the control condition and during drug administration. In NBQX, the ratio of the null to preferred direction responses increased 10-fold and averaged 100%, indicating a symmetrical response. Preferred responses in NBQX were only slightly less than control values, averaging 86% ± 34% (mean ± SD; n = 10). The quinoxaline antagonist DNQX at 10 μM caused a similar loss of DS (Table 1; Fig. 3*B*) (29). However, in comparison to NBQX, DNQX was less effective at blocking DS and exerted a more suppressive effect on the light-evoked responses of DSGCs. Part of this suppression may be due to DNQX's reported antagonism of the glycine binding site on the *N*-methyl-D-aspartate (NMDA) receptor (unlike NBQX) (30, 31). For comparison, we tested the effects of an NMDA antagonist, DAP7 (200–250 μM), on DS (7 On–Off; 1 On-center DSGCs). Consistent with previous published results (32), DAP7 did not abolish DS (8/8 cells); however, DAP7 reduced cell firing in both directions.

The receptive field centers of On–Off DSGCs are spatially discrete, extending over an area only slightly larger than their dendritic field (33). The dendrites of DSGCs arborize in both sublaminae of the IPL and thus receive input from both On- and Off-center cone bipolar cells (7). The responses of On–Off DSGCs to moving bars are composed of responses to both the leading edge (LE) and the trailing edge (TE) of the bar that reflect synaptic inputs from On and Off bipolar cells. These responses can be temporally separated by using wide bar stimuli. Fig. 3 shows PSTHs of the average response of these cells to 500-μm bars moving in the preferred and null directions. The effects of the three EAA antagonists and one agonist were tested on the LE and TE responses of DSGCs in the control condition (Fig. 3 *Left*), in the presence of the drug (Fig. 3 *Right*). The LE and TE responses to the bar persisted in both the preferred and null directions in 1 μM NBQX (n = 9) and also 10 μM NBQX (n = 9) (Figs. 3*A* and 4*D*). The effect of DNQX (10 μM) was similar, although light-evoked responses were more reduced (n = 6) (Fig. 3*B*). Fig. 3*C* shows PSTHs of the response of an On–Off DSGC to DAP7 (200 μM) during passage of the wide bar. In the presence of DAP7, the directional preference of the cell was maintained and both the LE and TE responses to the bar remained (32).

We tested the effect of blocking On bipolar cell input to DSGCs by applying the metabotropic agonist APB (50–100 μM) (n = 14) (34). APB blocks acetylcholine release from starburst amacrine cells arborizing in the On sublamina of the IPL (35, 36). The effects of APB were examined on 12 On–Off DSGCs using thin or wide bar stimuli. Wide bars revealed that the LE responses were strongly reduced in the presence of APB to only 24% ± 11% (n = 6; mean ± SD) of control values, yet DS nonetheless remained on all cells. In contrast, in two cases in which On-center DSGCs were tested, their bar responses were completely eliminated. Thus, there appear to be two separate DS mechanisms that subserve On and Off inputs to DSGCs.

Table 1. Effects of EAA agonists and antagonists on DS of On–Off DSGCs

Drug (μM)	Spike response ratio, mean ± SD: Control, null/preferred	Drug, null/preferred	Total spikes, drug/control	n
NBQX (10)	0.10 ± 0.11	1.00 ± 0.21*	1.63 ± 0.96	10
DNQX (10)	0.24 ± 0.20	0.73 ± 0.20†	1.23 ± 0.80	5
DAP7 (200–250)	0.12 ± 0.10	0.01 ± 0.02	0.58 ± 0.14	7
APB (50–100)	0.09 ± 0.15	0.14 ± 0.21	0.86 ± 0.42	6

*$P \leq 0.0001$.
†$P \leq 0.05$.

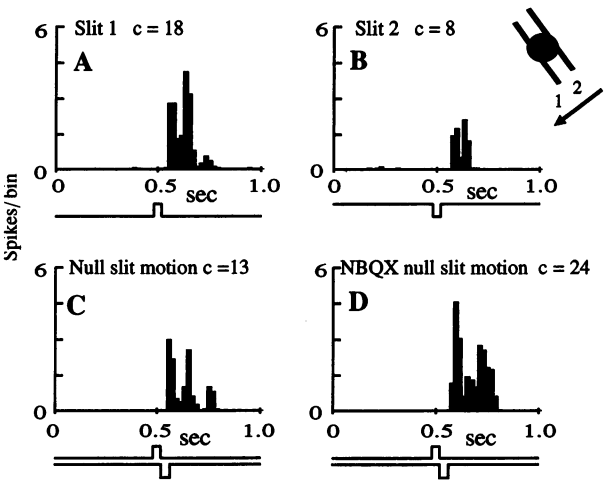

FIG. 4. Apparent motion DSGC experiments. Two stationary bars (100 μm wide) were positioned along the preferred–null axis (arrow) at opposite ends of the receptive field center (see diagram). Bars were individually flashed for 50 msec (*A* and *B*) or sequentially (50-msec delay), stimulating apparent bar motion (*C* and *D*), and the average response (c=) from 15 stimulus presentations is illustrated as PSTHs (20-msec bin width). Bar timing is indicated by lower lines. (*A* and *B*) Individual bar responses in the control condition. (*C*) Null direction apparent motion in the control condition. Number of impulses was reduced 52% from the sum of *A* and *B*. (*D*) Null direction apparent motion in NBQX (10 μM). Response increased 85% from null motion control. Bar stimulus times were taken from ref. 19.

Since the light-evoked responses of DSGCs remained in the presence of quinoxalines during the loss of DS, it was likely that these light-evoked responses were transmitted to third-order neurons through other EAA pathways. When the NMDA antagonist DAP7 (200 μM) was applied in combination with NBQX (10–20 μM), the firing of DSGCs to moving bars was either totally abolished (n = 6) or a few, 1–3, spikes intermittently remained (n = 4) and for all cells averaged only 0.4% ± 1.4% of the control response. Thus, in the presence of NBQX, a major portion of the light-evoked responses of On–Off DSGCs is mediated through NMDA receptors.

One theory of DS proposes that a bar stimulus moving in the null direction activates a feedforward delayed inhibition. This inhibitory mechanism has been described as sustained or time delayed and is thought to extend over a larger area than the receptive field center of the DSGC (18, 19, 36). We tested whether the effects of NBQX blocked this delayed spatial inhibition on 4 On–Off DSGCs by using two-bar apparent motion experiments (Fig. 4). In the control condition, PSTHs were generated for flashes of two stationary bars individually (Fig. 4 *A* and *B*), positioned on opposite sides of the receptive field center along the preferred–null axis. In control conditions, when the stimuli were sequentially presented for null direction motion (Fig. 4*C*), responses averaged 48% ± 18% (mean ± SD) less than the sum of the two bars presented individually. In the presence of NBQX (Fig. 4*D*), null motion sequence responses increased an average of 104% ± 39% over the null control (mean ± SD). This suggests that NBQX removes a spatially extensive inhibitory process, which is critical for sustaining the null direction inhibition in DSGCs.

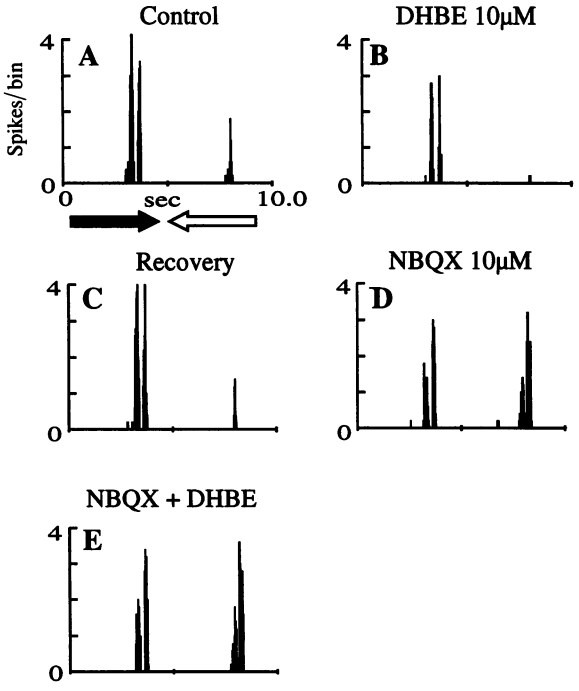

FIG. 5. PSTHs of effects of application of the nicotinic antagonist DHBE (10 μM) on the light-evoked responses of DSGCs in the presence and absence of NBQX (conventions as in Fig. 3). (*A*) Control response. (*B*) Bar response of this cell was strongly reduced (38% of control) by DHBE, but DS persisted. (*C*) Recovery 14 min later. (*D*) Application of NBQX caused a loss of DS. Addition of DHBE to NBQX did not reduce the ganglion cell's bar responses (113% of NBQX control). Wide bar speed, 7°/sec.

Although an increase in excitation could also account for the increase in the null motion sequence response observed in NBQX, no increases in the spontaneous firing of DSGCs were observed in NBQX.

DSGCs have been reported to be highly sensitive to acetylcholine and nicotinic antagonists (37). Nicotinic acetylcholine receptors have been reported on isolated ganglion cells in many species (38–40). Since starburst amacrine cells are the only cells in the retina that contain the neurotransmitter acetylcholine, we decided to test whether acetylcholine antagonists had any effect on DSGCs in the presence of 10 μM NBQX when DS was lost. Three different nicotinic antagonists [dihydro-β-erythroidine (DHBE), hexamethonium, and mecamylamine] were tested with similar results. Fig. 5 shows a PSTH of an On–Off DSGC in the control condition and in the presence of 10 μM DHBE (Fig. 5 *A* and *B*). In 10 μM DHBE, the bar response was reduced to 38% of the control bar response and, for seven cells, averaged 33% ± 26% of control values (mean ± SD). After recovery, NBQX was applied and DS was lost (Fig. 5*D*). In the presence of NBQX, DHBE was now reapplied and surprisingly no reduction was seen. Cells in DHBE averaged 118% ± 30% of the control response in NBQX ($P < 0.005$) (Fig. 5*E*). Similar results were seen on four cells when 100 μM hexamethonium was used (hexamethonium alone, 61% ± 18%; NBQX + hexamethonium, 105% ± 19%; mean ± SD of respective controls). Combinations of the potent noncompetitive antagonist mecamylamine (20 μM) and NBQX also did not depress bar responses of four cells tested (102% ± 12%; mean ± SD), while, on separate cells, mecamylamine alone caused a strong reduction (32% ± 22%; $n = 3$). Thus, the inhibitory actions of nicotinic antagonists are alleviated when DSGCs lose their DS in NBQX.

DISCUSSION

EAAs have revealed some insights into the mechanism of DS. The kainate/AMPA antagonists NBQX and DNQX both abolished the DS of all On–Off DSGCs and also of the few On-center DSGCs recorded. Thus, it appears that kainate/AMPA receptors appear to be critical to the normal functioning of the direction-selective mechanisms in DSGCs. In contrast, NMDA receptor antagonists had no effects on directional preference and only reduced the magnitudes of bar responses, similar to previous reports (32). The loss of DS by quinoxalines occurred at all bar stimulus speeds tested and displayed different properties from the previous GABAergic and cholinergic drugs reported to abolish DS in the rabbit (20, 21). Application of the EAA agonist APB (50–100 μM), which blocked the function of On bipolar cells, had no effect on DS, other than reducing the size of the LE bar response (34). The apparent lack of a loss of DS in APB suggests that DS for On–Off DSGCs may be composed of two independent direction-selective mechanisms operating in the On and Off sublaminae of the IPL.

Starburst amacrine cell function also appears to be blocked or strongly reduced in the presence of the kainate/AMPA antagonist NBQX. The only retinal neurons known to contain acetylcholine are starburst amacrine cells. The lack of depressant effects by nicotinic antagonists on DSGCs in the presence of NBQX implies that, in NBQX, the light-evoked depolarizing acetylcholine release by starburst amacrine cells onto DSGCs (and other retinal neurons) is markedly reduced. In the intact rabbit eye, the quinoxaline DNQX has been reported to block the light-evoked release of [^{3}H]acetylcholine under physiological concentrations of Mg^{2+}, while under these conditions NMDA antagonists had no effect (41). This implies that one action NBQX has in abolishing DS is to block the light-evoked responses of starburst amacrine cells. Yet, similar to previous reports, we found that nicotinic antagonists did not block DS (Fig. 5*B*; ref. 10). Since starburst amacrine cells contain the inhibitory neurotransmitter GABA in addition to acetylcholine, NBQX is likely to also block co-release of GABA (12, 13), as picrotoxin has been reported to block DS (9). This could correlate with the inhibitory process removed in apparent motion experiments by NBQX (see Fig. 4). In amacrine cells of the tiger salamander retina, the light-evoked EAA input to some cells is totally blocked by quinoxalines (42), while the light-evoked responses of others remain. Thus, this study supports a role for starburst amacrine cells in the direction-selective process. However, it is important to realize that the actions of a kainate/AMPA antagonist such as NBQX are likely to occur at several levels in the retinal neurocircuitry. Thus, in the future, it will be critical to find out what type of light-evoked responses starburst amacrine cells and other retinal neurons possess when DS is lost in NBQX.

We thank Dr. T. Honoré for donating NBQX, N. Daw for editorial comments, and M. Sikora for programming assistance. This work was supported by National Institutes of Health Grants EY10617 and EY03014 to E.D.C. and R.F.M., and the RPB (Research to Prevent Blindness) Foundation.

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