GABA Transporters Regulate Inhibition in the Retina by Limiting $GABA_C$ Receptor Activation

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Inhibition is mediated by two classes of ionotropic receptors in the retina, $GABA_A$ and $GABA_C$ receptors. We used the GABA transport blocker NO-711 to examine the role of GABA transporters in shaping synaptic responses mediated by these two receptors in the salamander retinal slice preparation. Focal applications (puffs) of GABA onto $GABA_C$ receptors on bipolar cells terminals or $GABA_A$ receptors on ganglion cells elicited currents that were enhanced by NO-711, demonstrating the presence of transporters in the inner plexiform layer (IPL). IPSCs were evoked in bipolar and ganglion cells by puffing kainate into the IPL. NO-711 enhanced the IPSCs only in bipolar cells, suggesting that, when GABA uptake was blocked, the $GABA_C$ receptors were more strongly activated by spillover transmission than the $GABA_A$ receptors on ganglion cells. NO-711 enhanced the light-evoked IPSCs mediated by $GABA_C$ receptors on bipolar cell axon terminals, which resulted in reduced transmission between bipolar and ganglion cells. NO-711 also shifted the intensity–response relationship of the ganglion cell, reducing its sensitivity to light. Surround illumination has been shown by others to produce similar shifts in ganglion cell light sensitivity. Our results show that GABA transporters limit the extent of inhibitory transmission at the inner retina during light-evoked signal processing.

Key words: retina; GABA; GABA transporter; spillover; surround inhibition; $GABA_C$ receptor; NO-711; patch clamp

The shape of inhibitory signals in the CNS is determined by the time course of transmitter release, the properties of the postsynaptic receptors, and clearance of transmitter from the synapse. GABA, the main inhibitory transmitter in the CNS, activates two types of ionotropic receptors, $GABA_A$ and $GABA_C$. These two types of GABA receptors are found in high abundance in the inner plexiform layer (IPL) of the retina in which they exhibit distinct functional properties and cellular distributions. $GABA_C$ receptors, which have a higher affinity for GABA, are located on bipolar cell axon terminals, whereas $GABA_A$ receptors, which have a lower affinity for GABA, are located postsynaptic to the bipolar cell, on amacrine and ganglion cell dendrites, and on bipolar cell terminals. Signaling mediated by each receptor subtype is distinct: $GABA_C$ receptors mediate sustained GABAergic currents, whereas $GABA_A$ receptors mediate transient currents (Lukasiewicz and Shields, 1998). The mechanisms that shape these distinct synaptic responses are not fully understood. Because $GABA_C$ receptors have been implicated in mediating surround inhibition and the formation of transient responses in the IPL, a more complete knowledge of factors that fashion these signals will be valuable.

In the CNS, higher-affinity receptors may be activated, which are distant from the transmitter release sites. This type of synaptic signaling has been called spillover or diffuse transmission in contrast to conventional point-to-point synaptic transmission. Inhibitory spillover transmission has been demonstrated in hippocampus (Isaacson et al., 1993) and cerebellar cortex (Rossi and Hamann, 1998; Mitchell and Silver, 2000), whereas excitatory spillover transmission has been demonstrated in hippocampus (Rusakov and Kullmann, 1998) and retina (Matsui et al., 1998).

Activation of GABA and glutamate receptors by spillover is often limited by the activity of transporters (Isaacson, 2000). Blockade of GABA or glutamate transporters results in enhanced receptor activation, an effect attributed primarily to spillover. We determined whether uptake limited synaptic signaling by $GABA_A$ and $GABA_C$ receptors by blocking the GABA transporter 1 (GAT-1), which is found predominantly in the inner retina (Johnson et al., 1996; Yang et al., 1997; Ekstrom and Anzelius, 1998). Using the selective GAT-1 blocker NO-711, we showed that blockade of GABA uptake resulted in increased activation of $GABA_C$ receptors, which reduced the light-elicited signaling from bipolar cells to ganglion cells. Because $GABA_C$ receptors at release sites were probably saturated, the enhanced response was most likely attributable to spillover activation of additional receptors. The suppression of light-evoked, bipolar cell to ganglion cell transmission by NO-711 shifted the ganglion cell sensitivity curve to the right, similar to the reported effect of surround inhibition (Sakmann and Creutzfeldt, 1969; Thibos and Werblin, 1978). These data suggest that $GABA_C$ receptors on bipolar cell terminals influence the synaptic transfer of light-evoked information to ganglion cells and that GABA transporters limit inhibitory signaling in the IPL.

MATERIALS AND METHODS

Slice preparation. Larval tiger salamanders were obtained from Charles Sullivan (Nashville, TN) and were kept in aquaria at 5°C on a 12 hr light/dark cycle. Retinal slices were prepared as described by Lukasiewicz et al. (1994). Under a dissection microscope, the retina was removed from an eyecup, placed on a 0.45 μm pore membrane filter

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(Millipore, Bedford, MA) with the vitreal side down, and sliced at 200–300 μm intervals. Each slice was transferred to a recording chamber and viewed through an upright, fixed-stage microscope (Eclipse E-600-FN; Nikon, Tokyo, Japan) equipped with a 40× water-immersion lens and Hoffman modulation contrast optics. For light stimulation experiments, dissection and recording procedures were done in infrared (IR) light using IR viewers. Using a gravity-fed perfusion system, the slice was continually superfused with a Ringer's solution containing (in mM): 112 NaCl, 2 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 5 glucose, and 5 HEPES, adjusted to pH 7.8 with NaOH.

Whole-cell recording. Electrodes were pulled from borosilicate glass (IB150F-4; World Precision Instruments, Sarasota, FL) with a P-97 Flaming/Brown micropipette puller (Sutter Instruments, Novato, CA). Whole-cell recordings were made from ganglion cells or amacrine cells by using 5 MΩ pipettes containing (in mM) 95.25 Cs-gluconate, 8 TEA-Cl, 0.4 $MgCl_2$, 1 EGTA, and 10 Na-HEPES 10, adjusted to pH 7.5 with HCl, or from bipolar cells with electrodes containing (in mM) 60 NaH_2PO_4, 10 NaCl, 10 EGTA, 10 HEPES, 1 $MgCl_2$, 2 Mg-ATP, 0.1 NaGTP, and 1 cGMP, adjusted to pH 7.4 with KOH (Nawy and Jahr, 1991). Lucifer yellow (0.1%) was added to intracellular solutions to identify cell types by visualization of their morphology after electrophysiological recordings. Membrane potentials were corrected for junction potentials (−14.9 mV for ganglion cell and amacrine cell solution; −10 mV for bipolar cell solution).

The voltage–clamp recordings were made with a 3900A Integrating Patch Clamp (Dagan, Minneapolis, MN). Data were digitized and stored with a Pentium personal computer (P5–90; Gateway, San Diego, CA) using TL-1 data acquisition system (Axon Instruments, Foster City, CA). Patchit software (White Perch Software, Somerville, MA) was used to generate voltage command outputs, acquire data, trigger the puffer, and control the drug perfusion system. Data were filtered at 1 kHz with the four-pole Bessel low-pass filter on the 3900A and were sampled at 500–10,000 Hz.

Drugs. A large area of the slice was superfused with control and drug solutions through a large-diameter pipette connected to a gravity perfusion system. During all experiments, glycine receptors were blocked with strychnine (5 μM). D-2-Amino-5-phosphonopentanoic acid (D-AP-5) and 3-aminopropyl(methyl)phosphinic acid (3-APMPA) were obtained from Precision Biochemicals (Vancouver, British Columbia, Canada). SKF89976A was obtained from Tocris Cookson (Ballwin, MO). All other chemicals were obtained from Sigma (St. Louis, MO).

Focal drug application. GABA (200–300 μM) or kainate (0.5 or 1 mM) was puffed onto the surface of the slice from a micropipette (~5 MΩ) connected to Picospritzer II (General Valve, Fairfield, NJ). GABA was puffed for 30 msec onto either dendrites or axons of bipolar and ganglion cells. Kainate was applied focally for 10–30 msec in the IPL 200–300 μm lateral from the recorded cell.

Light stimulation. For light response experiments, we used a red light-emitting diode (LED) (LN21RCPHL; Panasonic, Tokyo, Japan) mounted 5 cm from the retinal slice preparation to stimulate with full-field illumination. Different intensities of light were applied by using the computer to control the current through the LED. The maximum emission of the LED was 1.7×10^9 photons $\cdot \mu m^{-2} \cdot sec^{-1}$ at 700 nm (0 log unit attenuation), measured by using a Tektronix (Beaverton, OR) J16-TV Photometer. We stimulated cells with various intensities of light (−4 to 0 log units attenuated) presented in random order. A 5 sec light stimulus was presented every 30–60 sec.

For some experiments (see Figs. 3*A*, 4*A*), the light source for stimulation was a tungsten–halogen lamp (20 W; Ealing Electro-Optics, Holliston, MA). Spot white light stimuli (110 μm) were used. The intensity of the unattenuated light stimulus was equivalent to 3.6×10^8 photons $\cdot \mu m^{-2} \cdot sec^{-1}$ of a monochromatic light of 500 nm. We stimulated cells with the light attenuated by 2.5–6.0 log units using neutral density filters. Similar results were obtained with full-field LED illumination.

Analysis. For most of the experiments, peak amplitudes and charge transfers were measured by using Tack software (White Perch Software. D_{37} is the time of the peak current to the time at which the current reached 37% of peak amplitude. Data were normalized to control values. Spontaneous currents were analyzed using MiniAnalysis (Synaptosoft, Leonia, NJ). The threshold for event detection was 4 pA to minimize false-positive event detection. Measurements were taken from 48–1800 well separated events per cell. Events were considered well separated if the preceding and following interval events were >40 msec.

For the light intensity–response curve, responses were normalized to the maximal response in control solution, which was the peak amplitude of the EPSCs in response to the unattenuated LED light stimulus. Then normalized data were plotted versus the Log_{10} attenuation of the stimulus luminance (L). The values were fitted using the following sigmoid function: $Y = a/(1 + e^{-(X - L_{50})/b})$, where a is the maximum response, b is the difference in light intensity between 25 and 75% of the maximal response, and L_{50} is the Log_{10} of the light intensity at half-maximum response. The luminance evoking a half-maximal response (L_{50}) and the width of the dynamic range, which is the difference in light intensity between 10 and 90% of the maximal response (L_{10-90}) (Euler and Masland, 2000), were determined from the fit. The Student's t test was used to determine whether or not a parameter was significantly different between two groups of cells. In the text, values are presented as mean ± SE, and differences were considered significant if $p < 0.05$.

RESULTS

A GABA uptake inhibitor enhanced GABA-evoked currents in the IPL

To determine whether GABA transporters play a functional role in the IPL, we measured GABA-evoked currents in ganglion and bipolar cells under control conditions and in the presence of the GAT-1 transporter blocker NO-711. The control solution in all experiments contained strychnine (5 μM) to isolate the GABA inputs. At a holding potential of 0 mV, focal application (puff) of GABA (200–300 μM) onto ganglion cell dendrites elicited an outward current (Fig. 1*A*, control), which was abolished by the $GABA_A$ antagonist bicuculline (150 μM) (data not shown), in agreement with previous studies (Lukasiewicz and Shields, 1998). The addition of NO-711 (3 μM) enhanced and prolonged the $GABA_A$ receptor-mediated currents (Fig. 1*A*, NO-711), increasing the charge transfer of responses (196 ± 24%; $n = 11$) (Fig. 1*D*, *left*) and prolonging the decay (D_{37} of 156 ± 13%). The enhancement was reversed after switching back to normal solution. Similar results were obtained with SKF89976A (10 μM), another GAT-1-selective blocker (data not shown).

To determine the effects of GABA transporters on $GABA_C$ receptor-mediated responses, we recorded GABA-evoked currents in bipolar cells in the presence of NO-711. $GABA_C$ receptor-mediated currents were elicited by puffing GABA onto bipolar cell axon terminals in the presence of a control solution containing strychnine, bicuculline (150 μM), and D-AP-5 (30 μM, to reduce spontaneous activity). GABA-evoked currents recorded from bipolar cells (Fig. 1*B*, control) had a slower onset and a slower decay than the $GABA_A$ receptor-mediated current recorded from ganglion cells, consistent with previous observations of $GABA_C$ receptors (Qian and Dowling, 1993; Lukasiewicz and Shields, 1998). NO-711 significantly augmented the responses and slowed their decays. The charge transfer was increased (314 ± 58%; $n = 13$) (Fig. 1*D*, *middle*), and D_{37} was prolonged (150 ± 8%). The enhancement by NO-711 of inner retinal GABA responses recorded from bipolar and ganglion cells indicates that the GAT-1 transporter contributes to the clearance of GABA in the IPL.

GABA-evoked currents in the outer plexiform layer were not enhanced by uptake blockers

GABA receptors are also located on bipolar cell dendrites, which may receive inputs from horizontal cells and interplexiform cells in the outer plexiform layer (OPL) (Chun and Wässle, 1989; Vardi et al., 1992). To determine the effects of the GAT-1 transporter on responses in the OPL, we puffed GABA onto bipolar cell dendrites in the presence of NO-711. The evoked current had a rapid onset and a rapid decay, similar to $GABA_A$ receptor-mediated currents in ganglion cells (Fig. 1*C*). Bicuculline (150 μM) greatly suppressed these currents, indicating that they were mediated mainly by $GABA_A$ receptors. In contrast with our

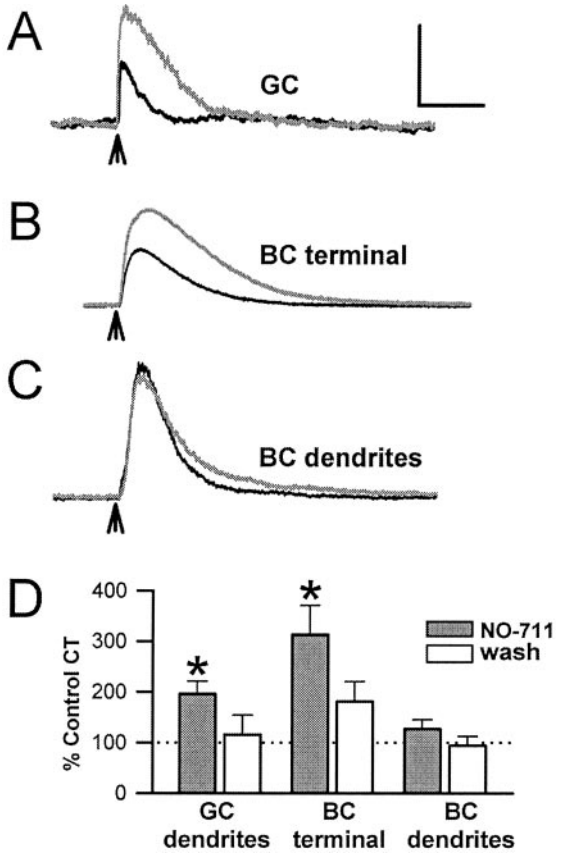

Figure 1. NO-711, a GAT-1 inhibitor, enhanced GABA evoked currents in the IPL but had little effect on GABA currents in the OPL. *A*, The outward current evoked by puffing GABA (200–300 μM) onto ganglion cell dendrites (*GC*) (*black trace*) was enhanced by NO-711 (3 μM) (*gray trace*). In this and in subsequent figures, the cell was held at 0 mV when measuring GABAergic responses, and the *arrows* beneath the *traces* indicate the timing of the puffs. Control currents are always represented by *black traces* and currents recorded in NO-711 by *gray traces* or *open circles*, here and below. *B*, Currents elicited by puffing GABA onto bipolar cell axon terminals (*BC terminal*) were also enhanced by NO-711. The time course of the GABA current was longer than in ganglion cells, consistent with its mediation by $GABA_C$ receptors (see Results). *C*, Currents evoked by puffing GABA onto bipolar cell dendrites (*BC dendrites*) had a shorter time course and were not significantly enhanced by NO-711. *D*, NO-711 (*gray bars*) enhanced the GABA-elicited charge transfer at ganglion cell dendrites (196 ± 24%; $n = 11$; $p < 0.01$) and at bipolar cell axon terminals (314 ± 58%; $n = 13$; $p < 0.01$) but not at bipolar cell dendrites (127 ± 18%; $n = 6$; $p = 0.19$). During washout of NO-711, responses recovered toward control levels (*white bars*). *Asterisks*, here and in subsequent figures, indicate a significant difference from control. Calibration: *A*, *C*, 40 pA, 0.5 sec; *B*, 40 pA, 1 sec.

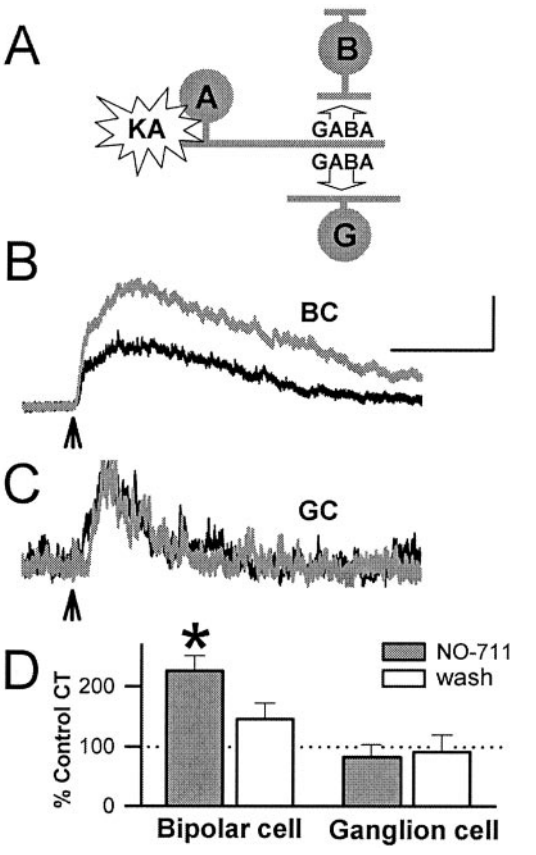

Figure 2. NO-711 enhanced monosynaptic IPSCs in bipolar cells but did not affect monosynaptic IPSCs in ganglion cells. *A*, Diagram of inner retina shows that IPSCs were evoked by kainate puffs (1 mM, 10–30 msec), applied focally in the IPL, 300 μm lateral from a recorded bipolar cell or ganglion cell. *B*, Bipolar cell. *KA*, Kainate; *A*, amacrine cell; *G*, ganglion cell. *B*, Kainate-evoked IPSCs in bipolar cells (*BC*) were enhanced by NO-711. *C*, Kainate-evoked IPSCs in ganglion cells (*GC*) were not affected by NO-711. *D*, NO-711 enhanced the charge transfer of the monosynaptic IPSCs in bipolar cells (226 ± 25%; $n = 5$; $p < 0.01$) but not in ganglion cells (83 ± 20%; $n = 5$; $p = 0.26$). Calibration: *B*, 10 pA, 2 sec; *C*, 10 pA, 1 sec.

results for the IPL, NO-711 had no significant effect on the currents elicited by GABA puffs onto bipolar cell dendrites in the OPL (127 ± 18%; $n = 6$) (Fig. 1*D*, *right*). These findings suggest that few, if any, GAT-1 transporters are found in the OPL. This is consistent with the GAT-1 immunohistochemical localization studies in tiger salamander retina by Yang et al. (1997), which showed this class of transporter was mainly in the IPL. Our results demonstrate that NO-711 reduced the clearance of GABA in the IPL but not in the OPL.

NO-711 enhanced monosynaptic IPSCs in bipolar cells but not in ganglion cells

To examine the effect of NO-711 on synaptic GABA signals in the IPL, we stimulated amacrine cells with a focal application of kainate (0.5 or 1 mM) in the IPL 300 μm lateral from the recorded cell (Fig. 2*A*). Previous studies of tiger salamander retina have shown that synaptic inputs to bipolar cells are mediated mainly by $GABA_C$ receptors and those to ganglion cells by $GABA_A$ receptors (Dong and Werblin, 1998; Lukasiewicz and Shields, 1998). At a holding potential of 0 mV, kainate puffs evoked IPSCs in both bipolar cells and ganglion cells (Fig. 2*B*,*C*, control). Bipolar cell responses were enhanced when GABA uptake was blocked with NO-711. The charge transfer was increased (226 ± 25%; $n = 5$) (Fig. 2*D*, *left*), and D_{37} was prolonged (150 ± 21%). On the other hand, NO-711 had no effect on ganglion cell IPSCs (charge transfer, 83 ± 20%; $n = 5$) (Fig. 2*C*,*D*, *right*). This result is in contrast to that obtained with puffs and may arise from how spillover transmission affects ganglion cell $GABA_A$ receptors and bipolar cell $GABA_C$ receptors.

The difference between the evoked and synaptic response enhancements might be explained by the different affinities of $GABA_A$ and $GABA_C$ receptors for GABA. To test for this possibility, we attempted to increase the sensitivity of the $GABA_A$ receptors with chlordiazepoxide hydrochloride (100 μM), pregnanolone [5α-pregnan-3α-ol-20-one (1 μM)], and pentobarbital (50 or 100 μM). Only pentobarbital was found to

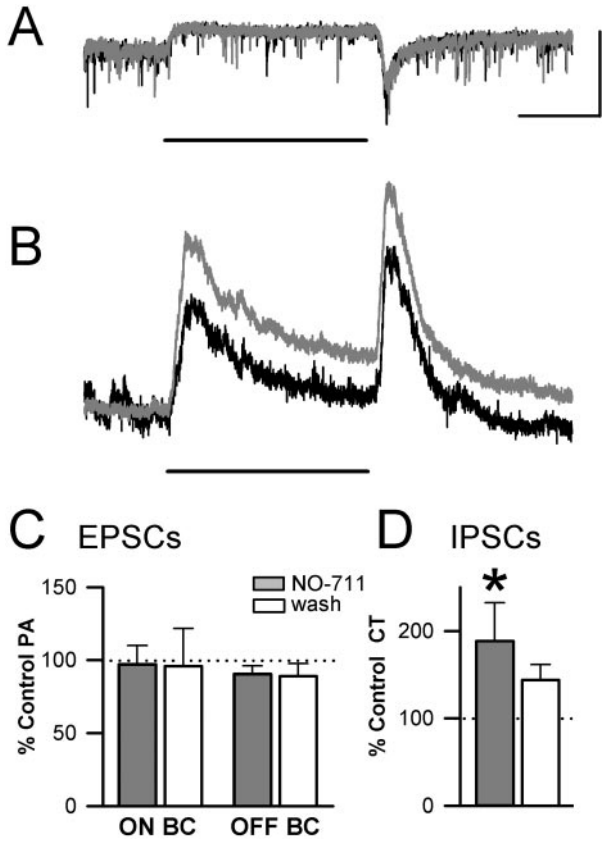

Figure 3. NO-711 enhanced light-evoked, $GABA_C$-mediated IPSCs in bipolar cells, but it did not affect light-evoked EPSCs. *A*, NO-711 did not affect the light-evoked EPSC in an OFF bipolar cell when voltage clamped to E_{Cl}. *B*, NO-711 enhanced the light-evoked IPSCs in a bipolar cell, which was voltage clamped to 0 mV. *C*, Normalized peak amplitude of EPSCs recorded in ON and OFF bipolar cells (*BC*). NO-711 did not affect the EPSCs recorded in ON (97 ± 13%; $n = 8$; $p = 0.16$) or OFF (91 ± 5%; $n = 8$; $p = 0.17$) bipolar cells. *D*, NO-711 enhanced the IPSC charge transfer in bipolar cells (188 ± 44%; $n = 6$; $p < 0.05$). In this and subsequent figures, the duration of the light stimulus is indicated by the *bar* below the *current traces*. Calibration: 10 pA, 2 sec.

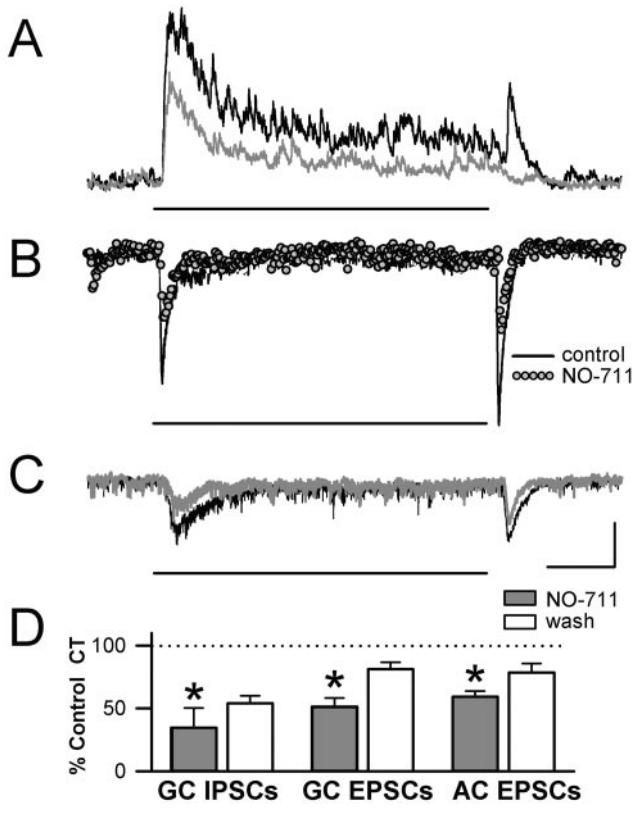

Figure 4. NO-711 reduced light-evoked EPSCs and IPSCs in ganglion cells. *A*, Light-evoked IPSC recorded from a ganglion cell held at 0 mV. NO-711 attenuated the light-evoked IPSC. *B*, Light-evoked EPSC was recorded from a ganglion cell held at E_{Cl}. NO-711 reduced the light-evoked EPSC. *C*, Light-evoked EPSC was recorded from an amacrine cell held at E_{Cl}. NO-711 reduced the light-evoked EPSC. *D*, Bar graph summarizing effects of NO-711 on normalized synaptic charge transfer. NO-711 (*gray bars*) reduced the ganglion cell (*GC*) IPSC charge transfer to 48 ± 17% of control ($n = 6$; $p < 0.05$), the ganglion cell EPSC charge transfer to 51 ± 7% of control ($n = 16$; $p < 0.05$), and the amacrine cell (*AC*) EPSC charge transfer to 59 ± 4% of control ($n = 5$; $p < 0.01$). Partial reversal occurred during washout of NO-711 (*white bars*). Calibration: 20 pA, 1 sec.

produce a consistent, long-lasting potentiation. Chlordiazepoxide and pregnanolone produced weak and/or transient enhancements, precluding their use. We recorded kainate-evoked IPSCs from ganglion cells after $GABA_A$ receptor sensitivity was enhanced by pentobarbital (Steinbach and Akk, 2001). Pentobarbital enhanced the IPSCs in ganglion cells (261 ± 60% of control charge transfer; $n = 5$), without affecting the baseline current. When NO-711 was applied in addition to pentobarbital, the $GABA_A$ receptor-mediated IPSCs were not enhanced (87 ± 6% of charge transfer in pentobarbital; $n = 5$; $p = 0.08$). Thus, the pentobarbital enhancement of $GABA_A$ receptor sensitivity was not sufficient to see an increase of ganglion cell IPSCs by NO-711.

Effects of NO-711 on light responses

To determine whether GABA transporters could affect visual signaling, we recorded light-evoked currents from bipolar cells and ganglion cells in dark-adapted retinal slices. Bipolar cells were identified by their voltage-gated currents and their morphology after filling with Lucifer yellow (Wu et al., 2000). Figure 3*A* shows an OFF bipolar cell EPSC evoked by light when the cell was held at the chloride reversal potential. NO-711 had no effect on EPSC peak amplitude in either ON bipolar cells or OFF bipolar cells (ON bipolar cells, 97 ± 13%, $n = 8$; OFF bipolar cells, 91 ± 5%, $n = 8$) (Fig. 3*A*,*C*), indicating that NO-711 had no effect on the transmission from photoreceptors to bipolar cells.

Light-evoked IPSCs were recorded from bipolar cells at the reversal potential for the excitatory inputs (0 mV). Blockade of GABA transporters by NO-711 enhanced the charge transfer (188 ± 44%; $n = 6$) and prolonged the decay (D_{37}, 181 ± 36%; $n = 6$) of light-evoked IPSCs recorded from both ON and OFF bipolar cells (Fig. 3*B*,*D*). These responses were eliminated by the $GABA_C$ receptor antagonist 3-APMPA (data not shown), confirming that the IPSCs were mediated primarily by $GABA_C$ receptors on the bipolar axon terminals (Dong and Werblin, 1998; Lukasiewicz and Shields, 1998).

Light-evoked IPSCs and EPSCs were also recorded from ganglion cells at 0 mV and E_{Cl}, respectively (Fig. 4*A*,*B*). Surprisingly, NO-711 reduced the peak amplitude and the charge transfers of both IPSCs and EPSCs (peak amplitude, IPSCs, 48 ± 17%, $n = 6$; EPSCs, 64 ± 8%, $n = 9$) (charge transfer, IPSCs, 34 ± 16%, $n = 6$; EPSCs, 51 ± 7%, $n = 14$, $p < 0.05$) (Fig. 4*D*). These data indicate that the enhancement of inhibitory inputs at bipolar cell terminals reduced the glutamate release onto both

amacrine and ganglion cells. The reduction of excitatory input to amacrine cells was suggested by the suppression of the GABAergic IPSC in ganglion cells. To confirm this, we recorded light-evoked EPSCs from amacrine cells at E_{Cl} (Fig. 4*C*). We found that NO-711 also reduced the charge transfer of amacrine cell EPSCs (59 ± 4%; $n = 5$; $p < 0.01$) (Fig. 4*D*). The decrease in glutamatergic input to ganglion cells and amacrine cells was observed directly as a reduction in their EPSCs.

We tested whether the decrease in the IPSCs in ganglion cells may have been a result of a direct inhibitory effect of NO-711 on the postsynaptic $GABA_A$ receptors. NO-711 was found to enhance and not inhibit current responses attributable to the direct application of GABA to ganglion cells (Fig. 1*A*), indicating that the uptake blocker did not block $GABA_A$ receptors. Another method to rule out a postsynaptic action of NO-711 is to measure its effects on the amplitudes of spontaneous IPSCs and EPSCs in ganglion cells. In control solution, the mean peak amplitude of miniature IPSCs (mIPSCs) and mEPSCs were 7.4 ± 1.3 and −4.9 ± 0.7 pA, respectively ($n = 5$ for mIPSCs; $n = 5$ for mEPSCs), and the mean charge transfer of mIPSCs and mEPSCs were 309 ± 56 and 116 ± 26 fC, respectively. NO-711 did not change the peak amplitude or the charge transfer of either mIPSCs (7.5 ± 0.5 pA; 344 ± 50 fC; $n = 5$) or mEPSCs (−4.8 ± 0.7 pA; 106 ± 20 fC; $n = 5$), nor did it alter the rise time, decay, or half-width, indicating that NO-711 did not affect the kinetics of postsynaptic AMPA or $GABA_A$ receptor. Therefore, the suppression of the responses in ganglion cells by NO-711 was not attributable to a postsynaptic action but was a result of reduced glutamate release from the bipolar cell onto amacrine and ganglion cells. We assessed whether NO-711 acted presynaptically by measuring its effects on mEPSC frequency. NO-711 suppressed the frequency of mEPSCs (74 ± 10% of control; $p = 0.03$; $n = 5$) but did not affect the frequency of mIPSCs (85 ± 20% of control; $p = 0.15$; $n = 5$). These data suggest that elevated extracellular GABA acted at bipolar cell axon terminals to reduce transmitter release but had little effect on amacrine cell transmitter release.

To determine which GABA receptor subtypes were involved in the suppression of light-evoked responses in ganglion cells, we measured the effects of NO-711 on light-evoked responses in the presence of various antagonists. To test whether $GABA_A$ receptors were involved, we added 150 μM bicuculline, a $GABA_A$ receptor blocker, to the control solution. As in previous experiments, glycinergic receptors were always blocked with strychnine. When $GABA_A$ receptors were blocked with bicuculline, the light-evoked IPSCs in bipolar cells were enhanced by NO-711, similar to that observed in the control solution (Fig. 5). NO-711 also suppressed the ganglion cell EPSCs in either the presence or the absence of bicuculline (Fig. 6*A*,*C*, *left*, *middle*). The inability of bicuculline to block the effects of NO-711 suggests that $GABA_A$ receptors did not play a major role in the light-evoked suppression.

To test for the involvement of $GABA_C$ receptors in the NO-711-mediated suppression of EPSCs requires the use of a specific antagonist. Unfortunately, a clean antagonist does not exist. As reported previously (Flores-Herr et al., 2001), we also found that (1, 2, 5, 6-tetrahydropyridin-4-yl) methylphosphinic acid (TPMPA) was not a potent blocker of $GABA_C$ receptors. At higher concentrations TPMPA also reduced $GABA_A$ receptor-mediated responses (Ragozzino et al., 1996). Another $GABA_C$ receptor antagonist 3-APMPA was not used because it activates $GABA_B$ receptors, which are present on bipolar cell axon terminals (Shen and Slaughter, 2001). Therefore, we blocked $GABA_A$ and $GABA_C$ receptors with a combination of picrotoxin (200 μM) and I4AA (20 μM). In the presence of these blockers, the EPSCs were not suppressed by NO-711 (Fig. 6*B*,*C*, *right*). Shen and Slaughter (2001) demonstrated recently that $GABA_A$ receptors have little influence on ganglion cell EPSCs and suggested that the effects of picrotoxin on EPSCs were mediated by $GABA_C$ receptors. Together, these data suggest that NO-711 increased the activation of $GABA_C$ receptors on bipolar cell terminals by enhancing GABA spillover, which suppressed bipolar cell to ganglion cell synaptic transmission.

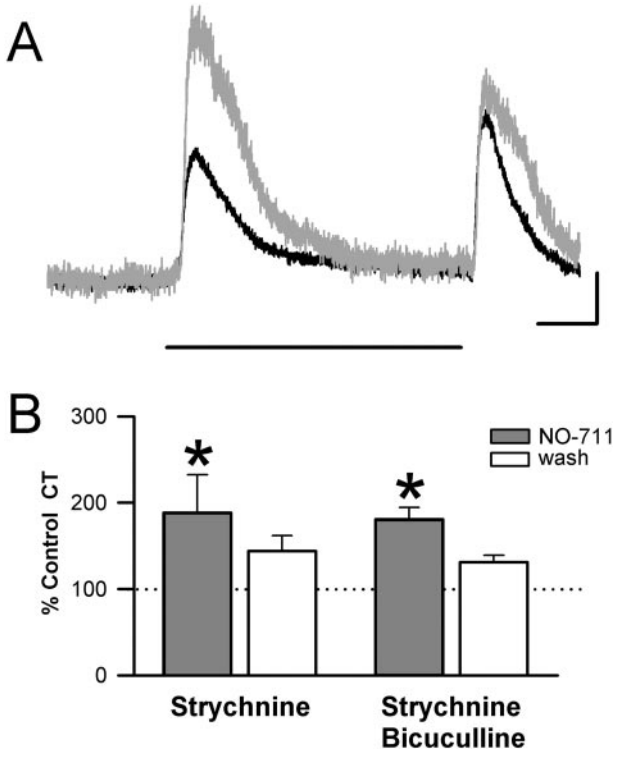

Figure 5. The enhancement of light-evoked IPSCs in bipolar cells by NO-711 was not mediated by $GABA_A$ receptors. *A*, IPSCs recorded from a bipolar cell in the presence of strychnine (5 μM) and bicuculline (150 μM) (*black trace*). NO-711 enhanced the IPSCs in the presence of the $GABA_A$ receptor antagonist bicuculline (*gray trace*). *B*, Light-evoked IPSC charge transfer in bipolar cells was enhanced in either the absence or the presence of $GABA_A$ receptor blocker (*gray bars*) (strychnine, 188 ± 44%, $n = 6$, $p < 0.05$; bicuculline and strychnine, 181 ± 14%, $n = 6$, $p < 0.05$). Partial reversal occurred during washout of NO-711 (*white bars*). Calibration: 10 pA, 1 sec.

GABA transporter inhibition shifts and broadens the ganglion cell intensity–response curve

We wanted to determine whether the accumulation of GABA caused by GAT-1 transporter blockade affected the responsiveness of ganglion cells over a range of light intensities. We recorded the EPSCs in response to full-field light stimulation from ganglion cells over a fivefold log unit range of light intensities and plotted the normalized peak amplitude of the ON response as a function of light intensity. In control Ringer's solution (Fig. 7*A*, *filled circles*), the intensity–response function was best fit with a sigmoidal function (see Materials and Methods), with an L_{50} (half-maximal response) of −2.18 ± 0.2 log ($n = 18$) and an L_{10-90} (width of dynamic range; see Materials and Methods) of 2.23 ± 0.6 log ($n = 18$). The addition of NO-711 caused the intensity–response relationship to shift the L_{50} to higher light

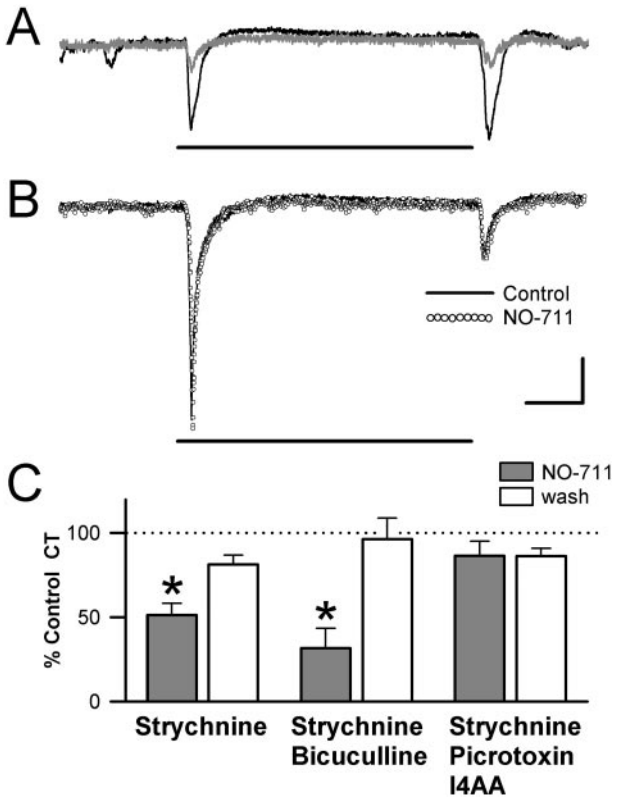

Figure 6. The suppression of light-evoked EPSCs in ganglion cells by NO-711 was mediated primarily by $GABA_C$ receptors. *A*, Light-evoked EPSCs recorded from a ganglion cell in the presence of bicuculline (150 μM) and strychnine (5 μM) (*black trace*). NO-711 reduced the EPSCs in the presence of these antagonists (*gray trace*). *B*, Light-evoked EPSCs recorded from a ganglion cell in the presence of $GABA_A$, $GABA_C$, and glycine receptor blockers (200 μM picrotoxin, 20 μM I4AA, and 5 μM strychnine) (*black trace*). NO-711 did not attenuate the currents in the presence of these antagonists (*open circles*). *C*, Light-evoked EPSCs in ganglion cells were attenuated by NO-711 (*gray bars*) in either the presence or the absence of a $GABA_A$ receptor blocker (without bicuculline, 51 ± 7% of control charge transfer, $n = 14$, $p < 0.01$; with bicuculline, 32 ± 12% of control charge transfer, $n = 7$, $p < 0.01$). In contrast, addition of $GABA_C$ receptor blockers eliminated the attenuation of the EPSCs (87 ± 9% of control charge transfer; $n = 7$; $p = 0.07$). Partial reversal occurred during washout of NO-711 (*white bars*). Calibration: 20 pA, 1 sec.

intensities (L_{50} was −0.65 ± 1.2 log; $n = 5$; $p < 0.05$) and broadened the dynamic range (L_{10-90} was 3.42 ± 0.3 log; $n = 5$; $p < 0.05$).

To confirm that the effects of NO-711 on the intensity–response relationship were attributable to accumulation of GABA in the IPL, we recorded EPSCs in the presence of $GABA_A$ and $GABA_C$ receptor blockers. If GABA receptor activation is responsible for the rightward curve shift observed in the presence of NO-711, then GABA receptor blockers should have the opposite effect, shifting the curves to the left. Normalized peak amplitude of the ON light response was plotted as a function of light intensity in control (Fig. 7*B*, *filled circles*; same as Fig. 7*A*), in the presence of bicuculline (Fig. 7*B*, *open triangles*), and in the presence of picrotoxin, I4AA, and bicuculline (Fig. 7*B*, *filled triangles*). Bicuculline shifted the L_{50} of the curve slightly to the right, but this effect was not significant ($p = 0.36$; L_{50} was −2.04 ± 0.3 log; $n = 9$). Bicuculline also did not change the width of the dynamic range ($p = 0.17$; L_{10-90} was 1.58 ± 0.3 log; $n = 9$), indicating that $GABA_A$ receptors do not play a major role in shifting or changing the dynamic range of the intensity–response curve. To determine whether $GABA_C$ receptors mediated the NO-711 effect, $GABA_C$ receptor blockers were applied in addition to bicuculline. Blockade of the $GABA_C$ receptors had the opposite effect of NO-711, shifting the curve to the left (L_{50} was −3.04 ± 0.3 log; $n = 4$; $p < 0.05$) and reducing the dynamic range (L_{10-90} was 0.86 ± 0.2 log; $n = 4$; $p < 0.05$). These results suggest that NO-711 enhanced the spillover of GABA, which activates $GABA_C$ receptors on bipolar cell terminals and shifts and broadens the light intensity–response curve measured in ganglion cells. Together, these results strongly suggest that the GABA transporter can modulate the light sensitivity of ganglion cells.

DISCUSSION

Our data indicate that the GABA transporter GAT-1 has a physiological role in the IPL. The GAT-1-selective blocker NO-711 enhanced currents evoked by puffing GABA onto ganglion cell dendrites and bipolar cell terminals. Monosynaptic IPSCs, however, were enhanced by NO-711 only in bipolar cells, suggesting that $GABA_C$ receptors, but not $GABA_A$ receptors, were activated by increased GABA spillover. NO-711 also augmented light-evoked $GABA_C$ receptor-mediated IPSCs in bipolar cells, which reduced light-evoked EPSCs in ganglion cells. The light intensity–response function for EPSCs was shifted to higher intensities by NO-711, suggesting that GABA accumulation reduced ganglion cell light sensitivity. These data suggest that GAT-1 transporters in the IPL regulate bipolar cell to ganglion cell transmission primarily by limiting $GABA_C$ receptor activation in bipolar cell terminals.

Mechanisms underlying NO-711 suppression of bipolar cell to ganglion cell transmission

NO-711 reduced both excitatory and inhibitory light responses in ganglion cells, suggesting that GAT-1 transporters can regulate bipolar cell to third-order neuron transmission through GABA receptors on bipolar cell terminals. It has been shown that $GABA_A$ receptor and $GABA_C$ receptors are present at synapses on the bipolar cell axon terminal (Koulen et al., 1998). The activation of $GABA_C$ receptors reduces bipolar cell to ganglion cell transmission in salamander retina, but $GABA_A$ receptor activation is ineffective (Lukasiewicz and Werblin, 1994; Shen and Slaughter, 2001). In our study, bicuculline failed to reverse the effects of NO-711, suggesting that $GABA_A$ receptors alone were not sufficient to account for the effects of NO-711. In contrast, picrotoxin and I4AA, which blocked both $GABA_C$ and $GABA_A$ receptors, completely eliminated the effect of NO-711. The simplest explanation of these results is that $GABA_C$ receptors played the major role in the NO-711-mediated modulation of bipolar cell to ganglion cell transmission. Because we were unable to reliably block $GABA_C$ receptors with TPMPA, we cannot rule out that $GABA_A$ receptors also played a role in the NO-711-mediated suppression of transmission and changes in ganglion cell light sensitivity. However, the role of $GABA_A$ receptors is probably minimal because IPSCs in salamander bipolar cells were mediated primarily (>80%) by $GABA_C$ receptors (Lukasiewicz and Shields, 1998), and $GABA_A$ receptors were shown to have minimal effects on salamander ganglion cell EPSCs (Shen and Slaughter, 2001).

NO-711 had little effect on transmission in the OPL. Currents evoked by GABA puffed onto bipolar cell dendrites and light-evoked EPSCs in the bipolar cells were not affected by NO-711.

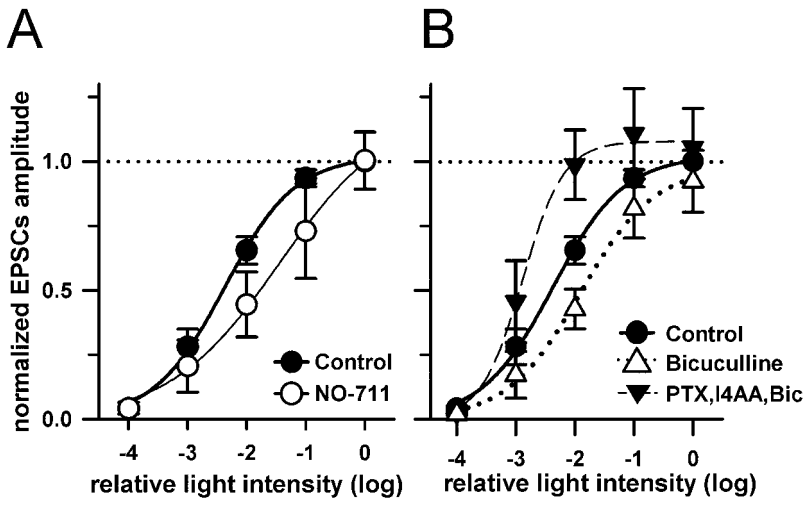

Figure 7. Light intensity–response curves for ganglion cell EPSCs were affected by NO-711 and GABA receptor antagonists. *A*, The peak amplitude of light-evoked EPSCs recorded from ganglion cells was normalized to the peak amplitude of the maximal EPSCs in response to the unattenuated LED light stimulus in control solution. Normalized, mean peak amplitude of ON response is plotted as a function of light intensity in control conditions (*filled circles*; $n = 18$) and in the presence of NO-711 (*open circles*; $n = 5$). The data were fitted with sigmoid curves (see Materials and Methods). NO-711 shifted the L_{50} of the curve to the right and increased its L_{10-90} (dynamic range) (see Results). *B*, Normalized peak amplitude of ON response is plotted as a function of light intensity in control (*filled circles*; same data as *A*), in the presence of bicuculline (*open triangles*; $n = 9$), and in the presence of picrotoxin, I4AA, and bicuculline (*filled triangles*; $n = 4$). Bicuculline did not significantly change the L_{10-90}. It slightly shifted the curve to the right, but the L_{50} was not significantly different from control (see Results). $GABA_C$ blockers in addition to bicuculline shifted the L_{50} to the left and reduced the L_{10-90}.

These results are consistent with the immunocytochemical studies in salamander, rat, and salmon retinas, which showed strong GAT-1-labeling in the IPL but only weak labeling in the OPL (Johnson et al., 1996; Yang et al., 1997; Ekstrom and Anzelius, 1998), supporting our observations that NO-711 acted primarily in the IPL.

NO-711 enhanced GABA spillover at the inner plexiform layer

Inhibitory transmission by GABA is usually considered to be point to point transmission, shaped by receptor desensitization and clearance, primarily attributable to rapid diffusion of transmitter away from the synaptic site and to a lesser extent by clearance attributable to uptake (Isaacson et al., 1993). A second type of transmission is mediated by spillover of transmitter from distant release sites. Spillover transmission is thought to activate high-affinity $GABA_B$ receptors in the hippocampus (Isaacson et al., 1993) and $\alpha 6$ subunit $GABA_A$ receptors in the cerebellar cortex (Rossi and Hamann, 1998). Spillover activation of receptors often results in slow, small-amplitude responses, which contribute significantly to the synaptic signal (Rossi and Hamann, 1998). Compared with point to point transmission, spillover transmission is more strongly limited by the uptake of GABA (Isaacson et al., 1993).

We found that the GABA transport blocker NO-711 enhanced kainate-evoked IPSCs mediated by $GABA_C$ receptors on bipolar cells but had no effect on IPSCs mediated by $GABA_A$ receptors on ganglion cells. This suggests that NO-711 caused spillover activation of additional $GABA_C$ receptors on bipolar cell terminals. One explanation for these findings is the different sensitivities of $GABA_A$ and $GABA_C$ receptors. For both native and expressed receptors, $GABA_C$ receptors are more sensitive (8 and 40×, respectively) than $GABA_A$ receptors (Amin and Weiss, 1994; Feigenspan and Bormann, 1994). We increased the sensitivity of $GABA_A$ receptors with pentobarbital to make them behave more like $GABA_C$ receptors. Although $GABA_A$ responses were increased by pentobarbital, NO-711 was still ineffective, probably because the sensitivity could not be increased sufficiently (Steinbach and Akk, 2001) to detect spillover. Another interpretation of our results is that the localization of GAT-1 transporters limits the synaptic activation of $GABA_C$ receptors but not $GABA_A$ receptors.

If spillover transmission activated $GABA_A$ receptors, then it is possible that GABA accumulation could lead to desensitization of these receptors. Our results suggest that $GABA_A$ receptors were not desensitized in the presence of NO-711. Evoked and spontaneous $GABA_A$ receptor-mediated IPSCs amplitudes were not affected by NO-711. Thus, NO-711 neither reduced nor enhanced these responses, suggesting that spillover transmission does not affect $GABA_A$ receptors.

Because bipolar cells make excitatory glutamatergic synapses onto both amacrine and ganglion cells, one would expect NO-711 to reduce EPSCs in both of these cell types. We found that EPSCs were reduced in both cell types. Decreasing the excitatory drive to amacrine cells should result in a reduction in GABA release onto bipolar cell terminals and onto ganglion cell dendrites. In the presence of NO-711, we observed the predicted decrease in the light-evoked GABAergic IPSCs of ganglion cells. Surprisingly, light-evoked IPSCs in bipolar cells were enhanced by NO-711, although the excitatory drive to amacrine cells was reduced. This enhancement most likely reflects the stronger sensitivity of $GABA_C$ receptors to spillover.

NO-711 altered the sensitivity of ganglion cells to light-evoked input

Many studies have examined the effects of illumination on ganglion cell sensitivity. Sakmann and Creutzfeldt (1969) demonstrated that a ganglion cell intensity–response function for a small spot was shifted to brighter intensities with increasing background illumination. Werblin and colleagues (Werblin, 1974; Thibos and Werblin, 1978) demonstrated that steady surround illumination caused a similar shift in the response–intensity relationship, and they attributed this shift to lateral interactions of horizontal cells in the OPL. Recent evidence has shown that the receptive field surround of amacrine and ganglion cells also involves a steady lateral inhibition at the inner retina (Cook et al., 1998; Taylor, 1999; Bloomfield and Xin, 2000; Flores-Herr et al., 2001).

Our results indicate that NO-711 enhanced GABA spillover, increasing the activation of this inner retinal lateral pathway. NO-711 altered the intensity–response relationship of ganglion cells in a manner consistent with the effects of surround illumination described by others. We demonstrated that NO-711 enhanced currents in bipolar cells mediated by $GABA_C$ receptors, which have been suggested previously to contribute to surround inhibition in amacrine cells and ganglion cells (Bloomfield and Xin, 2000; Flores-Herr et al., 2001). Surround illumination has also been shown to directly inhibit ganglion cells (Belgum et al.,

1987; Cook and McReynolds, 1998), but it is unlikely that this portion of surround inhibition would be affected by NO-711 because it is mediated by $GABA_A$ receptors.

This hypothesis is also supported by our evidence, which shows that the blockade of $GABA_C$ and $GABA_A$ receptors shifted the L_{50} of the ganglion cell intensity–response curve to the left, in the opposite direction to the NO-711-induced shift. Interestingly, blockade of $GABA_A$ receptors produced a small but insignificant shift of the L_{50} to the right, similar to the action of NO-711. This rightward shift was most likely attributable to bicuculline disinhibiting the amacrine cells, resulting in enhanced $GABA_C$ receptor signaling to bipolar cell terminals (Zhang et al., 1997). Thus, the L_{50} of the intensity response curve was shifted to the right by increasing $GABA_C$ signaling and was shifted to the left by blocking the activation of $GABA_C$ receptors, demonstrating that presynaptic $GABA_C$ receptors modulate ganglion cell light sensitivity. This is consistent with previous work showing that a significant fraction of the steady-surround signal occurs in the inner retina.

Blockade of GABA transporters by NO-711 also increased the dynamic range (L_{10-90}) of the ganglion cell intensity–response curve. This was most likely attributable to the increased activation of $GABA_C$ receptors because, when these receptors were blocked, the L_{10-90} of the curve was decreased. We used full-field light stimuli, which activated both center and surround pathways. The increased L_{10-90} can be attributed to enhancement of inhibitory signaling by NO-711 and the decreased L_{10-90} to reduction in this signaling by the $GABA_C$ receptor blockers. In agreement with our findings, Euler and Masland (2000) showed in bipolar cells that either GABA receptor inhibition or axotomy, which reduces inhibitory inputs, decreased the L_{10-90} of the sensitivity curves to full-field illumination. Together, these findings suggest that bipolar cell $GABA_C$ receptors mediate the dynamic range changes that we observed in ganglion cell sensitivity curves.

In summary, our data show that NO-711 enhances light-evoked, inhibitory signals in bipolar cell terminals. This suggests that GABA transporters normally act to limit inhibitory signaling at the inner retina particularly at $GABA_C$ receptors. Without transporters, additional $GABA_C$ receptors would be activated by spillover transmission, which would degrade both the spatial and temporal properties of inhibitory signaling.

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