Spike-Dependent GABA Inputs to Bipolar Cell Axon Terminals Contribute to Lateral Inhibition of Retinal Ganglion Cells

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Shields, Colleen R. and Peter D. Lukasiewicz. Spike-dependent GABA inputs to bipolar cell axon terminals contribute to lateral inhibition of retinal ganglion cells. J Neurophysiol 89: 2449–2458, 2003. First published November 13, 2002; 10.1152/jn.00916.2002. The inhibitory surround signal in retinal ganglion cells is usually attributed to lateral horizontal cell signaling in the outer plexiform layer (OPL). However, recent evidence suggests that lateral inhibition at the inner plexiform layer (IPL) also contributes to the ganglion cell receptive field surround. Although amacrine cell input to ganglion cells mediates a component of this lateral inhibition, it is not known if presynaptic inhibition to bipolar cell terminals also contributes to surround signaling. We investigated the role of presynaptic inhibition by recording from bipolar cells in the salamander retinal slice. TTX reduced light-evoked GABAergic inhibitory postsynaptic currents (IPSCs) in bipolar cells, indicating that pre- and postsynaptic pathways mediate lateral inhibition in the IPL. Photoreceptor and bipolar cell synaptic transmission were unaffected by TTX, indicating that its main effect was in the IPL. To rule out indirect actions of TTX, we bypassed lateral signaling in the outer retina by either electrically stimulating bipolar cells or by puffing kainate (KA) directly onto amacrine cell processes lateral to the recorded cell. In bipolar and ganglion cells, TTX suppressed laterally evoked IPSCs, demonstrating that both pre- and postsynaptic lateral signaling in the IPL depended on action potentials. By contrast, locally evoked IPSCs in both cell types were only weakly suppressed by TTX, indicating that local inhibition was not as dependent on action potentials. Our results show a TTX-sensitive lateral inhibitory input to bipolar cell terminals, which acts in concert with direct lateral inhibition to give rise to the GABAergic surround in ganglion cells.

INTRODUCTION

Retinal ganglion and bipolar cells exhibit the antagonistic center-surround receptive field organization. That is, illumination of the receptive field surround antagonizes responses to illumination of the receptive field center. Horizontal cell activity in the outer plexiform layer (OPL) was thought to mediate the inhibitory surround of bipolar cells (Kaneko 1970; Werblin and Dowling 1969), which in turn gave rise surround inhibition of ganglion cells (Naka and Witkovsky 1972; Werblin and Dowling 1969). Inhibition in the inner plexiform layer (IPL), on the other hand, was thought to underlie more complex elements of visual processing, such as motion detection and directional sensitivity (Caldwell et al. 1978; Werblin et al. 1988).

To determine whether these synaptic interactions in the IPL also contribute to the surround inhibition of ganglion cells, recent studies have exploited a known difference in the membrane properties of laterally extending interneurons; amacrine cells fire sodium action potentials, whereas horizontal cells do not fire action potentials. These reports demonstrated that lateral inhibition in the IPL contributed a significant fraction of surround inhibition of third-order neurons in amphibian and mammalian retinas (Bloomfield and Xin 2000; Cook and McReynolds 1998a; Cook et al. 1998; Taylor 1999). This surround had a postsynaptic component arising from TTX-sensitive, inhibitory inputs mediated by ganglion cell GABA<sub>A</sub> receptors (Bieda and Copenhagen 1999; Cook and McReynolds 1998a; Flores-Herr et al. 2001; Lukasiewicz and Shields 1998). There may also be a presynaptic component generated by GABAergic inputs to bipolar cell terminals mediated by GABA<sub>C</sub> receptors (Flores-Herr et al. 2001; Ichinose and Lukasiewicz 2002; Lukasiewicz and Shields 1998).

Here, we show that the GABA receptor-mediated inhibitory postsynaptic currents (IPSCs) in bipolar cells and ganglion cells are reduced by TTX. Our results extend earlier studies on ganglion cell surround inhibition in the salamander retina (Cook and McReynolds 1998a) by demonstrating that presynaptic GABAergic interactions in the IPL contribute to the TTX-sensitive ganglion cell surround. Because it is possible that the spike dependence of GABA release differs between wide- and narrow-field amacrine cells or between long-range and local signaling within a single amacrine cell, we compare the action potential dependence of long-range versus local GABAergic transmission. Our data show that signals transmitted over longer lateral distances rely strongly on amacrine cell action potentials, whereas those spreading over shorter distances depend less on spiking.

METHODS

Whole cell patch recording in tiger salamander retinal slices

Whole cell patch recordings (Hamill et al. 1981) were made from bipolar cells and ganglion cells in tiger salamander retinal slice preparations (Werblin 1978) using an Axopatch 200B amplifier (Axon

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Instruments, Foster City, CA). The retinal slices were prepared under either dim white light or infrared illumination. Experiments were generally performed using dim white light for viewing retinal slices. Infrared illumination was sometimes used to preserve light-responsiveness. Slice thickness was generally 250 µm but ranged from a minimum of 150 µm to a maximum of 350 µm. The preparation of the retinal slices, the microscope system, and the recording procedures have been described in detail previously (Lukasiewicz and Roeder 1995; Lukasiewicz and Werblin 1994). Tiger salamanders were obtained from C. D. Sullivan (Nashville, TN) and kept at 5°C on a 12-h light-dark cycle. Experiments were performed at room temperature (19–22°C). Whole cell patch electrodes were pulled from borosilicate glass (1B150F-4 or MT150F-4, W.P.I., Sarasota, FL) with a Sachs-Flaming micropipette puller Model PC-84 or a Flaming/Brown micropipette puller Model P-97 (Sutter Instruments, Novato, CA) and had measured resistances of <5 MΩ. Patchit software (White Perch Software, Somerville, MA) was used to generate voltage command outputs, acquire data, gate the drug perfusion valves, and trigger the Picospritzer and the Grass Stimulator. The data were digitized and stored with a 486 PC or a Pentium-90 PC using a Labmaster DMA data acquisition board (Scientific Solutions, Solon, OH). Responses were sampled between 0.7 and 2 kHz and were filtered at 1 kHz with the four-pole Bessel low-pass filter on the Axopatch 200B.

Data analysis

Tack software (White Perch Software, Somerville, MA) was used to average records and to determine the peak amplitude and charge transfer. Leak-subtracted responses (n ≥ 2) were averaged to obtain the current traces depicted in the figures. Sigma Plot software (SPSS, Chicago, IL) was used to create the graphs in the figures. Data in text and figure legends are expressed as means ± SE. Levels of significance were determined using one-tailed Student’s t-test.

Intracellular solutions

The cesium gluconate intracellular electrode solution used to record GABAergic IPSCs in ganglion and bipolar cells consisted of (in mM) 95.25 cesium gluconate, 8 tetraethylammonium chloride (TEA), 0.4 magnesium chloride, 1 ethylene glycol-bis(β-aminoethyl ether)-N,N,N’,N’-tetraacetic acid (EGTA), and 10 N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid hemisodium salt (HEPES hemisodium salt), adjusted to pH 7.5 with hydrochloric acid.

For bipolar cell recordings of light-evoked excitatory PSCs (EPSCs), one of the following solutions was used. No differences were observed between the two solutions. 1) EPSCs from five bipolar cells were recorded using an intracellular solution similar to that described by Nawy and Jahr (1991). This solution consisted of (in mM) 60 NaH2PO4, 10 NaCl, 10 EGTA, 10 HEPES, 1 MgCl2, 2 MgATP, 0.1 Na2GTP, and 1 cGMP, adjusted to pH 7.4 with KOH. 2) EPSCs from four bipolar cells were recorded using gramicidin perforated patches (Ebihara et al. 1995). The perforated-patch electrode solution consisted of the following (in mM): 107.5 cesium chloride and 10 HEPES, adjusted to pH 7.5 with Tris base. No differences were found for one cell in which potassium chloride was substituted for cesium chloride in the intracellular solution. Gramicidin stock solution was prepared by dissolving 10 mg/ml in methanol and was diluted to its final concentration of 75 µM with the electrode solution. The electrode tip was filled with gramicidin-free electrode solution to enhance seal formation.

Bipolar cell responses to voltage steps were recorded using either the CsCl/gramicidin (2nd solution; see preceding text) or a cesium gluconate plus regenerating ATP solution, which contained (in mM) 76 CsOH, 46 gluconic acid, 4.5 MgCl2, 10 BAPTA, 10 glucose, 10 HEPES, 10 TEA-OH, 4 Mg1.5ATP, 3 Na2GTP, 14 Na2 phosphate, and 500 U/ml phosphokinase, adjusted to pH 7.5 with CsOH. No differences in responses were observed between the two solutions.

Extracellular solutions

The bathing medium (normal salamander Ringer, NSR) contained (in mM) 112 sodium chloride, 2 potassium chloride, 2 calcium chloride, 1 magnesium chloride, 5 glucose, and 5 HEPES, adjusted to pH 7.8 with NaOH. Membrane potential values given in this paper were corrected for junction potential, which was calculated using Junction Potential Calculator software (Cell Micro Controls, Virginia Beach, VA). [Standard cesium gluconate solution = −15 mV; Nawy and Jahr (1991) = −10 mV; cesium chloride for gramicidin patch solution = −5 mV; cesium gluconate plus ATP regeneration cocktail = −16 mV.]

Unless otherwise indicated, all chemicals were obtained from Sigma Chemical (St. Louis, MO), 3-aminopropyl[(methyl)phosphonic acid (3-APMPA) and imidazole-4-acetic acid hydrochloride (I4AA) were obtained from Research Biochemicals (Natick, MA), and 2-aminophosphonopentanoic acid (α-AP5) was purchased from Precision Biochemicals (Vancouver, BC). Tetrodotoxin (TTX) was obtained from Sigma, Precision Biochemicals, or Alexis Biochemicals (San Diego, CA).

The control bathing solution for recording IPSCs was formulated to pharmacologically isolate the GABA receptor-mediated component of IPSCs. Glycine-evoked inhibitory synaptic responses were always blocked with strychnine (2–5 µM) (Belgum et al. 1984). When indicated, N-methyl-D-aspartate (NMDA) receptors were blocked with D-AP5 (40 µM) (Mittman et al. 1990). GABAa and GABAc receptors were antagonized with bicuculline (100–200 µM) and picrotoxin (100–200 µM). 3-APMPA (500 µM) or I4AA (15 µM) were sometimes included as GABAa receptor antagonists. Action potentials were abolished with the voltage-gated sodium channel blocker TTX (0.4 – 1 µM). The control bathing solution for recording bipolar cell EPSCs was formulated to pharmacologically isolate AMPA receptor-mediated currents by including antagonists of glycine, NMDA, and GABAa receptors. Antagonists were applied to a region of the slice under study (several mm in width) by a gravity-driven superfusion system as described previously (Lukasiewicz and Roeder 1995). The control solution for recording voltage step-evoked responses in bipolar cells and kainate-evoked excitatory currents in amacrine cells included 4 mM cobalt chloride to block voltage-gated calcium channels.

Light stimulation

The light stimulation and procedures were described in previous publications of this laboratory (Lukasiewicz and Roeder 1995; Lukasiewicz et al. 1995). A tungsten-halogen lamp (20 W; Ealing Electro-Optics, Holliston, MA) provided the white light stimuli (~800 µm in diameter) with unattenuated intensity equivalent to either 2.9 × 109 or 3 × 108 quanta µm−2 s−1 of a monochromatic light of 500 nm, which was attenuated with neutral density filters. For some bipolar cell recordings of EPSCs, full-field light stimuli were generated with a Digi-Key ( Thief River Falls, MN) 3750A Ultra-Bright LED (Apeak = 700 nm, maximum intensity 1.75 × 109 quanta µm−2 s−1). Patchit software (White Perch Software, Somerville, MA) was used to vary the current through the LED to control the light output.

Evoking GABAergic IPSCs in bipolar and ganglion cells

ELECTRICAL STIMULATION OF BIPOLAR CELLS. GABAergic IPSCs recorded in bipolar cells were evoked by electrically activating bipolar cell synaptic inputs to amacrine cells. Multiple bipolar cells were stimulated with positive current pulses applied to the extracellular patch electrode filled with normal salamander Ringer.
solution. (see Higgs and Lukasiewicz 1999 for details.) A silver/silver chloride electrode (separate from the bath ground) placed next to the slice served as the return path for the stimulating current. The stimuli were generated by a constant current stimulator (Grass S48 stimulator with stimulus isolation unit PSI U6; West Warwick, RI) that was triggered by the data acquisition program. The stimulating electrode, identical to the recording electrodes, was inserted into the OPL ≤0.5 μm or ≥300 μm from the soma of the recorded bipolar cell. The duration and magnitude of the stimuli were adjusted to minimize synaptic fatigue and to elicit reproducible responses (typically 1 ms, 1–10 μA).

FOCAL APPLICATION OF KAINATE. Amacrine cells were activated by puffing kainate (1 mM) into the IPL with a Picospritzer (General Valve, Fairfield, NJ) at ~40-s intervals. The puff pressure and duration were typically 5–10 lbs/in² and 10–15 ms, respectively. Cells were voltage clamped to between −10 and 0 mV, the reversal potential for nonspecific cation currents (Ecation). (The slightly negative holding potential was sometimes used in ganglion cells to permit the detection of currents directly evoked by the kainate puff).

RESULTS

Light-evoked GABAergic IPSCs in bipolar cells depend on action potentials

Several types of amacrine cell in salamander retina use sodium action potentials to propagate lateral inhibitory signals in the IPL (Cook and McReynolds 1998a; Cook and Werblin 1994; Cook et al. 1998). Inhibitory signaling to bipolar cell terminals in the inner retina is mediated by GABAergic amacrine cells (Lukasiewicz and Shields 1998; Lukasiewicz et al. 1994). Because GABAergic inhibition at bipolar cell terminals could contribute to surround inhibition of ganglion cells, we investigated whether action potentials were necessary for the transmission of lateral inhibitory signals to bipolar cells.

Figure 1A shows that IPSCs were elicited in a bipolar cell at light ON and at light OFF. The IPSCs were GABAergic because they were blocked by a combination of picrotoxin and bicuculline (data not shown) in agreement with previous studies (Lukasiewicz and Shields 1998; Roska et al. 1998). Because glycinergic inhibition depends on action potentials (Cook et al. 1998), GABAergic inhibition was isolated by blocking glycine receptors with strychnine (3 μM). IPSCs were recorded when cells were voltage clamped to 0 mV, the reversal potential for nonspecific cation conductances (Ecation). NMDA receptors were antagonized with d-AP5 (40 μM) to minimize the occurrence of spontaneous IPSCs. TTX suppressed the light-evoked IPSCs, indicating that lateral inhibition depended on sodium action potentials. Figure 1B shows for a population of bipolar cells that TTX significantly and reversibly suppressed the charge transfer and peak amplitude of responses to light ON and to light OFF.

Because amacrine cells express NMDA receptors, which are thought to amplify excitatory inputs (Dixon and Copenhagen 1992), the inclusion of d-AP5 in the control bath solution may have biased the system toward a greater reliance on action potentials to evoke transmitter release. Figure 1C shows that when d-AP5 was absent from the bath solution, TTX still reduced the light-evoked, ON, and OFF IPSCs in bipolar cells but was slightly less effective. These data suggest that both NMDA receptors and TTX-sensitive sodium channels may play a role in amplifying the excitatory amacrine cell responses that elicit GABAergic transmission to bipolar cells.

![FIG. 1. Light-evoked GABAergic inhibitory postsynaptic currents (IPSCs) in bipolar cells depend on action potentials. A: light-evoked bipolar cell GABAergic IPSCs recorded in the absence or presence of TTX (0.4 μM). The black line above the current traces indicates the duration of the light flash in this and subsequent figures. B: the effects of TTX on the charge transfer and peak amplitude of GABAergic IPSCs in a population of bipolar cells. TTX reduced the charge transfer of ON (n = 6) and OFF (n = 5) responses to 11.1 ± 3% and 9.4 ± 2.6% of control levels, respectively. Responses recovered to 78 ± 14% and 75 ± 17% after washout. C: the effects of TTX (0.4–1 μM) on the charge transfer and peak amplitude of GABAergic IPSCs in a population of bipolar cells in the absence of the N-methyl-D-aspartate (NMDA) antagonist t-2-amino-5-phosphonopentanoic acid (d-AP5). TTX reduced the charge transfer of ON (n = 11) and OFF (n = 12) responses to 34 ± 4 and 32 ± 7% of control values, respectively. Responses recovered to 88 ± 8% and 78 ± 6% after washout. The peak amplitudes were reduced to 53 ± 6% and 49 ± 9% of control levels. IPSCs recovered to 108 ± 13% and 91 ± 6% after washout. All responses in TTX were significantly different from controls (P < 0.0001). The percent suppression by TTX was significantly greater when d-AP5 was included in the control solution (P = 0.007, determined by 1-tailed, nonpaired t-test).]

Amacrine cells can make serial GABA_A receptor-mediated synaptic contacts onto other amacrine cells. This serial inhibition can reduce the GABA_C receptor-mediated IPSCs in bipolar cells (Roska et al. 1998; Zhang et al. 1997). Because TTX could potentially alter this serial circuitry, we included the GABA_A receptor antagonist bicuculline in the control solution. Under these conditions, TTX still suppressed the bipolar cell GABA_C receptor-mediated IPSCs (ON, n = 8; OFF, n = 7; data not shown). This reduction was reversible and not significantly different from that observed in the presence of strychnine alone. These data suggest that TTX acted directly on presynaptic amacrine cells to reduce the bipolar cell IPSCs and not indirectly by interfering with serial inhibitory circuits.

Our data show that GABAergic transmission to bipolar cell terminals relies strongly on sodium action potentials in ama-
These data suggest that voltage-gated sodium channels were
of TTX on the voltage-activated currents (data not shown).
We suspected that sodium currents might be involved in the
inward current, observed no effects between (2000).
In the presence of cobalt (4 mM) to block calcium
presence of voltage-gated sodium currents in bipolar cells,
crine cells. To test for this possibility, we looked for the
arise, in part, from decreased excitatory transmission to ama-
salamander bipolar cells express voltage-gated sodium chan-
Voltage-gated sodium channels have been identified on iso-
lated horizontal cells (Shingai and Christensen 1983; Ueda et
al. 1992) and on human rod photoreceptors (Kawai et al. 2001),
but there is no evidence that sodium currents shape light
responses of these neurons. If salamander photoreceptors or
horizontal cells express voltage-gated sodium channels, then
TTX could alter transmission from photoreceptors to second-
order neurons. TTX could act at photoreceptors to reduce
glutamate release and/or at horizontal cells, which are believed
to modulate glutamate release from cones through an ephaptic
feedback mechanism (Kamermans et al. 2001). To test for
these possibilities, we monitored photoreceptor output by rec-
ording light-evoked EPSCs from bipolar cells in the absence
and presence of TTX. The photoreceptor inputs to bipolar cells
were isolated by including (in μM) 5 strychnine, 200 bicucul-
line, 200 picrotoxin, and 40 d-AP5 in the bath. A subset of the
recordings was performed with the additional GABA-C an-
tagonist, I4AA (15 μM), in the control solution. When ON or OFF
bipolar cells were voltage clamped at either −60 or −70 mV,
their light-evoked EPSCs were unaffected by TTX (Fig. 2, A
and B). Figure 2C shows that TTX did not affect the light-
evoked EPSCs in six ON bipolar cells and in three OFF bipolar
cells. Although response fatigue sometimes occurred with
time, it was not coincident with application of TTX. These
results suggest that voltage-gated sodium channels do not alter
synaptic transmission from photoreceptors to bipolar cells in
agreement with previous work (Cook and McReynolds 1998b).
These data indicate that the reduction of light-evoked
GABAergic IPSCs in bipolar cells by TTX was not caused by
a decrease in transmission from photoreceptors to bipolar cells.
Salamander bipolar cells do not express voltage-gated sodium
channels
Voltage-gated sodium channels are expressed on certain
types of dissociated cone bipolar cells in rat and goldfish (Pan
and Hu 2000; Zenisek et al. 2001). If subpopulations of
salamander bipolar cells express voltage-gated sodium chan-
nels, then the effects of TTX on light-evoked IPSCs could
arise, in part, from decreased excitatory transmission to ama-
crine cells. To test for this possibility, we looked for the
presence of voltage-gated sodium currents in bipolar cells,
using a protocol similar to that described by Pan and Hu
(2000). In the presence of cobalt (4 mM) to block calcium
channels, we stepped from a holding potential of −80 mV to
between −35 and 0 mV. In 16 bipolar cells, we found no
evidence for an inward sodium current and observed no effects
of TTX on the voltage-activated currents (data not shown).
These data suggest that voltage-gated sodium channels were
not present on salamander bipolar cells we tested.

**TTX does not reduce glutamate release from photoreceptor terminals**

Voltage-gated sodium channels are identified on isolated horizontal cells (Shingai and Christensen 1983; Ueda et al. 1992) and on human rod photoreceptors (Kawai et al. 2001), but there is no evidence that sodium currents shape light responses of these neurons. If salamander photoreceptors or horizontal cells express voltage-gated sodium channels, then TTX could alter transmission from photoreceptors to second-order neurons. TTX could act at photoreceptors to reduce glutamate release and/or at horizontal cells, which are believed to modulate glutamate release from cones through an ephaptic feedback mechanism (Kamermans et al. 2001). To test for these possibilities, we monitored photoreceptor output by recording light-evoked EPSCs from bipolar cells in the absence and presence of TTX. The photoreceptor inputs to bipolar cells were isolated by including (in μM) 5 strychnine, 200 bicuculline, 200 picrotoxin, and 40 d-AP5 in the bath. A subset of the recordings was performed with the additional GABA-C antagonist, I4AA (15 μM), in the control solution. When ON or OFF bipolar cells were voltage clamped at either −60 or −70 mV, their light-evoked EPSCs were unaffected by TTX (Fig. 2, A and B). Figure 2C shows that TTX did not affect the light-evoked EPSCs in six ON bipolar cells and in three OFF bipolar cells. Although response fatigue sometimes occurred with time, it was not coincident with application of TTX. These results suggest that voltage-gated sodium channels do not alter synaptic transmission from photoreceptors to bipolar cells in agreement with previous work (Cook and McReynolds 1998b). These data indicate that the reduction of light-evoked GABAergic IPSCs in bipolar cells by TTX was not caused by a decrease in transmission from photoreceptors to bipolar cells.

**Salamander bipolar cells do not express voltage-gated sodium channels**

Voltage-gated sodium channels are expressed on certain types of dissociated cone bipolar cells in rat and goldfish (Pan and Hu 2000; Zenisek et al. 2001). If subpopulations of salamander bipolar cells express voltage-gated sodium channels, then the effects of TTX on light-evoked IPSCs could arise, in part, from decreased excitatory transmission to amacr ine cells. To test for this possibility, we looked for the presence of voltage-gated sodium currents in bipolar cells, using a protocol similar to that described by Pan and Hu (2000). In the presence of cobalt (4 mM) to block calcium channels, we stepped from a holding potential of −80 mV to between −35 and 0 mV. In 16 bipolar cells, we found no evidence for an inward sodium current and observed no effects of TTX on the voltage-activated currents (data not shown). These data suggest that voltage-gated sodium channels were not present on salamander bipolar cells we tested.

**TTX does not reduce spontaneous glutamate release from bipolar cells**

To obtain additional evidence that sodium channels were not present on bipolar cells, we determined whether TTX affected bipolar cell output. To assay glutamate release from bipolar cells, we recorded multiquantal spontaneous EPSCs (sEPSCs) from ganglion cells in the retinal slice. AMPA/KAIN receptor-mediated sEPSCs were isolated by including (in μM) 5 strychnine, 200 bicuculline, 200 picrotoxin, and 40 d-AP5 in the bath. Figure 3A shows that TTX had no effects on either the frequency or the amplitude of sEPSCs, suggesting that glutamate release from bipolar cells did not depend on regenerative sodium currents (control solution: 22.4 ± 4.7 events/s and 9.6 ± 1.5 pA; TTX solution: 25.1 ± 5.4 events/s and 9.7 ± 1.4 pA; n = 12). Similar results were reported by Tian et al. (1998)
TTX reduced spontaneous GABA release from amacrine cells

To determine whether the suppression of the light-evoked IPSCs by TTX was attributable to a reduction of amacrine cell signaling, we assayed GABA release from amacrine cells by recording spontaneous multiquantal GABAergic IPSCs in ganglion cells. Ganglion cell recordings were used to assess GABA release because the spontaneous IPSCs mediated by GABA<sub>C</sub> receptors on bipolar cells were difficult to resolve. GABA<sub>C</sub>-receptor-mediated sIPSCs were isolated by voltage clamping to 0 mV, the reversal potential for the EPSCs. Strychnine (5 μM) was included in the bath to block glycine receptors and D-AP5 (40 μM) was present reduce to occurrence of large spontaneous IPSCs. TTX reduced both the frequency and amplitude of spontaneous IPSCs in ganglion cells (Fig. 3B) in agreement with earlier studies (Protti et al. 1997; Tian et al. 1998) (control solution: 3.7 ± 1.3 events/s and 9.7 ± 2.8 pA; TTX solution: 0.98 ± 0.5 events/s and 5.4 ± 0.3 pA; n = 6). The apparent effect on amplitude most likely arises from presynaptic reduction in spontaneous multiquantal release not from a postsynaptic effect on GABA<sub>A</sub> receptors. These results confirm that GABA release from amacrine cells is highly dependent on spiking, whereas spontaneous glutamate release from bipolar cells does not appear to rely on TTX-sensitive sodium currents. Together, these data suggest that the suppression of evoked GABAergic IPSCs by TTX can be attributed to the blockade of sodium channels on amacrine cells and not to a reduction of bipolar cell glutamate release.

**Electrically evoked GABAergic IPSCs in bipolar cells depend on action potentials**

To more conclusively exclude the possibility of TTX acting upstream of the IPL, we used electrical stimuli (zaps) to directly depolarize bipolar cells, bypassing interactions in the OPL. Relative to the recorded bipolar cell, we administered zaps in either a local or lateral position in the OPL to determine whether there were differences in the degree of dependence on action potentials for signals transmitted over longer or shorter distances. Local and lateral zaps were ~60 and 300 μm from the recorded bipolar cell, respectively. GABAergic IPSCs were isolated by including strychnine (2 μM) in the control solution while voltage clamping to 0 mV.

Our data show that TTX strongly suppressed bipolar cell GABAergic IPSCs evoked by the lateral stimulus (Fig. 4, A and B, groups marked −bic). Interestingly, compared with laterally evoked responses, IPSCs evoked by the local zap depended significantly less on action potentials (Fig. 4, C and D, groups marked −bic). As in our light-evoked IPSC experiments, we controlled for potential network effects of TTX by blocking GABA<sub>A</sub> receptors with bicuculline (200 μM; see Fig. 4, B and D, groups marked +bic). As predicted, we found that zap-evoked GABA<sub>A</sub>-receptor-mediated IPSCs relied on action potentials and that this dependence was stronger for laterally than for locally evoked responses. These results confirm our findings that a significant portion of light-evoked GABAergic transmission in the IPL requires amacrine cell spiking and suggests a greater dependence on action potentials when the signal must travel over longer distances. Furthermore the very low sensitivity of locally evoked responses to TTX (compared with laterally evoked responses) suggests that bipolar to amacrine cell transmission does not strongly depend on action potentials.

Kainate puff-evoked GABAergic IPSCs in bipolar cells depend on action potentials

To directly determine whether the propagation of inhibitory signals in the IPL depended on spiking, we stimulated amacrine cell processes with focal puffs of kainate (1 mM) in the IPL. We applied kainate at sites local to (60 μm) and lateral to (300 μm) the recorded cell. Strychnine (3–5 μM) and D-AP5 (40 μM) were included in the control solution. Cells were voltage clamped close to the reversal potential for excitatory currents. Figure 5, A and B, shows IPSCs evoked with the lateral (A) and local (B) kainate puffs. In a sample of bipolar cells (Fig. 5C), TTX significantly suppressed the charge transfer and peak amplitude of laterally evoked IPSCs but had no significant effects on locally evoked currents. TTX did not
directly reduce excitatory currents in amacrine cells evoked by the kainate puffs (n = 7, data not shown), verifying that its main effects were to reduce the propagation of inhibitory signals. These data show that the suppression of light-evoked GABAergic inputs to bipolar cell terminals by TTX most likely arises from blockade of sodium channels on amacrine cell processes and not by a mechanism upstream of the amacrine cells. Furthermore, we demonstrated a difference in TTX sensitivity between local and lateral responses, suggesting that local signals do not depend on action potentials, whereas the transmission of lateral signals does.

Kainate puff-evoked GABAergic IPSCs in ganglion cells depend on action potentials

As noted in the preceding text, light-evoked GABAergic IPSCs in ganglion cells are sensitive to TTX (Bieda and Copenhagen 1999; Flores-Herr et al. 2001). In salamander, the propagation of lateral inhibition may be different for bipolar cells and ganglion cells. The GABA receptors, which mediate these two types of inhibition, are different. IPSCs in ganglion cells are mediated by GABA<sub>A</sub> receptors, whereas IPSCs in bipolar cells are mediated mainly by GABA<sub>C</sub> receptors (Dong and Werblin 1998; Ichinose and Lukasiewicz 2002; Lukasiewicz and Shields 1998). In addition, the amacrine cell types that contact bipolar and ganglion cells may be different. To compare lateral inhibition of ganglion cells to that of bipolar cells, we determined the TTX-sensitivity of ganglion cell GABAergic IPSCs evoked by locally or laterally stimulating amacrine cells with kainate puffs. Figure 6, A and C,
Our results suggest that the circuitry underlying the TTX-sensitive GABAergic lateral inhibition of salamander ganglion cells involves presynaptic inhibition of bipolar cell terminals in addition to the previously reported postsynaptic inhibition in ganglion cells (Bieda and Copenhagen 1999; Cook and McReynolds 1998a; Flores-Herr et al. 2001). Previous work has suggested an IPL component to bipolar cell surround responses. Łukasiewicz and Werblin (1994) recorded light-evoked GABA_C receptor-mediated IPSCs in bipolar cells, and subsequent studies demonstrated that depolarization of amacrine cells evoked synaptic GABAergic currents in salamander and ferret bipolar cells (Łukasiewicz and Shields 1998; Shields et al. 2000). Here, we extend these findings by showing that blockade of amacrine cell action potentials with TTX reversibly suppressed light-evoked GABAergic IPSCs in bipolar cells. This is the first demonstration of such a dependence on sodium spikes and suggests that the generation of GABAergic lateral inhibition in ganglion cells involves amacrine cell inputs to bipolar cell terminals. It is possible that other mechanisms besides spiking also contribute to the TTX dependence of lateral inhibitory signaling. Some amacrine cells also possess tonic TTX-sensitive sodium currents (Koizumi et al. 2001), which could boost their excitatory postsynaptic potentials (EPSPs), contributing to enhanced GABA release. These data, together with results from ganglion cells, are consistent with the existence of both pre- and postsynaptic components to the TTX-mediated suppression of ganglion cell surround inhibition.

Our results suggest that the main action of TTX was to block voltage-gated sodium channels on amacrine cells. First, when synaptic interactions in the OPL were bypassed by electrically stimulating bipolar cells, lateral inhibitory signaling was still suppressed by TTX. Second, IPSCs evoked by lateral kainate puffs were suppressed by TTX. The direct activation of amacrine cell inputs with lateral puffs of kainate allowed us to circumvent the bipolar cells. Finally, TTX reduced both the frequency and amplitude of sIPSCs, which reflect multiquantal release of GABA from amacrine cells. Taken together, these results suggest that TTX acted primarily on amacrine cells. However, as considered in the following text, neurons up-

**DISCUSSION**

Many ganglion cells display the classic center-surround antagonistic receptive field organization (Barlow 1953; Kuffler 1953). Traditionally, the ganglion cell surround was attributed to horizontal cell activity in the OPL (Werblin and Dowling 1969). This hypothesis was supported by experiments, which showed that injection of hyperpolarizing current into horizontal cells mimicked the surround response in ganglion cells (Mangel 1991; Naka and Witkovsky 1972). Although earlier studies suggested a role for amacrine cells in the formation of complex ganglion cell receptive fields (Caldwell et al. 1978; Werblin et al. 1988), the idea of an IPL component to the ganglion cell surround became widely accepted only recently. Several studies have demonstrated a TTX sensitivity of the inhibitory surround of third-order neurons in rabbit and mudpuppy retinas (Bloomfield and Xin 2000; Cook and McReynolds 1998a; Taylor 1999). These observations implicate amacrine cell interactions in surround inhibition, because horizontal cells do not spike, but many types of amacrine cells are known to utilize sodium action potentials (Bloomfield 1996; Cook and Werblin 1994; Miller and Dacheux 1976; Werblin 1977).

**Postsynaptic and presynaptic components of the TTX-sensitive ganglion cell lateral inhibition**

FIG. 6. Ganglion cell IPSCs evoked by stimulation of amacrine cells with KA depend on action potentials. A and B: TTX decreased IPSCs evoked by lateral (A) and local (B) KA puffs in the IPL. C: for laterally evoked currents, TTX reduced the charge transfer and peak amplitude to 10.6 ± 3.1 and 23.9 ± 5.4% of control values, respectively (n = 13, P < 4 × 10⁻⁶). Responses recovered to 70 ± 7% (charge) and 83 ± 5% (peak). The TTX-resistant component was larger for locally than for laterally evoked responses. Relative to control, TTX significantly decreased responses to 36.9 ± 13.0% (charge, P < 0.002, n = 6) and 48.4 ± 12.4% (peak, P < 0.002). Charge transfer and peak amplitude recovered to 85 ± 7 and 89 ± 7%, respectively. Compared with locally evoked GABAergic IPSCs, laterally evoked responses in the same ganglion cells (n = 6) were significantly more sensitive to TTX (charge, P < 0.03; peak, P < 0.02).

shows that TTX strongly suppressed laterally evoked IPSCs. Figure 6, B and C, demonstrates that TTX was less effective in suppressing GABAergic responses evoked by the local kainate puff, indicating that spiking is more important for lateral than local GABAergic transmission. Similar to our findings with bipolar cells, laterally evoked IPSCs in ganglion cells were more sensitive to suppression by TTX than were locally evoked responses. Compared with responses evoked with the lateral stimulus, the average charge transfer and peak amplitude of the TTX-insensitive component of locally evoked IPSCs were greater (n = 6). In both bipolar cells and ganglion cells, transmission of signals over longer distances relied strongly on spiking, but local signaling showed a lesser dependence on action potentials. Compared with locally evoked IPSCs in bipolar cells, however, these local inhibitory responses in ganglion cells were more sensitive to TTX.
stream of amacrine cells may possess voltage-gated sodium channels.

Voltage-gated sodium channels in other retinal neurons

There are reports of voltage-gated sodium channels in certain classes of photoreceptors, horizontal cells, and cone bipolar cells in other species (Kawai et al. 2001; Pan and Hu 2000; Shingai and Christensen 1983; Ueda et al. 1992; Zenisek et al. 2001). In the salamander, these channels have been observed on ganglion and amacrine cells but never on other retinal neurons (Barnes and Werblin 1986; Lukasiewicz and Werblin 1988). Our results suggest inhibitory, lateral transmission in the IPL depended on spiking in wide-field amacrine cells and not on sodium channel activity in photoreceptors, horizontal cells, or bipolar cells, as we detail in the following text.

Photoreceptors and horizontal cells. Because functional voltage-gated sodium channels have been found on photoreceptors (Kawai et al. 2001) and isolated horizontal cells (Shingai and Christensen 1983; Ueda et al. 1992), we considered the possibility that TTX could alter photoreceptor output. Our recordings from bipolar cells showed that TTX did not affect their light-evoked EPSCs, indicating that photoreceptor to bipolar cell transmission did not depend on regenerative sodium currents. Consistent with these findings, Cook and McReynolds (1998) showed that TTX did not affect voltage responses to light in second-order retinal neurons in salamander.

Bipolar cells. Certain classes of cone bipolar cells in the rat and goldfish express voltage-gated sodium channels (Pan and Hu 2000; Zenisek et al. 2001). It was postulated that these channels could aid in boosting graded excitatory synaptic potentials. We never observed voltage-gated sodium currents in salamander bipolar cells, but we routinely observed these currents in amacrine and ganglion cells. We cannot exclude the possibility that some salamander bipolar cells possess TTX-sensitive sodium channels. If these channels are present in salamander bipolar cells, then TTX should reduce excitatory signaling between bipolar cells and third-order cells. The evidence from experiments on salamander retina does not support this notion. Cook and colleagues (Cook and McReynolds 1998a; Cook et al. 1998) showed that light-evoked excitatory responses in ganglion cells were not reduced by TTX. Our results also suggest that bipolar cell transmission did not depend on regenerative sodium channels. Spontaneous glutamate transmission from bipolar cells to ganglion cells was not affected by TTX in agreement with earlier studies in mouse (Tian et al. 1998). Also, locally evoked amacrine cell IPSCs, elicited by electrical stimulation of bipolar cells, were not suppressed by TTX (Fig. 4C), suggesting that bipolar cell to amacrine cell transmission did not depend on regenerative sodium currents. These findings suggest that glutamate release from salamander bipolar cells does not strongly depend on voltage-gated sodium channels.

Lateral signaling

The GABAergic amacrine cell population in salamander is morphologically diverse with the processes of different amacrine cells extending laterally over a narrow or wide region of the IPL (Yang et al. 1991). Distinct amacrine cell subtypes may make connections with bipolar and ganglion cells in the salamander retina. Furthermore, wide- and narrow-field classes of amacrine cells may vary in their reliance on action potentials for transmitter release. Here we show that GABAergic signaling to bipolar cells depended more strongly on action potentials when amacrine cells were excited distally. It is likely that the lateral kainate puff and electrical stimulation predominantly evoked GABA inputs from wide-field amacrine cells, which utilize spikes to propagate signals across their dendritic arborers (Barnes and Werblin 1987; Bloomfield 1996; Cook and Werblin 1994).

A general feature of lateral inhibition in the salamander inner retina may be its dependence on action potentials. GABAergic, lateral transmission to ganglion cells (Cook and McReynolds 1998a) and to bipolar cells (reported here) depend on action potentials. Change-sensitive inhibition in salamander ganglion cells is another type of lateral inhibition that depends on action potentials (Cook et al. 1998). Wide-field, glycinergic amacrine cells mediate this inhibition. Similar to our results, glycinergic transmission elicited by lateral electrical stimulation was more dependent on spiking compared with locally evoked transmission (Cook et al. 1998).

Synaptic inputs to amacrine cells may influence lateral inhibitory signaling. Serial inhibitory circuits composed of GABAergic and glycinergic amacrine cells limit the extent of inhibition at bipolar cell terminals (Roska et al. 1998; Zhang et al. 1997). It is possible that these serial circuits may be sensitive to TTX. However, when serial inhibition was blocked with bicusculine and strychnine, TTX still reduced lateral inhibitory signals to bipolar cells, indicating that serial signaling did not play a large role in our preparation. Glycine receptors were always blocked in our experiments to isolate the GABA-mediated IPSCs. Its possible that glycine receptor blockade may have biased inner retinal signaling to favor TTX sensitive lateral signaling. Although we cannot rule out this possibility, recent work suggests this is not the case. The spike-dependent surround inhibition to ganglion cells, which was mediated by GABAergic amacrine cells, was shown to be insensitive to strychnine (Cook and McReynolds 1998a). This demonstrates that GABAergic lateral signaling was not affected by glycinergic inputs.

Local signaling

The local stimulus elicited GABAergic signals, which did not depend strongly on regenerative sodium currents. Local inhibition, which was relatively insensitive to TTX, could be attributed to either the activation narrow-field amacrine cells or the activation of distal processes of wide-field amacrine cells. The effect of TTX on locally (kainate puff) evoked IPSCs in bipolar cells was insignificant, whereas the effect on ganglion cell responses was significant. This could reflect different amacrine cell populations, which provide local inputs to bipolar and ganglion cells. Amacrine cells making local contacts onto ganglion cell dendrites may depend on sodium spikes for GABA release, whereas amacrine cells making local synapses onto bipolar cell terminals do not depend on action potentials for transmitter release. A second explanation is that ganglion cell dendritic arborers are typically much broader than bipolar cell axon terminals. The local stimulus may activate amacrine cell processes that traverse some distance before contacting the

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ganglion cell. The local stimulus for ganglion cells therefore may be more distal than it is for bipolar cells.

In summary, our results suggest that lateral inhibition at bipolar cell axon terminals depends on TTX-dependent, voltage-gated sodium currents. Previous work has shown that a wide field GABA_A receptor-mediated input to ganglion cells depends on action potentials and mediates a large component of the surround response (Cook and McReynolds 1998a). Our results suggest that a wide-field inhibition of bipolar cell terminals may also contribute to the ganglion cell surround.

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