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Retinal input to efferent target amacrine cells in the avian retina

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Abstract

The bird visual system includes a substantial projection, of unknown function, from a midbrain nucleus to the contralateral retina. Every centrifugal, or efferent, neuron originating in the midbrain nucleus makes synaptic contact with the soma of a single, unique amacrine cell, the target cell (TC). By labeling efferent neurons in the midbrain we have been able to identify their terminals in retinal slices and make patch clamp recordings from TCs. TCs generate Na⁺ based action potentials triggered by spontaneous EPSPs originating from multiple classes of presynaptic neurons. Exogenously applied glutamate elicited inward currents having the mixed pharmacology of NMDA, kainate and inward rectifying AMPA receptors. Exogenously applied GABA elicited currents entirely suppressed by GABAzine, and therefore mediated by GABA_A receptors. Immunohistochemistry showed the vesicular glutamate transporter, vGluT2, to be present in the characteristic synaptic boutons of efferent terminals, whereas the GABA synthetic enzyme, GAD, was present in much smaller processes of intrinsic retinal neurons. Extracellular recording showed that exogenously applied GABA was directly excitatory to TCs and, consistent with this, NKCC, the Cl⁻ transporter often associated with excitatory GABAergic synapses, was identified in TCs by antibody staining. The presence of excitatory retinal input to TCs implies that TCs are not merely slaves to their midbrain input; instead, their output reflects local retinal activity and descending input from the midbrain.

Keywords

retina; centrifugal neurons; amacrine cell; neurotransmitter; chicken

Introduction

In the retinas of birds, a large calyx synapse is formed between the “restricted” type of efferent fiber (rEF) and the soma of an unusual amacrine cell, originally called an “association amacrine cell” by Cajal (Ramón y Cajal, 1889; Uchiyama & Stell, 2005). More recently these amacrine cells have been called efferent target amacrine cells, or simply, target cells (TCs) and it is now known that their presynaptic partners, the rEFs, originate in the isthmo-optic nucleus (ION) of the midbrain (Cowan *et al.*, 1961; Cowan & Powell, 1963). The ION takes its input largely from the ipsilateral optic tectum and, in ground feeding birds, sends out approximately 8000 myelinated efferent fibers to the contralateral retina where they fan out to make one-to-one synapses with TCs (Cowan & Powell, 1963; Hayes & Holden, 1983; Fritzsche *et al.*, 1990; Nickla *et al.*, 1994; Lindstrom *et al.*, 2009). The light responses and receptive field properties of ION neurons have been characterized in a number of studies and, based on these, it seems that the centrifugal, or efferent, system is part of a homotopic loop for rapid feedback of visual signals to the retina (Holden, 1968;

Holden & Powell, 1972; Miles, 1972c). Some direct studies examining the influence of the ION on retinal responses support this view (Miles, 1972a; Pearlman & Hughes, 1976b; Uchiyama & Barlow, 1994; Li *et al.*, 1998) but the true function of this efferent system, nevertheless, remains uncertain.

This efferent system, by providing input to a lower processing level, the retina, from a higher level, the ION, constitutes an example of the descending input that forms a common motif within the central nervous system and about which little is understood. It remains to be seen how much the organization and function of this loop within the visual system typifies those seen elsewhere in the brain, but as a model it has the practical advantages of accessibility and well-defined anatomy.

In this study we report the first physiological recordings from TCs in retinal slices and combine these with immunohistochemistry to uncover the likely identity of the transmitter used by efferent fibers. Suggested candidates for this role are acetylcholine (Nickla *et al.*, 1994), glutamate (Miles, 1972a; Fischer & Stell, 1999) or, alternatively, an inhibitory transmitter (Pearlman & Hughes, 1973; Holden, 1978). In addition to a conventional transmitter whose presence is indicated by the clouds of vesicles surrounding the numerous active zones of the rEF terminals (Lindstrom *et al.*, 2009), there is evidence that nitric oxide also plays a role at this synapse. Both the rEF and TC express nitric oxide synthase (Morgan *et al.*, 1994; Goureau *et al.*, 1997; Fischer & Stell, 1999; Posada & Clarke, 1999; Rios *et al.*, 2000; Lindstrom *et al.*, 2009).

The anatomy of TCs is extraordinary. They do not have a typical dendritic tree, instead the large soma gives rise to a compact basket network of dendrites extending no more than a few μm . This diminutive dendritic zone constitutes a private neuropil since it lies in the Inner Nuclear Layer (INL), the cellular layer of the inner retina, rather than the Inner Plexiform Layer (IPL) where neurons characteristically make synapses (Lindstrom *et al.*, 2009). A single axon leaves the soma, which is invariably located in the ventral retina, and typically runs a great distance in the retina before terminating in a small field of synapses with neurons that are probably amacrine cells (Ramón y Cajal, 1995; Uchiyama *et al.*, 2004). Cajal classified TCs as amacrine cells but their uniqueness has prompted others to suggest they deserve their own class (Uchiyama & Stell, 2005). The function of these neurons is unknown but they clearly constitute a crucial component of the efferent system. TCs have commonly been thought of as slave or relay neurons, driven exclusively by input from efferent fibers (Uchiyama & Stell, 2005) and in turn passing these signals to their postsynaptic partners in the dorsal retina (Uchiyama *et al.*, 2004). In a recent study, however, we showed ultrastructural evidence for at least one additional type of synaptic input to TCs originating from neurons within the retina (Lindstrom *et al.*, 2009). From the pleomorphic appearance of synaptic vesicles in these other presynaptic structures, we speculated that they might be GABAergic. We show here, using pharmacology, that there is indeed more than one synaptic input to TCs, and at least part of the retinal input is mediated by GABA, even though it is excitatory.

Methods

Ethical approval

A total of 141, 2-3 week-old white leghorn chickens (*Gallus gallus*) were used in this study. Chickens were hatched from eggs obtained from the Avian Sciences Facility of the University of California, Davis. All methods were reviewed and approved by the Institutional Animal Care and Use Committee.

Labeling of efferent fibers and preparation of slices

The labeling of efferent fibers by injection of Fluoro-Ruby into the ION followed the procedure described previously (Lindstrom *et al.*, 2009) and shown diagrammatically in Figure 1A. Following a minimum survival period of 3 days, the chicken was euthanized by intraperitoneal injection of a lethal dose of pentobarbital (Beuthanasia-D, Webster Veterinary, 047305). The right eye was quickly removed and chilled on ice for approximately 30 sec, then hemisected just posterior to the ora serata. The anterior chamber and vitreous were removed, and the posterior eyecup was placed in chilled, Ca^{2+} -free Hank's solution (GIBCO, 14170). Two squares, corresponding to the regions of the retina where rEFs are found in highest density, were cut out of the eye cup using a scalpel. They were created by making a dorsal-ventral cut along each side of the pecten and a temporal-nasal cut 1 mm dorsal to the end of the pecten. The most ventral and peripheral portions of the eyecup were removed, leaving two 5 mm $\times$ 5 mm squares; one from the nasal retina, and one from the temporal retina. Each square was further cut into 4 equal squares, which were stored in oxygenated (5% carbon dioxide, 95% oxygen) normal external solution ([mM]: NaCl 100, KCl 6, CaCl_2 1, MgSO_4 2, NaHCO_3 30, Glucose 50) at room temperature until slicing.

Immediately prior to slicing, each retinal square was first attached to a piece of Millipore filter (Millipore; mixed cellulose ester, 0.8 μm pore; AAB02500), ganglion cell side down. After removing the sclera and retinal pigmented epithelium, a manual tissue slicer was used to create retinal slices of 100-300 μm thickness. Slicing was performed in Ca^{2+} -free Hank's solution. Slices of the desired thickness were positioned on a coverslip using Apeizon T grease, mounted in a recording chamber (Warner Instruments, RC26GLP), and rinsed with oxygenated (5% carbon dioxide, 95% oxygen) normal external solution.

Slice recording

The recording chamber was mounted in an Olympus upright fluorescence microscope (BX51W1) and continuously perfused with warmed (34°C) and oxygenated (5% carbon dioxide, 95% oxygen) external solution, either Normal or a High Mg^{2+} /0 Ca^{2+} external solution ([mM]: NaCl 90, KCl 6, MgCl_2 8, MgSO_4 2, NaHCO_3 30, Glucose 50) that was used to block synaptic transmission (Masland & Ames, 1976). External solutions could be rapidly changed using a multi-well gravity fed perfusion system. Drug solutions were prepared as stock solutions in water (except GYKI-53655, which was prepared in DMSO), then diluted in the appropriate external solution. Stock solutions were aliquotted and stored at -20°C until use.

GABA receptor activity was examined using 10-100 μM (-)-Bicuculline methobromide (Biomol, EA-109) or methiodide (Sigma, B6889), a GABA_A receptor antagonist; 20 μM GABAzine (SR 95531 hydrobromide), a competitive GABA_A receptor antagonist (Fischer or Sigma); and 10-50 μM TPMPA ((1,2,5,6-tetrahydropyridin-4-yl)methylphosphinic acid), a competitive GABA_C receptor antagonist (Tocris, 1040). Glutamate receptor activity was examined using 10 μM GYKI 53655 (the generous gift of Dr. Matt Frerking) which, at this concentration, will block >90% of the AMPA current without significantly affecting the kainate or NMDA currents (Paternain *et al.*, 1995; Wilding & Huettnner, 1995); 10 μM NBQX (1,2,3,4-Tetrahydro-6-nitro-2,3-dioxobenzo[f]quinoxaline-7-sulfonamide; Sigma, N183), an antagonist of AMPA and kainate receptors; and 20 μM APV (D-(-)-2-Amino-5-phosphonopentanoic Acid; Calbiochem), a competitive NMDA receptor antagonist. DNQX (6,7-Dinitroquinoxaline-2,3-dione; Tocris, 2312), was also used as an antagonist for AMPA and kainate receptors at a concentration of 20 μM , substantially lower than the IC_{50} for binding at the glycine site of native NMDA receptors (Randle *et al.*, 1992). In order to

examine the action potentials produced by TCs, Na⁺ channels were reversibly blocked with 0.1 μ M Tetrodotoxin (TTX).

Images of slices were acquired using a cooled infrared sensitive camera (Q Imaging; Rolera) and displayed using QCapture Pro software. Fluoro-Ruby labeled efferent terminals were detected using illumination from a mercury source (OSRAM, HBO 103W/2) and a rhodamine filter cube (Chroma 41002a; 535/50 BP excitation filter, 620/60 BP emission filter). Once an efferent terminal was located, infrared differential interference contrast (IR-DIC) was used to locate the soma of the TC and position the patch-electrode and puff-pipet as illustrated in Figure 1B.

Patch-electrodes pulled from borosilicate glass tubing (1.5 mm O.D., 0.86 mm I.D.; Sutter Instruments) had a tip resistance of 5-10 M Ω when measured in the bath. Typically, patch-electrodes were filled with Cs methanesulfonate (CsMs) internal solution ([mM]: CsCl 9, Cs methanesulfonate 131, EGTA 1.1, HEPES 10, MgCl₂ 2, CaCl₂ 0.1). In a few initial recordings, patch-electrodes were filled with KCl internal solution ([mM]: KCl 140, EGTA 1.1, HEPES 10, MgCl₂ 2, CaCl₂ 0.1). To confirm the identity of recorded cells, all internal solutions also contained Lucifer Yellow (0.02 %; Sigma), allowing imaging with a Lucifer Yellow filter cube (Chroma 31010; 425/40 BP excitation filter, 540/50 BP emission filter). The experimentally determined liquid junction potential between normal external and CsMs internal solutions was approximately -8.5 mV. All voltages have been corrected for this junction potential. The measured series resistance for these experiments was 17.1 ± 6.6 M Ω (mean $\pm$ StDev).

Puff-pipets were filled with normal external solution containing either 100 μ M glutamate (Fischer); 150 μ M GABA (Fischer); or 100 μ M Epibatidine (Sigma, E1145), a nicotinic acetylcholine receptor antagonist. In the High Mg²⁺ control experiments the puffed drug was dissolved in High Mg²⁺/0 Ca²⁺ external solution.

Data analysis

Except for recordings of the relatively slow responses to puffs of glutamate, GABA, or epibatidine, which were low-pass filtered at 100 Hz, all intracellular recordings were filtered with a 1500 Hz low-pass Gaussian filter implemented in software. Extracellular recordings were typically band-pass filtered at 40-1000 Hz. Event timing and the peak amplitude of PSCs were determined using Clampfit's threshold search function.

PSC rise times and decay constants (τ) were determined by first averaging the traces of 10 or more individual PSCs (aligned by their peaks). The decay phase of the resultant trace was fitted with the sum of two exponentials, using SigmaPlot's fitting function to determine τ_1 and τ_2 . The rise time was the time required for the average PSC trace to rise from 10-90% of the peak amplitude.

To determine the reversal potentials of the GABA- and glutamate-puff responses, the current-voltage (I-V) relation for each cell was fitted with a linear (GABA) or quadratic polynomial function (glutamate), and the point where the fit crossed the voltage axis was taken as the reversal potential. In the case of GABA, the entire I-V relation was fitted. However, for glutamate, only the portion of the I-V relation from the negative peak produced by the voltage dependence of the Mg²⁺ block of NMDA receptors (approx. -30 mV) to 50 mV was fitted.

SigmaStat software was used for all statistical analyses. The mean response values (frequency, amplitude, and latency) were calculated for each condition (Normal, Drugs, and Wash) for each cell. Given the large variability in Normal frequency and amplitude between

cells, these two response values were typically normalized prior to statistical analysis. Unless indicated otherwise, one-way repeated measures ANOVAs were performed to compare the mean response values for each condition for the number of cells indicated (n). Where appropriate, data are presented as mean $\pm$ standard deviation. For those experiments in which we examined the effect of glutamate receptor antagonists on PSC frequency, the distribution of frequency values did not pass the Normality test, so a Friedman repeated measures ANOVA on ranks was used (results are reported as the median and 25th–75th percentile range).

Tissue preparation for fluorescence microscopy

Following a minimum survival period of 3 days from the injection of label, animals were euthanized with a lethal dose of pentobarbital (Beuthanasia-D, Webster Veterinary, 047305). Once the animal was deeply anesthetized, as assessed by a lack of the toe pinch reflex, it was transcardially perfused with phosphate buffered saline (PBS; Sigma) followed by 4% paraformaldehyde (Sigma, P6148) in PBS. The right eye was removed and hemisected. The anterior chamber and vitreous were discarded, while the retina and sclera were postfixed in chilled 4% paraformaldehyde for 20 min (for GluR1, GluR2/3, or GABA_A receptor antibodies) or 1-2 hrs (for all other antibodies). In cases where Fluoro-Ruby labeled efferent terminals were not required (e.g. double labeling with parvalbumin antibodies), the left eye was also removed and fixed in the same manner.

The eyecup was rinsed with PBS and cut into the high EF density squares described in slice preparation, separated from the sclera, and placed in 30% sucrose solution at 4°C until equilibrated (typically overnight). The following day they were embedded in OCT medium (Ted Pella, 27050), frozen on dry ice, and stored at –20°C until sectioning. Sections of 12–20 μ m were cut on a cryostat (Leica CM1900) and stored at –20°C.

Retinal sections were processed using standard immunohistochemistry techniques. Autofluorescence was quenched using 1% glycine in 0.3% Triton-PBS for 15 min. A blocking solution containing 2–10 % normal goat serum, 2–4% bovine serum albumin, and 0.3 % Triton in PBS was applied for 1 hr at room temperature. Sections were incubated with primary antibodies diluted in blocking solution for 16–24 hrs at 4°C, washed 3x in PBS (40, 20, 20 min), then incubated with secondary antibodies diluted in PBS or blocking solution for 1–2 hrs at room temperature. The sections were then washed with PBS and coverslipped with VectaShield hardset mounting medium. There were three cases in which this standard procedure was slightly modified. First, in sections labeled with anti-GABA_A-R α 1-subunit or anti-vGluT1 antibodies, 0.1% Tween-20 was substituted for Triton. Second, for sections labeled with the anti-ChAT antibody, 8% normal donkey serum was used instead of normal goat serum. Third, sections labeled with the NKCC antibody underwent a 5-min pre-treatment with 1% SDS (Bio-Rad Laboratories, 161-0300) in PBS between the quenching and blocking steps. Sections were blocked for 30 min in a solution containing 10% normal goat serum, 2% BSA, 0.3% Triton, 1% Saponin (Sigma, 16109) and 0.2% SDS, and were incubated with the NKCC antibody diluted in block for 1 hr. All subsequent washes and secondary antibody application followed the standard procedure outlined above. Typically, rabbit anti-tetramethylrhodamine (TMR) or mouse anti-Rhodamine was used to amplify the signal associated with Fluoro-Ruby labeled rEFs. Secondary controls (in which retinal sections were only incubated with the secondary antibody) were performed with each procedure to provide a measure of background fluorescence.

A list of the primary antibodies used, their dilutions and their source can be found in Table 1. All secondary antibodies (Goat anti-Mouse, Goat anti-Guinea Pig, Goat anti-Rabbit, Donkey anti-Goat, Donkey anti-Mouse, and Donkey anti-Rabbit) were obtained from

Invitrogen and were used at a dilution of 1:500. Alexa 488 or 568 conjugates were used as appropriate.

Immunohistochemistry imaging

Stained sections were examined using an inverted confocal microscope (Olympus FLUOVIEW) with krypton (488 nm) and argon (568 nm) lasers. Images were converted to 24-bit TIF images in Fluoview software, and then transferred to Adobe Photoshop for adjustment of brightness, contrast and sharpness. Images of antibody labeled tissue were always processed with images of the corresponding secondary control to assure that these adjustments did not produce false results. The images of the secondary controls are not shown, but they typically look black, except for some autofluorescence in the photoreceptor outer segments and oil droplets.

Results

Identification of target cells

To acquire patch-clamp recordings from TCs, we made retinal slices from 141 chickens, using the regions of high rEF density as defined in the density map of Lindstrom *et al* (2009). Three days before retinal slices were made, the contralateral ION was labeled with Fluoro-Ruby, (Figure 1), which allowed sufficient time for the label to be transported to the rEF terminals in the retina. Depending on the fraction of ION neurons labeled, a typical retinal slice contained between 2 and 10 labeled terminals that were accessible to the patch electrode. Since rEF terminals form an encircling pericellular nest around their postsynaptic partner (Lindstrom *et al.*, 2009), we were able to infer the location of the postsynaptic element and image its outline with IR-DIC optics. As we expected from anatomical studies, the postsynaptic element (revealed by Lucifer yellow filling from the patch pipet) was the soma of a TC. These unusual cells were unambiguously distinguished by their unique morphology; a relatively large, prolate soma, a compact basket of short, anastomosing dendrites, and an axon that typically ran along the border of the INL and IPL until reaching an edge of the slice (Figure 1C).

Although IR-DIC optics clearly revealed TCs, a relatively low success rate in patching these cells was largely due to their tight investment by Muller cells, as shown by electron microscopy (Lindstrom *et al.*, 2009). Nevertheless, 181 TCs were patch clamped, of which 89 survived long enough to contribute data to one or more of the experiments described below. Only in one instance did we observe (but did not record from) another cell type as the postsynaptic element to a labeled rEF. From its large, round, pale soma this cell was a displaced ganglion cell. This finding reveals that DGCs do receive rEF input, but only rarely (only 1 in >500 observed rEF terminals), meaning that DGCs are not a principal recipient of rEF input as was claimed in pigeon (Maturana & Frenk, 1965).

TCs are spiking neurons

Upon membrane rupture, TCs were seen to have a resting membrane potential (V_{Rest}) of -62 ± 7 mV ($n=7$ cells, with KCl internal) and a membrane capacitance of 7.4 ± 2.3 pF ($n=76$). A characteristic feature of these cells is that in current clamp they typically displayed a barrage of spontaneous, depolarizing synaptic potentials upon which action potentials frequently rode (Figure 2A).

We analyzed the characteristics of spontaneous postsynaptic potentials (PSPs) in seven current-clamped TCs using KCl internal solution ($E_{Cl} +3.3$ mV). The mean PSP amplitude and frequency were 6.2 ± 1.6 mV and 6.6 ± 2.1 Hz, respectively. We also examined the properties of action potentials (APs), which in 4 of these cells were readily distinguished

from PSPs by their rapid kinetics and larger amplitudes (inset in Figure 2A). Rise times for each cell were determined by averaging isolated events aligned to their peaks and measuring time from 10-90% of peak. The mean PSP rise time was 2.8 ± 0.9 msec. Action potentials had a significantly larger amplitude (43.2 ± 13.1 mV) and shorter rise time (0.6 ± 0.1 msec) than the PSPs. AP frequency was 2.5 ± 1.7 Hz, approximately one-third of the PSP frequency ($n=4$ cells). Prior to membrane rupture, action potentials could be recorded in loose-patch configuration (Figure 2B) and were seen at a frequency of 2.2 ± 1.4 Hz ($n=6$ cells), not significantly different from that seen in the whole-cell current-clamp recordings described above ($p=0.768$). We infer from this that membrane rupture, when using the KCl internal solution, did not substantially change the excitability of these cells or their synaptic input.

In voltage clamp mode, spontaneous post synaptic currents (PSCs) were seen, and some of the larger ones generated action currents, though at a much reduced frequency of 0.4 ± 0.5 Hz (same 4 cells used to analyze AP frequency in current clamp). A +20 mV step from the holding voltage (V_H) of -70 mV (Figure 2C) typically produced an action current that could be blocked with $0.1 \mu\text{M}$ TTX (Figure 2C; $n=4$ cells). TTX was also capable of blocking action currents generated by spontaneous PSCs. We infer that a sodium based action potential is initiated in a poorly voltage-clamped region, very likely the narrow tapering initial segment of the axon, and thus it is not blocked by the V_H of -70 mV. TTX did not eliminate all synaptic input to the TC (Mean PSC frequency in TTX relative to Normal: 0.32 ± 0.1 , Wash 0.83 ± 0.72).

TCs respond to putative transmitters

For the remainder of this study we used CsMs internal solution, which had the effect of linearizing the I-V relations for TCs by suppressing a large outward current elicited by depolarization. Cesium also caused the zero current potential (resting membrane potential) to shift from approximately -60 mV (reported above) to about -20 mV, presumably by suppressing K^+ current activated at rest. Spontaneous inward PSCs were visible at -70 mV with CsMs internal solution. An important attribute of the CsMs internal solution was that by having low internal Cl^- ($E_{\text{Cl}} = -56$ mV), it allowed the separation of cation and anion carried currents, although, as we show, the undisturbed value for E_{Cl} is likely to be significantly more positive than this.

The external solution for many of the experiments contained High $\text{Mg}^{2+}/0 \text{ Ca}^{2+}$ so that we could be assured that drug effects represented direct effects on TCs. This external solution reversibly eliminated virtually all spontaneous PSCs in our cells (Figure 2D). PSC frequency, 9.3 ± 7.7 Hz in Normal, was reduced to 0.34 ± 0.42 Hz in High Mg^{2+} external solution ($p<0.01$; $n=11$ cells). PSC frequency returned to 6.0 ± 7.3 Hz in Wash.

To determine which neurotransmitter receptors are expressed by TCs, voltage-clamp recordings were obtained from a TC while puffs of neurotransmitter agonists (glutamate, GABA, or epibatidine) were applied to the soma at a minimum interval of 1 sec. Peak currents were calculated from the average of 2 - 10 responses in each cell and, in order to determine whether these drug responses were direct or indirect effects, we compared these responses to those obtained in High Mg^{2+} external solutions in which synaptic transmission was suppressed.

TCs have functional glutamate receptors—Brief (20 msec) puffs of glutamate were applied to the soma. In Normal external solution, puffs of $100 \mu\text{M}$ glutamate reliably evoked inward currents (-90 ± 78 pA) at the holding potential of -20 mV ($n=12$ cells). A typical puff response is shown in Figure 3A. Since some of the recorded TCs were on the surface of the slice, while others were as many as three cell layers deep, some of the variability in puff

response amplitude is attributable to differences in the concentration of glutamate reaching the TC soma. Figure 3B shows that the peak amplitude of the glutamate-puff response was not significantly reduced in High $Mg^{2+}/0\ Ca^{2+}$ external solution ($p=0.309$, $n=5$ cells, data normalized to the response in High Mg^{2+}). Thus the glutamate-puff response, at least the slow component illustrated in Figure 3A, is due to activation of glutamate receptors on the TC itself, not through an indirect interaction with a neighboring cell.

We evaluated the contribution of AMPA, kainate, and NMDA receptors to the glutamate puff response using antagonists alone and in combination. For the cell shown in Figure 3A, 10 μM GYKI-53655, an AMPA receptor selective antagonist (Paternain *et al.*, 1995; Wilding & Huettner, 1995), had little effect on the amplitude of the glutamate puff response at $-20\ mV$, but 20 μM DNQX (an antagonist of both AMPA and kainate receptors) significantly reduced the response amplitude when applied alone. When 20 μM APV (an NMDA receptor selective antagonist) was subsequently applied in the presence of DNQX, the glutamate puff response was completely eliminated. Washing reversed the effects of these antagonists.

Similar results were recorded from 7 other TCs in two sets of experiments; one performed in Normal external and another in High $Mg^{2+}/0\ Ca^{2+}$ external. Two-way repeated measures ANOVA applied to the results obtained in Normal and High $Mg^{2+}/0\ Ca^{2+}$ external showed that all significant differences were attributable to the drug condition (i.e. the presence or absence of one or more of the glutamate antagonists mentioned; $p<0.001$). No significant differences were associated with the type of external solution used (i.e. Normal or High Mg^{2+} ; $p=0.245$), nor was there an interaction between the type of external solution and the drug condition ($p=0.378$). For this reason, the effects of these glutamate antagonists on the glutamate puff response were evaluated using data pooled from both Normal and High $Mg^{2+}/0\ Ca^{2+}$ experiments and are summarized in Figure 3C. These results would seem to indicate that although kainate and NMDA receptors are present on TCs, AMPA receptors are absent. However, we will show that AMPA receptors are in fact present but are not apparent at all voltages.

Current-voltage (I-V) relations for the glutamate-evoked current were obtained using voltage ramps ($-90\ mV$ to $+50\ mV$ in 200 msec) before, during (250 msec after the start), and ~ 3 sec after a puff of glutamate (inset Figure 3D), when the evoked current had returned to baseline. This protocol was typically repeated twice and averaged for each condition (normal, antagonist, and wash). Prior to each voltage ramp, TCs were held at $-20\ mV$, thereby inactivating the voltage-gated Na^+ current. The glutamate-evoked current was determined as a subtraction of averaged ramp-currents with and without glutamate puff.

In normal external, the glutamate I-V showed strong outward rectification (black trace in Figure 3D), and a reversal potential (E_{Glu}) of $-6.3 \pm 3.4\ mV$ ($n=10$ cells). This was somewhat more negative than the E_{Glu} of 0 mV expected if the glutamate channels present were equally permeable to all cations. This discrepancy is probably due to the somewhat greater permeability of glutamate channels to Cs^+ relative to Na^+ (Hille, 2001). Although in the prior experiments at a fixed V_H of $-20\ mV$ we saw no evidence for the presence of AMPA receptors, in these voltage ramp measurements, GYKI-53655 clearly reduced the glutamate puff response at voltages negative to $-40\ mV$ (blue trace in Figure 3D). By appropriate current subtractions we obtained the I-V relations for the AMPA-mediated current (Figure 3E) which is strongly inward rectifying. Glutamate receptors, including GluR1, GluR3 and GluR4 subunits, show strong inward rectification and Ca^{2+} permeability if these subunits are expressed in the absence of GluR2 receptors, or if the GluR2 receptors have been edited to have a glutamine (Q) at the Q/R site (Hollmann *et al.*, 1991; Verdoorn *et*

al., 1991). We infer from the observed strong inward rectification of AMPARs in TCs that they exhibit Ca^{2+} permeability.

Blocking the AMPA-mediated component revealed a negative slope, between -80 and -30 mV (Figure 3D, blue trace), indicative of the voltage dependent relief from Mg^{2+} -block that is typical of NMDA receptors (Mayer *et al.*, 1984). Blocking AMPA, kainate, and NMDA receptors with the combination of DNQX and APV practically eliminated the glutamate-evoked current, except at the most positive voltages (Figure 3D). In all cells but one, the effect of DNQX and APV was reversed in Wash. Based on these results it appears that TCs express functional AMPA, kainate, and NMDA receptors, and that as a consequence of their complementary I-V relations, glutamate produces a current that is largely independent of voltage over most of the TC's operating range.

TCs fail to respond to a nicotinic agonist—A study by Nickla *et al* (1994) suggests that TCs and/or rEFs express the $\alpha 7$ subunit of the nicotinic acetylcholine receptor (nAChR). We therefore examined TC responses to puffs of epibatidine, a nicotinic agonist. In 4 cells with normal rates of spontaneous PSCs recorded in Normal external solution, TCs failed to respond to brief (20 msec; Figure 4A) or long (up to 540 msec) puffs of $100 \mu\text{M}$ epibatidine. Given that epibatidine is a strong agonist (activation in the nM and low μM range) of all nAChRs (Gerzanich *et al.*, 1995) we conclude that functional nAChRs are not expressed in TCs.

TCs have functional GABA_A receptors—In Normal external solution, brief (20 msec) puffs of $150 \mu\text{M}$ GABA, applied to the soma at a V_H of -20 mV, reliably evoked a large outward current ($n=10$ cells, Figure 4B), whose peak amplitude varied greatly from cell to cell ($38 - 799$ pA; mean 285 pA). As with the glutamate puff experiments, much of the variability in puff response amplitude is attributable to differences in the concentration of GABA reaching the TC soma. As shown in Figure 4C, the amplitude of the GABA-puff response was not significantly reduced in High $\text{Mg}^{2+}/0 \text{ Ca}^{2+}$ external solution ($p=0.825$; $n=5$ cells; data normalized to the response in High Mg^{2+}). Thus the GABA-puff response is mediated by GABA receptors on the TC itself.

We evaluated the contribution of GABA_A receptors to the GABA-puff response using the selective antagonist GABAzine ($20 \mu\text{M}$) (Ueno *et al.*, 1997). For the typical cell shown in Figure 4B, GABAzine completely blocked the GABA-puff response, but this effect was only partially reversed in Wash. Similar recordings were obtained from 9 other TCs in two sets of experiments; one done in Normal external and another in High Mg^{2+} external. As with glutamate, we pooled data after showing with two-way repeated measures ANOVA that all significant differences were attributable to the drug condition (i.e. before, during, or after GABAzine; $p<0.001$, Figure 4C). These pooled data confirmed the efficacy of GABAzine in inhibiting the GABA-puff response (normalized amplitude, 0.01 ± 0.004 in GABAzine) and the partial recovery in Wash (0.31 ± 0.07).

In 3 cells, we tested whether the addition of TPMPA to GABAzine could further reduce the already very small amplitude of the GABA-puff response. Since no significant effect was seen (normalized amplitude 0.05 ± 0.04 in GABAzine, 0.05 ± 0.03 in GABAzine & TPMPA; $p=0.927$), we conclude that TCs express functional GABA_A receptors, but not GABA_C receptors.

The I-V relation of the GABA-evoked response, obtained using the same protocol described for glutamate, was slightly outward rectifying with a reversal potential of -52 ± 5 mV (Figure 4E, $n=10$ cells), near the calculated E_{Cl} of -56 mV.

GABA is an excitatory neurotransmitter in TCs

Extracellular recordings of TC APs were occasionally acquired prior to patching a TC. In six cells examined in this way, application of 20 μ M GABAzine decreased spontaneous AP frequency ($p < 0.01$), although the degree of reduction varied greatly from cell to cell. AP frequency in GABAzine ranged from 1%-66% of AP frequency in Normal external (2.16 ± 1.44 Hz), with a mean of 30% (Figure 5A&B). Reversal of this effect in Wash was not always seen however. Consistent with this effect of GABAzine, we found that puffs of GABA ranging in duration from 20 msec to 1.5 sec typically evoked 1-3 APs (mean, 1.8; $n = 8$ cells), at a mean latency of 40 ± 40 msec. Although the latency measured in any one cell was consistent from trial to trial (Figure 5C), variability between cells was large, presumably because of differences in accessibility to drug.

The clear excitatory effect of GABA described above could be indirect through disinhibition of an excitatory input to the TC for example, or it could be direct. To resolve this issue we repeated the extracellular GABA-puff experiment in High $Mg^{2+}/0$ Ca^{2+} external solution. As illustrated by the example shown in Figure 5C, High $Mg^{2+}/0$ Ca^{2+} external solution practically eliminated spontaneous APs but did not change the latency or number of APs elicited by the GABA-puff ($p = 0.329$). GABAzine, on the other hand, completely suppressed the burst ($p < 0.01$, $n = 3$ cells). The mean number of evoked APs was 1.9 in Normal, 1.5 in High Mg^{2+} , and 0.2 in GABAzine (Figure 5D). These results show that GABA has a direct excitatory effect on TCs rather than the more typical inhibitory effect. Moreover, the fact that GABA elicits the same number of action potentials in the presence or absence of Mg^{2+} suggests that any indirect effect of GABA, acting via disinhibition for example, is small relative to its direct effect on TCs. A reasonable interpretation of the reduction in spontaneous action potential frequency by GABAzine (Figure 5A) is that the drug abolishes a continuous stream of GABAergic PSPs in TCs. This view receives support from the following experiments in which we examined the effects of antagonists on PSC frequency.

Spontaneous PSC frequency is reduced by GABA and glutamate antagonists

The GABA_A receptor antagonists, Bicuculline (10-100 μ M) or GABAzine (20 μ M), were used in combination with the GABA_C receptor antagonist, TPMPA (10-50 μ M), to determine if some of the observed PSCs were caused by GABA. In the 11 cells tested (4 with Bicuculline and TPMPA, and 7 with GABAzine and TPMPA), the GABA antagonists significantly reduced spontaneous PSC frequency to $55.1 \pm 47.5\%$ of Normal ($p = 0.005$, Fig. 5E) though, as expected, the reduction in PSC frequency was only partially reversed after washing with Normal external solution ($70.4 \pm 52.2\%$ of Normal). Clearly, some non-GABA input is also present in TCs. Some fraction of this spontaneous input might be glutamatergic but this has been difficult to demonstrate since, as we will show in the next section, exogenously applied glutamate, and possibly its antagonists, act in a way that has a large indirect component.

Applied by themselves, glutamate antagonists produced a small, reversible but non-significant reduction in PSC frequency. The non-NMDA receptor antagonists, NBQX (10 μ M) or DNQX (20 μ M), were used in combination with the NMDA receptor antagonist, APV (20 μ M), in 9 cells (6 with NBQX and APV, and 3 with DNQX and APV). The median spontaneous PSC frequency was reduced to 76.9% (68.9-85.0%) of Normal by the glutamate antagonists (Fig. 5E) and recovered in wash (100.0%; 82.3-137.6% of Normal). When glutamate antagonists were applied together with GABA antagonists, spontaneous PSC frequency was significantly reduced ($21.2 \pm 15.6\%$ of Normal; $p = 0.001$; $n = 4$; Fig. 5E) but importantly we found it was never abolished entirely, implying that another neurotransmitter must be released on to TCs. The fraction of PSCs contributed by glutamate directly is uncertain, since glutamate likely modulates the frequency of other PSCs.

Glutamate activates retinal input to the TC

Thus far, our results suggest that TCs receive both a glutamatergic and an excitatory GABAergic input and probably at least one other additional input. Close examination of TC responses to glutamate puffs, recorded both extra and intracellularly, provides evidence that GABAergic input comes from an intrinsic retinal neuron.

When intracellular recordings from the glutamate puff experiments were viewed singly at high temporal resolution, rather than averaged and low-pass filtered as they are in Figure 3A, it was possible to see two distinct responses. In addition to the large inward current, which we have shown is chiefly due to kainate and NMDA receptors at the V_H of -20 mV, there are typically brief outward PSCs whose frequency increased following a puff of glutamate (Figure 6A). In 10 cells recorded in Normal external, the glutamate puff increased outward PSC frequency to 57.2 ± 31.2 Hz, more than 5x the frequency of spontaneous outward PSCs recorded just prior to the puff (11.9 ± 8.45 Hz, $p=0.001$; Figure 6B). These outward PSCs must be generated by an intrinsic retinal neuron and, in support of this, we found that High Mg^{2+} abolishes these currents, leaving only the large inward current (Figure 6A&B).

The outward nature of both the spontaneous PSCs and the glutamate evoked (GE)-PSCs suggests that they are likely GABA/glycinergic, because E_{Cl} in these experiments is negative to the V_H . Consistent with this, we found that GABAzine decreased the frequency of GE-PSCs to ~40% of normal (36.9 ± 26.4 Hz in Normal; 15.2 ± 14.0 Hz in GABAzine; $n=3$; Figure 6B), but in no cells were all PSCs abolished.

Extracellular recordings of the TC response to glutamate puffs also revealed both direct and indirect effects on AP frequency. Brief (20 msec) glutamate puffs caused a burst of action potentials, typically 4 APs at a latency of 15 ± 3 msec in Normal external solution ($n=7$ cells, Figure 6D). In High $Mg^{2+}/0$ Ca^{2+} external solution, the latency to the first AP was not significantly affected (15 ± 3 msec in Normal, 20 ± 5 msec in High Mg^{2+} ; $n=5$ cells; $p=0.189$), but the number of APs in the burst was reduced (4.6 ± 2.5 in Normal, 1.0 ± 1.2 in High Mg^{2+} ; $n=5$ cells; $p<0.01$; Figure 6 C&D). The action potentials abolished by High $Mg^{2+}/0$ Ca^{2+} must be due to the input from intrinsic retinal neurons (i.e. the GE-PSCs discussed in the previous section). In extracellular recordings, where the normal Cl^- gradient has not been disturbed, these inputs are excitatory, as we showed in Figure 5C. Some fraction of this input is contributed by GABA and some is contributed by another transmitter, possibly glycine.

In summary, our physiological recordings show that exogenously applied glutamate stimulates intrinsic retinal neurons to produce excitatory input to TCs. Some, but not all, of this input is GABAergic. While TCs clearly possess glutamate receptors, the evidence for direct glutamatergic input to TCs is weak. We now show that immunohistochemistry supports the idea that GABAergic retinal neurons contribute excitatory input to TCs, and furthermore, we find good evidence that rEFs provide glutamatergic input.

Expression of neurotransmitter receptors

We examined TCs for the presence of receptors for acetylcholine (ACh), GABA, and glutamate using a variety of antibodies (Table 1). While the picture painted by these antibodies is consistent, the relative scarcity of antibodies immunoreactive in chicken necessarily means the picture is incomplete and provides no data on NMDA or kainate receptor expression, for example.

To identify TCs in retinal sections, we double labeled with an antibody against parvalbumin, since this has been shown to label TCs strongly (Fischer & Stell, 1999; Lindstrom *et al.*,

2009). While some other amacrine cell types are also parvalbumin-positive, TCs can be readily distinguished based on the location of their somata at the INL-IPL border, larger size, prolate shape, and more intense staining (Lindstrom *et al.*, 2009). The parvalbumin-positive processes found in the IPL (particularly clear in Figure 8Da) arise from the other amacrine cell types (Fischer & Stell, 1999), whereas the dendrites of the TCs are restricted to a small private neuropil at the base of each TC soma lying within the INL (Lindstrom *et al.*, 2009). In those sections in which we wanted to identify the rEF terminals, we employed an antibody to the Fluoro-Ruby (anti-TMR or anti-Rhodamine) injected into the ION and transported to the retina.

In agreement with Nickla *et al.* (1994), we found that TCs appear weakly immunopositive for the $\alpha 7$ subunit of the nAChR (Figure 7A). We also found that they are immunonegative for the $\alpha 3$ and $\alpha 8$ subunits of the nAChR (data not shown). The lack of colocalization between $\alpha 3$ and $\alpha 8$ nAChR antibodies was not due to an inability of these antibodies to detect $\alpha 3$ and $\alpha 8$ nAChRs, as we observed positive staining of a number of cells in the amacrine cell layer, consistent with previous reports (Schoepfer *et al.*, 1990; Hamassaki-Britto *et al.*, 1994).

Evidence that TCs express GABA_A receptors was provided by an antibody against the $\alpha 1$ subunit of the GABA_A receptor. Figure 7B shows a typical parvalbumin-positive TC that is also immunoreactive for the $\alpha 1$ subunit of the GABA_A receptor. While this result is not unexpected, in view of our physiological findings, it differs from the briefly reported negative result of Fischer and Stell (1999) who used an antibody against GABA_A $\beta 2$ & $\beta 3$ subunits.

We also found that TCs are immunopositive for the AMPA receptor subunit, GluR2 and/or GluR3 (Figure 7C). The region of colocalization appears to be primarily the dendritic basketwork of the TC, although some expression can be seen in the upper soma. Using the same antibody to GluR1 as Fischer and Stell (1999) we found, unlike their report, no evidence of immunoreactivity in TCs or rEFs (data not shown).

Staining patterns for presynaptic markers reveal that rEFs likely release glutamate

Using an antibody to ChAT, the synthetic enzyme for ACh, we found strong labeling of starburst amacrine cells and their dendrites in lamina 2 and 4 of the IPL, as has been widely reported in this retina (Spira *et al.*, 1987; Drenhaus *et al.*, 2003). TCs and their immediate vicinity were, by contrast, clearly immunonegative (Figure 8A). This accords well with the lack of TC responses to epibatidine, but raises the question of why an apparently inactive $\alpha 7$ subunit would be found on the TC. It is unlikely that the antibody (mAb306) is recognizing an epitope on another protein, because a different clone (mAb319), which binds to a neighboring region of the $\alpha 7$ -nAChR protein (AAs 365-384 rather than AAs 380-400), exhibits a similar staining pattern (data not shown).

In looking for evidence of presynaptic glutamate, we found an antibody against the vesicular glutamate transporter, vGluT2, to be particularly specific and unambiguous. The distribution of this transporter within the chicken retina has not been previously described, but in the rodent retina it is found in ganglion cells (Sherry *et al.*, 2003) including melanopsin containing cells, and a subset of cones. Within the INL of the chicken, we found the only structure that stains for vGluT2 to be the rEF terminal. vGluT2 immunostaining defines a region closely investing the lower half of the TC soma, as identified by parvalbumin staining (data not shown), corresponding to the rEF terminal as seen in EM images (Lindstrom *et al.*, 2009). Close inspection of vGluT2 staining shows a lumpy distribution in which it is often possible to see roughly spherical structures of about 2 μ m diameter (Figure 8B,a). These are undoubtedly the presynaptic boutons of the rEF terminal that collectively resemble a bunch

of grapes when stained with the NADPH-diaphorase method, and in EM have been shown to be packed with clear, round synaptic vesicles (Lindstrom *et al.*, 2009). When rEF terminals were visualized in Fluoro-Ruby labeled preparations, it was clear that the vGluT2 immunostaining overlapped with the terminal, and individual boutons were often seen to be coincident in the two color channels (Figure 8B). These results strongly suggest that glutamate is the neurotransmitter released at the synapse between the rEF and TC.

In contrast to the correspondence of vGluT2 staining with the presynaptic structure of the rEF, an antibody against the GABA synthetic enzymes GAD 65/67 presents a quite different picture. As shown in Figure 8C a few very small puncta of strong GAD immunoreactivity colocalize with or adjacent to the diminutive dendritic processes of the TC (arrows, Figure 8C). Double labeling for GAD and Fluoro-Ruby revealed that the pattern of these GAD-positive puncta was distinct from that of the large presynaptic boutons of the rEF terminal and generally the GAD-positive puncta were seen in the interstices between rEF terminal boutons (data not shown). We believe that these puncta are not part of the rEF terminal, but instead represent the synaptic boutons of a retinal neuron. Two points argue for this interpretation. First, the diameters of the GAD puncta are typically close to, or below, the point spread function of our optics, significantly smaller than the diameter of rEF presynaptic boutons (Lindstrom *et al.*, 2009). In their sparsity and small size they resemble the putative GABAergic terminals seen in electron microscopy images (Lindstrom *et al.*, 2009). Second, when GAD puncta are compared to Fluoro-Ruby labeled rEF terminals there is no close correspondence. Instead, puncta are often seen to correspond to “holes” in the rEF terminal structure (data not shown).

TCs express NKCC

Since our electrophysiological experiments show an excitatory GABA_A input to TCs, we looked for the Na-K-Cl cotransporter (NKCC) characteristically associated with such synapses and responsible for an unusually positive Cl⁻ equilibrium potential (Kakazu *et al.*, 1999; Russell, 2000; Jang *et al.*, 2001; Marty *et al.*, 2002; Marty & Llano, 2005; Reisert *et al.*, 2005; Zhang *et al.*, 2007). Using a monoclonal antibody to NKCC (Lytle *et al.*, 1995), we have found that TCs express NKCC (Figure 8D) chiefly in their dendritic processes but, to a lesser extent, also in the upper region of the soma. In these experiments we used rabbit (rather than mouse) anti-parvalbumin (Figure 8D,a) to identify the position of TCs.

Discussion

In this study we have identified rEF terminals in retinal slices by virtue of the fluorescent label transported to them from their somata in the ION. By patching and labeling their postsynaptic partners with Lucifer yellow, we confirm that these are the atypical amacrine cells referred to as target cells. In only one instance out of more than 500 observations was another cell type seen to be the postsynaptic partner. Interestingly, this was a displaced ganglion cell, which in the pigeon retina has been claimed as a recipient of rEF input (Maturana & Frenk, 1965). In a subsequent careful study in pigeon, Hayes and Holden (1983) estimated that 2.7% of rEFs may synapse with displaced ganglion cells, and we conclude that in chicken the percentage is probably even smaller. Our patch clamp recordings from TCs, the first from this cell type, reveal the likely transmitters to which they respond, and carry implications concerning the function of the centrifugal visual system.

Glutamate is the transmitter of the rEF

Several results indicate that glutamate is the likely transmitter of efferent fibers. In the absence of synaptic input, TCs respond to exogenously applied glutamate with inward currents. Consistent with this, we discovered, using antibodies to GluR 2/3, that AMPA

receptors are found over the basal portion of the TC soma where efferent fiber boutons generally make their synapses (Lindstrom *et al.*, 2009). Particularly significant is our finding that the vesicular glutamate transporter vGluT2, though seemingly as uncommon in the inner retina as it appears to be in other species (Sherry *et al.*, 2003), is strongly expressed in rEF terminals, where it can be seen in their characteristic large presynaptic boutons. The pharmacology of responses to exogenously applied glutamate shows that, unusually, receptors from all 3 of the ionotropic families, AMPA, NMDA and kainate, conduct current, but because of the different rectification shown by these channels, their relative contributions depend on voltage. Since the currents we attribute to AMPARs are inward rectifying, it is probable that these are carried through Ca^{2+} permeable channels (Hollmann *et al.*, 1991; Verdoorn *et al.*, 1991). NMDA and potentially kainate receptors (Burnashev *et al.*, 1995) also conduct Ca^{2+} , which is therefore likely to show a concentration rise in the TC cytosol during synaptic activation. In this context it is significant that nitric oxide synthase is found in abundance in the somata of TCs as well as in the boutons of efferent terminals. Calcium influx into both these structures is likely to stimulate nitric oxide synthase (Garthwaite & Boulton, 1995), though it is unclear what nitric oxide does in this system.

Excitatory GABA input comes from an intrinsic retinal neuron

In addition to a glutamate input, we have evidence that GABA is also a transmitter to TCs. Roughly half of all spontaneous PSCs are abolished by GABAzine, which also completely abolishes the current responses to exogenously applied GABA. The sensitivity of these responses to GABAzine argues that they are mediated entirely by GABA_A receptors. GABA is excitatory to TCs, as shown by extracellular recordings in which the natural value of E_{Cl} is undisturbed. Further, our experiments show that this effect of GABA is a direct one since exogenously applied GABA increases action potential frequency, even when synaptic transmission has been blocked with extracellular Mg^{2+} . Though less well known as an excitatory transmitter than as an inhibitory one, there is substantial precedent for this role not just in embryonic CNS (Marty *et al.*, 2002), but also in the adult nervous system (Marty & Llano, 2005), including the retina (Vardi *et al.*, 2000; Dmitriev *et al.*, 2007; Li *et al.*, 2008). Where GABA has been found to be excitatory this has generally been attributed to an unusually positive E_{Cl} , achieved through the action of the Cl^- transporter, NKCC. We have shown here with immunohistochemistry that this molecule is present on the soma of TCs.

What class of neuron provides GABAergic input to TCs? Quite apart from the arguments presented above that rEFs are glutamatergic, there are several pieces of evidence that indicate these structures are not the source of GABA. A study in pigeon showed GABA to be absent from the output neurons of pigeon ION (Miceli *et al.*, 1995). Consistent with this, our immunohistochemistry shows the GABA synthesizing enzymes GAD 65 and GAD 67 to be absent from the 2 μm diameter boutons of rEFs and instead restricted to only a few narrow processes present within the inner neuropil of the TC. Very likely these correspond to the relatively scarce processes we have previously described from electron microscopy that contain pleomorphic synaptic vesicles (Lindstrom *et al.*, 2009). These processes probably belong to an amacrine cell since a large fraction of amacrine cells are GABAergic (Mosinger *et al.*, 1986; Wulle & Wagner, 1990) and, with the possible exception of interplexiform cells whose presence has not been reported in this retina, are the only cell types present in the inner retina known to use GABA as a transmitter. If this conjecture is correct, being excitatory is a most unusual role for an amacrine cell, but of course this does not imply that this amacrine cell is anything other than an inhibitory interneuron with respect to its other postsynaptic partners.

Two findings indicate that at least one other transmitter provides input to rEFs. Glutamate antagonists, when applied together with GABA antagonists, fail to suppress all PSCs seen in TCs. Similarly, exogenously applied glutamate puffs elicited PSCs, only some of which are

suppressed by GABA_Azine. We suspect that glycinergic input may also be present but we have no direct evidence for this.

Implications for function

As has been widely assumed, we now confirm that TCs can generate action potentials, allowing signals to be transmitted from TC somata, located exclusively in the ventral retina (Hayes & Holden, 1983; Lindstrom *et al.*, 2009), to their axon terminals, often at a considerable distance away in the dorsal retina (Ramón y Cajal, 1995; Uchiyama *et al.*, 2004). While this might seem self-evident, it should be remembered that horizontal cells in the mammalian retina comprise dendritic and axonal elaborations connected by a thin axon that effectively keeps these two structures isolated from each other (Nelson *et al.*, 1975).

It has been argued that TCs are slave neurons driven exclusively by efferent fiber input (Uchiyama & Stell, 2005). Our finding that there are other synaptic inputs from intrinsic retinal neurons, probably amacrine cells, to TCs suggests that TCs are not merely slave neurons but rather, they combine their efferent input with local retinal input. It is significant that GABAergic input is excitatory, and we might therefore conclude that this local retinal input to TCs acts synergistically with efferent fiber input to produce spiking. Furthermore, the presence of NMDA receptors might imply that summation of these inputs is nonlinear and perhaps acts, to some extent, as a coincidence detector as argued at other synapses.

Several implications flow from these findings. The first strong implication concerns the receptive fields of some ganglion cells in the dorsal retina. Because, as we have shown here, ventrally located TCs receive excitatory retinal input, this must influence the responses of ganglion cells lying within their, mostly dorsally located, terminal fields. The neurons immediately postsynaptic to TC terminals are probably amacrine cells (Ramón y Cajal, 1972; Uchiyama *et al.*, 2004) rather than ganglion cells. Nevertheless, the activity of these amacrine cells will be reflected in ganglion cell spiking, implying that in addition to the classical center-surround organization, these ganglion cells will also possess a field in the ventral retina that influences their responses. Properties like this have not been reported in any of the experimental descriptions of either avian ganglion cells (Holden, 1969; Miles, 1972b; Pearlman & Hughes, 1976a; Holden, 1977; Uchiyama & Barlow, 1994) or ION cell properties (Holden & Powell, 1972; Miles, 1972c), though it is unclear whether these would have been detected using the experimental methods employed. The visual stimuli used in these studies were unlikely to reveal this kind of effect; moreover, it might be the case that the anesthetics employed depressed the proper functioning of the centrifugal visual system.

A second implication is that TCs are genuinely “association” amacrine cells as Cajal must have surmised when he named them (Ramón y Cajal, 1995). Specifically, they associate activity in the ventral retina with activity in the dorsal retina. This idea, however, poses a difficult problem since, in contrast to the orderly organization of the rest of the centrifugal visual system (Holden & Powell, 1972), the projection of TC axons, while generally proceeding from the ventral retina to the dorsal retina, is nevertheless apparently chaotic (Catsicas *et al.*, 1987; Uchiyama *et al.*, 2004). What could be achieved by the seemingly haphazard connection of regions of ventral retina with regions of the dorsal retina? It remains possible that a pattern might be revealed by a more systematic mapping of these connections and this might suggest an answer.

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Figure 1. Methods used to Label rEFs and Record from TCs

A: Schematic illustration of the labeling method. Under stereotaxic control 0.5 μ L of the anatomical tracer, Fluoro-Ruby, was injected unilaterally into the ION. IO-neurons took up Fluoro-Ruby and transported it to their axon terminals (red arrow). After a minimum survival of 3 days, sufficient Fluoro-Ruby accumulated in the rEF terminals to allow their visualization in retinal slices.

B: A retinal slice imaged in IR-DIC and fluorescence optics. A single rEF is shown (red) crossing the IPL and terminating in a pericellular nest in the INL. Identifying the postsynaptic TC from its DIC outline and contact with the rEF terminal, a patch clamp pipet (shown in cartoon) was used to fill the cell with Lucifer Yellow (LY). This cell could also be stimulated with exogenous neurotransmitters from a puff-pipet placed as illustrated.

C: A typical LY-filled TC with attached pipet (yellow), showing the TC axon (arrowheads), and close apposition between the stubby TC dendrites and rEF pericellular nest (red); features that were used to confirm identity of the recorded TC. The boundary between the INL and IPL is indicated with a dashed line.

Figure 2. TCs Produce APs that can be Triggered by Spontaneous PSPs

A: Sample traces from a current clamp recording of a TC showing many spontaneous PSPs, 5 are ridden by APs (arrows). Inset shows detail of a single AP (black) superimposed on a single PSP (grey). Recorded using KCl intracellular solution.

B: Extracellular recording of APs obtained during loose patch recording from a TC.

C: A voltage-step from -79.5 mV to -59.5 mV (top trace) elicited a rapid and transient current in a voltage-clamped TC (lower black trace). Application of $0.1 \mu\text{M}$ TTX completely suppressed this current (grey trace), implying that TCs have functional voltage-gated Na^+ -channels. Recorded using CsMs intracellular solution.

D: Voltage-clamp recordings from a TC held at -20 mV show that High Mg^{2+} external blocks most of the spontaneous PSCs recorded in the TC. In Normal external (upper black traces) this TC received a steady stream of spontaneous synaptic input. The vast majority of this input was blocked by High $\text{Mg}^{2+}/0 \text{ Ca}^{2+}$ external (grey traces). This effect was fully reversible by washing with Normal external (lower black traces). Recorded using CsMs intracellular solution.

Figure 3. Pharmacological Characterization of Glutamate Receptors on TCs

A: Voltage-clamp recordings from a TC showing the typical response (black trace) to a 20 ms puff of glutamate ($V_H = -20$ mV). 100 μ M glutamate (Glu, black bar) elicited a large inward current in Normal external (black trace). The glutamate puff response was significantly decreased by DNQX (red trace) and completely eliminated by DNQX & APV (green trace), but GYKI-53655 (blue trace) had little effect at this voltage. Full recovery was achieved in Wash (grey trace).

B: Comparison of the amplitude of the glutamate puff response in Normal and High $Mg^{2+}/0$ Ca^{2+} external. Prior to grouping, the mean amplitude of the glutamate-puff response in each cell was normalized to the mean amplitude of the response in High Mg^{2+} . No significant difference was detected ($n=5$ cells).

C: Summary of the effects of glutamate antagonists on the glutamate puff responses of 8 TCs. Data have been pooled from cells recorded in Normal and High $Mg^{2+}/0$ Ca^{2+} external (see text), thus the term “Initial” has been used to signify the response prior to application of any glutamate antagonist.

D: I-V relations of the glutamate puff response. The protocol used to determine the I-V relation of the puff response is illustrated in the inset; grey trace shows the timing of the puff of agonist (glutamate), black trace shows the timing of the voltage ramps. In Normal external (black), the I-V relation of the glutamate puff response showed strong outward rectification and reversed at -8.4 mV. Evidence for the presence of Ca^{2+} -permeable AMPA receptors is provided by the ability of GYKI-53655 (blue) to reduce current at more negative voltages but not at positive voltages. The isolated AMPA-mediated component of the I-V relation (i.e. the difference between Normal and GYKI-53655), is shown in E. The negative slope between -80 and -30 mV is indicative of a significant NMDA mediated component. The combined application of DNQX & APV blocked the glutamate puff response at all voltages (bright green trace). The Wash trace is omitted.

Figure 4. Pharmacological Characterization of GABA Receptors on TCs

A: Voltage clamp recording from a TC during puff application of 100 μ M epibatidine (Epibat, black bar). Trace shown is the average of 4 trials, in none of which was a response elicited. The two small events visible in this trace are the result of spontaneous PSCs that occurred during the third trial.

B: 150 μ M GABA (black bar) elicited a large outward current in Normal external (black trace), which was completely eliminated by 20 μ M GABA_A (light grey trace) and partially recovered after washing (dark grey trace).

C: Comparison of the amplitude of the GABA-puff response in Normal and High Mg²⁺/0 Ca²⁺ external, as in Figure 3B. No significant difference was detected ($n=5$ cells).

D: Summary of the effect of GABA_A (with or without TPMPA) on the GABA-puff response in 10 TCs. Data have been pooled as for the glutamate experiments (Figure 3C). The mean amplitude of the GABA-puff response was reduced to practically zero by GABA_A and further addition of TPMPA ($n=3$ cells) did not further reduce the amplitude. Partial reversal was seen in Wash.

E: I-V relations of the GABA puff response using the same protocol as illustrated in Figure 3D. In Normal external (black), the I-V relation of the GABA-puff response was weakly outward rectifying, reversing at -59 mV. GABA_A blocked the response at all voltages (light grey). This effect was only partially reversed in Wash (dark grey).

Figure 5. GABA input to the TC is Excitatory

A: Extracellularly recorded spontaneous APs from a TC. In this cell, APs were observed at a frequency of 3.1 Hz in Normal external (upper trace). GABAzine (middle trace) reduced AP frequency to 1.3 Hz; no significant recovery was observed in Wash (lower trace).

B: Summary of the reduction in spontaneous AP frequency following the application of GABAzine (Gz; $n=3$). GABAzine significantly decreased AP frequency; however, reversal of this effect was not seen. N=Normal; W=Wash.

C: Sample traces from a TC showing the burst of APs elicited by short (20 ms) puffs of GABA (bars). In Normal external, High Mg^{2+} , and Wash each puff evoked 1-2 APs. Spontaneous APs, common in Normal external, were eliminated by High Mg^{2+} , while GABA-puff evoked APs were unchanged by High $Mg^{2+}/0\ Ca^{2+}$ but eliminated by GABAzine.

D: Summary of the effect of High $Mg^{2+}/0\ Ca^{2+}$ (Mg) or GABAzine (Gz) on GABA-puff evoked APs for the cell illustrated in C and two others. Neither the number of APs (left) nor the latency (right) of the GABA-puff evoked burst were significantly altered by High Mg^{2+} . However, GABAzine significantly reduced the number of APs and increased the latency.

E: Spontaneous PSC frequency is reduced, but not eliminated, by GABA and glutamate antagonists. GABA antagonists alone (Gb) suppress approximately half of the spontaneous PSCs recorded in TCs ($n=11$), while Glutamate antagonists alone (GI) only suppress about 25% ($n=9$). Combined application of GABA and glutamate antagonists (Gb&GI) reduced spontaneous PSC by 80% ($n=4$), roughly the value expected from additive effects of the individual antagonists.

Figure 6. Exogenous Glutamate has Direct and Indirect Excitatory Effects on the TC

A: Intracellular recording of the glutamate puff response revealed that glutamate increased the frequency of the outward PSCs. Given the V_H of -20 mV, current through glutamate receptors is expected to be inward, while current through GABA and glycine receptors should be outward. Top, an example of a TC's response to a single glutamate-puff illustrates the relationship between the single, large inward current (associated with activation of glutamate receptors on the TC) and the multiple, small outward PSCs (associated with GABA or glycinergic input to the TC). The glutamate-evoked (GE)-PSCs observed during the decay of the large inward glutamate puff response (black trace) are likely mediated by synaptic transmission as they are eliminated by High $Mg^{2+}/0$ Ca^{2+} (grey trace) whereas, the inward current is not eliminated and thus is mediated by glutamate receptors on the TC itself. Bottom, PSTHs from 2 different TCs showing the dramatic increase in outward PSC frequency following a glutamate puff, and the significant reduction of spont- and GE-PSCs caused by application of High $Mg^{2+}/0$ Ca^{2+} (left) or GABAzine (right). Bars above the current traces and PSTHs show the timing of the glutamate puff (thick bar) and the TC's inward glutamate puff response (thin bar).

B: Summary of the effects of High $Mg^{2+}/0$ Ca^{2+} and GABAzine on spontaneous- and GE-PSCs. Spontaneous (spont) frequency was measured during a period preceding each glutamate puff; GE frequency was measured during the 50-100 msec following each puff. In Normal external solution, short puffs of glutamate cause a 5-fold increase in the frequency of PSC ($n=10$). High $Mg^{2+}/0$ Ca^{2+} external solution was able to virtually eliminate both spont- and GE-PSCs ($n=7$). GABAzine was able to suppress most of the spont- and GE PSCs, but the remaining PSCs suggest that some of these events might be glycinergic ($n=3$).

C: Sample traces from a TC showing the burst of APs elicited by short (20 ms) puffs of 100 μ M glutamate (thick bar). In Normal external (upper black traces) each glutamate puff evoked 3-5 APs, in addition spontaneous APs were observed (arrows). In High $Mg^{2+}/0$ Ca^{2+} external (grey traces) spontaneous APs were eliminated and the number of APs in the glutamate puff response was decreased to 1-2. In Wash (lower black trace), spontaneous APs returned and the number of APs in the glutamate puff response increased to 10.

D: Summary of the effect of High $Mg^{2+}/0$ Ca^{2+} on the glutamate puff evoked APs. High $Mg^{2+}/0$ Ca^{2+} significantly decreased the number of APs, but did not alter the latency. This effect was partially reversed in Wash ($n=5$).

Figure 7. Immunohistochemical Characterization of Neurotransmitter Receptors on TCs

Shown here are TC somata identified by an antibody to parvalbumin (red) together with antibodies to transmitter receptors (green). Images are collapsed confocal stacks showing the entire thickness of the retina with photoreceptors at the top and the ganglion cells or optic fiber layer at the bottom. Dotted lines indicate the INL-IPL border. The intensity and contrast of these images were adjusted to allow clear visualization of antibody labeling on and around the TC somata where all synapses to TCs are known to lie (Lindstrom *et al.*, 2009); TCs make no synapses in the IPL. Scale Bars are 20 μ m.

A: Soma of a TC (a) stains weakly for the nAChR $\alpha 7$ subunit (b), apparent colocalization can be seen in (yellow, c). The faintness of the green channel signal is indicated relatively high autofluorescence shown in the outer segments at the top of the images (d), a DIC image is shown here for orientation but omitted from subsequent figures.

B: GABA_A receptors colocalize with TCs. Double label immunohistochemistry for parvalbumin identifies a TC (a), that is also positive for the $\alpha 1$ subunit of the GABA_A receptor (b), merged in (c).

C: Retina double labeled for parvalbumin (red, a) and GluR2/3 (green, b) shows strong colocalization (c).

Figure 8. Cellular Localization of Transmitter Associated Molecules

A: ChAT staining is absent from the region surrounding the rEF terminal. Double labeling for rEF terminals and choline acetyltransferase, the synthetic enzyme for ACh, revealed no colocalization between the two. (a), ChAT staining (green) shows 2 strongly stained laminae in the IPL and regular arrays of starburst amacrine cells both above and below the IPL. The rEF terminal, labeled with antibodies against TMR (red), used to increase the signal of Fluoro-Ruby in rEF terminals, lies in a region of the INL that lacks ChAT staining. Scale bar is 20 μ m. (b&c) Higher magnification images of the region surrounding the rEF shown in the merged image, (a). Scale bar is 20 μ m.

B&C: Presynaptic markers for glutamate and GABA have distinct staining patterns. In both B and C the larger merged image (a) shows the entire thickness of the retina with photoreceptors at the top and the optic fiber layer at the bottom. Dotted lines indicate the INL-IPL border. The three images on the right (b-d) show higher magnification images of the region surrounding the TC so that colocalization between the two antibodies can be more closely examined. All images are compressed confocal stacks. Scale bars are 10 μ m.

B: Double labeling with antibodies against rhodamine (red), identifying the rEF terminal and vGluT2 (a presynaptic marker for glutamatergic synapses, green) shows a matching pattern. In b, c, & d, individual presynaptic boutons, approximately 2 μ m in diameter, can often be seen in both images (arrows).

C: Double labeling with antibodies against parvalbumin (red) and GAD65/67 (green) reveals that a few small, punctate GABAergic terminals contact thin, dendritic extensions of the TC (yellow in merged images and arrows in b & c). These GABAergic terminals do not have the same staining pattern as vGluT2 in B and do not correspond to the rEF terminals.

D: TCs express high levels of NKCC. A retinal slice double labeled with antibodies against parvalbumin, used to identify the TC (a) and NKCC (b). In the merged image (c) the soma and dendritic region of the TC is seen to be strongly NKCC positive. Scale bar is 20 μ m.

Table 1
Primary Antibodies

Antibody	Dilution	Source	Catalog #
Mouse anti-Parvalbumin	1:1000	Sigma	P3088
Rabbit anti-GABA _A -R α 1 subunit	1:150	Abcam	AB33299
Rabbit anti-GluR2/3 subunit	2:100	Chemicon	AB 1506
Rabbit anti-GluR1 subunit	2:100	Chemicon	AB 1504
Rat anti- α 3nAChR subunit (mAb313)	1:500	Abcam	AB 24666
Mouse anti- α 7nAChR subunit (mAb306)	1:500	Covance	MMS-627R
Rat anti- α 8nAChR subunit (mAb308)	1:500	Abcam	AB 24720
Goat anti-ChAT	1:100	Chemicon	AB 144P
Guinea Pig anti-vGluT 1	1:1000	Chemicon	AB 5905
Rabbit anti-vGluT 2	1:1000	Synaptic Systems	135403
Guinea Pig anti-vGluT 3	1:2500	Chemicon	AB 5421
Rabbit anti-GAD 65+67	1:1000	Abcam	AB11070
Mouse anti-NKCC (T4 ascites)	1:10000	DSHB*	
Rabbit anti-Parvalbumin	1:2000	Abcam	AB11427
Rabbit anti-Tetramethylrhodamine (TMR)	1:1000	Invitrogen	A-6397
Mouse anti-Rhodamine(11H10)	1:1000	Abcam	AB9093

* DSHB is the University of Iowa Developmental Studies Hybridoma Bank