

# Masked excitatory crosstalk between the ON and OFF visual pathways in the mammalian retina

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**Non-technical summary** An organizing principle of the visual system is the segregation of ON and OFF responses into parallel streams to signal light increment and decrement. This segregation begins in the retina where the output ganglion cells can be divided into ON and OFF subtypes based on their responses to light. Here we show that blockade of GABAergic inhibition reveals opposite polarity responses in ganglion cells whereby OFF cells display ON responses and ON cells display OFF responses. This crossover excitation was found in both the rabbit and mouse, indicating that it is a common synaptic mechanism in the mammalian retina. Overall, these results challenge the idea that light increment and decrement is signalled by different visual pathways. Moreover, our findings suggest that release of inhibition under certain light conditions can enable single ganglion cells to carry both ON and OFF signals, thereby allowing additional information to be propagated across the limited bandwidth of the optic nerve.

**Abstract** A fundamental organizing feature of the visual system is the segregation of ON and OFF responses into parallel streams to signal light increment and decrement. However, we found that blockade of GABAergic inhibition unmasks robust ON responses in OFF  $\alpha$ -ganglion cells ( $\alpha$ -GCs). These ON responses had the same centre-mediated structure as the classic OFF responses of OFF  $\alpha$ -GCs, but were abolished following disruption of the ON pathway with L-AP4. Experiments showed that both GABA<sub>A</sub> and GABA<sub>C</sub> receptors are involved in the masking inhibition of this ON response, located at presynaptic inhibitory synapses on bipolar cell axon terminals and possibly amacrine cell dendrites. Since the dendrites of OFF  $\alpha$ -GCs are not positioned to receive excitatory inputs from ON bipolar cell axon terminals in sublamina-*b* of the inner plexiform layer (IPL), we investigated the possibility that gap junction-mediated electrical synapses made with neighbouring amacrine cells form the avenue for reception of ON signals. We found that the application of gap junction blockers eliminated the unmasked ON responses in OFF  $\alpha$ -GCs, while the classic OFF responses remained. Furthermore, we found that amacrine cells coupled to OFF  $\alpha$ -GCs display processes in both sublaminae of the IPL, thus forming a plausible substrate for the reception and delivery of ON signals to OFF  $\alpha$ -GCs. Finally, using a multielectrode array, we found that masked ON and OFF signals are displayed by over one-third of ganglion cells in the rabbit and mouse retinas, suggesting that masked crossover excitation is a widespread phenomenon in the inner mammalian retina.

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**Abbreviations** L-AP4, L-2-amino-4-phosphonobutyric acid;  $\alpha$ -GC,  $\alpha$ -ganglion cell; CNS, central nervous system; DNDS, 4,4'-dinitro-stilbene-2,2'-disulphonic acid; 18 $\beta$ -GA, 18 $\beta$ -glycyrrhetic acid; INL, inner nuclear layer; IPL, inner plexiform layer; MFA, meclofenamic acid; PTX, picrotoxin; TPMPA, 1,2,5,6-tetrahydropyridin-4-yl-methylphosphinic acid.

## Introduction

A key organizing feature of the vertebrate retina is the segregation of responses signalling light increment and decrement into parallel ON and OFF pathways (reviewed by Wässle, 2004). Hartline (1938) first classified retinal ganglion cells as ON, OFF, or ON–OFF, based on their excitatory responses to the onset and/or offset of light stimuli. It is now clear that the ON and OFF pathways are generated at the very first synapse in the retina, resulting from the differential expression of ionotropic and metabotropic glutamate receptors on the second-order bipolar cells (Nomura *et al.* 1994; Masu *et al.* 1995; Vardi & Morigiwa, 1997; DeVries, 2000). In the inner retina, ON and OFF bipolar cell axons terminate in different sublaminae of the inner plexiform layer (IPL) where they selectively innervate ON and OFF ganglion cells (Famiglietti & Kolb, 1976; Nelson *et al.* 1978; Peichl & Wässle, 1981; Bloomfield & Miller, 1986). The ON and OFF signals generated in the retina thereby apparently remain separate as they propagate to the lateral geniculate nucleus and finally converge at the level of the visual cortex (Schiller, 1982; Knapp & Mistler, 1983; Horton & Sherk, 1984; Thurlow *et al.* 1993).

While the segregation of ON and OFF signals is supported by both morphological and physiological data, there is emerging evidence for significant inhibitory interactions between the two pathways. This ‘crossover’ inhibition between the ON and OFF streams has been found at the level of bipolar cells, amacrine cells and ganglion cells (Zaghloul *et al.* 2003; Roska *et al.* 2006; Molnar & Werblin, 2007; Hsueh *et al.* 2008; Manookin *et al.* 2008; Murphy & Rieke, 2008). These inhibitory interactions are thought to be mediated mainly by glycinergic, multistratified amacrine cells whose dendrites receive excitatory bipolar cell synaptic input in one sublamina of the IPL, but provide inhibition to ganglion cells stratifying in the other sublamina.

In addition, examples of excitatory interactions between the ON and OFF pathways in the mammalian retina have been reported sporadically over the years. However, these interactions, which have been reported to date only in ON ganglion cells, were often revealed after blockade of GABAergic inhibition (Ariel & Daw, 1982; Nirenberg & Meister, 1997; Roska & Werblin, 2001; Rentería *et al.* 2006; Ackert *et al.* 2009), thereby suggesting that the mixing of excitatory ON and OFF signals in retinal ganglion cells may be masked under common experimental conditions. Consequently, such excitatory interactions were often reported anecdotally as simple observations without follow-up analysis or even attributed to technical artifact.

Therefore, in the present study, we have investigated excitatory crosstalk between the ON and OFF pathways in the rabbit and mouse retinas, including the

circuitry responsible for its generation and masking. We initially focused on OFF  $\alpha$ -ganglion cells ( $\alpha$ -GCs), finding that blockade of GABAergic inhibition reveals a robust centre-mediated ON response sensitive to L-2-amino-4-phosphonobutyric acid (L-AP4). The ON response was abolished by gap junction blockers, suggesting that the electrical synapses between  $\alpha$ -GCs and neighbouring multistratified amacrine cells form the pathway for excitatory crosstalk between the ON and OFF pathways. We also found that the inhibition responsible for masking of ON responses in OFF  $\alpha$ -GCs is subserved by both GABA<sub>A</sub> and GABA<sub>C</sub> receptors on pre-synaptic bipolar and possibly amacrine cells, suggesting that the masked excitatory crosstalk may occur in many ganglion cell subtypes. Indeed, recordings made with a multielectrode array showed that blockade of GABAergic inhibition unmasks opposite polarity responses in over one-third of ON and OFF ganglion cells examined in both rabbit and mouse retinas. Our results therefore challenge the view that excitatory ON and OFF signals are transmitted centrally via strictly segregated parallel streams subserved by different subtypes of ganglion cells.

## Methods

### Flattened retina–sclera preparation

The rabbit and mouse retina–sclera preparations used in this study have been previously described (Hu *et al.* 2000; Hu & Bloomfield, 2003; Völgyi *et al.* 2004). Adult New Zealand White rabbits were anaesthetized with an intra-peritoneal injection of 40% ethyl carbamate (2.0 g (kg body weight)<sup>-1</sup>). For mouse experiments, wild-type adult C57BL/6 mice were anaesthetized with an intra-peritoneal injection of sodium pentobarbital (0.08 g (g body weight)<sup>-1</sup>). Following a local injection of 2% lidocaine hydrochloride to the eyelids and surrounding tissue, the eyes were removed under dim red illumination and hemisected anterior to the ora serrata. The vitreous humour was removed with an ophthalmic sponge (Bausch & Lomb, Rochester, NY, USA), and the resultant retina–eyecup was flattened by making radial cuts at the periphery in a Maltese-cross configuration. For all recordings from the rabbit, retina–eyecups or isolated retinas were placed in a superfusion chamber, which was mounted on the stage of an upright light microscope (Olympus BX51-WI; Olympus, Centre Valley, PA, USA) within a light-tight Faraday cage. The tissue was superfused at a rate of 30 ml min<sup>-1</sup> with a mammalian Ringer solution (in mM): 120.0 NaCl, 5.0 KCl, 25.0 NaHCO<sub>3</sub>, 0.8 Na<sub>2</sub>HPO<sub>4</sub>, 0.1 NaH<sub>2</sub>PO<sub>4</sub>, 10.0 glucose, 0.01 ascorbate, 1.0 MgSO<sub>4</sub> and 2.0 CaCl<sub>2</sub>. All chemicals were obtained from Fisher Scientific (Waltham, MA, USA) or Sigma-Aldrich (St Louis, MO, USA). The superfusate was kept at a

constant temperature of 34°C and a pH of 7.4 was maintained by bubbling with a gaseous mixture of 95% O<sub>2</sub>–5% CO<sub>2</sub>. After enucleations, animals were killed with either an intracardial injection of ethyl carbamate (rabbits) or cervical dislocation (mice). All surgical procedures were approved by the Institutional Animal Care and Use Committee at NYU School of Medicine. These procedures comply with *The Journal of Physiology* policy and UK regulations on animal experimentation. Animals were maintained in 12/12 h day–night cycle and all experiments were performed during daylight hours.

### Visualization of cells

To visualize ganglion cells in the rabbit retina, the superfusion was temporarily halted and 3–5 drops of 0.1% Azure B (Sigma), dissolved in Ringer solution, were placed on the retinal surface. After 60–90 s the superfusion was resumed and the Azure B suctioned off the retina and discarded. Optimal staining density occurred within 10–15 min and cells remained visible for the entire duration of the experiment (up to 10 h). A 780 nm cut-off filter allowed transmission of infrared (IR) light from below the stage and then up through a condenser and the glass coverslip mounted in the superfusion chamber. An IR sensitive CCD camera (IR-1000; Dage-MTI, Michigan City, IN, USA) captured the retinal image that was displayed on a video monitor outside the Faraday cage. This protocol allowed retinas to remain in the dark-adapted state during targeting and recording of cells.

### Extracellular and intracellular recordings

Extracellular recordings from rabbit retina neurons were made using carbon fibre microelectrodes (World Precision Instruments, Sarasota, FL, USA) attached to an isolated AC differential amplifier (ISO-80; World Precision Instruments). Intracellular recordings were obtained from neurons using sharp microelectrodes fashioned from borosilicate glass tubing with filament. Electrodes were filled with 4% *N*-(2-amino-ethyl)-biotinamide hydrochloride (Neurobiotin; Vector Laboratories, Burlingame, CA, USA) or 10 mM Po-Pro-1 iodide (Invitrogen, Carlsbad, CA, USA) in 0.1 M Tris buffer, pH 7.6, and then backfilled with a small amount of 3 M potassium chloride to produce a reversible junction with the Ag–AgCl connector. Final DC resistances of these electrodes ranged from 200 to 450 MΩ. After physiological characterization of a cell, Neurobiotin was injected into the cell with a combination of sinusoidal (3 Hz; 0.8 nA; peak-to-peak) and direct current (0.4 nA) applied simultaneously; this method allowed for passage of tracer through the microelectrode without polarization. For

some experiments, we added the chloride channel blocker 4,4'-dinitro-stilbene-2,2'-disulphonic acid (DNDS; Pfaltz and Bauer Inc., Waterbury, CT, USA) to the microelectrode (500 μM in 0.1 M Tris). All recordings were digitized online with an analog-to-digital board (Digidata 1200; Molecular Devices, Sunnyvale, CA, USA) and stored for offline analysis. All spike records were sorted and time-stamped offline using commercially available software (Offline Sorter; Plexon, Dallas, TX, USA). Peristimulus time histograms were generated using the Neuro-Explorer software (Nex Technologies, Littleton, MA, USA).

### Whole cell recordings

Whole-cell patch-clamp recordings were made from ganglion cells in an isolated rabbit retina. A piece of retina was transferred to the recording chamber and placed flat, ganglion cell layer up, over a cellulose acetate/nitrate membrane filter (Millipore, Billerica, MA, USA) that was mounted in the superfusion chamber. Responses to full-field light stimulation were recorded with low-resistance electrodes (5–6 MΩ), filled with a pipette solution consisting of (in mM): 100 potassium gluconate, 10 NaCl, 0.5 CaCl<sub>2</sub>, 2 MgCl<sub>2</sub>, 0.5 EGTA, 2 ATP, 0.1 GTP and 10 Hepes, buffered to pH 7.4. The retina was superfused at a rate of 4–5 ml min<sup>-1</sup> with a Ringer solution maintained at 34°C and equilibrated with 95% O<sub>2</sub>–5% CO<sub>2</sub> to pH 7.4. Voltage clamp data were acquired at a sampling rate of 2 kHz using pCLAMP 9.2 (Molecular Devices) software, low-pass filtered at 500 Hz and digitized. Series resistance was compensated 50–80% with the series resistance compensation circuitry of the patch-clamp amplifier (Axopatch 200B; Molecular Devices).

### Multielectrode array recordings

For multielectrode array experiments on rabbit and mouse, retinas were isolated, mounted on filter paper (8 μm pore size; Millipore), and placed ganglion side down on the grid of a 60-channel electrode array (Multi Channel Systems, Reutlingen, Germany). The isolated retinas were superfused with a Ringer solution at a rate of 5–7 ml min<sup>-1</sup>. Spike data were sorted and time-stamped using commercially available software as described above.

### Pharmacology

For pharmacological experiments, drugs were applied to the retina by switching from the control Ringer solution to one containing the drug. We utilized the following drugs in our experiments: picrotoxin (PTX; Tocris Bioscience, Ellisville, MO,

USA), L-2-amino-4-phosphonobutyric acid (L-AP4; Tocris Bioscience), SR-95531 (Tocris Bioscience), 1,2,5,6-tetrahydropyridin-4-yl-methylphosphinic acid (TPMPA; Sigma), baclofen (Sigma), CGP-55845 (Tocris Bioscience), strychnine (Sigma), 18 $\beta$ -glycyrrhetic acid (18 $\beta$ -GA; Sigma), and meclofenamic acid (MFA; Sigma).

### Light stimulation

Light stimulus intensities were kept within the scotopic range and retinas were maintained in a dark-adapted state. For most experiments, a green light emitting diode ( $\lambda_{\text{max}} = 525$  nm) focused onto the retinal surface provided full-field illumination. In some experiments, white light provided by a tungsten-halogen lamp was used to stimulate the retina. Light intensities used in different experiments are provided in the figure legends.

For area summation experiments, we utilized concentric green spots of varying sizes, but constant intensity, centred on the soma of the targeted cell. The stimuli were generated on a DLP projector (Samsung, Ridgefield Park, NJ, USA) using Matlab (The MathWorks, Natick, MA, USA) and Psychtoolbox-3 extensions (Brainard, 1997; Pelli, 1997). The stimuli were delivered to the camera port of the microscope by means of a precision, coherent fibre optic and projected onto the retina through the objective.

To measure the receptive field size of cellular responses, a 50  $\mu\text{m}$  wide/1.0 mm long rectangular slit of light was moved along its minor axis (parallel to the visual streak) in discrete steps in both directions from the central position. The position of the slit at which it evoked the largest response was considered to be centred over the cell. Peak spike frequency responses were plotted against stimulus position and the extent of a neuron's centre-receptive field was taken as the diameter of the Gaussian function fitted to the data using Origin software (OriginLab Corp., Northampton, MA, USA). The Gaussian diameter was defined as 0.849 times the width ( $w$ ) of the Gaussian at half height ( $w \approx 2\sigma$ ).

### Histology and immunocytochemistry

After electrophysiological experiments, retinas were fixed at room temperature in a solution of 4% paraformaldehyde in 0.9% phosphate buffered saline (PBS), pH 7.4, for 12–15 min. The retina was then detached, trimmed, and washed overnight in PBS at 4°C. Neurobiotin injections in retinas were visualized using a Cy3-conjugated streptavidin reagent (Sigma). After labelling with streptavidin-Cy3, retinas were washed in PBS for 1 h followed by incubation in a primary antibody solution of goat anti-choline acetyltransferase (ChAT) antibody (Chemicon, Temecula, CA, USA) at

a concentration of 1:100 for 72 h at 4°C. Retinas were then washed in PBS, before incubation in a secondary antibody solution of donkey anti-goat Cy2 (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) at a concentration of 1:200 overnight at 4°C. Retinas were subsequently washed in PBS and mounted in Vectashield mounting medium (Vector Laboratories). Retinas were imaged using a Zeiss 510 Meta confocal microscope (Zeiss, Thornwood, NY, USA).

### Results

To visually target  $\alpha$ -GCs for electrophysiological recordings it was necessary to unequivocally identify them in the superfused rabbit retina-eyecup. As reported previously, Azure B was found to stain a subset of the somata within the ganglion cell layer (GCL) of the living rabbit retina (Hu *et al.* 2000; Hu & Bloomfield, 2003). These included mosaics of cells easily identified by their regular spacing and particularly large somata when viewed under IR illumination. In this study, we targeted the largest somata in the GCL that, when stained with Neurobiotin, displayed the morphological features described previously for  $\alpha$ -GCs in a number of mammalian species, including the rabbit (Boycott & Wässle, 1974; Wässle *et al.* 1975, 1981; Peichl *et al.* 1987; Peichl, 1991; Hu & Bloomfield, 2003) (Fig. 1A). These features included: (1) relatively large somata (diameter range of 19–28  $\mu\text{m}$ ) and dendritic fields (diameter range of 585–1010  $\mu\text{m}$ ); (2) 4–6 stout primary dendrites; (3) dendrites with up to sixth-order radiate branching at acute angles; (4) rare overlap of dendrites; (5) relatively long terminal dendrites; (6) a narrowly stratified arbour in either sublamina *a* or *b* of the inner plexiform layer (IPL), which corresponded to the cells' OFF or ON receptive fields, respectively (Famiglietti *et al.* 1977; Nelson *et al.* 1978; Bloomfield & Miller, 1986) (Fig. 1B); and (7) tracer coupling to neighbouring  $\alpha$ -GCs, as well as an array of different amacrine cell subtypes (Xin & Bloomfield, 1997; Hu & Bloomfield, 2003; Mills *et al.* 2007). We recorded from a total of 124 OFF  $\alpha$ -GCs in this study.

### GABA blockade unmask an ON response in OFF $\alpha$ -GCs

Under control, dark-adapted conditions, recordings from OFF  $\alpha$ -GCs ( $n = 92$ ) showed a brisk and transient response to the offset of light stimuli ( $19 \pm 11$  spikes) (Fig. 2A and E) as previously described in a number of mammalian species (Peichl & Wässle, 1981; Saito, 1983; Fukuda *et al.* 1984; Stanford & Sherman, 1984; Hu & Bloomfield, 2003). The OFF  $\alpha$ -GCs showed little to no spontaneous activity in dark-adapted retinas and therefore the light-evoked responses were unambiguous.

Application of the non-specific GABA blocker picrotoxin (PTX;  $100 \mu\text{M}$ ) had two prominent and reversible effects on the responses of OFF  $\alpha$ -GCs (Fig. 2B and F). First, it produced an increase in the response to light offset, indicating a release from tonic inhibition. Second, PTX revealed a robust response at light onset that had brisk and transient properties similar to those of the OFF response ( $14 \pm 9$  spikes). We found that PTX unmasked an ON response in every OFF  $\alpha$ -GC examined in this study ( $n = 41$ ). The unmasked ON response showed a latency of  $180 \pm 28$  ms.

To determine whether the unmasked ON response is in fact generated by the ON pathway we applied the mGluR6 agonist L-AP4 ( $100 \mu\text{M}$ ), which effectively abolishes the responses of ON bipolar cells (Slaughter & Miller, 1981). Whereas the application of L-AP4 reversibly blocked the ON response of OFF  $\alpha$ -GCs ( $n = 13$ ), the OFF response remained (Fig. 2C). These results indicate that the unmasked ON response is dependent on a functionality of the ON pathway. Interestingly, we found that L-AP4 often enhanced the OFF response of OFF  $\alpha$ -GCs, a finding consistent with crossover inhibitory interactions between the ON and OFF pathways (Zaghloul *et al.* 2003; Renteria *et al.* 2006; Roska *et al.* 2006; Molnar & Werblin, 2007; Hsueh *et al.* 2008; Manookin *et al.* 2008; Molnar *et al.* 2009).

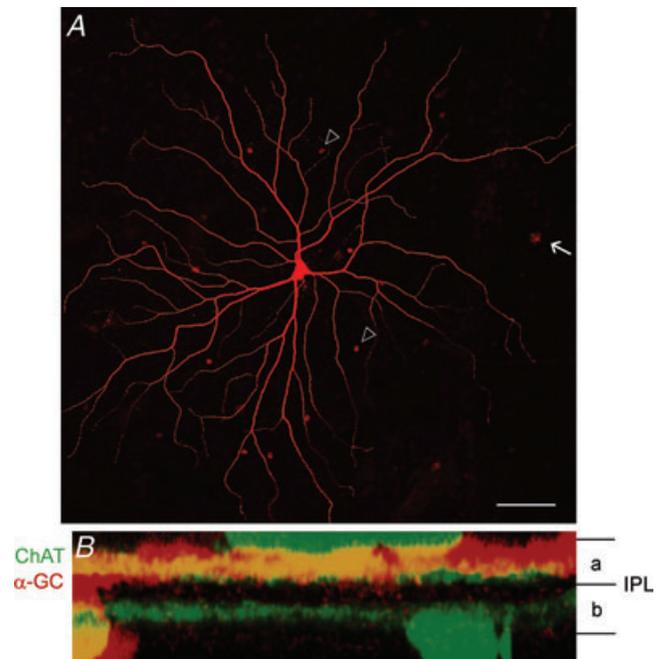
To determine the receptive field structure of the ON and OFF responses of OFF  $\alpha$ -GCs, we computed the area summations by evoking them with concentric spots of light of changing diameter, but of constant dim intensity ( $n = 5$ ) (Fig. 3A). Both ON and OFF responses were saturated by spots of light between about  $500$  and  $750 \mu\text{m}$ , whereas larger spots produced a slight attenuation of activity, presumably due to activation of a reduced antagonistic surround under our dark-adapted conditions (Jensen, 1991; Muller & Dacheux, 1997). The similar profiles of the ON and OFF responses suggest that the former is not a surround response, but rather that both signals are mediated by centre receptive field mechanisms.

To further evaluate the receptive field sizes of the ON and OFF responses, we computed Gaussian widths using a  $50 \mu\text{m}$  wide/ $1 \text{ mm}$  long slit of light displaced in discrete lengths along its minor axis. Consistent with the area summation measures, we found that the ON and OFF receptive fields of individual OFF  $\alpha$ -GCs were comparable in size (Fig. 3B and C). These data indicate that the ON and OFF receptive fields were co-extensive and occupied similar space, consistent with the idea that they are both centre-mediated.

### Pharmacology of the unmasked ON response

Since PTX blocks both GABA<sub>A</sub> and GABA<sub>C</sub> receptors, we used antagonists specific for the receptors to determine

the role of each in the inhibition masking ON responses. Application of the GABA<sub>A</sub> receptor blocker SR-95531 in doses of  $5$ – $20 \mu\text{M}$  ( $n = 7$ ) did not unmask any ON spikes in OFF  $\alpha$ -GCs (Fig. 4B). Similarly, blockade of GABA<sub>C</sub> receptors with TPMPA in doses of  $50$ – $100 \mu\text{M}$  ( $n = 5$ ) also failed to unmask the ON response (Fig. 4C). In current clamp experiments, we did find that application of SR-95531 ( $20 \mu\text{M}$ ) and TPMPA ( $100 \mu\text{M}$ ) produced a small depolarization at light onset,  $4.2 \pm 1.3 \text{ mV}$  and  $5.6 \pm 1.8 \text{ mV}$ , respectively. Thus, while the selective GABA blockers could unmask a subthreshold, excitatory synaptic response at light onset, neither alone could unmask the robust spike response revealed by PTX. However, when SR-95531 and TPMPA were applied together at the lowest doses ( $5 \mu\text{M}$  and  $50 \mu\text{M}$ , respectively), the robust ON spike response was reversibly unmasked in OFF  $\alpha$ -GCs ( $n = 7$ ) (Fig. 4D). These results indicate that whereas both GABA<sub>A</sub> and GABA<sub>C</sub> receptors subserve the inhibition masking the ON response in OFF  $\alpha$ -GCs, activation of each receptor alone was sufficient to mask it. We also examined whether metabotropic GABA<sub>B</sub> receptors are involved in the masking of the ON response. However, neither the application of the GABA<sub>B</sub> receptor agonist baclofen



**Figure 1. Coupling pattern and dendritic stratification of OFF  $\alpha$ -GCs in the rabbit retina**

A, photomicrograph showing the flatmount image of an OFF  $\alpha$ -GC injected with Neurobiotin. The somata of tracer-coupled amacrine cells (arrowheads) and  $\alpha$ -GCs (arrow) can be visualized. Scale bar:  $100 \mu\text{m}$ . B, a z-stack (vertical) confocal image of the dendrites of a Neurobiotin-injected OFF  $\alpha$ -GC (red). The dendrites stratify within sublamina-a of the IPL. The two ChAT bands (green) were labelled using an antibody to provide landmarks for the boundaries of sublamina-a and -b.

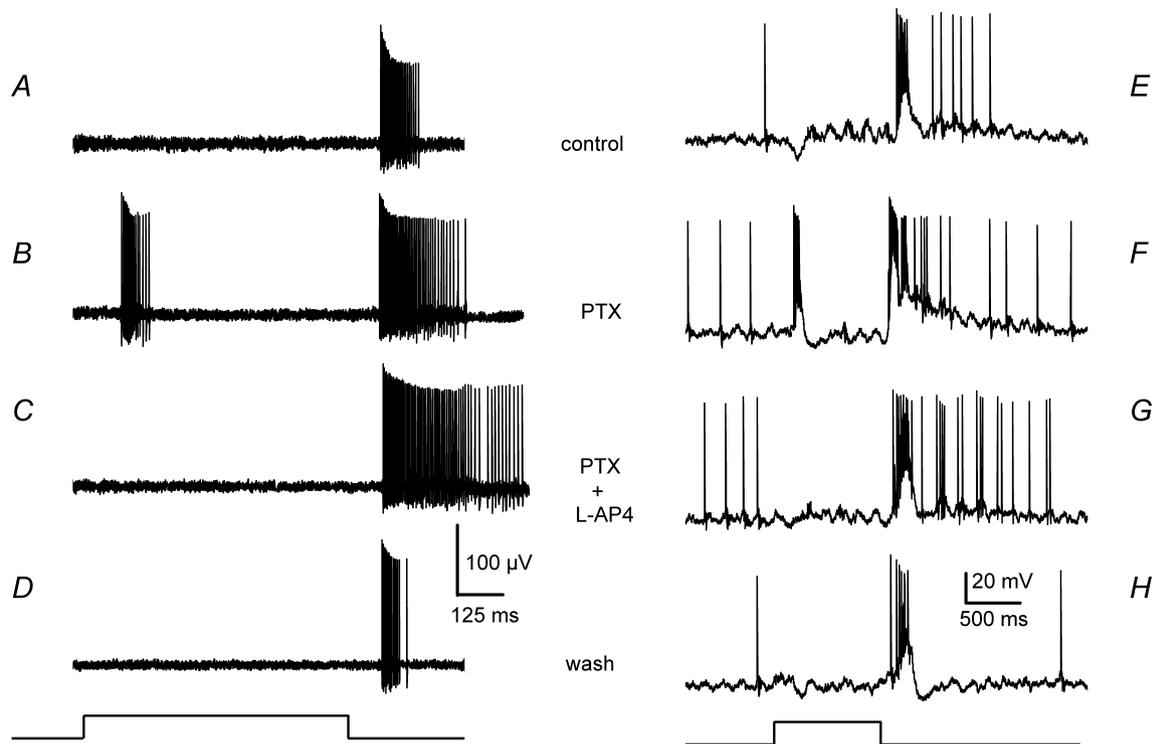
(100  $\mu\text{M}$ ;  $n=8$ ) nor the GABA<sub>B</sub> receptor antagonist CGP-55845 (50  $\mu\text{M}$ ;  $n=6$ ) was able to unmask an ON response in OFF  $\alpha$ -GCs (Fig. 4G and H).

Picrotoxin has been shown to block glycine receptors in retinal neurons (Wang & Slaughter, 2005; Li & Slaughter, 2007), suggesting that at least part of the effects of PTX could have reflected blockade of glycinergic inhibition. However, while application of strychnine (1  $\mu\text{M}$ ;  $n=9$ ) increased the spontaneous activity of OFF  $\alpha$ -GCs and evoked bursts of spikes, it never unmasked an ON response (Fig. 4J).

### Localization of inhibitory circuits masking the ON response

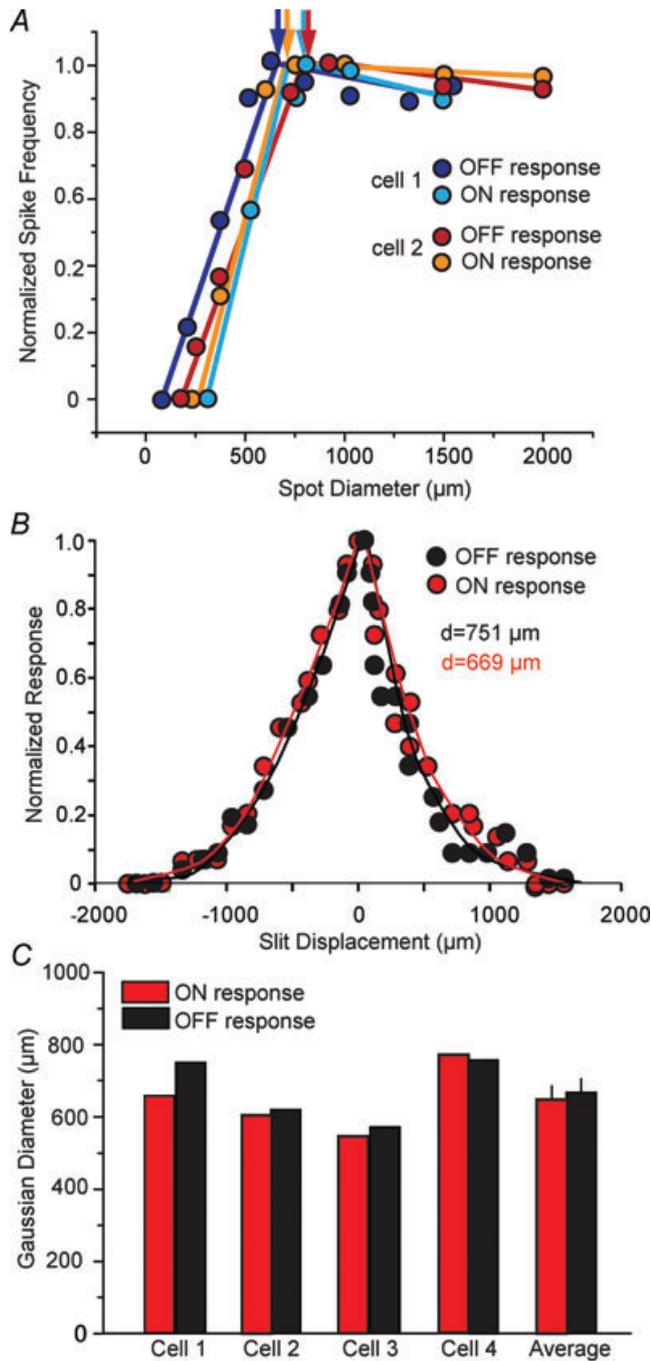
There are two major types of inhibition found in the inner retina: feedforward inhibition in which amacrine cells synapse directly onto ganglion cells and feedback

inhibition where amacrine cells synapse onto the axon terminals of bipolar cells or dendrites of other amacrine cells (Dowling & Boycott, 1966). The finding that GABA<sub>C</sub> receptors play a role in the masking of ON responses in OFF  $\alpha$ -GCs implicates feedback inhibition to bipolar cells, whereas GABA<sub>A</sub> receptors can subserve either feedback or feedforward inhibition (Enz *et al.* 1996; Wässle *et al.* 1998; Rotolo & Dacheux, 2003; Zhou & Dacheux, 2005; Eggers *et al.* 2007; Eggers & Lukasiewicz, 2010). To differentiate between these two possibilities we examined whether the chloride channel blocker DNDS could unmask the ON response in OFF  $\alpha$ -GCs. Delivered to OFF  $\alpha$ -GCs via an intracellular microelectrode, DNDS eliminates the effects of feedforward GABA<sub>A</sub> receptor activation by blocking the chloride channel complex (Dudek & Friedlander, 1996; Shao & Burkhalter, 1996; Völgyi *et al.* 2002). Therefore, if GABA<sub>A</sub>-mediated feedforward inhibition to OFF  $\alpha$ -GCs was responsible for masking the ON response, then



**Figure 2. Blockade of GABAergic inhibition unmasks a robust ON response in OFF  $\alpha$ -GCs in the rabbit retina**

A, extracellularly recorded responses of an OFF  $\alpha$ -GC consists of a brisk and transient burst of spike activity at stimulus offset. Presentation of the light stimulus is indicated by the stimulus trace at the bottom of the figure. The increase in spike activity at light offset is likely to reflect the removal of a tonic inhibition at light offset. B, blockade of GABAergic inhibition with PTX (100  $\mu\text{M}$ ) unmasks a robust brisk and transient ON response at stimulus onset. The increase in spike activity at light offset likely reflects the removal of a tonic inhibition. C, blockade of the ON pathway using the mGluR6 receptor agonist L-AP4 (100  $\mu\text{M}$ ) abolishes the unmasked ON response, indicating that the ON component is produced by the ON pathway. The increase in spike activity at stimulus offset is likely to reflect the removal of a crossover inhibition by ON amacrine cells; see text for details. D, after wash, the OFF  $\alpha$ -GC once again shows the classic response at stimulus offset. Light intensity of green stimulus for extracellular recordings was 0.21  $\text{Rh}^* \text{rod}^{-1} \text{s}^{-1}$ . E–H, current clamp recordings from an OFF  $\alpha$ -GC using the same pharmacological protocol as in A–D. Light intensity for white light stimulus =  $\log -5.5$ . Maximum irradiance ( $\log 0.0$ ) = 2.37  $\text{mW cm}^{-2}$ .



**Figure 3. The classic OFF and unmasked ON responses of OFF  $\alpha$ -GCs show similar area summation and receptive field profiles**

A, area summations were computed from extracellular spike recordings evoked by concentric spots of light of varying diameter, but of constant intensity. The curves show the area summation profiles of the ON (unmasked by PTX) and OFF responses of two OFF  $\alpha$ -GCs in the rabbit retina. The area summation of all the responses fell within a common range suggesting that both ON and OFF signals were centre mediated. Reductions in the response profiles to larger spots of light are likely due to surround inhibitory mechanisms. Light intensities for green stimulus =  $0.21 \text{ Rh}^* \text{ rod}^{-1} \text{ s}^{-1}$ . B, receptive field profiles of the OFF and

intracellular application of DNDS, together with bath-applied TPMPA, to block GABA<sub>C</sub>-mediated inhibition, should abolish the GABAergic inhibition and reveal the response. Conversely, if feedback inhibition was responsible for masking the ON response, then application of DNDS should have no effect.

The intracellularly recorded responses of OFF  $\alpha$ -GCs consisted of a hyperpolarization at light onset, likely to be due to glycinergic crossover inhibition from the ON pathway (Manookin *et al.* 2008; Murphy & Rieke, 2008) and a depolarizing response at light offset (Fig. 5A). Application of DNDS ( $500 \mu\text{M}$ ;  $n = 5$ ) had two major effects including an increase in spontaneous spike activity, presumably due to release from a tonic inhibition, and a reduction of the inhibitory ON hyperpolarization (Fig. 5B). However, DNDS did not unmask an ON response in OFF  $\alpha$ -GCs ( $n = 5$ ) indicating that blockade of feedforward inhibition via GABA<sub>A</sub> receptors was insufficient to produce unmasking. To determine whether a combination of GABA<sub>C</sub>-mediated feedback inhibition and GABA<sub>A</sub>-mediated feedforward inhibition masked the ON response, we applied TPMPA ( $50 \mu\text{M}$ ) together with DNDS ( $500 \mu\text{M}$ ) ( $n = 3$ ). This combination also did not unmask an ON response in OFF  $\alpha$ -GCs (Fig. 5C). In contrast, application of DNDS together with PTX ( $n = 2$ ), which blocks both the GABA<sub>A</sub> and GABA<sub>C</sub> receptors presynaptic to the OFF  $\alpha$ -GCs, successfully unmasked the ON response (Fig. 5D). Taken together, these results suggest that feedback inhibition, mediated by both GABA<sub>A</sub> and GABA<sub>C</sub> receptors, is responsible for masking ON responses in OFF  $\alpha$ -GCs and that feedforward inhibition plays little or no role.

To further verify the presynaptic origin of the inhibition masking the ON responses, we performed whole-cell voltage clamp recordings from OFF  $\alpha$ -GCs ( $n = 4$ ). At  $-60 \text{ mV}$  and more positive holding potentials, the light evoked response included an inhibitory outward current at light onset as previously reported (Manookin *et al.* 2008; Murphy & Rieke, 2008) and an excitatory inward current at offset (Fig. 5E). Under voltage clamp conditions, with the membrane potential held at  $-70 \text{ mV}$ , near the reversal potential for chloride, the outward current at light onset was eliminated, but this did not reveal an excitatory inward current (Fig. 5E). Our results are consistent with those of Molnar *et al.* (2009) who reported that OFF  $\alpha$ -GCs in the

unmasked ON response following application of PTX to an OFF  $\alpha$ -GC. The computed Gaussian diameters were very similar for the two responses. C, the receptive field Gaussian diameters computed for the OFF and unmasked ON responses for 4 OFF  $\alpha$ -GCs and the composition averages. Like for the area summation measures, the receptive fields of the ON and OFF responses were very similar. Light intensity for white light stimulus =  $\log -5.0$ . Maximum irradiance ( $\log 0.0$ ) =  $2.37 \text{ mW cm}^{-2}$

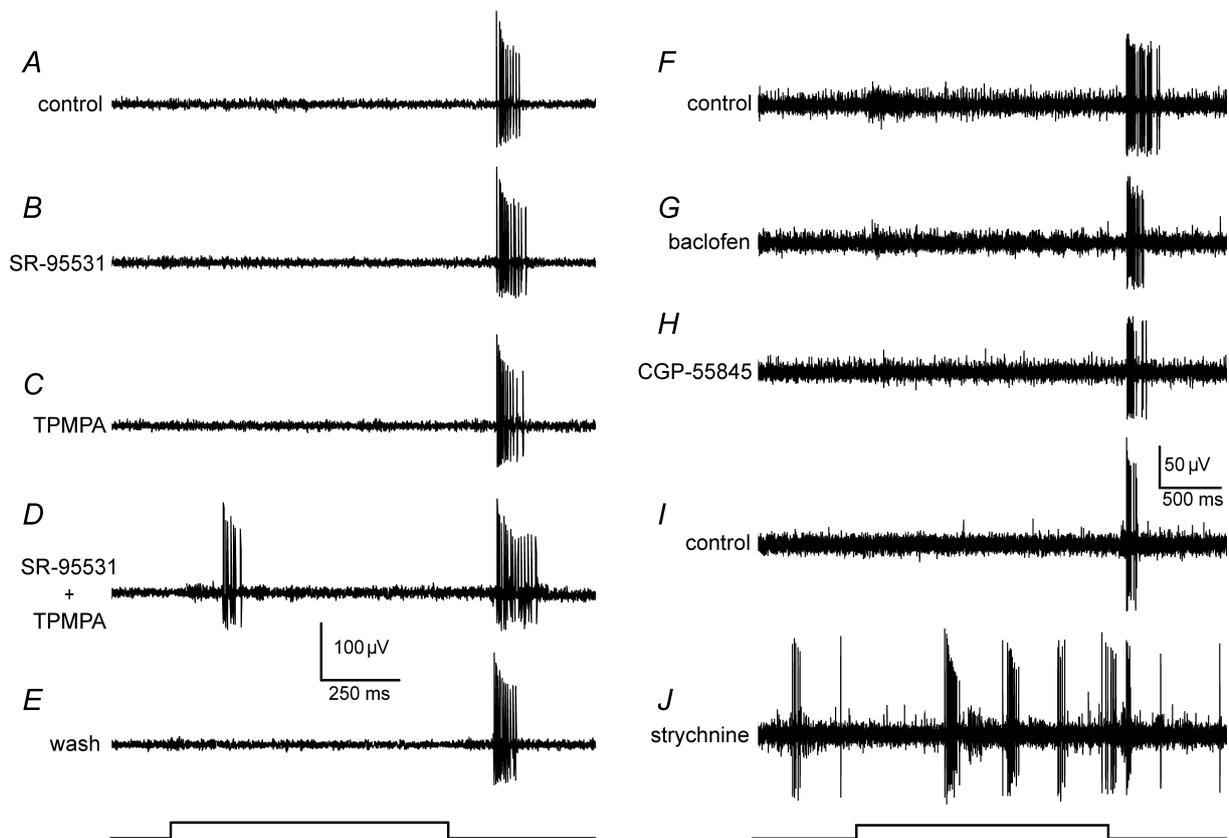
rabbit do not receive excitation at light onset. In contrast, bath application of PTX ( $100\ \mu\text{M}$ ) or a combination, but not separate, application of SR-95531 ( $20\ \mu\text{M}$ ) and TPMPA ( $100\ \mu\text{M}$ ) (data not shown) unmasked an excitatory current in response to light onset in these cells (Fig. 5F). These findings indicate that OFF  $\alpha$ -GCs only receive an excitatory ON input after the blockade of GABAergic inhibition with PTX, further supporting the conclusion that the excitatory ON signals are masked by inhibition positioned presynaptically.

### Coupling via gap junctions is required for expression of the ON response

Since the dendrites of OFF  $\alpha$ -GCs are restricted to sublamina-*a* of the IPL, they are positioned to receive excitatory inputs only from OFF bipolar cells and not

from ON bipolar cells whose axons stratify in sublamina-*b* (Nelson *et al.* 1978). This organization raises the important question: how do OFF  $\alpha$ -GCs receive excitatory signals from the ON pathway? Although it would appear that OFF  $\alpha$ -GCs cannot receive excitatory ON inputs via conventional chemical circuitry, an alternative pathway is the gap junctions formed with a number of amacrine cell subtypes (Xin & Bloomfield, 1997; Hu & Bloomfield, 2003; Mills *et al.* 2007).

To test this hypothesis we used  $18\beta$ -glycyrrhetic acid ( $18\beta$ -GA), which has been shown to effectively block gap junctional transmission in the nervous system (Davidson & Baumgarten, 1988; Ackert *et al.* 2009). We first unmasked the ON response in OFF  $\alpha$ -GCs with PTX ( $100\ \mu\text{M}$ ) and then applied  $18\beta$ -GA ( $25\ \mu\text{M}$ ) to block gap junctions (Fig. 6A–E). We found that  $18\beta$ -GA reversibly abolished the unmasked ON response, but had



**Figure 4. Both GABA<sub>A</sub> and GABA<sub>C</sub> receptors are involved in inhibitory circuits responsible for masking of the ON responses of OFF  $\alpha$ -GCs**

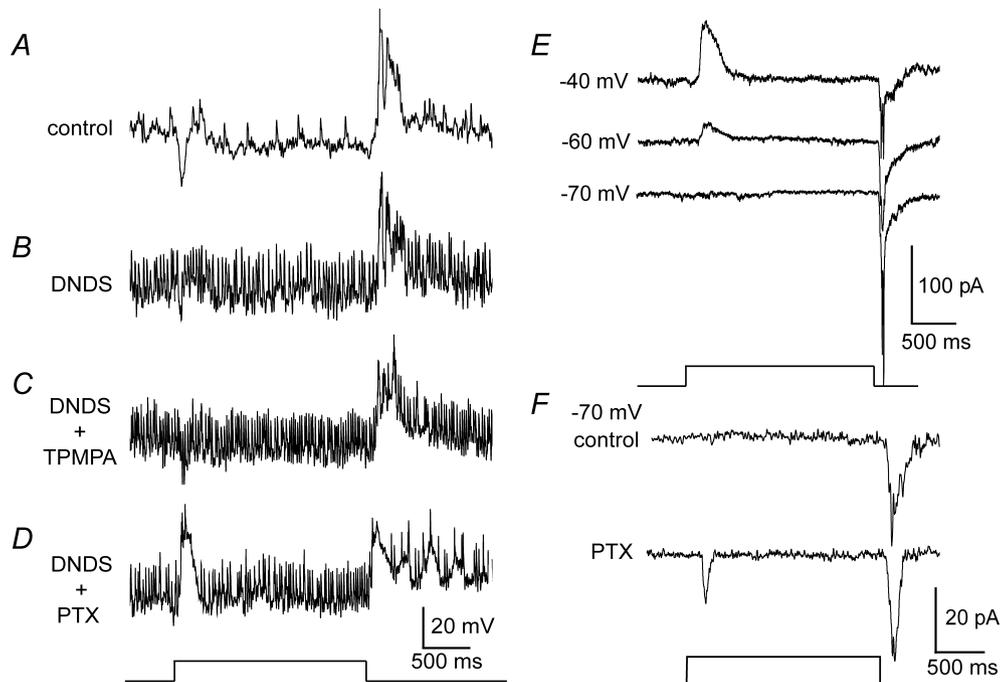
A, response of an OFF  $\alpha$ -GC in the rabbit retina under control conditions. Trace at bottom indicates onset and offset of the light stimulus. Neither the blockade of GABA<sub>A</sub> receptors using SR-95531 (B,  $5\ \mu\text{M}$ ), nor the blockade of GABA<sub>C</sub> receptors using TPMPA (C,  $50\ \mu\text{M}$ ) alone unmask an ON response in the OFF  $\alpha$ -GC. D, however, the simultaneous blockade of both receptors unmask the ON response. E, the effects of the selective GABA receptor blockers were reversible after wash. F, response of another OFF  $\alpha$ -GC under control conditions. Neither the GABA<sub>B</sub> receptor agonist baclofen (G,  $100\ \mu\text{M}$ ), nor the GABA<sub>B</sub> receptor antagonist CGP-55845 (H,  $50\ \mu\text{M}$ ) unmask an ON response and neither has a significant effect on the OFF light response of the OFF  $\alpha$ -GC. I, response of another OFF  $\alpha$ -GC under control conditions. J, blockade of glycine receptors using strychnine ( $1\ \mu\text{M}$ ) increased spontaneous burst activity of the OFF  $\alpha$ -GC, but failed to unmask an ON response at stimulus onset. Light intensity of green stimulus =  $0.21\ \text{Rh}^* \text{rod}^{-1} \text{s}^{-1}$ .

no significant effect on the OFF response ( $n = 7$ ). We have recently shown that it takes approximately 20 min for  $18\beta$ -GA to block gap junctions in the retina (Ackert *et al.* 2009). Consistent with the timing of the uncoupling action of  $18\beta$ -GA, we found that it took about 20 min of exposure of the drug to completely block the ON response in OFF  $\alpha$ -GCs. To verify the results with  $18\beta$ -GA, we utilized a second gap junction blocker, meclofenamic acid (MFA; Pan *et al.* 2007). Identical to the effects of  $18\beta$ -GA, administration of MFA ( $100 \mu\text{M}$ ) completely abolished the ON response unmasked with PTX, yet had no effect on the OFF response ( $n = 3$ ) (Fig. 6F–H).

These results suggest that electrical synapses form the pathway for crossover excitation of OFF  $\alpha$ -GCs. To further test this idea, we investigated crossover excitation in ON  $\alpha$ -GCs, which, unlike OFF  $\alpha$ -GCs, are not electrically

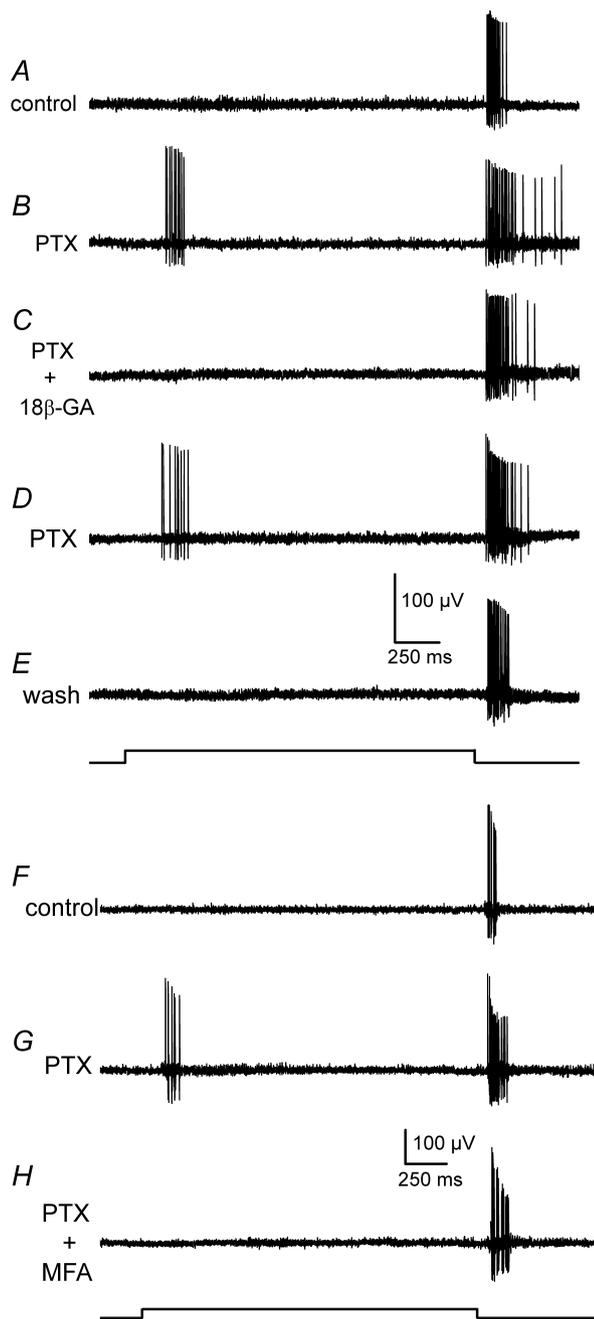
coupled (Hu & Bloomfield, 2003). If gap junctions are required for reception of excitatory crossover inputs, we would expect that ON  $\alpha$ -GCs would not display an OFF response under GABAergic blockade. Indeed, we found that blockade of GABAergic inhibition with PTX ( $100 \mu\text{M}$ ) did not unmask an excitatory OFF response in ON  $\alpha$ -GCs ( $n = 4$ ) (Fig. 7).

Our results indicate that functional gap junctions, presumably those between OFF  $\alpha$ -GCs and amacrine cells, are required for the transmission of excitatory ON signals to OFF  $\alpha$ -GCs. In this scheme, amacrine cells receive excitatory inputs from ON bipolar cell terminals in sublamina-*b* of the IPL and subsequently relay them to OFF  $\alpha$ -GCs via gap junctions that lie in sublamina-*a*. To test this idea, we examined the stratification of amacrine cells coupled to OFF  $\alpha$ -GCs. We first injected OFF  $\alpha$ -GCs



**Figure 5. The inhibition responsible for masking the ON response acts presynaptic to OFF  $\alpha$ -GCs**

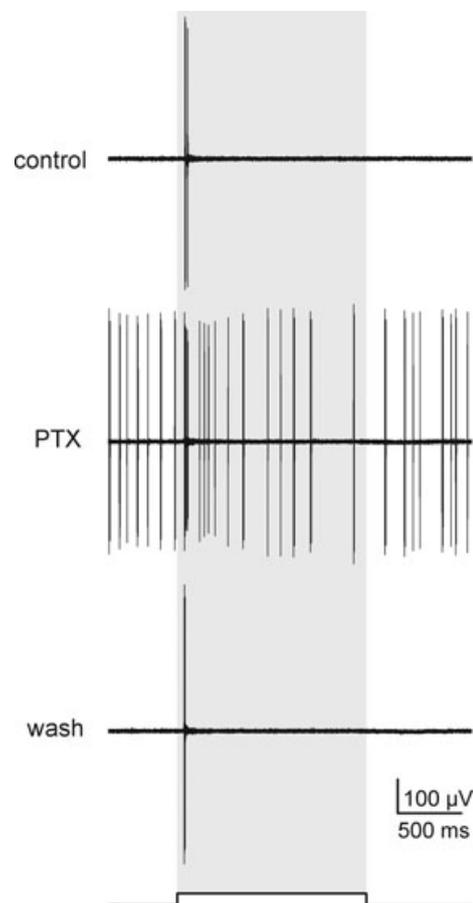
A, intracellular recording of an OFF  $\alpha$ -GC shows a hyperpolarizing component at light onset and a depolarization at light offset. Light trace indicating stimulus onset and offset is below panel D. B, intracellular blockade of chloride channels using a DNDS-filled ( $500 \mu\text{M}$ ) microelectrode increases spontaneous spiking and reduces the hyperpolarization at stimulus onset indicating a blockade of tonic and light-evoked inhibition of the OFF  $\alpha$ -GC. However, DNDS did not unmask an excitatory ON response. C, simultaneous application of intracellular DNDS and bath-applied TPMPA ( $50 \mu\text{M}$ ), which blocked  $\text{GABA}_A$ -mediated feedforward inhibition and  $\text{GABA}_C$ -mediated feedback inhibition, did not unmask an ON response in the OFF  $\alpha$ -GC. D, however, the simultaneous application of intracellular DNDS and bath-applied PTX ( $100 \mu\text{M}$ ), which blocked both  $\text{GABA}_A$ - and  $\text{GABA}_C$ -mediated feedback inhibition, unmasked a robust excitatory ON depolarization at light onset. Resting membrane potentials for recordings in panels A–D were  $50$ – $55$  mV. E, whole cell recording from an OFF  $\alpha$ -GC voltage clamped to  $-40$ ,  $-60$ , and  $-70$  mV. At a membrane potential of  $-40$  mV, the cell shows an outward inhibitory current at light onset and an inward excitatory current at light offset. At the approximate reversal potential for chloride of  $-70$  mV, the outward current at light onset is eliminated, but no excitatory inward current is unmasked. Light-evoked excitatory postsynaptic currents recorded from an OFF  $\alpha$ -GC indicate the presence of excitatory inputs at light offset, but not light onset, under control conditions. F, whole cell recording of an OFF  $\alpha$ -GC voltage clamped at  $-70$  mV before and after bath application of PTX ( $100 \mu\text{M}$ ). PTX unmasked an excitatory inward current in response to light onset. Light intensity of green stimulus =  $0.21 \text{ Rh}^* \text{ rod}^{-1} \text{ s}^{-1}$ .



**Figure 6. Gap junctions are required for the presence of excitatory ON responses in OFF  $\alpha$ -GCs**

A, the typical extracellularly recorded response of an OFF  $\alpha$ -GC in the rabbit retina under control conditions. B, application of PTX (100  $\mu$ M) unmasks a robust ON response in the OFF  $\alpha$ -GC. C, application of the gap junction blocker 18 $\beta$ -GA (25  $\mu$ M) blocks the unmasked ON response, but has no significant effect on the OFF response. D, the ON response returns after washout of 18 $\beta$ -GA. E, the typical response of the OFF  $\alpha$ -GC is restored when all drugs are washed out. F, the typical extracellularly recorded response of another OFF  $\alpha$ -GC in the rabbit retina under control conditions. G, application of PTX (100  $\mu$ M) unmasked an ON response at stimulus onset. H, application of the gap junction blocker MFA (100  $\mu$ M) blocks the unmasked ON response, but has no significant effect on the OFF response. Light intensity of green stimulus = 0.21 Rh\* rod<sup>-1</sup> s<sup>-1</sup>.

with the gap junction-permeant fluorescent dye Po-Pro-1 (Hoshi *et al.* 2006) to visualize the somata of coupled amacrine cells in the living rabbit retina (Fig. 8A). We have shown previously that OFF  $\alpha$ -GCs in the rabbit are coupled to a number of different amacrine cell subtypes (Hu *et al.* 2010). Here, we targeted the Po-Pro-1-labelled amacrine cell somata of intermediate size, and injected them with Neurobiotin to determine their dendritic/axonal morphology ( $n = 12$ ). Figure 8B shows an example of a Neurobiotin-injected intermediate amacrine cell coupled to an OFF  $\alpha$ -GC. The amacrine cell displays radiate dendritic branching that narrows to form thin axon-like structures running for nearly 2 mm. A vertical rotation of the confocal Z-stack image of the amacrine cell shows that the cell maintains processes that stratify in sublamina-*a* (Fig. 8C) and sublamina-*b* (Fig. 8D) of the IPL. We examined the dendritic morphology of 12



**Figure 7. PTX does not unmask crossover excitation in ON  $\alpha$ -GCs**

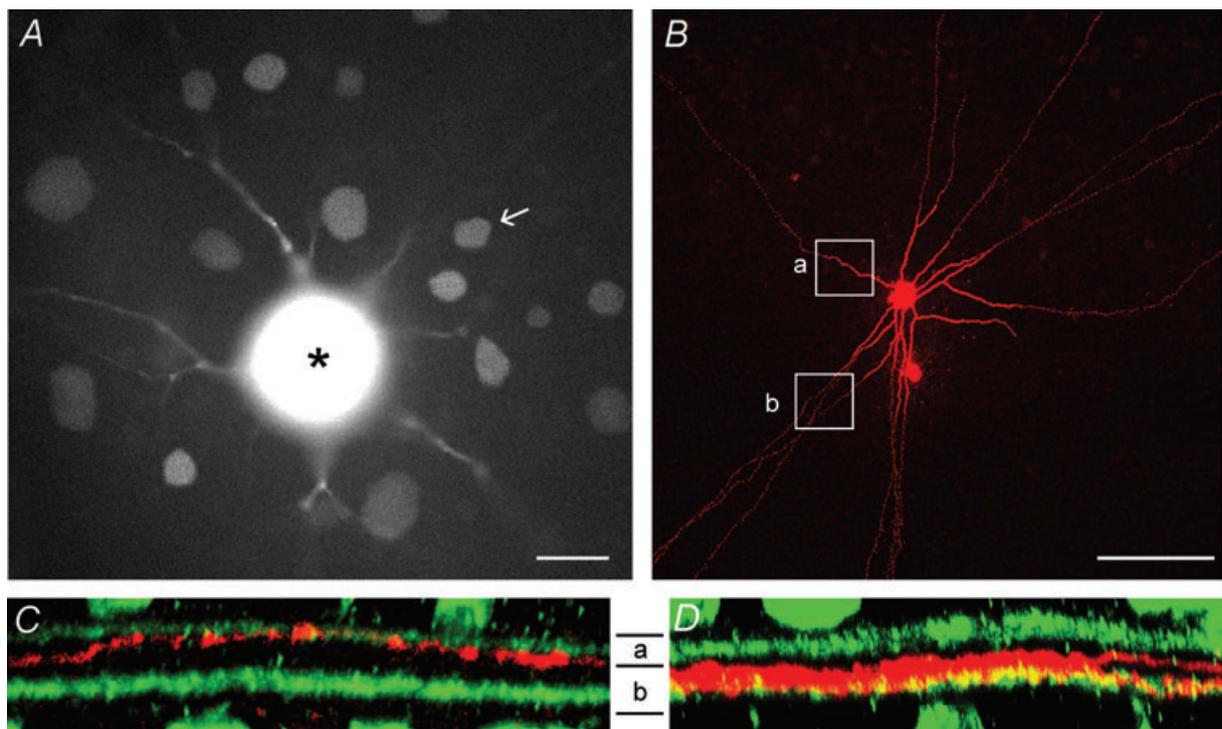
Extracellular recordings from an ON  $\alpha$ -GC before, during and after application of PTX (100  $\mu$ M). PTX increased the spontaneous and light-evoked spike activity of the cell, but did not unmask an OFF response at stimulus offset. Shaded bar indicates duration of the light stimulus. Light intensity of green stimulus = 0.21 Rh\* rod<sup>-1</sup> s<sup>-1</sup>.

intermediate-sized amacrine cells coupled to OFF  $\alpha$ -GCs and found that each maintained processes in both sublaminae of the IPL. These results indicate that the amacrine cells coupled to OFF  $\alpha$ -GCs can serve as the necessary anatomical substrate for receiving excitatory inputs from ON bipolar cell terminals in sublamina-*b* of the IPL and the subsequent transfer of these ON signals to OFF  $\alpha$ -GCs via gap junctions made in sublamina-*a*.

### Excitatory crosstalk between the ON and OFF pathways is widespread in mammalian ganglion cells

Considering the fact that many ganglion cell subtypes are coupled to neighbouring amacrine cells (Vaney, 1991; Xin & Bloomfield, 1997; Völgyi *et al.* 2009), our results for OFF  $\alpha$ -GCs indicating that these gap junctions may form the conduit for the mixing of excitatory ON and OFF signals suggest that excitatory crosstalk between the ON and OFF pathways may be widespread amongst

mammalian ganglion cells. To test this idea we used a 60-channel multielectrode array to determine the effects of PTX on a large number of ganglion cells in both the rabbit and mouse retinas. In the rabbit, we found that the blockade of GABA receptors with PTX (100  $\mu$ M) unmasked robust ON responses in 44% of OFF ganglion cells we recorded ( $54 \pm 13$  spikes;  $n = 34$ ) and unmasked an OFF response in 31% of ON ganglion cells ( $49 \pm 9$  spikes;  $n = 35$ ) (Fig. 9A). Latencies of the unmasked ON and OFF responses were  $174 \pm 24$  ms and  $125 \pm 23$  ms to stimulus onset and offset, respectively. In the mouse retina, PTX (100  $\mu$ M) revealed an ON response in 19% of OFF ( $26 \pm 15$  spikes;  $n = 21$ ) ganglion cells, whereas PTX unmasked OFF responses in 44% of ON ganglion cells ( $17 \pm 13$  spikes;  $n = 25$ ) examined (Fig. 9B). Latencies of the unmasked ON and OFF responses were  $187 \pm 34$  ms and  $155 \pm 32$  ms to stimulus onset and offset, respectively. Overall, these results indicate that a significant number of mammalian ganglion cells receive masked excitatory crossover inputs.



**Figure 8.** OFF  $\alpha$ -GCs are coupled to amacrine cells that stratify within both sublamina-*a* and sublamina-*b* of the IPL

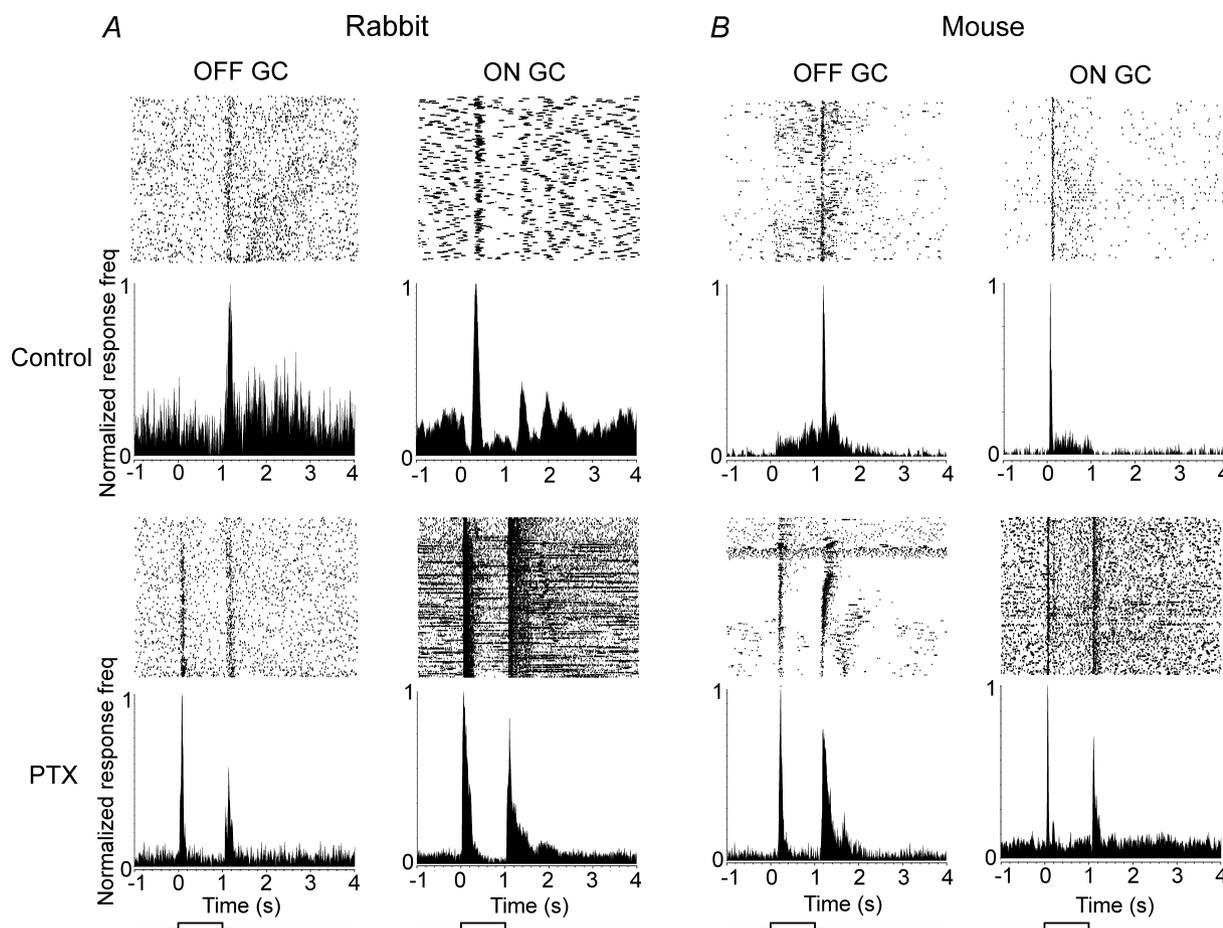
A, photomicrograph of the living rabbit retina showing an OFF  $\alpha$ -GC injected with the gap junction-permeant fluorescent tracer Po-Pro-1. The soma of the OFF  $\alpha$ -GC with bloomed fluorescence is indicated by the asterisk. The somata of coupled amacrine cell and ganglion cell neighbours can also be clearly visualized. One amacrine cell (arrow) was labelled with Neurobiotin. Scale bar = 25  $\mu$ m. B, confocal micrograph of the amacrine cell in panel A labelled with Neurobiotin after *post hoc* histological processing. This subtype of amacrine cell typically showed long radiate branches that became narrow and axon-like, running for up to 2 mm. The rectangles show the portion of the processes in the confocal images in the subsequent panels. Scale bar = 100  $\mu$ m. C, Z-stack vertical confocal images of the amacrine cell process (red) in rectangle 'a' in panel B showing that it stratifies in sublamina-*a* of the IPL. ChAT bands are shown in green. D, Z-stack vertical confocal image of the amacrine cell process (red) in rectangle 'b' in panel B showing that it stratifies within sublamina-*b* of the IPL.

## Discussion

Work over the past 70 years has shown the segregation of visual information into parallel ON and OFF pathways to be a principal organizing feature of the visual system (Hartline, 1938; Werblin & Dowling, 1969; Famiglietti & Kolb, 1976). Here, we found that over one-third of the ganglion cells in the rabbit and mouse retinas with classic ON and OFF responses also display robust opposite polarity responses following blockade of GABAergic inhibition. Interestingly, reports of masked opposite polarity responses in mammalian ganglion cells have surfaced intermittently over the years (Ariel & Daw, 1982; Nirenberg & Meister, 1997; Roska & Werblin, 2001; Rentería *et al.* 2006; Ackert *et al.* 2009). However, these results were often cited anecdotally, even attributed to technical artifact, and so the extent of crossover excitation in the retina has remained unclear. Further, previous investigations had all focused on the masked OFF inputs

of ON ganglion cells and it therefore remained an open question as to whether OFF ganglion cells could receive excitatory crossover ON inputs. The present report is thus the first to examine crossover excitation in both ON and OFF ganglion cells in the mammalian retina and to examine the synaptic circuitry underlying its generation and masking. Overall, our results indicate a significant interaction between excitatory ON and OFF signals in the inner retina and thus call into question the traditional view that these signals are largely segregated within parallel retinal circuits innervating different ganglion cell subtypes.

Our spatial measures showed that the area summation and receptive fields of the ON responses in OFF  $\alpha$ -GCs were co-extensive with those of the OFF responses, suggesting that the unmasked ON response was centre-mediated. This assertion is supported by our finding that the ON responses were blocked by application



**Figure 9. Crossover excitation is displayed by many ganglion cells in the rabbit and mouse retinas**

*A*, raster plots and peristimulus time histograms (PSTHs) of an OFF and an ON ganglion cell in the rabbit retina recorded with a multielectrode array. Blockade of GABAergic inhibition with PTX ( $100 \mu\text{M}$ ) unmasks robust responses of opposite polarity. *B*, raster plots and PSTHs of an OFF and an ON ganglion cell in the wild-type mouse retina recorded with a multielectrode array. Similar to the rabbit, blockade of GABAergic inhibition with PTX unmasks robust responses with polarity opposite to that of the cells' classic centre-mediated responses. Light intensity of green stimulus =  $0.18 \text{ Rh}^* \text{ rod}^{-1} \text{ s}^{-1}$ .

of L-AP4, indicating that they were generated by the ON pathway. Consistent with our findings, Roska & Werblin (2001) found that, following PTX application, OFF responses could be evoked in certain ON ganglion cells in rabbit with a light stimulus restricted to the centre receptive field.

Recently, Rentería *et al.* (2006) reported a late ON response in certain OFF ganglion cells in the mouse retina. However, those responses were not blocked by application of L-AP4, indicating that they were not generated by the ON pathway, and showed latencies >1000 ms from stimulus onset. The unmasked ON responses reported here were sensitive to L-AP4 and showed latencies <200 ms and thus were different from the late ON responses reported previously.

### Feedback inhibition masks crossover excitation

Our results show that a GABAergic inhibition, subserved by both GABA<sub>A</sub> and GABA<sub>C</sub> receptors, masks the ON signals in OFF  $\alpha$ -GCs under our experimental conditions. Two lines of evidence indicate that the site(s) of the masking inhibition is likely to be presynaptic to ganglion cells. First, we found that application of the intracellular chloride channel blocker DNDS to OFF  $\alpha$ -GCs, either alone or together with TPMPA, did not unmask an ON response. Second, no excitatory ON signals were recorded in voltage-clamped OFF  $\alpha$ -GCs even when held at the reversal potential for the inhibitory response evoked at stimulus onset. Consistent with our finding, Molnar *et al.* (2009) also reported that OFF  $\alpha$ -GCs in the rabbit do not receive excitation at light onset. Taken together, these data suggest feedback and not feedforward inhibition in the masking of crossover excitation.

While there is both anatomical (Enz *et al.* 1996; Wässle *et al.* 1998), and physiological (Rotolo & Dacheux, 2003; Zhou & Dacheux, 2005; Eggers *et al.* 2007; Eggers & Lukasiewicz, 2010) evidence showing that GABA<sub>C</sub> receptors are expressed exclusively on bipolar cell terminals, GABA<sub>A</sub> receptors are found on both bipolar cell terminals and amacrine cell processes (Greferath *et al.* 1994). This indicates that the masking inhibition can be limited to ON bipolar cells that provide excitatory drive to the amacrine cells that are coupled to OFF  $\alpha$ -GCs and, in addition, could involve direct inhibition of the coupled amacrine cells. Irrespective of which of these scenarios is correct, it is important to note that the blockade of both GABA<sub>A</sub> and GABA<sub>C</sub> receptors is necessary for unmasking the ON response in OFF  $\alpha$ -GCs. This suggests a certain level of redundancy in the masking inhibitory circuit in that activation of either receptor alone is sufficient to mask the crossover excitation.

Finally, the fact that the masking inhibition appears not to be directly on  $\alpha$ -GCs, but is acting presynaptically on

bipolar cell terminals and possibly amacrine cells suggests that crossover excitation is a phenomenon expressed by numerous ganglion cell subtypes due to the divergent circuitry subserved by these presynaptic cells in the IPL. This idea is clearly supported by our finding that many of the ganglion cells recorded in the rabbit and mouse retinas showed crossover excitation when GABAergic inhibition was blocked.

### Gap junctional coupling with amacrine cells mediates crossover excitation in ganglion cells

Although our results indicate a convergence of ON and OFF signals within individual ganglion cells, there is no question, regarding the anatomical and physiological evidence, of the segregation of ON and OFF signals in presynaptic circuits within the retina. This is most clearly exemplified by the parallel ON and OFF bipolar cells, whose terminals end within different sublaminae of the IPL.

The fact that OFF  $\alpha$ -GCs' dendrites adhere to this sublamination scheme, stratifying exclusively in sublamina-*a* to receive excitatory drive from OFF bipolar cells (Fig. 1A), raises the issue as to how these cells receive crossover ON excitation. Although it is plausible that OFF  $\alpha$ -GCs dendrites receive direct synaptic drive from ON bipolar cells as they ascend through sublamina-*b*, such inputs have not been observed in electron microscopic studies (Kolb & Nelson, 1993; Owczarzak & Pourcho, 1999). In addition, it has recently been shown that certain ON bipolar cells can make ectopic synapses in the OFF sublamina-*a* of the IPL (Dumitrescu *et al.* 2009; Hoshi *et al.* 2009), providing a second plausible route by which the coupled amacrine cells in this study could receive ON inputs. Although this possibility cannot be completely discounted, our present results provide strong evidence that the ON excitation is derived from electrical synapses, likely those formed between OFF  $\alpha$ -GCs and wide-field amacrine cells. First, we found that application of gap junction blockers abolished the unmasked ON response in OFF  $\alpha$ -GCs, but had no significant effect on the classic OFF response. Second, we found that it took approximately 20 min for gap junction blockers to abolish the ON response, which is the same time necessary to show uncoupling between ganglion cells and amacrine cells (Ackert *et al.* 2009). Third, we found that ON  $\alpha$ -GCs, which are not electrically coupled, do not receive excitatory crossover inputs. Finally, coupled amacrine cell dendrites were found to be bistratified within both sublamina-*a* and -*b*, and thus can provide the morphological substrate for the crosstalk between ON and OFF channels. We therefore posit that the gap junctions between ganglion cells and amacrine cells form the conduit for ON signal transmission to OFF  $\alpha$ -GCs. In this scheme, multistratified

amacrine cells receive ON signals via chemical synapses with ON bipolar cell axon terminals in sublamina-*b* of the IPL and then deliver these signals to OFF  $\alpha$ -GC dendrites in sublamina-*a*.

A number of functional roles have been attributed to retinal gap junctions (Bloomfield & Völgyi, 2009), and our present results suggest a novel one: forming conduits for excitatory interaction between ON and OFF channels in the inner retina. Recently, we found a similar function for gap junctions as mediators of excitatory OFF inputs to ON direction selective GCs (Ackert *et al.* 2009). Taken together, these results suggest that gap junctions between amacrine cells and ganglion cells form symmetrical circuits for the crossover excitation of both ON and OFF ganglion cells. Many ganglion cell subtypes in mammalian retinas have been shown to be coupled to neighbouring amacrine cells (Vaney, 1991; Xin & Bloomfield, 1997; Völgyi *et al.* 2009), including 40% of ganglion cells in the mouse retina. An extensive morphological substrate therefore exists for the interaction between ON and OFF channels in the inner retina, clearly consistent with our finding that many ganglion cells in rabbit and mouse retinas show crossover excitation.

In addition to the crossover excitation described here, amacrine cells also play an important role in crossover inhibition between the ON and OFF pathways (Molnar & Werblin, 2007; Manookin *et al.* 2008; Murphy & Rieke, 2008; Liang & Freed, 2010; Werblin, 2010). This inhibition is mediated by amacrine cells that carry signals across the ON and OFF sublamina boundary of the IPL. It has been suggested that crossover inhibition can compensate for non-linear rectification at chemical synapses, can suppress anomalous ON signals in OFF ganglion cells, and can extend the light responses of OFF ganglion cells via disinhibition (Rentería *et al.* 2006; Manookin *et al.* 2008; Liang & Freed, 2010; Werblin, 2010). It is at present uncertain whether the inhibition responsible for masking the crossover excitation is also a form of crossover inhibition. However, crossover inhibition appears to be mediated by glycinergic amacrine cells (Werblin, 2010) and so it is likely the GABAergic masking inhibition is mediated by amacrine cells with the same ON or OFF physiology as their target neurons. In any event, it appears that amacrine cells play extensive roles in communication between the ON and OFF pathways, with small-field glycinergic cells affording crossover inhibition via conventional chemical synapses and wide-field, presumably GABAergic, cells subserving crossover excitation via gap junction-mediated electrical synapses.

### Masking of excitatory inputs is widespread in the CNS

Overall, our results show that ganglion cells with classic ON and OFF responses can express excitatory opposite polarity signals when GABAergic inhibition is blocked.

This raises the obvious question concerning the wiring strategy of the retina: why is the excitatory crosstalk between the ON and OFF pathways masked by inhibition under control experimental conditions? To address this question, it is important to note that the masking of synaptic inputs is a phenomenon found throughout the CNS and appears to be highly plastic. For example, manipulation of sensory afferents has been found to produce rapid and dramatic changes in the receptive field structure of neurons in somatosensory cortex (Metzler & Marks, 1979; Merzenich *et al.* 1983; Calford & Tweedale, 1988; Turnbull & Rasmusson, 1990; Calford, 2002; Foeller & Feldman, 2004), visual cortex (Gilbert & Wiesel, 1992), inferior colliculus (Snyder *et al.* 2000), motor cortex (Sanes *et al.* 1988), thalamus (Nicolelis *et al.* 1993; Faggin *et al.* 1997) and brainstem (Dostrovsky *et al.* 1976; Faggin *et al.* 1997). The rapid timeframe, within minutes, in which the receptive field changes occur virtually eliminates axonal sprouting and creation of new synapses as potential underlying mechanisms. Instead, it is believed that the receptive field changes reflect a release from tonic inhibition that unmasks previously latent synaptic inputs (Batuev *et al.* 1982; Garraghty *et al.* 1991; Jacobs & Donoghue, 1991; Wellman *et al.* 2002; Foeller & Feldman, 2004). Masking can therefore be considered a component of the normal dynamics of the plastic brain in which neuronal responses are modified under changing stimulus conditions.

In the retina, Geffen *et al.* (2007) observed that many salamander OFF ganglion cells can reversibly switch their response polarity to ON following a peripheral image shift. Importantly, their study implicated inhibitory synaptic circuitry in the mechanism underlying the change in response polarity. In addition, Sagdullaev & McCall (2005) reported that certain mouse OFF ganglion cells could be changed to ON-OFF or ON with changes in the size or intensity of the light stimulus, possibly due to alterations in inhibition. We therefore posit that GABAergic inhibition is relieved under certain stimulus conditions allowing crosstalk between the ON and OFF channels in the inner retina. What would be the consequence of this crosstalk? One idea is that the unmasking of crossover excitation in the retina would result in ON and OFF signals being carried by a larger number of output ganglion cells. Since the optic nerve forms a bottleneck in the visual system, the multiplexing of signals across a larger contingent of ganglion cell subtypes can increase valuable bandwidth. Crossover excitation can thereby enhance the efficiency and capacity of visual information flow to the brain.

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