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DIRECT INPUTS TO OFF A AND G9 GANGLION CELLS FROM ALL AMACRINE CELLS IN RABBIT RETINA

Wei-Li Liu

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**DIRECT INPUTS TO OFF A AND G9 GANGLION CELLS FROM AII
AMACRINE CELLS IN RABBIT RETINA**

A

THESIS

Presented to the Faculty of

The University of Texas

Health Science Center at Houston

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The University of Texas

M. D. Anderson Cancer Center

Graduate School of Biomedical Sciences

in Partial Fulfillment

of the Requirements

for the Degree of

MASTER OF SCIENCE

by

Wei-Li Liu B.S.

Houston, Texas

August, 2010

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Direct inputs to OFF α and G9 Ganglion Cells from

All amacrine cells in Rabbit Retina

Publication No. _____

Wei-Li Liu, B.S.

Supervisory Professor: Stephen C. Massey, Ph.D.

In the mammalian retina, All amacrine cells are essential in the rod pathway for dark-adapted vision. But they also have a “day job”, to provide inhibitory inputs to certain OFF ganglion cells in photopic conditions. This is known as crossover inhibition. Physiological evidence from several different labs implies that All amacrine cells provide direct input to certain OFF ganglion cells. However, previous EM analysis of the rabbit retina suggests that the dominant output of the All amacrine cell in sublamina a goes to OFF cone bipolar cells (Strettoi et al., 1992).

Two OFF ganglion cell types in the rabbit retina, OFF α and G9, were identified by a combination of morphological criteria such as dendritic field size, dye coupling, mosaic properties and stratification depth. The All amacrine cells (Alls) were labeled with an antibody against calretinin and glycine receptors were marked with an antibody against the $\alpha 1$ subunit. This material was analyzed by triple-label confocal microscopy. We found the lobules of Alls made close contacts at many points along the dendrites of individual OFF α and G9 ganglion cells. At these potential synaptic sites, we also found punctate labeling for the glycine receptor $\alpha 1$ subunit. The presence of a post-synaptic marker such as the $\alpha 1$ glycine receptor at contact points between All lobules and OFF ganglion cells supports a direct inhibitory input from Alls. This pathway provides for crossover inhibition in the rabbit retina whereby light onset provides an inhibitory signal to OFF α and G9 ganglion cells. Thus, these two OFF ganglion cell types receive a mixed excitatory and inhibitory drive in response to light stimulation.

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List of Abbreviations

Ach:	acetylcholine
AGB:	AMPA-activated 1-amino-4-guanidobutane
All:	All amacrine cell
AMPA:	alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
APB:	2-amino-4-phosphonobutyric acid
BC:	bipolar cell
ChAT:	choline acetyltransferase
CNQX:	6-cyano-7-nitroquinoxaline-2, 3-dione
Cx36:	connexin 36
DAPI:	4, 6-diamino-2-phenylindole dihydrochloride
GABA:	γ -aminobutyric acid
GC:	ganglion cell
GJ:	gap junction
GluR4:	glutamate receptor 4
GlyR α1:	α 1 glycine receptor
INL:	inner nuclear layer
IPL:	inner plexiform layer
KA:	kainite
MFA:	meclofenamic acid
mGluR6:	metabotropic glutamate receptor 6
NBQX:	2, 3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2, 3-dione
OFF BC:	OFF cone bipolar cell
ON BC:	ON cone bipolar cell
OPL:	outer plexiform layer

PBS: Phosphate buffered saline

Introduction

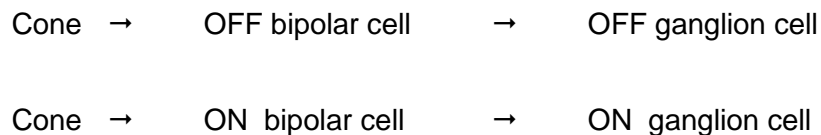
Rod and Cone Pathways

There are two types of photoreceptor in the mammalian retina that feed distinct pathways of differing sensitivity. Cones, which account for 3-5% of photoreceptors, support high acuity and color vision when light is abundant in daylight or sunlight. In contrast, rods far outnumber cones accounting for 95-97% of photoreceptors. Rods are specialized for high sensitivity under dark or starlight conditions and they can respond to the absorption of a single photon. Additional sensitivity is achieved by convergence in the rod pathways through the retina.

Cones contact approximately ten kinds of cone bipolar cell which may be subdivided into ON and OFF bipolar cells. ON and OFF bipolar cells produce opposing responses to light stimulation due to the expression of different post-synaptic glutamate receptors. OFF bipolar cells express conventional AMPA/KA receptors (DeVries, 2000; Haverkamp et al., 2001) whereas ON bipolar cells express the sign-inverting mGluR6 receptor (Slaughter and Miller, 1981; Nomura et al., 1994; Vardi et al., 2000). In turn, OFF bipolar cells synapse with OFF ganglion cells in sublamina a of the inner plexiform layer (IPL) and ON bipolar cells contact ON ganglion cells in sublamina b. Recently, certain exceptions to this rule have been identified such that melanopsin ganglion cells and

dopaminergic amacrine cells receive ON input in the OFF sublayer (Hoshi et al., 2009).

However, the large α ganglion cells and most other types obey the stratification rules of the IPL. Thus, the simplest cone pathways run in parallel:



In contrast to cones, rods contact a single morphological type of rod bipolar cell (Fig. 1). Rod bipolar cells produce ON responses and they express mGluR6 receptors (Nomura et al., 1994). However, rod bipolar cells do not contact ganglion cells directly. Instead, the rod bipolar output goes to two types of post-synaptic amacrine cells. One type, either S1 or S2, makes reciprocal inhibitory synapses mediated by γ -aminobutyric acid (GABA) back to the rod bipolar cell (Vaney, 1986; Sandell and Masland, 1989; Zhang et al., 2002). These are widefield GABA amacrine cells bearing many independent varicosities (Grimes et al., 2010). The other major output target for rod bipolar cells is the All amacrine cell, also known as the rod amacrine cell (Famiglietti and Kolb, 1975).

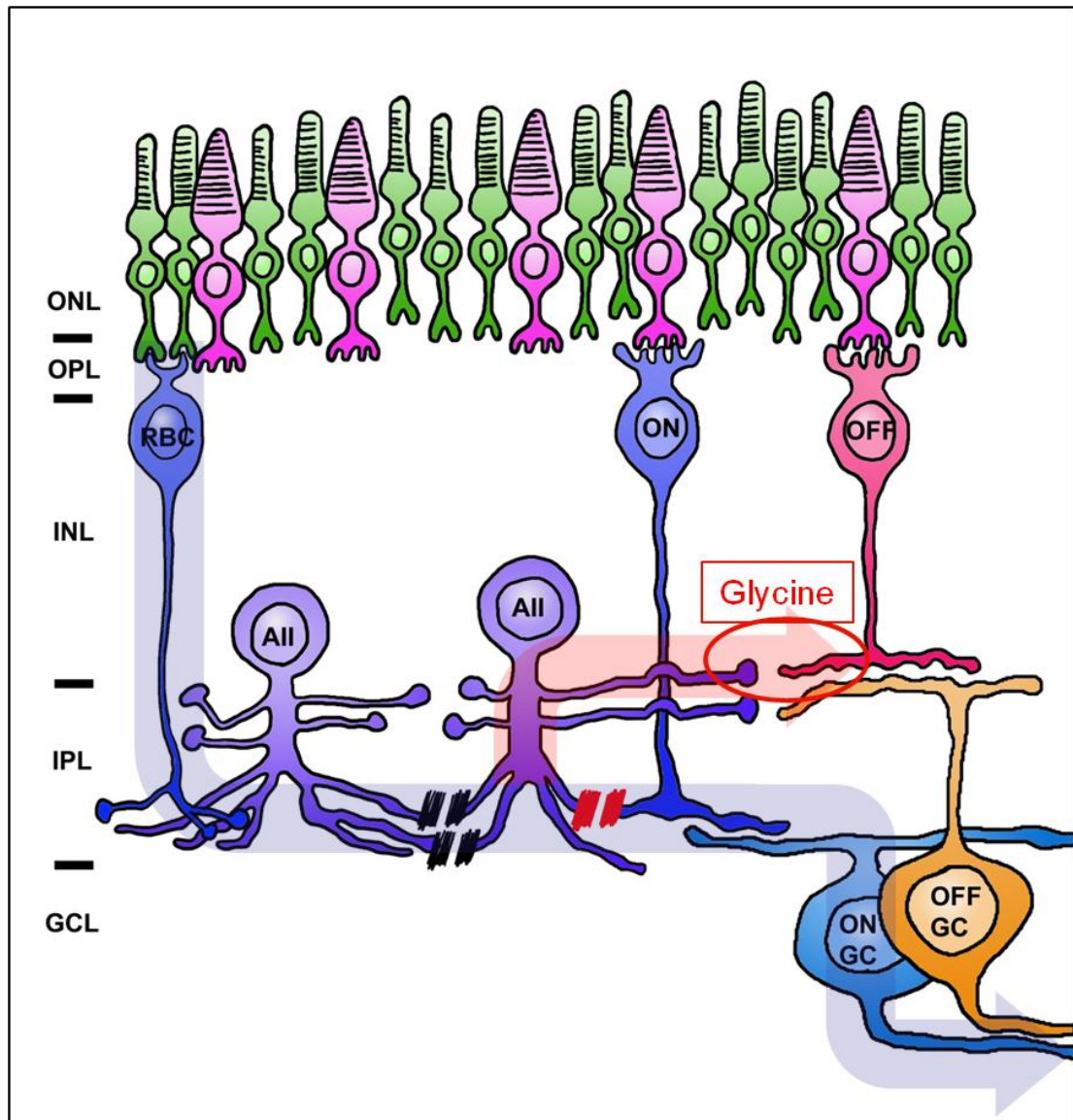


Fig. 1: Cartoon: the Rod Pathway.

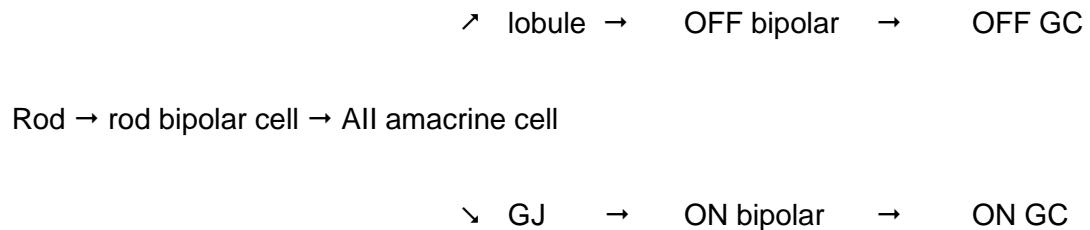
All amacrine cells play an essential role in the rod pathway. Rod signals split at the AII amacrine cell: ON signals enter ON cone bipolar cells via gap junctions in sublamina b (blue arrow) while OFF signals are mediated via glycine release from the lobules of AII amacrine cells in sublamina a (red arrow). In contrast, both ON and OFF cone bipolar cells contact ganglion cells directly.

All Amacrine Cells

All amacrine cells are glycinergic bistratified amacrine cells with distinctive lobules tethered by very fine processes in sublamina a and an overlapping network of dendrites in sublamina b where they receive input from rod bipolar terminals. The dominant input from rod bipolar cells to All amacrine cells is mediated by AMPA receptors (Li et al., 2002; Singer and Diamond, 2003; Trexler et al., 2005). Importantly, All amacrine cells are extensively coupled via Cx36 gap junctions (Feigenspan et al., 2001; Mills et al., 2001) and this coupled network is thought to function as a signal averaging network to reduce noise in the primary rod pathway (Vardi and Smith, 1996).

The output from All amacrine cells bifurcates such that the lobules provide an inhibitory glycinergic input to OFF cone bipolar cells and certain ganglion cells (Fig. 1). Of these the output to bipolar cell terminals is numerically dominant (Strettoi et al., 1992). The ON outputs from AIs are made via additional gap junctions with ON cone bipolar cells (Famiglietti and Kolb, 1975). The opposing responses generated by the glycinergic chemical synapses versus the sign conserving connections mediated by gap junctions provide the appropriate signals to OFF and ON bipolar cells respectively. As before, the ON and OFF cone bipolar cells contact ON and OFF ganglion cells and, in this way, the rod pathway is said to “piggy-back” on the cone pathways (Dacheux and Raviola, 1995).

Thus the primary rod pathway can be summarized as follows (Fig. 1):



Another All pathway: crossover inhibition

While recording from certain OFF ganglion cells, in the presence of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) to block conventional glutamate receptors, a light evoked glycinergic inhibitory input was discovered (Manookin et al., 2008; Münch et al., 2009). Because bipolar cell output was blocked by CNQX, it was proposed that a gap junction pathway was involved such as that between ON cone bipolar cells and All amacrine cells. These gap junctions are well known to be bidirectional (Trexler et al., 2001; Veruki and Hartveit, 2002; Trexler et al., 2005). Further experiments showed this pathway was blocked by 2-amino-4-phosphonobutyric acid (APB) which is a metabotropic glutamate receptor 6 (mGluR6) agonist, indicating ON pathways were involved. Secondly, the pathway was blocked by gap junction antagonists such as meclofenamic acid (MFA) (Pan et al., 2007) and finally, the signals were blocked by the glycine antagonist strychnine, consistent with All output. In addition, paired recordings showed a direct connection

between All amacrine cells and OFF ganglion cells (Münch et al., 2009). In summary, the evidence suggests the following pathway (Fig. 2):

Cone → ON cone bipolar cell → GJ → All → lobule → OFF GC

This pathway provides crossover inhibition whereby ON circuits not only excite ON ganglion cells but simultaneously inhibit OFF ganglion cells. It also suggests that All amacrine cells have a daytime role driven by cone inputs as well as their better known function in the primary rod pathway (Oesch and Diamond, 2009).

Previous work has suggested that the primary output from the lobules of All amacrine cells is passed to OFF cone bipolar terminals (Fig. 3). However, the evidence summarized above suggests that All amacrine cells must make direct connections with certain OFF ganglion cells despite evidence which suggests the primary output from All lobules is via OFF cone bipolar cells (Strettoi et al., 1992). If the pathway led from All → OFF bipolar cell → OFF ganglion cell, light responses would be blocked by glutamate antagonists (Fig. 3). The resistance to 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX) implies a direct connection between All lobules and OFF ganglion cells. The present work is designed to test the hypothesis that All amacrine

cells make direct glycinergic contacts with certain OFF ganglion cells.

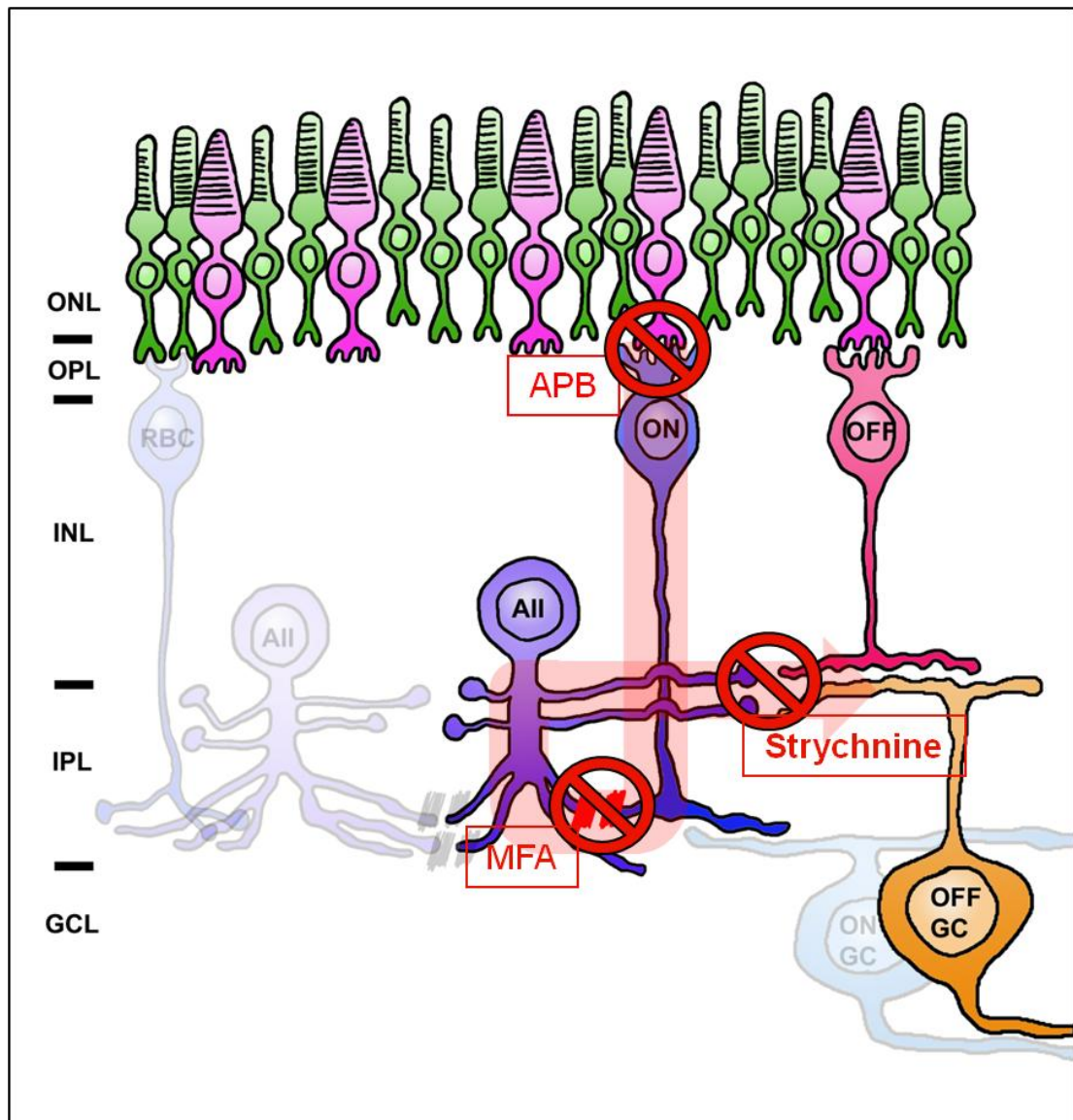


Fig.2: Cartoon: Crossover Inhibition Pathway.

A “day job” for AII amacrine cells in photopic conditions. Cone driven signals from ON bipolar cells run “backwards” through the AII/bipolar gap junctions to modulate OFF pathways via glycine release from the AII lobules. This crossover inhibition can be blocked at certain points by antagonists: 1. APB blocks the mGluR6 receptor; 2. MFA blocks gap junctions; 3. Strychnine blocks glycine receptors. Rod bipolar cells and ON GC, grayed out.

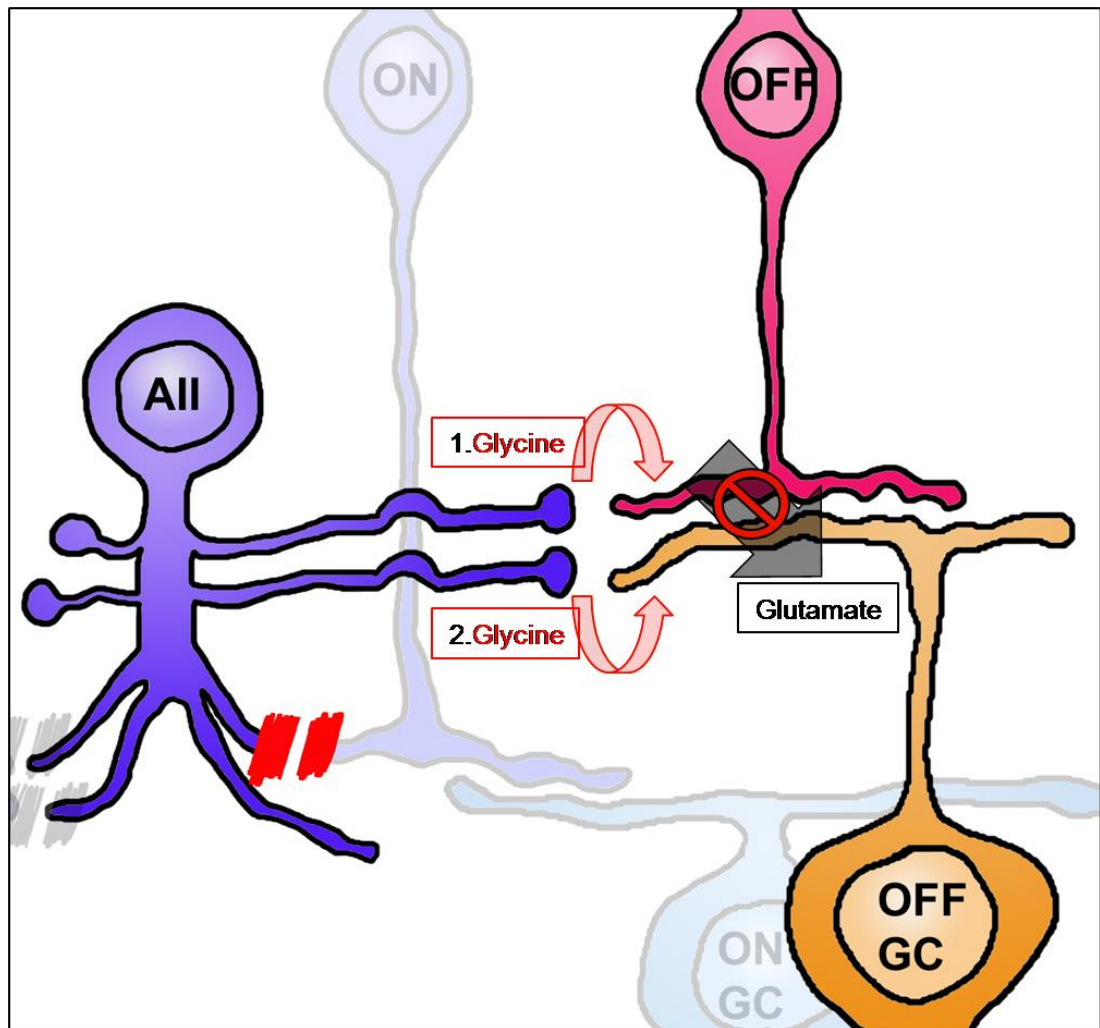


Fig. 3: Cartoon: All Outputs.

The glycinergic output of All amacrine cell lobules may pass by two possible pathways: 1. Directly onto OFF bipolar cell terminals (Strettoi et al., 1992); 2. Directly onto OFF ganglion cell dendrites. The pharmacology supports the second pathway because the output from OFF bipolar cells would be blocked by glutamate antagonists. The aim of this project is to provide morphological evidence for pathway 2, direct contacts between All amacrine cells and OFF ganglion cells.

Ganglion Cell Classification

Ganglion cells provide the output of the retina, transferring signals to the brain.

There are approximately 12 - 20 different ganglion cell types in the retina which are thought to carry parallel channels of visual information (Xin and Bloomfield, 1997; Rockhill et al., 2002; Dacey et al., 2003). The classification of ganglion cell types is based on the combination of three criteria: morphology (Rockhill et al., 2002; Roska and Werblin, 2003), electrophysiology (Devries and Baylor, 1997; Roska and Werblin, 2003), and biochemistry (Marc et al., 1998). However, a direct correspondence across these classification schemes has not yet been achieved.

Morphologically, different cell types have distinct properties. The most useful variables for the purpose of ganglion cell classification include the dendritic branching pattern, the dendritic field size, the dendritic density, the stratification level in the IPL, and the dye coupling patterns (Kong et al., 2005; Volgyi et al., 2005; Volgyi et al., 2009).

Individual ganglion cell types are distributed in distinct non-random mosaics such that the surface of the retina is tiled by each ganglion cell type. Thus each point on the retinal surface is sampled by every ganglion cell type. This morphological property has been confirmed by electrophysiological sampling of the retina with multi-electrode arrays (Devries and Baylor, 1997; Chichilnisky and Baylor, 1999).

Physiologically, ganglion cells may be classified as ON or OFF, transient or sustained, brisk or sluggish. In addition, some cell types have more complex receptive fields such as the ON and ON/OFF directionally selective types. Finally, the inhibitory and excitatory inputs to different ganglion cell types were compared in voltage clamp and correlated with cellular morphology (Manookin et al., 2008; Münch et al., 2009).

Recordings from nearby pairs of ganglion cells of the same type showed that certain ganglion cell types exhibit correlated firing patterns. In other words, they are synchronized (Mastrorade, 1983; Xin and Bloomfield, 1997; Volgyi et al., 2005; Volgyi et al., 2009). The substrate for synchronized firing is thought to be gap junction coupling between ganglion cells of the same type. Furthermore, ganglion cell types known to be physiologically coupled reveal dye coupling patterns when filled with a neuronal tracer such as Neurobiotin. In addition, ganglion cell coupling is mostly absent in the Cx36 knockout mouse. A few cell types are still modestly coupled and this implies the use of another neuronal connexin, such as Cx45. Some ganglion cell types are never coupled.

A biochemical classification scheme against a panel of antibodies was used to identify all the cells in the ganglion cell layer of the rabbit retina. This included 13 different ganglion cell types, as well as displaced amacrine cells. Statistical separation between

cell type was demonstrated by cluster analysis. There is a further correlation in that ganglion cell types shown to be synchronized and dye coupled also showed a biochemical signature for small inhibitory transmitters such as GABA and glycine. These molecules are small enough to diffuse through the gap junctions from coupled amacrine cells. These examples show there is some consistency between the different criteria by which we classify ganglion cell types although a complete correspondence has not been obtained.

The total number of ganglion cell types has not been reliably established.

Estimates vary from 11 to around 20. The most comprehensive survey of the rabbit retina identified 13 different ganglion cell types with a few more samples that were unclassified. However, it is clear that there are additional cell types which are not included in the catalog such as the bistratified diving ganglion cell described by Hoshi et al., 2009. In addition, recent evidence suggest that there are multiple types of melanopsin ganglion cells and perhaps two morphologically distinct kinds of ON DS ganglion cell. The four different directional axes of ON/OFF and three axes of ON DS ganglion cells present an additional complication although these are not usually classified as different cell types.

Alpha Ganglion Cells

Alpha ganglion cells are the largest ganglion cells in the mammalian retina and cells of the same basic morphology are found across all mammalian species (Peichl et al., 1987a). They account for 1-4% of all ganglion cells and seem to constitute a specific cell type which is utilized across species. α ganglion cells were first described in the cat retina where the complete population was labeled with a reduced silver or neurofibrillar stain and the correlation was made between the alpha morphology and brisk transient or Y ganglion cells (Enroth-Cugell and Robson, 1966; Peichl and Wässle, 1979). Furthermore, it was established that OFF α ganglion cells stratified in sublamina a while the dendrites of ON α ganglion cells were restricted to sublamina b. Thus, they conform to the stratification rules of the inner retina (Famiglietti and Kolb, 1975; Famiglietti et al., 1977; Bloomfield and Miller, 1986). More specifically, in the rabbit retina, the OFF α ganglion cells were shown to ramify just below the cholinergic a band and the ON α ganglion cells were immediately below the cholinergic b band (Peichl et al., 1987b). This was later confirmed in a confocal study of the rabbit retina (Zhang et al., 2005).

Neurofibrillar staining of the complete population was used to establish that each α ganglion cell type forms an evenly spaced non-random mosaic with distinct territorial properties (Wässle and Riemann, 1978; Wässle et al., 1981). OFF α ganglion cell type tiles the retina with a coverage factor of approximately 1.85 (Peichl et al., 1987b). This work

established methods to characterize the properties of a neuronal mosaic by nearest neighbor analysis and suggested that different ganglion cell types form independent mosaics. Thus, close neighboring pairs of α ganglion cells were composed of one ON cell and one OFF cell. These characteristics were used in the present study to differentiate between ganglion cell types.

In the rabbit retina, there is one further difference between ON and OFF α ganglion cells: the OFF cells are coupled and the ON cells are not (Hu and Bloomfield, 2003; Volgyi et al., 2005; Mills et al., 2007). Neurobiotin injections into OFF α ganglion cells produce a stereotyped and repeatable labeling pattern (Fig. 4). OFF α ganglion cells are coupled to neighboring OFF α cells and to a set of wide field amacrine cells. Furthermore, OFF α ganglion cells have synchronized firing patterns consistent with the presence of gap junction coupling (Hu and Bloomfield, 2003). In contrast, the spikes of neighboring ON ganglion cells were not synchronized. Thus, there is consistency between the cross-correlation analysis and the presence of dye coupling. The presence of dye coupling provides an additional diagnostic criterion to identify OFF α ganglion cells.

The coupling pattern of α ganglion cells seems to be variable across mammalian species. For example, in the mouse retina, OFF α ganglion cells are coupled

homologously to other OFF α ganglion cells as well as heterologously to several amacrine cell types. Mouse ON α ganglion cells are coupled only to amacrine cells. There is variable evidence concerning the role of Cx36 in ganglion cell coupling. In the rat retina, Cx36 plaques were located at dendritic crossings between α ganglion cells (Hidaka et al., 2002) and in the Cx36 knock-out mouse retina, OFF α ganglion cell coupling was still present, although ganglion cell to amacrine cell coupling was abolished (Volgyi et al., 2005). However, in another study, OFF α ganglion cell coupling was abolished in mice without Cx36 (Schubert et al., 2005). The disagreement may result from the difficulty of identifying specific ganglion cell types, especially in the mouse retina.

In another study of the mouse retina, OFF α ganglion cells were identified as PV-5 in a mouse line expressing GFP driven by the parvalbumin promoter (Münch et al., 2009). These cells also had light driven inhibitory responses thought to originate via the AII /gap junction pathway described above. In support of this pathway, dual recordings showed direct synaptic inputs from AII amacrine cells to PV-5 ganglion cells (Münch et al., 2009).

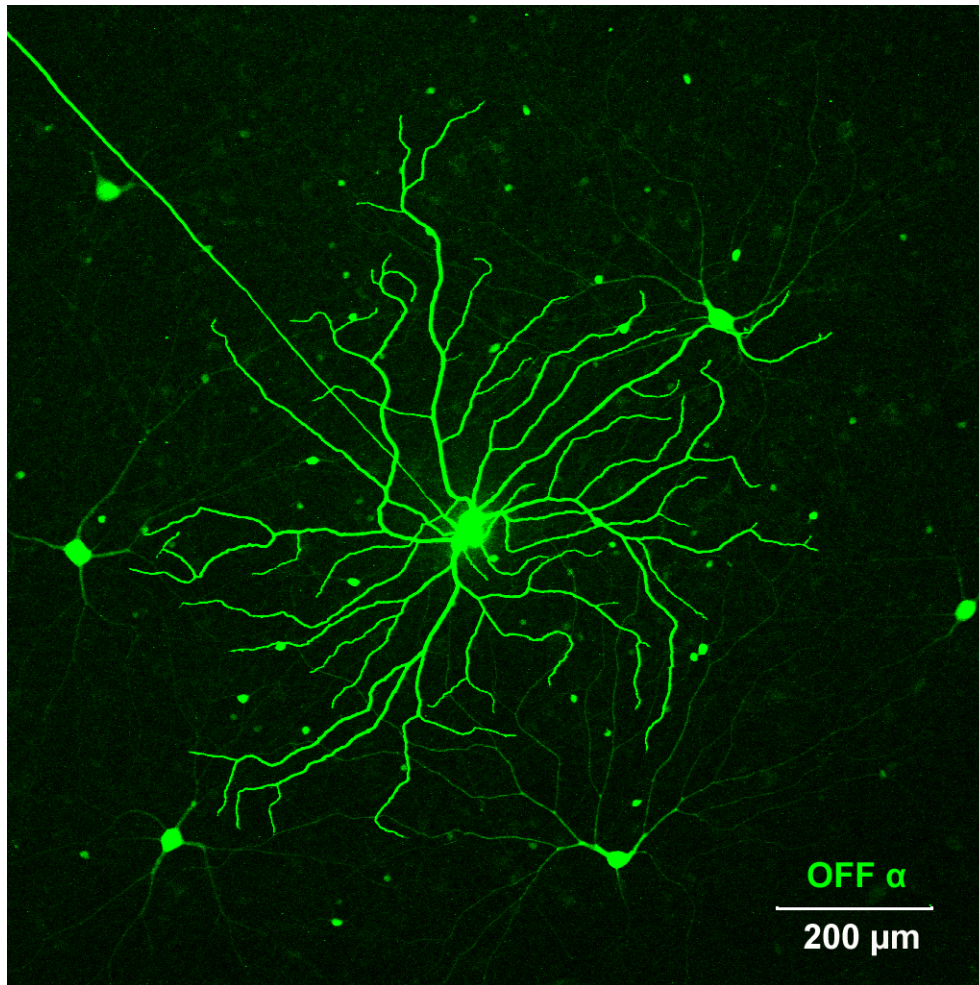


Fig. 4: Dye-injected OFF α ganglion cell.

This ganglion cell has the typical OFF α morphology with radial dendrites and acute branch points. It is stratified in sublamina a of the IPL. Note the dye coupling to a ring of neighboring OFF α cells and to 50 or 60 wide-field amacrine cells.

G9 Ganglion Cells

In the Rockhill catalog, an additional OFF ganglion cell, called G9, was described with similar characteristics to the OFF α ganglion cell as it was narrowly stratified in sublamina a (Rockhill et al., 2002). However, G9 ganglion cells had a smaller dendritic field and they were stratified just above the cholinergic a band, in contrast to OFF α

ganglion cells whose dendrites were located just below the cholinergic a band. G9 ganglion cells also have a similar dendritic morphology to α ganglion cells which has made them difficult to distinguish (van Wyk et al., 2009) (Fig. 5). In the present work, we have developed several different criteria to reliably identify OFF α ganglion cells and G9 ganglion cells.

There have been few reliable recording from G9 ganglion cells. Again, in large part, this results from the difficulty in identifying and targeting specific ganglion cell types. However, in an extensive survey of the guinea pig retina, the OFF δ cell ganglion cell appears to be the morphological homolog of the rabbit OFF α ganglion cells (Manookin et al., 2008). OFF δ ganglion cell were characterized as OFF sustained cells with a large crossover inhibitory input (Manookin et al., 2008). In the mouse retina, 3 ganglion cell types were identified as α -like, despite the fact that mammalian species have a paramorphic pair of α ganglion cells, one ON and one OFF. The third ganglion cell type, regarded as indistinguishable in wholemount appearance from an α ganglion cell, was stratified above the cholinergic a band and may be the mouse homolog of the rabbit G9 ganglion cell. It was reported to produce sustained OFF responses to light stimulation (van Wyk et al., 2009). In another study of mouse retina, OFF α ganglion cells were also divided into two groups, one transient and one sustained, and these may reflect the two

cell types described above (Pang et al., 2003). G9 ganglion cells may also correspond to PV-6 ganglion cells in a mouse line expressing GFP driven by the parvalbumin promoter (Münch et al., 2009).

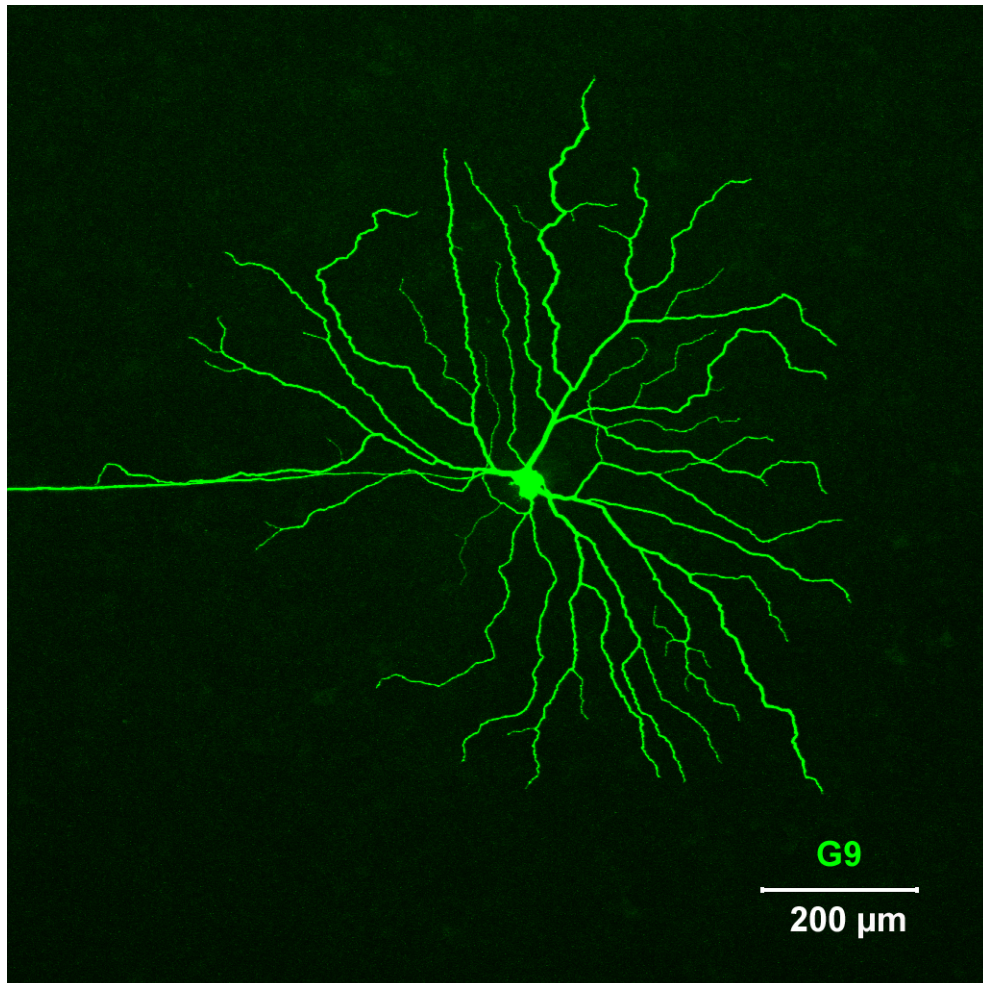


Fig. 5: Dye-injected G9 OFF ganglion cell.

The morphology is similar to the OFF α cell but at the same eccentricity, the dendritic field is smaller. It is also stratified in sublamina a of the IPL. However, there is no coupling to either ganglion cells or amacrine cells.

ON/OFF Directionally Selective Ganglion Cells (G7)

ON/OFF DS ganglion cells were discovered in the rabbit retina nearly 50 years ago (Barlow et al., 1964). Physiologically, they display a null/preferred axis with a total of 4 different directions. The correlation between the physiology and morphology was first determined by Amthor who identified the ON/OFF DS cell as a bistratified ganglion cell with a distinct retroflexive dendritic pattern (Amthor et al., 1984). They are exquisitely sensitive to cholinergic agonists and stratify exactly within the two cholinergic bands of the IPL. For this reason, they are sometimes used as reference markers to gauge the relative depth of processes within the IPL. In the present work, the OFF dendritic trees of several ON/OFF DS cells were used as controls to compare the relative strength of All input with reference to OFF α and G9 ganglion cells.

The mechanism of directional selectivity has been a subject of continuous debate since the discovery of this cell type. There is general agreement that asymmetrical GABA inhibition is the principal determinant of directional selectivity because GABA antagonists unmask responses in the null direction (Wyatt and Daw, 1975; Taylor et al., 2000). The source of GABA comes from starburst amacrine cells and in paired recordings, depolarizing a starburst amacrine cell caused a GABA-mediated inhibitory input to an ON/OFF DS ganglion cells (Fried et al., 2005). Directional responses may originate in

individual starburst dendrites due to the asymmetrical distribution of voltage dependent channels or chloride (Mangel, 1998; Euler et al., 2002; Hausselt et al., 2007). However, starburst amacrine cells also release acetylcholine (Ach) but the role of this transmitter is not so obvious. Despite the general agreement on the basic mechanism, there are many specific details and connections to be worked out and this is still an active research area.

Local Edge Detector (G1)

The smallest and most numerous ganglion cell type in the rabbit retina is the local edge detector (G1) in the Rockhill catalog. These cells have small cell bodies recognizable in material stained with acridine orange so they may be readily targeted for intracellular dye injection. The density of local edge detectors has been calculated as sufficient to explain the psychophysically determined visual acuity of the rabbit (van Wyk et al., 2006).

Glycine Receptors

Glycine is one of the major inhibitory transmitters in the CNS. In the mammalian retina GABA and glycine account for the vast majority of amacrine cells and approximately half the amacrine cells release glycine (Wässle et al., 2009). More than 10, primarily small-field glycinergic amacrine cells have been identified (Wässle et al., 2009). Of these, the best known and most numerous is the AII amacrine cell (Famiglietti and Kolb, 1975; Vaney,

1985; Mills and Massey, 1991).

Glycine receptors are pentameric structures composed of two α and three β subunits (Grudzinska et al., 2005). They are ligand-gated chloride channels. There are four isoforms of α subunits (α 1, α 2, α 3, and α 4) but only one β subunit. In general, only one post-synaptic α -subunit was found at any particular post-synaptic cluster. In other words, the glycine receptors are not colocalized at mixed synapses. All four glycine receptor subtypes are found in the mammalian retina but their distribution is quite different (Heinze et al., 2007). Narrow field and wide field amacrine cells express α 2 subunits and ON starburst amacrine cells express α 4 subunits, consistent with a heavy band of labeling in cholinergic b. Inhibitory post-synaptic currents in displaced amacrine cells were unchanged in the α 1 glycine receptor knock-out mouse (Majumdar et al., 2009). In contrast, All amacrine cells have glycine receptors utilizing α 3 subunits (Majumdar et al., 2009).

Finally, and most relevant for the present work, α 1 glycine receptors are predominantly distributed in sublamina a of the IPL (Sassoe-Pognetto et al., 1994). This is consistent with the expression by OFF ganglion cells and OFF cone bipolar cells (Jusuf et al., 2005). In the rabbit retina, OFF α ganglion cells were sensitive to exogenous glycine (Rotolo and Dacheux, 2003). α 1 glycine receptors were shown to be post-synaptic to All

amacrine cells in the primate retina (Jusuf et al., 2005). The post-synaptic targets were primarily identified as DB3 and OFF midget bipolar cells (Jusuf et al., 2005). Similar results were reported for the rat retina with the All input to recover in labeled OFF cone bipolar cells mediated by $\alpha 1$ glycine receptors (Sassoe-Pognetto et al., 1994).

A-type or α -like ganglion cells in the mouse retina express kinetically fast glycine receptors composed of $\alpha 1$ subunits (Majumdar et al., 2007). Both ON and OFF A-type ganglion cells showed fast responses to glycine. Thus, it is likely that $\alpha 1$ glycine receptors can be used as post-synaptic markers when looking for All input to certain OFF ganglion cells. This will form the basis of the strategy adopted in the present experiments.

Statement of Hypothesis

The evidence reviewed above suggests that All amacrine cells receive cone-driven ON bipolar cell input via a gap junction pathway and in turn provide direct glycinergic inhibitory inputs to certain OFF ganglion cells. These inputs provide a form of crossover inhibition from ON to OFF pathways. To investigate this pathway, we will use triple label confocal microscopy to reconstruct the neuronal contacts. OFF ganglion cells will be dye injected and identified by their morphological properties. All amacrine cells will be stained with an antibody against calretinin and, to confirm the synaptic nature of the contacts, we will localize glycine receptors at the contact points.

The hypotheses to be tested are; 1) that the lobules of All amacrine cells in sublamina a make direct contacts with OFF ganglion cells and 2) that $\alpha 1$ glycine receptors occur at the contact points. The occurrence of glycine receptors exactly at the contact points will suggest that these are synaptic sites. In addition, we will develop statistical tests to show that the observed labeling pattern cannot occur by chance.

Methods and Materials:

Research Animals and Retina Preparation:

Adult New Zealand albino rabbits were used for these experiments. All procedures were performed in accordance with the guidelines of the University of Texas at Houston Animal Welfare Committee. Light-adapted rabbits were deeply anesthetized with urethane (1.5 g/kg, i.p.) and were killed by intracardiac injection of 3ml urethane after removal of both eyes. Retinas were removed from the sclera, separated into several pieces and mounted on black cellulose filters. Rectangular pieces of retina, cut to include a portion of the myelinated band as a reference point, were mounted in a perfusion chamber (Warner Instruments) and superfused via an in-line heater with Ames solution (Sigma), bubbled with 95% O₂/5% CO₂. The temperature was maintained between 34 and 36 degrees Celsius.

Cell Recognition and Microinjection:

A few drops of acridine orange solution (1%) (Invitrogen) were applied to stain ganglion cells. Several types of ganglion cells could be identified and targeted for injection. Targeted cells were injected with thick wall glass electrodes, tip filled with 5% Lucifer Yellow (Invitrogen) and 4% Neurobiotin (Vector laboratories) in ddH₂O and backfilled with 3M LiCl. All cells were injected for 10 min and perfused for at least 30 mins to permit diffusion of the Neurobiotin. After the last injected cell, all tissues were fixed with 4%

paraformaldehyde in 0.1M phosphate buffer for 30 mins at room temperature. To visualize the cells, tissues were incubated in 1:200 streptavidin-Cy3 (Jackson ImmunoResearch) or 1:200 streptavidin-Alexa Fluor 488 (Invitrogen) overnight at 4 C°. Immunostained tissues were rinsed several times with 0.1M PBS /0.3% Triton X-100 and then mounted in Vectashield (H-1000, Vector) to prevent fluorescent fading.

Immunohistochemistry:

Antibody labeling was carried out using indirect immunofluorescence. For immunolabeling, retinas were blocked with 3% donkey serum (Jackson ImmunoResearch)/ 0.1M PBS/ 0.05% sodium azide for 2 hrs at room temperature or overnight at 4 C°. After blocking , tissues were rinsed several times with 0.1M PB Triton X-100 and incubated in primary antibodies in 1% donkey serum /0.05% sodium azide/ 0.1 M PBS/ 0.3% Triton X-100 for 7 days at 4 C°. Tissues were rinsed several times in 0.1M PBS /0.3% Triton X-100 after primary incubation and then incubated in secondary antibodies to visualize primary antibodies overnight at 4 C°. To visualize All amacrine cells and their lobules, a goat polyclonal antibody, against calretinin (1:5000; Millipore, AB1550) was used. This was particularly effective in superior or peripheral inferior retina. Glycine receptors were labeled using a mouse monoclonal antibody, against the glycine receptor $\alpha 1$ subunit (1:1000, Synaptic Systems, 146111). Alternatively, another rabbit polyclonal antibody against the $\alpha 1$

subunit glycine receptor (1:500; Millipore, AB5052) was used. These two antibodies labeled the same small puncta, primarily in sublamina a of the IPL which indicates antibody specificity. However, the polyclonal antibody produced greater background staining.

For stratification studies, a goat polyclonal antibody against, choline acetyltransferase (ChAT) (1:100; Millipore) was used to visualize the two cholinergic bands, which were used as reference points. Donkey anti-goat Cy3 (1: 200; Jackson ImmunoResearch), donkey anti-goat Alexa 488 (1:200; Invitrogen), donkey anti-goat Cy5 (1:200; Jackson ImmunoResearch), donkey anti-rabbit Cy5 (1:200; Jackson ImmunoResearch), donkey anti-mouse Cy3 (1:200, Jackson ImmunoResearch) were used as secondary antibodies in double or triple label studies.

Confocal Microscopy and Quantification of contacts:

A Zeiss LSM 510 Meta laser scanning confocal microscope was used to analyze the immunolabeled samples. Low power images with a 10x or 20x objective at 1 μ m intervals were obtained to document ganglion cell morphology in wholemount retinas. Survey images were taken with 40X (NA 1.3) oil immersion objects to show the contacts between ganglion cell dendrites and the lobules of All amacrine cells. For detailed contact information in triple labeled preparations, high resolution images were obtained using a

63X (NA 1.4) oil immersion object with 0.3 μm optical sections. Some images were reconstructed as mini-stacks, (3-6 sections; 1-2 μm) as required. Brightness and contrast of the photos were adjusted with Adobe Photoshop. Lobular contacts, glycine receptor clusters and the length of ganglion cell dendrites were examined by animating through image series in Zeiss LSM Image Browser.

The point spread function of the instrument was measured using fluorescent latex beads (Invitrogen). We imaged 0.1, 0.2, 0.5 and 1 μm beads. When set to just less than saturation, the 0.5 and 1 μm beads appeared almost exactly as the calibrated size. The two smaller beads appeared substantially larger than their true size. A line profile was taken across the 0.1 and 0.2 μm beads and the width measure at half height. This gave the same value, 300nm, for both beads except the smaller bead was much dimmer, only just above the background. This value was taken as the point spread function. It is a measure of the resolution limit for this instrument. Objects smaller than the point spread function may be observed under optimal conditions but below this limit, they drop rapidly into the background noise because the intensity scales as the square of the diameter. Practically speaking, it is very difficult to view objects smaller than 150nm and, of course, they appear as 300nm diameter images.

Colocalization analysis

To examine the contact points in an objective way, Image J software was used to analyze the series of images in an LSM file. A colocalization analysis plug-in was downloaded from the image J website. All files used the same size threshold setting which was just below the point spread function. To verify the colocalization, a rotation analysis was performed (see results section). By using the colocalization plug-in in Image J, glycine receptors in image files were rotated 90 degree clockwise and the receptors in individual optical sections were analyzed. Images were taken for each of 5 identified ganglion cells of 3 specific cell types (OFF α , G9, ON/OFF DS) with a 63X oil objective at zoom 2.5 from 4 different, near-terminal dendrites of the injected ganglion cell. 6 OFF α ganglion cells, 5 G9 ganglion cells, and 3 G7 ganglion cells were used for this analysis. Images of G7 were only scanned and analyzed for the OFF layer dendrites.

After analyzing the files, all data were exported into Excel files and the calculation were performed in a spreadsheet.

Dendritic length measurements

The images of injected cells were taken by confocal microscopy with 10X or 20X objectives. Dendritic length was measured with LSM software and exported into an Excel file.

RESULTS

Previous electrophysiology studies imply that All amacrine cells make direct contacts with certain OFF ganglion cells (Manookin et al., 2008; Münch et al., 2009). To test this hypothesis, we used triple-label confocal microscopy to visualize potential contacts between OFF ganglion cells and All amacrine cells. Furthermore, All amacrine cells release glycine so it is expected that glycine receptors will occur at the contact sites. There are four subtypes of glycinergic receptor which are differentially distributed throughout the inner plexiform layer (Jusuf et al., 2005). Immunolabeling and electrophysiology both suggest that large α -like ganglion cells express $\alpha 1$ glycine receptors. Therefore, we will examine the distribution of $\alpha 1$ glycine receptors and determine if they occur at the sites of All contact with OFF ganglion cells. If $\alpha 1$ glycine receptors are found at the contact sites, this will suggest that All amacrine cells provide a glycinergic inhibitory input to certain OFF ganglion cells.

Thus, there are three requirements to test the hypotheses: 1) to identify specific ganglion cell types in the rabbit retina; 2) to label All amacrine cells; and 3) to localize $\alpha 1$ glycine receptors at the contact sites between All amacrine cells and OFF ganglion cells.

OFF α Ganglion Cells (G11)

α ganglion cells are a common cell type found across all mammalian species (Peichl et al., 1987a). There are two subtypes; ON α ganglion cells respond to light increments and have dendrites in sublamina b of the IPL while OFF α ganglion cells fire to light decrements and stratify in sublamina a. Thus, α ganglion cells form a paramorphic pair which obeys the stratification rules of the IPL.

OFF α ganglion cells have the largest cell bodies of all ganglion cells in the rabbit retina (Marc and Jones, 2002) which makes them relatively easy to target for intracellular dye injection. Their primary dendrites rose steeply to sublamina a and the somas were oval in shape, as opposed to the ON α ganglion cells which tend to be polygonal due to the exit of lateral primary dendrites at each vertex. These characteristics were used to target individual cells for dye injection in retinæ stained with acridine orange.

An example of a Neurobiotin filled OFF α ganglion cell is shown in figure 4. This cell had a typical radial branching pattern with very thick primary dendrites. The branch angles were acute; there were relatively few dendritic crossings, few high order branches and low dendritic density. The lack of fine branches left wide spaces between the dendrites. This is the classic wholemount appearance of an OFF α ganglion cell. At high

resolution, it was seen that the dendrites ramified just below the cholinergic a band (Zhang et al., 2005). OFF α ganglion cells were well dye-coupled both to a ring of immediate neighbors just outside the dendritic field. It should be noted that the dendrites of the injected cell approached but did not touch the cell bodies of the neighboring dye-coupled ganglion cells. In addition, in the amacrine cell layer, numerous wide-field amacrine cells, approximately 60, were also dye-coupled. The morphological appearance, stratification in sublamina a and the dye-coupling pattern served as primary identifiers for this cell type.

ON α ganglion cells had a similar wholemount appearance but could be readily distinguished from OFF α ganglion cells by the stratification in sublamina b and the lack of dye coupling (Hu and Bloomfield, 2003).

G9 OFF Ganglion Cells

In the Rockhill catalog (Rockhill et al., 2002), another OFF ganglion cell was classified as G9. The somas appeared smaller than those of OFF α ganglion cells when stained with acridine orange and the nucleus was usually on one side of the soma. These features were used to target them for intracellular dye injection. A Neurobiotin-filled example is shown in figure 5. The dendritic branching pattern was similar to OFF α ganglion cells with a radial pattern and few high order branches. The mouse homolog of this cell type was regarded as indistinguishable from the OFF α ganglion cell in

wholemound view (van Wyk et al., 2009). However, in the rabbit retina, the dendritic field was smaller than those of OFF α ganglion cells and the stratification depth was above the cholinergic a band. Importantly, G9 OFF ganglion cells were not dye-coupled to any other ganglion cells or amacrine cells. Morphological homologs of G9 were classified as OFF δ ganglion cells in guinea pig retina and sustained OFF cells in mouse retina (Manookin et al., 2008; van Wyk et al., 2009).

A Patch of Four Large Ganglion Cells

Due to the similarity of their morphologies, it may be difficult to distinguish between OFF α ganglion cells and G9 ganglion cells, especially in the wholemount view (van Wyk et al., 2009). Therefore, we dye-injected four nearby, overlapping ganglion cells with large cell bodies to compare the dendritic field size, stratification, coupling pattern and mosaic properties. The cells were separated by approximately the dendritic radius of an OFF α ganglion cell to make it likely that adjacent neighbors would be obtained. In a representative patch shown in figure 6 A, the four ganglion cells have similar α -like dendritic features and it was not possible to classify the cell types by their wholemount appearance. Next, individual ganglion cells were colorized with reference to a high resolution confocal series, as necessary, to single out specific dendrites (Fig. 6B,C). The ability to scroll up and down through the series was important to follow individual dendrites.

This procedure identified the contiguous dendritic tree of each ganglion cell so they could be separated for a clear comparison (Fig. 6D). Dye-coupled amacrine cells were colored white. While all the injected cells showed a similar morphology, the magenta cell to the lower right was noticeably smaller than the other three ganglion cells. This is probably significant because these neighboring cells are all from the same eccentricity.

An ellipse was drawn around the terminal dendrites of each cell and they were reassembled into the original configuration (Fig. 7A). Not only is the red ellipse around the lower right cell smaller than the others but now it is possible to compare the territorial properties of each cell (Fig. 7A and 8). The top three cells all had dendrites that approached but did not overlap the neighboring somas, excluding the magenta cell. This is the same pattern seen with OFF α ganglion cells whereby the dye coupled somas of neighboring OFF α cells were located just outside the dendritic field of the injected cell (Fig. 4) (Wässle and Riemann, 1978; Peichl and Wässle, 1981). In contrast, the dendrites of the cyan cell completely bypassed the cell body of the magenta cell. This indicates that the magenta cell is not part of the mosaic formed by the other three injected ganglion cells; it is a different ganglion cell type.

The dendrites of these ganglion cells overlap in many places and this presents a

favorable situation to assess the depth of stratification at high magnification. The terminal dendrites of the magenta cell always ran above the dendrites of the green and cyan colored cells, higher in sublamina a towards the inner nuclear layer. In contrast, the other three cells ramified at the same level. Finally, the top three cells were dye coupled to amacrine cells within their dendritic fields. However, within the dendritic field exclusive to the magenta cell, there were no dye-coupled amacrine cells. The only coupling in this area was common to one of the other injected cells (Fig. 7B). Again, this difference indicates that the magenta cell to the lower right is a different cell type. The cells in this patch of dye injected ganglion cells were unambiguously identified as three OFF α ganglion cells and one G9 ganglion cell based on the criteria outlined above.

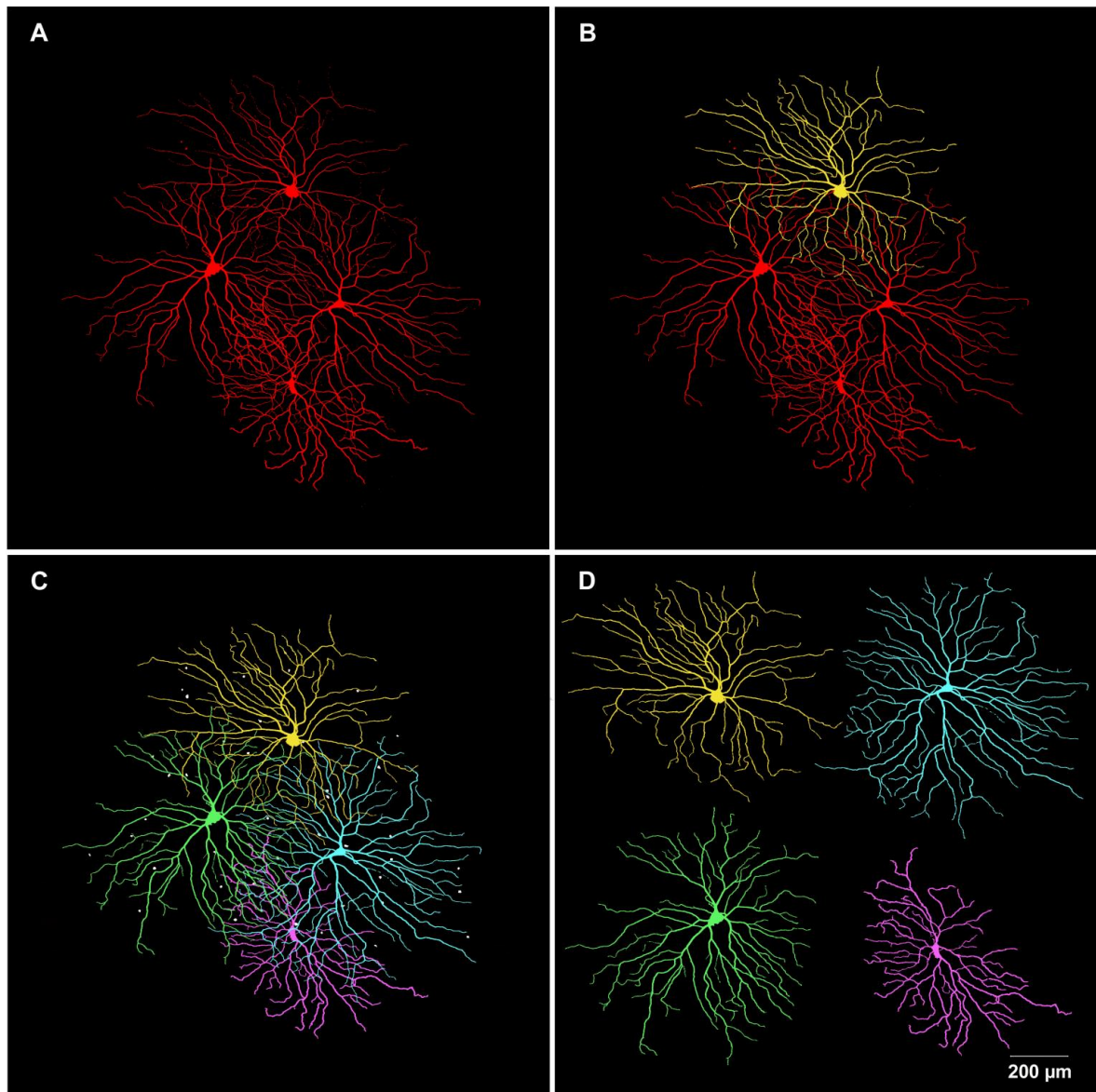


Fig. 6: Patch of Four Large OFF Ganglion Cells.

A: A patch of 4 nearby OFF ganglion cells was dye injected

B: Each separate cell was color coded. In the original patch, it is difficult to appreciate the differential morphology.

C: Colorized ganglion cells reassembled. Dye coupled amacrine cells shown in white.

D: The individual cells were separated. The lower right cell (magenta) had finer primary dendrites and a smaller dendritic field

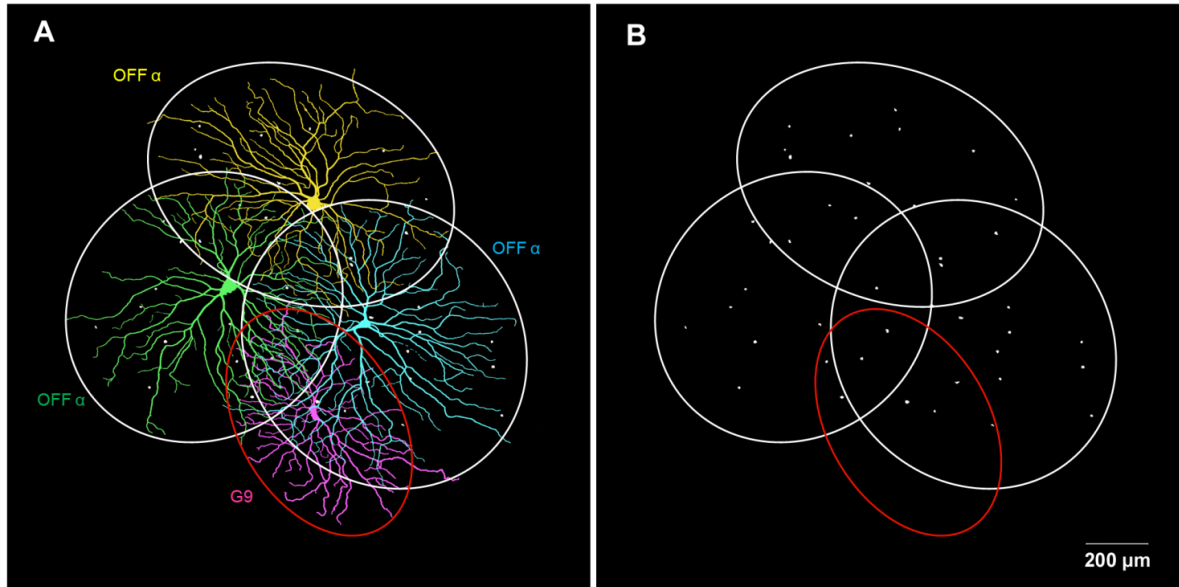


Fig. 7: Dendritic Fields of Four Large OFF Ganglion Cells.

A: The dendritic field of each cell was outlined with an ellipse. The cell in the red ellipse broke the mosaic pattern of the other three cells. It is a different ganglion cell type.

B: The pattern of dye coupled amacrine cells was restricted to the white ellipses. There were no dye coupled cells in the red ellipse except where it overlapped with the others.

Conclusion: This patch has 3 OFF α Ganglion Cells and 1 G9 Ganglion Cell.

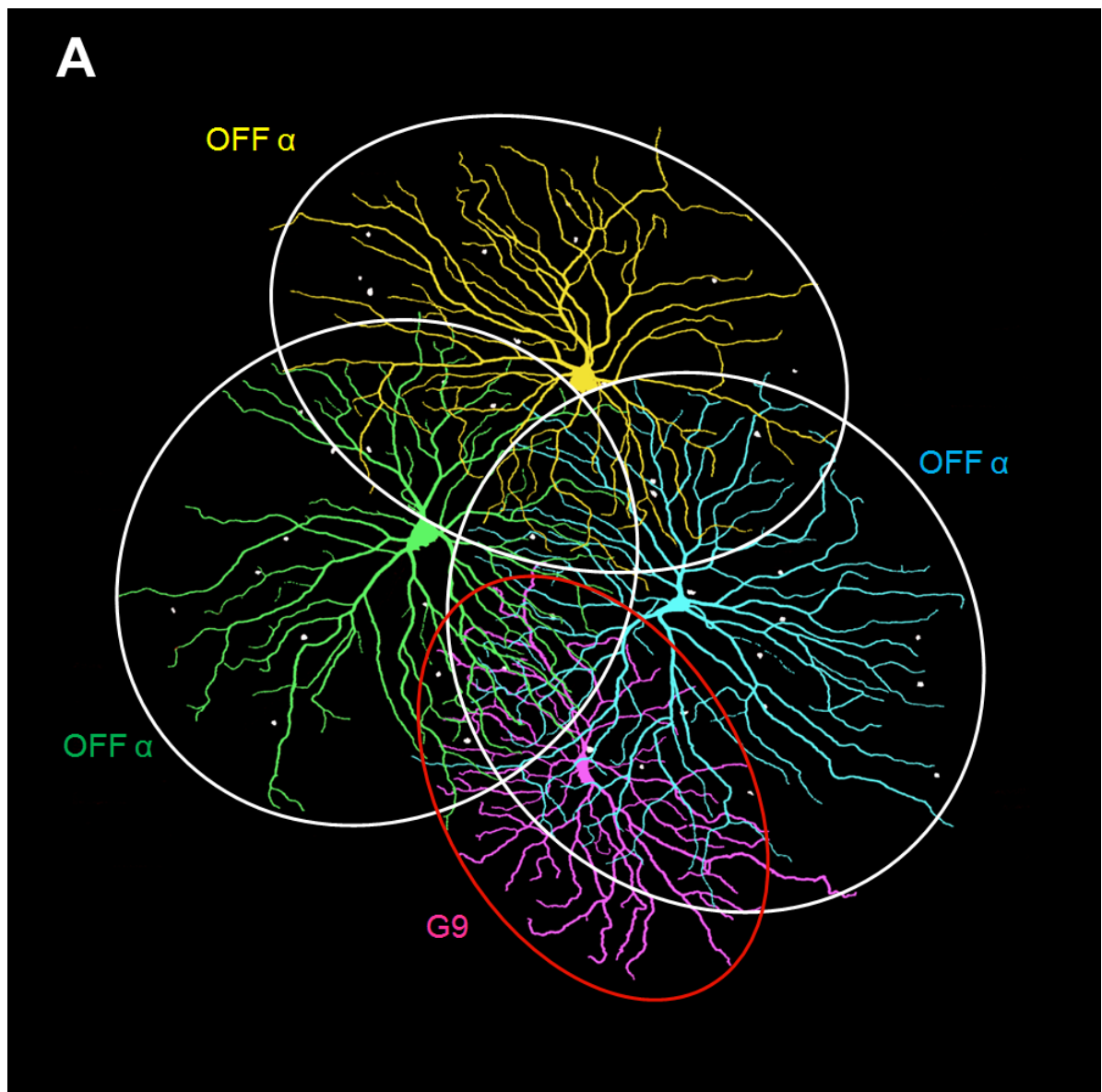


Fig. 8: Patch of Four Large OFF Ganglion Cells, High Resolution.
Several different criteria were used to identify 3 OFF α Ganglion Cells and 1 G9 Ganglion Cell.

Stratification of OFF α and G9 Ganglion Cells

Stratification is among the most important variables to classify different ganglion cells (Kong et al., 2005). To compare the stratification of OFF α ganglion cells with G9 ganglion cells, a pair of close neighbors with overlapping dendritic fields were dye injected. It was important that the two ganglion cells were close enough so that their dendritic fields overlapped (Fig. 9). As in the patch of large ganglion cells described above, the OFF α ganglion cell was larger than the G9. The ring of coupled OFF α ganglion cells was faint but visible and within the dendritic field of the OFF α cell, there were many dye coupled amacrine cells. In the zone exclusive to the G9 dendrites, there were no coupled amacrine cells.

This preparation was also labeled with an antibody against choline acetyltransferase to mark the two cholinergic bands in the IPL as reference markers. Two overlapping dendrites were selected for confocal imaging at high resolution. In a confocal series using the 63 oil objective and 0.3 μ steps, the vertical dendrite, which could be traced back to the OFF α cell, was in focus just below the cholinergic a band at a depth of -1.2 μ (Fig. 9 B). In the next image, the focus was at the level of the cholinergic a band designated 0 μ , and all the ganglion cell dendrites were visible but partly out of focus. Finally, in the last panel, the plane of focus was just above the cholinergic a band at a

depth of +1.6 μ and the horizontal dendrites were in focus while the vertically oriented OFF α was blurred. At this level, most of the cholinergic dendrites are lost and some of the cholinergic cell bodies in the amacrine cell layer became visible.

This focal series demonstrated that the dendrites of OFF α ganglion cells and G9 ganglion cells were stratified at different levels in the IPL. The G9 dendrites were always higher in the IPL than the OFF α dendrites. The focal plane of the cholinergic a band lay between the ganglion cell dendrites such that the G9 dendrites were just above the cholinergic a band and the OFF α dendrites were just below as previously reported (Peichl et al., 1987b; Zhang et al., 2005). The Z-axis measurements indicated there was a gap of 2.8 μ between the dendrites of the two ganglion cell types.

A Z-axis reconstruction was performed on overlapping dendrites from a separate pair of cells. The G9 dendrites ran on top of the cholinergic a band while the OFF α dendrites ran underneath (Fig. 9E). This confirms findings in the Rockhill catalog of the rabbit retina. Similar findings suggest that these two ganglion cell types have homologs in the mouse and guinea pig retina van (Manookin et al., 2008; van Wyk et al., 2009).

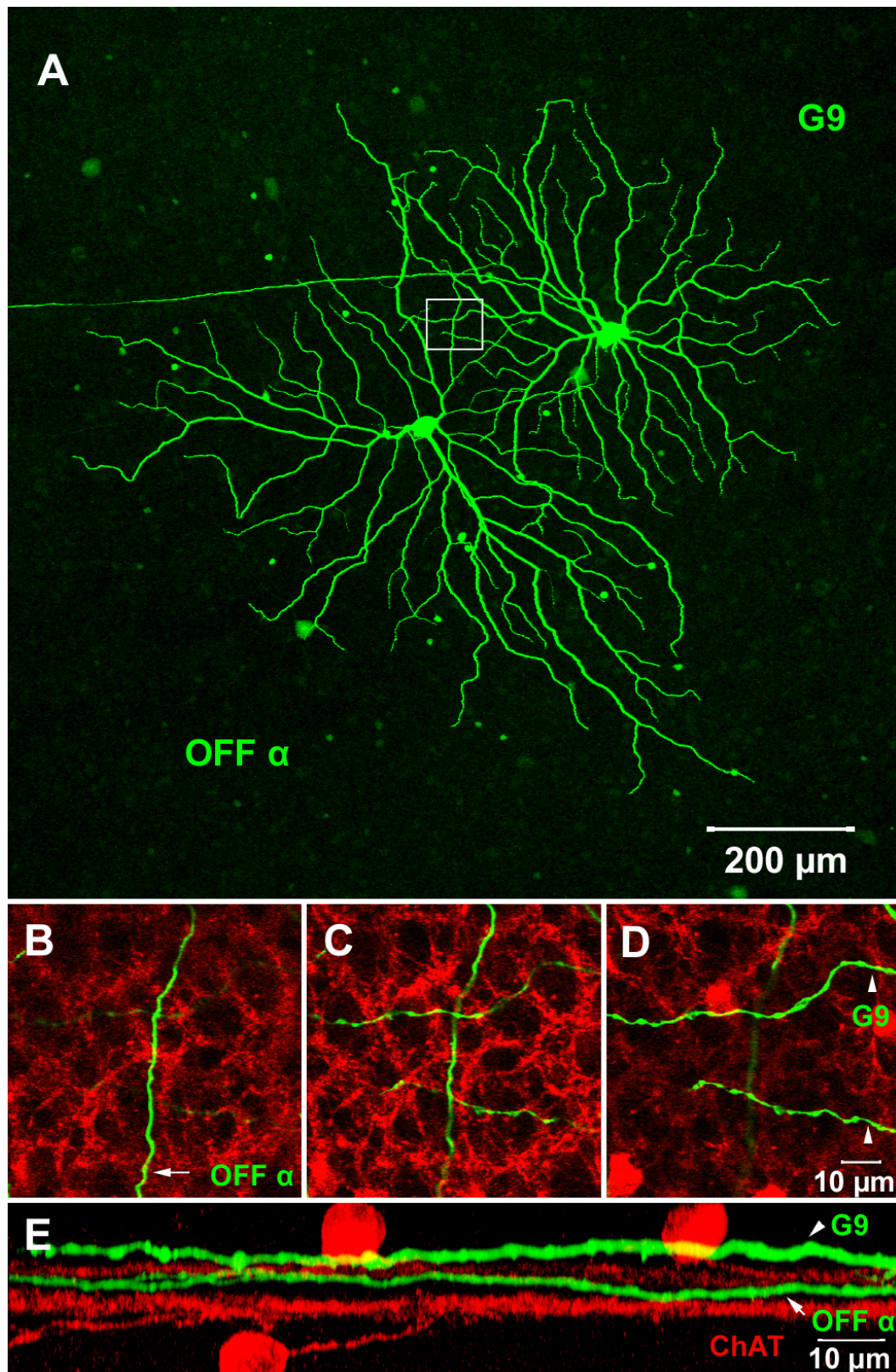


Fig.9: An OFF α Ganglion Cell and a G9 Ganglion Cell Pair: Stratification

Fig.9: An OFF α Ganglion Cell and a G9 Ganglion Cell Pair: Stratification

A: A pair of overlapping cells was dye injected, one OFF α ganglion cells and one G9 ganglion cell. The G9 was smaller with no dye coupling. The OFF α ganglion cell was dye coupled to neighboring OFF α cells and wide-field amacrine cells. Two overlapping dendrites were selected for a focal series (box).

B – D: A focalseries through the overlapping dendrites from the box in A. The cholinergic band in sublamina a (red) was stained with an antibody to choline acetyltransferase. The OFF α dendrite was in focus below cholinergic a (B). The G9 dendrite was in focus above cholinergic a.

E.: A Z-axis reconstruction from a different pair of cells. The G9 dendrite was just above the cholinergic a band while the OFF α dendrite was just below.

Conclusion: OFF α and G9 ganglion cells were stratified at different depth in the IPL, as also reported by Rockhill et al., (2002).

Properties of OFF α and G9 ganglion cells

Different ganglion cell types can be classified with multi-variate analysis (Badea and Nathans, 2004; Kong et al., 2005; Coombs et al., 2006). The reason for doing this is that the use of one criterion may be insufficient to separate two similar cell types. For example, G9 and OFF α ganglion cells are known to have a similar morphology, even if the dendritic field size is different (Rockhill et al., 2002). Of course, the dendritic field size also varies with retinal eccentricity. By using several additional variables, it is possible to distinguish those two cell types reliably.

The two most useful variables to recognize G9 ganglion cells were the stratification and the lack of dye coupling. In addition, a size analysis was conducted in inferior retina

from a total of 14 OFF α ganglion cells and 18 G9 ganglion cells. On average, the OFF α ganglion cells were larger at all eccentricities but there some overlap between the two populations (Fig. 10). The trend lines were significantly different and it was common experience that in OFF α /G9 pairs or in patches of dye coupled cells the G9 ganglion cells were smaller. In addition, OFF α ganglion cells had thicker primary dendrites and, in dye-coupled patches formed a regular mosaic (Peichl et al., 1987b). The G9 ganglion cells clearly broke the OFF α ganglion cell mosaic, a further indication of a different cell type. Finally, the G9 ganglion cells had more branches compared to OFF α ganglion cells and due to the smaller size of the G9 ganglion cells, a higher dendritic density (total dendritic length/dendritic field area). These variables are summarized in Table 1 and the application of these criteria allowed the reliable identification of OFF α and G9 ganglion cells.

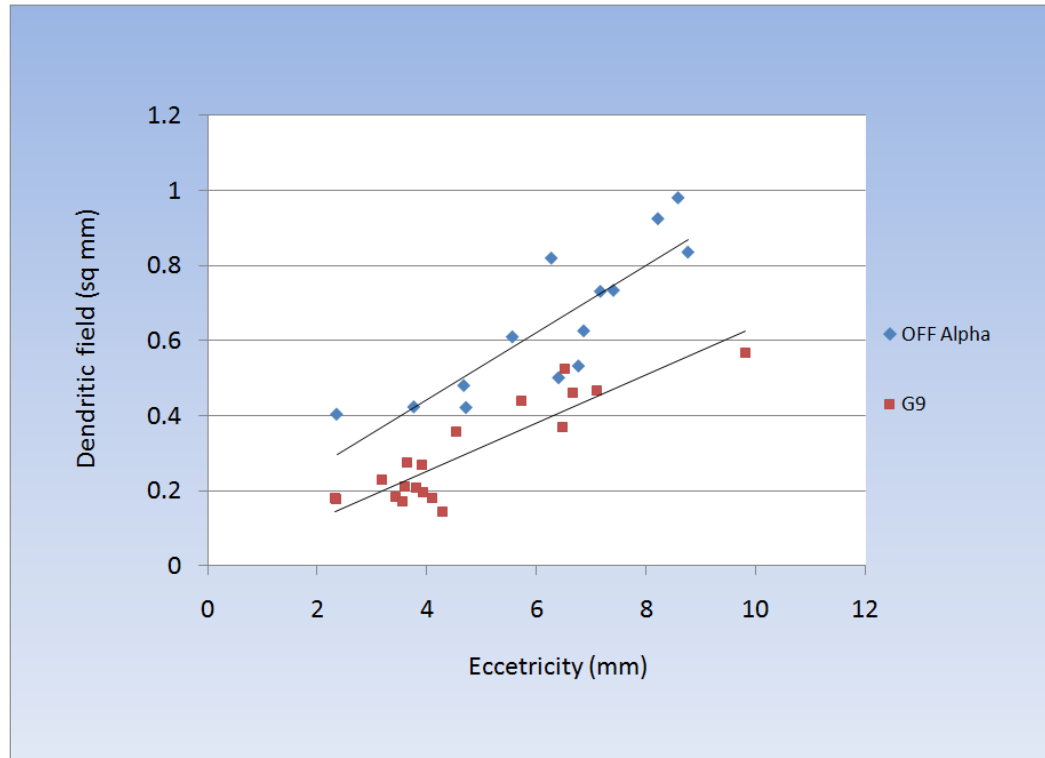


Fig. 10: Size Analysis for OFF α and G9 Ganglion Cells vs Eccentricity.

At a given eccentricity, the OFF α cells were larger than G9 ganglion cells.

Regression analysis showed these two lines were significantly different ($p < 0.0001$).

Table 1:

Morphology	OFF α Ganglion Cell	G9 Ganglion Cell
Dendritic field	Very large	Large
Primary Dendrites	Thicker	Thin
Stratification	Below cholinergic a	Above cholinergic a
Dye Coupling	Yes	No
Mosaic/Tiling	Standard pattern	Breaks OFF α pattern
Dendritic Density	Lower	Higher

Additional Ganglion Cell Types

Two other ganglion cell types could be easily recognized and provided control examples for comparison with the OFF ganglion cells described above. ON/OFF directionally selective ganglion cells has large round somas and were easily recognized by their bi-stratified appearance and their retroflexive space-filling dendrites (Amthor et al., 1984; Vaney, 1994; He et al., 1999) (Fig. 11). Local edge detectors had small round somas and they were very numerous. They had the smallest dendritic field area, characteristic thorny dendrites and they were coupled to a small number of amacrine cells within the dendritic field. Local edge detectors were stratified in sublamina 3, just below the level of All lobules. These results are in agreement with previous descriptions of this cell type (van Wyk et al., 2006) (Fig.12).

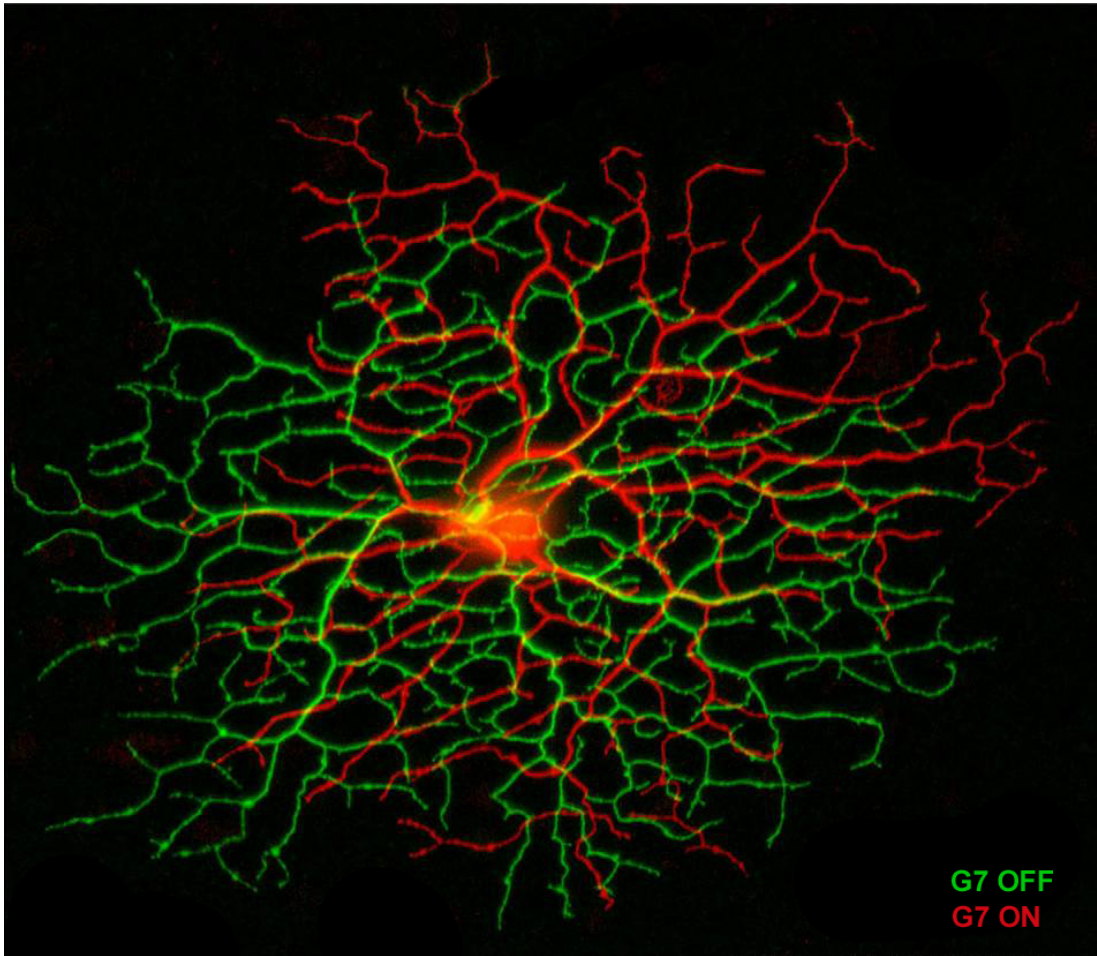


Fig. 11: ON/OFF Directionally Selective Ganglion Cell (G7)

A classic ON/OFF DS ganglion cell. These cells are bistratified with retroflexive, space filling dendrites. Dendrites in sublamina a, green; dendrites in sublamina b, red.

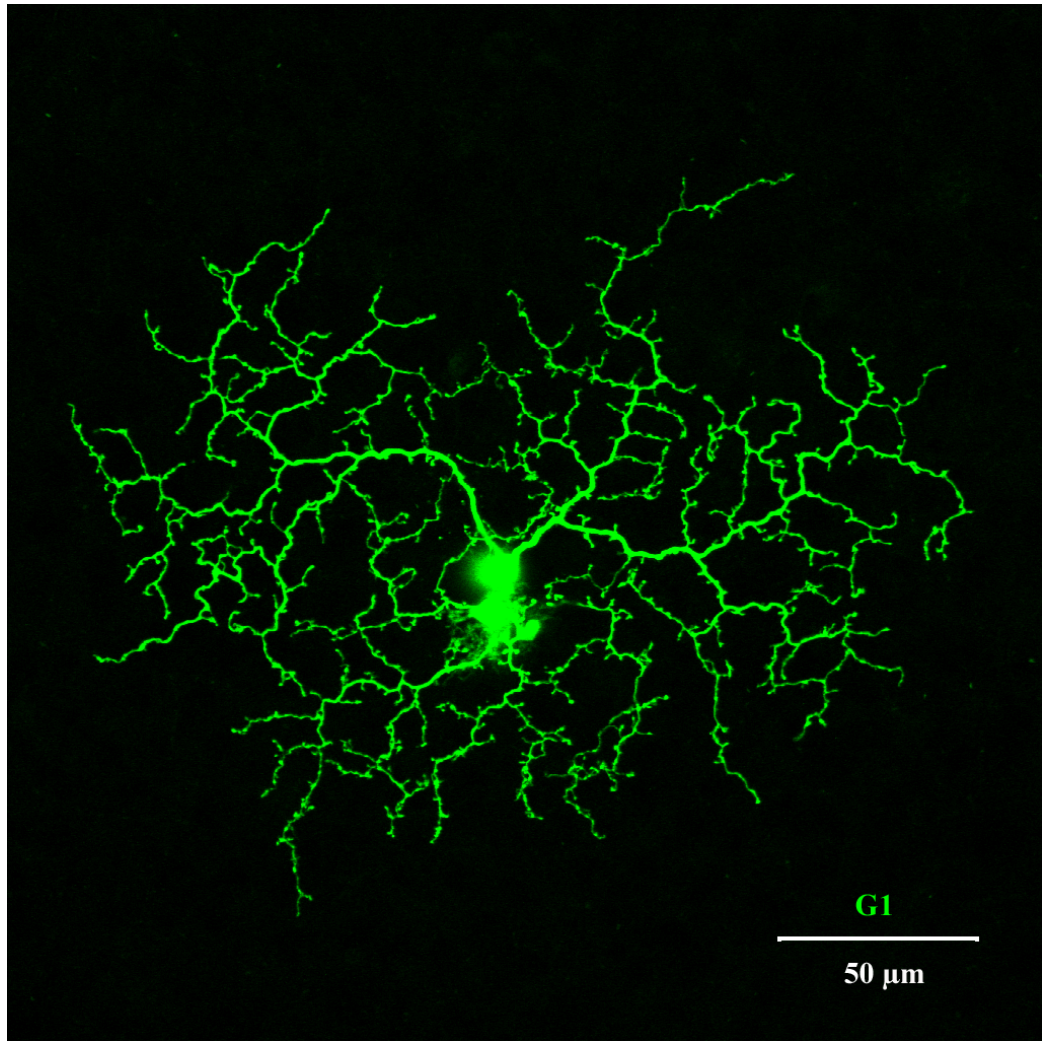


Fig. 12: Local Edge Detector (LED, G1)

These cells were the smallest ganglion cells encountered. They had typical thorny dendrites and were dye- coupled to a few amacrine cells (van Wyk et al., 2006).

All Amacrine Cells

All amacrine cells are well known small bistratified amacrine cells with a distinct morphology which is easily recognizable. The somas are located in the inner nuclear layer with prominent stalks descending into the IPL. The lobular structures in sublamina a are the sites of glycine release onto OFF bipolar cell terminals which form the predominant target (Strettoi et al., 1992). A portion of the All output goes to OFF ganglion cells and these contacts are the subject of the present work. The dendrites in the ON layer form a well-connected dendritic system coupled to other AIs and ON bipolar cell terminals via gap junctions.

There were two primary choices to label All amacrine cells: 1) intracellular dye injection in DAPI labeled retina; 2) labeling the whole All population with an antibody against calretinin (Massey and Mills, 1999). We chose to use the calretinin antibody because the labeling was bright and specific enough to identify individual lobules and many fine details of the All structure. The best calretinin labeling was obtained in superior retina and for this reason we filled many OFF ganglion cells in this area of the retina. In addition, the use of a calretinin antibody raised in goat made it convenient to conduct triple label studies in combination with other antibodies. An example of a field of calretinin labeled All amacrine cells is shown in figure 13. An evenly spaced population of small amacrine cells

was labeled and each soma was surrounded by a spray of small lobules. This is the classic and diagnostic appearance of All amacrine cells as described by previous authors (Mills and Massey, 1991; Vaney et al., 1991; Massey and Mills, 1999).

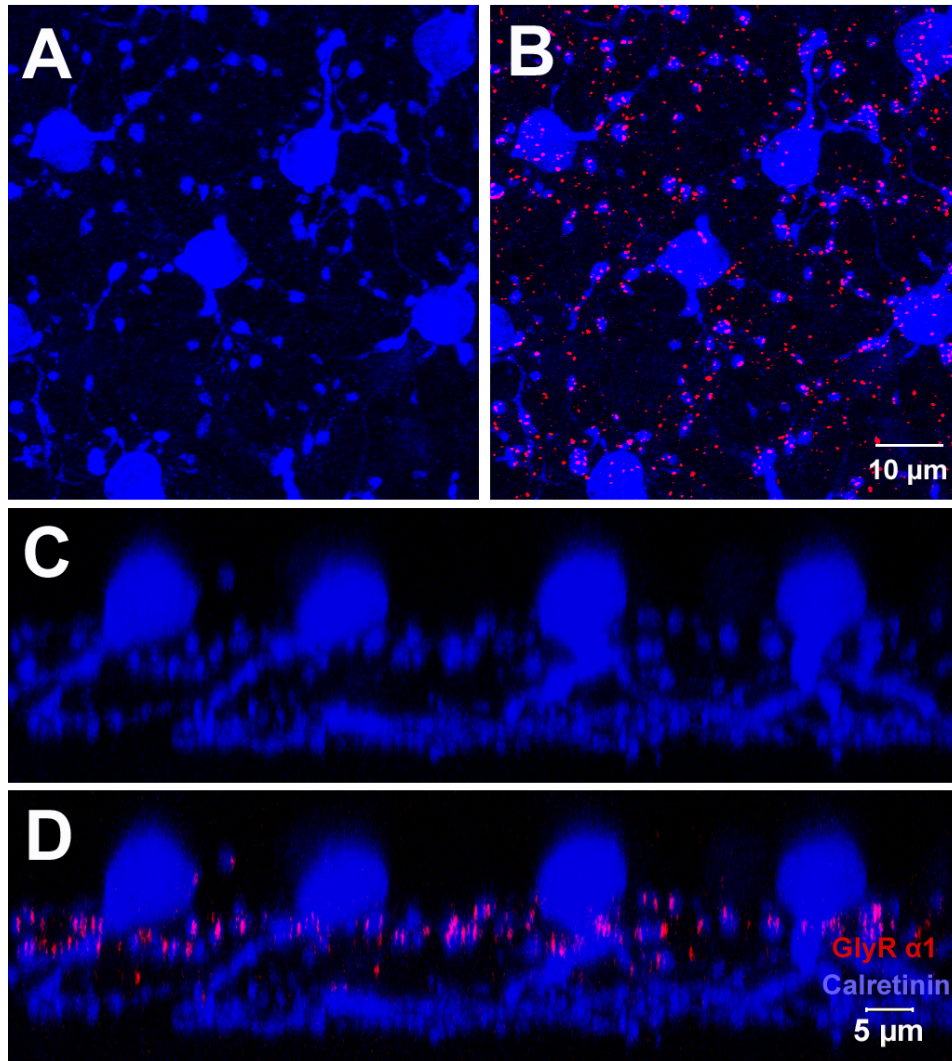


Fig. 13: Calretinin labeled All Amacrine Cells and $\alpha 1$ Glycine Receptors; Z-axis Reconstruction

A: All amacrine cells stained with an antibody against calretinin. The spray of fine dendrites around each soma terminated in lobules in sublamina a. This feature is diagnostic for All amacrine cells.

B: Double label with an antibody against the glycine receptor $\alpha 1$ subunit (red). Glycine receptors were associated with nearly every All lobule. They occurred at the edge of each All lobule because the lobules are pre-synaptic structures and the glycine receptors are post-synaptic.

C: Z-axis reconstruction shows the bistratified appearance of All amacrine cells.

D: Most $\alpha 1$ glycine receptors were found in sublamina a, associated with All lobules.

α 1 Glycine Receptors

Previous studies suggest that A-type ganglion cells in mouse retina (homolog to OFF α ganglion cells in rabbit retina.) express glycine receptor α 1 subunit (Sassoe-Pognetto et al., 1994; Majumdar et al., 2009). To test the specificity of the glycine receptor antibody, we compared two antibodies against the α 1 glycine receptor subunit which were obtained from different vendors. One antibody was a monoclonal antibody raised against the first 10 amino acids of the N-terminus of the α 1 subunit. This antibody (Synaptic Systems, 146111), developed and characterized by the Betz laboratory (Grenningloh et al., 1990) was used at a dilution of 1:1000. The other antibody was a rabbit polyclonal raised against the N-terminus of the α 1 subunit receptor (1:500; Millipore, AB5052). These two antibodies were combined with the goat calretinin antibody in a triple label experiment to test antibody specificity (Fig. 14).

Many of the most prominent clusters of α 1 glycine receptors were double labeled with both antibodies (Fig. 14). In addition to the previous description of the antibodies, including Western blots, this provides strong support for antibody specificity. However, the rabbit polyclonal α 1 glycine receptor antibody was associated with greater background noise leading to a poorer signal-to-noise ratio. For this reason, the cleaner monoclonal antibody was preferred and used in the majority of experiments. In the triple label panel, All amacrine cells were labeled with the calretinin antibody. Most of the double labeled puncta

were associated with All lobules and this provides further evidence of antibody specificity (Fig. 14C). The background labeling with the rabbit $\alpha 1$ glycine receptor antibody was not associated with Alls further suggesting it was non-specific.

Using the monoclonal $\alpha 1$ glycine receptor antibody, we found that nearly every All lobule was associated with $\alpha 1$ glycine receptors. There was a tendency for the $\alpha 1$ clusters to lie at the edge or just outside the lobules. This was because the All lobules are pre-synaptic structures while the $\alpha 1$ glycine receptors were expressed by post-synaptic structures (Jusuf et al., 2005). At high resolution (Fig. 15), it was more obvious that the $\alpha 1$ clusters lay apposed to the surface of individual lobules although the view was often complicated by the depth of focus and the convoluted surface of All lobules. Animating through a series of high resolution images confirmed the surface location of $\alpha 1$ glycine receptors. This was consistent with the release of glycine from All lobules and the expression of $\alpha 1$ glycine receptors by post-synaptic neurons, including both OFF bipolar cell terminals and ganglion cells.

Some $\alpha 1$ glycine receptors were not associated with All amacrine cells and these were presumed to be post-synaptic to other unidentified glycinergic amacrine cells, of which there are many. Quantitative analysis showed that in sublamina a of the IPL 62.5%

of the $\alpha 1$ glycine receptors were associated with All amacrine cells. A Z-axis reconstruction through a line of four All amacrine cells confirmed that $\alpha 1$ glycine receptors were mostly confined to sublamina a, as previously reported (Fig. 13C, D) (Sassoe-Pognetto et al., 1994; Jusuf et al., 2005). Furthermore, this view also showed a clear association of $\alpha 1$ glycine receptors with the lobules of All amacrine cells.

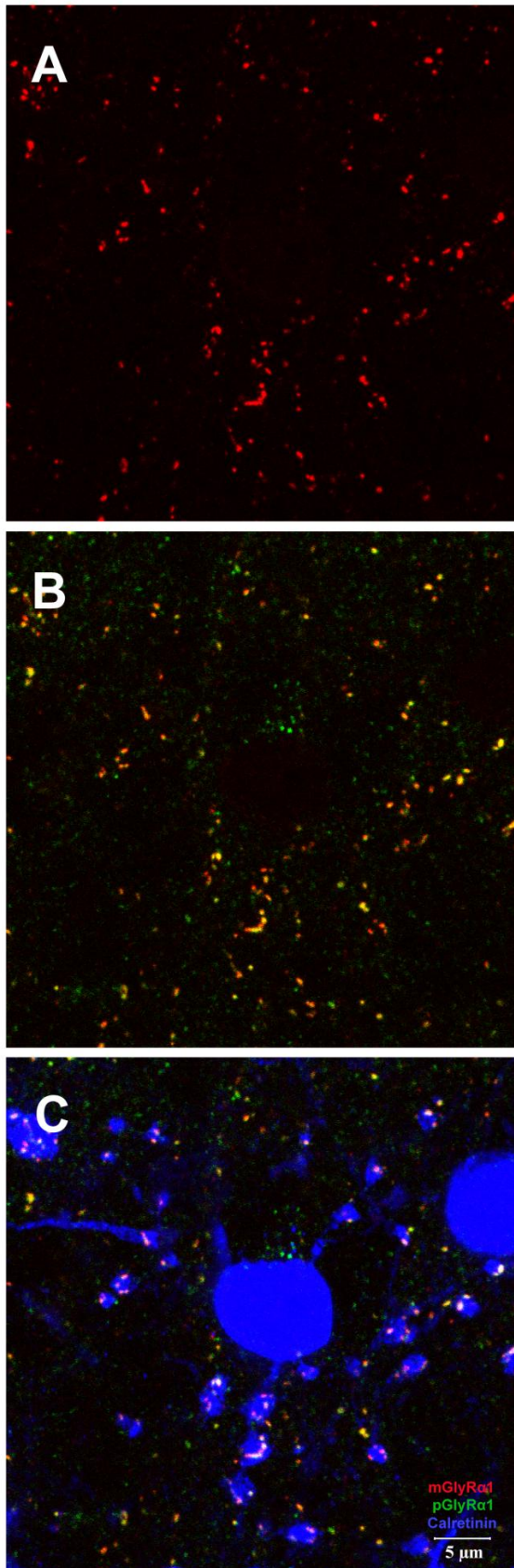


Fig.14: Double-label: Monoclonal and Polyclonal $\alpha 1$ Glycine Receptor Antibodies Plus All Amacrine Cell

A: Monoclonal antibody against $\alpha 1$ glycine receptors (red).

B: Double-label with a polyclonal antibody against $\alpha 1$ glycine receptors (green). The large puncta are double labeled often yellow. The non-specific background labeling is green.

C: Triple -label with a calretinin labeled All (blue). The double labeled $\alpha 1$ glycine receptors were associated with All lobules.

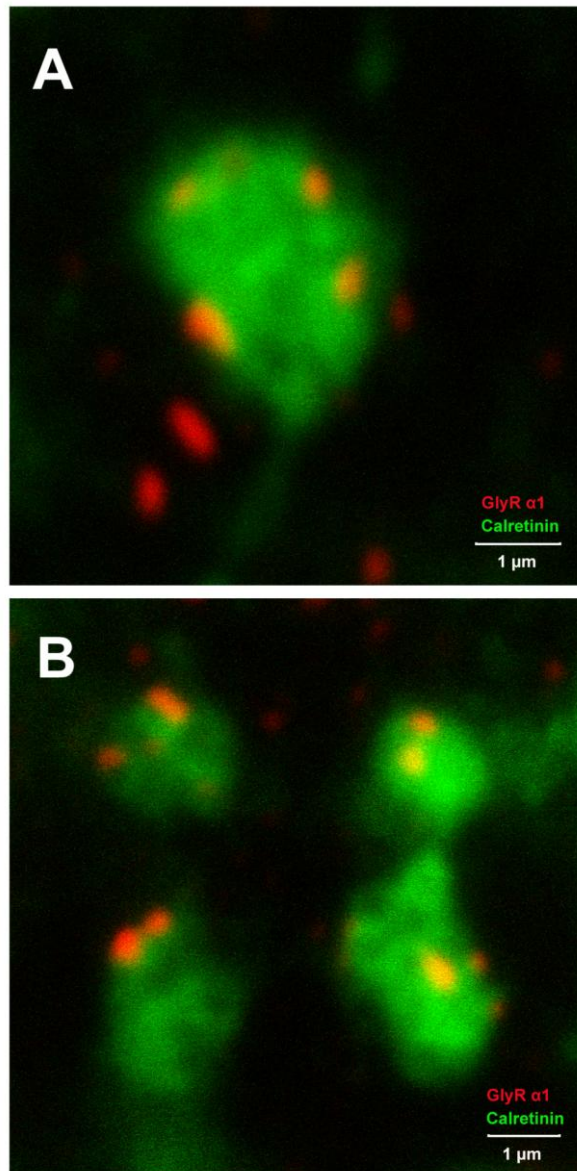


Fig.15: All Lobules and $\alpha 1$ Glycine Receptors at High Resolution

All amacrine cell lobules (green) and $\alpha 1$ glycine receptors (red). The $\alpha 1$ glycine receptors are located at the edge of the All lobules because they are post-synaptic.

All Amacrine cell Contacts with OFF α and G9 Ganglion Cells

Individual dye injected ganglion cells, which were clearly identified as specific ganglion cell types using the criteria outlined above were selected for immunolabeling with the combination of calretinin and $\alpha 1$ glycine receptor antibodies. Even in low power images (40x, zoom 1), many appositions between All lobules and the ganglion cell dendrites were observed (Fig. 16A,C). These figures also emphasized the large size of the dye-injected ganglion cells; hundreds of All amacrine cells fell within the dendritic field of OFF α and G9 ganglion cells. At higher resolution (63x, zoom 3-4), it was possible to observe the distribution of $\alpha 1$ glycine receptors in this material.

Fig. 17A shows a dendritic branch point from a dye injected G9 ganglion cell. Proximal dendrites were not used in this analysis because they traverse the IPL and may not make appropriate synaptic contacts. The cell body of an All amacrine cell was located between the branches and a series of small lobules were aligned with the G9 dendrites (Fig. 17C). Most of the fine dendrites giving rise to the lobules were too fine to image or they were outside the plane of focus so the lobules could not always be traced to the parent All. However, most of the lobules in this frame arise from the central All while some lobules to the lower left came from an All amacrine cell outside the field.

Panel E shows the distribution of $\alpha 1$ glycine receptors in this material. As before, essentially every AII lobule was associated with post-synaptic glycine receptors. Importantly, $\alpha 1$ glycine receptors were nearly always found exactly at the contact points between AII lobules and the G9 dendrite (circles, Fig. 17E). An individual AII lobule could be apposed to several $\alpha 1$ receptors on any face suggesting the presence of multiple post-synaptic targets, probably including OFF bipolar terminals as expected. However, on the side in contact with the G9 dendrite an $\alpha 1$ glycine receptor was located between the AII and the G9 dendrites within the resolution limit of the confocal microscope.

A similar length of dendrite, not close to the soma, was selected from a dye-injected OFF α ganglion cell (Fig. 17B). A nearby AII amacrine cell gave rise to a cluster of 6-8 lobules which were aligned along the OFF α dendrite (Fig. 17D). In the triple-label image, it can be seen that $\alpha 1$ glycine receptors were present at the contact points between AII lobules and the OFF α dendrite (circles, Fig. 17F). At first sight, the largest profile appears to be the AII soma but actually this was the descending primary dendrite. The cell body was much larger and was out of focus at a higher level in the inner nuclear layer. An $\alpha 1$ glycine receptor cluster was found along the primary stalk exactly at the focal plane of the OFF α dendrite. Thus, one of the potential synaptic contacts onto the OFF α was made by the primary dendrite of the AII.

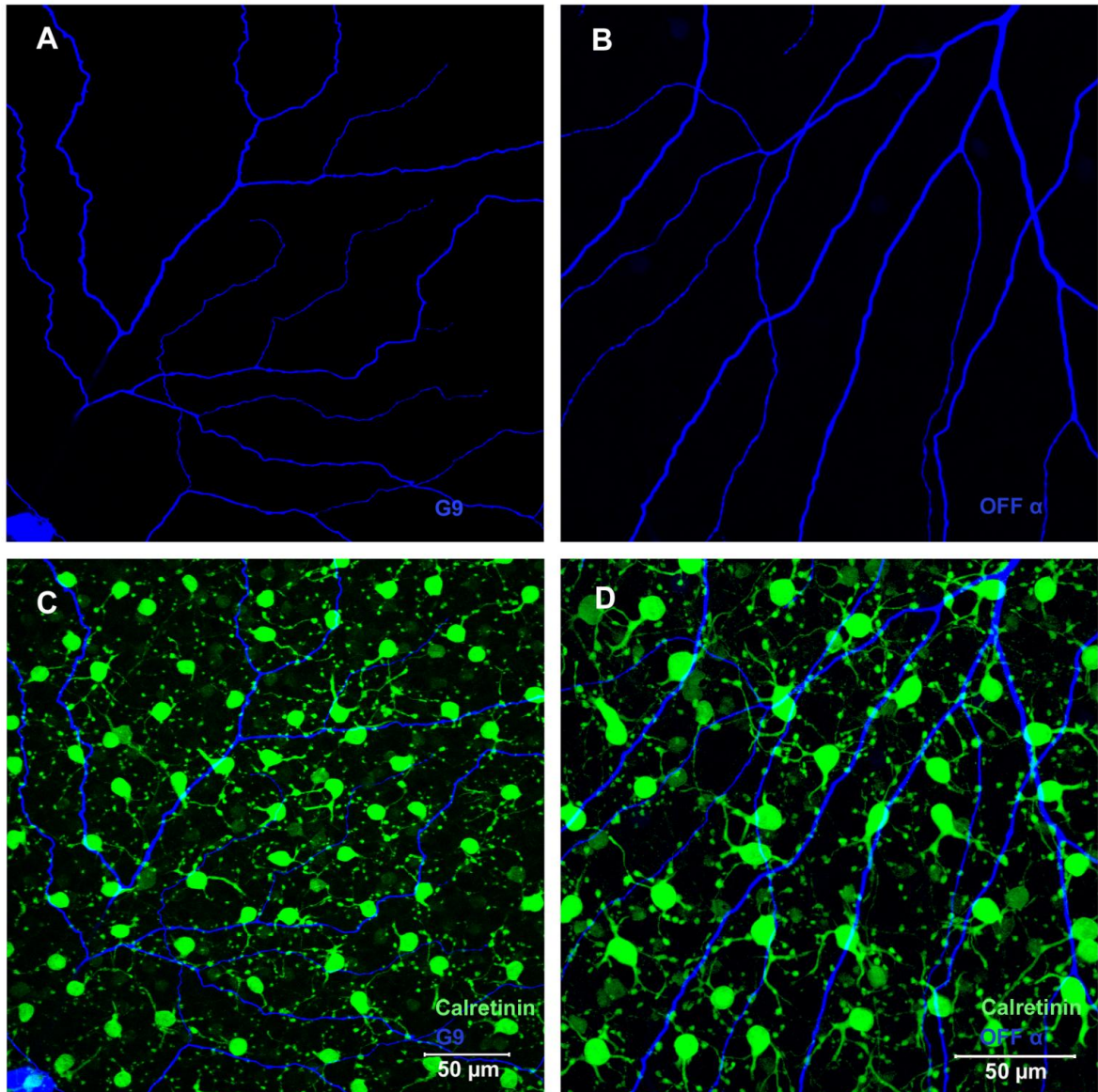


Fig. 16: Low Magnification: G9 and OFF α Ganglion Cells with All Amacrine Cells

A: The dendrites of a dye-injected G9 ganglion cell. This cell was not dye coupled and stratified above the cholinergic a band. These features positively identify this cell as a G9 ganglion cell.

C: Double label image showing many close contacts between All lobules (green) and the dendrites of the G9 ganglion cell (blue).

B: The dendrites of a dye-injected OFF α ganglion cell. This cell was identified by the criteria outlined above.

D: Double label, OFF α ganglion cell dendrites (blue) and All amacrine cells (green). It be seen that many All lobules are apposed to the OFF α cell dendrites.

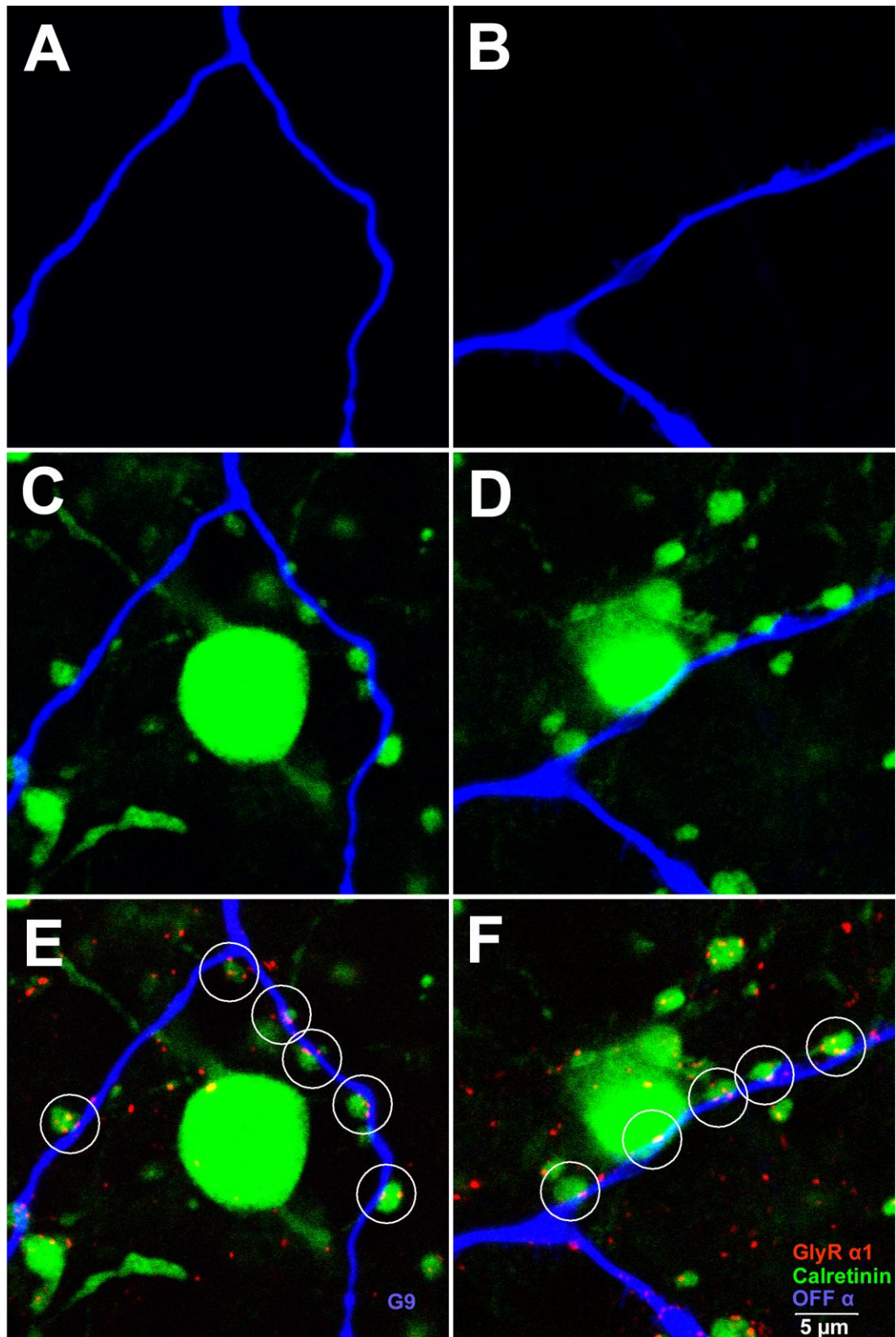


Fig. 17: Resolution Triple Label: G9 and OFF α Ganglion Cells

Fig. 17: Resolution Triple Label: G9 and OFF α Ganglion Cells

A: Dye-injected G9 ganglion cell dendrites (blue).

C: Many appositions between All lobules (green) and G9 dendrites.

E: $\alpha 1$ glycine receptors (red) were found at contact points between All lobules and G9 dendrites.

B: Dye-injected OFF α ganglion cell dendrites (blue).

D: Many appositions between All lobules (green) and OFF α dendrites.

F: $\alpha 1$ glycine receptors (red) were located at contact points between All lobules and OFF dendrites. Isolated glycine receptors may be associated with other unmarked glycinergic amacrine cells.

Other Ganglion Cell Types

A similar analysis was carried out for ON/OFF DS ganglion cells and local edge detectors. The left three panels in Fig. 18, show the results for a local edge detector. The dendrites of this dye-injected cell had the typical thorny appearance of a local edge detector. However, there were very few All lobules in the same focal plane because the LED was stratified in sublamina 3, slightly below most of the All lobules. The bright profiles in figure 18C were the descending primary dendrites of several neighboring All amacrine cells. In the triple label panel (Fig. 18E), there were very few $\alpha 1$ glycine receptors and no evidence for lobular All input to the LED. As shown above and previously reported, the majority of $\alpha 1$ glycine receptors were found in sublamina a just above the LED level.

We also examined the OFF dendritic tree of several ON/OFF DS ganglion cells which are stratified between G9 and OFF α ganglion cells coincident with the cholinergic a band. The dendrites showed a space –filling pattern with many 90 degree branches, in contrast to the acute angle branch pattern of G9 and OFF α ganglion cells (Fig. 18B). At this level, there were numerous All lobules and $\alpha 1$ glycine receptors but relatively few All contacts. Some examples were located in figure 18D, F where potential contacts coincident with an $\alpha 1$ glycine receptor are marked by arrows. There were more non-All $\alpha 1$ glycine receptors at this level also but a clear impression that ON/OFF DS ganglion cells

received less All input than the OFF ganglion cells described above.

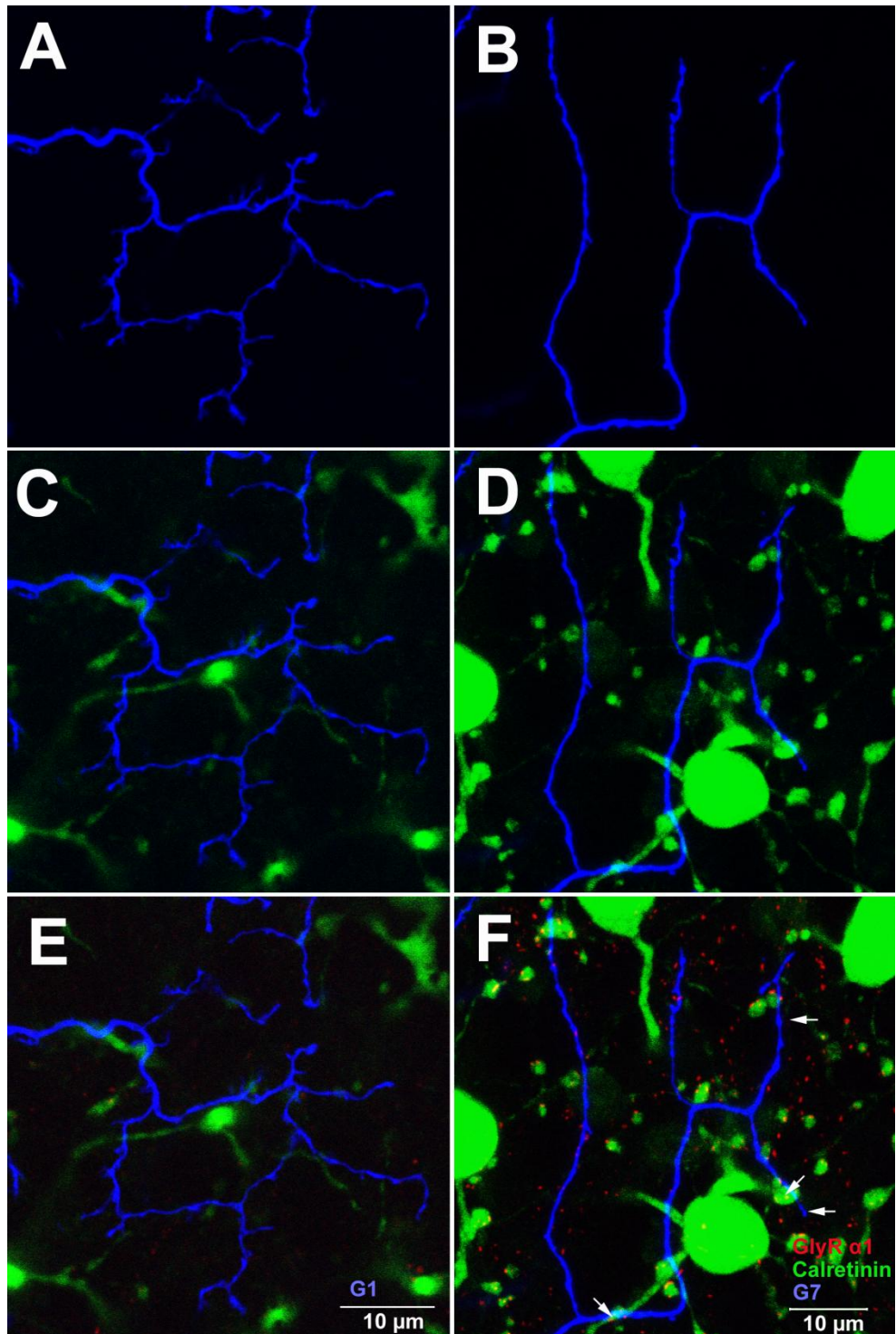


Fig. 18: Resolution Triple Label: LED and ON/OFF DS Ganglion Cells

Fig. 18: Resolution Triple Label: LED and ON/OFF DS Ganglion Cells

A: LED dendrites, with typical thorny appearance (blue)

C: Calretinin labeled All amacrine cells (green). At this level, the descending stalks were in focus.

E: $\alpha 1$ glycine receptors (red). There are very few $\alpha 1$ glycine receptors at this level, below the All lobules.

B: ON/OFF DS ganglion cell dendrites (blue). The dendrites were retroflexive and space filling.

D: Calretinin labeled All amacrine cells (green). There were many lobules at this level.

F: $\alpha 1$ glycine receptors (red). A few potential contacts were marked with arrows.

Quantitative Analysis

To establish that the potential synaptic contacts described above were not due to random overlap, we used Image J to compare the images above with similar constructs where the spatial relationships in the original image have been destroyed. This was most easily accomplished by taking the triple label images and rotating the channel of $\alpha 1$ glycine receptors through 90, 180 and 270 degrees. Then, the numbers of triple label contacts were counted in a procedure sometimes known as rotation analysis. The procedure was outlined in figure 19.

A confocal image series of an OFF α ganglion cell dendrites, (the same cell shown in figure 17B,D,F), together with the $\alpha 1$ glycine receptors was used for this analysis (Fig. 19). $\alpha 1$ glycine receptors colocalized with the ganglion cell dendrite were highlighted for

each optical section and for display purposes stacked to a single image plane. The colocalized receptors were high-lighted in panel C. The colocalized $\alpha 1$ receptors on the ganglion cell dendrite were in turn colocalized with the AII lobules, again for each optical section. These triple labeled points represent $\alpha 1$ glycine receptors at the contact points between AII lobules and the OFF α dendrite. The results are displayed in panel G and they were counted in Image J.

The same procedure was followed in the adjacent panel except the $\alpha 1$ glycine receptor channel was rotated out of phase by 90 degrees. Because of the number and density of the $\alpha 1$ receptors, some random overlap with the ganglion cell was still present and these few colocalizations are high-lighted in panel 19D. However, in the next step, colocalization with the AII lobules, the random number fell very close to zero. The colocalizations at each step were calculated in Image J and the procedure was repeated for 90, 180 and 270 degree rotations.

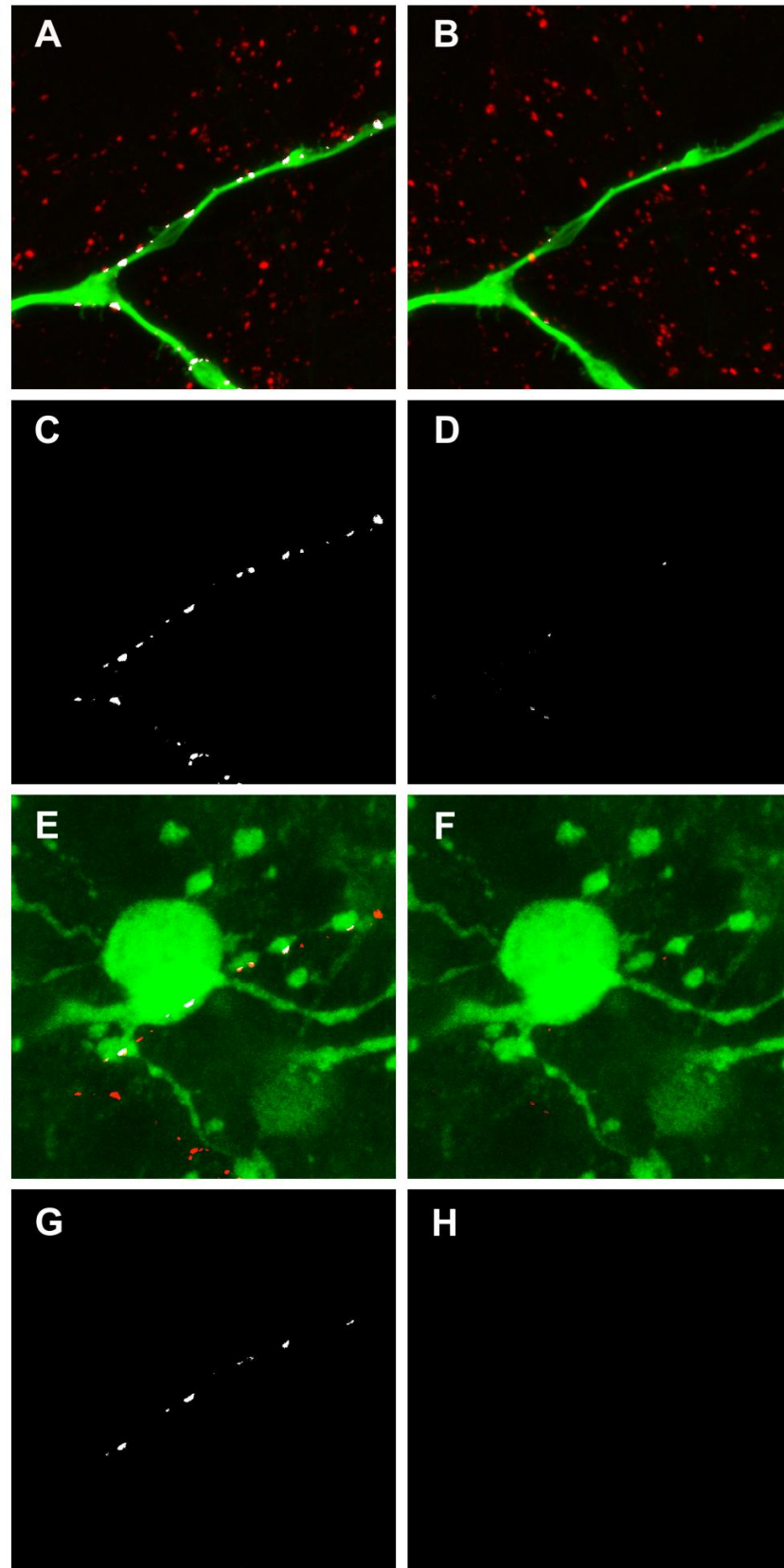


Fig. 19: Demonstration: Rotation Analysis with Image J

Fig. 19: Demonstration: Rotation Analysis with Image J

A, C, E, G: Analysis for an OFF α ganglion cell (green). Colocalized $\alpha 1$ glycine receptors (red) were high-lighted in white. These were the $\alpha 1$ receptors on the OFF α dendrite. In turn, these were colocalized with the All lobules for display in the bottom panel. These highlights were the contact points between All lobules and the OFF α dendrite where there were $\alpha 1$ glycine receptors .

B,D,F,H: Same analysis with $\alpha 1$ glycine receptors rotated out of phase by 90 degrees. There were a few apparent $\alpha 1$ glycine receptors still associated with the OFF α dendrite but when they were colocalized with the All lobules, the chance coincidence fell close to zero. This suggested that the colocalization of All contacts at OFF α dendrites with an $\alpha 1$ glycine receptors may highlight potential synaptic contacts.

The results of the rotation analysis were displayed in figure 20. Rotating the $\alpha 1$ glycine receptors out of phase reduced the apparent colocalization of $\alpha 1$ receptors with the OFF α dendrite to approximately 30% of the control value. This indicates that the $\alpha 1$ glycine receptors are not randomly distributed, rather they occur along the OFF α cell dendrite in numbers far greater than should occur by random chance. With the addition of the third channel, the All lobules, the apparent colocalization of all three channels was reduced by more than 90% compared to the control value. This suggests that the probability of a random overlap of all three channels is very low. Furthermore, this result validates the use of triple label analysis and suggests that the use of a third marker, such as the $\alpha 1$ glycine receptor, can be used to label potential synaptic sites such as the contact points between All lobules and OFF α ganglion cell dendrites.

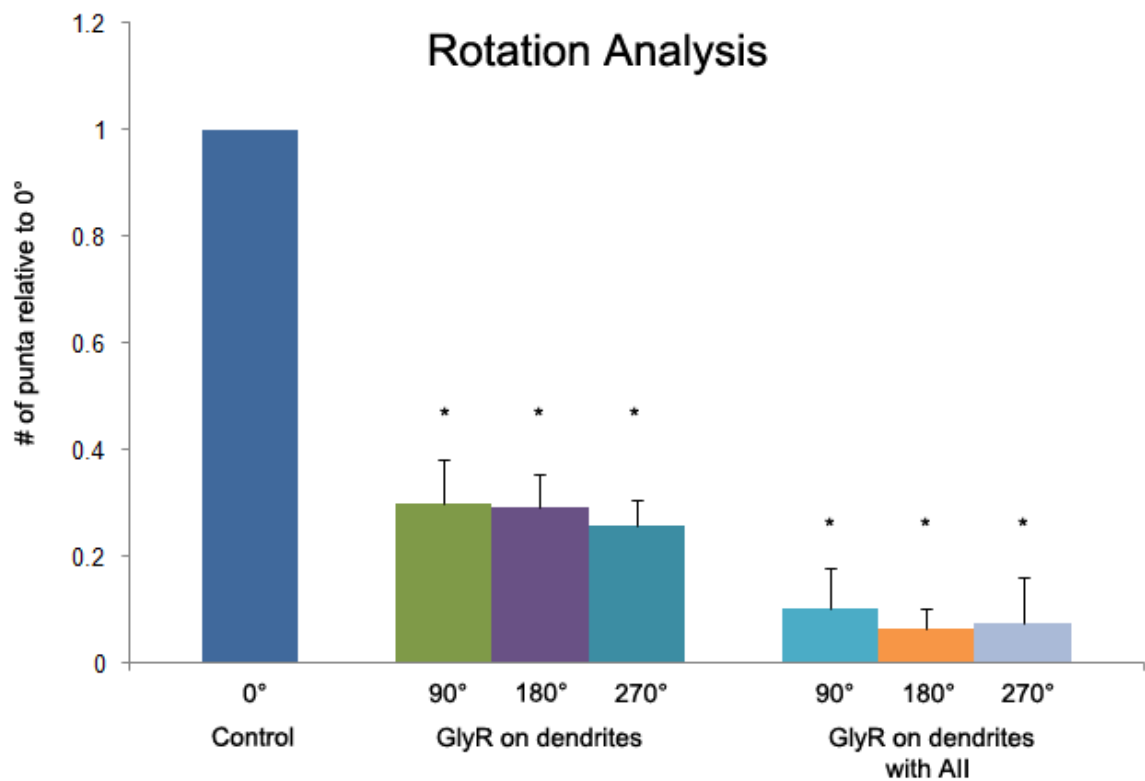


Fig. 20: Rotation Analysis Results

A summary of the rotation analysis showed that the random coincidence with two markers (dendrites and $\alpha 1$ glycine receptors) fell to 30% on rotation. With three markers (dendrites, $\alpha 1$ glycine receptors and All lobules) the random coincidence fell to less than 10%.

The results of the rotation analysis provided convincing evidence that the triple labeled hot spots, consisting of $\alpha 1$ glycine receptors at All contact points with OFF ganglion cell dendrites were All synapses. Therefore, to assess the relative strength of All input to the different ganglion cell types, we used Image J to count the triple labeled hotspots in a confocal series of images. This was conducted on randomly selected, near-terminal dendritic fields of five ganglion cells for each cell type and the results were

normalized to 100 μ of dendritic length (Fig. 21). OFF α ganglion cells received the highest number of lobular All contacts at 7 per 100 μ m of dendritic length. This was approximately double the number of All contacts with G9 ganglion cells, 3.5 per 100 μ m of dendritic length. The least number of All inputs was found for ON/OFF DS ganglion cells at less than 2 per 100 μ m of dendritic length.

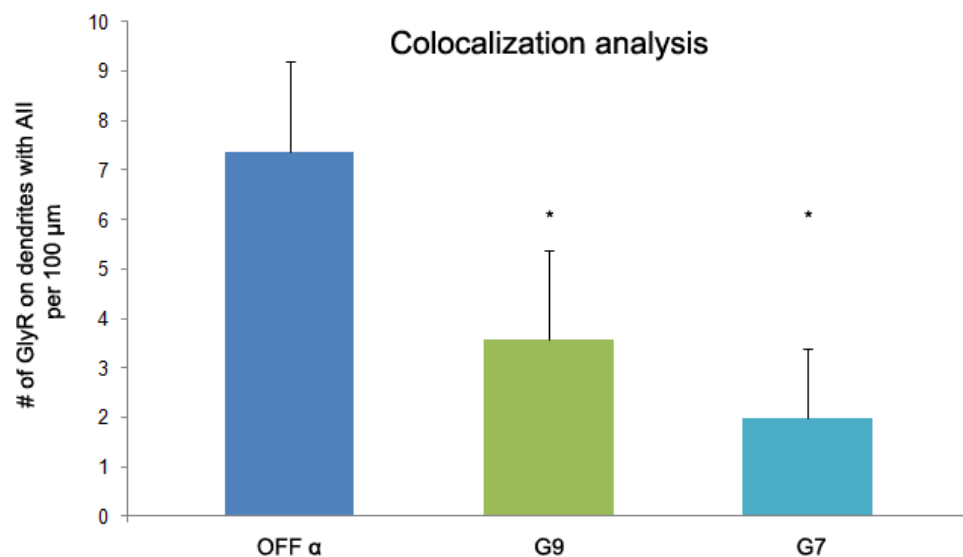


Fig. 21: Colocalization Analysis for OFF α , G9 and ON/OFF DS Ganglion Cells

The number of All lobular contacts with dendrites and α 1 glycine receptors per 100 μ m of ganglion cell dendrite. OFF α dendrites had the most contacts followed by G9 and ON/OFF DS ganglion cell had the least.

The original goal of this project was to compare the All input to OFF ganglion cells.

Multiplying by the total dendritic length by the normalized numbers above yielded an

estimate of the total number of All contacts for each OFF ganglion cell type. We calculated

that OFF α ganglion cell dendrites received 827 All inputs while G9 ganglion cells received 406 All inputs. These numbers take into account the smaller size but higher dendritic density of the G9 ganglion cells.

Finally, the All inputs account for only a fraction, (G9, 21%; OFF α , 26%) of the $\alpha 1$ glycine receptors found on OFF ganglion cells. The remaining $\alpha 1$ glycine receptors were not associated with All amacrine cells and may represent sites of glycinergic input from other glycinergic amacrine cells.

All Input to Dye-Coupled Amacrine Cells

Some of the OFF α ganglion cell patches were extremely well coupled and in these preparations, the dendrites of three different OFF α cells overlapped in some areas, shown in a low power montage (3 20x images, Fig 22A). This was consistent with the coverage factor of approximately 1.85 calculated for OFF α ganglion cells (Peichl et al., 1987b). These images were extremely complex but the different OFF α cell dendrites could be identified by tracing back to the ganglion cell bodies in the low power image and the remaining coupled amacrine cell dendrites were highlighted. In the stacked image (Fig.22B,C), a dye coupled wide-field amacrine cell soma was present near the center of the image. As expected, All inputs to the OFF α dendrites were relatively common.

However, we were unable to identify evidence of All input to the dye-coupled amacrine cells.

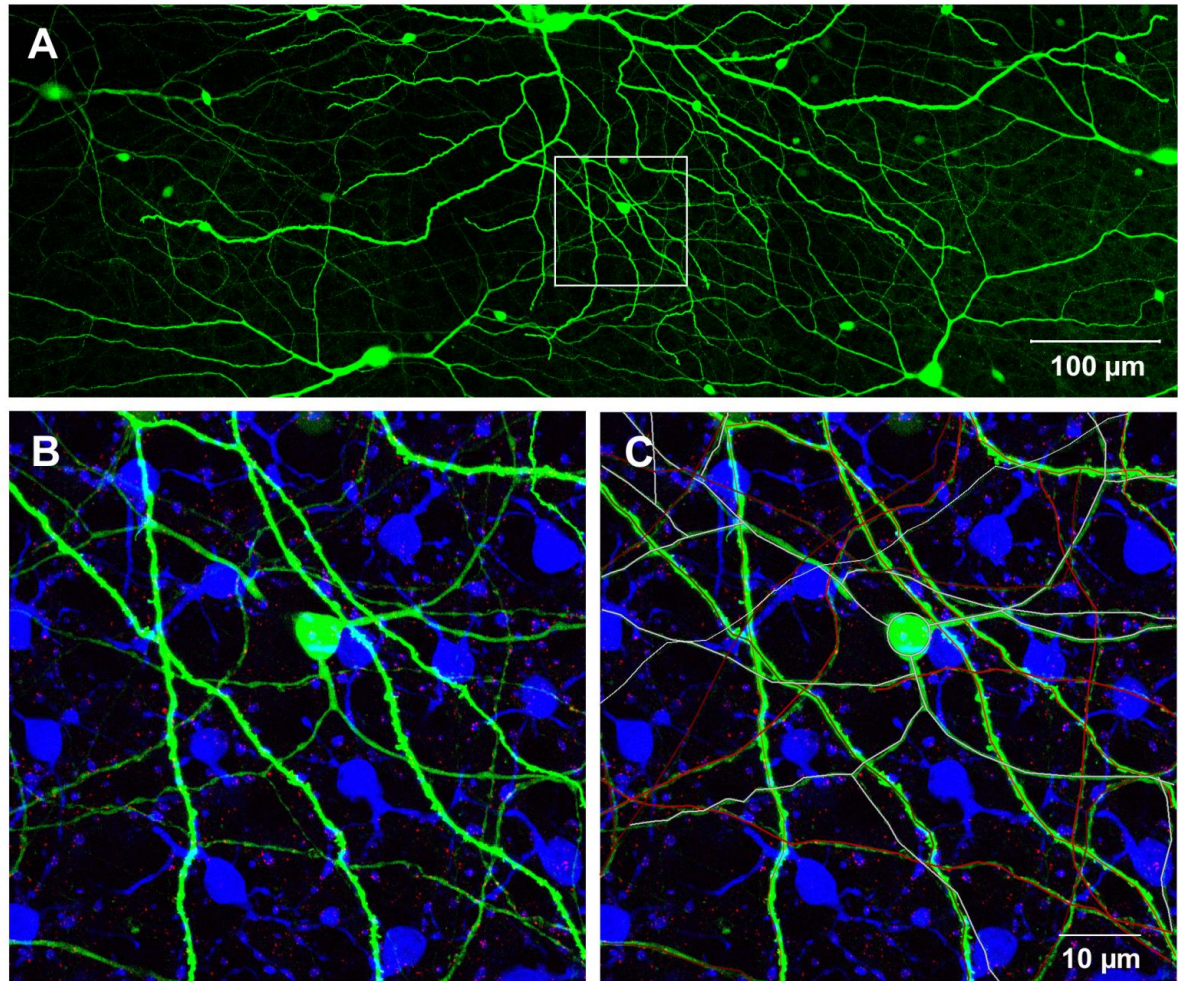


Fig. 22: Dye Coupling in a Patch of OFF α Ganglion Cells

A: A montage of 3 20x fields to show a large patch of dye coupled OFF α Ganglion Cells. The square shows a region with overlapping dendrites of three OFF α Ganglion Cells. In addition, there were many dendrites from dye-coupled amacrine cells.

B: 63x field confocal stack to show all the dye coupled dendrites in this region.

C: OFF α dendrites were identified by tracing back to the appropriate cell body in the low power image and marked red in the overlay. Dye coupled amacrine dendrites, many from the soma close to the middle, were marked in gray.

Discussion

Physiological evidence from several different labs implies that All amacrine cells have direct input to certain OFF ganglion cells. However, previous EM analysis of the rabbit retina suggests that the dominant output of the All amacrine cell in sublamina a goes to OFF cone bipolar cells (Strettoi et al., 1992). Using a combination of intracellular dye injection, immunolabeling and confocal reconstruction, we identified specific OFF ganglion cell types and found evidence for direct synaptic contacts from the lobules of All amacrine cells. We developed statistical methods to validate the triple label strategy used in these experiments and a quantitative analysis revealed that OFF α and G9 ganglion cells receive a major input from All amacrine cells via $\alpha 1$ glycine receptors. The glycinergic output of All lobules is the final step in a crossover inhibition pathway whereby ON signals inhibit certain OFF ganglion cells.

All input to OFF Ganglion Cells

The conventional wisdom, following the classic EM reconstruction of Strettoi et al. 1992, was that most output from All lobules goes to OFF cone bipolar terminals, which, in turn, synapse with OFF ganglion cells. The discovery that crossover inhibition, first described in the mudpuppy retina 30 years ago (Belgum et al., 1982; Arkin and Miller, 1987), was mediated in part via a gap junction pathway through All amacrine cells was

based on the finding that OFF ganglion cells have inhibitory inputs that were resistant to the block of AMPA/KA receptors. This left the cone → ON BC→GJ →All→OFF GC as the most likely pathway. The All input could not be routed via the OFF bipolar cell because the OFF bipolar input to OFF ganglion cells would be blocked by AMPA/KA antagonists.

Additional evidence in support of this pathway showed that APB, which blocks ON pathways, MFA, which blocks gap junctions, and strychnine, which blocks glycine receptors, all blocked the light-driven inhibitory input by actions at different points along the pathway (Fig. 2), (Wu et al., 2004; Manookin et al., 2008; Münch et al., 2009; van Wyk et al., 2009). Therefore, we set out to test the hypothesis that All amacrine cells make direct contacts with certain OFF ganglion cells.

The major finding here is that we found unequivocal evidence for direct input from the lobules of All amacrine cells to OFF ganglion cells mediated via $\alpha 1$ glycine receptors. This directly supports the crossover inhibition pathway described above. These results also confirm the presence of a direct input from All to OFF ganglion cells as demonstrated by the heroic paired recordings of Münch et al 2009 in the PV/GFP mouse retina. Numerically, we found that OFF α ganglion cells received over 800 All inputs so this is a major retinal pathway, supporting cross-over inhibition. G9 ganglion cells received half the number of All inputs approximately 400 per cell. The OFF dendritic tree of ON/OFF DS

ganglion cells received even fewer AII inputs, suggesting this pathway makes a smaller contribution to this ganglion cell type. Local edge detectors (G1) received no detectable AII inputs which is unsurprising because they were stratified just below the level of the AII lobules.

The most detailed physiology has been reported for the mouse and guinea pig retinas. In the guinea pig retina, the homologs of rabbit OFF α and G9 ganglion cells appear to be OFF α and OFF δ ganglion cells respectively. The OFF δ cell has a particularly prominent ON driven inhibitory input which provides the main drive to this cell type (Manookin et al., 2008). Thus, it was surprising that the G9 ganglion cells in this study received less AII input than the OFF α ganglion cells. However, it may be that the relative strength of the inhibitory input to the OFF δ ganglion cell more closely reflects the balance between excitatory bipolar drive and crossover inhibition via the AII. In future experiments, this could be tested by measuring the number of GluR4/AMPA receptor inputs in comparison to the $\alpha 1$ glycine receptors counted here. There is supporting evidence for this idea because excitation mapping with AMPA-activated 1-amino-4-guanidobutane (AGB) suggested that OFF sustained ganglion cells, perhaps equivalent to G9, receive less excitatory AMPA driven input than OFF transient cells, probably equivalent to the OFF α ganglion cells reported here (Marc and Jones, 2002).

Crossover Inhibition

For cone signals, the straight-through pathway is from cones→cone bipolar cells→ganglion cells and there are two parallel channels of opposite sign known as ON and OFF pathways. This has been accepted as the simplest pathway since it was first established that bipolar cells use glutamate as a neurotransmitter and they provide an excitatory drive (Slaughter and Miller, 1981). However, as first reported by Belgium (Belgium et al., 1982), OFF ganglion cells can also be light driven by ON inhibition. The sum of these two drives is additive though temporally separate and the ratio may vary according to ganglion cell type. This seems to be a common mechanism because it has been reported across species, from salamanders to mammals. Further evidence supporting crossover inhibition comes from the application of APB. When APB is applied, not only are ON channels blocked, but OFF ganglion cells usually show increased firing due to the block of cross over inhibition (Massey and Miller, 1988).

The gap junction pathway is only one of many potential crossover inhibition pathways in the retina. There are many diffuse or multi-stratified amacrine cells that could transmit signals between the ON and OFF layers of the IPL. However, in the Cx36 knock-out mouse, in which the gap junctions between AII cells and between AII cells and ON bipolar cells are absent (Deans et al., 2002) the ON driven glycinergic inhibition is dramatically

diminished (Wu et al., 2004; Münch et al., 2009; van Wyk et al., 2009). This implies that the All gap junction pathway is a major contributor of crossover inhibition to OFF ganglion cells. Munch et al 2009 have proposed that one advantage of the All/gap junction pathway is speed, so that OFF ganglion cells may provide a rapid approach-sensitive function to avoid looming predators. This would provide a clear advantage to survival.

Finally, this circuit may confer major benefits for retinal function. It has been proposed that the dual drive to OFF ganglion cells may provide a greater dynamic range, Furthermore, if certain OFF ganglion cells have spontaneous activity, then crossover ON inhibition will allow them to signal light increments by a reduction in firing rate, in addition to the increased signal at light OFF. It has also been proposed that crossover inhibition provides other useful functions such as to partially compensate for the rectification provided by excitatory bipolar inputs (Molnar et al., 2009). In summary, we have found morphological evidence in support of a crossover inhibition pathway in the retina which may fulfill several desirable functions increasing retinal performance.

Identification of Specific Ganglion Cell Types

The number of ganglion cell types in mammalian species has not been definitively identified. However, most estimates lie in the range of 12 – 20 different types (Rockhill et al., 2002; Kong et al., 2005). This constitutes a major obstacle for work on ganglion cells. If the specific ganglion cell type is not identified, then ensuing measurements may be derived from a variety of different cell types with unknown variation. With recent efforts in several mammalian species to catalog ganglion cell types and unravel the retinal circuits involved, it has become increasingly clear that reliable identification of specific cell types is a necessity for further progress. In the present work, we used multivariate analysis to distinguish between OFF ganglion cells in the rabbit retina. OFF α ganglion cells could be reliably separated from G9 ganglion cells, despite their similar morphology, on the basis of size, stratification and dye coupling (Rockhill et al., 2002; van Wyk et al., 2009).

This work was conducted in the rabbit retina which has some particular experimental advantages for the analysis of retinal circuitry. Based in large part on the work of Masland and colleagues, most of the major cell types in the rabbit retina have been identified. Morphological catalogs of bipolar cells, amacrine cells and ganglion cells are available, in addition to physiological and biochemical classifications (Rockhill et al., 2002; MacNeil et al., 2004; Masland, 2004). Arguably, we know more about the rabbit retina than

any other region of the CNS. The size of the rabbit retina is also a significant advantage, not just because the preparation is larger. In smaller species such as the mouse retina, the packing density means that the ganglion cell layer is completely full. In fact, there are more displaced amacrine cells than ganglion cells in the ganglion cell layer (Strettoi and Masland, 1996). Furthermore, the cell density means that the closely packed cells are small with a relatively uniform round shape. In contrast, there is a wide variation in cellular appearance for the rabbit retina following simple staining protocols using DAPI or acridine orange. These cues enable specific ganglion cells to be targeted with a high rate of success based on their gross appearance. Finally, a large data base of previously identified neurons is readily available for the rabbit retina.

Most recently some new molecular markers have been developed for retinal ganglion cells. These include junctional adhesion molecule B (JAM-B) (Kim et al., 2008) and calretinin driven mouse-GFP lines. The availability of genetic mouse strains expressing cell specific markers such as GFP is rapidly changing the equation and further advances may be expected in this direction. However, the small and uniform size of mouse cells is a major experimental handicap. Thus, there are solid reasons for conducting the present study in the rabbit retina. In many ways, it is the species of choice

for the analysis of retinal circuitry.

Triple Label Confocal Analysis of Synaptic Contacts

When two neurons contact each other in a repetitive manner, this may indicate a synaptic contact. But this may be a complex issue, especially between dense arrays of neurons where this is a significant degree of random overlap. The addition of a third synaptic marker such as a transmitter receptor really changes the picture because now random overlap may be differentiated from synaptic contacts by the requirement for the colocalization of three different markers. The rotational analysis conducted in this paper suggests that the random colocalization of three different markers is very low. In other words, the presence of a synaptic marker at the contact points between two neurons rarely occurs by chance. Our calculations suggest that rotating the $\alpha 1$ glycine receptors by 90 degrees reduces the triple label colocalization by more than 90% compared to the original orientation. Thus, the coincidence of three separate markers is a reliable indicator of synaptic contacts.

The size of the $\alpha 1$ glycine receptor clusters was 0.5 to 1 μ in diameter. This

exceeds the point spread function for the confocal microscope which we estimated to be 300nm in the XY dimension. The point spread function effectively sets the lower limit of resolution such that clusters smaller than this limit still appear as 300nm objects. However, below the point spread function, the intensity falls with the square of the radius and smaller receptor clusters rapidly drop into the background noise. We previously estimated that the smallest structure that can be detected using the confocal was approximately 150nm in diameter, under ideal conditions (Pan and Massey, 2007).

There is also some background noise in the images presented here. This takes at least two forms. Pixel noise, smaller than the point spread function cannot represent biological signals as discussed above. Rather, this very small scale noise was contributed by instrumentation noise, most often the background from the photomultipliers, generated by setting the gain too high. This noise was removed by median filtering or setting a size threshold below 300nm in the Image J routines for colocalization. Another form of noise may be generated by clumps of secondary antibody or non-specific binding of the primary or secondary antibodies. This type of noise may have the same size distribution as the specific labeling pattern and is thus difficult to remove objectively. However, the secondary antibodies used in this study were very reliable and the primary antibodies were previously

shown to have high specificity. Therefore, non-specific labeling was a very minor issue and no attempt was made to edit or filter the images presented here.

Future directions

Further progress in identifying the synaptic circuits leading to ganglion cells will be facilitated by the ability to recognize specific ganglion cell types. In this regard, the development of new ganglion cell markers of GFP-mouse lines will be of great benefit. Eventually, the advent of single cell libraries may provide more tools to identify specific cell types. The expression of $\alpha 1$ glycine receptors may be a useful marker to identify certain OFF ganglion cell profiles. As specific ganglion cell types are identified by confocal microscopy, it may become possible to correlate this information with large scale EM reconstruction projects that are already in progress (Denk lab, Marc lab). Finally, it may be possible to match the morphological classification of ganglion cell types with their physiological counterparts as we try to understand the roles of each individual ganglion cell.

Ganglion cell axons carry the visual output of the retina to the rest of the CNS via

the optic nerve and it is a great challenge to understand the diversity of ganglion cell types.

It may be that each ganglion cell type has stereo-typed central connections and a specific role in vision. The melanopsin ganglion cells, which reset the circadian clock and drive the pupillary light reflex may be the best known example of a ganglion cell with a specific job.

But the remarkable progress achieved in understanding the accessory optic pathway has been almost completely dependent on the identification of the intrinsically photosensitive retinal ganglion cells due to their unique expression of melanopsin, either by antibody labeling, by the development of cell markers driven by the melanopsin promoter or by the use of melanopsin knock-out lines. Thus, the seemingly simple identification of ganglion cell types is something of a bottleneck to further progress in this area.

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Poster