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Morphology and connectivity of the small bistratified A8 amacrine cell in the mouse retina

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Abstract

Amacrine cells comprise ~30 morphological types in the mammalian retina. The synaptic connectivity and function of a few GABAergic wide-field amacrine cells have recently been studied, however, with the exception of the rod pathway-specific AII amacrine cell the connectivity of glycinergic small-field amacrine cells has not been investigated in the mouse retina. Here, we studied the morphology and connectivity pattern of the small-field A8 amacrine cell. A8 cells in mouse retina are bistratified with lobular processes in the ON sublamina and arboreal dendrites in the OFF sublamina of the inner plexiform layer. The distinct bistratified morphology was first visible at postnatal day 8, reaching the adult shape at P13, around eye opening. The connectivity of A8 cells to bipolar cells and ganglion cells was studied by double and triple immunolabeling experiments using various cell markers combined with synaptic markers. Our data suggest that A8 amacrine cells receive glutamatergic input from both OFF and ON cone bipolar cells. Furthermore, A8 cells are coupled to ON cone bipolar cells by gap junctions, and provide inhibitory input via glycine receptor (GlyR) subunit $\alpha 1$ to OFF cone bipolar cells and to ON A-type ganglion cells. Measurements of spontaneous glycinergic postsynaptic currents and GlyR immunolabeling revealed that A8 cells express GlyRs containing the $\alpha 2$ subunit. Taken together, the bistratified A8 cell makes very similar synaptic contacts with cone bipolar cells as the rod pathway-specific AII amacrine cell. However, unlike AII cells, A8 amacrine cells provide glycinergic input to ON A-type ganglion cells.

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Introduction

There are five neuronal classes in the mammalian retina, photoreceptors, horizontal cells, bipolar cells, amacrine cells, and ganglion cells. Within each of these classes there are numerous types that can be distinguished morphologically and physiologically. Amacrine cells form the most diverse group by having over 30 morphological types in mammalian species (MacNeil and Masland, 1998; Badea and Nathans, 2004), and they can be broadly subdivided into two major groups, glycinergic and γ -aminobutyric acid (GABA)ergic amacrine cells.

GABAergic amacrine cells generally have large dendritic fields and their processes are often confined to a single stratum within the inner plexiform layer (IPL). These amacrine cells use their large receptive fields to mediate integration of signals from distant regions of the retina (de Vries et al., 2011). Glycinergic amacrine cells are generally narrow-field and have processes stratifying across multiple sublaminae within the IPL (Pourcho and Goebel, 1985; Menger et al., 1998). They are thought to be involved in local signal modulation and crossover inhibition between ON and OFF pathways (Hsueh et al., 2008; Manookin et al., 2008; van Wyk et al., 2009; Werblin, 2010).

The specific function for each of the many types of glycinergic amacrine cells in visual processing is still unclear. A recent study in the mouse retina has physiologically characterized a number of narrow-field amacrine cells and found at least 12 different types (Pang et al., 2012) but did not include the A8 amacrine cell. The bistratified A8 amacrine cell was first morphologically described in cat (Kolb and Nelson, 1981; Kolb et al., 1981) and was subsequently found in other mammalian species (ground squirrel: Linberg et al., 1996; rabbit: MacNeil and Masland, 1998; mouse: Badea and Nathans, 2004; Heinze et al., 2007; primate retina: Kolb et al., 1992; Neumann and Haverkamp, 2013). The A8 amacrine cell has processes in both the OFF (S1 stratum) and the ON (S4 stratum) layers of the IPL. This suggests a convergence and possible crossover modulation of OFF and ON pathways.

An EM study in the cat retina showed the A8 amacrine cell receives two types of input: bipolar cell input via ribbon synapses and amacrine cell input via conventional synapses (Kolb and Nelson, 1996). In addition, gap junctions were observed between the A8 and other amacrine cells, and between the A8 and ON cone bipolar cells. In the mouse retina, where all major bipolar cell types have been characterized (Wässle et al., 2009b), the specificity of the cone bipolar cell inputs can be determined. Axons terminals of OFF bipolar cell types 1 and 2 and ON bipolar cell types 6 and 7 can be found in S1 and S4 strata where A8 processes stratify and therefore are potential synaptic partners. The A8 cell may provide glycinergic output to bipolar cells, other amacrine cells and/or ganglion cells.

Postsynaptic glycine receptors (GlyRs) are pentameric ion channels consisting of three beta (β) and two alpha (α) subunits (Betz and Laube, 2006). Whereas only one β subunit is known, 4 different isoforms of the α subunit (α 1-4) of the GlyR are described (Matzenbach et al., 1994). All four α subunits have been localized to specific synapses within the mammalian retina (Sassoè-Pognetto et al., 1994; Haverkamp et al., 2003, 2004; Jusuf et al., 2005; Heinze et al., 2007), and the subunit composition of the GlyR was found to influence the GlyR's kinetic properties, e.g., GlyRs with the α 1 subunit were found to respond with fast kinetics whereas GlyRs containing α 2 exhibit slow kinetics (Ivanova et al., 2006; Wässle et al., 2009a). The differential distribution of GlyR subunits in addition to their diverse kinetics suggests that they provide inhibition in different retinal circuits (Zhang and McCall, 2012).

In the present study, we identified bipolar cell types providing putative input to A8 and GlyRs associated with A8 using transgenic mice that express GFP in A8 amacrine cells. In combination with immunohistochemical techniques, we found that A8 cells receive both ON and OFF cone bipolar input, are gap-junctionally coupled to ON cone bipolar cells, and provide inhibition via GlyR α 1 to OFF cone bipolar cells. In addition, we found putative glycinergic output to ON A-type ganglion cells.

Material and Methods

Animals and tissue preparation

All experiments were conducted in accordance with institutional guidelines of the Max-Planck-Institute for Brain Research (Frankfurt) and guidelines of the Universities of Oldenburg and Tübingen, and following the standards described by the German animal protection law (Tierschutzgesetz).

Three transgenic mouse lines were used. The *thy1*-GFP-O mice are available from Jackson Laboratory (B6.Cg-Tg(Thy1-EGFP)OJrs/GfngJ, stock number 007919) and were developed in the laboratory of Joshua R. Sanes (Feng et al., 2000). The GFP expression in this mouse line is random and sparse in the retina resulting in isolated cells ideal for morphometric analyses and receptor quantification (see Fig. 1A and Majumdar et al., 2007). However, the sparse nature of the GFP expression made it difficult to target A8 cells for tracer injections and patch clamp recordings. For injections and patch clamp recordings we used *Ier5*-EGFP transgenic mice carrying the gene for enhanced-GFP (EGFP) driven by a promoter upstream from the *immediate early response 5 gene* (*Ier5*), kindly provided by Botond Roska (FMI, Basel, Switzerland, Donor: Nathaniel Heintz, GENSAT project; Gong et al., 2003) and are available from the Mutant Mouse Regional Resource Center supported by NIH (MMRRC: 030081-UCD). These mice likely express EGFP in all A8 amacrine cells (Siegert et al., 2009). Therefore it was much easier to use this line for tracer injections and patch clamp recordings. In this mouse, A8 cells had the brightest fluorescent cell bodies (see Fig. 1C, arrows). It was not feasible to use this mouse for morphometric analyses and receptor quantification due to the high number of overlapping processes in the OFF layer. The GUS8.4-GFP mouse line carrying the gene for GFP driven by an 8.4-Kb promoter region upstream from the α -gustducin gene (kindly provided by Robert F. Margolske; Huang et

al., 2003) was crossed with *thy1-GFP-O* mice to establish whether type 7 ON cone bipolar cells provide input to A8 amacrine cells.

For immunocytochemistry, mice (aged P60 to P150) were anesthetized with isoflurane inhalation and euthanized by decapitation. Eyes were then removed and immersed in 4% (w/v) paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB), pH 7.4 at 20°C. While immersed in PFA, the cornea and lens were removed and the posterior eyecup was fixed for a total time of 12 or 30 minutes. After fixation, eyecups were rinsed in 0.1 M phosphate buffered saline (PBS) and retinas were dissected with four radial relieving cuts to flatten the tissue for mounting on a piece of filter paper (MF-Millipore). For Neurobiotin injections, *Jer5-EGFP* mice (2 – 6 months old) were killed with CO₂ and subsequent cervical dislocation. Eyes were enucleated, and cornea, lens, and vitreous body were removed in Ringer's solution (in mM: 110 NaCl, 2.5 KCl, 1 CaCl₂, 1.6 MgCl₂, 10 glucose, 22 NaHCO₃, adjusted to pH 7.4 with 95% O₂ and 5% CO₂) at room temperature. For some cells, the Ringer's solution contained only 12 mM NaHCO₃ and was heated to 30°C to enhance the diffusion of the tracer. Retinas were isolated from the eyecup and cut into pieces that were mounted, ganglion cell side up, on black filter paper (MF-Millipore).

Antibody characterization

The primary antibodies used in this study including their working dilution, source and the immunogen they are directed against, are listed in Table 1. The chicken anti-GFP antibody recognizes GFP at 30 kDa (manufacturer's data sheet). The rabbit antibody against the C-terminal binding protein 2 (CtBP2) recognizes a 110–120 kDa protein band corresponding to the size of the RIBEYE protein and a 50 kDa protein band corresponding to the transcription repressor CtBP2 (tom Dieck et al., 2005). The monoclonal antibody against the GlyR α 1 subunit (mAb2b, kindly provided by Heinrich Betz, Frankfurt, Germany) recognizes a single band of 48 kDa in Western blots of purified spinal cord GlyR preparation (Pfeiffer et al., 1984) and has been shown to be specific for the N-terminal 10 residues of the GlyR α 1 subunit (Schröder et al., 1991). In *Gla1^{ot/ot}* mice, immunostaining for mAb2b was abolished both in the spinal cord and in the retina (Wässle et al., 1998). The goat anti-GlyR α 2 antiserum has been shown to solely stain HEK293T cells that express the rat GlyR α 2 but not the rat or human GlyR α 1, - α 3, or - α 4 subunits, and to decorate glycinergic postsynapses in the IPL of the mouse retina (Haverkamp et al., 2004). The goat anti-GlyR α 3 antibody recognizes in transfected HEK293T cells only the recombinant rat GlyR α 3 protein but not GlyR α 1, - α 2, and - α 4, and all postsynaptic anti-GlyR α 3 staining of retinal sections is lost in GlyR α 3-deficient mice (Haverkamp et al., 2003). The rabbit anti-GlyR α 4 antibody specifically stains HEK293T cells that express mouse GlyR α 4 but not any of the other GlyR α subunits (Heinze et al., 2007). The rabbit anti-PKC α antibody detects an 80 kDa band on Western blots of rat brain that is blocked by preincubation with the antigen but not by preincubation with corresponding peptides of other PKC isoforms; a minor band at 45 kDa is possible (manufacturer's datasheet). The sheep anti-secretagogin (SCGN) antibody recognizes a single band of the predicted size (~32 kDa) on Western blots of mouse retinal lysates (Puthussery et al., 2010). The SMI-32 antibody is a mouse monoclonal IgG1 directed at a non-phosphorylated site on neurofilament H; it recognized a double band of 180 and 200 kDa, which merged into a single neurofilament H line on two-dimensional blots

(Sternberger and Sternberger, 1983). In mouse retina, the antibody stains ON-sustained and OFF-transient, but not OFF-sustained A-type ganglion cells (Bleckert et al., 2014). The mouse anti-synaptotagmin-2 (SYT2) antibody detects a single band of 60 kDa protein on Western blots of zebrafish embryos and mouse cerebellum, but not of mouse liver (Fox and Sanes, 2007). The guinea pig anti-vGluT1 antibody detects a 62 kDa band on Western blots of rat brain lysates (manufacturer's data sheet).

Immunohistochemistry

Immunolabeling was performed on whole retinas flat-mounted on filter paper (MF-Millipore). Retinas were incubated for four days in a cocktail of primary antibodies (see Table 1 for dilutions) in 3% normal donkey serum (NDS) and 0.5% Triton X-100 in PBS. They were then rinsed in PBS and incubated overnight in secondary antibodies diluted in the same incubation solution as above. Secondary antibodies are conjugated either to Alexa Fluor 488, Alexa Fluor 594, Alexa Fluor 647 (Molecular Probes, Eugene, OR, USA), Cy3 or Cy5 (Dianova, Hamburg, Germany). After a final set of rinses with PBS, retinas were mounted with VectaShield HardSet mounting medium (Vector Laboratories, CA, USA). For co-labeling of A-type ganglion cells with SMI32 and GlyR α 1 in *Ier5*-EGFP retinas, it was necessary to use a serial staining protocol, as both primary antibodies are from the same host. We incubated the wholemounts with rabbit anti-GFP and mouse anti-GlyR α 1 first. The staining was then visualized with Alexa Fluor 488 and Cy5 secondary antibodies. Next, the sections were incubated with mouse anti-SMI32 and the antibody was visualized with Cy3.

Tracer injections in A8 amacrine cells

Neurobiotin injections were performed as described previously (Pérez de Sevilla Müller et al., 2010). Fluorescent A8 amacrine cells in the light-adapted *Ier5*-EGFP retina were injected under epifluorescence using sharp microelectrodes (120–180 M Ω resistance) with 5 mM Alexa Fluor 594 and 4% (w/v) Neurobiotin diluted in 0.2 M KCl. The dye was iontophoresed with –0.5 nA square pulses of 500 ms at 1 Hz for 1–2 min to verify correct impalement of the cell. The current was then reversed to inject positively charged Neurobiotin for up to 10 min. Retinas were fixed in 4% PFA in 0.1 M PB, washed, and incubated overnight at 4°C with DyLight549-conjugated streptavidin to visualize Neurobiotin.

Some retinas were blocked using 10% NDS in PB overnight, and incubated with sheep anti-SCGN antibodies (1:2,000 in PB containing 1% NDS, 0.3% Triton X-100, 0.05% NaN₃) for 4–5 days at 4°C. After several washes, the retinas were incubated with Alexa Fluor 647-conjugated donkey anti-sheep secondary antibodies overnight at 4°C.

Whole-cell recordings and data analysis

For whole-cell recordings, retinal slices from *Ier5*-EGFP animals were prepared following Eggers and Lukasiewicz, 2006. Retinae were dissected from the eyecup, cut into half and put onto a filter paper (0.22 μ m pore size, Millipore) with the photoreceptor layer facing up. Vertical retinal slices of 200 μ m thickness were cut using a manual retina slicer (Werblin, 1978). Slices attached to the filter paper were subsequently stored in carbogenated extracellular solution at room temperature (RT, 20–22°C) and then transferred to the

recording chamber. The extracellular solution contained (in mM): 125 NaCl, 2.5 KCl, 1 MgCl₂, 1.25 NaH₂PO₄, 26 NaHCO₃, 2 CaCl₂ and 20 glucose, adjusted to pH 7.4 with 95% O₂ and 5% CO₂.

Whole-cell recordings were made from fluorescently labeled cells in light-adapted mouse retinal slices as previously described (Schubert et al., 2008). Slices were harvested from the central retina along the nasal-temporal axis. To account for possible inter-specimen variability, we recorded 14 A8 cells in 8 mice. For each recording a new slice was used. The resistance of the electrodes with standard solutions usually ranged between 6 and 8 MΩ. To block GABA_{A/C} receptors, SR-95531 (Gabazine, 5 μM) and (1,2,5,6-tetrahydropyridine-4yl) methylphosphinic acid (TPMPA, 75 μM) were added. The intracellular solution contained (in mM): 120 cesium gluconate, 1 CaCl₂, 1 MgCl₂, 10 Na-HEPES, 11 EGTA, 10 TEA-Cl, sulforhodamine B (0.005%) and was adjusted to pH 7.2 with CsOH. Under our experimental conditions, the calculated chloride equilibrium potential was -59.3 mV. Liquid junction potentials of 15 mV were corrected before the measurement with the pipette offset function of the amplifier. Series resistance (25.2 ± 1.8 MΩ, n = 14 cells) and cell capacitance were not compensated. Seal resistances of >1.5 GΩ were routinely obtained. Amacrine cells were voltage-clamped at 0 mV, the reversal potential for currents through cation channels, and glycinergic spontaneous inhibitory postsynaptic currents (sIPSCs) were recorded at RT. Amacrine cells were filled during the recording with sulforhodamine B for subsequent identification of A8 cells.

Data acquisition was performed with an Axopatch 200B amplifier at a sampling frequency of 10 kHz using the Clampex software (Axon Instruments, Foster City, CA). Signals were Bessel filtered at 2 kHz. We analyzed sIPSCs with amplitudes >4 pA that could be distinguished from background noise with the MiniAnalysis program (Synaptosoft, Leonia, NJ). To quantify parameters of glycinergic synaptic events, the mean frequency ± SE and the mean amplitude ± SE were determined. The decay time constants (determined by using a single exponential fit) for those synaptic events were quantitatively analyzed in detail for 13 A8 cells (fast sIPSCs in all 13 cells and slow sIPSCs in 9 cells; 4 cells showed fast but no slow sIPSCs).

Image analysis

Images were taken using an Olympus FV1000 confocal microscope equipped with argon and HeNe lasers. Z-stack of images were performed with Plan-Apochromat UPlanSApo 60x/1.35 Olympus oil immersion objective and scanned at a minimum resolution of 1024×1024. The image stacks were taken with a z-distance of 0.5 μm between optical sections. Images of injected cells were taken with a Leica TCS SL confocal microscope with a 40x/1.25 HCX PL APO oil immersion objective.

Images stacks were then analyzed and viewed with the ImageJ distribution, Fiji (<http://fiji.sc/wiki/index.php/Fiji>, RRID: SciRes_000137) and Imaris (Bitplane, RRID: nif-0000-00314) software. For quantitative analysis of synaptic puncta (CtBP2 and GlyRα1-4) associated with A8 dendrites individual optical slices from Z-stacks were analyzed by using the ImageJ Cell Counter plugin (RRID: nif-0000-30467). The total number of puncta was manually assessed by marking and counting all puncta in contact with A8

dendrites. Specific bipolar cell input was determined by quantifying the proportion from the total puncta that also colocalize within a bipolar cell marker. Imaris software was used to repeat the quantification in a semi-automated manner on the same image stacks. Volume masks around A8 cells (using an automated threshold determined by the software) were created and spots were created for all puncta within the regions of interest (using software-determined automatic threshold), and then finally, all spots that are located less than 0.25 μm from the surface of the A8 mask were quantified. As control, the image channel with the receptor stain was flipped on the x-axis and subsequently re-analyzed as described above. A paired t-test was used to determine significance ($p < 0.05$) between quantified samples and flip controls (Table 2). For analyzing the amount of A8 synapses on A-type ganglion cells in *Ier5*-EGFP retina, only manual counts were possible, as SMI-32 and GlyR α 1 are both monoclonal mouse antibodies and the GlyR α 1 puncta appeared – due to the serial staining protocol – in two channels. Images for figures were adjusted for brightness and contrast using Adobe Photoshop (San Jose, CA, USA, RRID: SciRes 000161).

Results

Morphology of the A8 cell in the mouse retina

In each *thy1*-GFP-O mouse retina, a total of approximately 50 single ganglion cells and 15 single narrow-field amacrine cells of different types express GFP (Fig. 1A; see also Fig. 1 in Majumdar et al., 2007). A previous study using these mice observed a narrow-field bistratified amacrine cell (Figs. 5 and 6 in Heinze et al., 2007) morphologically comparable to the A8 amacrine cell described by Kolb et al. (1981) in the cat retina. These bistratified amacrine cells have processes that narrowly stratify in two regions of the IPL, one in the OFF layer at S1 and one in the ON layer at S4 (Fig. 1B). In addition, we found similar looking EGFP-labeled amacrine cells in the *Ier5* line (Siegert et al., 2009) (Fig. 1C). These mice express EGFP in likely all A8 amacrine cells, recognizable by the brightest fluorescent cell bodies (Fig. 1C, arrows). EGFP is also weakly expressed in other amacrine cell types in the INL and in displaced amacrine cells and ganglion cells in the GCL (Arndt Meyer, Timm Schubert, unpublished observations).

Using the basic criterion of having distinct bistratified processes in S1 and S4 of the IPL, isolated A8 amacrine cells were imaged in flat-mounted *thy1*-GFP-O retinas (2–3 cells per retina). The cells typically have thin dendrites in the OFF plexus that extend directly from the soma (Fig. 1D). The ON (inner) plexus is formed from a single process extending to S4 which then branches into thicker dendrites with more varicosities compared to the OFF (outer) plexus (Fig. 1E). A total of 51 of these A8 cells ($n = 19$ animals) were imaged and convex polygons were drawn around the most distal tips of the dendrites for each plexus to measure their areal coverage. The dendrites from the outer OFF plexus covered an average area of $2,031 \pm 561 \mu\text{m}^2$ per cell and were on average larger than the ON plexus, which covered an average area of $1,487 \pm 474 \mu\text{m}^2$ per cell (Fig. 1F).

Developmental morphology of the A8 cell

To observe the development of the distinct bistratified morphology of the A8 cell, retinas were harvested from *Ier5*-EGFP (for population stratification patterns in vertical view; Fig.

2A–D) and *thy1*-GFP-O (for single cell morphology in wholemount view; Fig. 2E–H) mice at ages between postnatal day 7 and postnatal day 15. At postnatal day 7, no bistratified amacrine cells were found in the retinas of three *thy1*-GFP-O animals. Narrow-field amacrine cells were present at this age (not shown) but none of these cells exhibited the distinct bistratified morphology of A8 cells. The same holds true for the *Ier5*-EGFP retina. GFP-expression is already visible in several amacrine cells at postnatal day 7; however, the cells do not have recognizable processes and therefore cannot be identified as A8 cells. At postnatal day 8, weak bands are seen in the IPL of *Ier5*-EGFP mice (Fig. 2A) and we found examples of individual amacrine cells with processes stratifying close to the cell body and another smaller band stratifying closer to the ganglion cell layer in the *thy1*-GFP-O mouse (Fig. 2E). At postnatal day 10, stratification in ON and OFF layers was more evident (Fig. 2B) and we found bistratified amacrine cells with thin processes in the outer layer and thick processes in the inner layer (Fig. 2F). By the time of eye opening at P13, the morphology of A8 cells was comparable to an adult state. We did not observe any differences in the developmental pattern of A8 cells across the *Ier5*-EGFP retina, therefore indicating that all the A8 cells mature within the same timeframe.

A8 cells receive inputs from multiple bipolar cell types

Since both A8 and AII amacrine cells are small-field and bistratified in morphology (Kolb et al., 1981), we wondered if the connectivity patterns of their inputs showed similarities. AII cells have essentially the opposite morphology of the A8 cells and receive their main synaptic input at the arboreal dendrites in the ON layer and have their synaptic outputs at the lobular processes in the OFF layer (Kolb and Famiglietti, 1974; Tsukamoto and Omi, 2013; Marc et al., 2014). To reveal whether A8 cells receive synaptic input from mainly ON or OFF bipolar cells, antibodies against CtBP2, a ribbon synapse marker (tom Dieck et al., 2005; Jusuf et al., 2006), were used in combination with different bipolar cell markers. The *thy1*-GFP-O mouse was used to quantify the total number of CtBP2 puncta associated with the outer (Fig. 3A) and inner dendrites (Fig. 3B) of single isolated GFP-positive A8 cells. An automated approach using Imaris software to quantify CtBP2 puncta in 10 cells found on average 322 ± 37 puncta associated with S1 dendrites and 234 ± 36 puncta associated with S4 dendrites (see Table 2 for Imaris counts, SEM and p-values), showing clearly that A8 cells receive strong input from both OFF and ON bipolar cells.

To determine the proportion of the total input from a single type of OFF bipolar cell, we labeled type 2 bipolar cells with antibodies against SYT2 (Fox and Sanes, 2007). CtBP2 puncta that were found within $0.25 \mu\text{m}$ of both SYT2-positive bipolar axons and A8 processes (arrows in Fig. 3C, E) were considered as input from type 2 bipolar cells to the A8 cell. Remaining puncta associated with A8 cells but not within the $0.25 \mu\text{m}$ mask of SYT2-positive axons were classified as input from unidentified bipolar cells. For seven A8 cells we found that about 37% of the A8-associated CtBP2 puncta belonged to the axons of SYT2-positive type 2 bipolar cells (see Table 2). This suggests that A8 amacrine cells receive additional input from other OFF bipolar cells in the S1 layer, presumably type 1 and type 4 OFF bipolar cells.

ON bipolar cell input to the S4 plexus of the A8 cell was determined using antibodies against the alpha subunit of protein kinase C (PKC α ; for rod bipolar cells, Greferath et al., 1990) and antibodies against secretagogin (SCGN; for type 6 cone bipolar cells, Puthussery et al., 2010). We found insignificant rod bipolar cell input from the four A8 cells analyzed (p-value > 0.05 compared to flipped controls). Less than 3% of the CtBP2-positive ribbons associated with the ON plexus were also 0.25 μ m from rod bipolar cell axons (Table 2). For four additional A8 cells we found that at least 31% of the CtBP2-positive ribbons associated with the ON plexus are derived from SCGN-positive type 6 axons (Fig. 3D, F and Table 2).

These results indicate that at least one other type of ON cone bipolar cell provides input to the A8 amacrine cell. The type 7 bipolar cell, which stratifies in layer S4 of the IPL, is one possible candidate. There are no known markers for type 7 bipolar cells, therefore we crossed *thy1*-GFP-O mice with GUS8.4GFP mice, which express GFP in type 7 cone bipolar cells and weakly in rod bipolar cells (Huang et al., 2003). Due to the patchy expression of GFP in both cell types of interest, we were only able to observe qualitatively that type 7 bipolar cells provide input to A8 amacrine cells (not shown).

A8 cells are coupled to ON bipolar cells

To test whether A8 cells are coupled to other cells via gap junctions, we injected Neurobiotin, which has been shown to pass through gap junctions in the mammalian retina (Vaney, 1991), into GFP-positive cells in the *Ier5*-GFP mouse. This mouse line was used because it has a high number of GFP-positive A8 cells, this facilitates dye injections and allows for the detection of putative homologous A8 coupling. In the INL, all brightly GFP-labeled injected cells were confirmed as A8 cells, based on their distinct bistratified morphology (Fig. 4A–C). Out of 20 Neurobiotin-injected A8 cells, tracer coupling to ON cone bipolar cells could be detected for 8 cells (Fig. 4B, F). The number of coupled bipolar cells ranged from 1–8 cells (mean: 4.25); coupling never exceeded the dendritic field of the injected A8. Additional immunolabeling for SCGN showed that coupled cells comprised SCGN-positive type 6 ON cone bipolar cells (Fig. 4G–I, arrows; 3/8 and 4/7 in two retinas) and some SCGN-negative ON cone bipolar cells (Fig. 4G–I, arrowheads). In contrast to AII amacrine cells, which form extensive electrically coupled networks (Vaney, 1991), injected A8 cells did not display homocellular coupling to other A8 cells.

Glycinergic receptors associated with A8 dendrites

Glycinergic amacrine cells are known to make synapses with bipolar, other amacrine and ganglion cells and to receive input from other amacrine cells (Hendrickson et al., 1988). We investigated if the glycinergic output of A8 cells or, vice versa, if the glycinergic input onto A8 cells is selectively transmitted by a certain GlyR subtype. For this, sections of the *Ier5*-EGFP retina were double labeled against GFP and subunit-specific antibodies against GlyR α 1, GlyR α 2, GlyR α 3 and GlyR α 4 (Fig. 5A–H). The α 1 subunit is preferentially expressed in large puncta in the OFF layer and shows reduced density in the ON layer, whereas the α 2 subunit shows a more uniform distribution across the IPL. The GlyR α 3 subunit shows four bands of higher density of puncta, and the GlyR α 4 subunit shows a band of higher density at the border between stratum 3 and 4 (Fig. 5A–D; see also: Heinze et al., 2007). The double labeling with GFP in the *Ier5* mouse did not reveal any subunit to be

preferentially associated with the A8 cell (Fig. 5E–H); but as expected the GlyR α 4 puncta in stratum 3/4, which are preferentially expressed by ON cholinergic amacrine cells (Heinze et al., 2007), were not associated with the A8 dendrites in the ON plexus (Fig. 5H).

To determine the GlyR subunits associated with A8 cells more quantitatively, the outer (Fig. 5I–L) and inner plexus (Fig. 5M–P) of single cells were analyzed in *thy1*-GFP-O wholemounts. The association with GlyR α 4 puncta was very low (Fig. 5L, P), whereas for the other three subunits we found several puncta in each optical slice associated with A8 dendrites (Fig. 5I–K, 5M–O). We quantified the amount of associated GlyR subunits using Imaris software and we also quantified flip controls using the same method (see Table 2 for Imaris counts and p-values). We found significant difference ($p < 0.05$) between automated counts and flip controls for GlyR α 1 and GlyR α 2 in both inner and outer layers. Only the inner layer showed significance for GlyR α 3. GlyR α 4 does not seem to be closely associated with A8 cells as quantified puncta were not significantly different to flip controls. In theory, puncta with complete overlap with the A8 amacrine cell represent putative input synapses while puncta with partial overlap represent putative output synapses, however, these distinctions were not quantified due to imaging limitations. Therefore our anatomical results suggest A8 amacrine cells are involved in complex glycinergic pathways, receiving input and sending output via different glycine receptors.

A8 cells receive glycinergic input

Recent studies showed that morphologically distinct amacrine cells express different sets of GlyRs: AII amacrine cells express α 3 β GlyRs with fast kinetics (decay time constant $\tau \sim 11$ ms), other narrow-field and wide-field amacrine cells contain predominantly α 2 β GlyRs with slower kinetics ($\tau \sim 27$ ms) but also express α 4 β GlyRs with very slow kinetics ($\tau \sim 70$ ms) (Weiss et al., 2008; Majumdar et al., 2009). Whole cell recordings performed from A8 cells in slices of the *Ier5* retina enabled us to study the GlyR composition of the input synapses. By analyzing glycinergic sIPSCs in the presence of GABA receptor antagonists we found that A8 cells reveal synaptic glycinergic events (Fig. 6A) with a mean frequency of 0.26 ± 0.07 Hz ($n = 14$ cells) (Fig. 6B) and a mean amplitude of 17.1 ± 3.3 pA ($n = 14$ cells) (Fig. 6C). In 13 out of 14 A8 cells, glycinergic sIPSCs could be segregated into two distinct groups of currents (example cell shown in Fig. 6D,E) with a fast mean decay constant (25.2 ± 1.8 ms, 185 sIPSC events in 13 cells) or currents with a slow mean decay constant (114.1 ± 12.5 ms, 92 sIPSC events in 9 cells) (Fig. 6F). Thus, we suggest that A8 cells receive glycinergic input via two distinct GlyRs (see discussion).

A8 amacrine cells synapse onto OFF bipolar cells

The majority of the large GlyR α 1 puncta in the OFF layer (Fig. 5A) represents synapses between AII amacrine cells and OFF bipolar cells (Sassoe-Pognetto et al., 1994). Here we asked if OFF bipolar cells which stratify in S1 get additional glycinergic input from A8 amacrine cells. We used antibodies to label GlyR α 1 receptors (anti-GlyR α 1) and OFF bipolar axon terminals (anti-vGluT1) on *thy1*-GFP-O retinas to test for putative A8 synapses onto OFF bipolar cells (Fig. 7A, B). We observed GlyR α 1 puncta (Fig. 7B, magenta) associated with the OFF processes of A8 cells (green). Most of the GlyR α 1 puncta appeared to be colocalized with vGluT1-positive OFF bipolar axons (cyan), and a subpopulation of

these overlapped with an A8 cell process (arrowheads). We also stained vertical sections of the *Ier5*-EGFP retina with antibodies against GFP, GlyR α 1 and vGluT1 (Fig. 7C, D) and found 88 examples of putative A8 synapses onto OFF bipolar axons in image stacks of 100 – 140 μ m IPL length and 3 – 6 μ m thickness ($n = 11$).

A8 amacrine cells provide glycinergic input to A-type ganglion cells

Next we investigated ganglion cells as potential postsynaptic partners to the A8 cell using both *thy1*-GFP-O and *Ier5*-EGFP mice. The retinas were stained with antibodies against GlyR α 1, because A-type ganglion cells have been shown to express this glycine receptor subunit (Majumdar et al., 2007). As already mentioned, numerous types of ganglion cells express GFP in *thy1*-GFP-O mice (in addition to narrow-field amacrine cells) including A-type ganglion cells (Majumdar et al., 2007). We focused on sustained A-type ganglion cells as their dendrites stratify within the vicinity of A8 OFF and ON stratification bands (van Wyk et al., 2009) and searched for A8 cells that were located within the dendritic fields of either OFF-sustained or ON-sustained A-type ganglion cells. For OFF A-type ganglion cells we found only one example (see discussion). For ON A-type ganglion cells we analyzed four cells with GFP-positive A8 amacrine cells within their dendritic field (Fig. 8). Although GlyR α 1 immunoreactive puncta are sparser in the ON layer compared to the OFF layer, labeled puncta were found to be associated with ON A-type ganglion cell dendrites and some of these were found at junctions where A8 processes overlapped with the ganglion cell dendrites (see arrows in Fig. 8, C1 – C4). For one A8 cell in the dendritic field of an ON A-type ganglion cell we found that 70% (14/20) of the puncta associated with the A-type ganglion cell were also adjacent to the ON stratifying A8 dendrites (Fig. 8D).

An additional approach was necessary to quantify the proportion of glycinergic A8 input onto A-type ganglion cells. We used SMI32 antibodies to label all ON A-type ganglion cells (Bleckert et al., 2014) and quantified the amount of GlyR α 1 puncta in *Ier5*-EGFP retinas (see Fig. 9 for triple labeling of SMI32, GlyR α 1 and GFP). We first quantified the total densities of GlyR α 1 puncta on the dendritic ganglion cell arbors by manual counts (0.42 puncta/ μ m) and found that ON A-type ganglion cells received ~46% of their GlyR α 1 input from A8 cells (Table 3). We then asked how many of the A8 output synapses were in contact with ON A-type dendrites and analyzed the GlyR α 1 puncta associated with A8 dendrites and with A8/SMI32. We found that ~40% of GlyR α 1 puncta postsynaptic to A8 dendrites were colocalized with ON A-type dendrites whereas 60% were not colocalized (Table 3). This suggests that ON A-type ganglion cells receive glycinergic input from at least one additional glycinergic amacrine cell, and that A8 amacrine cells synapse onto at least two cell types in the ON layer.

Discussion

A8 amacrine cells were first illustrated in Golgi studies by Kolb et al. in the cat (1981) and human retina (1992). A similar cell was revealed in rabbit retina by photofilling and was called flat bistratified amacrine cell (MacNeil and Masland, 1998). Pourcho and Goebel (1985) were first showing that A8 amacrine cells are glycinergic. We recently used an immunocytochemical marker (SYT2) to analyze the distribution and synaptic connectivity

of A8 amacrine cells in the macaque retina (Neumann and Haverkamp, 2013). Here we took advantage of two transgenic mouse lines to study A8 amacrine cells at the single cell level in the mouse retina.

A8 amacrine cells appear bistratified as they mature

We analyzed the appearance of the distinct bistratified morphology over the course of an A8 amacrine cell's development. The first bistratified A8 amacrine cells become apparent at P8, and their processes mature over the next 5 days. Studies of other known small-field amacrine cells show a similar developmental pattern. For example, AII amacrine cells are not bistratified at P7 but their appearance matures with bistratification by the end of postnatal week 2 (Rice & Curran, 2000). In addition, a type of narrow-field amacrine cell recently described appears bistratified at P7 but refines the arbors until P14 (Kay et al., 2011). In contrast, some wide-field amacrine cells, like the starburst amacrine cell, target their appropriate ON and OFF sublaminae earlier (within the first postnatal week) and provide laminar cues for direction-selective ganglion cells (Stacy & Wong, 2003).

A8 amacrine cells receive nonselective bipolar cell input and couple to ON bipolar cells

We then analyzed the synaptic connections of the A8 cells and compared them with AII amacrine cells. It is well established that AII amacrine cells receive a majority of their input from rod bipolar cells at their arboreal dendrites in the ON layer and make glycinergic output synapses at their lobular appendages in the OFF layer (Famiglietti and Kolb, 1975; Strettoi et al., 1992; Chun et al., 1993; Wässle et al., 1995). This connectivity was confirmed by a recent EM reconstruction of AII amacrine cells in both mouse and rabbit (Tsukamoto and Omi, 2013; Marc et al., 2014). Since A8 cells are bistratified amacrine cells with morphological characteristics similar to AII amacrine cells, we wondered if the arboreal dendrites in the OFF layer received excitatory input while the lobular processes in the ON layer formed the main inhibitory output synapses. That was clearly not the case: A8 amacrine cells receive both ON and OFF cone bipolar cell input in both layers, and our data suggest that A8 cells may receive input from any OFF cone bipolar cell, which stratifies in S1 and from any ON cone bipolar cell, which stratifies in S4. A8 cells also form gap junctions with the ON cone bipolar cells they receive glutamatergic input from. Our results are consistent with findings in cat retina where ribbon synapses and gap junction contacts between A8 dendrites and ON cone bipolar axon terminals were observed by EM reconstruction and were discussed as signal amplification (Kolb and Nelson, 1996). Signal amplification may occur when the depolarization of the A8 cell – caused by glutamatergic ON bipolar cell input – spreads back to the bipolar cell through the gap junction and thus enhances glutamate release. Gap junctional contacts may also serve local signal facilitation as the signals mediated by gap junctions are thought to be faster than those mediated by chemical synapses (Connors and Long, 2004). Consequently, the tonic current spread through gap junctions may slightly depolarize the A8 dendrite prior to the arrival of the glutamate signal and thus enhance the response. Conversely, a depolarization of the A8 cell due to excitatory OFF bipolar cell input would attenuate an incoming hyperpolarization from ON bipolar cells. In any case, the functional consequences resulting from the A8-ON bipolar cell coupling and the mixed excitatory ON- and OFF-input remain to be determined.

Tracer coupling to ON bipolar cells was not observed for all injected A8 cells. This is likely a result of the technical difficulties to inject A8 cells in the wholemount retina because the somata are small and were easily damaged during the injection, which may have led to a closure of gap junctions.

Earlier EM reconstruction (Kolb and Nelson, 1996) also revealed amacrine to amacrine cell coupling that could not be confirmed by our Neurobiotin injections.

A8 amacrine cells receive glycinergic input via GlyR α 2

It has been shown that GlyRs are comprised of a combination of two α and three β subunits (Grudzinska et al., 2005). Most GlyRs in the retina contain only one α subunit and a minority contains two different types (Haverkamp et al., 2003; Heinze et al., 2007). The type of α -subunit in a GlyR determines the kinetics of the receptor and therefore predicts the speed of spontaneous inhibitory postsynaptic currents. From the four α -subunits found in the retina, α 1 has the fastest kinetics followed by α 3, then α 2 and α 4 the slowest (reviewed in Wässle et al., 2009a). Immunohistochemical and electrophysiological studies have demonstrated that different retinal neurons generally have specific GlyR α -subunits for glycinergic input. OFF bipolar cells receive glycinergic input solely via GlyR α 1 (Ivanova et al., 2006); A-type ganglion cells receive their glycinergic inputs primarily via GlyR α 1 (Majumdar et al., 2007), although they may receive input via GlyR α 2 as well (Nobles et al., 2012). Some narrow-field glycinergic amacrine cells (Weiss et al., 2008), GABAergic wide-field, and displaced amacrine cells receive GlyR α 2-mediated input (Veruki et al., 2007; Majumdar et al., 2009). GlyR α 3 appears to be responsible for synaptic glycinergic currents in AII amacrine cells (Weiss et al., 2008), and ON-starburst amacrine cells express GlyR α 4 (Heinze et al., 2007; Majumdar et al., 2009). Our recordings of glycinergic sIPSCs from A8 cells indicate that the cells express predominantly α 2 β GlyRs with a decay time constant of ~25 ms. The additional sIPSCs with longer decay time constants of ~114 ms (Fig. 6F) may argue for a second GlyR containing the α 4 subunit. So far, the kinetics of sIPSCs of synapses containing GlyR α 4 can only be estimated indirectly (see Wässle et al., 2009a). In *Gla2*^{-/-} mice, narrow-field amacrine cells still received a small number of slow glycinergic sIPSCs (Weiss et al., 2008). The decay time constants of these slow sIPSCs were slower (τ ~70 ms) than those recorded from synapses containing GlyR α 2 (τ ~27 ms) and therefore most likely represent synapses containing GlyR α 4. Furthermore, in *Gla2*^{-/-} mice, glycine-evoked currents in ON-starburst amacrine cells were reduced by approximately 50% which suggest that both, α 2 and α 4 subunits contribute to the glycine-induced currents of ON-starburst cells (Majumdar et al., 2009). In wild-type mice, they observed a mean decay time constant of 66 ms, which is close to what we observed for A8 cells (55 ms; calculated by a mean of all sIPSC events rather than segregating into two groups).

A8 amacrine cells provide glycinergic inhibition to OFF-bipolar cells and to A-type ganglion cells

The synaptic connections of AII and A8 cells show interesting parallels. It has been suggested that small-field amacrine cells convey crossover inhibition between the different functional layers of the IPL, e.g., receiving excitatory input from bipolar cells within the ON layer, and in turn releasing glycine within the OFF layer or vice versa (Werblin, 2010). Both,

AII and A8 cells provide glycinergic input to OFF bipolar cells and possibly also to OFF-sustained (S) A-type ganglion cells via GlyR α 1. van Wyk et al. (2009) demonstrated that OFF-S cells receive a significant portion of their glycinergic input from AII amacrine cells. However, their results also suggested that the additional inhibition recorded at high background light level might come from another glycinergic amacrine cell. We believe that the A8 cell is a good candidate that provides this additional glycinergic input. We observed a few GlyR α 1 immunoreactive puncta at regions where A8 processes overlapped with dendrites of an OFF-S cell (not shown). Additionally, we saw GlyR α 1 puncta not associated with vGluT1-immunostained OFF bipolar cell axons but postsynaptically expressed at A8 dendrites (Fig. 7D, arrows). These are likely candidates to be expressed on OFF-S cells.

In contrast to AII amacrine cells, A8 amacrine cells make glycinergic output synapses at their lobular appendages in the ON layer. In fact, they seem to be responsible for half of the glycinergic input onto ON A-type ganglion cells. EM reconstructions revealed local feed-forward inhibition for ~42% of the amacrine cell contacts onto ON A-type ganglion cells (Bleckert et al., 2013). Therefore, our results here suggest the A8 amacrine cell as a possible candidate to provide both, local feed-forward inhibition and crossover inhibition onto ON A-type ganglion cells.

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Figure 1. Morphology of A8 amacrine cells in the mouse retina

A low magnification flatmount view showing single cells expressing GFP in *thy1-GFP-O* mouse retinas, including narrow-field amacrine cells that have visibly smaller cell bodies (arrows in A). Vertical views of A8 cells are shown in B and C from transgenic *thy1-GFP-O* and *Ier5-EGFP* mice, respectively. Wholemount views of the outer and inner plexus of an A8 cell in *thy1-GFP-O* retina are shown in D and E, a summary of the dendritic field size of single A8 cells is shown in F. Scale bars = 100 μm (A), 10 μm (B–E)

Figure 2. A8 cells in the mouse retina have bistratified morphology early in development
 A population of A8 cells in vertical view from *Ier5*-EGFP retinas (A–D) and isolated single A8 cell examples from *thy1*-GFP-O mouse retinas (E–H) aged between postnatal day 8 (P8) to postnatal day 15 (P15). Two weak strata of processes are observed at P8 (A) and a bistratified morphology can be found in amacrine cells of the *thy1*-GFP-O mouse (E). The stratification pattern of A8 cells in the IPL becomes more distinct with age and coverage of each plexus grows before reaching an adult-like morphology by P13. Scale bars = 10 μm

Figure 3. Excitatory input to the A8 cell in the *thy1*-GFP-O retina

Bipolar inputs to the outer (A) and inner (B) processes of the A8 cell (green) are visualized via CtBP2-labeled synapses (magenta). Higher magnifications of selected regions are shown in C and D. Cone bipolar axons (cyan) of type 2 OFF bipolar axons were labeled with SYT2 (C) and type 6 ON bipolar axons were labeled with SCGN (D). Imaris 3D rendering of the same reconstructed regions as in C and D are shown in E and F, respectively. Putative OFF bipolar input from type 2 bipolar cells and ON bipolar input from type 6 ON bipolar cells are indicated by the arrows. Scale bars = 10 μm (A, B); 5 μm (C–F)

Figure 4. A8 cells are coupled to ON bipolar cells

A8 cells were injected with Alexa594 and Neurobiotin in the *Ier5-EGFP* retina. Vertical (A–C) and wholemount (D–F) views show the typical A8 morphology (A) and the colocalization of the Alexa dye with EGFP (D, E). The gap junction-permeable tracer Neurobiotin spread to putative ON cone bipolar cells (B, C) lying within the dendritic area of the A8 (F, indicated with dashed lines; the asterisk marks the position of the injected A8 soma). Neurobiotin injections followed by immunostaining for SCGN (G–I) revealed that the coupled cells are from at least two cell types, as shown by SCGN-positive (arrows) and SCGN-negative (arrowheads) coupled bipolar cells in the wholemount view of the outer INL. Scale bars = 10 μ m.

Figure 5. Glycine receptor alpha subunits associated with A8 cells

The distributions of glycine receptor alpha subunits (GlyR α 1-4) in vertical retina sections from *Ier5*-EGFP mice are shown with respect to A8 cells (A – H panels). High magnification, wholemount views from *thy1*-GFP-O mice showing A8 cells and colocalization with glycine receptor alpha subunits for both outer (I – L) and inner (M – P) processes. Scale bars = 10 μ m (A – H); 5 μ m (I – P).

Figure 6. Glycinergic sIPSCs in A8 amacrine cells

A, Representative whole-cell recordings showing glycinergic sIPSCs of an A8 amacrine cell in presence of Gabazine (GZ, 5 μ M) and TPMPA (75 μ M). Inset shows single sIPSC at higher temporal resolution. B, C, Plots showing mean frequency (B) and mean amplitude (C) of recorded A8 amacrine cells (n=14). D, Example recording of another A8 cell with sIPSCs with slow (dark grey circle) and fast (bright grey circle) decay time constants. E, Plot showing distribution of decay time constants of the example A8 cell shown in D (n=67 sIPSCs). Dark grey circles indicate sIPSCs with small amplitude and slow decay time constants; bright grey circles indicate sIPSCs with large amplitude and fast decay time constants. F, Plot showing mean decay times for fast sIPSCs (185 sIPSCs recorded in 13

cells) and slow sIPSCs (92 sIPSCs recorded in 9 cells), grey circles indicate means for each cell).

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Figure 7. Glycine receptors $\alpha 1$ found on OFF bipolar cells are putative postsynaptic targets for the A8 cell

The OFF plexus of an A8 cell in the *thy1-GFP-O* mouse is shown in A, a high magnification of an example area with putative output synapses is shown in panel B. Output synapses are visualized by antibodies against GlyR $\alpha 1$ (magenta) and examples of puncta found at A8 cells are indicated by arrowheads (B1). In B2, GlyR $\alpha 1$ puncta colocalize at OFF bipolar axons labeled with vGluT1 (cyan). B3 shows all three channels merged and the arrowheads indicate A8 dendrites forming a putative output synapse via GlyR $\alpha 1$ that is on OFF bipolar cell axons. A vertical section of the *Ier5-EGFP* retina stained with antibodies against GFP (green), GlyR $\alpha 1$ (magenta) and vGluT1 (cyan) is shown in C, a high magnification of another section in panel D. Arrowheads indicate putative output synapses of the A8 onto OFF bipolar cell axons, arrows indicate GlyR $\alpha 1$ puncta not associated with vGluT1-immunostained OFF bipolar cell axons but postsynaptically expressed at A8 dendrites. Scale bars = 20 μ m (A); 10 μ m (C); 5 μ m (B, D)

Figure 8. ON A-type ganglion cells are putative targets for A8 cells

An example of an A8 cell with putative output onto an ON A-type ganglion cell is shown in this projected image. Both the ganglion cell and the A8 cell express GFP in the *thy1*-GFP-O mouse retina. The boxed region in A is shown in higher magnification in B and arrows indicate areas where the processes of the amacrine cell overlap with the ganglion cell. Single optical planes are shown in the C subpanels highlighting the overlapping areas. GlyR α 1 puncta (magenta) are found at these overlapping areas. D is a summary drawing of the ganglion cell dendrites and A8 cell shown in B forming putative contacts via GlyR α 1 (magenta circles). A convex polygon (red) outlines the area covered by the A8 cell processes, and the GlyR α 1 labeled puncta not adjacent to an A8 cell process found within the polygon (blue circles) and outside the polygon (white circles) are indicated. Scale bars = 50 μ m (A); 10 μ m (B, D); 5 μ m (C)

Figure 9. ON A-type ganglion cells receive putative input from A8 cells

An example of an SMI32-labeled A-type ganglion cell (cyan) with glycinergic input (magenta) is shown in the projected image in A. The ON plexus of EGFP-expressing A8 cells (green) is clearly visible in the *Ier5*-EGFP mouse retina. The boxed regions in A are shown in higher magnification as single optical sections with putative synapses in panels B and C. Glycinergic synapses are visualized by antibodies against GlyR α 1 (magenta). In B1 and C1, GlyR α 1 colocalize with ganglion cell dendrites (cyan), in B2 and C2, GlyR α 1 puncta colocalize with A8 dendrites (green). B3 and C3 show all three channels merged, arrows indicate A8 dendrites forming putative output synapses via GlyR α 1 onto A-type ganglion cell dendrites, arrowheads indicate additional glycinergic synapses on A-type dendrites and open arrowheads indicate A8 output synapses onto unknown profiles. Scale bars = 20 μ m (A); 10 μ m (B, C)

Table 1

Primary antibodies used in this study

Antibody	Antigen/immunogen	Species/dilution	Source, Cat#, RRID
GFP:Alexa 488	Green fluorescent protein isolated from Aequoria Victoria. IgG fraction was purified by ion-exchange chromatography and conjugated to the Alexa 488 dye.	Rabbit polyclonal 1:1,000	Invitrogen (Carlsbad), Cat# A-21311 RRID: AB_10058149
GFP	His-tagged green fluorescent protein of Aequorea victoria.	Chicken polyclonal 1:1,000	Millipore (Billerica), Cat# 06-896 RRID: AB_11214044
CtBP2	C-terminal binding protein 2, aa 361-445	Mouse monoclonal 1:10,000	BD Biosciences (San Jose), Cat# 612044 RRID: AB_399431
CtBP2	Synthetic peptide corresponding to aa 431-445 of rat CtBP 2 coupled to key-hole limpet hemocyanin via an added N-terminal cysteine residue.	Rabbit polyclonal 1:2,000	Synaptic Systems (Göttingen), Cat# 193 003 RRID: AB_2086768
GlyRa1	Purified rat spinal cord glycine receptor	Mouse monoclonal 1:100	Heinrich Betz, (Frankfurt), mAb2b
GlyRa2	Raised against the 18 N-terminal amino acids of the human GlyRa2 subunit	Goat polyclonal 1:100	Santa Cruz Biotech (Heidelberg), Cat# SC-17279 RRID: AB_2110230
GlyRa3	Raised against a peptide corresponding to the 15 C-terminal amino acids of the human GlyRa3 subunit	Goat polyclonal 1:100	Santa Cruz Biotech (Heidelberg), Cat# SC-17282 RRID: AB_2278934
GlyRa4	Synthetic peptide corresponding to the C-terminal 15 aa of mouse GlyRa4	Rabbit polyclonal 1:1000	Millipore (Billerica), Cat# AB9696 RRID: AB_673015
PKCa	Protein kinase C, regulatory subunit α , Peptide sequence: KVNPFVHPILQSAV	Rabbit polyclonal 1:1,000	Sigma (St. Louis), Cat# P4334 RRID: AB_477345
SCGN	Full length (276AA) recombinant human Secretagogin + 10AA N-term Histidine-tag	Sheep polyclonal 1:2,000	BioVendor (Heidelberg), Cat# RD184120100 RRID: AB_2034062
SMI-32	Neurofilament heavy chain (nonphosphorylated)	Mouse monoclonal 1:1,000	Sternberger Monoclonals Incorporated Cat# SMI-32P RRID: AB_2314912
SYT2 (ZNP-1)	Synaptotagmin II 1–5 day zebrafish embryo	Mouse monoclonal 1:250	ZIRC (Eugene), Cat# znp-1 RRID: AB_10013783
vGluT1	Synthetic peptide from rat vGluT1	guinea pig polyclonal 1:50000	Millipore (Billerica), Cat# AB5905 RRID: AB_11213019

Table 2

Statistics for bipolar cell input onto A8 cells and GlyR subunits associated with A8 cells.

	Total CtBP2 OFF	Total CtBP2 ON	Syt2 CtBP2 OFF	SYT2/Total CtBP2 OFF	SCGN CtBP2 ON	SCGN/Total CtBP2 ON	PKCα CtBP2 ON	PKCα/Total CtBP2 ON
data	322 ± 37	234 ± 23	121 ± 18	37%	63 ± 11	31%	5 ± 2	2.6%
flipped	245 ± 36	237 ± 20	70 ± 18		46 ± 7		7 ± 4	
n	10	10	7		4		4	
Paired t-test data/flipped	0.0003	0.8375	0.0015		0.046 [*]		0.361 [*]	

	GlyRa1 OFF	GlyRa1 ON	GlyRa2 OFF	GlyRa2 ON	GlyRa3 OFF	GlyRa3 ON	GlyRa4 OFF	GlyRa4 ON
data	143 ± 17	50 ± 5	112 ± 20	96 ± 24	67 ± 21	163 ± 26	10 ± 4	9 ± 1
flipped	103 ± 14	37 ± 4	79 ± 13	72 ± 20	47 ± 15	96 ± 13	30 ± 9	17 ± 3
n	5	5	5	5	4	4	3	3
Paired t-test data/flipped	0.0063	0.0082	0.0286	0.0135	0.1752 [*]	0.0271 [*]	0.8088 [*]	0.3117 [*]

Imaris counts on the OFF and ON plexus of single A8 cells in the *thyl-L*-GFP-O mouse. Numbers are mean values and SEM from all cells. Red numbers indicate no significance between quantified samples and flip controls (p > 0.05).

^{*} Since n = 4 normal distribution could not be determined; nonetheless paired t-test is feasible (de Winter, 2013).

Table 3
Statistics for GlyRα1 puncta in the ON layer in association with A-type ganglion cells and A8 amacrine cells

	GlyRα1 on A-type ganglion cells					GlyRα1 postsynaptic to A8		
	Dendritic length in μm	Total GlyRα1	GlyRα1/μm	GlyRα1 with A8	GlyRα1 with A8 [%]	Total GlyRα1	GlyRα1 with A-type RGC	GlyRα1 with A-type RGC [%]
DATA	229.8	60	0.26	36	60.0	51	20	39.2
	119.8	56	0.47	31	55.5	63	24	38.1
	166.8	81	0.48	39	48.1	79	37	46.8
	180.8	60	0.33	27	45.0	54	24	44.4
	158.8	85	0.53	40	47.1	54	23	42.6
	198.5	72	0.36	29	40.3	61	25	41.0
	159.8	78	0.49	33	42.3	59	21	35.6
	120.3	53	0.44	23	43.4	54	23	42.6
	169.2	65	0.38	24	36.9	61	24	39.3
	142.5	64	0.45	30	46.9	56	20	35.7
MEAN			0.42		46.5			40.5

Manual counts on SMI32-labeled ON A-type ganglion cells in the *Jer5*-EGFP retina. Ten image stacks were taken at different areas from retinae of four animals.