Amacrine cells are interneurons composing the most diverse cell class in the mammalian retina. They help encode visual features, such as edges or directed motion, by mediating excitatory and inhibitory interactions between input (i.e., bipolar) and output (i.e., ganglion) neurons in the inner plexiform layer (IPL). Like other brain regions, the retina also contains glial cells that contribute to neurotransmitter uptake, metabolic regulation, and neurovascular control. Here, we report that, in mouse retina (of either sex), an abundant, though previously unstudied inhibitory amacrine cell is coupled directly to Müller glia. Electron microscopic reconstructions of this amacrine type revealed chemical synapses with known retinal cell types and extensive associations with Müller glia, the processes of which often completely ensheathe the neurites of this amacrine cell. Microinjecting small tracer molecules into the somas of these amacrine cells led to selective labeling of nearby Müller glia, leading us to suggest the name “Müller glia-coupled amacrine cell,” or MAC. Our data also indicate that MACs release glycine at conventional chemical synapses, and viral retrograde transsynaptic tracing from the dorsal lateral geniculate nucleus showed selective connections between MACs and a subpopulation of retinal ganglion cell types. Visually evoked responses revealed a strong preference for light increments; these “ON” responses were primarily mediated by excitatory chemical synaptic input and direct electrical coupling with other cells. This initial characterization of the MAC provides the first evidence for neuron-glia coupling in the mammalian retina and identifies the MAC as a potential link between inhibitory processing and glial function.

Key words: amacrine; gap junctions; glia; retina; synapse; vision

Significance Statement

Gap junctions between pairs of neurons or glial cells are commonly found throughout the nervous system and play multiple roles, including electrical coupling and metabolic exchange. In contrast, gap junctions between neurons and glial cells have rarely been reported and are poorly understood. Here we report the first evidence for neuron-glia coupling in the mammalian retina, specifically between an abundant (but previously unstudied) inhibitory interneuron and Müller glia. Moreover, viral tracing, optogenetics, and serial electron microscopy provide new information about the neuron’s synaptic partners and physiological responses.
Introduction
The circuitry of the mammalian retina uses diverse, parallel circuits to encode many distinct features of the visual world. Amacrine cells, the largest class of inhibitory cell types in the retina, play a critical role in such processing. Amacrine cells are interneurons with cell bodies in either the inner nuclear layer (INL) or ganglion cell layer (GCL), and their processes are typically confined to the most extensive synaptic region of the retina, the inner plexiform layer (IPL; see Fig. 1). They use a diverse array of synaptic mechanisms, including the release of many different neurotransmitters (e.g., GABA, glycine, glutamate, acetylcholine, nitric oxide, dopamine, neuropeptides), as well as gap junctions (i.e., electrical synapses) (for review, see Kolb, 1995; Wu and Maple, 1998; Masland, 2012). Understanding the connectivity and function of amacrine cells is essential to our understanding of how the retina performs computations required for effective and rapid visual processing.

Here, we examine physiological and anatomic characteristics of a previously unstudied high-density, narrow-field amacrine cell. In addition to presenting cell-specific tools (i.e., antibodies and mouse lines), and describing the cell’s synaptic connections with other retinal neurons in the IPL (i.e., bipolar, amacrine, and ganglion cells), we provide evidence for previously unknown, direct coupling between this amacrine cell type and Müller glia. Electron microscopic reconstructions of this amacrine revealed that Müller glia extend processes that physically ensheathe this amacrine cell type’s neurites in the IPL. Small tracer molecules injected into this amacrine cell diffused into nearby Müller glia, indicating gap junction connections between the two cell types and leading us to suggest the name “Müller glial-coupled amacrine cell” (MAC) for this amacrine cell type. Together, the results presented here suggest that MACs provide local glycine inhibition within retinal neural circuits and also mediate interactions with glial cells.

Materials and Methods
Electrophysiology. Experiments were conducted in accordance with Institutional Animal Care and Use Committee at the University of Washington or in accordance with National Institutes of Health guidelines, as approved by the National Institute of Neurologic Disorder and Stroke Animal Care and Use Committee (ASP 1220). WT and genetically modified (Table 1) C57/B16 mice of either sex were dark-adapted overnight before death. Eyes were then immediately enucleated and submerged in warm (−32°C) bicarbonate-buffered Ames medium (Sigma Millipore) that was continuously bubbled with carbogen (95% O2/5% CO2). Small scissors were used to remove the cornea. The lens and vitreous were then removed with forceps before storing the retinal cups for up to 6 h in a customized light-proof storage chamber containing warm oxygenated Ames. To prepare the retina for electrophysiology recordings, we took half an eye cup, isolated the retina from the pigment epithelium, trimmed the retina into a rectangle, and mounted it photo- receptor-side down on a poly-L-lysine-coated microscope slide. A mounted retina was placed in the recording chamber, where it was superfused continuously with −32°C oxygenated Ames. For experiments that probed visual responses, retinal dissections and mounting were conducted exclusively under infrared illumination.

Voltage-clamp whole-cell recordings were conducted with electrodes (retinal ganglion cell [RGC]: 2-3 mΩ; AC-A: 5-6 mΩ) containing the following (in mM): 105 Cs methanesulfonate, 10 TEA-Cl, 20 HEPES, 1 MgCl2, 1 CaCl2, 2 EGTA, 4 Mg-ATP, 0.5 Tris-GTP, and 0.1 Alexa (594) hydrazide (−280 mOsm; pH 7.3 with CsOH). Current-clamp whole-cell recordings were conducted with electrodes (ACs: 5-6 mΩ) containing the following (in mM): 123 K-aspartate, 10 KCl, 10 HEPES, 1 MgCl2, 1 CaCl2, 2 EGTA, 4 Mg-ATP, 0.5 Tris-GTP, and 0.1 Alexa (594) hydrazide (−280 mOsm; pH 7.2 with KOH). NBQX (10 μM; Tocris Bioscience), APV (25 μM), and MFA (100 μM; Sigma Millipore) were added to the perfusion solution as indicated in Figure 6. APB (10 μM; Tocris Bioscience), UBP (25 μM; Tocris Bioscience), APV (25 μM), and NBQX (10 μM) were bath-applied for >3 min before testing for ChR2-evoked responses in RGCs. To isolate excitatory or inhibitory synaptic input, cells were held at the estimated reversal potential for inhibitory or excitatory input of −60 and −10 mV, respectively. Absolute voltage values were corrected for liquid junction potentials (K+-based = −10.8 mV; Cs+-based = −8.5 mV).

Full field illumination (diameter: 500-560 μm) was delivered to the preparation through a customized condenser from short wavelength (peak power at 405 or 460 nm) LEDs. Light intensities (photons/μm2/s) were converted to photosensitization rates (R2/photoreceptor/s) using the estimated collecting area of rods and cones (0.37 and 0.5 μm2, respectively), the LED emission spectra, and the photoreceptor absorption spectra (Govardovskii et al., 2000). Flashes were 10 ms in duration, except for ChR2 experiments, which used a 50 ms flash. In Figure 6A, B, the video monitor was set to a mean of −200 R2/rod/s and contrast steps were 0.5 s in duration.

Electrophysiology example traces presented throughout the figures represent the average of 5-20 raw responses to the same stimuli for a single cell. All averaged traces were baseline-corrected before amplitude assessment. For flash responses (from darkness), the mean amplitude was assessed for a 50 ms time window centered on the peak of the largest control response for a given cell (and held constant for all other conditions). For 0.5 s steps (increments and decrements) on a −200 R2/rod/s background, mean amplitude was assessed for a 0.5 s window that was delayed from the onset of the step by 50 ms.

Immunohistochemistry. For immunohistochemical experiments, retinas were isolated in cold oxygenated mouse ACSF, pH 7.4, containing the following (in mM): 119 NaCl, 2.5 KCl, 2.5 CaCl2, 1.3 MgCl2, 1 NaH2PO4, 11 glucose, and 20 HEPES. Retinas were fixed in 4% PFA in ACSF for 20 min, washed in 0.1 M PBS, and were either incubated with primary antibodies in blocking solution (5% donkey serum and 0.5% Triton in PBS) or were embedded in 4% agar (low-gelling agarose, Sigma Millipore) and sectioned at 120 μm in the vibratome. Retina slices were used for immunolabeling with the glycine antibodies (rabbit and rat, gifts from D. Pow). GFP was amplified with an anti-chicken GFP antibody from Abcam, and retina slices were incubated overnight with
primary antibodies in blocking solution followed by washes in PBS and a 3 h incubation with anti-isotypic secondary antibodies (Invitrogen or Jackson ImmunoResearch Laboratories). Labeling of Dact2-GFP or Plxcs2-GFP retinas for GAD67 (mouse monoclonal, Millipore), glycine transporter GlyT1 (goat, Millipore), GlyRα1 (mouse, Synaptic Systems), syntaxin 1 (mouse, Sigma Millipore), Crabp (mouse, Abcam), and PRR1R17 (rabbit, Sigma Millipore) was performed by incubating whole retinas over 3 nights in primary antibody solution followed by washes in PBS and an overnight incubation with anti-isotypic fluorescently conjugated secondary antibodies.

RGCs were either biolabelled using the CMV·dTomato plasmid as described previously (Morgan et al., 2008) or injected with 4% Neurobiotin. For tracer injection experiments, 4% Neurobiotin or 4% Neurobiotin and 2% Lucifer yellow in 200 mM KCl were injected into GFP-positive amacrine cells or Müller glia endfeet in the retinas. Injection sites were matched to a bank of previously reconstructed bipolar cell locations and synaptic organization matched that of the MAC. Subsequently, two additional MACs were found by looking for cells with tight association with the RGCs. To visualize potential synaptic contacts within RGC processes, the RGC processes were first masked in 3D using the Labelfield function in Amira. Thereafter, the presynaptic amacrine GFP signal was multiplied with the RGC cell mask (using the Arithmetic function in Amira) to isolate the points of apposition between the GFP amacrine processes and RGCs. The immunolabelled glycine receptor signal was also multiplied with the RGC 3D mask to isolate the receptor signal exclusively within the RGC processes. Thereafter, the GFP signal apposed to RGC processes and the glycine receptor signal within RGC processes was covisualized in 3D to ascertain synaptic appositions. The masked RGC was divided into soma, ON dendritic and OFF dendritic compartments for ascertaining number of GFP boutons apposed with each RGC compartment and the fraction of GFP-labeled glycine receptor signal was also multiplied with the RGC 3D mask to determine the number of GFP amacrine processes within the RGC cell soma and at the RGC layer. After NIBR-ing, the retina was unmounted and fixed in 4% glutaraldehyde overnight, and then processed for SBSEM. The tissue was washed 3 × 5 min (all washes) in 0.1 m sodium cacodylate buffer and incubated in a solution containing 1.5% potassium ferrocyanide and 2% osmium tetroxide (OsO4) in 0.1 m cacodylate buffer (0.66% lead in 0.3 m aspartic acid, pH 5.5) for 1 h. After washing, the tissue was placed in a freshly made thiocarbohydrazide solution (0.1 × TCH in 10 ml double-distilled H2O heated to 60°C for 1 h) for 20 min at room temperature. After another rinse, at room temperature, the tissue was incubated in 2% OsO4 for 30 min at room temperature. The samples were rinsed again and stained en bloc in 1% uranyl acetate overnight at 4°C, washed and stained with Walton’s lead aspartate for 30 min. After a final wash, the retinal pieces were dehydrated in a graded ice-cold alcohol series, and placed in propylene oxide at room temperature for 10 min. Finally, the samples were embedded in Durcupan resin. Semithin sections (0.5-1 μm thick) were cut and stained with toluidine blue, until the fiducial marks (box) in the GCL appeared. The block was then trimmed and mounted in the SBSEM microscope (GATAN/Zeiss, 3View). Serial sections were cut at 70-80 nm thickness and imaged at an xy resolution of 6.0-8.0 nm; 3 × 3 thicknesses, each ~40 μm × μm was obtained with an overlap of ~10%. The image stacks were concatenated and aligned using TrackEM (National Institutes of Health). The MAC was traced using the tracing tools in Track EM.

Two additional SBSEM datasets were used to explore glial ensheathment in more detail. The first block, K0725, covers a volume size of ~50 × 210 × 260 μm, with a voxel size of 13.2 × 13.2 × 26 nm (Ding et al., 2016). This resolution allows for the visualization of vesicles and chemical synapses but does not allow for identification of gap junctions. The second block used, K0731, was of the approximate size 60 × 80 × 80 μm with a similar voxel size to the K0725 block. The preparation of K0731 block was optimized to preserve extracellular space within the retinal tissue, allowing for identification of gap junctions (Palotto et al., 2015).

Müller glia were identified by dense internal filament structures that are characteristic of Müller glia and are absent from other cell types in the IPL. From Müller glia reconstructions in K0725, neuronal ensheathments were identified and ensheathed cells were reconstructed, leading to the identification of two narrow-field amacrine cells whose morphology and synaptic organization matched that of the MAC. Subsequently, two additional MACs were found by looking for cells with tight association with Müller processes in the central layers of the IPL. Inputs and outputs on these four cells were then counted and categorized. Synaptically connected bipolar cells were matched to a bank of previously reconstructed bipolar cells (same block) to determine type (Ding et al., 2016).

All cells from these two blocks were reconstructed and annotated using the EM analysis software Knossos (https://knossos.app/), and skeleton renderings for figures were created using the visualization software Paraview (Ahrens et al., 2005).
Neurod6-Cre (NEX-Cre) mice (a generous gift from Klaus Nave from University of Gottingen) were described previously (Goebl et al., 2006). NEX-Cre mice were maintained on a C57BL/6 background, and crossed to conditional TVA-expressing (cTVA) mice generated either by the Saur laboratory (Seidler et al., 2008) or by the C.C. laboratory (Beier et al., 2013). During initial studies, a conditional tdTomato reporter mouse (The Jackson Laboratory, stock #007909, commonly known as A9) was also bred to the cTVA mice to obtain a Cre-driven tdTomato reporter and TTA receptor expression. Zero viral transmissions were observed from an infected RGC to an amacrine cell that was not tdTomato , confirming our previous findings of the specificity of envA-TVA transmission (Beier et al., 2013).

As an alternate way to deliver TTA, we used plasmids or AAV viruses that encode Cre-dependent TTA. Plasmids or AAVs were subretinally injected into conditional NEX-Cre mice at P0–1; ~0.3 μl of virus, AAV-flex-Tcb (2/8) (10^{13} gc/ml), or 0.3 μl of either one of the plasmids, pAAV-flex-Tc66t or pAAV-flex-Tcb (1 μg/μl) (Miyamichi et al., 2013) was injected using a pulled angled glass pipette controlled by a Femtojet (Eppendorf) into the right eye. In the case of plasmids, an electric pulse was delivered right after the injections, to electroporate the construct (AAV preparation and electroporation strategies described previously in detail: Matsuda and Cepko, 2007; Xiong et al., 2015). For the final analysis, data derived from the different methodologies used for TTA delivery were combined.

In an effort to increase viral transmission efficiency, 11 of 74 mice injected in the studies were bred to IFNAR−/− mice (032045-JAX; MRC Diagnostics, 1127703910). VSV construction and production strategies used in this study were previously described (Beier et al., 2013). Briefly, a recombinant VSV with a chimeric glycoprotein (A/G) that consists of the extracellular and transmembrane domains of EnvA and cytoplasmic domain of rabies virus glycoprotein (RABV-G) was pseudotyped with RABV-G. All experiments were performed using mice of either sex after weaning, typically around 2-3 months of age.

The pseudotyped VSV(A/G)RABV-G was injected unilaterally into the left dorsal lateral geniculate nucleus, using a stereotaxic instrument (Narishige International) and pulled capillary microdispensers (Drummond Scientific). Injection volume was 250-500 nl (at a 100 nl/min rate, using an UltraMicroPump III (WPI). VSV(A/G)RABV-G had a concentration of 1-2 × 10^{7} ffu/ml.

Injection coordinates used were: AP −2.5 from bregma, LM 2, DV −2.75.

After 2-4 d following VSV injections, mice were killed with CO_{2}, and eyes enucleated. Corneas were removed with small scissors, lenses were removed, and retinalae were isolated in cold PBS. Freshly dissected retinas were fixed in ice cold 4% formaldehyde for 30 min before 3× wash with PBS. Whole retinal cups were blocked in 5%-6% donkey serum in PBS and were fixed in ice cold 4% formaldehyde for 30 min before 3× wash with PBS. Whole retinal cups were blocked in 5%-6% donkey serum in PBS and were fixed in ice cold 4% formaldehyde for 30 min before 3× wash with PBS. Then, the retinae were passed through a sucrose gradient until they sank in cold 30% sucrose in PBS. Retinae were transferred to 1:1 30% sucrose:OCT and incubated for 1 h in a cold room with gentle shaking. Retinae were embedded in the 1:1 30% sucrose:OCT solution for sectioning; 50-μm-thick sections were adhered to Fisher Superfrost Plus microscope slides coated with poly-ω-lysine in 1× borate buffer, washed 2× with ddH_{2}O, and dried completely. After the sections were air-dried, they were treated with Protease K (1.5 μg/ml, NEB, P8107S), postfixed in 4% PFA followed by acetic anhydride treatment. After probe hybridization, the GlyT1 probe was detected using anti-DIG-HRP (1:750) and tyramide amplification (PerkinElmer TSA Cy3). This was followed by the immunohistochemistry protocol to amplify the GFP signal in Dact2-GFP retinae. Chick anti-GFP (Abcam; ab13970, 1:200) and donkey anti-chick AlexaFluor-488 (1:250, Jackson Immunoresearch Laboratories) were used, as described in Viral tracing. These sections were analyzed as described for glycine labeling experiments described in Immunohistochemistry.

The effects of pharmacological manipulations in our electrophysiology experiments were compared with control responses in the same cell, thus allowing for paired statistical comparisons. Data are presented as mean ± SEM unless otherwise noted. For statistical comparisons, we first determined whether the distributions were random, had equal (or unequal) variance, and were normally distributed using the Runs, Kolmogorov-Smirnov, and Jarque-Bera, respectively. We then used a Wilcoxon Rank test or a Student’s t test (depending on the results of the above-mentioned tests) to determine significance; statistical tests and p values are explicitly stated for each instance in the results.

For assessment of IHC or FISH labeling, we quantified glycine immunoreactivity on retinal slices (taken from the Dact2-GFP and Plxcl2-GFP lines) by making ~50 separate measurements of average pixel intensity (within a ~15 μm ROI) from somas in the outer nuclear layer (ONL) and INLs. The ONL does not contain glycineric cells, and was thus used as a null distribution for determining whether glycine levels in cells from the INL, in particular GFP+ cells, were significantly different from zero. The mean of the null/ONL distribution was subtracted from all measurements taken from a given slice. Dact2-GFP slices usually contained only a single MAC, so measurements from multiple slices were combined after null subtraction. We determined the appropriate statistical analysis using the same approach described above for our electrophysiology experiments. GAD67 and GlyT1 antibodies were tested on...
Figure 2.  Identification of a high-density, narrow-field amacrine cell, the MAC (also known as AC51-70 and Type 23).  
A. The 10 amacrine cell types with the highest spatial density in the mouse retina ranked according to density (Helmstaedter et al., 2013).  
B. The top 10 amacrine cell types (as in A) color-coded according to what is known regarding their inhibitory neurotransmitters (i.e., GABAergic, glycinergic, unknown).  
C. Left, Reconstructions of MACs, adapted from Helmstaedter et al. (2013). Right, Example of a single MAC. Blue arrow points to the corresponding cell in the population.  
D. MACs are uniquely and sparsely labeled in the Dact2-GFP line. Top and side views of an MAC labeled with GFP in the Dact2-GFP mouse line. Three isolated and strongly labeled MACs (from the Dact2-GFP line) were used to assess stratification within the IPL. Thick line indicates the mean. Shaded region represents SEM.  
E. Anti-syntaxin, an antibody specific to amacrine cells, labeled GFP<sup>+</sup> cells in the Dact2-GFP line (7 of 7 Dact2-GFP<sup>+</sup> cells).  
F. Anti-PPP1R17 labeled amacrines at a density similar to that of MACs found in serial EM literature; furthermore, all GFP<sup>+</sup> cells in the Dact2-GFP line were PPP1R17<sup>+</sup>.  
G. Other mouse lines used in this study, such as Plxnd2-GFP, NEX-Cre-Tdt, and Dact2-cre-Tdt-ChR2, labeled various fractions of the PPP1R17<sup>+</sup> cells (e.g., MACs).
Figure 3. Serial block face EM reconstructions reveal canonical synaptic connectivity and Müller glia (MG) ensheathment. A, Left, NIRB marks were used to target a GFP+ cell in the Dact2-GFP line. Right, The resulting MAC reconstruction revealed conventional output synapses onto amacrine cells (ACs, A1), bipolar cells (BCs, A2), and RGs (GCs, A3), and chemical synaptic inputs from bipolar (shown in Fig. 7) and amacrine cells (A4). B, Reconstructions also revealed MG ensheathment (red) of MAC processes (blue, B5,B6) that lacked obvious chemical synaptic structures. Left, Skeleton and partial reconstruction of MG that ensheathed the identified MAC at multiple locations. Middle, Expanded view of the 3D Müller ensheathment from the white box in the left panel. Right, Electron micrographs of the Müller ensheathments denoted in the left panel with numbers 5 and 6. C, MG reconstructed in another, nonlabeled EM block (K0725) (Graydon et al., 2018) also ensheathed MACs (see Materials and Methods). Left, An example of a MG reconstruction (red) with multiple ensheathments of an amacrine cell (blue) with morphology matching the MAC. Middle, This particular MAC is ensheathed by the red MG at 4 locations within its arbor (red ensheathments), and also with another MG (not shown) at 2 additional locations (yellow ensheathments). The cell bodies for these MG and amacrine cell are missing from this thinner block, which only contains a small fraction of the INL (where their cell bodies typically reside). D, Exemplary electron micrograph of an MG ensheathment of an MAC in K0725. E, Bar graph summarizes the ultrastructural analysis of 5 reconstructed MACs (mean ± SD).

Results
A recent inventory of the neuronal constituents of the mouse IPL using SBFSEM reconstructions (Helmstaedter et al., 2013) revealed >45 morphologically distinct types of amacrine cells. When ranked by density (no. of cell bodies per square millimeter of retina), the majority of the well-studied amacrine cells, including those involved in night vision (AII, A17) and the encoding of directed motion (ON and OFF starburst amacrine cells, or SACs) are among the top 10 (see Fig. 2A). Also, near the top of this list are several amacrine types for which very little is known. More than half of the high-density amacrine types display narrow-field morphologies typically associated with a glycnergic neurotransmitter phenotype (see Fig. 2A,B) (Menger et al., 1998; Pourcho and Goebel, 1983), although this has not been confirmed for all types. We focused on the second most densely distributed amacrine type, initially termed AC51-70 (Helmstaedter et al., 2013), and referred to here as the MAC. These cells are easily recognized by their unique morphology (see Fig. 2C), as they are the only narrow-field amacrine cells that stratify exclusively in the ON layers (inner half) of the IPL (Fig. 1), and have only 1 or 2 processes that connect the synaptic arbor to the soma. Their functional characteristics and circuit wiring, however, remain largely unknown (Pang et al., 2012). Cells with similar morphology have been observed in the primate retina (Polyak, 1941), but as with mouse, we know little about them. Here we combine several techniques to explore the synaptic connectivity and physiology of MACs in the mouse retina.

We identified a mouse line (Dact2-GFP) in the GENSAT (www.gensat.org) database that selectively labels a sparse but homogeneous population of amacrine cells with morphology matching the AC51-70 from Helmstaedter et al. (2013) (Type 23; Fig. 2C, D). Closer inspection of the cells' morphology revealed a monopolar neuron with a small bushy arbor restricted primarily to the inner half of the IPL, and immunohistochemical (IHC) experiments showed that this GFP+ cell expresses the amacrine marker, syntaxin (7 of 8 Dact2-GFP+ cells, n = 3 animals; Fig. 2E) (Voinescu et al., 2009). Additionally, we mined the single-cell transcriptomic database for the mouse retina with the purpose of finding markers for the MAC (Macosko et al., 2015). In their initial paper, Macosko et al. (2015)
identified a cluster (#20) that strongly expressed mRNA for the protein phosphatase regulatory subunit, PPP1R17. A follow-up study determined that an antibody to PPP1R17 labels several types of narrow-field amacrine cells whose neurotransmitter and physiology were unknown (Yan et al., 2020). We found that this antibody labeled 100% of the GFP1 cells in the Dact2-GFP line (Fig. 2F,G), suggesting that it effectively labels MACs. Furthermore, anti-PPP1R17 labeled cell bodies were observed at a similar density to that reported for AC51-70 in Helmstaedter et al. (2013) (Fig. 2G), suggesting that this antibody might label a large population of AC51-70s. We later use this antibody to identify MACs in the absence of a genetically encoded fluorescent label.

MACs make connections with neurons and Müller glia

We targeted a GFP+ cell (i.e., MAC) in the Dact2-GFP line for ultrastructural analysis by performing SBFSEM on tissue in which an MAC had been identified and fiducially marked by NIRB (see Materials and Methods) (Bishop et al., 2011; Della Santina et al., 2016). Partial reconstruction of this MAC revealed a total of 26 synaptic inputs and 20 synaptic outputs, as indicated by clusters of vesicles adjacent to electron-dense regions of membrane (Fig. 3A). In addition to conventional synapses, the reconstruction also revealed that MAC processes were frequently ensheathed by Müller glial processes (ensheathment defined here as a Müller process surrounding 50% of the circumference of a neuronal process) which lacked obvious synaptic structures (Fig. 3B). These glial ensheathments were found on the descending neuritic stalk and branches of the small bushy arbor of the MAC. Glial ensheathment was not simply a consequence of overlapping processes between high-density cell types, because glial ensheathment was rarely observed in complete reconstructions of two AII amacrine cells, the most numerous amacrine cell type (data not shown). To examine more closely the extent to which glial ensheathment is a true feature of MACs, we reconstructed Müller glia in a larger, previously obtained block-face dataset (K0725) (Graydon et al., 2018) that did not have genetic labels or NIRB. Each reconstructed Müller glia (n = 3) in this dataset exhibited multiple neuronal ensheathments throughout the IPL;
Figure 5. Weak coupling between Müller glia and GFP<sup>+</sup> neurons in the Plcxz2-GFP line. A, Side view of a Müller glia loaded with neurobiotin through the end feed (GCL) in the Plcxz2-GFP line. B, Maximum projection of the INL from the stack shown in A. Neurobiotin signal was quantified in the somas of the GFP<sup>+</sup> cells surrounding the injected Müller and at two null locations without GFP<sup>+</sup> (n1 and n2). C, To minimize contamination from signals originating outside the GFP<sup>+</sup> cells, average neurobiotin signal intensity measurements were taken from each GFP<sup>+</sup> soma (ROI = 14 μm<sup>2</sup> circle) in the single-image plane with the largest somatic diameter. Examples of 4 GFP<sup>+</sup> cells with significant levels of neurobiotin coupling (left column) and 4 GFP<sup>+</sup> without significant neurobiotin signal (right column). D, Scatter plot represents the relationship between neurobiotin and GFP signals for 38 GFP<sup>+</sup> cells and 2 null locations. E, Histogram represents the distribution of average neurobiotin signal for the 40 ROI measurements. Positive examples from C are labeled numerically. F, Histogram represents the number of tracer-coupled GFP<sup>+</sup> cells from 6 separate Müller injections. G, Electron micrographs of contact (putative “gap junction,” arrow) (Pallotto et al., 2015) between Müller glia (red) and amacrine cells (blue) in the IPL. Yellow shading represents ensheathed neurons without obvious gap junction connectivity. H, Potential gap junctions were quantified by examining 10 ensheathments in each of the 5 sublamina (20% increments; 50 ensheathments in total) for contact between Müller glia ensheathments and neuronal processes.
by reconstructing some of the ensheathed neurons (~30), we found two amacrine cells that mirrored the morphology and synaptic organization of the MAC identified using the NIRB technique (Fig. 3C). These putative MACs (including 2 others; see Materials and Methods) were ensheathed by additional Müller glia, with each MAC consistently exhibiting a total of 6 or 7 ensheathments, which in each case could be traced back to a total of 2 Müller glia (n = 4; Fig. 3D,E). These data indicate that Müller ensheathment is a feature of MACs, and suggest that neurons other than MACs are also ensheathed by Müller glia.

We hypothesized that these ensheathments might enable interactions between neuronal and glia networks, and that gap junctions, which are not easily resolved in conventional SBFSEM reconstructions (Pallotto et al., 2015), may play a role. To test for gap junctions between MACs and Müller glia, we injected gap junction-permeable (neurobiotin) and -impermeable (Lucifer yellow) tracer molecules into MACs (under light-adapted conditions) and analyzed the diffusion of these tracers into coupled cells using IHC (see Materials and Methods) (Mills and Massey, 1995; Vaney et al., 1998; Pang et al., 2010, 2013). These experiments were conducted on another transgenic mouse line (Plcxd2-GFP; Fig. 2F) that labels a larger number of MACs than the Dact2-GFP line (for more information on overlap between PPP1R17 labeling and fluorescent protein expression in the

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**Figure 6.** Visually evoked signals and origins of excitatory synaptic input to the MAC. A. Top, Current-clamp recording of visual responses to flashed spots (light increments) of increasing size. Bottom, Response amplitudes plotted as a function of spot size for the example cell. Open markers represent responses to light increments. Closed markers represent responses to light decrements. B, Responses to a 100 µm spot of various contrasts (n = 4). C, Responses to a 500 µm spot of various contrasts (n = 4). D, Voltage-clamp recordings (Vhold — reversal potential for chloride) assessing flash sensitivity from darkness. Normalized (to the brightest flash in control) response amplitudes are plotted as a function of flash strength. After control recordings, APV and NBQX were bath-applied to block NMDA and AMPA receptors, respectively. Responses to flashes ≤1 R'/rod/flash were blocked by the cocktail, but further increasing the flash strength under these conditions revealed cocktail-insensitive responses. E, Dim flash responses in darkness reversed at the theoretical reversal potential for excitatory glutamate receptors (~0 mV). APV- and NBQX-insensitive light responses emerged as flash strength was increased, and reversed at positive potentials indicating a lack of voltage clamp.
various mouse lines used in this study, see Fig. 2G). These experiments consistently (14 of 14 injections) produced neurobiotin labeling in Müller glia (Müllers labeled per injected MAC: median = 2, range = 2-4; Fig. 4A,B) surrounding the injected MAC (MAC-Müller intersomal distance: $13.9 \pm 1.6 \mu m$, $n = 26$; Fig. 4C), but not in neighboring MACs (Fig. 4A). In contrast, Lucifer yellow labeling (included in a subset of injections; $n = 4$) was constrained to the injected MAC (Fig. 4A). To further
confirm the identity of putative Müller glia, we also used IHC for CRALBP, a known marker for Müller glia (Bunt-Milam and Saari, 1983). Indeed, suspected Müller glia were immunoreactive for CRALBP (Fig. 4D). This experimental approach occasionally showed additional tracer-coupling to cone bipolar cells (CBCs) and wide-field amacrine cell processes, but these coupling patterns tended to be variable across samples and thus were not pursued further (Fig. 4B). The observed coupling with Müller glia was not a feature common across amacrine cell types, as similar injections into AII amacrine cells (n = 2) labeled ON CBCs and a few AII amacrine cells but did not label Müller glia (Fig. 4E) (see also Mills and Massey, 1995; Trexler et al., 2001).

If MACs are coupled to Müller glia via gap junctions, then tracer molecules should, in theory, transmit bidirectionally. However, this notion is likely complicated by the widely different resting membrane potentials of Müller glia (−80 mV) and neurons (−40 mV) (Jorstad et al., 2017). When applied to a static conductance (e.g., a gap junction), this difference in membrane potential should preferentially drive cations (e.g., neurobiotin, Na⁺, K⁺) from neurons into Müllers and anions (e.g., Cl⁻, glycine) from glial cells into neurons. With this potential source of rectification in mind, we tested for tracer coupling from Müller glia to MACs in the Plcxd2-GFP line. For these experiments, we patched the endfeet of Müller glia (in the GCL) with a neurobiotin-filled electrode (Fig. 5A) and used 5–10 s current injections of 1 nA to facilitate neurobiotin transfer from Müllers to MACs. Tracer coupling was assessed by measuring the average neurobiotin signal (14 μm² ROI) within individual GFP somas from single image planes (see Materials and Methods; Fig. 5C–E). Although the strength of neurobiotin coupling was modest compared with MAC injections (Fig. 4A), we found significant neurobiotin signals in Plcxd2-GFP somas of 5 of 6 Müller glia injections (Fig. 5F). Together, these results indicate that MACs and Müller glia are directly coupled to one another, likely through gap junctions.
Figure 9. Conventional markers for glycinergic (GlyT1) and GABAergic (GAD67) amacrine cells fail to label MACs in standard immunohistochemistry assays, but FISH reveals low levels of GlyT1 transcript. A, An antibody against GAD67 failed to label MACs in the Dact2-GFP line (0 of 18 Dact2-GFP<sup>+</sup> cells). B, Dact2-GFP<sup>+</sup> cells were also negative for GlyT1 (0 of 8 Dact2-GFP<sup>+</sup> cells) using traditional IHC methods. C, Low levels of GlyT1 transcript were detected in MACs. A FISH probe was used against GlyT1 in Dact2-GFP retina sections. Left, MAC cell body (green) merged with the GlyT1 signal (magenta). Middle, GlyT1 signal alone. Right, Histograms showing analysis of GlyT1 labeling in ONL, IPL, and Dact2-GFP<sup>+</sup> cells.

Depending on retinal fixation conditions, gap junctions can be observed in electron micrographs as plaques or other electron-rich structures at adjoining membranes (Tsukamoto and Omi, 2017; Sigulinsky et al., 2020). Unfortunately, these structures were not apparent in our NIRBed block (Fig. 3A), nor the publicly available K0725 block (Fig. 3C) (Ding et al., 2016), even at contacts between cells known to be coupled by gap junctions (e.g., AII-AII, AII-ON CBC) (Graydon et al., 2018). In an effort to determine whether gap junctions are located within glial ensheathments, we turned to another published SBFSEM dataset where gap junctions are identifiable because of preservation of the extracellular space (Pallotto et al., 2015). Under normal conditions, nearly 20% of the brain’s volume consists of extracellular space, which creates a separation between the membranes of neighboring cells, except when a synapse (chemical or electrical) is present. Typical EM fixation protocols use hypo-osmotic solutions that lead to a collapse of extracellular space, which can leave gap junctions, which are not typically surrounded by clusters of intracellular vesicles, difficult to resolve. Although we were unable to find a complete MAC in this much smaller block (see Materials and Methods), partial reconstruction of a Müller glia cell revealed numerous neuronal ensheathments in the IPL. Müller ensheathments (Fig. 5G, red) occasionally contained continuous appositions with amacrine cells (blue) that, in the absence of synaptic vesicles in these fixation conditions, indicates putative gap junctions (Pallotto et al., 2015). Other ensheathed neurons in the IPL (e.g., bipolar and ganglion cells) (Fig. 5G, yellow) did not form continuous contacts with the Müller glia. We quantified the percentage of ensheathments that contained putative neuronal gap junctions by examining 10 examples of neuronal ensheathment within each of the 5 sublayers for continuous membrane apposition (Fig. 5H). These data indicate that only a fraction of a Müller glia’s neuronal ensheatments contain putative gap junctions (7 of 50 sampled ensheathments, primarily in the central layers of the IPL), and that these connections seem to be made preferentially with amacrine cells.

MAC light responses are primarily driven by on excitatory synaptic inputs

The light response properties of MACs were investigated by targeting GFP<sup>+</sup> cells for whole-cell recordings using two-photon microscopy (980 nm excitation laser) in dark-adapted Plexd2-GFP retinas. We began by probing the receptive fields of MACs with brief presentations (0.5 s) of spots of various sizes (50-1000 μm) and contrasts (±100% on a 200 R*/rod/s background). Current-clamp recordings revealed a clear center-surround receptive field (Fig. 6A). We also probed the contrast response function (−100% to 400%) of MACs for two different spot sizes (diameters: 100 and 300 μm). MACs exhibited a strong preference for positive contrasts (i.e., light increments) regardless of spot size or stimulus strength (Fig. 6A–C), consistent with their stratification within the ON layer of the IPL (Fig. 2D).

We next explored the synaptic mechanisms mediating flash sensitivity from darkness using voltage-clamp recordings. At a holding potential of −68 mV, MACs were sensitive to dim flashes (10 ms, 500 μm spot) that elicited ~0.1 photoisomerizations per rod per flash (R*/rod-flash); saturating responses were elicited by flashes >1 R*/rod/flash (Fig. 6D). Flash strength was then held constant while varying the holding potential. Flash-evoked signals reversed near 0 mV (~0.05 ± 3.3 mV, n = 7; Fig. 6E,G), the reversal potential for EPSCs mediated by AMPA and/or NMDA receptors, indicating that MACs receive primarily excitatory synaptic input under these conditions. The current–voltage relationship for the EPSCs exhibited outward rectification (Fig. 6G), a characteristic of responses mediated at least in part by NMDA receptors (Dingledine et al., 1999; Manookin et al., 2010). EPSCs evoked by flashes producing 1 R*/rod or less were almost entirely abolished by a combination of NMDA and AMPA receptor antagonists (to 3 ± 2% of control, n = 9, p = 4 × 10<sup>−6</sup>, paired Student’s t test; Fig. 6D). However, subsequent increases in flash strength recruited an additional response component with a shallower current–voltage relationship that reversed at significantly larger holding potentials (20.7 ± 3.6 mV, n = 5, p = 0.002, paired Student’s t test; Fig. 6E). A weakly voltage-dependent conductance with a positive reversal potential is consistent with gap-junction-mediated input from a retinal cell type with a higher visual threshold (Trexler et al., 2005; Murphy and Rieke, 2011; Grimes et al., 2014; Ke et al., 2014). Accordingly, subsequent application of the gap junction blocker, MFA (100 μM), reduced the remaining amplitudes by 61 ± 14% within 10 min (n = 5, p = 0.006, paired Student’s t test; data not shown). Light-evoked signals transmitted through gap junctions to MACs could come...
from ON CBCs, ON amacrine cells, or from Müller glia (Figs. 4 and 5).

Which cells provide excitatory synaptic input to MACs? Our SBFSEM reconstructions revealed both conventional (Figs. 3A4, 7A1) and ribbon-type synaptic input to MACs (Fig. 7B2). This observation, along with a lack of light-evoked inhibitory inputs (Fig. 6), led us to hypothesize that MACs receive glutamatergic input from bipolar cells and possibly a glutamatergic amacrine cell. Recent studies have described an amacrine cell that expresses VGlut3 (Haverkamp and Wassle, 2004; Johnson et al., 2004; Grimes et al., 2011) and releases glutamate (Lee et al., 2014, 2016; T. Kim et al., 2015). Overlapping stratification in the central layers of the IPL suggests that VGlut3 ACs could provide input to MACs (Fig. 2D). We tested for these connections using both anatomic and physiological approaches. First, we crossed the Plcxd2-GFP line with a mouse line that expresses YFP tagged to the glutamate receptor scaffold PSD95 and labeled these retinas with the VGlut3 antibody. Despite the substantial overlap in stratification, PSD95 was rarely observed at appositions between VGlut3 ACs and MACs (an average of three examples per MAC, \( n = 2 \)). This suggests that VGlut3 ACs provide, at most, a minor input to MACs. Second, paired recordings (\( n = 6 \)) between overlapping MACs and VGlut3 ACs (in a Plcxd2-GFPxVGlut3-cre-Tdt mouse line) failed to show obvious signal transmission between these two amacrine cell types (data not shown).

Closer inspection the 5 MACs identified in our SBFSEM datasets revealed that the conventional synaptic inputs to MACs come exclusively from narrowly stratified wide-field amacrine cells (17 ± 5 synapses per MAC) whose processes tended to extend entirely through the SBFSEM volumes (Fig. 7A,G). According to our reconstructions, a majority of the wide-field amacrines receive ribbon-type synaptic input from Type 5t (ON) CBCs (Fig. 7G). One might predict that these wide-field amacrines would therefore provide GABAergic input to MACs in response to light increments, but we found no evidence for light-evoked inhibition in our dark-adapted MAC recordings (Fig. 6D–G), nor did we observe robust hyperpolarizing responses to varying spot sizes at higher light levels (Fig. 6A–C). The characteristics and signaling roles of the synaptic connections with these wide-field amacrine cells remains a topic for investigation.
future studies. MACs in the K0725 block received excitatory inputs from bipolar cells previously identified as Type 5o and Type 6 CBCs (Ding et al., 2016; Graydon et al., 2018) (Fig. 7E–G; see Materials and Methods).

MACs likely make conventional glycinergic output synapses
Across all reconstructed MACs (n = 5), we found 53 ± 12 conventional chemical output synapses onto amacrine, bipolar, and RGCs (Fig. 3E). We used IHC and optogenetics to determine the molecular identity of the neurotransmitter released at these conventional synapses.

Previous anatomic studies have correlated amacrine arbor size with neurotransmitter phenotype: narrow-field amacrine cells are typically glycinergic, whereas wider-field amacrine cells are GABAergic (Pourcho and Goebel, 1983; Menger et al., 1998). To determine whether narrow-field MACs contain elevated levels of glycine, we performed IHC on retinal slices from the Dact2-GFP line (Fig. 8A,B), and compared the glycine immunoreactivity within GFP+ MAC somas to measurements taken from locations throughout the INL and ONL within the same slices (see Materials and Methods; Fig. 8C). The INL contains a mixture of bipolar, amacrine, and horizontal cells and represents a very diverse set of neurons with various levels of expected glycine immunoreactivity (e.g., some ON CBCs express glycine because of their gap junctions with glycinergic amacrine cells) (Vaney et al., 1998). As expected, we observed a broad distribution of glycine expression across 50 somatic ROIs (Fig. 8C, purple bars). The ONL, on the other hand, contains somas of photoreceptors that do not express glycine. We therefore compared immunoreactivity in the GFP+ somas (green bars) to the distribution of immunoreactivity for 50 equivalent measurements taken from the ONL of the same slice (Fig. 8C, black bars). Glycine immunoreactivity in GFP+ somas fell well outside the distribution of ONL glycine expression in the same slices (Fig. 8C), indicating the MACs do express significant levels of glycine (p = 7 × 10⁻⁷ for 6 Dact2-GFP+ cells vs 300 ONL control measurements, Wilcoxon Rank test). We also examined glycine immunoreactivity in retinal slices taken from the Plcxd2-GFP line (Fig. 8D–I). For these experiments, a second glycine antibody (rat) was also used. Measurements from the ONL showed that the rat-derived glycine antibody exhibited less background noise than the rabbit antibody, but both antibodies labeled GFP+ somas significantly (rabbit: p = 2 × 10⁻⁶ for 6 Dact2-GFP+ cells vs 50 ONL control measurements, Wilcoxon Rank test, Fig. 8F; rat: p = 2.2 × 10⁻¹⁰ for 6 Plcxd2-GFP+ cells vs 50 ONL control measurements, Wilcoxon Rank test, Fig. 8I).

...
Together with the pattern of chemical output synapses we observed in our SBFSEM reconstructions (Fig. 3E), these data suggest that MACs may release glycine onto multiple cell types in the IPL, including RGCs. To test for functional glycine release, we made recordings from RGCs in a mouse line that expresses ChR2 in a sparse subset of MACs (Dact2-cre/Tdt-ChR2). Unlike the Dact2-GFP line, which exclusively and sparsely labeled MACs, the Dact2-cre/Tdt-ChR2 retinas also expressed tdTomato-tagged ChR2 in wide-field (presumably GABAergic) amacrine cells and RGCs. The wider repertoire of labeling with Tdt-ChR2 in the Dact2-cre line likely reflects the sensitivity and history of the activity of the Cre allele at this locus, but another cross (i.e., Dact2-cre x YFP-tagged ChR2) failed to label MACs entirely. We made whole-cell recordings from a random collection of RGCs in a flat-mount retinal preparation (Fig. 10), and used a cocktail of APB, NBQX, APV, and UBP to block glutamatergic synaptic transmission throughout the retina while leaving direct inhibitory synaptic transmission intact (Lee et al., 2014, 2016; Park et al., 2015; Tien et al., 2016). A spot of...
UV light was then delivered to the retina to test for ChR2-driven responses in RGCs. Of 48 RGCs tested, only one showed a robust ChR2-evoked response. Synaptic currents recorded from this RGC (Fig. 10A,B) reversed near the expected reversal potential for a CI-mediated (ECI) conductance and were completely blocked by the glycine receptor antagonist, strychnine (1 μM; Fig. 10B). Subsequent imaging of the RGC and the ChR2-Tdt+ cells revealed a single MAC within the proximal arbor of a narrowly stratified small arbor RGC with morphology similar to one of the W3 RGC types (i.e., Local Edge Detector; Fig. 10A) (Helmstaedter et al., 2013). Other RGCs, like the ON-OFF direction-selective (OODS) cell (n = 9), failed to respond to ChR2 activation (Fig. 10C, D). The low success rate of these experiments likely reflects a combination of several features: sparse MAC labeling in the

**Figure 13.** Serial block face EM reconstructions of postsynaptic targets of the MAC. A, MACs make chemical synapses onto CBC5i and CBC6. Colored dots represent synapses. B, Electron micrograph of an identified synapse between the MAC and CBC6. C, MACs synapse onto a narrow-field amacrine type we identify as Type 21 from Helmsmaedter et al. (2013) (H21). D, Electron micrograph of an identified synapse between the MAC and H21. E, MACs make synapses onto A17 neurites in sublamina 4 of the IPL. Green dots represent synapses from the 4 reconstructed MACs onto A17s. Magenta dots represent ribbon input to the A17. F, Electron micrograph of an identified synapse between an MAC and A17. G, Average number of identified output synapses per reconstructed MAC (n = 4), broken down by postsynaptic cell type. Inconsistent postsynaptic partners (i.e., <1 synapse per MAC on average) are excluded from this graph but are included in the analysis in E.

**Bipolar**

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**MACs provide outputs to bipolars, amacrine, and RGCs in a highly selective manner**

We used a viral tracing technique to more broadly assess MAC connectivity with RGCs (Fig. 12). For this purpose, we used a mouse line with Cre expression in a more substantial number of MACs (NEX-Cre) (Kay et al., 2011). This line also drives Cre expression in other amacrine cell types; thus, visual identification of the MAC’s unique morphology and/or the PPP1R17 antibody (Fig. 2) was used to determine whether the connected amacrine cells were indeed MACs (see Materials and Methods). We used a variant of VSV that encodes within its TVA, either by crossing to a floxed-TVA mouse (Seidler et al., 2016).
al., 2008; Beier et al., 2013) or by introduction of a flexed-TVA-mCherry construct (Miyamichi et al., 2013). VSV was pseudo-typed with the rabies virus glycoprotein (RABV-G) to allow the injected virus to be taken up by any cell at the inoculation site. VSV-A/G pseudo-typed with RABV-G was injected into a retinorecipient brain area (the lateral geniculate nucleus), where it was taken up by RGC axon terminals (Fig. 12A). Retrogradely transported virus replicates once it is in the cell body of a RGC, and then spreads transsynaptically from the RGC to amacrine cells that express TVA. This method allowed us to probe many RGC types for connectivity to MACs. Subsequent confocal imaging of these retinas showed that RGCs connected to MACs were enriched for several RGC types with distinct, known morphologies (Fig. 12B–F) as follows: (1) RGCs with small arbor (e.g., HD1, HD2 and W3; Fig. 12B) (Jacoby and Schwartz, 2017; T. Kim and Kerschensteiner, 2017); (2) RGCs with highly asymmetric dendrites (e.g., JamB, F-mini; Fig. 12C) (I. J. Kim et al., 2008; Rousseau et al., 2016); and (3) RGCs that are asymmetrically bistratified (e.g., Sbc) (Jacoby et al., 2015; Tien et al., 2015). Some RGCs with virally connected MACs were not easily identified based on morphology, and were thus lumped into the “unknown” category (Fig. 12D). RGCs with virally connected MACs sometimes had multiple amacrine cells labeled within their arbors (Fig. 12G). Because of the heterogeneity of the NEX-Cre line, there were a few virally infected RGCs that had only non-MAC ACs connections; these RGCs were excluded from the final analysis and cell counts. Notably, the exemplary cell for the small arbor RGC in Fig. 12B is (to us) morphologically indistinguishable from the RGC showing the robust connectivity via ChR2 strategy in Fig. 10A, further supporting this connectivity pattern. Although many different RGC types were labeled with this approach, all encountered OODS RGCs (n = 10), a well-known RGC type that costratifies with the MAC, lacked trans-synaptically labeled MACs (Fig. 12E,F). This lack of apparent connectivity between MACs and OODS RGCs is further supported by our IHC experiments, which showed a lack of colocalization between NB-injected OODS RGCs, GlyRα1 puncta, and GFP-labeled amacrine cells in the Plxna2-GFP line (Fig. 11C). Previous work has confirmed that this approach effectively identifies synaptic partners of OODS cells and exhibits limited false-positives (Beier, et al., 2013). Together, the data in Figures 8, 10–12 argue that MACs provide feedforward inhibition onto specific RGC types by way of conventional glycinergic synaptic transmission, while avoiding others (e.g., OODS).

Our initial SBFSEM results indicated that the majority of MAC output synapses are made onto bipolar and amacrine cells (Fig. 3E), but our circuit-tracing techniques were impractical for broad assessment of MAC connectivity with the other ~60 cell types residing in the INL. In lieu of this, we returned to the large SBFSEM dataset (Ding et al., 2016) and partially reconstructed all neurons postsynaptic to the MACs. From these reconstructions, we confirmed the identity of two amacrine cell types and one bipolar cell type that consistently received direct chemical synaptic output from the MACs. Each MAC (n = 4) makes an average of 8 output synapses onto Type 6 CBCs (Fig. 13A,B,G). This result, combined with the finding that MACs receive substantial input from Type 6 CBCs (Fig. 7E–G), suggests a feedback inhibition role. In support of this notion, we also found multiple examples of direct reciprocal connectivity between each MAC/type 6 CBC pair. MACs receive substantial input from Type 5 CBCs (in particular Type 5o), but these CBCs are only a minor output target of the MAC. MACs also made chemical synapses onto a narrow-field amacrine cells that we identified as Type 21 (18 synapses per MAC) in Helmsaedter et al. (2013) (Fig. 13C,D). MACs also synapsed onto A17 amacrine cell dendrites in sublamina 4 (5 synapses per MAC; Fig. 13E–G), a well-known amacrine that participates in visual processing under dim lighting conditions. This suggests that MACs might also play a role in visual processing at night, when mice are most active.

Discussion

Here we present our initial findings regarding the physiology and synaptic connectivity of the MAC (Fig. 14), the second-most numerous amacrine cell type in the mouse retina. The results indicate, unexpectedly, that MACs are directly coupled to Müller glia. Direct coupling between neurons and glia is unusual (see Alvarez-Maubein et al., 2000; Pakhotlin and Verkhrotsky, 2005) and has not previously been reported in the mammalian retina, but few studies have tested this possibility directly. We speculate that MACs might play an important role in retinal functions involving neuron-glia interactions. For example, recent work (Biesecker et al., 2016) has revealed neuron-glia interactions in the IPL that lead to vasodilation of retinal capillaries (within the INL) in response to increased neural activity, although the identity of the neuron(s) participating in this functional hyperemia remain unclear. Biesecker et al. (2016) found that this neurovascular coupling pathway involves IP3-dependent calcium release...
within the Müller glia, which could, in theory, be triggered by gap junction transmission of IP$_3$ signals generated in the MAC (Boitano et al., 1992). Another possibility is that Ca$^{2+}$ signals generated in the MAC are transmitted through gap junctions to Müller glia, where they could then trigger intracellular signaling pathways, either directly or by boosting ER calcium stores, previously shown to enhance IP$_3$R sensitivity to IP$_3$ (Missaia et al., 1992).

Another interesting feature of the MAC is their poor expression of traditional GABAergic and glycineric markers (e.g., GAD67 and GlyT1) that label most amacrine types in a mutually exclusive manner. This observation is consistent with published transcriptomic data on PPP1R17$^+$ ACs (Macosko et al., 2015) and suggests that MACs are members of a recently characterized amacrine subpopulation originally termed non-GABAergic, nonglycinergic (nGnG) amacrine cells (Kay et al., 2011). Our data indicate, however, that these cells do release glycine via conventional synaptic transmission. We also note that our ISH did reveal low levels of GlyT1 within these cells (Fig. 9C) and that this observation is consistent with a recent publication examining gene expression across amacrine types (in which the MAC seemingly corresponds to cluster #30) (Yan et al., 2020). Some apparent non-GABAergic, nonglycinergic amacrine cells may therefore release glycine despite low levels of expression of the commonly associated synaptic and transporter genes/proteins. It remains unclear how MACs acquire the appropriate levels of glycine, but glycine can pass through gap junctions (Vaney et al., 1998; Deans et al., 2002), and Müller glia, which we show are gap junctionally coupled to MACs, take extracellular glycine using GlyT1 in culture (Hosoya et al., 2010).

Our data also indicate that MACs receive light-evoked glutamatergic input but provided little evidence for inhibition in response to the visual stimuli used in our experiments (Fig. 6). This physiological result is surprising given that, according to our EM reconstructions, the majority of input synapses to the MAC come from wide-field amacrine cells (Figs. 3E and 7A,G). One possibility is that these presynaptic amacrine cells release glutamate onto the MAC. Recent work has shown that one amacrine cell type expresses VGLUT3 and releases glutamate (Lee et al., 2014, 2016), which led us to test for possible connections with the MAC. However, both paired physiology recordings and immunohistochemical analysis failed to show substantial connectivity between MACs and VGLUT3 amacrine cells. A recent publication on sequencing data of the amacrine population has revealed an amacrine cell type that expresses VGLUT1, but an initial screening suggests that the VGLUT1$^+$ amacrine cell arbor is confined to sublamina 1 of the IPL (Yan et al., 2020). Since our serial EM reconstructions of the MAC (n = 5) showed no evidence for synaptic connections in sublamina 1 and 2 (Figs. 3 and 7), synaptic inputs from the VGLUT1$^+$ amacrine cell seems unlikely. Other possibilities are that we have not yet found a visual stimulus that drives these presynaptic wide-field amacrine effectively, or that these interneurons provide tonic, but not phasic, inhibition to the MAC.

The cell type-specific connections with the MAC (Figs. 7, 12, and 13) suggest enticing targets for future physiology studies. For example, our reconstructions indicate that MACs make reciprocal connections with Type 6 CBCs. Type 6 CBCs provide the majority of feedforward excitation to the ON α RGC (Schwartz et al., 2012), a known intrinsically photosensitive RGC (M4) (Estevez et al., 2012; Schroeder et al., 2018) with dynamic receptive fields (Farrow et al., 2013; Grimes et al., 2014). We were also intrigued by the MAC’s synaptic outputs to A17 amacrine cells. These synaptic contacts were restricted to sublaminae 4 and 5 of the IPL, where A17 amacrine cells make reciprocal GABAergic synapses with rod bipolar cells (Nelson and Kolb, 1985; Hartveit, 1999; Grimes et al., 2010). Serial inhibition of this nature could dis-inhibit the rod bipolar cell axon terminal when the MAC is active and thereby influence signalizing near visual threshold.

In conclusion, the results and approaches presented here raise intriguing questions regarding potential roles for MACs in visual processing and neurovascular coupling and suggest a number of powerful tools (transgenic mouse lines, antibody labels, circuit-tracing) to address those questions in future studies.

References


