RESEARCH ARTICLE

Identification of Amacrine Neurons with a Glycinergic and Gabaergic Phenotype in the Mouse Retina

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Abstract
The amacrine neurons in the mammalian retina comprise a large variety of cell types with distinct properties and functions that serve to integrate and modulate signals presented to output neurons. The majority of them use either glycine or GABA as inhibitory neurotransmitters and express the glycine transporter 1 (GlyT1) or glutamic acid decarboxylase (GAD67) and GABA transporters (GAT1 and GAT3), as a glycinergic or GABAergic marker respectively. We report here a novel subpopulation of amacrine neurons expressing both, GABAergic and glycinergic markers, in retinas from wild-type C57BL/6J mice and two transgenic lines. In retinal sections from the transgenic line expressing eGFP under the control of the glycine transporter 2, eGFP expression was exclusively found in cell bodies and dendrites of inhibitory amacrine neurons, identified for their immunoreactivity to syntaxin 1A. All of the glycinergic and a large portion of the GABAergic amacrine neurons contained eGFP; of these, 8-10% of GlyT1 positive neurons were also labeled either with GAD67, GAT1 or GAT3. These findings were confirmed in retinas from a wild-type and a mouse line expressing eGFP under the GAD67 promoter and two different anti-GlyT1 antibodies, showing the presence of a subpopulation with a dual phenotype. Moreover, eGFP-positive dendrites on both mouse lines were found juxtaposed to GlyR subunits and the scaffold protein gephyrin in several areas of the inner plexiform layer, demonstrating the glycinergic character of these neurons. This dual phenotype was also demonstrated in primary retina cultures, in which isolated neurons were positive for GlyT1 and GAD67 or GAT1/3. Altogether, these data provide compelling evidence of a subpopulation of dual inhibitory, glycinergic/GABAergic amacrine neurons. The co-release of both neurotransmitters may serve to strengthen the inhibition on ganglion cells under synaptic hyperexcitability.

Keywords: GABA, amacrine neuron, glycinergic, glycine receptor, Glycine Transporter 1

1. Introduction

In the mature mammalian central nervous system, inhibitory neurotransmission is achieved mainly by the release of GABA or glycine from the presynaptic neuron followed by binding and activation of ionotropic $GABA_{A/C}$ or glycine receptors ($GABA_{A/C}R$ or GlyR respectively) (1-3). In addition, glycine participates at excitatory glutamatergic synapse, where it serves as a co-agonist with glutamate for the activation of the NMDA receptor (4). GABAergic and glycinergic neurons are widespread in many areas of the forebrain, brain stem and spinal cord; in the retina, the majority of these inhibitory cells are amacrine neurons (ACs). Their cell bodies are localized in the inner nuclear layer with dendrites extending to the inner plexiform layer, where they make synaptic contacts with bipolar, other amacrines cells and ganglion cells (5,6). In addition to these classical amacrine neurons, a small set of inhibitory interplexiform amacrine neurons extend their dendritic processes to both, the inner and outer plexiform layer, providing a feedback loop to transmit information from the inner to the outer retina. It had been reported that glycinergic and GABA amacrine neurons in the mammalian retina comprise about 35-40% and 40-50%, respectively (7-10). These inhibitory neurons together modulate the message delivered to the ganglion cells. In the rod pathway, the release of glutamate by the rod bipolar cells (RBC) drives the glycinergic AII amacrine cell (AC) to release glycine onto axons of cone bipolar and dendrites of OFF-ganglion cells. At the same level, the gap junctions between the AII AC and the ON-Cone bipolar cells relay the excitatory impulse provided by the RBC, thus the rod signal diverges onto the ON and OFF pathways with the main participation of the glycinergic AII (6,11). There are another nine types of small field glycinergic amacrine cells which could also shape the light signal before it leaves the retina. The GABAergic amacrine cells, classified as wide field AC, send inputs to other ACs, ganglion and bipolar cells (12). For instance, the GABAergic A17 AC makes reciprocal synapses onto the RBC, activating $GABA_AR$ as well as $GABA_CR$ to modulate the scotopic signal. Thus, GABA and glycine act as the main inhibitory neurotransmitters within the retina and the localization of the corresponding receptor subunits at the level of proteins and mRNA transcripts has been extensively described (13).

The termination of glycinergic neurotransmission in the CNS is achieved by the reuptake of glycine from the synaptic cleft back into the presynaptic neurons by the action of two Na^+, Cl^--dependent transporters GlyT1 and GlyT2, encoded by two different genes. These GlyTs belong to the SCL6 gene family, which includes the dopamine, serotonin, nor-epinephrine and GABA transporters (14,15). It has been postulated that GlyT1 is mainly expressed in glia cells whereas GlyT2 is strictly neuronal and juxtaposed to GlyR containing neurons (16). Nonetheless in recent years, GlyT1 has been shown to be expressed in neurons and areas devoid of GlyT2 (17-20). In the retina, GlyT1 is clearly expressed in glycinergic AC mainly localized to cell bodies of the inner nuclear layer and neuronal processes of the inner plexiform layer where it appears in puncta contacting any of the $GlyR_{(\alpha1-\alpha4)}$ subunit isoforms (2,21,22). Unlike CNS regions where GlyT2 is enriched in glycinergic neurons such as the brain stem, cerebellum and spinal cord; in the mammalian retina GlyT2 protein does not seem to be expressed or occurs at levels undetected by conventional fluorescence microscopy and biochemical techniques (16). Nonetheless, a pharmacological study showed that 50-70% of glycine uptake in complete rat retinas and retinal synaptosomes was inhibited by incubation with amoxapin, a GlyT2 inhibitor (23). Additional studies in single amacrine cell expression profiling from mice at P5 and P11

showed the presence of GlyT2 mRNA in every amacrine neuron analyzed (24). Although the experimental evidence suggests the presence of mRNA for GlyT2, more direct evidence for protein expression is yet to be reported.

The co-existence and co-release of neurotransmitters have been well established, in particular for GABA and glycine. In the spinal cord, the co-release of GABA and glycine was demonstrated in spinal cord slices and cultured neurons (25,26). In addition, several neurons in the auditory brainstem nuclei were identified as GABAergic and glycinergic by the presence of both neurotransmitters and the vesicular inhibitory amino acid transporter (VIAAT), thus suggesting the co-release of GABA and glycine. The neurons in these nuclei displayed synaptic currents sensitive to strychnine and bicuculline, mediated by GlyRs and $GABA_AR$, respectively (27). In the retina, some ACs that showed immunoreactivity for glycine, GlyT1 and the vesicular glutamate transporter vGluT3 co-release two neurotransmitters. They were called dual transmitter amacrine cells (28). Moreover, it has been recently shown that ACs comprising this cell type, known as Glutamate Amacrine Cells (GACs), actually co-release glycine and glutamate in such a manner that they perform a cross-over excitation-inhibition between the OFF and the ON channels (29,30). To investigate if GlyT2 protein is expressed in the retina and whether dual ACs GABAergic/glycinergic exist, we examined retina sections from a transgenic mouse expressing the enhanced green fluorescent protein (EGFP) under control of GlyT2 promoter, pGlyT2-EGFP. The results suggest that GlyT2 promoter is active in amacrine neurons but the GlyT2 protein may be expressed at low levels undetected by conventional fluorescence microscopy methods. On the other hand, we also report a novel subpopulation of AC expressing GlyT1 and GAD67 or GAT1/3 demonstrating the presence of dual transmitter amacrine cells in the mouse retina. These dual glycinergic and GABAergic markers were also observed in amacrine neurons from wild-type mice and confirmed in retinas from the transgenic line expressing eGFP under GAD67 promoter (pGAD67-GFP).

2. MATERIALS AND METHODS

2.1 Antibodies

The rabbit polyclonal anti-GlyT1 antibodies were kindly donated by Detlev Boison and Dietmar Benke (University of Zurich); these highly specific antibodies recognized the rat, mouse and human GlyT1 protein in multiple applications, as shown in published manuscripts (19,31-34). The rat anti-GlyT1 antibodies were custom-ordered and raised against the same synthetic peptide corresponding to the last 19 amino acids of the mouse GlyT1, *N*-AQIPIVGSNGSSRFQDSRI-*C*, reported by Gabernet et al., 2005. The synthetic peptide was conjugated to KLH and used for rat immunization and affinity purification (Invitrogen, Waltham, MA). The results from the custom-made GlyT1 antibodies were confirmed by staining with the rabbit polyclonal anti-GlyT1 antibody from Synaptic Systems (Goettingen, Germany). The affinity purified rabbit polyclonal anti-GlyT2 antibodies were raised against the first 201 amino acids of the human *N*-terminus fused to the *C*-terminus of GST. The PCR fragment encoding amino acids 1-201 of the human GlyT2 was inserted into the plasmid pGEX-4T-1 followed by transformation into *E.coli* BL21 cells (Agilent Technologies, Santa Clara, Ca). The construct was subjected to automated DNA sequencing to confirm the correct frame prior to protein purification. The recombinant protein was purified by affinity chromatography following the manufacture recommendations (GE Healthcare Bio-

Sciences, Pittsburgh, PA) and used for custom antibody preparation (ThermoFisher Scientific, Waltham, MA). The anti-GlyT2 antibody was affinity purified in a Sepharose A column coupled to the GlyT2 protein. The antibody specifically recognized the human GlyT2 expressed in porcine aortic endothelial cells but not the human GlyT1b (35). A complete list of primary and secondary antibodies is shown in Table 1.

Table I. List of antibodies used in this study

Antibodies	Host Species	Supplier	References
GlyT1	Rabbit	Drs. Detlev Boison and Dietmar Benke, University of Zurich	(19,31-34)
GlyT1	Rat	Custom	This study
GlyT1	Rabbit	Synaptic Systems, 272103	(17,36)
GlyT2	Rabbit	Custom	This study, (35)
GAD67	Mouse	Millipore, MAB5406	(37-40)
HPC-1	Mouse	Sigma, S0664	(41-43)
GAT-1	Rabbit	Millipore, AB1570	(40,44,45)
GAT-3	Rabbit	Millipore, AB1574	(45-47)
GlyR (clone mAB4a)	Mouse	Synaptic Systems, 146011	(17,37,48)
Gephyrin (clone mAb7a)	Mouse	Synaptic Systems, 147011	(21,37,49)
Glycine	Rabbit	Dr. David Pow, Australia	(41,49,50)
Anti-Mouse (CY3)	Donkey	Jackson Immunoresearch Laboratories, 715-165-150	
Anti-Rabbit (CY3)	Donkey	Jackson Immunoresearch, Laboratories 711-165-152	
Anti-Rabbit (Cy5)	Donkey	Jackson Immunoresearch, Laboratories 111-175-144	
Anti-Rat (ALEXA 647)	Goat	Invitrogen, A-21247	
Anti-Rat (ALEXA 546)	Goat	Invitrogen, A-11081	

2.2 Animals and tissue preparation

Adult transgenic mice expressing EGFP under control of the GlyT2 promoter were used for this study (kindly provided by Dr. David Ladle, Wright University, Dayton, OH, with the permission from Dr. H. U. Zeilhofer, University of Erlangen-Nurnberg, Germany, (51)). Heterozygote pGlyT2-EGFP adults and newborn mice (Postnatal day 1-3, P1-3) were used for histology or preparation of primary cultures, respectively. Adult C57BL/6J black mouse and the heterozygote GAD67-EGFP expressing EGFP under the GAD67 promoter (CB6-Tg(GAD67-EGFP)G42Zjh/J) were obtained from Jackson Laboratories (Bar Harbor, ME). The animal use procedures were in conformance with the Guide for Care and Use of Laboratory Animals (National Institute of Health). These procedures were performed under a protocol approved by the University of Texas at El Paso (UTEP) Institutional Animal Care and Use Committee (IACUC). Two

months old animals were deeply anesthetized by intraperitoneal injection of pentobarbital (100 mg/kg) and the eyes enucleated and hemisected at the equator. After the vitreous was removed, the posterior eyecups were fixed for 15 min by immersion in freshly prepared 4% paraformaldehyde/PBS. The eyecups were rinsed in PBS and cryoprotected by step-wise immersion in 10%-, 20%- followed by overnight incubation in 30% sucrose/PBS. The eyecups were mounted in embedding medium (Tissue-Teck OCT), frozen and sectioned in a cryostat at 14-µm-thick vertical sections which were collected on gelatin-coated slides and frozen at -20 C until use.

2.3 Immunofluorescence staining and quantitation of cell numbers

Sections from the central retina were thawed and blocked for 30 minutes in 5% normal goat serum (NGS), 0.1% Triton X-100, 1% BSA in PBS, followed by overnight incubation of the two primary antibodies prepared in the blocking solution. Next day, the sections were washed three times, incubated for 2 hours with the respective secondary antibodies diluted in 5% NGS 1% BSA in PBS, and washed again. Finally, the sections were mounted with Mowiol (Sigma-Aldrich St. Louis, MO) and visualized by confocal laser scanning microscopy (Zeiss LSM 700). To determine the cell numbers, confocal Z-stack images were obtained from sections of the central retina and selected optical planes used for quantitation. The cell numbers in the plots are presented as the percentage of HPC-1 positive neurons and represent the average of a minimum of 100 HPC-1 positive cells per eye, from at least three different eyes. The final images were adjusted with Adobe-Photoshop.

2.4 Primary Neuronal Culture

Neuronal primary culture was prepared from transgenic heterozygote mice expressing EGFP/GlyT2 neurons. Animals were sacrificed by rapid decapitation and the eyes removed to obtain the retina. The retinas were collected and transferred to a Dissection Saline solution with 1.5 mM $CaCl_2$, 100 mM NaOH, Papain 20 U/ml (Worthington, Lakewood, NJ), 1mM ethylenediaminetetraacetic acid (EDTA) and 1 mM cysteine. After an incubation period of 20 minutes, the cells were plated in glass coverslips previously coated with Poly-D-lysine (Sigma-Aldrich St. Louis, MO) and ECL cell attachment matrix (Millipore, Temecula, CA). The cells were plated using DMEM (Gibco, Grand Island, NY) without L-glutamine, containing 10% fetal bovine serum (Atlanta Biologicals Lawrenceville, GA), penicillin/streptomycin and the following day the media was replaced by Neurobasal medium (Gibco, Grand Island, NY) without L-glutamine, containing Glutamax (Gibco, Grand Island, NY), B27 (Gibco Grand Island, NY), antibiotics and anti-mitotics.

2.5 Immunofluorescence staining and microscopy of cultured cells

The neurons grown on glass coverslips were washed three times with PBS and fixed with 4% paraformaldehyde for 15 minutes at room temperature. Then, the cells were permeabilized using 0.01% Saponin, 1% BSA in PBS at room temperature for 3 minutes. The cells were incubated for one hour at room temperature in the cocktail of primary antibodies made in 0.05% Saponin, 1% BSA/ PBS. After the incubation period, the cells were washed and incubated for an additional hour with the corresponding secondary antibodies prepared in 0.05% Saponin, 1% BSA/ PBS, followed by three washes. After a final rinse in deionized water, the coverslips were mounted on slides using Mowiol (Calbiochem) and analyzed by confocal laser scanning microscopy (Zeiss LSM 700). Confocal Z-stack images were obtained from the bottom to the top of the cultured neuron and a selected focal plane through the middle

of the stack used for presentation. Final arrangement of the images was performed with Adobe-Photoshop CS5.

2.6 Tissue lysis and western blotting

The brain stem or complete retina tissue from rat and mouse were homogenized in 1 ml lysis buffer (1% Triton X-100, 1% Deoxycholic acid, 0.1% SDS, 25 mM HEPES, pH 7.6, 10% glycerol, 100 mM NaCl, 10 mM sodium fluoride, 1mM phenylmethylsulfonyl fluoride), using a dounce homogenizer at 4 °C. Additional tissue was homogenized in the same lysis buffer containing 1 mM Dithiothreitol. The lysate was nutated for 10 min followed by centrifugation at 15,000 g for 15 min. The cleared lysate was separated from insoluble material and 50 μg of protein subjected to 8% SDS-gel electrophoresis followed by western blotting with rabbit GlyT2 and actin antibodies. The lysate was not boiled before SDS-PAGE to prevent GlyT1 or GlyT2 aggregation.

3. Results

3.1 GFP expression in amacrine neurons of the mouse pGlyT2-EGFP retina

The presence of glycinergic neurons in the inner nuclear layer of the mammalian retina has been extensively characterized and they are marked by the presence of glycine and GlyT1, but not GlyT2. To examine whether these neurons express GlyT2 protein, we used a transgenic mouse line pGlyT2-EGFP, kindly donated by Prof. H.U. Zeilhofer (University of Nurëmberg, Germany). This mouse line has been shown to express eGFP in neurons labeled by glycinergic markers in several encephalic nuclei and known to stimulate synaptic currents mediated by the GlyR (51,52), demonstrating to be a reliable model for studying glycinergic neurons/circuits in the mammalian central nervous system. However, ectopic expression of eGFP was reported in the piriform cortex and thalamus in the homozygote which was absent in the heterozygote mouse (51). To this extent, we prepared vertical sections from the central retina of the heterozygote pGlyT2-GFP mice and analyzed by confocal microscopy. We observed intense accumulation of eGFP in cell somata and lower levels in processes restricted to the inner nuclear (INL) and plexiform (IPL) layers (Fig. 1A); based on their morphologies and positions in the INL, these cells are classified as the AC's, according to Wässle *et al* (53). This pattern of distribution was previously reported for GFP-positive cells in the retina of this transgenic line (54). To characterize this neuronal population, we stained sections with HPC-1, a general amacrine cell marker and found that all eGFP-positive cells were positive for HPC-1, accounting for ~59 % of the total amacrine cell population (Fig.1B and E). Given that the majority of amacrine neurons are represented by inhibitory neurons, we further stained retinal sections with antibodies directed to a variety of pre- and post-synaptic glycinergic markers such as GlyT1, glycine, glycine receptor (GlyR) and gephyrin. In sections stained with anti GlyT1 antibodies, we observed specific staining at the plasma membrane of glycinergic neurons and their dendritic arbor, all of them showed also expression of eGFP, representing 38% of the total HPC-1 ACs (Fig. 1C and E). Moreover, an identical pattern of staining was obtained with a commercially available anti-GlyT1 antibody that recognizes the last 44 amino acids of the mouse GlyT1 from Synaptic Systems (Goettingen, Germany).

Given that ~59% HPC-1-positive cells expressed eGFP and only 38% were GlyT1 positive, there is a ~21% of eGFP-positive neurons devoid of GlyT1 that remain to be characterized (Figs. 1C, arrowheads and 4D). To get further insight into these neurons, we subjected sections to staining with antibodies against the neurotransmitter glycine. As shown in Figure 1D, strong immunoreactivity

was observed in cell bodies of the amacrine cells, in close proximity to the inner nuclear-plexiform layer border. Moreover, weaker glycine reactivity was detected in neighboring cell bodies toward the middle of the INL corresponding to bipolar cells that received glycine from amacrine cells through gap junctions but do not express GlyT1 (55). This pattern of staining for glycine has been previously described (49). It is worth noting that every amacrine cell with strong reactivity for glycine was also an AC expressing eGFP. Similar to the results obtained for GlyT1, some amacrine cells were devoid of glycine reactivity, confirming that 21% of the eGFP-positive ACs were devoid of GlyT1 or glycine immunostaining (Fig. 1D, arrowheads).

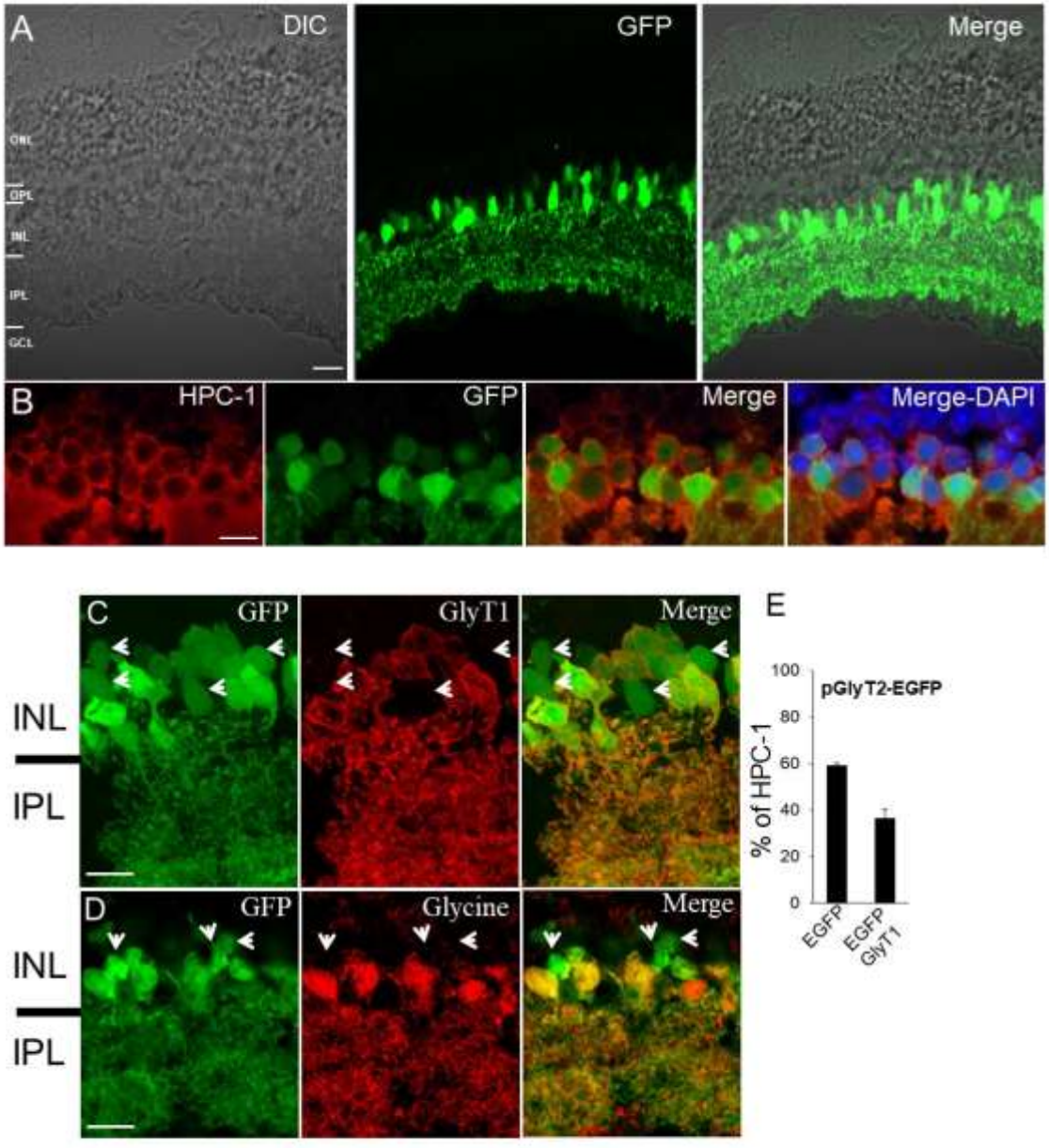


Figure 1. Characterization of inhibitory amacrine neurons in the retina of the pGlyT2-EGFP transgenic mouse. A) Vertical sections from the central retina of the pGlyT2-eGFP mouse were subjected to immunostaining and analyzed by confocal microscopy. Z-stack images were acquired and an optical section from the middle of the stack selected for presentation. Left panel: bright field showing the retinal layers; middle panel, eGFP expression; right panel, merge image. *Scale bar*, 20 µm. **B)** Vertical section stained with anti-Syntaxin 1 (HPC-1) antibodies and DAPI followed by CY-3 labeled secondary antibodies. **C)** Same as B but sections labelled with rabbit antibodies against GlyT1 or **D)** anti-glycine. *Scale bar*, 10 µm. Abbreviations: *INL*, inner nuclear layer; *IPL*, inner plexiform layer; *GCL*, ganglion cell layer. **E)** Quantitation of EGFP-positive or EGFP/GlyT1 neurons. Cell counts were obtained from a minimum of 100 HPC-1 positive cells per retina, for a total of three retinas from three different mice. The plots represent the percent of HPC-1 positive cells +/- SE. Open arrowhead in C and D indicate eGFP labeled cells devoid of GlyT1or Glycine immunoreactivity, respectively.

Consistent with other reports, GlyT2 staining with antibodies to the *N*-terminus did not show labeling at any cell layer in retina sections, suggesting low abundance, at levels undetectable by conventional fluorescence microscopy or poor reactivity of the antibody in mouse tissue (not shown). To detect the possible GlyT2 protein by a more sensitive biochemical approach, we subjected tissue lysates from the brain stem, a region enriched in GlyT2, and retina lysates to western blot analysis. Blotting was carried out with a rabbit N-GlyT2 antibody directed against the *N*-terminus (amino acids 1-201) of the human transporter. This antibody detects the endogenous mouse and rat GlyT2 by western blot and heterologous expressed human GlyT2 in *P*orcine *A*ortic *E*ndothelial (PAE) cells (Fig 2, (35)). The specificity of the N-GlyT2 antibody was also tested by western blotting of total lysates of PAE cells and stably transfected PAE-hGlyT2 cells. As shown in Figure 2B, the antibody recognized a single ~100 kDa band corresponding to the human GlyT2, absent in the empty vector transfected cells. The antibody also recognized a ~45 kDa band corresponding to the fusion protein GST-GlyT2 which was used as an antigen (Fig. 2B). Due to a large amount of GST-GlyT2 protein loaded in the gel, a minor amount of aggregation was also detected around 90 kDa, this could be because of dimer formation. Initial experiments in brain stem lysates showed an immunoreactive band at the predicted molecular weight for GlyT2 which was absent in the lane for the retina lysate, as previously reported (16). It is worth noting that in the retinal lysate, a discrete GlyT2 signal was observed at the interface between the stalking and the separating gel, suggesting possible aggregation of GlyT2 (Fig. 2C). To investigate whether that high molecular weight signal corresponded to GlyT2, we lysed the brain stem tissue and retinas in RIPA buffer containing 1 mM dithiothreitol. The blot revealed an immunoreactive band that appeared at the predicted molecular weight for GlyT2 (100 kDa) but with low abundance compared to the levels observed in the brain stem lysate (Fig. 2D). However, a reactive signal in the retina lysate remains at ~190 kDa, potentially representing SDS-resistant dimers, as shown for several SLC6 family members including GlyT2 (56-59). It is intriguing that the mRNA for GlyT2 has been detected in the retina but the protein still not identified by conventional immunofluorescence (16,24,60). The development of a highly specific antibody that recognize the rodent GlyT2 in nerve tissue by immunofluorescence should shed light into the possible expression of GlyT2 protein in the retina.

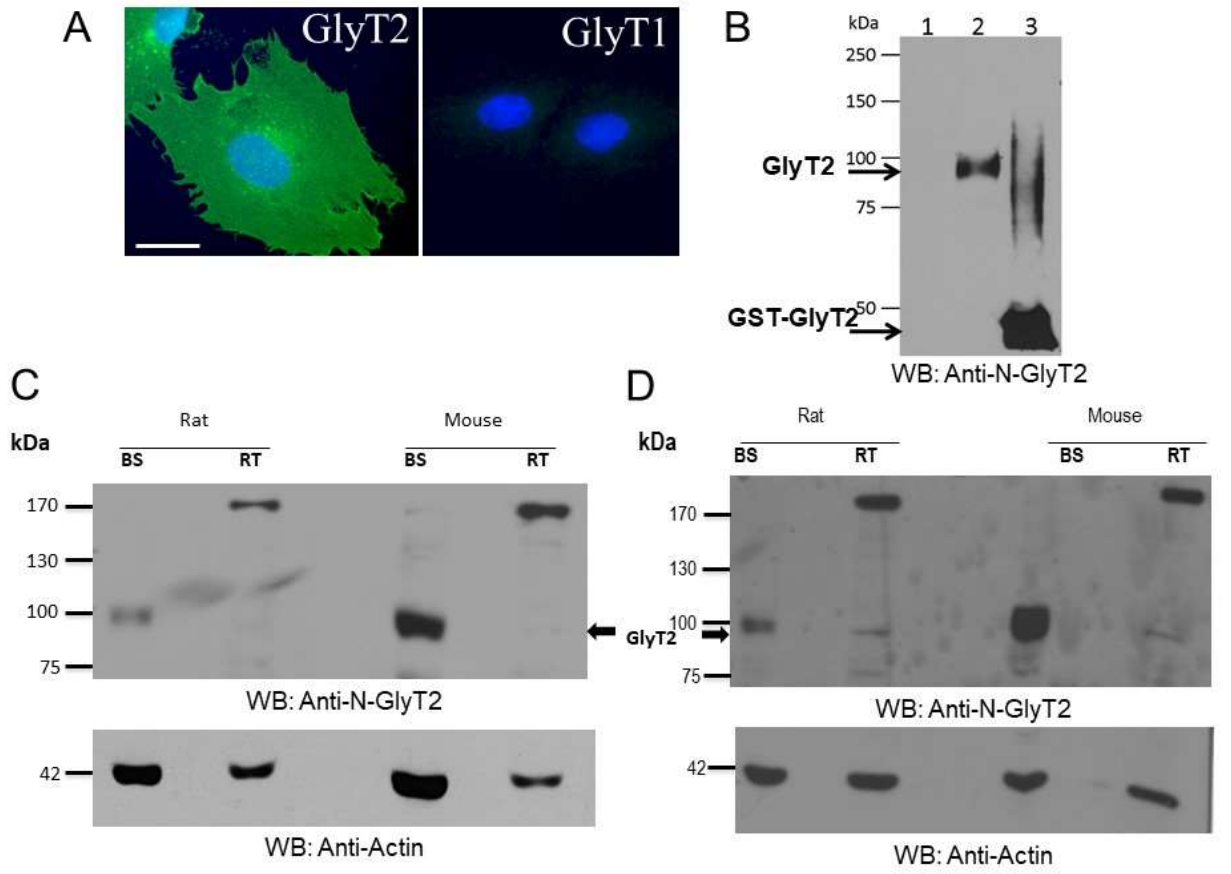


Figure 2. GlyT2 protein detected in the rat and mouse retina. A) The human GlyT2 or the GlyT1b were expressed in PAE cells and immunostained with purified rabbit anti-GlyT2 antibodies against the N-terminus of GlyT2 (Anti-N-GlyT2) followed by Alexa-488 labeled secondary antibodies and DAPI, *Scale bar*, 10 µm. **B)** Cell expressing the human GlyT2 were lysed and subjected to western blot with anti-N-GlyT2 antibodies. Lane 1, parental cells; lane 2, PAE cells expressing GlyT2; lane 3, 100 ng of the fusion protein GST-N-GlyT2. **C)** Brain stem and retinas were homogenized in RIPA buffer and cleared by centrifugation. Cleared lysates were quantitated and subjected to 8% SDS-PAGE and western blotting with a rabbit GlyT2 and actin antibodies. **D)** Same as "**A**" but the tissue was lysed in buffer containing 1 mM Dithiothreitol. The blots are representative of at least three experiments.

We also subjected additional sections to staining with the GlyR subunits and the scaffolding protein gephyrin. As shown in Fig. 3, dense labeling for gephyrin appeared all over the inner plexiform layer (IPL), particularly many overlapping areas labeled for eGFP along the AC dendrites. Other gephyrin-positive areas devoid of eGFP likely correspond to strictly GABA-receptor containing fibers. In addition, staining with the mAb4a antibody, recognizing all the known GlyR subunits, showed widespread labeling in the IPL, and dense labeling in the outer strata of IPL (OFF sublamina) juxtaposed to eGFP-positive dendrites. These results clearly demonstrated that the majority of eGFP-labeled cells correspond to glycinergic neurons that synapse with postsynaptic neurons containing GlyR and gephyrin.

3.2 Dual Glycinergic and GABAergic phenotype in a small population of amacrine neurons.

It was observed that about 27% of eGFP-positive ACs did not stain for GlyT1 or glycine antibodies in several experiments, also shown in a previous study (54). To characterize this eGFP-positive subpopulation, we stained retinal sections with GABAergic markers given that GABA neurons are abundant in the INL. As shown in Fig 4A, staining of pGlyT2-EGFP sections from the central retina with the GABA synthetizing enzyme glutamic acid decarboxylase 67 (GAD67) labeled GABA neurons in the INL and dendrites extending toward the IPL. The staining showed GABA neurons containing and devoid of eGFP expression (Fig. 4A). This staining pattern corresponds to GABAergic AC neurons in the INL and accounts for about 50% of total amacrine cell population, as demonstrated by staining with HPC-1 and quantitative analysis, values consistent with previous reports for the mammalian retina (Fig. 4D) (7,9,10,42,61-63). Quantitative analysis of the HPC-1 positive showed an average of 27% of eGFP-expressing neurons immunoreactive for GAD67 (Fig. 4E). These results suggest that in the mouse pGlyT2-eGFP the majority, if not all, of eGFP containing cells are represented by glycinergic and GABAergic neurons. It is worth noting that within the GAD67/eGFP-positive population, a small number of neurons were also labeled for GlyT1, suggesting a dual phenotype glycinergic and GABAergic, accounting for 3-5% of the HPC-1 positive amacrine cells, equivalent to 8-10 % of the total glycinergic amacrine neurons (Fig. 4A, arrowheads and 4E). To provide additional support of these results, we subjected sections to staining with antibodies against the plasma membrane GABA transporter, isoforms 1 and 3 (GAT1 and GAT3 respectively). As shown in Fig. 4B, GAT1 labeled cell bodies of AC in the INL that extended their dendrites to the IPL; additional labeling was observed in the ONL, extending to membranes located in the outer nuclear layer, consistent with GAT-1 distribution reported in previous studies (64,65). As depicted in the merged image, a small percentage of GAT-1 positive neurons in the INL showed co-localization with GlyT1 (Fig. 4B, see arrows). Likewise, co-localization was observed also for GAT-3 and GlyT1, providing additional evidence of this dual, glycinergic and GABAergic phenotype (Fig. 4C, marked by arrows).

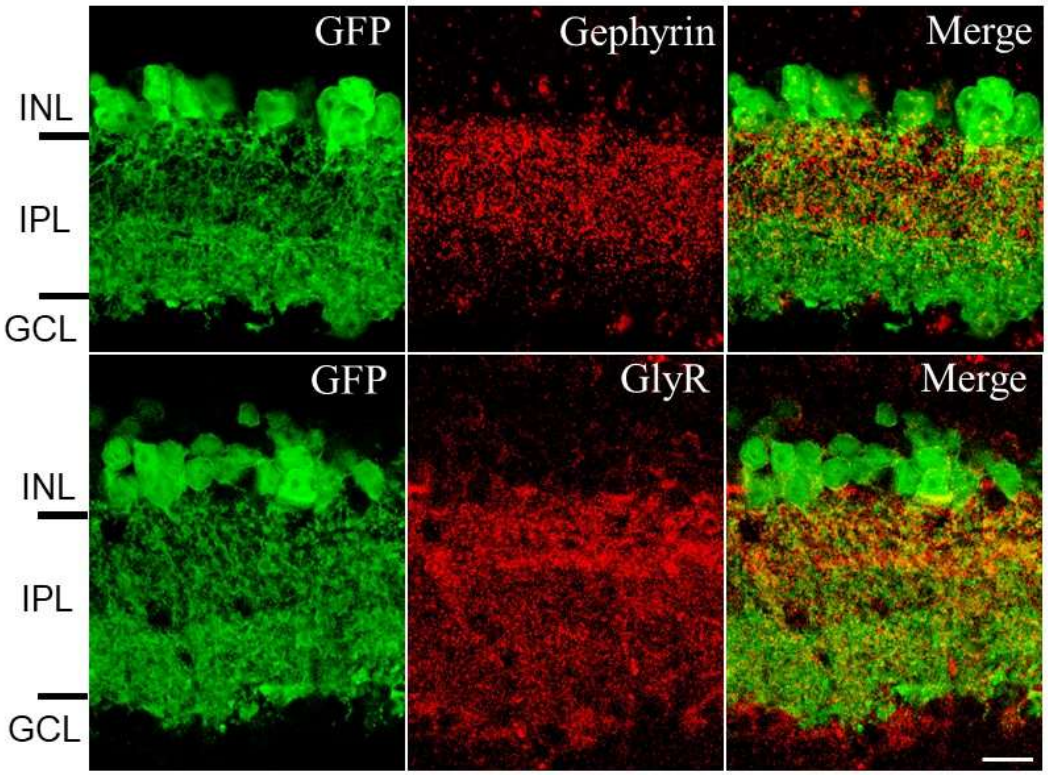
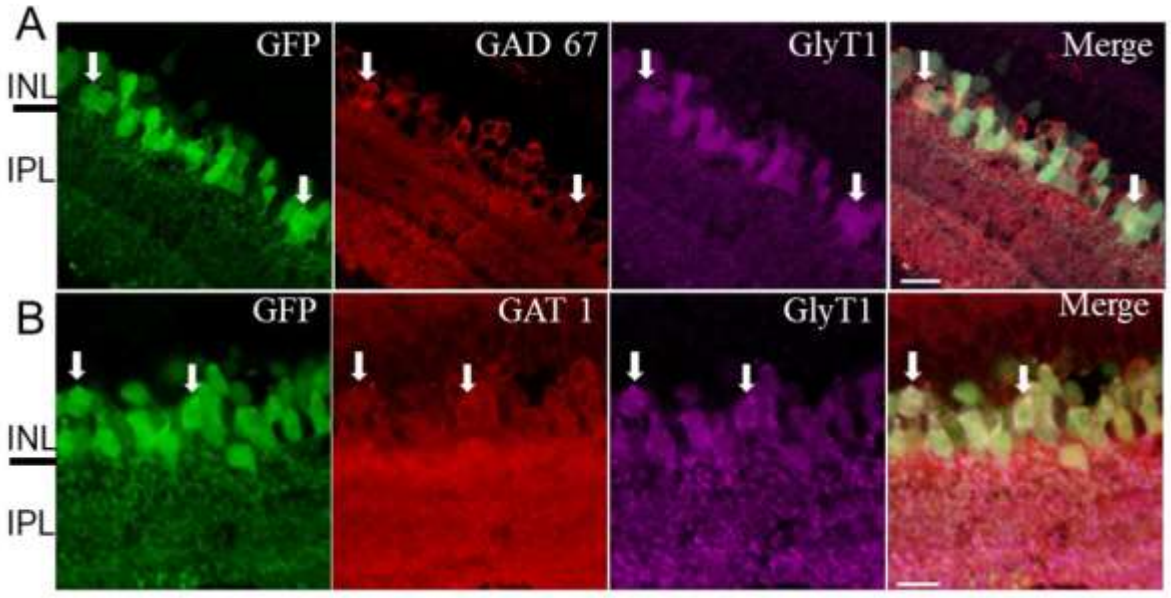


Figure 3. Localization of eGFP-positive neurons with post-synaptic markers in retinas of the pGlyT2-EGFP transgenic mouse. Vertical section of the central mouse retina were labelled with antibodies against the protein gephyrin (upper panel) or the GlyR subunits using the mAb4a antibody that recognizes all the receptor subunits, followed by staining with CY-3 labeled secondary antibodies. Confocal Z-stack images were acquired and an optical section from the middle of the stack selected and used for presentation. *Scale bar*, 10 µm.



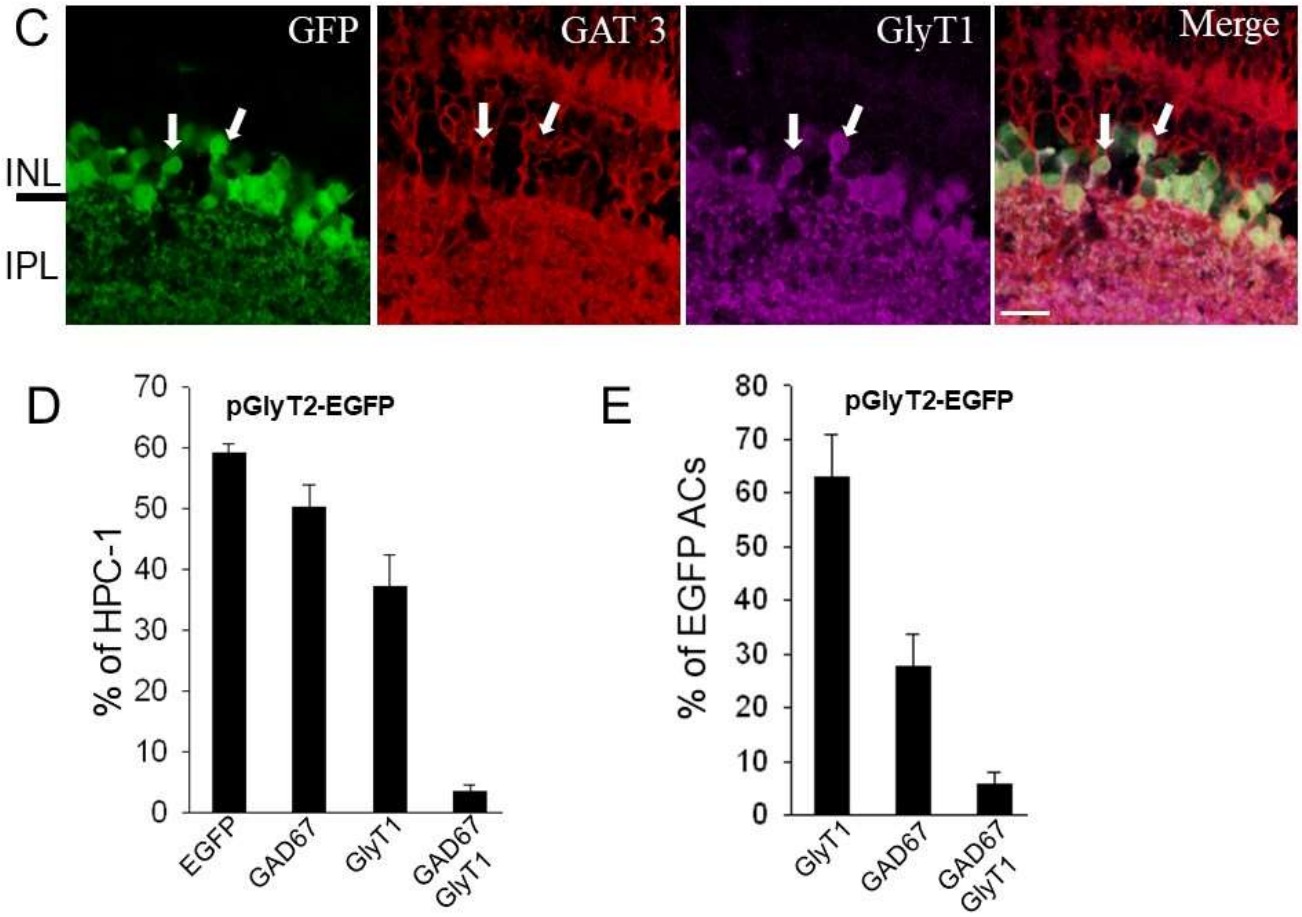
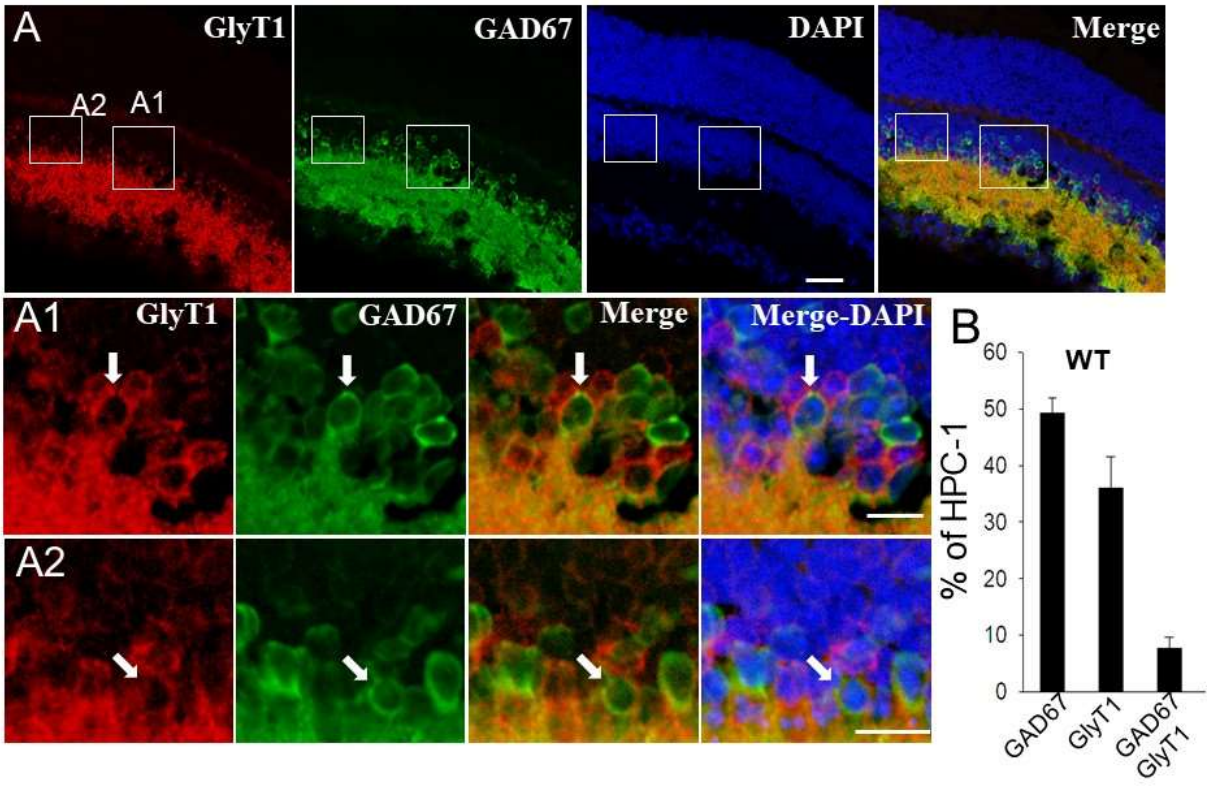


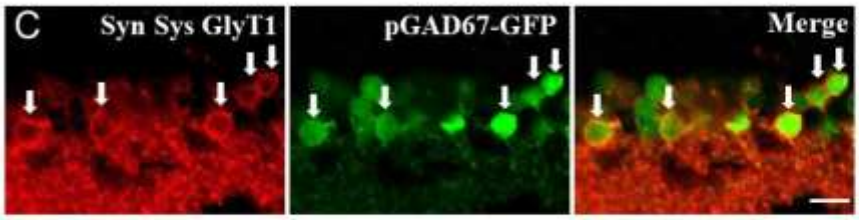
Figure 4. The mouse retina contains a subpopulation of dual GABAergic and glycinergic amacrine neurons. A) Vertical sections from the central retina of the pGlyT2-eGFP mouse were immunolabeled with antibodies recognizing the GABA synthetizing enzyme GAD67 and rabbit GlyT1 antibodies or synaptic systems or followed by the corresponding secondary CY-3 and CY-5 labeled antibodies. Z-stack images were acquired through the corresponding channels and an optical section from the middle of the stack selected and used for this figure panels. **B)** Sections were stained as in **A** but using the rabbit GABA transporter anti-GAT1 and rat anti-GlyT1 antibodies or **C)** GAT3 and rat GlyT1. *Scale bar* 10 µm. Abbreviations: *INL*, inner nuclear layer; *IPL*, inner plexiform layer. Open arrowhead in A-C indicate eGFP labeled cells containing GlyT1 and GAD67, or GAT1 or GAT3. **D)** A minimum of 100 HPC-1 positive cells per retina from three different eyes were used to quantify the number of GAD67 or GlyT1 positive cells and those containing both markers. The plots represent the percent of GAD67 or/and GlyT1 positive cells +/- SE. **E)** Same as D but normalized by 100 EGFP positive ACs.

It was reported earlier that GABAergic and glycinergic amacrine neurons are mutually exclusive in the mouse and macaque retinas (41,49). The group Kay *et al* (2011) and many others have reported their findings using a goat anti-GlyT1 antibody, which is currently not available. On the other hand, Haverkamp and Wassle (2000) based their conclusion on the results obtained by staining mouse retinal sections with GABA and Glycine antibodies and did not present results by combining GAD and GlyT1. They found variations in the staining of GABA neurons that were dependent on the specific antibody used for immunofluorescence (49). We therefore validated our findings with a different anti-

GlyT1 antibody and decided to test a different commercially available antibody from synaptic systems (Synaptic Systems, Gottingen, Germany). To get further insights into these dual inhibitory neurons, we obtain sections of a wild-type C57BL/6J and subjected to staining with GAD67 and our GlyT1 or ssGlyT1 antibodies. In sections from three different wild-type mice, antibodies for GAD67 and GlyT1 stained amacrine neurons in the INL (Fig 5A, insets A1 and A2, highlighted by arrows). After analyzing the localization and quantity of cells with both markers, we identified a consistent 8-10% of ACs expressing both markers, GAD67 and GlyT1, providing additional evidence of the presence of dual –glycinergic/GABAergic population of neurons (Fig. 5B). Consistent with our findings in the mouse retina, the presence of subpopulations of GlyT1/GAD65 or GABA/glycine containing neurons have been reported in the human, macaque and marmoset retinas, accounting for 3-10% of the total inhibitory ACs (8,66-68).

Given that the majority of studies characterizing the glycinergic and GABAergic amacrine neurons have been performed with anti-GAD or anti-GAT antibodies, we took the advantage of available transgenic lines expressing reporter genes in GABA neurons. These mouse lines have allowed the discovery of new subpopulations of neurons with poor expression of target proteins. To this extent, we subjected retinas from the transgenic line expressing the EGFP under control of the GAD67 promoter (pGAD67-GFP) to staining with GlyT1. As shown in Figure 5C, ssGlyT1 antibody stains the plasma membrane of glycinergic cell bodies and their dendritic arbor whereas eGFP accumulates in the cell bodies of neurons of the INL. As depicted in the merged image, some cell bodies of GFP-positive AC’s were surrounded by GlyT1 reactivity (Fig 5C, see arrows). Immunostaining with HPC-1 and quantitation showed that an average of 70-73% AC's expressed eGFP and 30% of these were positive for GlyT1 (Fig. 5D). The high percentage of GFP-positive cells is probably given by ectopic expression of the transgene in non-GABAergic neurons since staining of these sections with GAD67 antibody labeled about 50% of HPC-1 cells, all of them GFP-positive cells (not shown). Altogether, these data demonstrate the co-existence of the GABA-synthesizing enzyme and GlyT1, thus providing evidence for the presence of GABA and glycine as neurotransmitters in this subpopulation of AC.





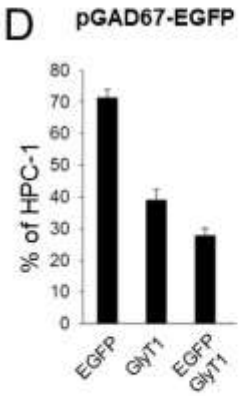


Figure 5. Co-expression of GAD67 and GlyT1 in amacrine neurons from wild-type retinas and the transgenic mouse expressing eGFP under GAD67 promoter. A) Vertical sections from the central retina of C57BL/6J mice were subjected to immunostaining with GlyT1 and GAD67 antibodies followed by incubation with CY-3 and Alexa-488 labeled secondary antibodies, respectively. *Scale bar*, 50 µm. **A1 and A2)** Magnification of the areas enclosed in panel A, cells labeled for GAD67 and GlyT1 are marked by an arrow. **B)** Vertical sections were stained with HPC-1, GlyT1 and GAD67 and the resulting images used for quantitation as described in 4D. **C)** Vertical sections from the central retina of the transgenic line pGAD67-EGFP were labeled with GlyT1 antibody from Synaptic Systems followed by incubation with CY-3 labeled secondary antibodies. Nuclei were stained with DAPI. Arrows indicate co-expression of GlyT1 and eGFP in amacrine neurons. *Scale bar*, 10 µm for B and C. **D)** Sections from the pGAD67-EGFP were subjected to quantitation analysis, as described in 4D. *Scale bar*, 10 µm.

3.3 Identification of glycinergic and GABAergic markers in dissociated retinal neurons.

To seek for more direct evidence of the co-presence of glycinergic and GABAergic makers in individual AC, we prepared primary cultures from retina of the heterozygote pGlyT2-GFP transgenic C57BL/6 mice at P1-P3. The retinas were dissociated and the cells cultured and maintained for 10-15 days to allow differentiation. On the day of the experiment, the cultures were fixed with paraformaldehyde and subjected to immunostaining and confocal microscopy. As shown in Fig. 6A, eGFP containing neurons were readily identified and displayed strong eGFP fluorescence in the cell body and less accumulation in the dendritic arbor. Staining of the neurons with GAD67 allowed the identification of three set of neurons, one of them was labeled with GAD67 and eGFP-positive (Fig. 6A, arrows) and other two populations contained either GAD67 or eGFP (Fig. 6A, open or solid arrows, respectively). Finally, GlyT1 staining revealed a few neurons also positive for eGFP and GAD67, providing evidence of amacrine neurons with dual phenotype at the single cell level (Fig 6A, open arrowheads). Likewise, these eGFP-containing neurons showed immunoreactivity to GlyT1 and GAT1 or GlyT1 and GAT3 antibodies (Fig. 6B and C). It is worth mentioning that GlyT1 staining and eGFP expression was restricted to neurons and completely absent in glia cells, as demonstrated by staining with anti-GFAP antibody (not shown). Altogether, these findings demonstrate the presence of a population of GABA/Glycinergic amacrine neurons marked by co-expression of GlyT1, GAD67, GAT1 and GAT3.

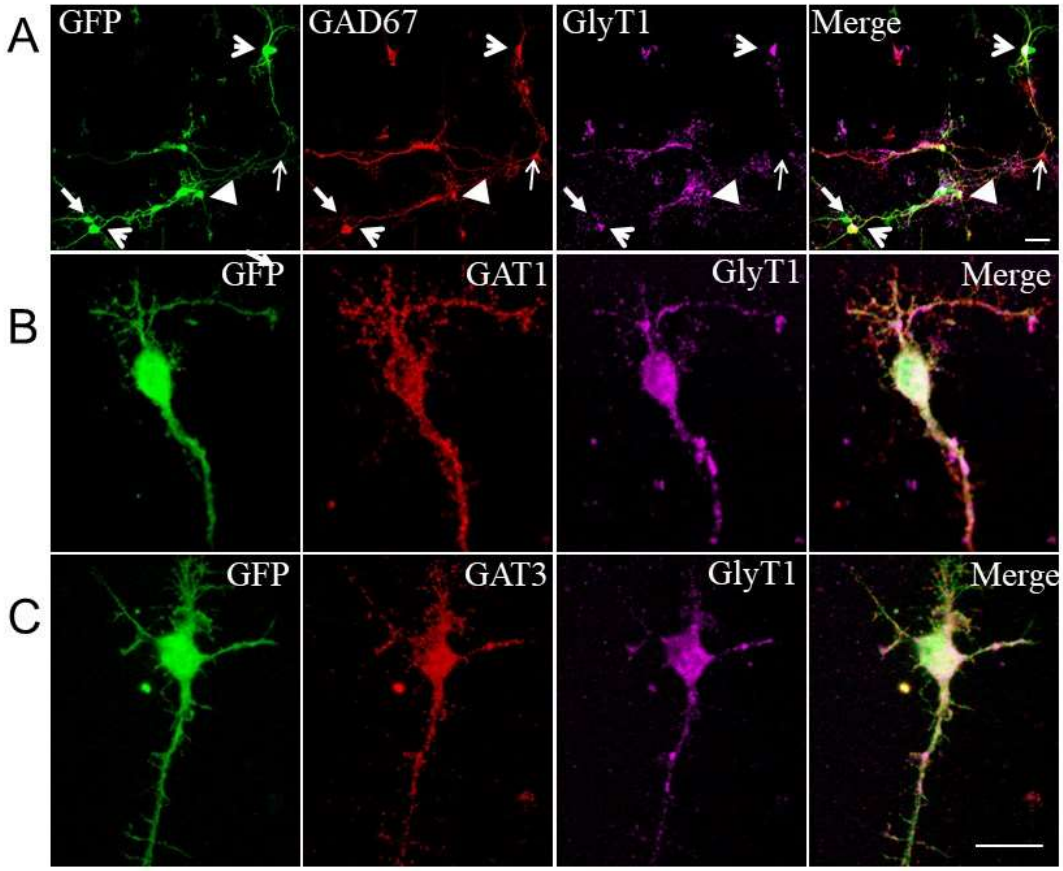


Figure 6. GABAergic and glycinergic markers co-expression in cultured cells isolated from pGlyT2-eGFP transgenic mouse. A) Primary dissociated retinal cultures were fixed with paraformaldehyde and incubated with antibodies recognizing GAD67 and GlyT1 followed by staining with the corresponding CY-3 and CY-5 labeled antibodies, respectively. The inhibitory neurons were heterogeneous and contained eGFP and GlyT1 (long arrows), or eGFP, GAD67 and GlyT1 (open arrowheads), or GAD67 (thin open arrows). **B)** Cells were subjected to immunostaining with antibodies against GAT1 and GlyT1 or **C)** against GAT3 and GlyT1 as described under Experimental Procedures. *Scale bar*s, 10 µm.

4. Discussion

In the mammalian central nervous system, glycinergic neurotransmission is controlled by the action of two closely related glycine transporters, GlyT1 and GlyT2. It has been postulated that GlyT2 is restricted to the presynaptic glycinergic neurons, where its main function is to replenish glycine into the neuronal cytoplasm to be used as substrate for the vesicular inhibitory amino acid transporter (VIAAT) (69-71). On the other hand, glycinergic neurons in the retina are known to express GlyT1 but the detection of GlyT2 with a variety of antibodies has failed. In this study, we used the pGlyT2-EGFP mouse line where eGFP expression is driven by the GlyT2 promoter and look for GlyT2 protein within the retina. Previous studies with this mouse line have shown that the eGFP expressing neurons also express the GlyT2 protein and/or the neurotransmitter glycine in the brain stem, demonstrating their glycinergic phenotype (26,51,72). Additional published work and the current data describe that eGFP expressing cells in the mouse retina correspond to glycinergic ACs (54,60), with a morphology and position similar to ACs described in the rat retina (53). These ACs are positive for glycine

and GlyT1, similar to those glycinergic neurons described for the spinal cord and the encephalon (26,51). Given that the expression of eGFP is driven by the GlyT2 promoter, our data suggest the expression of this glycine transporter in the rodent retina. The expression of GlyT2 is also supported by identification of the mRNA from transcriptomic data in the rat retina, northern blot from isolated mouse retina or single amacrine cell genome-wide expression profiling in mice retina, discussed below (24,60,73).

Previous studies have not detected the GlyT2 protein by conventional immunofluorescence and microscopy techniques using antibodies against the *N*- or *C*-terminus (16,60). From these results, we speculate about the possibility that: (1) GlyT2 is expressed at levels to be detected by pharmacological methods but below the threshold of detection by antibodies (23). A similar scenario of detection has been reported for some ipRGCs subtypes in which melanopsin could not be detected by traditional immunohistochemistry with the use of highly sensitive antibodies (74). Remarkably, the larger variety of ipRGCs was only detected and characterized by the use of transgenetic mouse models (75-77). Based on these findings, we can also hypothesize that the GlyT2 directed antibodies are not sensitive enough to detect low levels of the GlyT2 protein by immunofluorescence. However, we were able to detect GlyT2 protein by western blot and consistent with our hypothesis, the levels of protein expression at the predicted molecular weight are low compared to those observed in the brain stem. On the other hand, (2) we can also speculate that the fixation method induces modifications on the exposed GlyT2 epitopes preventing binding of the antibody. Nonetheless, the transgenic mouse pGlyT2-eGFP provides evidence of an active GlyT2 promoter in a subpopulation of glycinergic AC in the retina, suggesting that this transgenic line is more sensitive than immunofluorescence for detection of GlyT2 containing neurons. This hypothesis is also supported by the presence of GlyT2 transcripts in the rat and mouse retina (60,78), and by the effects of GlyT1 and GlyT2 inhibitors such as amoxapine and sarcosine on glycine uptake from rat retinas (23). Additional evidence that supports GlyT2 expression came from single cell profiling analysis of mouse amacrine neurons isolated at 6 different environmental ages. Of 32 single profiled amacrine neurons, 14 were shown to express GlyT1 and 16 Gad1, demonstrating the inhibitory character of these characterized interneurons (24,73). Interestingly, these results of glycinergic AII amacrine neurons demonstrated the presence of the GlyT2 mRNA at postnatal days (PD) 5 and PD11. In addition, they reported three glycinergic outliers in the cluster with GABAergic cells expressing low levels of Gad1 (24). More recent evidence on the GlyT2 expression in the rodent retina came from next-generation sequence analysis in rat retinas. This RNA-Seq analysis allowed the identification of 15442 expressed genes, among them, GlyT2 was identified with a minimum of 10 counts (73). These findings combined with our results provide evidence of the expression of the mRNA and GlyT2 protein in the rodent retina.

The data presented here about eGFP expression in neurons devoid of GlyT1 suggests a degree of ectopic expression of the transgene in the pGlyT2-EGFP. This is supported by the identification of individual GAD67 positive neurons expressing GFP and devoid of GlyT1, but within the inhibitory amacrine neurons. This ectopic expression can be explained by the fact that some neurons from the brain stem and amacrine cells can express GABA/glycinergic markers, as well as both neurotransmitters, and therefore some transcription factors in selective GABA neurons likely recognize the GlyT2 promoter in the pGlyT2-GFP transgenic line (26,42,79).

It is worth mentioning that a highly sensitive antibody to the mouse GlyT2 needs to be developed to better explore whether GlyT2 is expressed in the mammalian retina.

To date, the generation of transgenic lines labeling GABAergic cells has allowed the identification of populations of inhibitory neurons releasing GABA and glycine and therefore called dual GABAergic and glycinergic neurons (80-82). In this study, we also report the co-existence of GABAergic protein markers within a subpopulation of glycinergic GlyT1-positive ACs in the retina of wild-type and transgenic mice. These neurons were labeled by the VIAAT, GAD67, the GABA transporters GAT1 and GAT3 and the glycine transporter GlyT1, either in retinal sections or in retinal primary cultures. Consistent with these findings, a number of studies have described a high proportion of the GABAergic and glycinergic populations co-occur along with other neurotransmitters in the auditory brain stem, such as neuro-active peptides and biogenic amines (27,83-85). In addition, recent work demonstrated the co-release of GABA and glycine in cultured neurons from the spinal cord, prepared from the transgenic pGlyT2-eGFP line (26). Nonetheless, published work supports the presence of two separate population of GABA or glycine ACs in the mammalian retina, using antibodies against the neurotransmitters or anti-GlyT1 antibodies currently not commercially available (41,49). We believe that the specificity and affinity of the antibodies for their target is a factor that could lead to confounding results and previously discussed by Haverkamp and Wassle (2000). Since we were unable to test the antibodies used in previous studies, we provided additional support to our hypothesis by staining with two anti-GlyT1 antibodies, anti-GAT and -GAD antibodies, in retinas from two transgenic lines and wild-type black mouse.

In the rodent retina, it is known that AC and ganglion cells receive simultaneously glycine and GABA and the currents have been recorded from retinal slices using the patch clamp technique (86,87). These studies support the co-release of both GABA and glycine from a single presynaptic AC neuron onto a single postsynaptic neuron and the presence of dual GABA/glycine amacrine neurons. This hypothesis is supported by the co-localization of the amino acids glycine and GABA in the same amacrine neurons in the primate retina, accounting for 3-4% of the amacrine cells in those studies (8,67). Additional evidence come from the detection of GABA and Glycine receptors onto a single neighbor AC neuron (88) or ganglion cells in the mouse and rat retina (86,87). The co-release of two inhibitory neurotransmitters and the presence of their receptors on postsynaptic neurons could be important for the postsynaptic neuron at the time of integration of excitatory and inhibitory inputs. The small amplitude, short duration, synaptic current of one of the inhibitory transmitters would lengthen the interspike period, whereas the large amplitude long time decay of the second inhibitory transmitter would have a shunting effect, preventing the postsynaptic neuron from spiking at all. Not surprising, recent evidence has shown that glutamate amacrine cells which are positive for GlyT1 and vGluT3 release glycine and glutamate onto two different types of ganglion cells. Glycine activates glycine receptors on uniformity detectors and glutamate excites ganglion cells engaged in contrast sensitivity, which makes GACs dual excitatory/inhibitory transmitter cells (29,30). Finally, we propose that the presence of neurons handling two different neurotransmitters combined with their corresponding transporters could be critical for the duration and the amplitude of the signal onto the receiving amacrine and ganglion cells under different light intensities.

5. Conclusions

This study demonstrates the presence of a subpopulation of amacrine glycinergic neurons co-expressing GAD67 and GABA transporters in the mouse retina. Given that GABA and glycine are recognized by the same vesicular transporters for vesicle refilling, this dual phenotype is determined by the GlyT1 and GABA markers. The co-release of two inhibitory neurotransmitters is likely to be critical for fast and prolong inhibition of excitation because of the corresponding kinetics of the glycine and GABA receptors, respectively.

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