

Diversity of Glycine Receptors in the Mouse Retina: Localization of the $\alpha 2$ Subunit

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ABSTRACT

γ -Aminobutyric acid (GABA) and glycine are the major inhibitory neurotransmitters in the retina, glycine being produced in approximately half of all amacrine cells. Whereas retinal cell types expressing the glycine receptor (GlyR) $\alpha 1$ and $\alpha 3$ subunits have been mapped, the role of the $\alpha 2$ subunit in retinal circuitry remains unclear. By using immunocytochemistry, we localized the $\alpha 2$ subunit in the inner plexiform layer (IPL) in brightly fluorescent puncta, which represent postsynaptically clustered GlyRs. This was shown by doubly labeling sections for GlyR $\alpha 2$ and bassoon (a presynaptic marker) or gephyrin (a postsynaptic marker). Synapses containing GlyR $\alpha 2$ were rarely found on ganglion cell dendrites but were observed on bipolar cell axon terminals and on amacrine cell processes. Recently, an amacrine cell type has been described that is immunopositive for glycine and for the vesicular glutamate transporter vGluT3. The processes of this cell type were presynaptic to GlyR $\alpha 2$ puncta, suggesting that vGluT3 amacrine cells release glycine. Double labeling of sections for GlyR $\alpha 1$ and GlyR $\alpha 2$ subunits showed that they are clustered at different synapses. In sections doubly labeled for GlyR $\alpha 2$ and GlyR $\alpha 3$, approximately one-third of the puncta were colocalized. The most abundant GlyR subtype in retina contains $\alpha 3$ subunits, followed by those containing GlyR $\alpha 2$ and GlyR $\alpha 1$ subunits. *J. Comp. Neurol.* 477:399–411, 2004. © 2004 Wiley-Liss, Inc.

Indexing terms: mouse retina; glycine receptors; $\alpha 1$ subunit; $\alpha 2$ subunit; $\alpha 3$ subunit

Glycine and γ -aminobutyric acid (GABA) are the major inhibitory neurotransmitters of the mammalian retina. Approximately half of the amacrine cells release glycine, whereas the other half release GABA (Marc, 1989; Pourcho, 1996; Pow and Hendrickson, 1999). Glycinergic amacrine cells comprise more than 10 morphological types (Vaney, 1990; MacNeil and Masland, 1998; Menger et al., 1998) but share relatively small dendritic trees. The diffuse and often bistratified organization of these dendritic trees suggest that they perform local circuit operations between the different sublaminae of the inner plexiform layer (IPL). Glycinergic amacrine cells receive synaptic input from bipolar cells at ribbon synapses and from other amacrine cells—both GABAergic and glycinergic—at con-

ventional synapses. Their output synapses contact bipolar cells, other amacrine cells and ganglion cells (Pourcho and Owczarzak, 1991a,b; Sassoè-Pognetto et al., 1994). Glycinergic amacrine cells have a high-affinity uptake system for

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glycine (Pourcho and Goebel, 1985; Marc, 1989) and express the glycine transporter GlyT1 (Zafra et al., 1995; Menger et al., 1998; Pow and Hendrickson, 1999). The most numerous glycinergic amacrine cell is the AII cell, the interneuron of the rod pathway (Pourcho and Goebel, 1985). Recently, two further glycinergic amacrine cells were identified by their selective immunolabeling for either aquaporin (Kim et al., 2002) or the vesicular glutamate transporter vGluT3 (Haverkamp and Wässle, 2004). In the rabbit retina, DAPI 3-positive cells have been identified as a further glycinergic amacrine cell type (Wright et al., 1997).

The postsynaptic glycine receptor (GlyR) is prominently expressed in the spinal cord (Becker et al., 1988), in the brainstem (Friauf et al., 1997), and in the retina (Wässle et al., 1998). The GlyR is a ligand-gated chloride channel composed of three copies of ligand binding α subunits and two copies of the structural β subunit. The β subunit binds to the clustering protein gephyrin (for review see Vannier and Triller, 1997; Harvey and Betz, 2000; Legendre, 2001). Molecular cloning has revealed four genes encoding the α subunits ($\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 4$) and only one gene encoding the β subunit (Matzenbach et al., 1994; Laube et al., 2002).

The immunocytochemical localization of GlyRs in the retina and in other areas of the CNS has so far mainly been studied with three monoclonal antibodies (Pfeiffer et al., 1984; Schröder et al., 1991). Antibody mAb2b raised against the N-terminus of the $\alpha 1$ subunit recognizes the $\alpha 1$ subunit; mAb4a raised against a peptide (96–105) common to all subunits recognizes the $\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 4$ (and to a lesser extent the β subunit); and mAb7a recognizes most isoforms of gephyrin. Recently, a polyclonal antibody has been raised in rabbits against the C-terminus of the $\alpha 3$ subunit (Haverkamp et al., 2003a). When these antibodies were applied to the mammalian retina, they revealed a clustering of GlyRs in the IPL (Grünert and Wässle, 1993). Electron microscopy showed that the GlyR clusters represent densely packed GlyRs at postsynaptic sites (Sassoè-Pognetto et al., 1994; Sassoè-Pognetto and Wässle, 1997). Both light and electron microscopy showed also the presence of gephyrin aggregates at postsynaptic densities (Pourcho and Owczarzak, 1991a,b). It appears that all retinal GlyRs depend on gephyrin for their synaptic clustering, because, in gephyrin knockout mice (Feng et al., 1998), GlyRs are no longer aggregated at synapses (Fischer et al., 2000).

The GlyR $\alpha 1$ subunit is found at the synapses between AII amacrine cells and OFF-cone bipolar cells (Sassoè-Pognetto et al., 1994) and also on amacrine and ganglion cell dendrites (Koulen et al., 1996). Some $\alpha 1$ clusters have also been detected in the outer plexiform layer (OPL) and possibly represent the postsynaptic targets of glycinergic interplexiform cells (Smiley and Yazulla, 1990). GlyR $\alpha 3$ subunit clusters are found in four bands of high density across the IPL but are rarely colocalized with GlyR $\alpha 1$ clusters, suggesting that they are localized at different synapses. Double labeling with additional markers has suggested that GlyR $\alpha 3$ subunits are located on bipolar cell axon terminals and amacrine cells (Haverkamp et al., 2003a).

The present study describes the localization of the GlyR $\alpha 2$ subunit in the mouse retina. GlyR $\alpha 2$ transcripts have been found in adult rat retina by *in situ* hybridization (Greferath et al., 1994). This is in contrast to the spinal cord, where GlyR $\alpha 2$ subunits are observed only in embry-

onic and juvenile animals (up to postnatal day 14) and are gradually replaced by the “adult” GlyR $\alpha 1$ and $\alpha 3$ subunit isoforms (Becker et al., 1988; Malosio et al., 1991; Singer et al., 1998). To study the expression of GlyR $\alpha 2$ in the retina at the protein level, a novel polyclonal antiserum against the C-terminus of the subunit was raised. However, this antiserum showed some cross-reactivity with the $\alpha 1$ subunit. By contrast, an additional polyclonal antiserum against the N-terminus of the GlyR $\alpha 2$ subunit showed no cross-reactivity with other GlyR subunits. As previously described for $\alpha 1$ and $\alpha 3$ subunits, numerous GlyR $\alpha 2$ clusters were observed throughout the IPL. The colocalization of the three GlyR subunits and their expression by bipolar, amacrine, or ganglion cells were studied in double-labeling experiments.

MATERIALS AND METHODS

Retinae of adult wild-type (C57BL/6J) mice were used for these studies. Retinae of transgenic mice expressing green fluorescent protein (GFP) under the control of the Thy1 promoter were used to study ganglion cell labeling (Feng et al., 2000). They were a kind gift of Dr. J. Sanes (St. Louis, MO). Thy-1 is a major cell-surface glycoprotein of rat thymocytes (Letarte-Muirhead et al., 1975) and has been localized to ganglion cells of the retina (Barnstable and Dräger, 1984). Retinae of transgenic mice expressing enhanced GFP (EGFP) under the control of the promoter of the glycine transporter GlyT2 were used to study the labeling of glycinergic amacrine cells (Zeilhofer et al., 2003). The animals were deeply anesthetized with halothane and killed by cervical dislocation. All procedures were approved by the local animal care committee and were in accordance with the law for animal experiments issued by the German Government (Tierschutzgesetz). The eyes were removed and dissected, and the posterior eye cup containing the retina was immediately immersed in 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4, for 8–10 minutes. After fixation, the retina was dissected from the eye cup and cryoprotected in graded sucrose solutions (10%, 20%, and 30% w/v, respectively). Cryostat sections were cut at 14 μ m, mounted, and stored at -20°C .

Antibodies

Three mAbs raised against purified GlyRs were used in the present study (kindly provided by Heinrich Betz, Frankfurt, Germany): mAb2b is specific for the N-terminal 10 residues of the GlyR $\alpha 1$ subunit (Schröder et al., 1991); mAb4a recognizes an epitope between positions 96 and 105 of the GlyR $\alpha 1$ subunit (Schröder et al., 1991), which is highly conserved in all α subunits and the β subunit (Grenningloh et al., 1987, 1990; Kuhse et al., 1990; Harvey et al., 2000); mAb7a is specific for the GlyR clustering protein gephyrin (Pfeiffer et al., 1984). These antibodies were diluted 1:100. A rabbit polyclonal antibody against the C-terminal 13 amino acids of the mouse GlyR $\alpha 3$ subunit was used as previously described (Haverkamp et al., 2003a) at a dilution of 1:400.

Two polyclonal antibodies against the GlyR $\alpha 2$ subunit were used. A rabbit polyclonal antibody was raised against the peptide CTYKIIRHEDVHKK, which comprises the C-terminal 13 amino acids of the mouse GlyR $\alpha 2$ subunit, with an additional N-terminal cysteine residue. High-performance liquid chromatography-purified peptide (95% purity) was coupled to keyhole limpet hemocyanin by means

of the N-terminal cysteine thiol and used to immunize New Zealand white rabbits. Affinity purification was performed by using the synthetic peptide coupled to sulfo-link gel (Multiple Peptide Systems, Inc., San Diego, CA). The other antibody against the GlyR $\alpha 2$ subunit was purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and was raised in goat against the N-terminal 18 residues of the human GlyR $\alpha 2$ subunit. Specificity of both anti-GlyR $\alpha 2$ antisera was assessed by immunohistochemistry of HEK 293T cells transfected with human GlyR $\alpha 1$, rat GlyR $\alpha 2$, rat GlyR $\alpha 3L$, and mouse GlyR $\alpha 4$ subunit cDNAs. Coverslips carrying transfected cells were fixed by immersion in 4% paraformaldehyde (w/v) in phosphate-buffered saline (PBS; pH 7.4) for 2.5 minutes and then permeabilized for 20 minutes [0.1% Triton X-100 in PBS, 1% bovine serum albumin (BSA)], followed by 30 minutes of incubation in blocking buffer [10% normal donkey serum (NDS), 1% BSA in PBS] before processing for immunocytochemistry. In double-labeling experiments, incubations were performed with the goat (gt) anti-GlyR $\alpha 2$ antiserum (1:200 in 1% BSA, 3% NDS, PBS) and mAb4a (1:100), followed by secondary antibody incubation (as described in detail below for retinal sections). Coverslips were mounted with Aqua Poly/Mount (Poly Sciences, Inc., Eppenheim, Germany) and analyzed by fluorescence microscopy. In cultures transfected with the GlyR $\alpha 1$, $\alpha 3$, or $\alpha 4$ cDNAs, expression of GlyR subunits was detected with mAb4a (Fig. 1A,E,G) but not with the gt anti-GlyR $\alpha 2$ antiserum (Fig. 1B,F,H). However, in cultures transfected with the GlyR $\alpha 2$ cDNA, both mAb4a (Fig. 1C) and the gt anti-GlyR $\alpha 2$ antiserum (Fig. 1D) detected the expression of GlyR $\alpha 2$. This finding shows that the gt anti-GlyR $\alpha 2$ antiserum recognizes the expression of GlyR $\alpha 2$ and does not cross-react with the other known α subunits.

We also tested the new rabbit (rb) anti-GlyR $\alpha 2$ antiserum in the same way (dilution 1:2,000). In cultures transfected with the GlyR $\alpha 2$ cDNA, both mAb4a (Fig. 1K) and the rb anti-GlyR $\alpha 2$ antiserum (Fig. 1L) detected the expression of GlyR $\alpha 2$. In cultures transfected with GlyR $\alpha 3$ cDNA (Fig. 1M,N) and GlyR $\alpha 4$ (Fig. 1O,P), the $\alpha 3$ and $\alpha 4$ subunits were detected by mAb4a but not with the rb anti-GlyR $\alpha 2$ antiserum. However, in cultures transfected with GlyR $\alpha 1$ cDNA (Fig. 1I,J), the rb anti-GlyR $\alpha 2$ antiserum also showed detectable staining of GlyR $\alpha 1$ cDNA transfected cells (Fig. 1J). This shows that the rb anti-GlyR $\alpha 2$ antiserum recognizes the expression of GlyR $\alpha 2$ but shows some cross-reactivity with GlyR $\alpha 1$. The reason for this is that the C-terminal sequence (last 13 amino acids) of GlyR $\alpha 2$ and GlyR $\alpha 1$ differs by only four amino acids. Because of the cross-reactivity of the rb anti-GlyR $\alpha 2$ antiserum, it was applied only in controls, and the data presented were obtained by using the gt anti-GlyR $\alpha 2$ antiserum (Santa Cruz Biotechnology).

Bipolar axon terminals were labeled with a guinea pig antiserum against the vesicular glutamate transporter vGluT1 (1:50,000; Chemicon, Temecula, CA). Amacrine cells were labeled with rabbit polyclonal antisera against glutamic acid decarboxylase GAD65/67 (1:8,000; Sigma, St. Louis, MO) and with a guinea pig antiserum against vGluT3 (1:2,000; a kind gift from Dr. E. Weihe, Marburg, Germany). Conventional synapses in the IPL were labeled with an mAb against the presynaptic cytomatrix protein bassoon (1:5,000; Stressgen, Victoria, British Columbia, Canada). The GFP signal in the GFP knock-in retinae was increased with rabbit polyclonal antisera against GFP (1:2,000; Molecular Probes, Eugene, OR).

Antibodies were diluted in PBS, pH 7.4, containing 5% Chemiblocker (Chemicon) and 0.5% Triton X-100. Immunocytochemical labeling was performed with the indirect fluorescence method. The sections were incubated overnight in the primary antibodies, followed by incubation (1 hour) in the secondary antibodies, which were conjugated either to Cy3 (red fluorescence; Dianova, Hamburg, Germany) or Alexa TM 488 (green fluorescence; Molecular Probes). In double-labeling experiments, sections were incubated in a mixture of primary antibodies, followed by a mixture of secondary antibodies. In some cases, the staining of the GlyR $\alpha 2$ was intensified by using a secondary antibody raised in rabbit, followed by a tertiary antibody donkey anti-rabbit that carried the same fluorophore.

Light microscopy

Fluorescent specimens were viewed with a Zeiss (Oberkochen, Germany) Axiophot microscope equipped with a fluorescent filter set that was wedge corrected; i.e., shifting from one filter to the other did not introduce spatial displacements. Errors of misalignment could be detected by a fluorescence filter set designed for simultaneous viewing of two fluorochromes (51004v2; Chroma, Brattleboro, VT). Black-and-white digital images were taken using a cooled CCD camera (Spot 2; Diagnostic Instruments, Sterling Heights, MI). With the Metaview software (Universal Imaging, West Chester, PA), images taken with the red and green fluorescent filters were pseudocolored and superimposed. Confocal micrographs were taken using a Zeiss LSM5 Pascal confocal microscope equipped with an argon laser and an HeNe laser. High-resolution scanning was performed with a Plan-Apochromat $\times 63/1.4$ objective and with $1,024 \times 1,024$ or $2,048 \times 2,048$ pixels. Single optical sections are shown. The brightness and the contrast of the final images were adjusted in Adobe Photoshop 5.5.

Measurements of densities and colocalization

Colocalizations of immunofluorescent puncta were quantified in the following way. Two micrographs of the double-labeled sections were taken with the $\times 100$ objective, using red and green fluorescence filters, and printed at a final magnification of $\times 5,000$. The immunofluorescent puncta of the micrographs were transferred onto tracing paper. Even in this first step there is an intrinsic failure rate, because puncta are difficult to detect when they are weakly stained or covered by a cloudy background. To test the positional errors that might have been made, we transferred puncta of the same micrograph twice onto separate transparencies. The two images were superimposed at their correct position, and the number of puncta that coincided was counted. In theory the coincidence rate should be 100%, however, the coincidence rate measured in this way was approximately 80%. The two images were also superimposed randomly, and the number of puncta that coincided was counted. The coincidence rate for such random superpositions was up to 10%. After these two test trials, the two images of the micrographs of the double-labeled sections were superimposed at their correct position, and the numbers of coincidences were counted. For each pair of colocalizations, more than 1,000 puncta were sampled that were taken from at least four sections. The coincidence rates refer to the percentage of GlyR $\alpha 2$ puncta and not the second label.

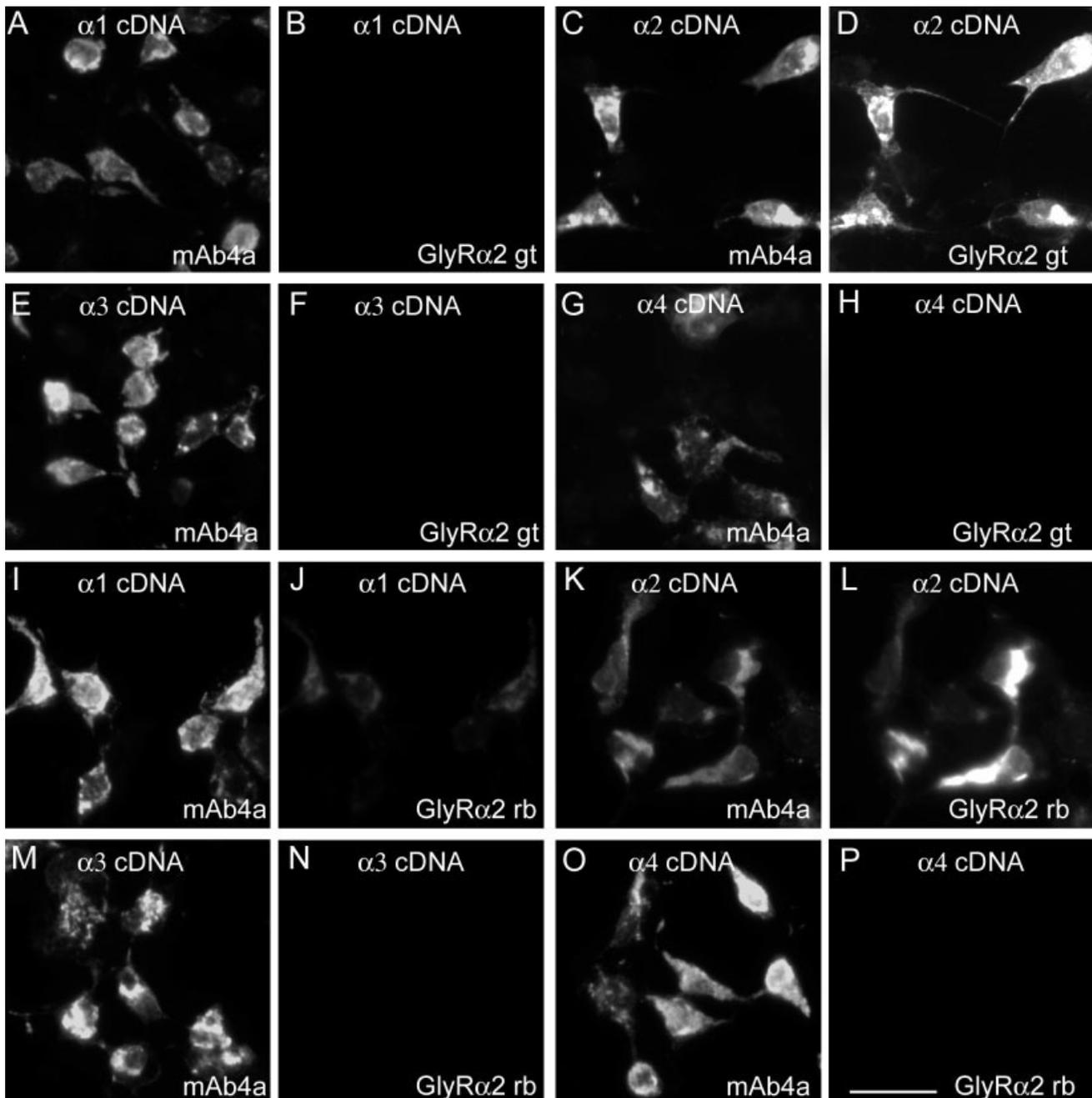


Fig. 1. Fluorescence photomicrographs of HEK 293T cells transfected with equal amounts of cDNAs and doubly immunostained with mAb4a and two different antiglycine receptor $\alpha 2$ antisera. **A,B:** mAb4a (A) but not the goat anti-GlyR $\alpha 2$ (GlyR $\alpha 2$ gt) antiserum (B) recognizes the GlyR $\alpha 1$ subunit. **C,D:** mAb4a (C) and the GlyR $\alpha 2$ gt antiserum (D) recognize the GlyR $\alpha 2$ subunit. **E,F:** mAb4a (E) but not the GlyR $\alpha 2$ gt antiserum (F) recognizes the GlyR $\alpha 3$ L subunit.

G,H: mAb4a (G) but not GlyR $\alpha 2$ gt antiserum (H) recognizes the GlyR $\alpha 4$ subunit. **I,J:** mAb4a (I) recognizes the GlyR $\alpha 1$ subunit, and the rabbit anti-GlyR $\alpha 2$ antiserum (GlyR $\alpha 2$ rb) also cross-reacts (J). **K,L:** mAb4a and GlyR $\alpha 2$ rb antiserum recognize the GlyR $\alpha 2$ subunit. **M,N:** mAb4a (M) but not GlyR $\alpha 2$ rb antiserum (N) recognizes the GlyR $\alpha 3$ subunit. **O,P:** mAb4a (O) but not GlyR $\alpha 2$ rb antiserum (P) recognizes the GlyR $\alpha 4$ subunit. Scale bar = 25 μ m.

RESULTS

Immunocytochemical staining of mouse retina with subunit-specific antibodies against GlyRs

A low-power fluorescence micrograph of a vertical section through a mouse retina immunostained for the GlyR

$\alpha 2$ subunit is shown in Figure 2A. The retinal structure of the same section is indicated in the Nomarski micrograph shown in Figure 2B. Dense punctate immunofluorescence was detected for the $\alpha 2$ subunit within the IPL, suggestive of synaptic localization in postsynaptic densities (Brandstätter et al., 1998; Wässle et al., 1998). The distribution of GlyR $\alpha 2$ puncta across the outer four layers of the IPL is

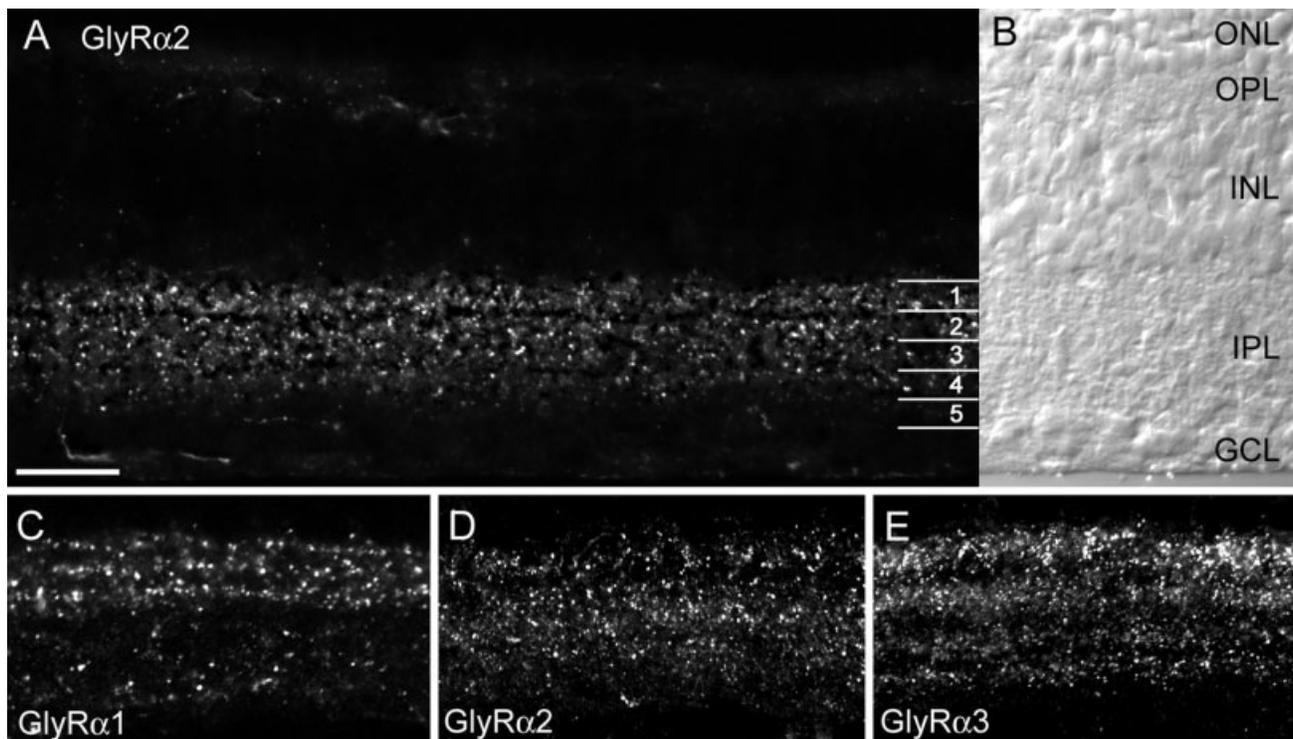


Fig. 2. Photomicrographs of vertical sections through mouse retinae that were immunolabeled for the $\alpha 2$ subunit of the glycine receptor (GlyR $\alpha 2$). Retinal layers are indicated (ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer). **A:** Conventional fluorescence photomicrograph. Sublaminae 1–5 of the IPL are indicated. **B:** Nomarski micrograph of the same section showing the retinal

layers. **C:** Confocal fluorescence photomicrograph showing punctate GlyR $\alpha 1$ distribution in the IPL. **D:** Confocal fluorescence photomicrograph showing punctate GlyR $\alpha 2$ distribution in the IPL. **E:** Confocal fluorescence photomicrograph showing the punctate GlyR $\alpha 3$ distribution in the IPL. Scale bar = 25 μm in A (applies to A,B); 14 μm for C; 16 μm for D,E.

fairly uniform, with an indication of a reduced density of puncta at the border between sublaminae 1/2, and 3/4. This corresponds to the strata where cholinergic amacrine cells ramify.

Figure 2C–E compares the distributions of the GlyR $\alpha 1$ – $\alpha 3$ subunits across the IPL. The $\alpha 1$ subunit is preferentially expressed in large puncta in the OFF-sublamina and shows a reduced density in the ON-sublamina (Fig. 2C). The $\alpha 2$ subunit shows a more uniform distribution (Fig. 2D), whereas four bands of high density can be discriminated in the case of the $\alpha 3$ subunit. This suggests that the three subunits are clustered at different synapses and are involved in different glycinergic circuits.

Synaptic localization of the GlyR $\alpha 2$

To demonstrate that GlyR $\alpha 2$ puncta represent aggregates of GlyRs at postsynaptic sites, sections were double-labeled for GlyR $\alpha 2$ and selected synaptic markers (Fig. 3). Bassoon is a cytomatrix protein found in the presynaptic terminals of conventional synapses in the IPL (Brandstätter et al., 1999). Figure 3A–C shows a section doubly labeled for GlyR $\alpha 2$ (Fig. 3A) and bassoon (Fig. 3B). Both markers show a punctate fluorescence, but bassoon labeling is more widespread; this presynaptic marker is also expressed at non-GlyR $\alpha 2$ -containing synapses. Despite this, superposition of GlyR $\alpha 2$ and bassoon labeling shows that many bassoon puncta are closely associated with GlyR $\alpha 2$ puncta (arrows in Fig. 3C). This we interpret as

synaptic labeling: Bassoon is present in the presynaptic terminals, and GlyR $\alpha 2$ is located at postsynaptic sites. However, it is also noteworthy that not all GlyR $\alpha 2$ clusters are associated with bassoon puncta. This is because not all presynaptic terminals of the IPL express bassoon (Brandstätter et al., 1999).

Gephyrin is expressed together with GlyRs at postsynaptic sites, where it links the receptors to the cytoskeleton (Kneussel and Betz, 2000). Figure 3D–F shows a section that was doubly labeled for GlyR $\alpha 2$ (Fig. 3D) and gephyrin (Fig. 3E). Superposition (Fig. 3F) shows that many of the GlyR $\alpha 2$ puncta coincide with gephyrin clusters (boxes in Fig. 3D–F). This was also assessed quantitatively in six sections (see Materials and Methods), and a mean coincidence rate of $46.7\% \pm 4.4\%$ (mean \pm SD) was found.

Our next aim was to discover whether the different GlyR subunits colocalize within the same postsynaptic densities or whether they occur at different synapses. Figure 3G–I shows a section that was doubly labeled for GlyR $\alpha 2$ (Fig. 3G) and for GlyR $\alpha 1$ (Fig. 3H). Their superposition in Figure 3I shows that they rarely colocalize. This result was corroborated in a larger sample of sections ($n = 8$), and a mean coincidence rate of $9\% \pm 3.1\%$ (mean \pm SD) was found. This rate is not significantly different from the coincidence rate found when the puncta were randomly superimposed ($6.6\% \pm 2.7\%$). This finding demonstrates that GlyR $\alpha 2$ and GlyR $\alpha 1$ clusters correspond—as a rule—to different synapses.

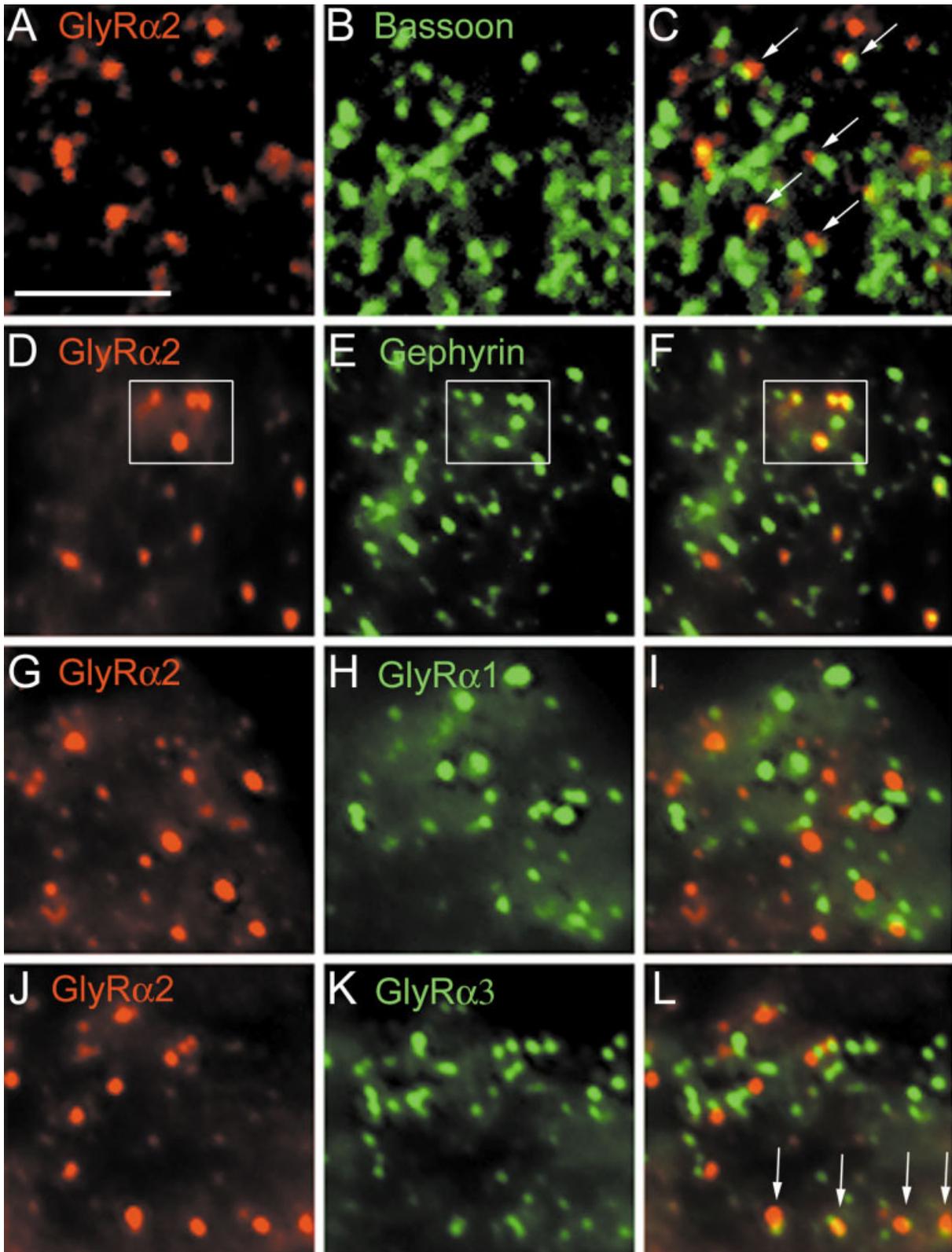


Fig. 3. High-power fluorescence micrographs of vertical sections through the inner plexiform layer of mouse retinae that were doubly labeled for GlyR $\alpha 2$ and synaptic markers. All four micrographs were taken from the outer half of the IPL, the OFF-sublamina. **A:** GlyR $\alpha 2$ -immunoreactive puncta. **B:** Same section as in A but showing bassoon immunoreactive puncta. **C:** Superposition of A and B; the arrows indicate bassoon spots closely associated with GlyR $\alpha 2$ puncta. **D:** GlyR $\alpha 2$ -immunoreactive puncta. **E:** Same section as in D but

showing gephyrin-immunolabeled puncta. **F:** Superposition of D and E; the boxed area indicates colocalized puncta. **G:** GlyR $\alpha 2$ -immunoreactive puncta. **H:** Same section as in G but showing GlyR $\alpha 1$ -immunolabeled puncta. **I:** Superposition of G and H. **J:** GlyR $\alpha 2$ -immunoreactive puncta. **K:** Same section as in J but showing GlyR $\alpha 3$ -immunolabeled puncta. **L:** Superposition of J and K. The four arrows indicate colocalizations. Scale bar = 5 μm .

We also doubly labeled sections through the IPL with anti-GlyR $\alpha 2$ (Fig. 3J) and anti-GlyR $\alpha 3$ (Fig. 3K). Superposition of the two micrographs (Fig. 3L) shows that some puncta coincide (arrows). This was also quantitatively assessed ($n = 14$ sections), and a coincidence rate of $26.7 \pm 3.6\%$ was observed. Because random colocalizations equal 6–10%, this indicates that, in about 20% of the GlyR $\alpha 2$ -expressing synapses, the GlyR $\alpha 3$ subunit is also found. Close inspection of Figure 3L (arrows) shows that GlyR $\alpha 2$ and GlyR $\alpha 3$ puncta were not in perfect register. This finding is discussed below.

We also measured the relative densities of the synaptic clusters in sections doubly labeled for GlyR $\alpha 2$ /GlyR $\alpha 1$ and GlyR $\alpha 2$ /GlyR $\alpha 3$. They were GlyR $\alpha 3$, 100; GlyR $\alpha 2$, 80; and GlyR $\alpha 1$, 67. Hence, in the retina, GlyR $\alpha 3$ is expressed at the most synapses, followed by GlyR $\alpha 2$, then GlyR $\alpha 1$. This is also illustrated in Figure 2C–E, where the lowest numbers of puncta are found in the GlyR $\alpha 1$ -labeled section (Fig. 2C), more puncta are present in the GlyR $\alpha 2$ -labeled section (Fig. 2D), and the highest density of clusters is revealed by GlyR $\alpha 3$ labeling.

Glycinergic amacrine cells and the GlyR $\alpha 2$ subunit

Glycinergic amacrine cells are the presynaptic partners of GlyRs at glycinergic synapses. However, they also receive glycinergic synapses from other glycinergic amacrine cells (Koontz and Hendrickson, 1987; Hendrickson et al., 1988; Pourcho and Owczarzak, 1991a,b). A close association of GlyRs and glycinergic amacrine cells is expected in both instances. Figure 4A shows a section through the inner retina of a transgenic mouse expressing EGFP under the control of the glycine transporter GlyT2 gene promoter (Zeilhofer et al., 2003). All glycinergic amacrine cells and a few GABAergic amacrine cells express EGFP in this transgenic mouse (Haverkamp and Wässle, unpublished observation). The section shown in Figure 4A was also immunolabeled for GlyR $\alpha 2$. The boxed area is shown at higher magnification in Figure 4B–D. Note that GlyR $\alpha 2$ -immunoreactive puncta (Fig. 4B) and the processes of the GFP-labeled amacrine cells (Fig. 4C) are in close proximity (Fig. 4D). This represents further evidence that GlyR $\alpha 2$ clusters represent glycinergic synapses.

Expression of the GlyR $\alpha 2$ subunit on ganglion cell dendrites

To assess whether ganglion cell dendrites receive glycinergic synapses expressing the GlyR $\alpha 2$ subunit, we used a transgenic mouse expressing GFP under the control of the Thy-1 promoter, which is active in all ganglion cells (Feng et al., 2000). Figure 4E shows a section through the inner retina of a Thy-1-GFP transgenic mouse, which shows labeling of ganglion cell perikarya and their dendrites in the IPL. The same section was also immunostained for the GlyR $\alpha 2$ subunit, and many red puncta are found throughout the IPL. Müller cell end feet and ganglion cell perikarya are also lightly labeled in this section. However, this results from cross-reactivity of the gt anti-GlyR $\alpha 2$ antiserum, which occasionally occurs in lightly fixed retinae. In sections that were doubly labeled for gt anti-GlyR $\alpha 2$ and rb anti-GlyR $\alpha 2$, most puncta in the IPL were doubly labeled. However, extrasynaptic labeling of Müller cell end feet and perikarya of amacrine and ganglion cells was observed only with the gt anti-GlyR $\alpha 2$ antiserum, suggesting that it represents cross-reactivity. The boxed area in Figure 4E is shown at higher

magnification in Figure 4F–H. The superposition of the GFP-labeled dendrites and the GlyR $\alpha 2$ hot spots (Fig. 4H) shows they are not in register. This lack of correlation becomes particularly obvious when Figure 4D and H are compared. We therefore conclude that synapses containing the GlyR $\alpha 2$ subunit are only rarely found on ganglion cell dendrites.

Expression of the GlyR $\alpha 2$ subunit on bipolar cell axons

Bipolar cell axons in the IPL were labeled with antibodies against the vesicular glutamate transporter vGluT1 (Fig. 5A), which labels OFF-cone, ON-cone, and rod bipolar cell axon terminals (Haverkamp et al., 2003b; Johnson et al., 2003b; Ghosh et al., 2004). The section shown in Figure 5A was also immunostained for the GlyR $\alpha 2$ subunit. Three selected fields (boxes) taken from OFF-cone axon terminals (1), the ON-cone axon terminals (2), and the rod bipolar axon terminals (3) are shown at higher magnification beneath Figure 5A. Close inspection of these fields shows that green GlyR $\alpha 2$ puncta coincide with red bipolar cell axon terminals in all three examples. This indicates that the three bipolar cell classes receive glycinergic input through GlyR $\alpha 2$ -expressing synapses. However, Figure 5A also shows many green puncta that are obviously *not* in register with bipolar cell axon terminals.

Expression of the GlyR $\alpha 2$ subunit on the processes of GABAergic amacrine cells

GABAergic amacrine cells were labeled with antibodies against the GABA-synthesizing enzyme glutamic acid decarboxylase GAD 65/67 (Fig. 5B), a reliable marker for this cell type in mammalian retinae (Vardi and Auerbach, 1995; Haverkamp and Wässle, 2000). The section in Figure 5B was also immunostained for the GlyR $\alpha 2$ subunit, and punctate immunofluorescence was observed throughout the IPL. Three selected fields (boxes) from the OFF-sublamina (4), from the middle of the IPL (5), and from the ON-sublamina (6) are shown at higher magnification beneath Figure 5B. Close inspection of these fields shows that green GlyR $\alpha 2$ puncta coincide with GAD65/67-labeled processes in all three examples. Thus, GABAergic amacrine cells also appear to represent targets of glycinergic synapses expressing the GlyR $\alpha 2$ subunit. Figure 5A,B strongly suggests that the GlyR $\alpha 2$ subunit is found at synapses where bipolar and GABAergic amacrine cells receive input from glycinergic amacrine cells. However, it will be necessary to label individual bipolar or amacrine cells selectively [for instance, by intracellular injection of Lucifer yellow (LY)] and study GlyR localization with high-resolution confocal microscopy (Ghosh et al., 2001; Li et al., 2002; Grünert et al., 2003) to validate this finding.

Amacrine cells expressing the vesicular glutamate transporter vGluT3 are presynaptic to GlyR $\alpha 2$ hot spots

Recently, an amacrine cell type has been identified by the expression of the vesicular glutamate transporter vGluT3 (Fremeau et al., 2002; Johnson et al., 2003a; Haverkamp and Wässle, 2004). The cells make up 1% of the amacrine cell population, and their processes occupy two narrow strata in the middle of the IPL. Because they express both glycinergic and glutamatergic markers, it was unclear whether they release glycine or glutamate, or

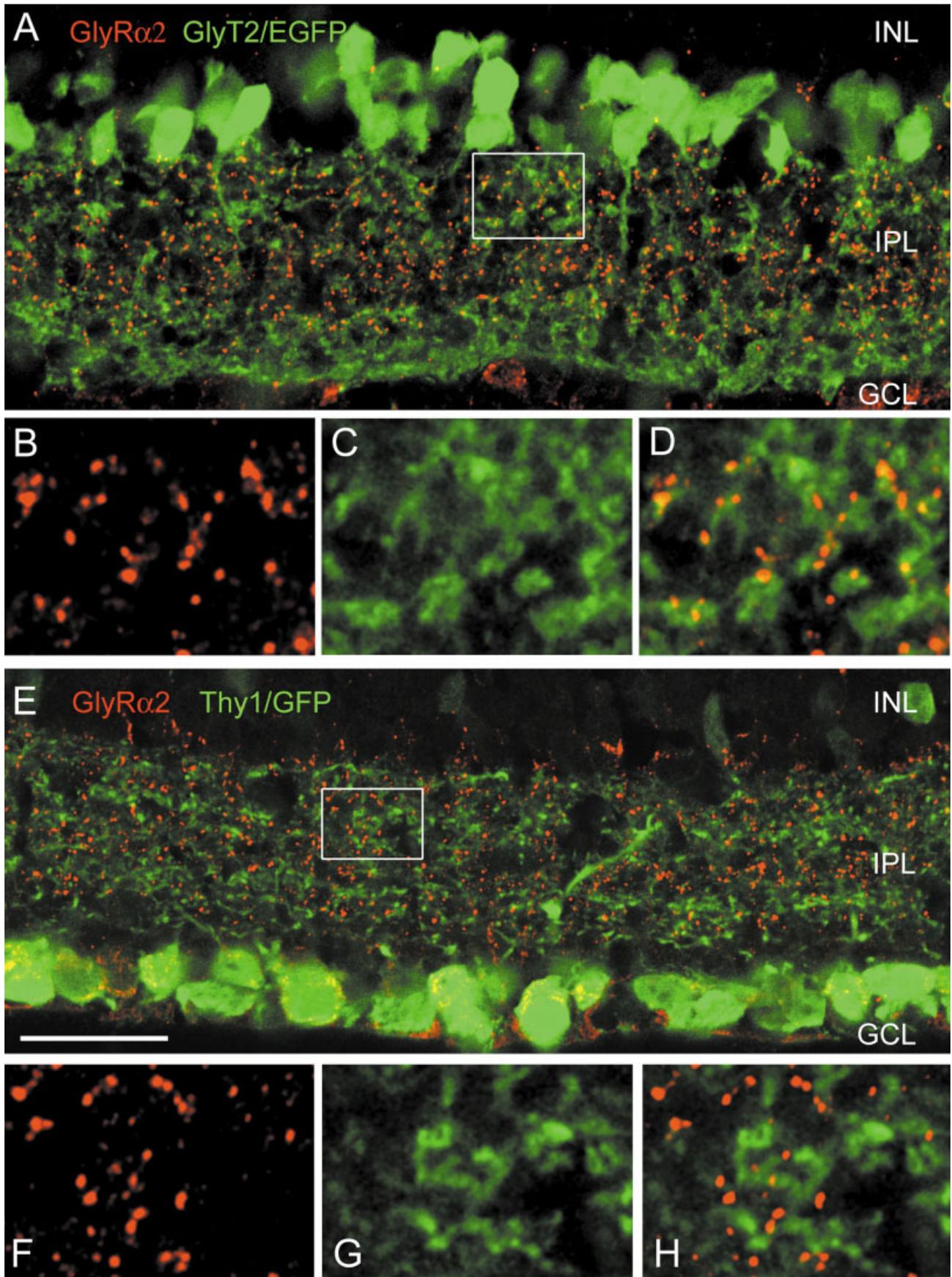


Figure 4

even both transmitters, at their synapses (Haverkamp and Wässle, 2004). To test the involvement of vGluT3-immunoreactive amacrine cells with glycinergic synapses, we doubly labeled sections for vGluT3 and GlyR $\alpha 2$ (Fig. 6). In the low-power micrograph (Fig. 6A), vGluT3-labeled processes overlap with the distribution of GlyR $\alpha 2$ -immunoreactive puncta in the middle of the IPL, and many instances of overlapping profiles (yellow spots) can be detected. The middle of the IPL is shown at higher magnification in Figure 6C, and the boxed area is shown in isolation in Figure 6D. The green vGluT3-labeled processes shown in Figure 6D are in close apposition to the red GlyR $\alpha 2$ clusters. However, they only partially overlap, suggesting the GlyR $\alpha 2$ clusters are not on the vGluT3-labeled processes but occupy a postsynaptic position. This result indicates that vGluT3-immunoreactive amacrine cells release glycine—possibly in addition to glutamate—and that the GlyR $\alpha 2$ subunit is expressed in the target synapses. This suggests that a close correlation may exist between the different types of glycinergic amacrine cells and the molecular composition of partnering postsynaptic GlyRs.

DISCUSSION

Distribution of glycinergic synapses in the retina

Glycinergic synapses expressing the $\alpha 1$, $\alpha 2$, or $\alpha 3$ subunits show characteristic distributions across the IPL of the mouse retina (Fig. 2C–E). The $\alpha 1$ subunit is found in large puncta in the OFF-sublamina of the IPL (Fig. 2C), representing the synapses between AII amacrine cells and the OFF-cone bipolar cells in the rod pathway (Sassoè-Pognetto et al., 1994; Grünert and Wässle, 1996; Haverkamp et al., 2003a). In addition, smaller GlyR $\alpha 1$ -immunoreactive puncta occur at lower density throughout the IPL. Intracellular injection with LY has shown that these correspond to synapses on alpha ganglion cells (Koulen et al., 1996), although the presynaptic partners of these synapses remain unidentified. Glycinergic synapses expressing the $\alpha 3$ subunit are aggregated in four bands of high density within the IPL (Fig. 2E; Haverkamp et al., 2003a). Two of these bands coincide with the axon terminals of cone bipolar cells (type 3 and type 5; Haverkamp et al., 2003a; Ghosh et al., 2004), although amacrine cells also show GlyR $\alpha 3$ immunoreactivity. By contrast, GlyR $\alpha 2$ -immunoreactive synapses are distributed evenly across the IPL (Fig. 2A,D). We have shown that ganglion cells are not the preferred target of such synapses (Fig.

4E), but we have observed them on bipolar axon terminals and amacrine cell processes (Fig. 5).

Taken together, our results show that there is no unique distribution of the different glycinergic synapses to classes of neurons, such as ganglion cells expressing the GlyR $\alpha 1$, bipolar cells the GlyR $\alpha 2$, and amacrine cells the GlyR $\alpha 3$ subunits. However, we cannot exclude that such specific expression holds for pairs of pre- and presynaptic partners. For example, it appears that AII amacrine cells prefer $\alpha 1$ subunit GlyRs at their synapses with OFF-cone bipolar cells. To resolve these questions, it will be necessary to label individual neurons by specific markers, such as the intracellular injection of LY, and study the GlyR hot spots that coincide with the processes of the labeled neurons. Such experiments may reveal whether different types of glycinergic amacrine cells prefer specific postsynaptic GlyRs. They may also show whether the many different types of postsynaptic neurons, bipolar, amacrine, and ganglion cells, have a preference for certain GlyR subtypes.

Subunit composition of synaptic GlyRs in the retina

Synaptic GlyRs in the retina are likely to be heteromeric receptors composed of α and β subunits. The GlyR β subunit must be an essential constituent of synaptic GlyRs in the retina, insofar as the β subunit interacts with gephyrin (Kneussel et al., 1999), and GlyR clustering is abolished in the retina of gephyrin knockout mice (Fischer et al., 2000). The α subunits are required for agonist binding (Schmieden et al., 1989; Sontheimer et al., 1989) and, therefore, are also essential for the functioning of synaptic GlyRs. There is good evidence that three α and two β subunits form the heteromeric receptor (Langosch et al., 1988; Griffon et al., 1999). In artificial expression systems, GlyRs assembled from different α subunits can contain variable α subunit ratios (Kuhse et al., 1993). If combinations of different α subunits occur in the assembly of synaptic GlyRs, a plethora of distinct GlyR complexes could theoretically be found. However, the results of the present study suggest that combinations of different α subunits within the same GlyR channel are rarely expressed at retinal synapses.

We have shown in the present study that GlyR $\alpha 2$ clusters do not coincide with GlyR $\alpha 1$ puncta (Figs. 3G–I). In a preceding study, this was also described for GlyR $\alpha 3$ and GlyR $\alpha 1$ labeling (Haverkamp et al., 2003a). Taken together, these results suggest that, for retinal GlyRs, 1) a mixture of the $\alpha 1\beta$ with $\alpha 2\beta$, or of the $\alpha 1\beta$ with $\alpha 3\beta$ does not occur at the same synapse and 2) heteromeric GlyRs of the form $\alpha 1\alpha 2\beta$ or $\alpha 1\alpha 3\beta$ are not found. A comparable result has also been described for retinal GABA_A receptors, where isoforms containing different α subunits were observed only infrequently (Koulen et al., 1996).

In sections doubly labeled for GlyR $\alpha 2$ and GlyR $\alpha 3$, a coincidence rate of 26.7% was observed (Figs. 3J–L). Close inspection of the double-labeled puncta (arrows in Fig. 3L) showed that the GlyR $\alpha 2$ and GlyR $\alpha 3$ puncta were not in perfect register but were positioned eccentrically. We interpret this finding as an indication that the $\alpha 2$ and $\alpha 3$ subunits do not coassemble into heteromeric $\alpha 2\alpha 3\beta$ GlyRs but are expressed in different subtypes ($\alpha 2\beta$ and $\alpha 3\beta$) that are distributed independently across the postsynaptic site. This would explain why the clusters are not in perfect register. However, a caveat has to be kept in mind. The z-axis resolution of the light microscope, irrespective of

Fig. 4. Confocal micrographs of vertical sections through the inner part of mouse retinae doubly labeled for green fluorescent protein (GFP) and GlyR $\alpha 2$. **A:** EGFP (green) under the control of the GlyT2 promoter is expressed in amacrine cell perikarya and their processes within the IPL; GlyR $\alpha 2$ -immunoreactive puncta (red) are found throughout the IPL. The boxed area is shown at higher magnification in B–D. **B:** GlyR $\alpha 2$ -immunoreactive puncta. **C:** GFP-labeled amacrine cell processes. **D:** Superposition of B and C. **E:** GFP (green) under the control of the Thy1 promoter is expressed in ganglion cell perikarya and their dendrites within the IPL; GlyR $\alpha 2$ -immunoreactive puncta (red) are found throughout the IPL. The boxed area is shown at higher magnification in F–H. **F:** GlyR $\alpha 2$ -immunoreactive puncta. **G:** GFP-labeled ganglion cell dendrites. **H:** Superposition of F and G. Scale bar = 25 μ m in E (applies to A, E); 5 μ m for B–D, F–H.

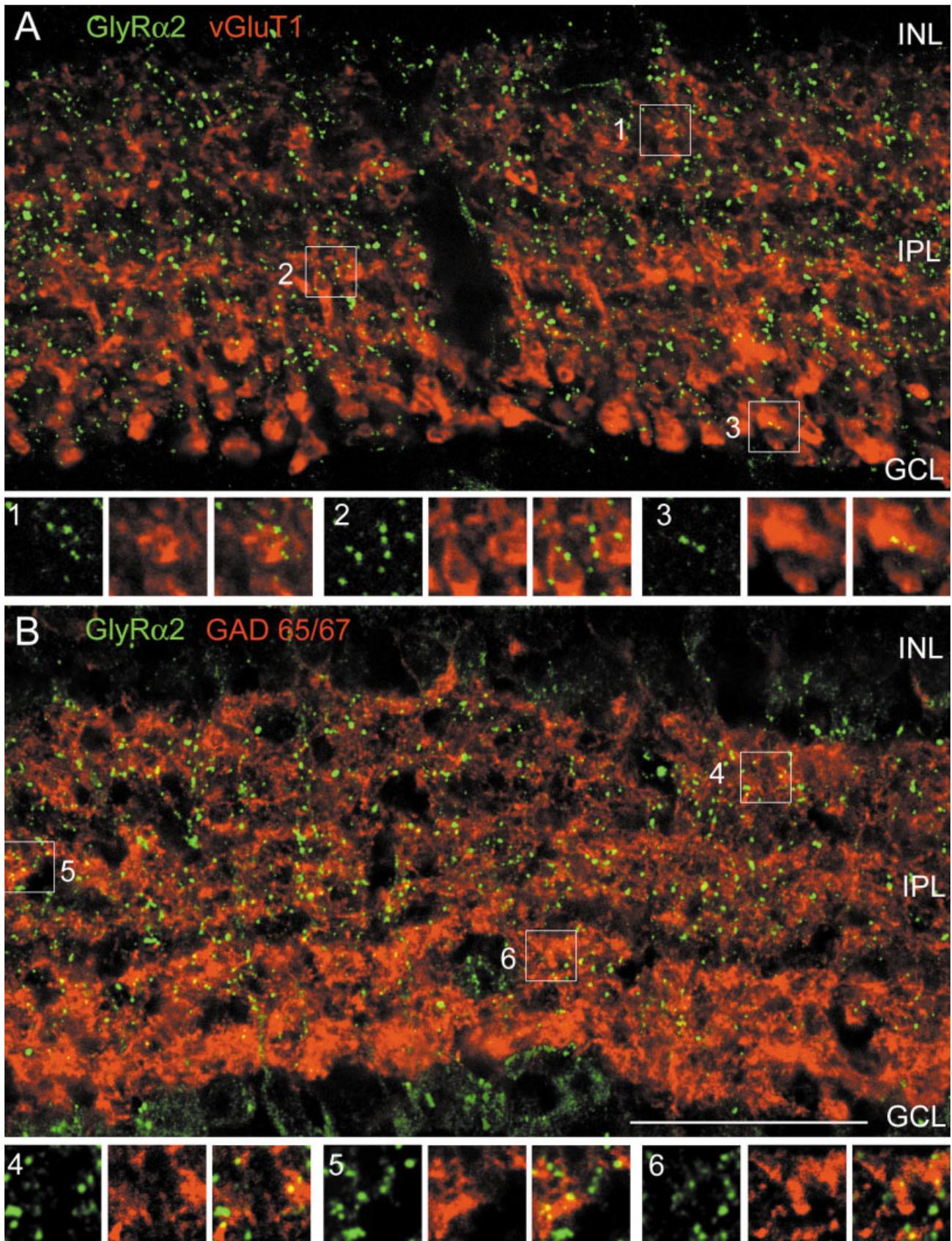


Fig. 5. Confocal micrographs of vertical sections through the inner part of mouse retinae doubly labeled for GlyR α 2 (green) and the vesicular glutamate transporter vGluT1 (red) and glutamic acid decarboxylase GAD 65/67 (red). **A:** GlyR α 2 puncta (green) are found throughout the IPL. The axon terminals of bipolar cells (red) express vGluT1 immunoreactivity. Selected areas are indicated by the boxes

(1, 2, 3) and are shown at higher magnification beneath **A**. **B:** GlyR α 2 puncta are found throughout the IPL. The processes of GABAergic amacrine cells (red) express GAD 65/67 immunoreactivity. Selected areas are indicated by the boxes (4, 5, 6) and are shown at higher magnification beneath **B**. Scale bar = 25 μ m.

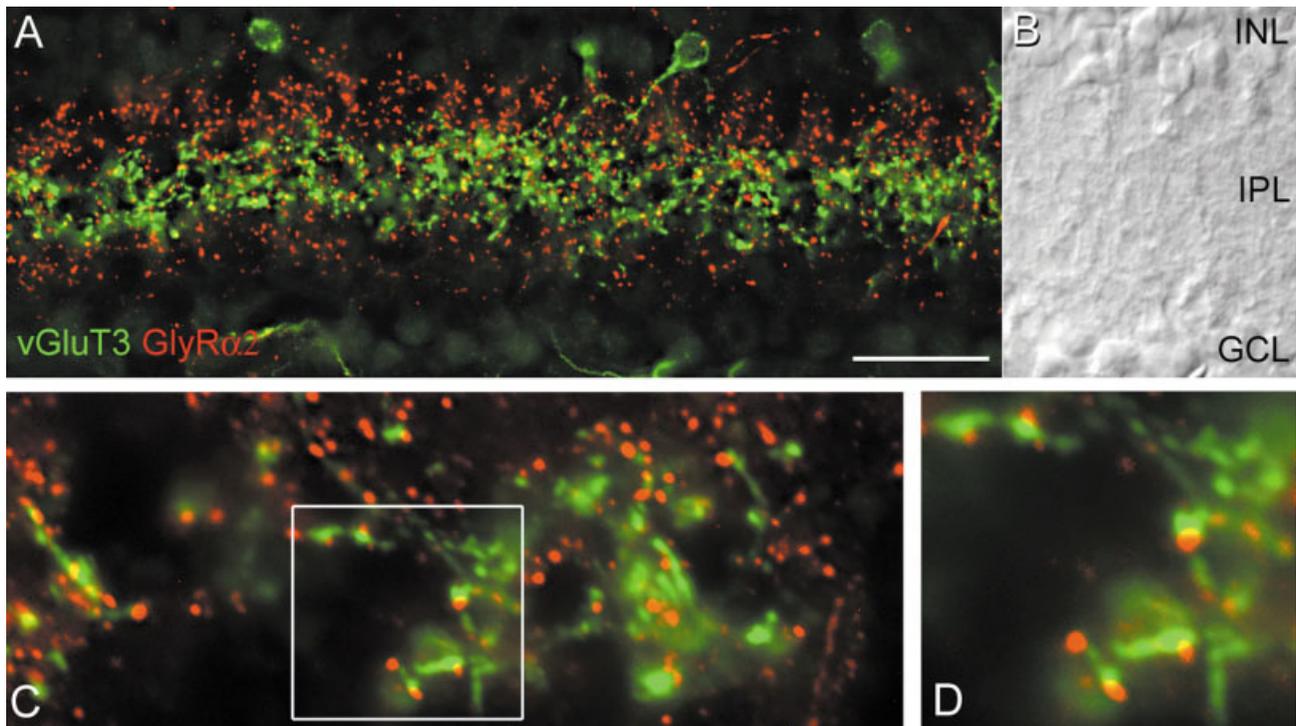


Fig. 6. Fluorescence micrograph of vertical sections through the inner part of mouse retinae doubly labeled for GlyR $\alpha 2$ (red) and the vesicular glutamate transporter vGluT3 (green). **A:** GlyR $\alpha 2$ puncta (red) are found throughout the IPL. The cell bodies of vGluT3-labeled amacrine cells (green) are in the INL, and their processes ramify in the middle of the IPL. **B:** Nomarski micrograph showing the retinal

layers. **C:** High-power fluorescence micrograph showing the middle part of the IPL. Many red GlyR $\alpha 2$ puncta are closely associated with the green amacrine cell processes. This is shown for the boxed area at higher magnification in D. Scale bar = 25 μm in A (applies to A,B); 6 μm for C; 3.6 μm for D.

whether conventional or confocal microscopy is applied, is not better than 1 μm . Puncta 1 μm or less apart in the z-direction would thus be fused. It is, therefore, possible that the GlyR $\alpha 2$ and GlyR $\alpha 3$ puncta may not overlap at all physically but exist side-by-side or even at the adjacent processes of different neurons. Only electron microscopic study can ultimately determine whether GlyR $\alpha 2$ and GlyR $\alpha 3$ are expressed within the same postsynaptic site. In conclusion, although many combinations of different α subunits within the same GlyR are theoretically possible, most retinal GlyRs appear to consist of only one kind of α subunit together with the β subunit. In addition, at most postsynaptic sites, only one type of α subunit appears to be expressed.

Gephyrin immunoreactivity was observed in only approximately half of the GlyR $\alpha 2$ clusters (Fig. 3D–F). It has been noted previously that GlyRs on bipolar cell axons do not colocalize with gephyrin (Sassoè-Pognetto et al., 1994). By contrast, all GlyRs clusters disappear in gephyrin knockout mice, suggesting that gephyrin is involved in the clustering of *all* GlyRs at synaptic sites. A likely solution for this apparent discrepancy is provided by analysis of the gephyrin gene, which predicts splice variants of gephyrin that are not recognized by the antibody mAb7a (Ramming et al., 2000). It is possible that such splice variants are preferentially expressed in bipolar cell axons.

Functional consequences of GlyR diversity in the retina

Studies of recombinant GlyRs have shown that the expression of the different α subunits results in channels

with different kinetic properties (Harvey et al., 2000; Legendre, 2001; Breiting and Becker, 2002). In the spinal cord and brainstem of neonates, synaptic GlyRs are composed of $\alpha 2$ and β subunits and are replaced in juveniles by the $\alpha 1\beta$ combination (Becker et al., 1988; Malosio et al., 1991; Singer et al., 1998). As a result of this switch, glycinergic inhibitory postsynaptic currents (mIPSCs) become faster (neonate τ decay = 14.2 msec, juvenile τ decay = 6.7 msec; Singer et al., 1998). Spontaneous glycinergic IPSCs have also been recorded from rod bipolar cells (τ decay = 13.6 msec; Ciu et al., 2003), from amacrine cells (τ decay = 24.3 msec; Frech et al., 2001), and from ganglion cells (τ decay = 20 msec; Protti et al., 1997). These differences in kinetics support the conclusions of the present anatomical study; i.e., bipolar, amacrine, and ganglion cells express different sets of GlyRs. Ciu et al. (2003) also observed that the amplitudes of the glycinergic mIPSCs recorded in rod bipolar cells fell into two groups, those that were blocked by TTX and those that persisted during TTX application. They interpreted this result as input from two different amacrine cell types, one with a spike triggered glycine release and the other with a graded release. These findings also support our suggestion that different presynaptic partners signal through specific GlyR subtypes expressed by the postsynaptic neuron.

Pharmacological studies of glycinergic inhibition in the tiger salamander retina have revealed two types of GlyRs, one sensitive to strychnine and the other to 5,7-dichlorokynurenic acid (Han et al., 1997; Han and Slaugher, 1998). They are also differentially modulated by Zn^{2+} (Han and Wu, 1999), suggesting the presence of different

α subunits harboring discrete Zn^{2+} binding sites (Laube, 2002).

Clearly, further electrophysiological and pharmacological experiments, particularly with the mouse retina, are needed before the correlation between the molecular diversity of GlyRs and the functional consequences can be made more conclusively. The molecular diversity will influence temporal characteristics of the receptors (τ decay) and define their sensitivity to glycine and also their possible modulation by other neuroactive substances.

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