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Selective Clustering of $GABA_A$ and Glycine Receptors in the Mammalian Retina

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Molecular cloning has revealed a multiplicity of neurotransmitter receptor isoforms with different subunit compositions. Additionally, there is growing evidence that such receptors are clustered at postsynaptic sites of neurons. Thus, the questions arise whether individual neurons express different receptor isoforms and, if so, whether different isoforms are present within the same cluster or are aggregated at distinct postsynaptic sites. We have studied with immunofluorescence methods and antibodies that recognize specific subunits the distribution of glycine and $GABA_A$ receptors in mammalian retinae. Alpha ganglion cells were injected in rat or rabbit retinae with a fluorescent marker and then immunostained for receptor localization. Clusters of glycine receptors and clusters of the $\alpha1$, $\alpha2$, $\alpha3$, and $\gamma2$ subunits of the $GABA_A$ receptor were found on the somatodendritic membranes of Alpha ganglion cells. Double-immunofluorescence experiments with different combinations of the subunit-specific antibodies showed that the $\alpha1$, $\alpha2$, and $\alpha3$ subunits of the $GABA_A$ receptor are not colocalized within the same clusters. These results indicate that an individual neuron can express several isoforms of the $GABA_A$ receptor and that these different isoforms are aggregated at distinct postsynaptic sites. This suggests individual sorting mechanisms of $GABA_A$ receptors at GABAergic synapses.

Key words: receptor clustering; $GABA_A$ receptors; glycine receptors; retinal ganglion cells; immunocytochemistry; Alpha ganglion cells; intracellular injection; Lucifer Yellow

Clustering of transmitter receptors at postsynaptic sites was shown first for the neuromuscular junction (for review, see Froehner, 1993). Acetylcholine receptor (AChR) density in such clusters is 1000-fold higher than in the extrasynaptic membrane; this is responsible for rapid signal transmission. Aggregation of transmitter receptors has been observed also at CNS synapses [AChR: Sargent and Pang (1989); glycine receptor: Triller et al. (1985), glutamate receptors: Jones and Baughman (1991), Baude et al. (1993, 1994), Aoki et al. (1994), Petralia et al. (1994); $GABA_A$ receptors: Baude et al. (1992), Craig et al. (1994), Nusser et al. (1995a,b)]. It appears to be developmentally regulated, and presynaptic terminals and postsynaptic sites specialize in parallel (Fletcher et al., 1991; Craig et al., 1993). However, in addition to clustered localization of transmitter receptors, nonsynaptic distribution has also been observed in the CNS (Somogy et al., 1989; Soltesz et al., 1990; Baude et al., 1994; Fritschy and Möhler, 1995; Nusser et al., 1995a).

Receptor localization for glycine, GABA, glutamate, and acetylcholine in retinal neurons has been performed using specific antisera, and synaptic as well as nonsynaptic localization has been reported. Clustering was first described for gephyrin, a protein that was originally purified with glycine receptors (Pfeiffer et al., 1982; Graham et al., 1985; Schmitt et al., 1987), but can be also localized to some GABAergic synapses of the retina (Sassoè-Pognetto et al., 1995). Immunofluorescence for gephyrin is concentrated to discrete puncta in the inner plexiform layer (IPL); these have been shown by electron microscopy to correspond to postsynaptic localization (Jäger and Wässle, 1987; Smiley and Yazulla, 1990; Yazulla and Studholme, 1991a,b; Zucker and Ehinger, 1992). Gephyrin links the receptors to subsynaptic microtubules (Kirsch et al., 1991; Kirsch and Betz, 1993, 1995). When antibodies against different subunits of the glycine receptor became available (for review, see Kuhse et al., 1995), clustering of glycine receptors at postsynaptic membranes was shown in detail (Sassoè-Pognetto et al., 1994). Bipolar, amacrine, and ganglion cells have been found to be $GABA_A$ receptor-positive by the use of antibodies specific to the $\alpha1$ and $\beta2/3$ subunits (Mariani et al., 1987; Richards et al., 1987; Hughes et al., 1989, 1991; Brecha and Weigmann, 1991; Vardi et al., 1992; Greferath et al., 1993, 1994a; Grünert and Hughes, 1993; Grünert et al., 1993; Vardi and Sterling, 1994). Recently, further antibodies specific for $GABA_A$ receptor subunits became available and, with these antibodies and the application of short fixation times, punctate distribution and concentration at postsynaptic sites of $GABA_A$ receptors were observed (Brandstätter et al., 1995; Greferath et al., 1995; Sassoè-Pognetto et al., 1995). Nomura et al. (1994) found the metabotropic glutamate receptor mGluR6 exclusively within the dendritic terminals of bipolar cells inserted into cone pedicles or rod spherules. Hartveit et al. (1994) localized the NMDA receptor subunit NR2A to discrete puncta in the IPL, which were shown by electron microscopy to represent postsynaptic sites at bipolar cell ribbon synapses. In contrast, Peng et al. (1995) found a more diffuse distribution of immunoreactivity for the glutamate receptors GluR1–4, GluR6/7, and mGluR1α. Localization of different subunits of the nicotinic AChR in the chick retina revealed a complex, diffuse labeling pattern in both the inner and the outer plexiform layers, and different populations of neurons expressed different subunits (Keyser et al., 1993). In earlier studies using α-bungarotoxin binding to label AChRs in goldfish (Zucker and

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Yazulla, 1982) and mouse (Pourcho, 1979) retinae, a concentration of the label at postsynaptic sites was described.

This molecular heterogeneity and the resulting multiplicity of receptor isoforms raise two general questions. First, can the same neuron express different $GABA_A$ or glycine receptor isoforms? If so, and because these receptors are clustered at postsynaptic sites, are different isoforms of the same receptor present within one cluster or at distinct postsynaptic sites? Such a specific clustering would require selective mechanisms of protein sorting to transport and cluster receptors with different subunit compositions to different synapses. The first question is addressed in the present paper for Alpha ganglion cells, which form a well defined retinal cell class and are easily recognized by their large cell bodies and dendritic branching pattern (Boycott and Wässle, 1974; Peichl et al., 1987; Peichl, 1989, 1991). Alpha ganglion cells were injected in rat or rabbit retinae with the fluorescent marker Lucifer Yellow (LY). The retinae were then immunostained for specific subunits of glycine or $GABA_A$ receptors using secondary antibodies with red fluorescence. A punctate distribution of immunofluorescence was observed, indicating clustering at postsynaptic sites. It was possible to decide whether the puncta coincided with LY-filled Alpha cell dendrites, that is, whether Alpha cells express the specific subunit. To address the second question, namely whether different isoforms of $GABA_A$ or glycine receptors are present within the same cluster or punctum, double-immunofluorescence experiments using two subunit-specific antibodies were performed.

MATERIALS AND METHODS

LY injections of Alpha ganglion cells. Adult albino rats were deeply anesthetized with halothane and then decapitated. Adult rabbits were killed by a lethal dose of sodium pentobarbital given intraveneously (60 mg/kg). The eyes were quickly enucleated, and an incision was made along the ora serrata. They were immersion fixed for 30 min in 2% paraformaldehyde (PA) in 0.1 M phosphate buffer (PB) at pH 7.4. Then the anterior part of the eye including the lens and the cornea was removed, and the eye cup was fixed in the same fixative for another 30 min. The retina was dissected from the eye cup and, after several washes in PB, was flattened on a slide, ganglion cells on top, and incubated for 1 min in a drop of 3 μM acridin orange [3,6-bis(dimethyl-amino)acridin, Sigma, St. Louis, MO] dissolved in PB. This caused a fluorescence labeling of ganglion cell bodies that could be selected for intracellular injection. After several washes in PB, the retina was spread flat in a Petri dish containing PB at room temperature. The dish was placed on a fixed-stage microscope (Zeiss, Thornwood, NY). Under visual control, labeled ganglion cell bodies were impaled by a micropipette containing a 5% aqueous solution of LY. The dye was iontophoretically injected by passing constant negative current of 1–3 nA for several minutes until the dendritic tree appeared completely filled. A detailed description of the LY injection procedure is given in Tauchi and Masland (1984) and Peichl et al. (1987, 1992). Alpha ganglion cells were preselected based on their large cell bodies and were positively identified from their dendritic branching pattern. For subsequent immunolabeling, the retinae were cryoprotected by immersion in ascending sucrose concentrations (10, 20, and 30%). Rat retinae were cut horizontally (60–70 μm thick) with a freezing microtome, and the sections were processed later free floating. Rabbit retinae are thinner and, thus, favorable for antibody penetration; they were processed as whole retinae. Before antibody staining, the retinae were frozen and thawed several times in liquid nitrogen.

Antibodies. For $GABA_A$ receptor immunocytochemistry, polyclonal antisera directed against $GABA_A$ receptor subunit-specific peptides were used [α1, residues 1–16; α2, 1–9; α3, 1–15 (Benke et al., 1991; Marksitzer et al., 1993; Gao et al., 1993); γ2, 1–15 (Stephenson et al., 1990)]. Antisera were raised in rabbit (α1, γ2) or in guinea pigs (α2, α3) and were used at dilutions of 1:10,000 (α1), 1:2000 (α2), 1:5000 (α3), and 1:5000 (γ2). They were a kind gift of Dr. H. Möhler (Zürich; α1, α2, α3) and Dr. A Stephenson (London; γ2).

Two monoclonal antibodies against glycine receptors were applied. They have been described in detail elsewhere (Schmitt et al., 1987; Schröder et al., 1991). Briefly, mAb2b is specific for the N-terminal sequence of the α1 subunit of the glycine receptor; mAb4a is specific for all α subunits known to date and the β subunit. They were kindly provided by Dr. H. Betz (Frankfurt). A third monoclonal antibody (mAb7a) is specific for gephyrin, a protein that links glycine receptors to the cytoskeleton (Kirsch et al., 1991; Kirsch and Betz, 1993). It was obtained from Boehringer Mannheim (Mannheim, Germany, Cat. No. 1170236). mAb2b was used diluted 1:100, mAb4a was used diluted 1:500, and mAb7a was diluted 1:50.

Immunolabeling protocol of LY-injected retinae. As described in detail by Grünert and Wässle (1993), the indirect immunofluorescence method was applied. Briefly, after blocking in 10% normal goat serum (NGS; in 0.01 M PBS containing 0.5% Triton X-100), the sections or whole retinae were incubated in the primary antibodies diluted in PBS with 3% NGS, 0.5% Triton X-100, and 0.05% sodium azide. Incubation in the primary antibodies was for 4 d at 4°C. The sections or whole retinae were then rinsed in PBS and incubated for 12 hr in the secondary antiserum (diluted in PBS with 3% NGS and 0.5% Triton X-100). After several rinses in PBS, sections or whole retinae were coverslipped with Mowiol (Hoechst, Frankfurt, Germany; see Harlow and Lane, 1988). Control sections were processed omitting the first antibody. This procedure always resulted in a complete lack of immunoreactivity. Because of the strong fluorescence of LY in the yellow–green range of the spectrum, secondary antibodies that show a red fluorescence were chosen. These were goat anti-mouse IgG conjugated to carboxymethylindocyanine (Cy3; Dianova, Hamburg, Germany; 1:1000), goat anti-rabbit IgG conjugated to Cy3 (Dianova; 1:1000), goat anti-guinea pig IgG conjugated to Cy3 (Dianova; 1:1000), and goat anti-mouse IgG conjugated to Texas Red (Amersham, Braunschweig, Germany; 1:50).

Double immunofluorescence of vertical sections. After fixation and cryoprotection as described for the LY-injected retinae, pieces of rat retinae were mounted and frozen in freezing medium (Reichert-Jung) on a cryostat (Reichert-Jung, Frigocut 2700). Vertical sections were cut at 12 μm, collected on gelatin-coated slides, and stored at −20°C before use.

Immunolabeling was performed as described previously (Grünert and Wässle, 1993; Grefcrath et al., 1995; Sassoè-Pognetto et al., 1994, 1995). Double immunolabeling was performed by incubating the sections in a mixture of two primary antibodies. The localization of the primary antibodies was revealed with specific secondary antibodies coupled to either red (Cy3, Texas Red) or green fluorescent markers (goat anti-rabbit/guinea pig IgG conjugated to Cy2 or FITC; Biotrend, Köln, Germany, 1:100).

Microphotography. The sections and whole mounts were examined and photographed with a Zeiss photomicroscope (Axiophot) using the appropriate fluorescence filters (LY: 395–400, FT 460, LP 470; FITC: 450–490, FT 510, LP 520; Cy3, Texas Red: BP 546, FT 580, LP 590). The fluorescence filters were wedge-corrected, so shifting from one filter to the other did not cause any displacements of the image. In some instances, very strong Cy3 fluorescence was also visible with the FITC filter. This could be blocked by an additional green interference filter (515–565) inserted into the microscope tube. The strong LY fluorescence was also visible with the FITC filter set and bled through the Cy3 filter set. However, this was an advantage, because it was possible to observe with the same filter (Cy3, red fluorescence) both the LY-filled dendrites and the immunoreactive puncta to study their possible colocalization. Black and white photomicrographs were taken on Kodak TMY 400 film, and color micrographs were taken on Kodak Ektachrome EPL 400 color-reversal film.

RESULTS

Alpha ganglion cells express glycine receptors

In the mammalian retina, glycine has been localized in about half of the amacrine cell population and in some bipolar cells (Pourcho and Goebel, 1985a,b, 1987a,b, 1990; Wässle et al., 1986; Koontz et al., 1993; Pow and Crook, 1994; Pow et al., 1995). Glycinergic amacrine cells are small-field amacrines and comprise several morphological types (Pourcho and Goebel, 1985a). Bipolar, amacrine, and ganglion cells are the postsynaptic elements at glycinergic synapses (Pourcho and Owczarzak, 1991a). Here we concentrate on the question of whether Alpha ganglion cells express the α1 subunit and/or other subunits of the glycine receptor. For this purpose, Alpha ganglion cells in rat and rabbit retinae were injected intracellularly with LY and, subsequently, the reti-

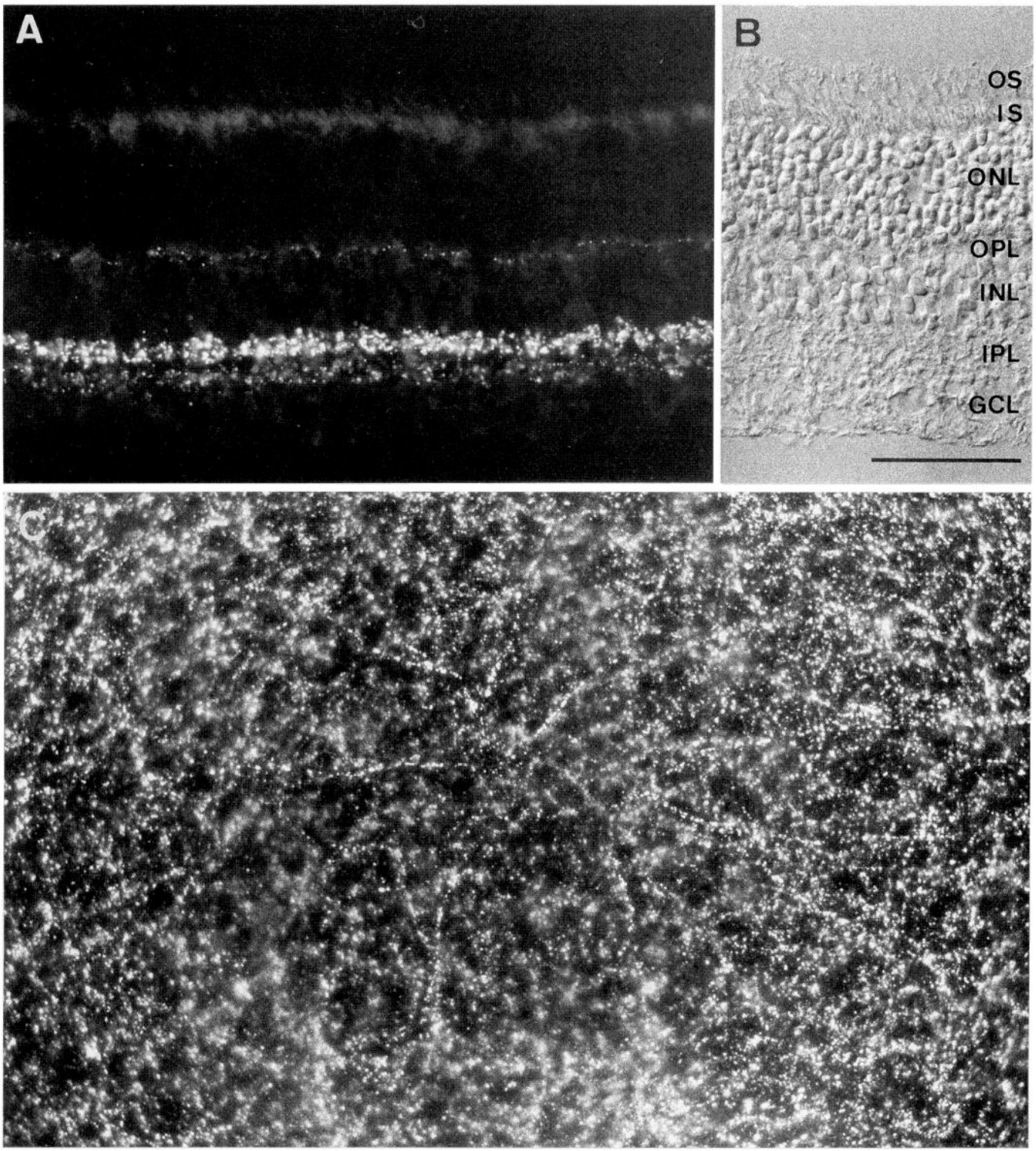

Figure 1. Micrographs of rabbit retinae immunostained for the α1 subunit of the glycine receptor (mAb2b). *A*, Fluorescence micrograph of a vertical section (Cy3, *red fluorescence*). *B*, Nomarski micrograph of the same section showing the retinal layers: *OS*, photoreceptor outer segments; *IS*, inner segments; *ONL*, outer nuclear layer; *OPL*, outer plexiform layer; *INL*, inner nuclear layer; *IPL*, inner plexiform layer; *GCL*, ganglion cell layer. Punctate immunofluorescence can be observed in *A*, which is concentrated in three bands. A band of rather large puncta is present in the outer half of the IPL (the OFF-sublamina), a band of smaller puncta occupies the inner half of the IPL (the ON-sublamina), and a thin band of puncta can also be seen in the OPL. *C*, Fluorescence micrograph of a whole mount of the rabbit retina. The plane of focus is in the inner half of the IPL. Scale bar (shown in *B*), 50 μm.

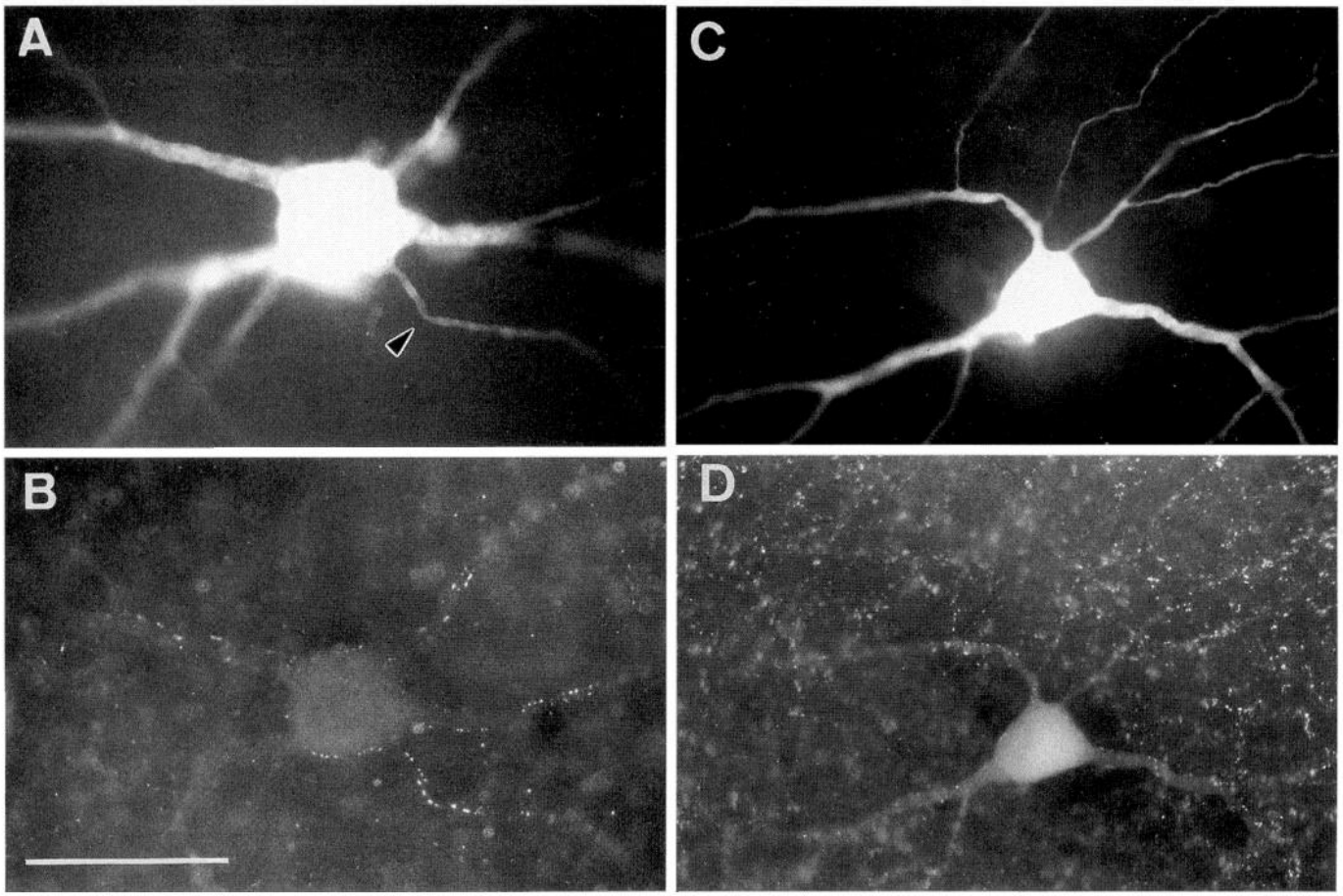

Figure 2. Two ON-Alpha cells of the rabbit retina, which were injected with LY and then immunostained for the α1 subunit of the glycine receptor (mAb2b). *A* and *C* show the two cells in retinal whole mounts using *yellow fluorescence*. The cell bodies and the primary dendritic trees become apparent. *B* and *D* show the two cells using *red fluorescence*. The α1-immunoreactive puncta and the LY-injected cell bodies and primary dendrites can be seen together. Scale bar: *A*, *B*, 50 μm; *C*, *D*, 60 μm.

nae were immunostained for glycine receptors. Figure 1*A* shows a vertical section of a rabbit retina that was immunolabeled for the α1 subunit of the glycine receptor. Strong punctate immunofluorescence can be observed in the IPL, and a sparse distribution of puncta can also be detected in the OPL (Grünert and Wässle, 1993). We have shown previously by electron microscopy that this punctate immunofluorescence represents a concentration of the α1 subunits at postsynaptic sites (Sassoè-Pognetto et al., 1994). The distribution of the immunoreactive puncta in a whole mount of the rabbit retina is shown in Figure 1*C*. The plane of focus is on the ON sublamina of the IPL. The immunoreactive puncta are not distributed homogeneously, and (with some imagination) one can see an alignment of the puncta along putative dendritic processes. Most puncta have diameters between 0.5 and 1.5 μm, which is close to the resolution limit of the light microscope. Electron microscopy has shown postsynaptic clusters of 0.3–1.5 μm diameter in the case of the α1 subunit of the glycine receptor (Sassoè-Pognetto et al., 1994).

Alpha ganglion cells were selected for intracellular injection with LY based on their large cell bodies made visible by acridin orange staining (Peichl, 1992). After the injection, the dendritic branching pattern was used as the major criterion for their identification. By changing the plane of focus in the IPL, it was possible to decide whether the dendritic branches were in the outer or inner half of the IPL and to classify the cells as OFF or ON types, respectively. Two ON-Alpha ganglion cells of the rabbit retina that were injected with LY and then immunostained for the α1 subunit of the glycine receptor are shown in Figure 2. Only the cell bodies and primary dendrites are visible in Figure 2. However, inspection under low power made it clear that both cells are Alpha ganglion cells. They have large cell bodies and rather thick primary dendrites that radiate away from the cell body without giving off many side branches (Boycott and Wässle, 1974). The focus in Figure 2*A* is more on the cell body, whereas that in Figure 2*C* is on the primary dendritic tree. Micrographs of the two cells taken at the same focal planes but with a Cy3 (red) fluorescence filter are shown in *B* and *D*, respectively. The puncta immunoreactive for the α1 subunit of the glycine receptor become apparent, and the dendrites of the Alpha ganglion cells are also just visible. The few puncta that are in the plane of focus in Figure 2*B* decorate the outline of the soma and of the thick primary dendrites of the Alpha ganglion cell. One of the smaller dendrites in focus (*arrowhead* in Fig. 2*A*) can be seen as a string of puncta in Figure 2*B*. In Figure 2*D*, the plane of focus is further toward the IPL, where the density of puncta is higher. Comparison of Figure 2, *C* and *D*, shows the alignment of strings of puncta along those dendrites that are in the plane of focus. Hence, Figure 2 suggests that many of the α1-immunoreactive puncta actually coincide with the dendritic and somatic membranes of Alpha ganglion cells. This is further analyzed by the fluorescence micrographs shown in Figure 3*A–D*.

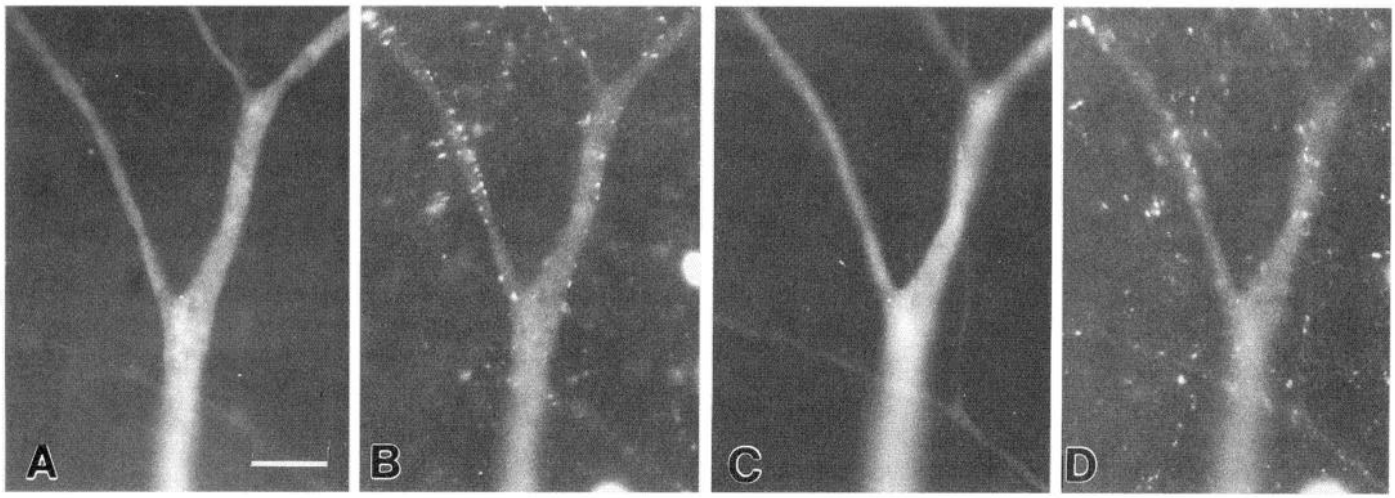

Figure 3. High-power fluorescence micrographs of a Y-shaped dendritic branch of an LY-injected ON-Alpha cell of the rabbit retina, which was then immunostained for the α1 subunit of the glycine receptor (mAb2b). *A* and *B* show the same focal plane and were taken with *yellow* and *red fluorescence*, respectively. Many immunoreactive puncta colocalize with the dendritic membrane. *C* and *D* show this dendritic branch at a focal plane taken 2 μm farther (outer) into the IPL. *C* was taken with *yellow fluorescence* and *D* with *red fluorescence*. In addition to the main Y-shaped dendritic branch, a small oblique branchlet can be seen at this focal plane. Both are decorated with α1-immunoreactive puncta. Scale bar (shown in *A*), 10 μm.

The use of high-power objectives (Plan Neofluar, X100/1.3) with a narrow plane of focus and viewing the LY-injected dendrites at different focal planes made it possible to determine whether immunoreactive puncta were positioned on the dendritic membrane. Both the yellow LY-injected dendrites and the red Cy3-immunofluorescent puncta could be observed using the same red fluorescence filter. Thus, the puncta appeared orange if and only if they were colocalized with dendrites. Figure 3 shows a Y-shaped dendritic branch of a LY-injected ON-Alpha ganglion cell of a rabbit retina immunostained for the α1 subunit of the glycine receptor. With yellow fluorescence (Fig. 3*A*, *C*), only the dendrites are apparent; with red fluorescence (Fig. 3*B*, *D*), both immunoreactive puncta and dendrites can be seen. Figure 3, *A* and *B*, shows the same focal plane; in Figure 3, *C* and *D*, the focal plane was taken 2 μm further into the IPL. The smaller sidebranch on the left is in focus in Figure 3, *A* and *B*; it is decorated (Fig. 3*B*) with immunoreactive puncta. This sidebranch and its immunoreactive puncta are out of focus in Figure 3, *C* and *D*. The vertically oriented dendritic branch is ~3–5 μm thick and, therefore, the two focal planes, 2 μm apart, represent different optical sections through this branch. Figure 3, *A* and *B*, cuts through the center of the branch; immunoreactive puncta are positioned on the membrane. Figure 3, *C* and *D*, shows the outer surface of the branch, and there also immunoreactive puncta are colocalized. Thus, by continuously changing the focal plane during observation on the microscope, it was possible to decide whether immunoreactive puncta coincided with the dendrites. In the case of thicker dendrites, one could see on which part of the surface of the dendrite puncta colocalized with the dendritic membrane. The shape of the puncta was an additional cue: those on the top or bottom surface were round, whereas those on the side of a dendrite were more elongated.

The same material was also observed with a confocal microscope. However, for this particular task, we find conventional microscopy with high-power objectives superior. Because immunofluorescence in the present experiments is punctate, small changes of focus in a conventional microscope cause them to disappear and, therefore, optical sectioning is very precise. The punctate fluorescence acts like the pinhole of the confocal microscope. The dynamic process of adjusting the best focus for each fluorescent dot or dendrite, as well as the hue information mentioned above, is a distinct advantage of conventional microscopy. Because LY and Cy3 fluorescence is very stable with respect to bleaching and fading, there was enough time to carefully analyze the cells and take a series of conventional fluorescence micrographs at different optical sections to reconstruct the complete dendritic tree of an Alpha ganglion cell. Such a reconstruction of an ON-Alpha ganglion cell of the rabbit retina is shown in Figure 4. The dendritic tree in Figure 4*A* has the typical branching pattern of an Alpha ganglion cell. In Figure 4*B*, the immunoreactive puncta within the dendritic tree are collapsed from three optical sections into a single plane. Their distribution looks similar to the micrograph in Figure 1*B*. Figure 4*C* shows finally those puncta immunoreactive for the α1 subunit of the glycine receptor that were colocalized with the dendrites of the Alpha cell. There is no apparent compartmentalization of puncta along the dendrites: both distal and proximal dendrites express immunoreactive puncta. Only few puncta are found directly on the surface of the soma.

Comparable experiments were also performed for the other two antibodies related to the glycine receptor: mAb4a, which recognizes all subunits, and mAb7a, which is specific for gephyrin. Both mAb4a and mAb7a produced a punctate immunofluorescence signal, and there was colocalization of the puncta with the dendrites of both ON and OFF Alpha ganglion cells in the rabbit. The three antibodies were also applied after LY injection into Alpha ganglion cells of the rat retina. Because the IPL of the rat retina is thicker, horizontal sections were cut through the retinae after LY injection into Alpha ganglion cells to ease penetration of the antibodies. Both ON- and OFF-Alpha cell dendrites were decorated—as described above for the rabbit—with immunoreactive puncta of all three antibodies against glycine receptors and gephyrin. The number of puncta that colocalized with the dendrites of Alpha cells was lowest for mAb2b (α1 subunit), it was 3–5 times higher for mAb4a (all subunits), and it was 4–7 times higher for mAb7a (gephyrin). The number of individual cells and the antibodies applied are listed in Table 1.

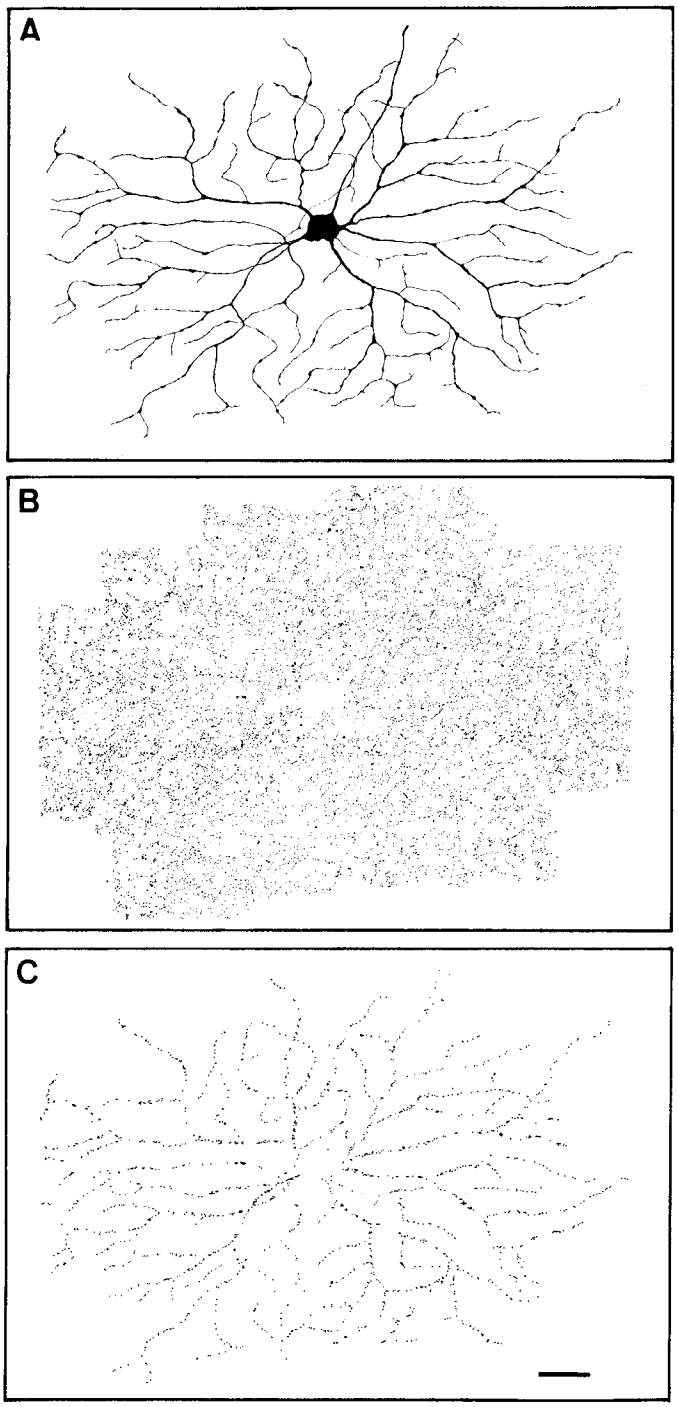

Figure 4. Reconstruction of the dendritic tree of an ON-Alpha ganglion cell of the rabbit retina and of the puncta immunoreactive for the α1 subunit of the glycine receptor. *A*, Drawing of the dendritic tree after injection with LY as viewed from the ganglion cell layer in a retinal whole mount. *B*, Drawing of the puncta immunoreactive for the α1 subunit of the glycine receptor. They were collapsed from three optical sections, which contained the Alpha cell dendrites, into a single plane. *C*, Drawing of those immunoreactive puncta that were found to be colocalized with the dendrites of the Alpha cell. The bright fluorescence of the LY-injected cell body masked the immunoreactive puncta there, and the absence of puncta from the area covered by the cell body is an artifact. Scale bar (shown in *C*), 50 μm.

Alpha ganglion cells express different isoforms of $GABA_A$ receptors

In the mammalian retina, GABA and the GABA-synthesizing enzyme glutamic acid decarboxylase (GAD) have been localized in different populations of amacrine cells and in horizontal cells (Brandon, 1985; Mosinger et al., 1986; Sarthy and Fu, 1989; Pourcho and Owczarzak, 1989; Wässle and Chun, 1989; Grünert and Wässle, 1990; Koontz and Hendrickson, 1990; Pow and Crook, 1994; Vardi et al., 1994; Vardi and Auerbach, 1995). GABAergic amacrine cells comprise many different morphological types and contain, in addition to GABA, a variety of different neuroactive substances (Vaney, 1990). Approximately 50% of all amacrine cells are GABAergic. $GABA_A$ receptors have been localized immunocytochemically in the retina of different mammals by the use of subunit-specific antibodies (for review, see Greferath et al., 1995). Punctate immunoreactivity, representing a clustering of receptors at postsynaptic sites, was observed with antibodies specific for the α1, α2, α3, β1, and γ2 subunits (Brandstätter et al., 1995; Greferath et al., 1995; Sassoè-Pognetto et al., 1995). We will test here whether Alpha ganglion cells of rat and rabbit retinae express clusters of α1, α2, α3, and γ2 subunits along their dendrites.

A vertical section through a rat retina that was immunostained for the γ2 subunit of the $GABA_A$ receptor is shown in Figure 5*A*. Punctate immunofluorescence can be observed in the IPL. A comparable staining pattern was also found in the rabbit retina. An Alpha ganglion cell of the rabbit retina that was injected with LY and immunostained for the γ2 subunit of the $GABA_A$ receptor is shown in Figure 5*C–E*. The low-power micrograph (Fig. 5*C*) illustrates the typical branching pattern of the Alpha cell dendritic tree. Figure 5*D* is a magnified view of one of the dendritic branches, and Figure 5*E* shows the γ2-immunoreactive puncta. The Alpha cell branch is only faintly visible in Figure 5*E*. However, comparison of *D* and *E* suggests that the puncta marked by arrows are colocalized with the dendrites of the LY-injected Alpha cell. The use of high-power objectives and direct microscopical analysis (as described above) showed this convincingly. This suggests expression of the γ2 subunit at postsynaptic sites located in the cell membrane of Alpha ganglion cells. To find out whether there is a uniform distribution of such synapses along Alpha cell dendrites, a reconstruction of γ2-immunoreactive puncta comparable with Figure 4 is shown in Figure 6. There is a rather uniform distribution of puncta along the dendrites of this Alpha cell (Fig. 6*B*). The relative density of puncta is lower than in Figure 4*B*, suggesting that there are fewer synapses expressing the γ2 subunit of the $GABA_A$ receptor than synapses expressing the α1 subunit of the glycine receptor.

Using the same approach, LY-injected Alpha ganglion cells were also immunostained for the α1, α2, and α3 subunits of the $GABA_A$ receptor. Punctate immunofluorescence was found with all three markers. For all three α subunits, many immunoreactive puncta were observed on Alpha cell dendrites. The numbers of cells injected and the subsequent immunocytochemical stainings are listed in Table 1.

There are two possible explanations for this expression of $GABA_A$ receptor subunits by Alpha ganglion cells. One is that Alpha ganglion cells are a homogeneous cell class and they express all these receptors. The other possibility is that Alpha ganglion cells are heterogeneous and comprise many different subtypes and that each of the subtypes expresses a specific type of $GABA_A$ receptor. We think this second explanation is unlikely

Table 1. Numbers of investigated retinal Alpha ganglion cells (GCs) expressing receptor subunits

Receptor components	Rat, ON Alpha GCs	Rat, OFF Alpha GCs	Rabbit, ON Alpha GCs	Rabbit, OFF Alpha GCs
$GABA_AR\ \alpha_1$	3	7	5	1
$GABA_AR\ \alpha_2$	8	7	1	2
$GABA_AR\ \alpha_3$	13	14	17	7
$GABA_AR\ \beta_1$	9	21	1	1
$GABA_AR\ \beta_{2/3}$	8	13	2	3
$GABA_AR\ \gamma_2$	14	32	21	10
GlyR α_1	21	33	21	16
GlyR all subunits	3	4	2	2
Gephyrin	2	3	3	1

because it would predict that some of the Alpha ganglion cells that we injected with LY would *not* express at least certain subunits, a result we never observed. Still, one might consider this indirect evidence. We therefore performed additional experiments in which individual Alpha ganglion cells of the rat retina were immunostained for two different subunits. LY-injected Alpha ganglion cells were classified as ON or OFF, and then horizontal sections (12 μm thick) were taken on a cryostat. The dendritic branches of the injected Alpha cell were thus cut up into at least two consecutive sections. One half of the sections was then immunostained for a certain subunit, and the other half was immunostained for another subunit. All three possible combinations of the three α subunits of the $GABA_A$ receptor were tested in this way: α1/α2, α1/α3, α2/α3. Results of the α1/α3 pair are presented in Figure 7*A–F*. Figure 7*A* shows a horizontal section of a rat retina that contains the cell body and the primary dendrites of the LY-injected Alpha ganglion cell. This section was immunoreacted for the α3 subunit of the $GABA_A$ receptor (Cy3-coupled secondary antibodies). A double-exposure micrograph with green and red fluorescence reveals the injected Alpha ganglion cell and the α3-immunoreactive puncta (Fig. 7*B*). Some of the puncta coincide with the primary dendrites of the Alpha ganglion cell. High-power micrographs, taken at two focal planes (1 μm apart) demonstrate this in more detail (*arrows* in Fig. 7*C*, *D*). Another section through the dendritic tree of this Alpha ganglion cell (Fig. 7*E*) was immunoreacted for the α1 subunit of the $GABA_A$ receptor (Fig. 7*F*). Some of the α1-immunoreactive puncta (*arrows* in Fig. 7*F*) coincide with the dendrite, suggesting that they are expressed at synapses made onto this dendrite. Table 2 summarizes the number of cells and combinations tested in this way. For all three combinations of these subunits, we always observed immunoreactive puncta on the dendrites of the LY-injected Alpha ganglion cells.

Specificity of $GABA_A$ receptor clusters

Double-label immunofluorescence was used to determine whether the individual subunits of the $GABA_A$ receptor are expressed and clustered at the same postsynaptic sites. Because of the strong fluorescence of LY in the yellow–green range of the spectrum, such double-labeling experiments using FITC- and Cy3-conjugated secondary antibodies could not be performed on LY-injected Alpha cells. Instead, vertical sections that did not contain LY-injected cells were used. Figure 8*A* explains the method applied and illustrates the colocalization of the α2 and γ2 subunits of the $GABA_A$ receptor (Sassoè-Pognetto et al., 1995). A vertical section of a rat retina was double-immunostained. Immunoreactivity for the γ2 subunit was visualized (Fig. 8*A*, *left*) using FITC-coupled secondary antibodies (*green fluorescence*), and α2 immunoreactivity (Fig. 8*A*, *right*) was visualized with Cy3-coupled secondary antibodies (*red fluorescence*). This latter micrograph (Fig. 8*A*, *right*) is printed as a mirror image; identical points of the section, therefore, are found at equal distances from the vertical midline. Comparison of the labeled puncta at both sides of the midline show mirror symmetric distribution indicating substantial colocalization. With some imagination, a symmetrical figure can be detected at the midline.

The same approach was chosen to detect colocalization of puncta that are immunoreactive for the different α subunits of the $GABA_A$ receptor. Vertical sections of rat retinae were double labeled for the α1 and α2 subunit of the $GABA_A$ receptor (Fig. 8*C*) and for the α1 and α3 subunit (Fig. 8*B*). No symmetry of immunoreactive puncta across the vertical midline can be detected in Figure 8, *B* or *C*, suggesting that the immunoreactive puncta are at noncorresponding positions. This search for the colocalization of the α1 subunit with the α2 and α3 subunits, respectively, was also performed more quantitatively. Photomicrographs of double-labeled sections were taken on color-reversal film with red and green fluorescence. The two slides were projected onto transparencies, and the immunoreactive puncta were drawn. First, the two transparencies were brought into precise register, and the coinciding puncta were counted. Then the two transparencies were superimposed with random horizontal shifts, and coinciding puncta were counted. The average number of colocalizations of puncta depended critically on the criterion applied. If the criterion was very stringent and only the puncta that precisely coincide were counted, only 5% of the puncta colocalized at random superposition of the transparencies. If we counted all of the cases in which puncta partially overlapped, this number increased to 25% for random superposition. In the cases of α1 versus α2 and α1 versus α3 subunits, the number of colocalizations at the correct superposition did not significantly exceed the random level.

The antibodies against the α2 and α3 subunits were raised in the same species, so a direct comparison could not be performed. However, the laminar distribution in the IPL and the sizes of α2 and α3 immunoreactive puncta differ (Greferath et al., 1995). Furthermore, α2 is strongly colocalized with gephyrin, whereas very few α3 puncta are gephyrin-immunoreactive (Sassoè-Pognetto et al., 1995).

We conclude that α1-, α2-, and α3-immunoreactive puncta are not colocalized. This suggests that these three subunits of the $GABA_A$ receptor are not clustered at the same postsynaptic sites, but are aggregated at different synapses.

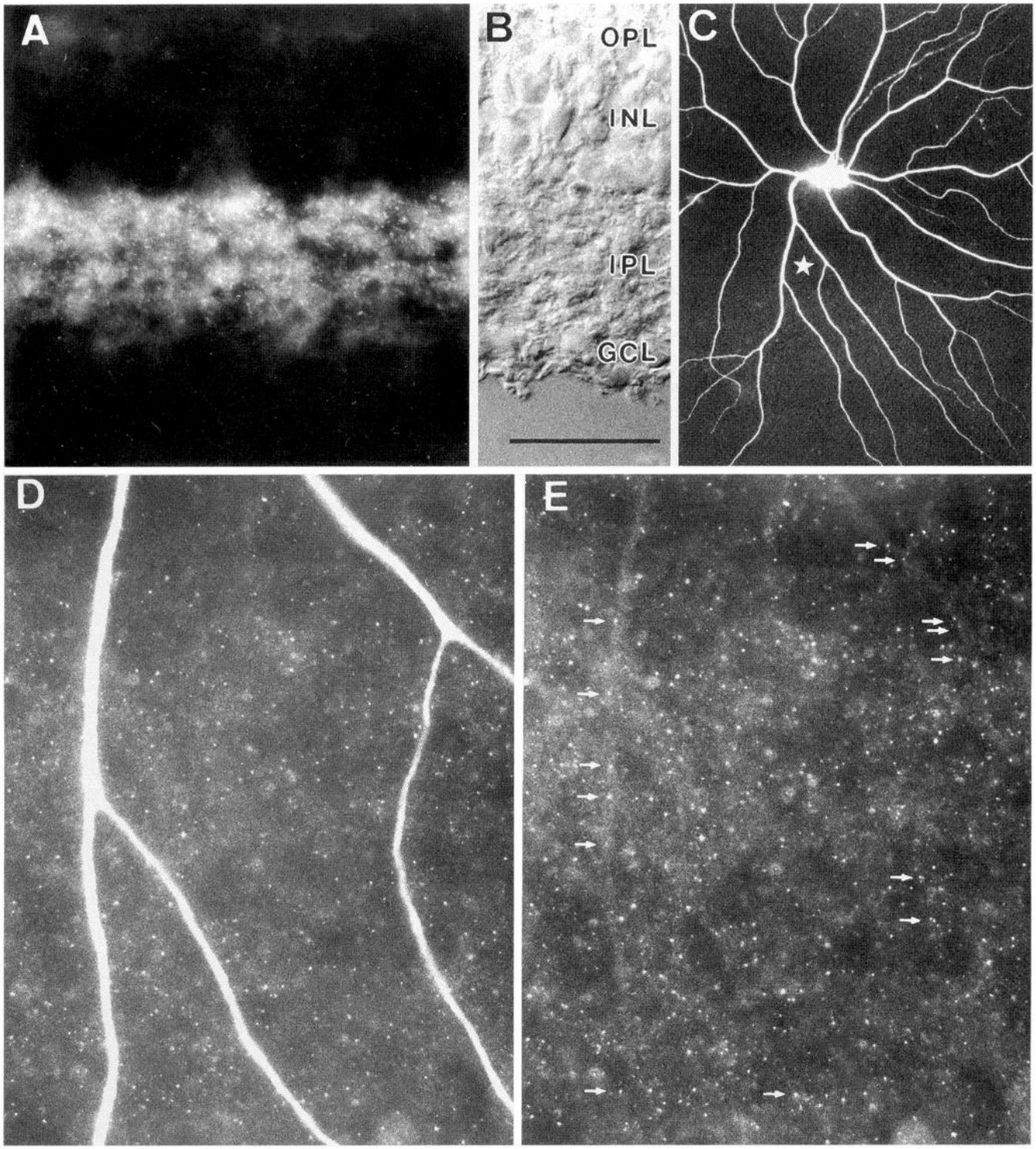

Figure 5. Localization of the γ2 subunit of the $GABA_A$ receptor in rat (*A*) and rabbit (*E*) retinae. *A*, Fluorescence micrograph of a vertical section of the rat retina showing punctate immunofluorescence in the IPL. The *white clouds* are groups of puncta that are out of focus (*green fluorescence*, FITC). *B*, Nomarski micrograph of the section in *A* showing the retinal layers (conventions as in Fig. 1*B*). *C*, Low-power fluorescence micrograph of an LY-injected Alpha cell in a whole mount of the rabbit retina, which was immunostained for the γ2 subunit of the $GABA_A$ receptor (*yellow fluorescence*). The branch encircling the *white star* is shown at higher magnification in *D*. *D*, Fluorescence micrograph taken with double exposure (*green* and *red* filters) showing a branch of the LY-injected Alpha cell and the punctate γ2 immunoreactivity. *E*, Single-exposure fluorescence micrograph (*red* filter) showing the same field as in *D*. The puncta indicated by the *arrow* coincide with the dendrites of the Alpha cell. Scale bar (shown in *B*): *A*, *B*, *D*, *E*, 25 μm; *C*, 250 μm.

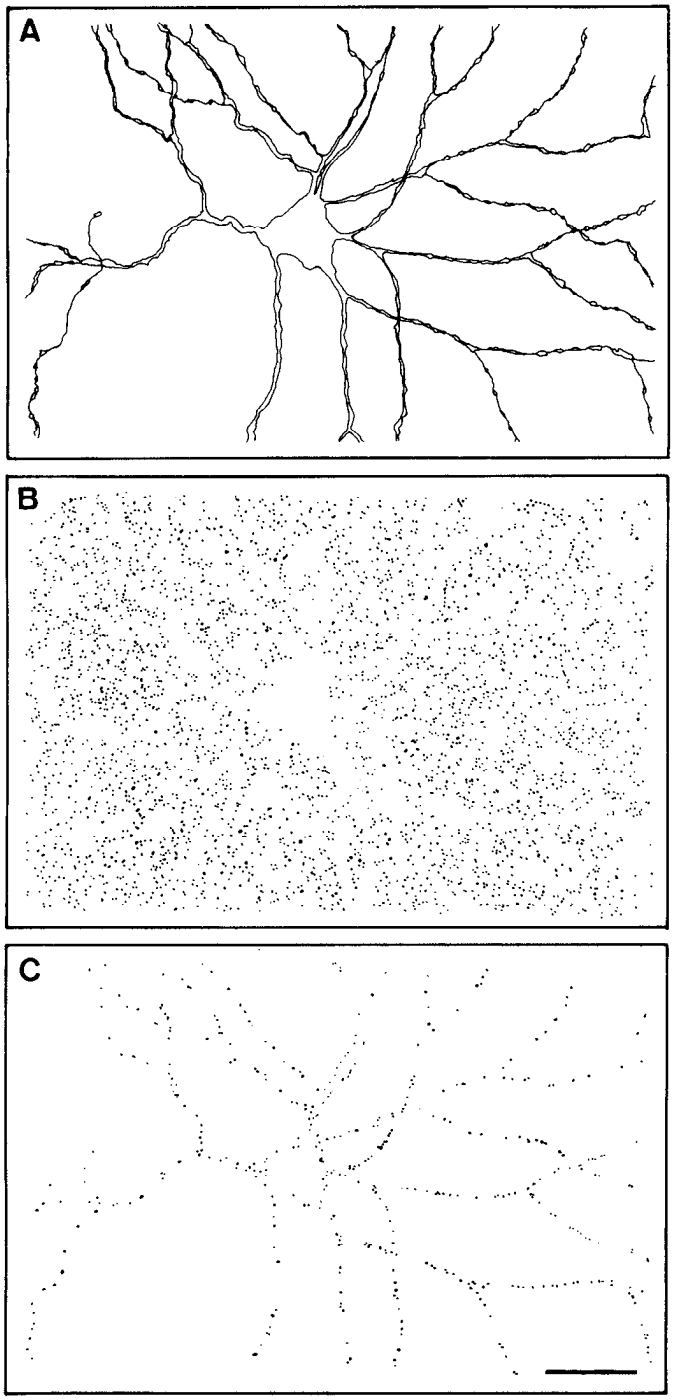

Figure 6. Reconstruction of the dendritic tree of an ON-Alpha cell of the rat retina and of the puncta immunoreactive for the $\gamma 2$ subunit of the $GABA_A$ receptor. *A*, Drawing of the central portion of the dendritic tree. *B*, Drawing of the immunoreactive puncta collapsed from four optical sections, which contained the Alpha cell dendrites, into a single plane. *C*, Drawing of those immunoreactive puncta that coincided with the dendritic tree of the Alpha ganglion cell shown in *A*. The bright fluorescence of the LY-injected cell body masked the immunoreactive puncta there. Scale bar (shown in *C*), 30 μm.

DISCUSSION

Immunofluorescent puncta on Alpha cell dendrites

Only electron microscopy can show definitely whether the immunofluorescent puncta that coincide with Alpha cell dendrites are actually postsynaptic aggregates of receptors expressed on the somatodendritic membrane of the LY-injected Alpha cells. However, as illustrated in Figures 2 and 3, there is strong evidence from light microscopy that some of the immunoreactive puncta coincide with the Alpha cell membrane. The antibodies recognize extracellular epitopes of the $GABA_A$ and glycine receptors, respectively (Schmitt et al., 1987; Benke et al., 1991, Kirsch et al., 1991, Schröder et al., 1991; Gao et al., 1993; Kirsch and Betz, 1993), and labeling in electron micrographs is confined to the synaptic cleft (Sassoè-Pognetto et al., 1994; 1995). The immunofluorescence is in close agreement with this labeling pattern, which is particularly apparent in the case of thick primary dendrites of Alpha cells that can be optically sectioned at different focal planes. With the focus at the top or bottom surface of the dendrites, immunoreactive puncta appear as round patches. With the focus cutting through the center of the dendrites, the puncta are exclusively on the side and appear as short strips. Hence, in a through-focus series the shape of the puncta changes from round to oval to linear, while at the same time their location moves from the center of the dendrite to the two sides. In the case of smaller dendrites, immunoreactive puncta often are in register with varicose swellings of the LY-injected dendrites. Freed and Sterling (1988) and Weber and Stanford (1994) have reconstructed Alpha ganglion cells of the cat retina from serial electron micrographs. They reported a rather uniform distribution of conventional synapses across the dendritic field of Alpha cells. Our finding of an even distribution of immunoreactive puncta (Figs. 4, 6) is in good agreement with these results.

Glycine receptors of Alpha ganglion cells

Two monoclonal antibodies that recognize the $\alpha 1$ subunit of the glycine receptor (mAb2b) and all subunits presently known (mAb4a), respectively, were applied in the present study. In previous studies, we found that both antibodies produced a punctate staining of the IPL; however, the structure and distribution of the puncta differed (Grünert and Wässle, 1993; Greferath et al., 1994b; Pinto et al., 1994; Sassoè-Pognetto et al., 1994, 1995). Puncta expressing the $\alpha 1$ subunit were usually larger, and their density was higher in the outer part of the IPL, and many large puncta were found to colocalize with the synaptic contacts between AII amacrine cells and OFF cone bipolar cells (Sassoè-Pognetto et al., 1994). Puncta immunolabeled by mAb4a were more numerous, smaller, and more evenly distributed throughout the IPL. In the present study, immunoreactive puncta on the somatodendritic membranes of Alpha ganglion cells were stained with both antibodies. However, between 3 and 5 times more puncta were labeled with mAb4a. Synapses expressing the $\alpha 1$ subunit, therefore, are only a minority of the total number of glycinergic synapses Alpha cells receive. No systematic differences were observed between OFF-and ON-Alpha ganglion cells. The results suggest at least two types of glycinergic synapses: those that express the $\alpha 1$ subunit, and those that apparently do not. The question arises which α subunit might be expressed in the latter synapses. So far it has been assumed that in the embryonic and neonatal CNS, the $\alpha 2$ subunit is the predominant subunit (Becker et al., 1988), which is later replaced by the adult $\alpha 1$ and $\alpha 3$ subunit isoforms (Malosio et al., 1991a,b; Kuhse et al., 1991). However, our *in situ* hybridization experiments (Greferath et al., 1994b) have shown that in the adult retina, $\alpha 1$, $\alpha 2$, and $\alpha 3$, as well as the β mRNAs, were expressed in retinal ganglion cells. The mRNA of the $\alpha 2$ subunit gave the strongest signal and, in contrast to the rest of the CNS, this subunit seems to be present also in the adult

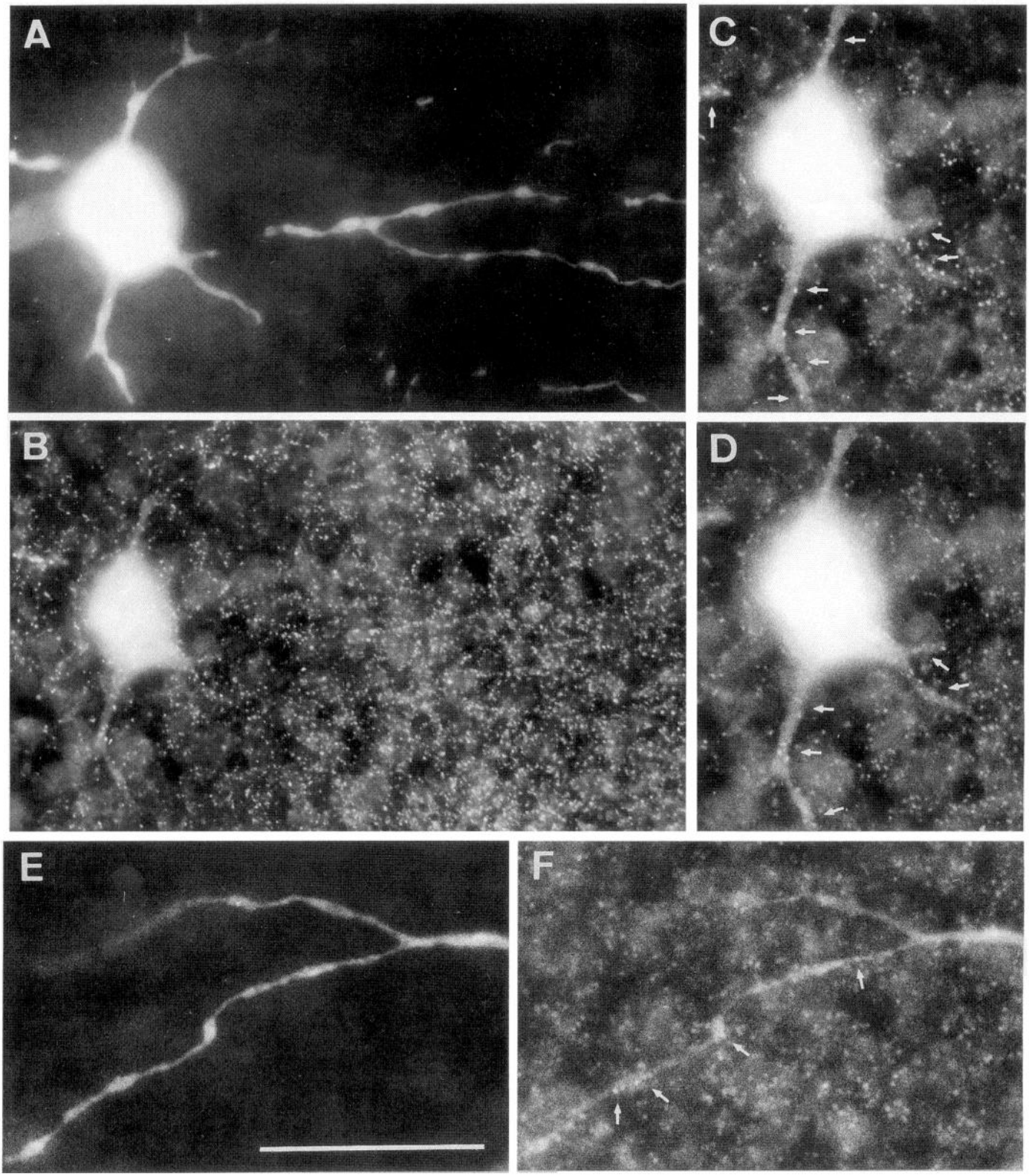

Figure 7. Localization of the $\alpha 3$ and of the $\alpha 1$ subunit of the $GABA_A$ receptor on the dendrites of an LY-injected ON-Alpha ganglion cell of the rat retina. *A*, Fluorescence micrograph of a horizontal section through the rat retina showing the cell body and the primary dendrites of the LY-injected Alpha cell (*yellow fluorescence*). *B*, Fluorescence micrograph of the same field as in *A* (double exposure with *green* and *red fluorescence*) showing the puncta immunoreactive for the $\alpha 3$ subunit as well as the injected Alpha cell. Some of the puncta coincide with the primary dendrites. *C*, *D*, High-power fluorescence micrographs taken at two focal planes and showing the LY-injected Alpha cell together with the $\alpha 3$-immunoreactive puncta. *Arrows* indicate puncta coinciding with the Alpha cell dendrites. *E* and *F* show another horizontal section through the same LY-injected Alpha ganglion cell. *E*, Fluorescence micrograph showing a dendritic branch of the injected Alpha cell (*yellow fluorescence*). *F*, Fluorescence micrograph of the same field as in *E* showing the $\alpha 1$-immunoreactive puncta and the Alpha cell dendritic branch. Some of the puncta (*arrows*) colocalize with this dendrite. Scale bar (shown in *E*): *A*, *B*, 40 μm; *C*, *D*, 30 μm; *E*, *F*, 20 μm.

Table 2. Numbers of investigated rat retinal Alpha ganglion cells (GCs) expressing two different $GABA_AR$ α subunits

Receptor subunit combinations	ON Alpha GCs	OFF Alpha GCs
$GABA_AR$ α_1/α_2	9	3
$GABA_AR$ α_1/α_3	7	2
$GABA_AR$ α_2/α_3	3	2

retina. It is possible, therefore, that Alpha ganglion cells express not only two, but several isoforms of the glycine receptor. Whether they are colocalized at the same synapses or, comparable to the $GABA_A$ receptors, are found at distinct synapses cannot be answered at present. It has been shown by Sassoè-Pognetto et al. (1995) that clusters of glycine receptors and $GABA_A$ receptors are not colocalized. This suggests that Alpha ganglion cells receive GABAergic and glycinergic input at distinct synapses.

$GABA_A$ receptors of Alpha ganglion cells

The current model of $GABA_A$ receptor stoichiometry assumes that five subunits are necessary for the formation of GABA-induced Cl^- channels (Macdonald and Olsen, 1994; Sieghart, 1995). In analogy with the nicotinic AchR, it is assumed that two α subunits may be present in the $GABA_A$ receptor–ion-channel complex. Results of immunoprecipitation of $GABA_A$ receptors suggest that as a rule identical α subunits assemble and two different α subunits are only rarely combined within the same receptor (Sieghart, 1995). The results presented here for the retina support this because puncta immunoreactive for the α1, α2, and α3 subunits were not colocalized. In addition, they also suggest that receptors with different α subunits do not occur within the same postsynaptic clusters.

The specific subunit composition of the $GABA_A$ receptor defines the pharmacological and physiological profile of the channel. Even if the clusters would have identical β or γ subunits, the α subunits themselves convey enough diversity to make the channels different. Varying the α subunits in combination with constant β and γ subunits resulted in differences in benzodiazepine pharmacology and steroid modulation of GABA responses (Macdonald and Olsen, 1994). In addition, the biophysical properties such as conductance levels or channel opening times are different for different α subunits. It is expected, therefore, that the clusters described here for the three α subunits of the $GABA_A$ receptor are functionally different.

Cell presynaptic to $GABA_A$ receptors in the IPL

Ultrastructural studies of the $GABA_A$ receptor-immunoreactive puncta in the IPL showed that they are located at conventional chemical synapses (Sassoè-Pognetto et al., 1995). Presynaptic at the $GABA_A$ receptor clusters, therefore, are amacrine cells, most likely GABAergic amacrine cells. The proportion of GABAergic amacrines in rabbits or cats is close to 50% of all amacrines (Vardi and Auerbach, 1995; Pow et al., 1995). They comprise many different morphological types and are generally wide-field amacrine cells (Vaney, 1990). In the context of the present study, the most remarkable feature of GABAergic amacrine cells is the great diversity of other neuroactive substances that they colocalize and perhaps corelease with GABA (Brecha et al., 1984, 1988; Massey and Redburn, 1987; Kosaka et al., 1988; Vaney and Young, 1988; Vaney, 1990; Wässle and Boycott, 1991). It has been shown that these neuroactive substances are able to modulate GABA responses through specific receptors and second-messenger systems.

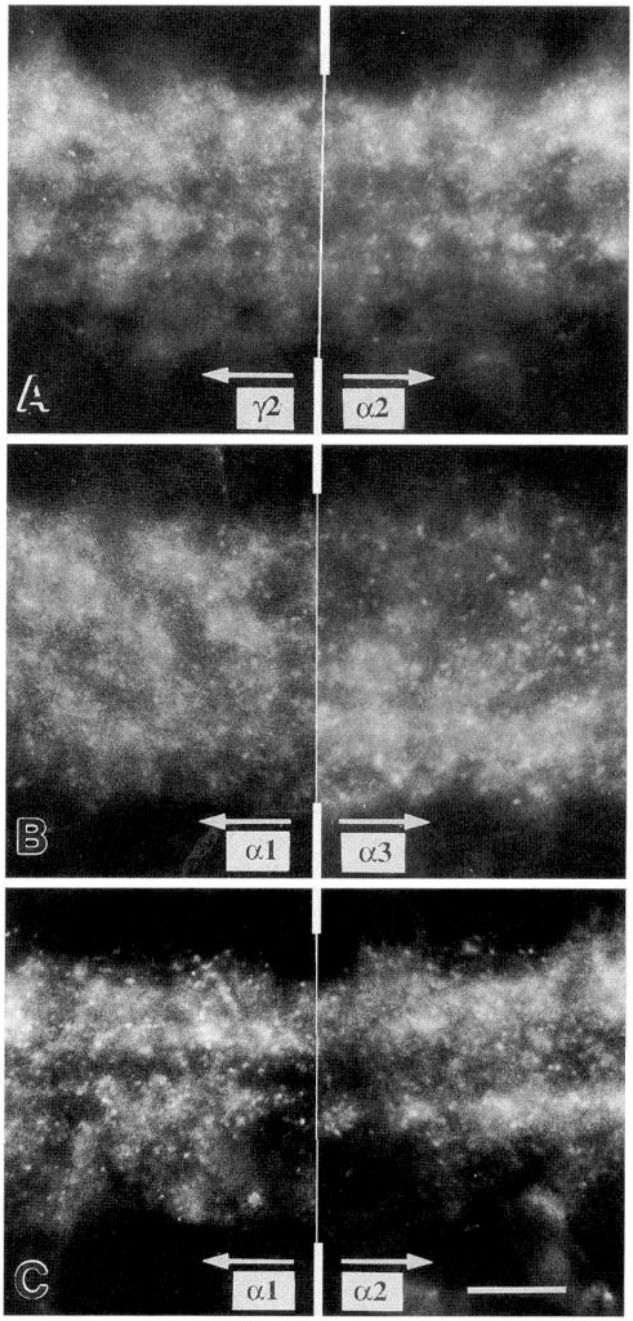

Figure 8. Micrographs of vertical sections showing the IPL of the rat retinae that were double immunostained for two subunits of the $GABA_A$ receptor. *A*, *Left*, Immunoreactivity for the γ2 subunit (FITC). *Right*, Immunoreactivity for the α2 subunit (Cy3). The two micrographs are printed as mirror images, sectioned along a common border (*vertical white midline*), and puncta at symmetrical positions represent colocalizations. *B*, *Left*, Immunoreactivity for the α1 subunit (FITC). *Right*, Immunoreactivity for the α3 subunit (Cy3). No puncta can be observed at symmetrical positions across the midline, indicating a lack of colocalization. *C*, *Left*, Immunoreactivity for the α1 subunit (FITC). *Right*, Immunoreactivity for the α2 subunit (Texas Red). No puncta are found at symmetrical positions. Scale bar (shown in *C*), 10 μm.

Veruki and Yeh (1992) have demonstrated modulation of $GABA_A$ receptor function in bipolar and ganglion cells by vasointestinal polypeptide (VIP). Feigenspan and Bormann (1994) found that dopamine, adenosine, VIP, somatostatin, and enkephalin increase GABA-induced currents of amacrine cells. In

these cases, modulation of $GABA_A$ receptor function may involve phosphorylation (Feigenspan and Bormann, 1994; Veruki and Yeh, 1994). The number and location of phosphorylation sites vary in the α, β, γ, and δ subunits and in the different subtypes of the same subunit (Macdonald and Olsen, 1994). All of this suggests that the specific synaptic clusters of $GABA_A$ receptors that we found on Alpha cells might receive synaptic input from different types of GABAergic amacrine cells. Moreover, because these amacrine cells colocalize other neuroactive substances, specific modulation might occur at the different synapses.

Specific aggregation of $GABA_A$ receptors

Craig et al. (1994) have discussed two possible mechanisms for generating neurotransmitter clusters at specific postsynaptic sites. In the first model, receptors are added along the entire somatodendritic surface and are clustered by trapping in the membrane. In the second model, the receptors are sorted into different intracellular transport vesicles that fuse only at the appropriate postsynaptic sites. The present results are in favor of the first model, because we found, in addition to the synaptic clusters of the receptors, a weak but specific immunofluorescence along the somatodendritic membrane. Similar extrasynaptic location of $GABA_A$ receptors has also been reported in the cerebellum (Somogyi et al., 1989; Soltez et al., 1990; Nusser et al., 1995a). Hence, one has to postulate that receptors are trapped at the postsynaptic sites. This must be a highly specific process, because different α subunits of the $GABA_A$ receptor cluster at different synapses. It is an attractive idea that the neuroactive substances mentioned above that colocalize with GABA in GABAergic amacrine cells may not only have a modulatory action on postsynaptic neurons, but also may act as signals for the selective clustering of specific subunits of the $GABA_A$ receptor. The recent findings of regulation of NMDA receptor clustering by phosphorylation (Ehlers et al., 1995) and the discovery of a postsynaptic density protein for this receptor (Kornau et al., 1995) show mechanisms by which the clustering might be regulated.

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