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Synaptogenesis in the Rat Retina: Subcellular Localization of Glycine Receptors, $GABA_A$ Receptors, and the Anchoring Protein Gephyrin

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ABSTRACT

The mechanisms by which neurotransmitter receptors are clustered at postsynaptic sites of neurons are largely unknown. The 93-kDa peripheral membrane protein gephyrin has been shown to be essential for the formation of postsynaptic glycine receptor clusters, and there is now evidence that gephyrin can also be found at gamma-aminobutyric acid (GABA)ergic synapses. In this study, we have analyzed the synaptic localization of glycine receptors, $GABA_A$ receptors, and the anchoring protein gephyrin in the inner plexiform layer of the developing rat retina, by using immunofluorescence with subunit specific antibodies. At early postnatal stages, the antibodies produced a diffuse staining, suggesting that early retinal neurons can express glycine and $GABA_A$ receptors. A clustered distribution of the subunits in "hot spots" was also observed. The number of "hot spots" increased during development and reached adult levels in about 2 weeks. Electron microscopy showed that synapses of the conventional type are present in the inner plexiform layer of the postnatal retina and that the hot spots correspond to an aggregation of receptors at postsynaptic sites. Gephyrin was also localized to "hot spots," and double immunofluorescence revealed a colocalization of gephyrin with the α2 subunit of the $GABA_A$ receptor. These results indicate that clustering of receptor subunits occurs in parallel with the formation of morphologically identifiable synaptic specializations and suggest that gephyrin may be involved in clustering of $GABA_A$ receptors at postsynaptic sites. J. Comp. Neurol. 381:158–174, 1997.

Indexing terms: synapse; development; mammalian retina; immunocytochemistry; electron microscopy

Aggregation of neurotransmitter receptors in the postsynaptic membrane is crucial for efficient signal transmission between neurons. Receptors of central neurons, such as acetylcholine receptors at the neuromuscular junction (reviewed in Froehner, 1993), are concentrated at postsynaptic sites. It was shown by using electron microscopic immunocytochemistry that glycine receptors (GlyRs) and the GlyR-associated protein gephyrin are precisely localized to the postsynaptic membrane of spinal neurons (Triller et al., 1985; Nicola et al., 1992). Further studies have shown that other receptor types, including glutamate and gamma-aminobutyric acid A ($GABA_A$) receptors, have a restricted spatial distribution on the postsynaptic neuronal surface (Jacob and Berg, 1983; Jacob et al., 1984; Aoki et al., 1987; Richards et al., 1987; Sargent and Pang, 1989; Somogyi et al., 1989; Petralia and Wenthold, 1992; Baude et al., 1993, 1994; Martin et al., 1993; Molnar et al., 1993; Craig et al., 1994; Petralia et al., 1994a,b). Some receptors are also present at extrasynaptic plasma membrane sites (Somogyi et al., 1989; Soltesz et al., 1990; Baude et al., 1992, 1994, 1995), but their concentration in the postsynaptic membrane is significantly higher (Nusser et al., 1995).

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Clustering of receptors at postsynaptic sites also occurs in retinal neurons. It has been shown by using specific antibodies that different subunits of glycine and $GABA_A$ receptors are concentrated at conventional synapses made by amacrine cells in the inner plexiform layer (IPL; Greferath et al., 1993, 1994b; Grünert and Wässle, 1993, 1996; Sassoè-Pognetto et al., 1994, 1995; Vardi and Sterling, 1994). Glutamate receptors are also clustered at postsynaptic membranes. Nomura et al. (1994) have demonstrated that the metabotropic glutamate receptor mGluR6 is concentrated in the dendritic tips of bipolar cells inserted into photoreceptor synaptic terminals, and we have shown that the NR2A subunit of the n-methyl-D-aspartic acid (NMDA) receptor is localized to ganglion cell dendrites that are postsynaptic at bipolar cell ribbon synapses (Hartveit et al., 1994).

The mechanisms by which receptors are transported to and anchored at the correct postsynaptic sites in central neurons are largely unknown. Some recent data seem to indicate that receptors may be initially localized extrasynaptically and become gradually restricted to synapses during development (Aoki et al., 1994; Craig et al., 1994; Nomura et al., 1994). This trapping mechanism, which also mediates receptor clustering at the neuromuscular junction (Froehner, 1993), requires signaling molecules released from the presynaptic terminal as well as local postsynaptic anchoring proteins that control receptor mobility in the membrane.

At the neuromuscular junction, agrin, a nerve-derived extracellular matrix protein, and rapsyn, a protein associated with the postsynaptic membrane, have been implicated in the molecular mechanisms underlying the formation and maintenance of acetylcholine receptor clusters (reviewed by McMahan et al., 1992; Hall and Sanes, 1993; Nastuk and Fallon, 1993; Apel and Merlie, 1995). Although there is no evidence that these molecules play similar roles at central nervous system (CNS) synapses, the peripheral membrane protein gephyrin is essential for the formation of glycine receptor clusters on CNS neurons (Kirsch et al., 1993b). There is considerable evidence that gephyrin could act as a bivalent linker protein that connects the β subunit of the GlyR with subsynaptic microtubules (Kirsch et al., 1991; Kirsch and Betz, 1995; Meyer et al., 1995). Gephyrin is not localized uniquely to glycinergic synapses (Bolhalter et al., 1994; Cabot et al., 1995; Todd et al., 1995; Craig et al., 1996) but can be associated with $GABA_A$-receptor subunits and may be involved with clustering of $GABA_A$ receptors (Kirsch et al., 1995; Sassoè-Pognetto et al., 1995). Therefore, gephyrin may act as an anchoring protein for different types of neurotransmitter receptors (Kirsch and Betz, 1993; Kirsch et al., 1993a).

Much of our knowledge about synaptogenesis and receptor clustering in central neurons is derived from studies on cell cultures (Basarski et al., 1994; Fletcher et al., 1994). This approach, however, does not preserve the anatomical continuity of the nervous tissue, and it is questionable whether cultured neurons develop normally and establish the appropriate connections in vitro. The mammalian retina could be an ideal system for elucidating in vivo some of the aspects of synapse formation. The cell types and connectivity of the retina are well characterized (Wässle and Boycott, 1991), and the patterns of retinal development, including cell birth and death, neuronal differentiation, and synaptogenesis, have been defined in several studies (reviewed by Robinson, 1991). Furthermore, recent immunocytochemical investigations using antibodies against neurotransmitter-receptor subunits have shown that individual synapses can be recognized at the light microscopic level as brightly stained puncta ("hot spots") that correspond to high densities of receptors localized to postsynaptic plasma membrane specializations (Grünert and Wässle, 1993; Hartveit et al., 1994; Nomura et al., 1994; Sassoè-Pognetto et al., 1994, 1995). The possibility of resolving individual synapses and of studying receptor clustering by light microscopy is a definite advantage.

We have used immunocytochemistry in the present study to analyze the expression and synaptic localization of GlyRs, $GABA_A$ receptors, and the anchoring protein gephyrin in the IPL of the developing rat retina. Electron microscopy was also performed to investigate the emergence of synapses at early stages of postnatal development. In particular, we wanted to determine whether clustering of receptor subunits and of gephyrin occurs in parallel with the formation of morphologically identifiable synaptic specializations. We also used double-label immunofluorescence to find out whether gephyrin is already associated with $GABA_A$-receptor subunits at the earliest stages of retinal synaptic development.

MATERIALS AND METHODS

Antibodies

Three monoclonal antibodies (mAb 7a, mAb 2b, and mAb 4a) against GlyRs or gephyrin (Pfeiffer et al., 1984) were provided by Dr. H. Betz (Department of Neurochemistry, Max-Planck-Institute for Brain Research, Frankfurt, Germany). The mAb 7a (also available from Boehringer, Mannheim, Germany) is specific for gephyrin (Schmitt et al., 1987) and was used diluted 1:50. The mAb 2b is specific for the N-terminal sequence of the α1 subunit of the GlyR (Schröder et al., 1991) and was used diluted 1:100. The mAb 4a recognizes all the GlyR subunits known (α1, α2, α3, β; Schröder et al., 1991) and was used diluted 1:500. Three polyclonal antibodies raised against specific peptides of the $GABA_A$-receptor subunits α1, α2, and α3 were provided by Dr. H. Möhler (Department of Pharmacology, University of Zürich, Zürich, Switzerland). These antibodies were raised in rabbits (α1) or in guinea pigs (α2, α3) and were diluted 1:10,000 (α1), 1:2,000 (α2), and 1:5,000 (α3).

The following secondary antibodies were used: goat anti-mouse conjugated to carboxymethylindocyanine (CY3; 1:1,000; Dianova, Hamburg, Germany); goat anti-mouse conjugated to fluorescein isothiocyanate (FITC; 1:50; Sigma, Deisenhofen, Germany); sheep anti-mouse conjugated to biotin (1:250; Vector, Burlingame, CA); goat anti-rabbit conjugated to CY3 (1:1,000; Dianova); goat anti-guinea pig conjugated to CY3 (1:1,000; Dianova); goat anti-guinea pig conjugated to FITC (1:50; Dianova); goat anti-guinea pig conjugated to biotin (1:100; Dianova). The biotinylated antibodies were used in combination with streptavidin-Texas Red (1:100; Amersham, Braunschweig, Germany), or the avidin-biotin-peroxidase complex (1:100; Vector).

Light microscopic immunocytochemistry

Neonatal rats were taken at 2-day intervals from postnatal day P1 to P21, defining the day of birth as P0. They were anesthetized with halothane and decapitated. The protocols were approved by the animal care officer of our

institute. The eyes were enucleated and opened by an encircling cut along the ora serrata. After the vitreous was removed, the posterior eyecups were fixed by immersion in 4% paraformaldehyde in phosphate buffer (PB; 0.1 M, pH 7.4). Because the antigenic epitopes of both glycine- and $GABA_A$-receptor subunits were very sensitive to prolonged fixation, the fixation time had to be reduced to only 10 minutes. Shorter fixation times further increased the intensity of the immunolabeling but were incompatible with an acceptable preservation of histological structure. The eyecups were rinsed in PB and cryoprotected in sucrose (10, 20, and 30%); the retina was dissected out and cut with a cryostat in 14-µm-thick vertical sections that were collected on gelatin-coated slides.

Synaptogenesis in mammalian retinae tends to be more advanced in the central retina than at the periphery, and most studies of synaptogenesis have concentrated on events in central retina (Robinson, 1991). Therefore, in the present study, all stainings were performed on sections taken from central retina. The sections were stained according to the indirect immunofluorescence method, as described in detail previously (Grünert and Wässle, 1993; Sassoè-Pognetto et al., 1994), except that all the incubation times were strongly reduced (preincubation in normal goat serum: 30 minutes; primary antibodies: 2 hours; secondary antibodies: 1 hour). The sections were then examined and photographed with a Zeiss photomicroscope (Axiophot) with a ×63 or ×100 objective.

Double immunolabeling was performed by incubating the sections in a mixture of one of the polyclonal antibodies against the $GABA_A$-receptor subunits (α2 or α3), with mAb 7a. The binding sites of the primary antibodies then were revealed with specific secondary antibodies coupled to red (CY3, Texas Red) or green (FITC) fluorescent markers (for details, see Sassoè-Pognetto et al., 1994). Black and white photomicrographs were taken with red or green fluorescence. The fluorescence filters were wedge corrected and did not cause any spatial displacement of the image. To visualize colocalizations, the negatives were printed as mirror images, and the prints were sectioned along a common border and mounted side by side (see also Sassoè-Pognetto et al., 1995; Koulen et al., 1996).

Control sections were processed at all developmental stages by omitting the incubation with the primary antibody. In young neonatal animals (P1–P7), we observed with all secondary antibodies a nonspecific, diffuse staining in the outer and inner plexiform layers (OPL and IPL, respectively; see also Hartveit et al., 1994). This is shown in Figure 1A for a P3 rat retina. Figure 1B shows the architecture of the retina at this developmental stage. There is a diffuse fluorescence in the IPL that might be due to leakage of the blood-brain barrier at early developmental stages, resulting in the presence of rat immunoglobulins in this retinal layer (Kapfhammer et al., 1994). However, this staining never had a punctate appearance. At later stages there was no background labeling in the OPL and IPL (Fig. 1C). Blood vessels were always found to be strongly fluorescent (Fig. 1A,C).

In summary, at early stages the IPL was nonspecifically labeled, i.e., labeled processes within the IPL could not be easily discriminated from the fluorescent background. However, labeling of puncta, which correspond to synapses (see later), and labeling of cell bodies located in the inner nuclear layer (INL) and in the ganglion cell layer (GCL) could be discriminated.

Electron microscopic immunocytochemistry

The ultrastructural localization of gephyrin and two $GABA_A$-receptor subunits (α2 and α3) was investigated in four rats aged P7. The eyecups were fixed by immersion in 4% paraformaldehyde, 0.05% glutaraldehyde, and 0.2% picric acid for 15 minutes. This fixation was found to be an acceptable compromise between ultrastructural preservation and staining intensity. After fixation, the central retina was cut in vertical vibratome sections (70 µm) that were processed free-floating according to the biotin-avidin-peroxidase method (for details, see Sassoè-Pognetto et al., 1994). After incubation in the primary and secondary antibodies, the sections were reacted in 3,3′-diaminobenzidine (DAB), and then the DAB reaction product was silver intensified and gold toned. Finally, the sections were postfixed in 1% osmium tetroxide, dehydrated in acetone, and embedded in Epon 812. Serial ultrathin sections were stained with uranyl acetate and lead citrate and observed with a Zeiss EM10 electron microscope.

Electron microscopy

The retinae of P3 and P7 rats were prepared for conventional electron microscopy to determine whether synaptic contacts can be already detected at these early stages of development. The eyecups were fixed overnight in a solution containing 4% paraformaldehyde, 2.5% glutaraldehyde, and 0.2% picric acid; they were then postfixed in 2% osmium tetroxide, dehydrated in ethanol, and embedded in Epon 812. Ultrathin sections were cut from regions of the retina corresponding to those used for light and electron microscopic immunocytochemistry.

RESULTS

The six primary antibodies used in the present study produce in the adult rat retina a punctate immunofluorescence with distinct patterns of stratification in the IPL (Grünert and Wässle, 1993; Greferath et al., 1995). There is strong evidence that each punctum corresponds to an aggregation of receptors at a postsynaptic site (Sassoè-Pognetto et al., 1994, 1995). In addition to the punctate immunofluorescence, there can be a diffuse labeling of cell bodies and processes, suggesting the presence of intracellular and extrasynaptic pools of receptor subunits.

We have observed in the present study diffuse cytoplasmic staining at the earliest stages of retinal postnatal development, indicating that early retinal neurons can express glycine and $GABA_A$ receptors. In the following sections, we concentrate on the appearance of the immunofluorescent puncta and examine the time course of receptor clustering for each of the receptor subunits.

Development of glycine receptors

The postnatal maturation of GlyRs was investigated with mAb 2b, which is specific for the α1 subunit, and mAb 4a, which binds all GlyR subunits. At all developmental stages, mAb 2b produced immunofluorescent puncta in the IPL, but little or no diffuse staining was observed. There was a progressive and continuous increase in the number of puncta from P1 to P21 (Fig. 2A–D). During the first postnatal week, only a few puncta were present in the outer half of the IPL. Figure 2A shows GlyR α1-immunoreactivity on a section of a P5 rat retina. This micrograph

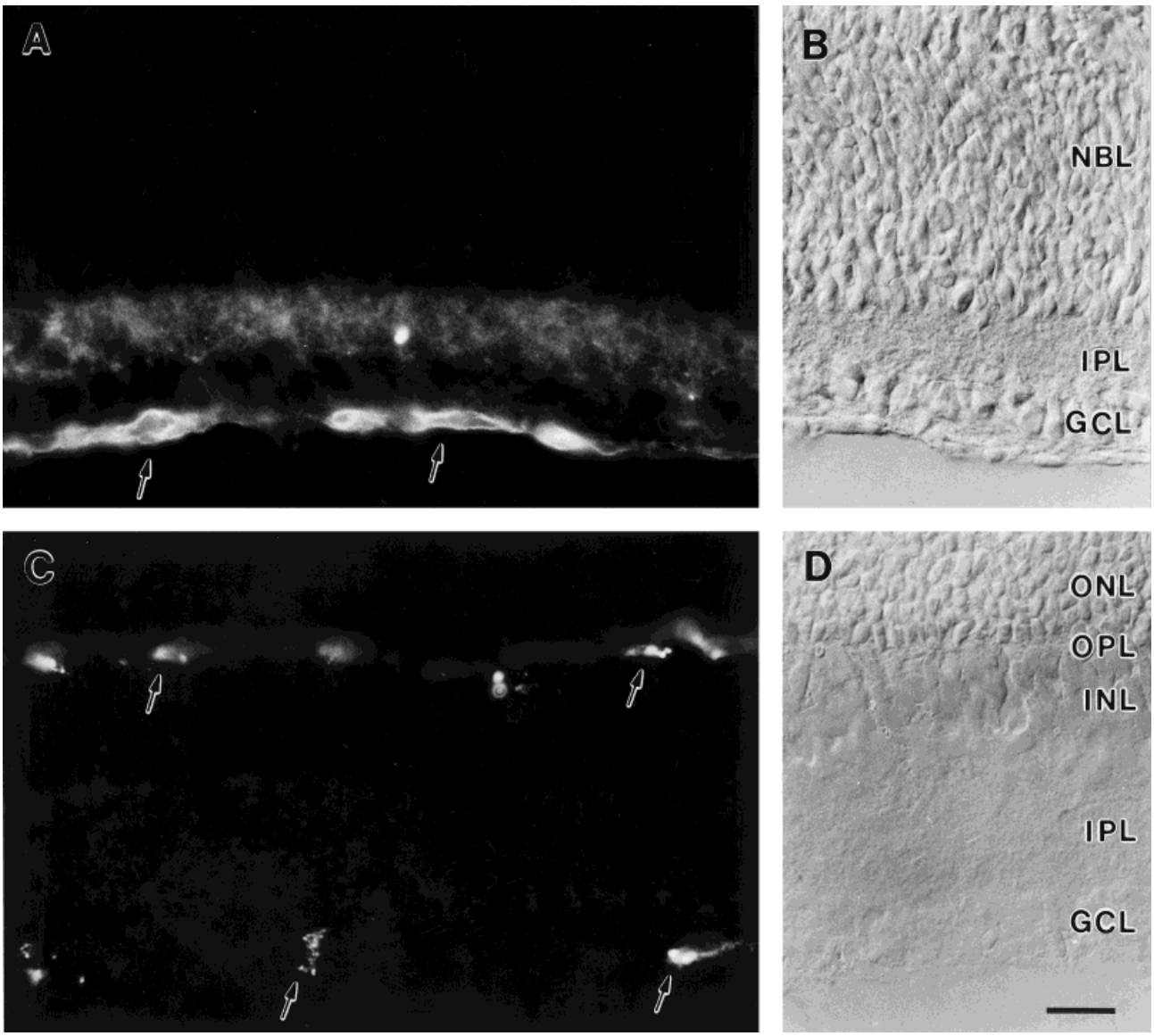

Fig. 1. Absence of punctate immunofluorescence on vertical sections of the developing rat retina (A: P3; C: P15) when the primary antibody was omitted. There is a diffuse fluorescence in the inner plexiform layer (IPL) at P3 (**A**) but not at P15 (**C**). Blood vessels (arrows) are fluorescent at all ages. **B,D:** Nomarski micrographs showing the layers of the retina at P3 and P15, respectively. The outer plexiform layer (OPL) is not visible at P3; it appears only at P5. P, postnatal; OPL, outer plexiform layer; INL, inner nuclear layer; ONL, outer nuclear layer; NBL, neuroblast layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bar = 20 μm.

was taken with a ×100 objective to better visualize the labeled puncta. All the other micrographs were taken with a ×63 objective. Starting from P9 (Fig. 2C), the density of the labeled puncta increased rapidly, and the labeling was organized in two bands, similar to the retinae of adult animals (Grünert and Wässle, 1993; Sassoè-Pognetto et al., 1994).

Monoclonal antibody 4a also produced immunofluorescent puncta that were preferentially localized in the outer part of the IPL and increased in number during development (Figs. 2E,F). In addition to the puncta, there was a labeling outlining cell bodies located in the putative amacrine cell layer and in the GCL, suggesting the presence of extrasynaptic receptors. Diffuse labeling of cells with this antibody has also been reported for the adult mammalian retina (Grünert and Wässle, 1993).

Development of $GABA_A$ receptors

Figure 3A–D illustrates the developmental pattern of the α2 subunit of the $GABA_A$ receptor. Hot spots immunoreactive for this subunit were detected in the IPL as early as P1. The density of the puncta increased with subsequent development (Fig. 3A), and from P5 on, the immunoreactivity was clearly concentrated in two bands (Fig. 3B). Cells in the GCL were stained diffusely, particularly during the first postnatal week (Fig. 3A,B). The diffuse staining was normally weaker than the punctate staining and is not always visible in the micrographs.

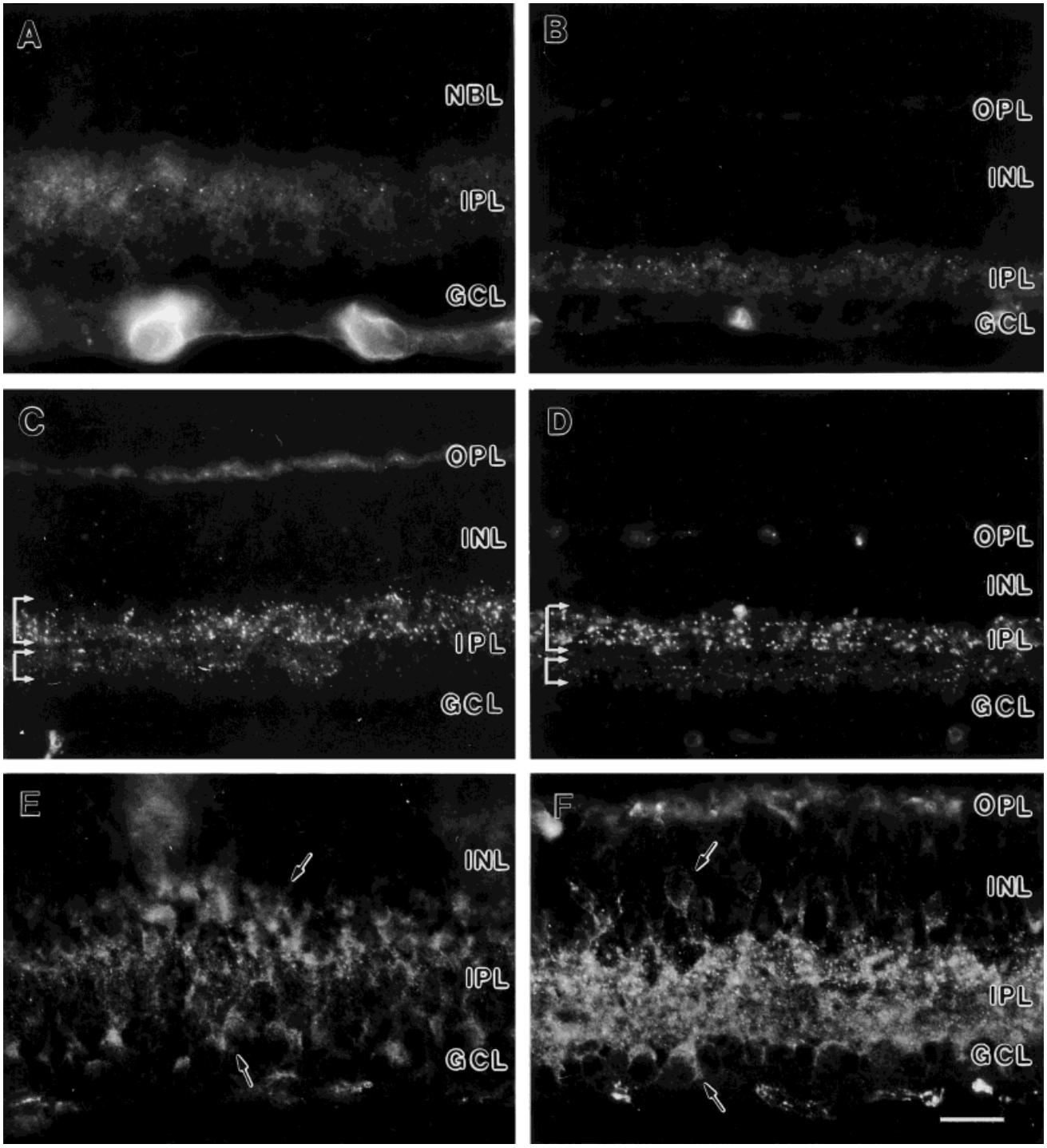

Fig. 2. Distribution of glycine receptor (GlyR) immunofluorescence in the developing rat retina. **A–D:** Immunofluorescence for the α1 subunit of the GlyR obtained with mAb 2b (A: P5; B: P7; C: P9; D: P17). Note the increase in the density of labeled puncta in the IPL from P5 (A) to P17 (D). Two broad bands of immunoreactive puncta (arrows) are first visible in the IPL at P9 (C) and are still visible at P17 (D). **E** (P7) and **F** (P15) show immunofluorescence for all GlyR subunits, obtained with mAb 4a. In addition to the puncta, there is a diffuse labeling in the IPL and in cells located in the INL and GCL (arrows). mAb 2b and mAb 4a are both monoclonal antibodies against GlyR. Abbreviations as in Figure 1, other conventions as in Figure 1. Scale bar = 13 μm in A, and 20 μm in B–F.

Hot spots immunoreactive for the α3 subunit of the $GABA_A$ receptor were already present in the IPL at P1 and gradually increased in number and density (Fig. 4A–D). At early postnatal stages (P1–P7) there was also a diffuse labeling of the IPL and of cells located in the putative amacrine and ganglion cell layers (Fig. 4A,B). This diffuse

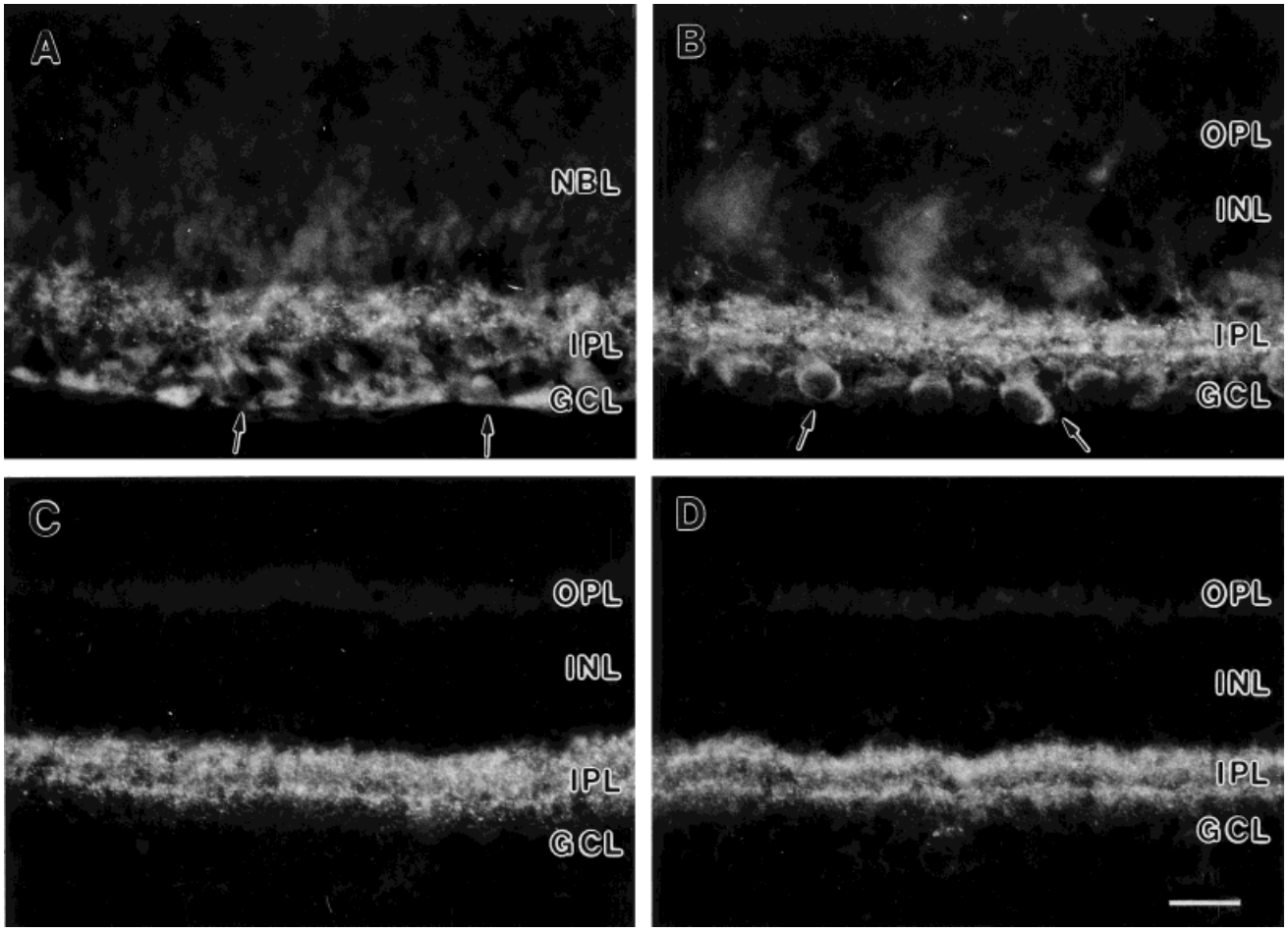

Fig. 3. Localization of the α2 subunit of the gamma-aminobutyric acid A ($GABA_A$) receptor in vertical sections of P3 (**A**), P7 (**B**), P9 (**C**), and P13 (**D**) rat retinae. There is punctate immunoreactivity in the IPL that increases with age. Starting from P5 and P7 (B), the immunoreactivity is clearly concentrated in two bands. Note the α2-positive cells (arrows) in the GCL at P3 (A) and P7 (B). Abbreviations as in Figure 1, other conventions as is Figure 1. Scale bar = 20 μm.

labeling was strongly reduced during further development. Stratification of α3-immunopositive bands in the IPL started at P9 (Fig. 4C) and reached the adult-like pattern (Fig. 4D) at P11.

The antiserum against the α1 subunit of the $GABA_A$ receptor labeled many cells located in the GCL at early postnatal stages (P1–P5; Fig. 5A). Although numerous, extremely fine puncta were visible in the IPL already at P1, clearly labeled puncta were first detected in the IPL and OPL only at P7 (Fig. 5B). The intensity of labeling in the IPL and OPL increased with subsequent development (Fig. 5B,C), and bands of higher receptor density were first visible in the IPL at P11. In adult rats, many cells located in the INL and in the GCL are positive for the α1 subunit (Greferath et al., 1995). This staining is not easily detected in the shortly fixed retinae of Figure 5C,D.

Early appearance of synapses in the IPL of the rat retina

The results so far indicate that hot spots containing high densities of glycine and $GABA_A$-receptor subunits are present in the IPL of young, postnatal rat retinae. It is conceivable that the appearance of the hot spots coincides with the formation of synaptic specializations in the IPL. However, Weidman and Kuwabara (1968), and later Horsburgh and Sefton (1987), reported that the first synapses in the IPL of the rat retina begin to form around P11. These are conventional synapses and, therefore, are likely to arise from amacrine cell processes. We examined whether morphologically identifiable synapses are present in the IPL already at P3 and P7. Figure 6 shows electron micrographs of transverse sections of the IPL of P3 (Fig. 6A–C) and P7 (Fig. 6D,E) rat retinae. At both stages, we found many specialized contacts between the processes, with thickening of the adjacent membranes, but without evidence of vesicles in either process (Fig. 6A). In addition, conventional synapses were observed. There were relatively few of these synapses at P3 (Fig. 6B,C) but were more numerous by P7 (Fig. 6D,E). They were characterized by a concentration of vesicles in the presynaptic terminal and membrane thickenings at both sides of the synaptic cleft. Usually, the number of synaptic vesicles in the presynaptic terminal was relatively low, suggesting that these synapses were not completely differentiated. Ribbon synapses were not present at these ages, but in a

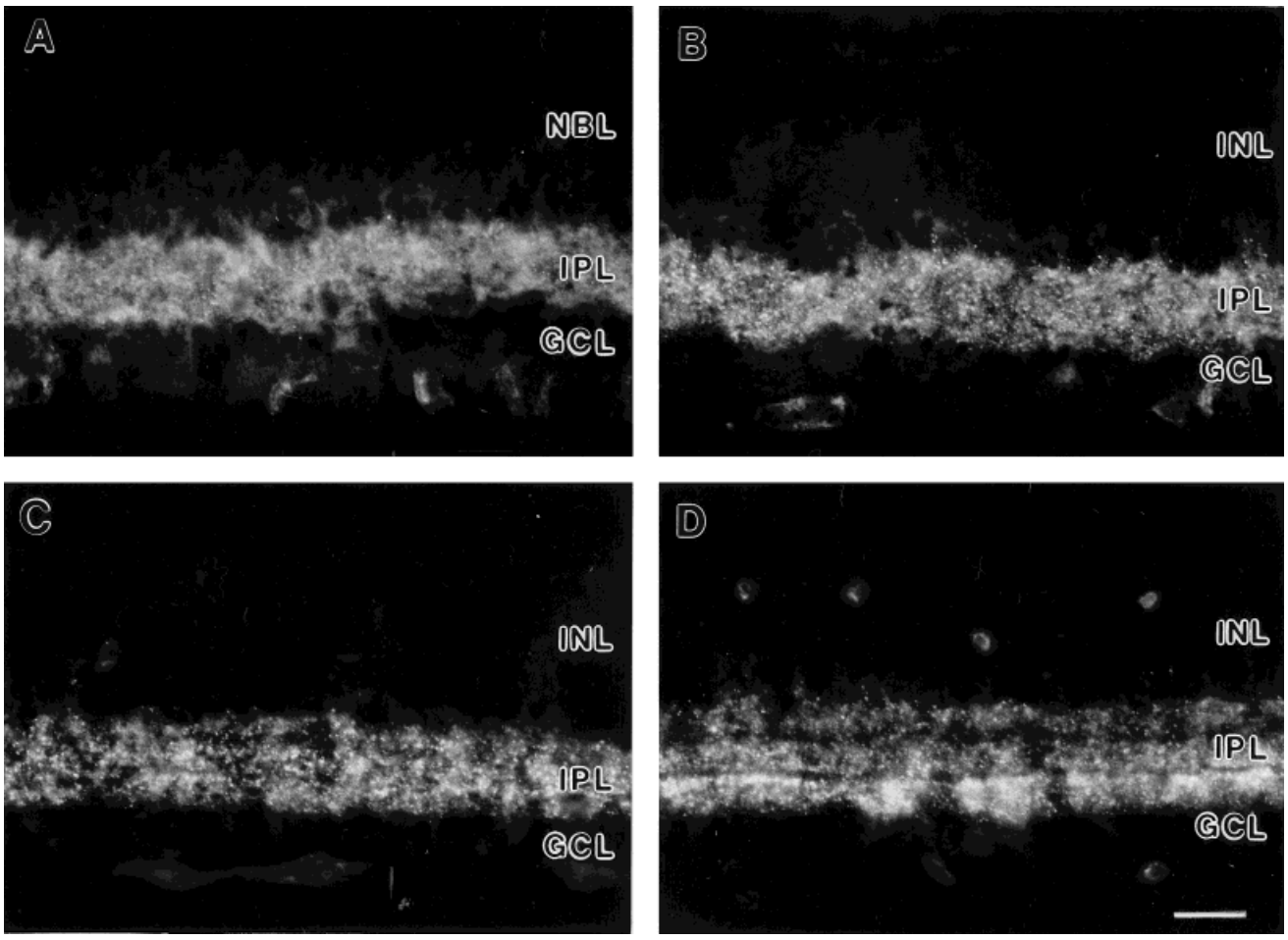

Fig. 4. Localization of the α3 subunit of the $GABA_A$ receptor in vertical sections of P3 (**A**), P7 (**B**), P9 (**C**), and P17 (**D**) rat retinae. At early stages (A), there is both punctate and diffuse staining in the IPL and in cells located close to the IPL. During development, the diffuse staining is reduced (B), and distinct bands of immunoreactive puncta appear in the IPL (C,D). Abbreviations as in Figure 1, other conventions as in Figure 1. Scale bar = 20 μm.

few instances we observed small ribbons floating in the cytoplasm of neuritic processes at P7 (not shown).

These results demonstrate that conventional synapses are already present in the IPL of the rat retina well before P11 and suggest that the hot spots detected by fluorescence microscopy may correspond to an aggregation of receptors at postsynaptic sites.

Synaptic localization of the $GABA_A$-receptor subunits α2 and α3 at P7

We performed electron microscopic immunocytochemistry on the retinae of four rats aged P7, to provide direct evidence that the hot spots correspond to high densities of receptor subunits clustered at synapses. Even with brief fixation, immunoreactivity for the GlyR subunits was completely abolished. We concentrated, therefore, on the investigation of the subcellular localization of the $GABA_A$-receptor subunits α2 and α3. For both subunits, we found immunolabeling of synaptic and extrasynaptic membranes (Fig. 7). These antibodies were raised against extracellular domains of the subunits (Benke et al. 1991; Gao et al., 1993; Marksitzer et al., 1993); thus, the immunoreactivities were restricted to the extracellular face of the plasma membrane. The immunoreactivity was particularly enriched in the synaptic cleft (Fig. 7), suggesting that the concentration of $GABA_A$-receptor subunits at synapses is significantly higher than on the extrasynaptic membrane. This is supported by the findings of Nusser et al. (1995) who quantified the relative densities of synaptic and extrasynaptic $GABA_A$ receptors on cerebellar granule cells of adult cats by using the postembedding immunogold method. They found that immunoreactivity for the α1 and β2/3 subunits was, respectively, about 127 and 55 times more concentrated in the synaptic junctions than on the extrasynaptic dendritic membrane. Therefore, we conclude that the hot spots seen in the light microscope correspond to high densities of receptors clustered at postsynaptic sites, whereas the diffuse staining likely reflects the presence of extrasynaptic receptors.

Development of gephyrin

The 93-kD protein gephyrin is essential for the formation of clusters of glycine and possibly of $GABA_A$ receptors (Kirsch et al., 1993b; Kirsch et al., 1995). Here we analyzed

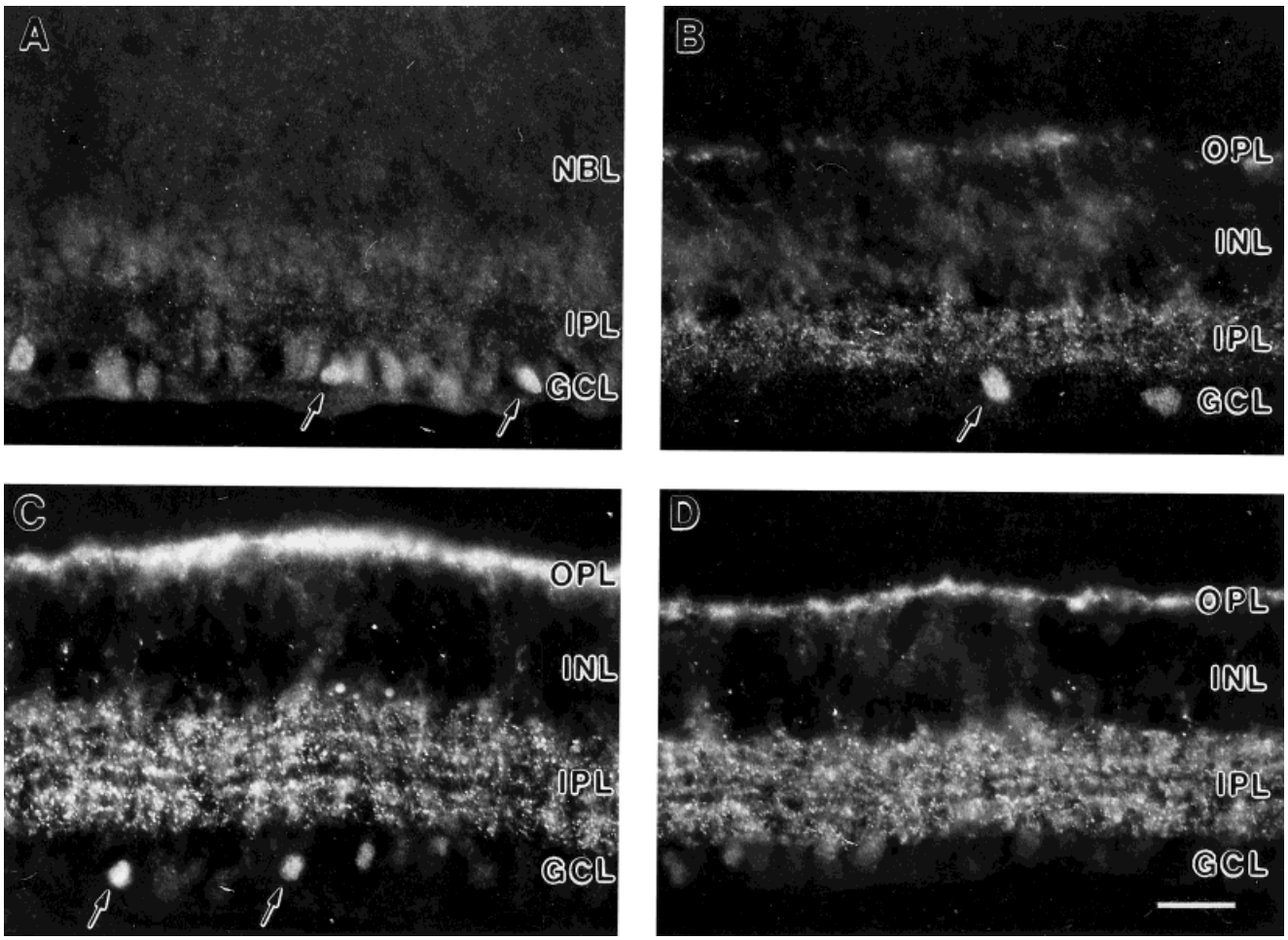

Fig. 5. Localization of the α1 subunit of the $GABA_A$ receptor in vertical sections of P3 (**A**), P7 (**B**), P11 (**C**), and P13 (**D**) rat retinae. Cells of the GCL are stained diffusely (arrows). There are relatively few puncta in the IPL at P7 (B). The puncta become more numerous and organized in bands at further stages (C,D). Note the intense staining of the OPL. Abbreviations as in Fig. 1, other conventions as in Fig. 1. Scale bar = 20 μm.

the developmental profile of gephyrin and compared it with the development of the receptor subunits. Overall, the development of gephyrin parallels that of the $GABA_A$-receptor subunit α2. There were a few gephyrin-immunoreactive puncta in the IPL at P1 and P3 (Fig. 8A). The puncta then increased in density, and from P5 on, they were organized in two bands (Fig. 8B–D). The intensity and pattern of labeling further increased and approximated adult levels around the time of eye opening (Fig. 8D).

Two unexpected results were obtained (not illustrated). We observed at P1 strong gephyrin immunoreactivity in cells located close to the retinal ventricular surface. These cells were elongated or round and may correspond to dividing cytoblasts (Robinson, 1991). The significance of gephyrin immunoreactivity in cells of the retinal outer border at the earliest postnatal stages remains obscure. Aggregates of gephyrin immunoreactivity were observed throughout the retina at all stages investigated. Strongly fluorescent puncta of large diameter were present in cells of the amacrine and ganglion cell layers (Figs. 8C, 10A,D) and in presumptive horizontal cells. Weakly fluorescent puncta of smaller diameter were localized in cells of the inner and outer nuclear layers. Electron microscopy revealed that these puncta correspond to large aggregates of the immunoreaction product located inside the cell nuclei (not shown). Nuclear staining with mAb 7a is not only present in the immature retina but also has been observed in the retina and in different brain regions of adult mammals (our unpublished observations and personal communication of J. Kirsch). It is likely that such staining is an artifact rather than corresponding to some specific function of gephyrin.

Close inspection with the electron microscope on P7 rat retinae revealed the subcellular pattern of gephyrin immunoreactivity (Fig. 9). Gephyrin immunoreactivity appeared in the cytoplasm of retinal neurons as randomly distributed clusters (Fig. 9A). Some of these clusters were localized close to the plasma membrane of cell bodies and processes (Fig. 9A,B); no apparent synaptic specializations were present. Gephyrin immunoreactivity was also found at conventional synapses, where it was localized to the cytoplasmic face of the postsynaptic membrane (Fig. 9C).

The results indicate that during development gephyrin is not exclusively localized at postsynaptic sites but can be present in the form of aggregates in the cytoplasm or close to the neuronal surface.

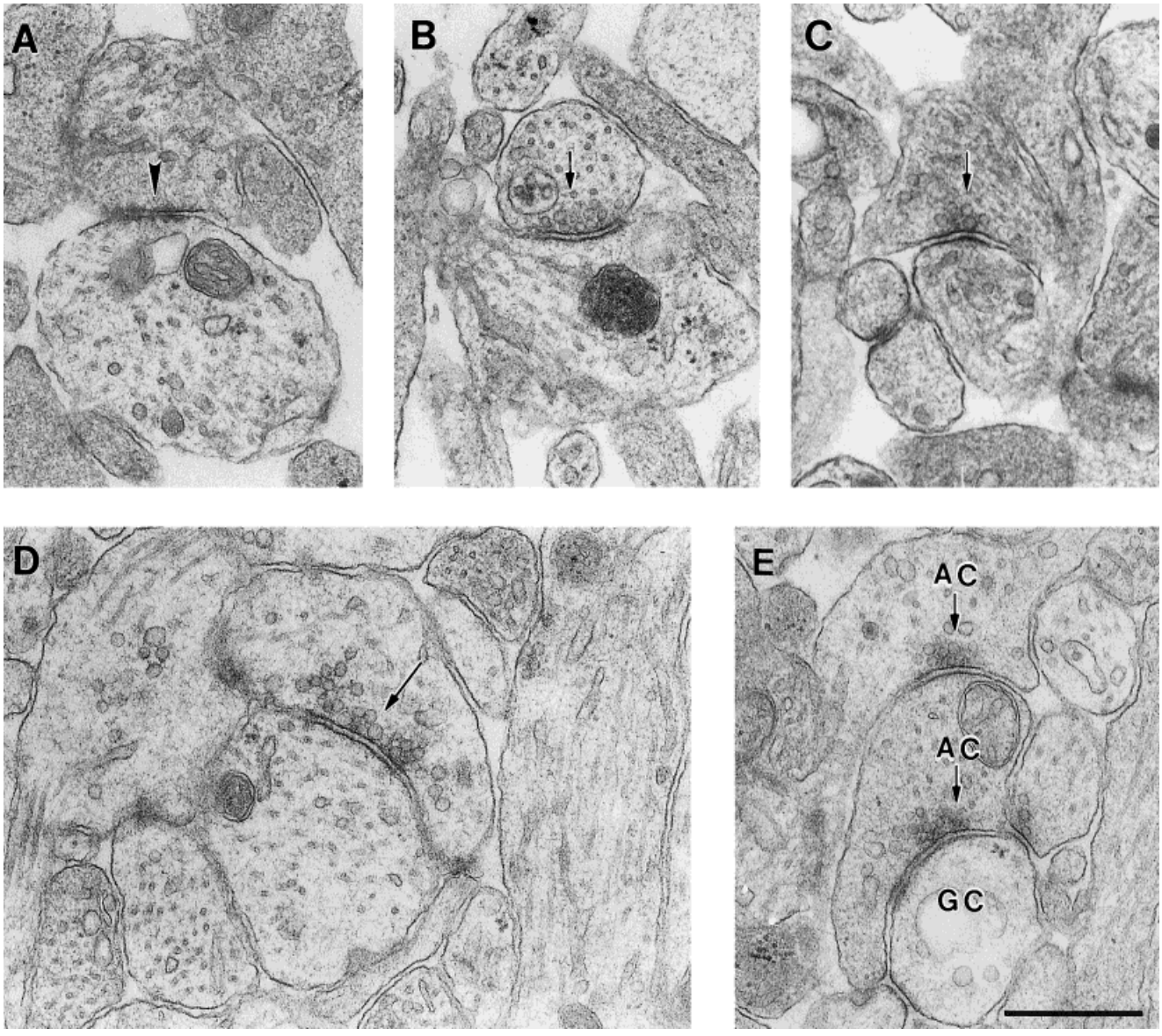

Fig. 6. Electron micrographs of the rat retina at P3 (A–C) and P7 (D,E), showing the appearance of conventional synapses in the IPL. **A:** Specialized contact (arrowhead) between two closely apposed processes containing no synaptic vesicles. **B,C:** Two examples of conventional synapses (arrows) at P3, exhibiting membrane specializations and vesicles associated with the presynaptic membrane. **D:** A conventional synapse (arrow) at P7. Note the large number of vesicles in the presynaptic terminal. **E:** Serial synapses (arrows) between two amacrine cell processes (AC) and a third putative ganglion cell dendrite (GC) at P7. Scale bar = 0.5 μm.

Colocalization of gephyrin and $GABA_A$ receptors

We have shown recently that in the IPL of the adult rat retina gephyrin is colocalized with some $GABA_A$-receptor subunits (Sassoè-Pognetto et al., 1995). Here we wanted to determine whether gephyrin is found at GABAergic synapses in the retinae of postnatal animals. Figure 10A,B presents a vertical section of a P7 rat retina that was immunolabeled for gephyrin (Fig. 10A) and the α2 subunit of the $GABA_A$ receptor (Fig. 10B). The two micrographs are printed as mirror images; therefore, corresponding points of the section are found in A and B at equal distances from the vertical midline. The distribution of the labeled puncta at both sides of the midline is strikingly symmetric, suggesting that gephyrin and the α2 subunit are present at the same synapses. The number of the gephyrin-immunofluorescent puncta is higher than the number of the α2-immunofluorescent puncta. Therefore, gephyrin is not exclusively localized to synapses expressing the α2 subunit.

Figure 10D,E compares the distribution of gephyrin (Fig. 10D) with the distribution of the α3 subunit of the $GABA_A$ receptor (Fig. 10E). In this case, the majority of the puncta are found at noncorresponding positions, indicating that gephyrin and the α3 subunit are only scarcely colocalized. Similar results were obtained on the retinae of

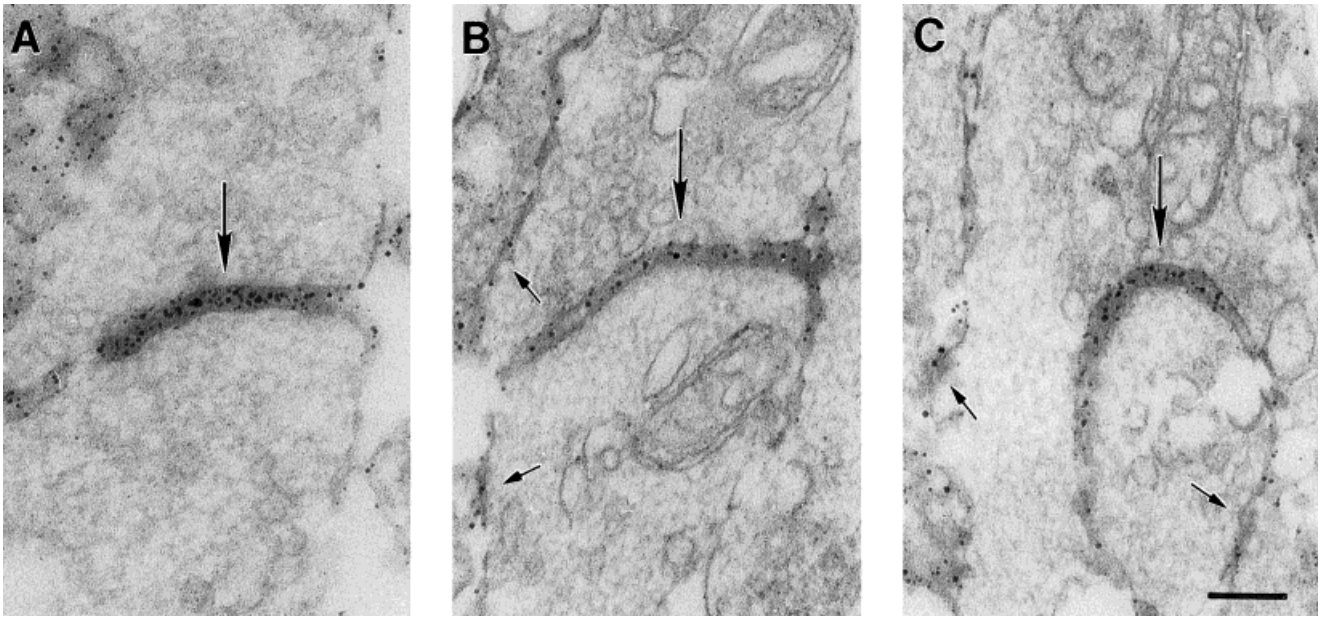

Fig. 7. Subcellular localization of the $GABA_A$-receptor subunits α2 (**A**) and α3 (**B,C**) in the IPL of a P7 rat retina. Three conventional synapses are shown (arrows). Strong immunoreactivity is visible in the synaptic cleft. Nonsynaptic plasma membrane domains are also labeled (small arrows). Scale bar = 0.1 μm.

P3 rats (not shown). Therefore, gephyrin is associated with $GABA_A$ receptors at the earliest stages of retinal synaptic development; as in the retinae of adult rats, there is a preferential association of gephyrin with $GABA_A$ receptors containing the α2 subunit.

DISCUSSION

The results of this study indicate that clustering of glycine and $GABA_A$-receptor subunits in retinal neurons occurs in parallel with the formation of conventional synapses in the IPL. Thus, the appearance of neurotransmitter receptor clusters is one of the first detectable signs indicating the presence of newly formed synapses. The results of this study also indicate that the peripheral membrane protein gephyrin is involved in early synaptogenetic events and may play a fundamental role in the differentiation of postsynaptic membranes at some types of glycinergic and GABAergic synapses.

Receptor clustering and synaptogenesis

We observed in the IPL of the postnatal rat retina hot spots immunoreactive for glycine and $GABA_A$-receptor subunits. The density of the hot spots increased during the first 2 postnatal weeks until adult-like patterns of immunoreactivity were reached. The hot spots detected by immunofluorescence microscopy likely correspond to high densities of receptors at postsynaptic sites. Thus, our results indicate that during postnatal development there is a continuous and progressive increase in the number of synapses expressing glycine and $GABA_A$ receptors. This is supported by the following observations: (1) previous investigations of the adult rat retina showed a correspondence between punctate immunofluorescence and synaptic localization of receptor proteins. This was shown for gephyrin and the α1 subunit of the GlyR (Sassoè-Pognetto et al., 1994), for the α3 subunit of the $GABA_A$ receptor (Sassoè-Pognetto et al., 1995), for the metabotropic glutamate receptor mGluR6 (Nomura et al., 1994), and for the NR2A subunit of the NMDA receptor (Hartveit et al., 1994; see also Koulen et al., 1996); (2) here we demonstrate by electron microscopy that conventional synapses are present in the IPL of the rat retina already at P3 and P7, contrary to previous reports indicating P11 as the age of emergence of the first conventional synapses (Weidman and Kuwabara, 1968; Horsburgh and Sefton, 1987); (3) our electron microscopic immunocytochemistry shows that the $GABA_A$-receptor subunits α2 and α3 are concentrated at conventional synapses in the IPL of the rat retina at P7. We, therefore, conclude that similar to the neuromuscular junction (Froehner, 1993; Hall and Sanes, 1993) clustering of transmitter receptors is an early event during the formation of synapses between retinal neurons. This also implies that immunofluorescence with subunit-specific antibodies can be effectively employed to monitor the emergence of synaptic specializations.

We also examined (unpublished results) the developmental expression and localization of the synaptic vesicle associated protein synaptophysin, which is usually regarded as a selective synaptic marker. Our results substantiate those previously published by Kapfhammer et al. (1994). Synaptophysin was not detected in the IPL prior to P9; however, in older animals there was an increase in expression. It is unclear why synaptophysin appears late in development when synaptic vesicles are clearly present at P3 and P7. Furthermore, immunofluorescence for synaptophysin was quite diffuse, and it was not easy to detect discrete puncta. Thus, in our hands, the localization of neurotransmitter receptors was the best marker to synaptic development.

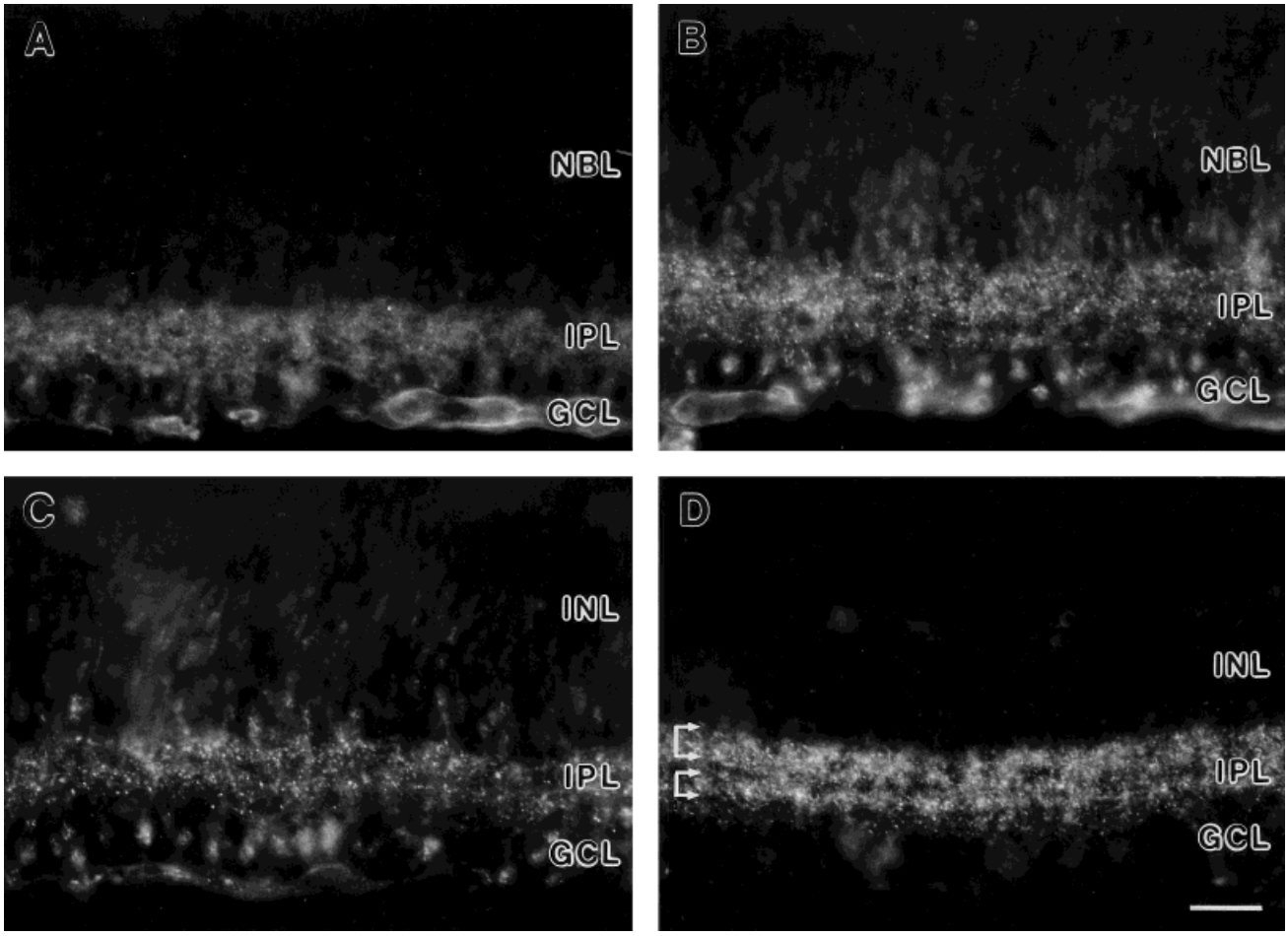

Fig. 8. Distribution of gephyrin immunoreactivity in the developing rat retina. **A–D:** An increase in the density of labeled puncta in the IPL from P3 to P13 (A: P3; B: P5; C: P7; D: P13). Two major bands of immunofluorescent puncta (see arrows in D) are first visible in the IPL at P5 (B) and P7 (C). Labeling of retinal neurons with mAb 7a is apparent in some of the micrographs (B,C). Abbreviations as in Figure 1, other conventions as in Figure 1. Scale bar = 20 μm.

Different developmental patterns of receptor subunits

The results here also indicate that the different receptor subunits are subject to different developmental sequences. Table 1 summarizes for each of the glycine and $GABA_A$-receptor subunits the approximate ages of appearance of the immunofluorescent puncta and their stratification within the IPL. For most subunits, a few hot spots are visible already at P1–P3, and the number of the hot spots increases with age; puncta immunoreactive for the α1 subunit of the $GABA_A$ receptor seem to appear considerably later. Interestingly, the expression of the $GABA_A$-receptor α1 subunit in brain is low at birth and increases with further development, whereas the α2 and α3 subunits are present at early stages but then disappear from many regions (Killisch et al., 1991; McKernan et al., 1991; Fritschy et al., 1994). Although in the retina we did not observe a decrease in the expression of the α2 and α3 subunits, the delayed appearance of α1-positive puncta is consistent with the emergence of pharmacologically distinct forms of $GABA_A$ receptors during development.

A developmental maturation has also been reported for the GlyR of rodent spinal cord. The predominant embryonic and neonatal GlyR isoform contains the α2 subunit (Becker et al., 1988), which is replaced by the α1 and α3 subunits within 2–3 weeks after birth (Takahashi et al., 1992). Such a switch in subunit composition is not likely to occur in the retina, because the GlyR α1 subunit is expressed at the earliest stages of retinal synaptic development. Moreover, mRNAs encoding the GlyR α2 subunit are widespread in the adult rat retina (Greferath et al., 1994a), suggesting the existence of functionally different isoforms of GlyRs in retinal synaptic circuitry.

The segregation of different glycine and $GABA_A$-receptor subunits into distinct sublayers within the IPL also occurs at different ages (Table 1). This may be the consequence of the extensive remodeling of dendritic processes that takes place during retinal development. It has been shown that the stratification of ganglion cell dendrites within the IPL is achieved by a remarkable reduction in the size and complexity of the dendritic trees, which ramify throughout the IPL early in development (Dann et al., 1988; Maslim and Stone, 1988; Ramoa et al., 1988; Wässle, 1988; Wong et al., 1992; Yamasaki and Ramoa, 1993). There is also evidence that the remodeling of ganglion cell dendrites during development is regulated by glutamatergic afferent

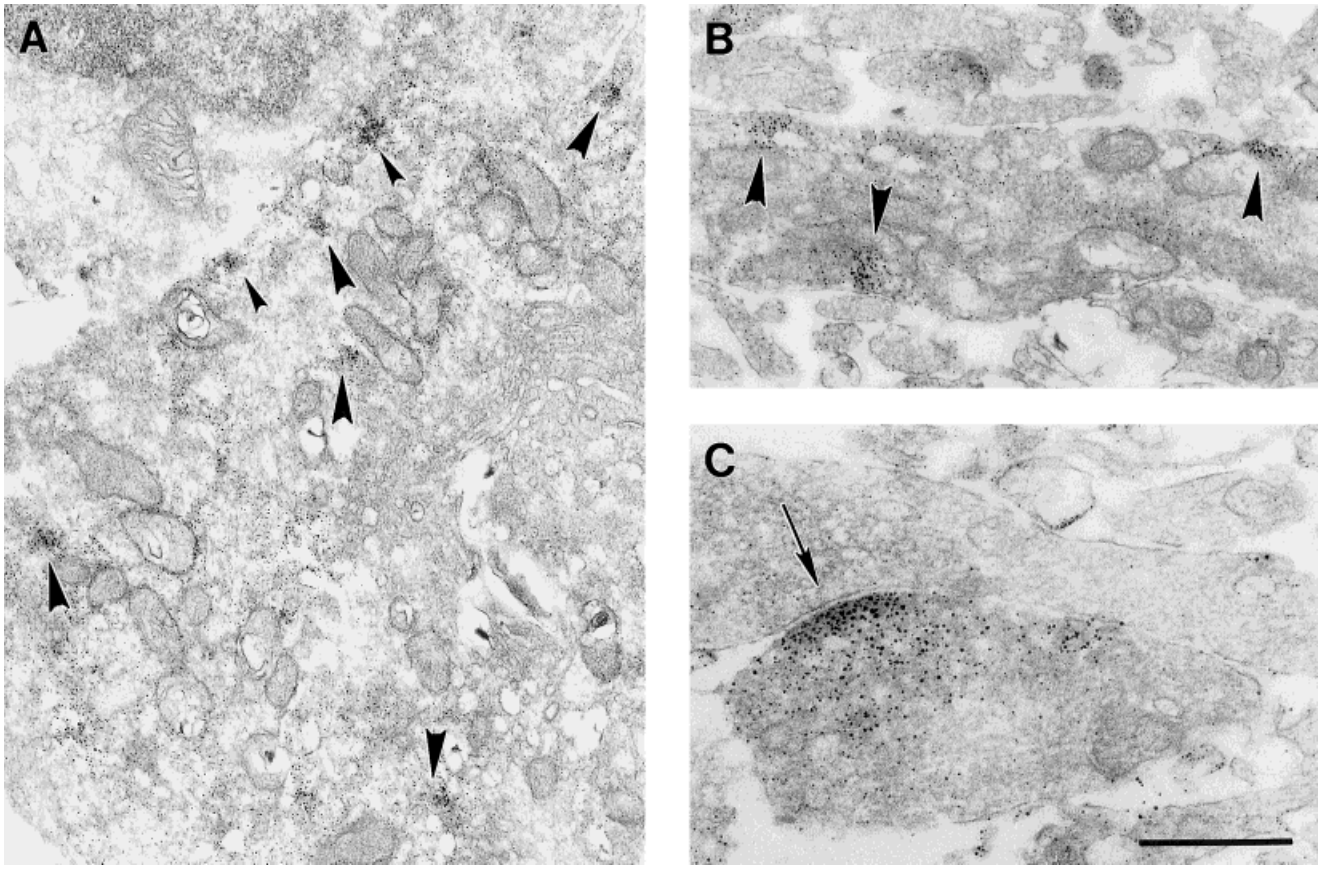

Fig. 9. Subcellular localization of gephyrin immunoreactivity at P7. **A:** Clusters of gephyrin-immunoreactive material (large arrowheads) in the cytoplasm of a neuron located in the GCL. Some clusters tend to be associated with the plasma membrane (small arrowheads). **B:** Example of a neuritic process in the IPL lacking apparent synaptic specializations. Gephyrin immunoreactivity occurs in clusters along the plasma membrane (arrowheads). **C:** Example of a conventional synapse (arrow) exhibiting gephyrin immunoreactivity. Scale bar = 0.7 μm in A, 0.5 μm in B, 0.35 μm in C.

activity (Lau et al., 1992; Bodnarenko and Chalupa, 1993). At present, there is no evidence that glycine and GABA receptors play a role in this process. However, GABA has been shown to be involved in the structural development of the outer retina (Messersmith and Redburn, 1993).

Developmental changes in the localization of receptor subunits

The immunocytochemical results here indicate that the distribution of glycine and $GABA_A$-receptor subunits during retinal postnatal development changes from one in which there is little punctate (e.g., synaptic) immunolabeling to one in which the number of receptor clusters increases in a way consistent with the formation of new synapses. Diffuse (e.g., nonsynaptic) somatodendritic labeling is widely expressed in neurons of the postnatal retina and is still detectable in adult animals (mAb 4a: Grünert and Wässle, 1993; $GABA_A$-receptors: Greferath et al., 1995; Koulen et al., 1996). Therefore, during ontogeny, the receptor subunits become increasingly more concentrated at postsynaptic sites but are not completely lost from the extrasynaptic areas of the plasma membrane. The presence of a readily recruitable pool of extrasynaptic receptors may be relevant for understanding how the different receptor subunits are sorted and targeted to appropriate synapses in the plasma membrane (Perez-Velazques and Angelides, 1993; Craig et al., 1994; Koulen et al., 1996). Craig et al. (1994) have discussed two general mechanisms for generating receptor clusters at specific postsynaptic sites. Receptors may be added along the entire somatodendritic surface and then clustered by lateral diffusion and trapping in the membrane. Alternatively, receptors may be sorted into different intracellular transport vesicles that fuse only at the appropriate postsynaptic sites. The presence of extrasynaptic receptors favors the clustering by trapping model, which also seems to be the mechanism for the aggregation of receptors at the neuromuscular junction (Hall and Sanes, 1993). This is supported also by previous studies that have examined the developmental expression of glutamate receptors. Aoki et al. (1994) have shown that during development the NR1 subunit of the NMDA receptor is associated with the plasma membrane prior to axons' arrival, whereas clustering of receptors to synapses may be promoted by axonal contact. Nomura et al. (1994) have described a progressive concentration of the metabotropic receptor mGluR6 at postsynaptic sites that is consistent with the establishment of synaptic connections in the developing retina. Hartveit et al. (1994) have shown that the NR2A subunit of the NMDA receptor is gradually localized to synapses during retinal develop-

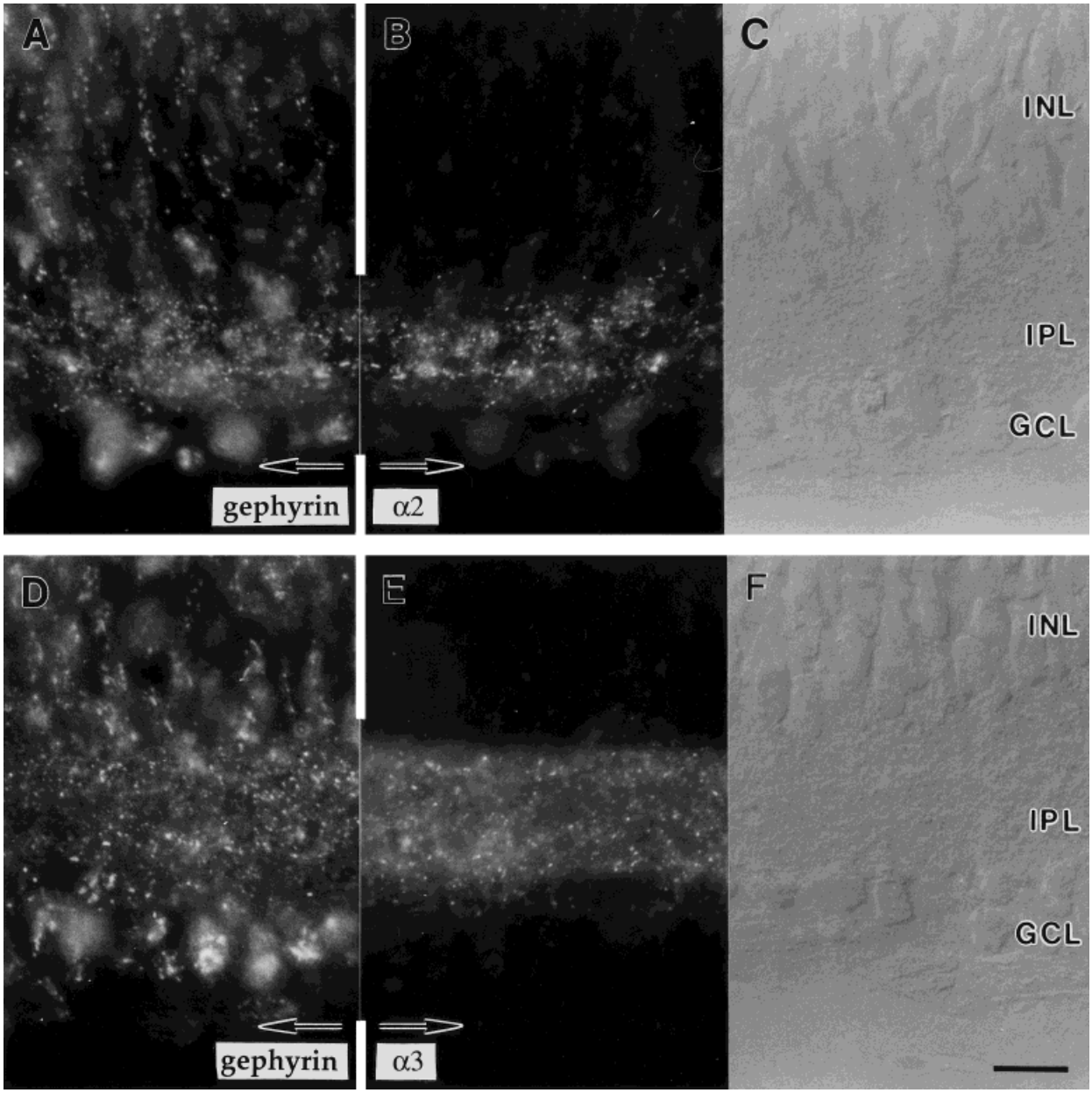

Fig. 10. **A–C:** Micrographs of a vertical section of a P7 rat retina that was double immunolabeled for gephyrin (A) and for the α2 subunit of the $GABA_A$ receptor (B). C is a Nomarski micrograph showing the retinal layers. A and B are printed as mirror images. The labeled puncta are found at symmetrical positions with respect to the vertical midline, suggesting strong colocalization. **D–F:** Micrographs of a vertical section of a P7 rat retina that was double immunolabeled for gephyrin (D) and for the a3 subunit of the $GABA_A$ receptor (E). F is a Nomarski micrograph showing the retinal layers. D and E are shown as mirror images. Very few puncta are found at symmetrical positions with respect to the vertical midline, indicating scarce colocalization. Note the gephyrin-immunoreactive puncta in neurons of the GCL and INL in A and D. Other conventions as in Figure 1. Scale bar = 10 μm.

ment. Taken together, these results indicate that the insertion of receptor subunits into the plasma membrane is an independent process, whereas the aggregation of the subunits at postsynaptic sites may be promoted by the establishment of synaptic junctions.

There are clear differences in the localization of different receptor subunits at synaptic or extrasynaptic membranes. For instance, immunoreactivity for the GlyR α1 subunit is predominantly localized to hot spots, suggesting that the extrasynaptic levels of this polypeptide may be too low to be detected by immunocytochemical methods. Conversely, the diffuse immunoreactivity observed with mAb 4a (Figs. 2E,F) suggests that other GlyR subunits (e.g., α2, α3, or β) are abundantly expressed at nonsynaptic sites. Similar results were obtained by Baude et al. (1992) who investigated the subcellular distribution of the $GABA_A$-receptor subunits α1, β2/3, and α6 on cerebellar granule cells. They showed that the α1 and β2/3 subunits are

TABLE 1. Developmental Profile of Glycine and $GABA_A$-receptor Subunits in the Inner Plexiform Layer of the Rat Retina

	GlyR[1] α1	Gephyrin	$GABA_A$[2] α2	$GABA_A$ α3	$GABA_A$ α1
First appearance of puncta	P1–P3[3]	P1–P3	P1–P3	P1–P3	P7
First appearance of bands	P9	P5	P5	P9	P7

[1]GlyR, glycine receptor.
[2]GABA, gamma-aminobutyric acid.
[3]P, postnatal day.

located at both synaptic and extrasynaptic sites, whereas the α6 subunit is present only at synapses. Furthermore, Vernallis et al. (1993) demonstrated that a class of acetylcholine receptors with a predominant synaptic localization on ciliary ganglion neurons contains the α3, β4, and α5 subunits, whereas a class of extrasynaptic receptors on the same neurons contains the α7 subunit but lacks the α3, β4, and α5 subunits. It is unknown whether these differences in localization reflect differences in function (Somogyi et al., 1989).

Function of gephyrin

One of the factors involved in the regulation of receptor localization is the interaction of receptor polypeptides with cytoskeletal elements (Apel and Merlie, 1995). The peripheral membrane protein gephyrin is the only intracellular component that has been shown to play a direct role in receptor clustering in the CNS (Kirsch et al., 1993b). Gephyrin binds to a large cytoplasmic loop of the GlyR β subunit (Meyer et al., 1995), and gephyrin clusters on the neuronal surface are disrupted after treatment with demecolcine, a microtubule-dissociating agent (Kirsch and Betz, 1995). Therefore, gephyrin serves as an anchoring molecule that links the GlyR to the subsynaptic cytoskeleton (Kirsch et al., 1991; Kirsch and Betz, 1995).

In the IPL of the mammalian retina, gephyrin is clustered at sites postsynaptic to amacrine cells (Pourcho and Owczarzak, 1991; Grünert and Wässle, 1993; Sassoè-Pognetto et al., 1994). Recently, we have shown that in the rat retina gephyrin is colocalized with some $GABA_A$-receptor subunits and have suggested that gephyrin might also be involved with the aggregation of $GABA_A$ receptors (Sassoè-Pognetto et al., 1995). The data of the present study indicate that gephyrin and $GABA_A$ receptors are colocalized at the same hot spots already at the earliest stages of retinal synaptic development. As in adult animals, there is a preferential association of gephyrin with specific subtypes of $GABA_A$ receptors containing the α2 subunit. This further supports the idea that gephyrin plays an important role in the formation and maintenance of some GABAergic synapses.

It has been shown recently that gephyrin can interact in vitro with the β3 subunit of the $GABA_A$ receptor (Kirsch et al., 1995). A direct comparison of the distribution of gephyrin and of the β3 subunit was not possible here. However, it was previously shown that the β2/3 and α2 subunits of the $GABA_A$ receptor colocalize in the rat retina (Greferath et al., 1995). Because the α2 subunit is also colocalized with gephyrin, we propose that gephyrin interacts with $GABA_A$ receptors that contain both the α2 and the β2/3 subunits.

An important finding of the present study is the transient appearance of gephyrin clusters in the cytoplasm of developing retinal neurons. These clusters are likely to result from an increased rate of gephyrin synthesis during the period of synaptogenesis. In many instances, the clusters were found in close vicinity of the plasma membrane, but no presynaptic endings were present (Fig. 9B). Similar results have been obtained with confocal-microscopic studies by Kirsch and co-workers (Kirsch et al., 1993b; Kirsch and Betz, 1995; Béchade et al., 1996), who suggested that the assembly of gephyrin clusters at the plasma membrane may precede the formation of glycinergic synapses. Taken together, these observations indicate that clustering of gephyrin may be the primary event in the formation of some types of synapses. This is consistent with the observation of Nishimura and Rakic (1987) that postsynaptic specializations in the IPL of the Rhesus monkey retina emerge prior to contacts with the presynaptic elements.

Intracellular clusters of gephyrin similar to those observed in the present study have been reported in the goldfish Mauthner cell following degeneration of some glycinergic afferents (Seitanidou et al., 1992). Accordingly, at least some of the gephyrin clusters that we observed in the cytoplasm of developing retinal neurons may be the consequence of protein recycling from degenerating synapses.

Synaptogenesis in the IPL of the rat retina

In the developing rat retina, the first synapse formation in the OPL has been reported around P5 (Weidman and Kuwabara, 1968). In the IPL, conventional synapses have been first described at P11, and ribbon synapses at P13 (Horsburgh and Sefton, 1987). The electron microscopic results of the present study indicate that many synapses of the conventional type are found in the IPL of the rat retina already during the first postnatal week. These synapses look immature, but the presence of synaptic vesicles in the presynaptic terminal (Fig. 6) and of neurotransmitter receptors on the postsynaptic membrane (Fig. 7) indicates that they are functional. In addition, we have recently shown the presence of morphologically and biochemically specialized synapses in organotypic slice cultures of the postnatal rat retina (Sassoè-Pognetto et al., 1996). Morest (1970) suggested the presence of presumed synaptic contacts between amacrine cells and ganglion cells in Golgi-impregnated retinae of rats aged P3. Horsburgh and Sefton (1987) also reported the presence of specialized junctions between neuritic processes in neonatal rats (see Fig. 8B of their article), but these junctions were not regarded as "unequivocal synapses."

At the developmental stages that we examined (P3 and P7), we also observed many examples of specialized contacts between processes containing membrane thickenings but lacking synaptic vesicles (Fig. 6A). Similar contacts have been described in the developing IPL of different mammalian species (monkey: Nishimura and Rakic, 1985, 1987; cat: Maslim and Stone, 1986; rat: Horsburgh and Sefton, 1987) and probably represent an early stage in the establishment of synaptic contacts.

No ribbon synapses were observed in the IPL at P3 and P7. This is consistent with the idea that the first synapses within the IPL are made by amacrine cells (Weidman and Kuwabara, 1968; Nishimura and Rakic, 1985, 1987; Maslim and Stone, 1986; Horsburgh and Sefton, 1987), at least in the rod-dominated retinal regions (Crooks et al., 1995).

Taken together, these results indicate that the sequence of synaptic differentiation in the IPL of the rat retina follows a scheme that is common to most mammalian

species, with a first phase characterized by the appearance of conventional synapses, a second phase of intense synaptogenesis and appearance of ribbon synapses prior to eye opening, and a third phase with a sharp decrease in the rate of synapse formation once the eyes have opened (Fisher, 1979; Robinson, 1991).

CONCLUSIONS

We have shown that retinal neurons possess the ability to express glycine and $GABA_A$ receptors and to associate them to the plasma membrane before functional synaptic contacts are formed. During development, there is a remarkable redistribution and clustering of receptor subunits, which is temporally correlated with the establishment of synapses. However, the molecular mechanisms that induce the formation of receptor clusters on CNS neurons remain to be determined. It has been demonstrated that the formation of postsynaptic receptor clusters on neurons is not regulated by synaptic activity but could be under the influence of some signaling molecule released presynaptically (Seitanidou et al., 1992; Craig et al., 1994). A recent study (Ehlers et al., 1995) has shown that the glutamate-receptor subunit NR1, when transfected in fibroblasts, spontaneously forms aggregates that associate with discrete plasma membrane domains in a manner that is regulated by alternative splicing and protein phosphorylation. In addition, the postsynaptic aggregation of $GABA_A$ receptors in cultured cerebellar granule cells seems to be independent of GABAergic innervation (Gao and Fritschy, 1995). Therefore, the initial formation of postsynaptic specializations may be regulated to a large extent by intrinsic factors, independently of presynatic influences. There is strong evidence that gephyrin is one of the factors promoting the differentiation and stabilization of postsynaptic membranes at some types of glycinergic and GABAergic synapses.

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LITERATURE CITED

Aoki, C., T.H. Joh, and V.M. Pickel (1987) Ultrastructural localization of β-adrenergic receptor-like immunoreactivity in the cortex and neostriatum of the rat brain. Brain Res. *437:*264–282.

Aoki, C., C. Venkatesan, C.G. Go, J.A. Mong, and T.M. Dawson (1994) Cellular and subcellular localization of NMDA-R1 subunit immunoreactivity in the visual cortex of adult and neonatal rats. J. Neurosci. *14:*5202–5222.

Apel, E.D., and J.P. Merlie (1995) Assembly of the postsynaptic apparatus. Curr. Opin. Neurobiol. *5:*62–67.

Basarski, T.A., V. Parpura, and P.G. Haydon (1994) Hippocampal synaptogenesis in cell culture: developmental time course of synapse formation, calcium influx, and synaptic protein distribution. J. Neurosci. *14:*6402–6411.

Baude, A., E. Molnar, D. Latawiec, R.A.J. McIlhinney, and P. Somogyi (1994) Synaptic and nonsynaptic localization of the GluR1 subunit of the AMPA-type excitatory amino-acid receptor in the rat cerebellum. J. Neurosci. *14:*2830–2843.

Baude, A., Z. Nusser, J.D.B. Roberts, E. Mulvihill, R.A.J. McIlhinney, and P. Somogyi (1993) The metabotropic glutamate receptor (mGluR1a) is concentrated at perisynaptic membrane of neuronal subpopulations as detected by immunogold reaction. Neuron *11:*771–787.

Baude, A., Z. Nusser, E. Molnár, R.A.J. McIlhinney, and P. Somogyi (1995) High-resolution immunogold localization of AMPA type glutamate receptor subunits at synaptic and non-synaptic sites in rat hippocampus. Neuroscience *69:*1031–1055.

Baude, A., J.M. Sequier, R.M. McKernan, K.R. Olivier, and P. Somogyi (1992) Differential subcellular distribution of the α6 subunit versus the α1 and β2/3 subunits of the $GABA_A$/benzodiazepine receptor complex in granule cells of the cerebellar cortex. Neuroscience *51:*739–748.

Béchade, C., I. Colin, J. Kirsch, H. Betz, and A. Triller (1996) Expression of glycine receptor a subunits and gephyrin in cultured spinal neurons. Eur. J. Neurosci. *8:*429–435.

Becker, C.-M., W. Hoch, and H. Betz (1988) Glycine receptor heterogeneity in rat spinal cord during postnatal development. EMBO J. *7:*3717–3726.

Benke, D., A. Cincin-Sain, S. Mertens, and H. Möhler (1991) Immunocytochemical identification of the α1- and α3-subunit of the $GABA_A$-receptor in the rat brain. J. Recept. Res. *11:*407–424.

Bodnarenko, S.R., and L.M. Chalupa (1993) Stratification of ON and OFF ganglion cell dendrites depends on glutamate-mediated afferent activity in the developing retina. Nature *364:*144–146.

Bolhalter, S., H. Möhler, and J.-M. Fritschy (1994) Inhibitory neurotransmission in rat spinal cord: colocalization of glycine-and $GABA_A$ receptors at GABAergic synaptic contacts demonstrated by triple immunofluorescence staining. Brain Res. *642:*59–69.

Cabot, J.B., A. Bushnell, V. Alessi, and N.R. Mendell (1995) Postsynaptic gephyrin immunoreactivity exhibits a nearly one-to-one correspondence with gamma-aminobutyric acid-like immunogold-labeled synaptic inputs to sympathetic preganglionic neurons. J. Comp. Neurol. *356:*418–432.

Craig, A.M., G. Banker, W.R. Chang, M.E. Mcgrath, and A.S. Serpinskaya (1996) Clustering of gephyrin at GABAergic but not glutamatergic synapses in cultured rat hippocampal neurons. J. Neurosci. *16:*3166–3177.

Craig, A.M., C.D. Blackstone, R.L. Huganir, and G. Banker (1994) Selective clustering of glutamate and γ-aminobutyric acid receptors opposite terminals releasing the corresponding neurotransmitters. Proc. Natl. Acad. Sci. USA *91:*12373–12377.

Crooks, J., M. Okada, and A.E. Hendrickson (1995) Quantitative analysis of synaptogenesis in the inner plexiform layer of the macaque monkey fovea. J. Comp. Neurol. *360:*349–362.

Dann, J.F., E.H. Buhl, and L. Peichl (1988) Postnatal dendritic maturation of alpha and beta ganglion cells in cat retina. J. Neurosci. *8:*1485–1499.

Ehlers, M.D., G.T. Whittemore, and R.L. Huganir (1995) Regulated subcellular distribution of the NR1 subunit of the NMDA receptor. Science *269:*1734–1737.

Fisher, L.J. (1979) Development of synaptic arrays in the inner plexiform layer of neonatal mouse retina. J. Comp. Neurol. *187:*359–372.

Fletcher, T.L., P. De Camilli, and G. Banker (1994) Synaptogenesis in hippocampal cultures: evidence indicating that axons and dendrites become competent to form synapses at different stages of neuronal development. J. Neurosci. *14:*6695–6706.

Fritschy, J.-M., J. Paysan, A. Enna, and H. Möhler (1994) Switch in the expression of rat $GABA_A$-receptor subtypes during postnatal development: an immunohistochemical study. J. Neurosci. *14:*5302–5324.

Froehner, S.C. (1993) Regulation of ion channel distribution at synapses. Annu. Rev. Neurosci. *16:*347–368.

Gao, B., and J.-M. Fritschy (1995) Cerebellar granule cells in vitro recapitulate the in vivo pattern of $GABA_A$-receptor subunit expression. Dev. Brain Res. *88:*1–16.

Gao, B., J.-M. Fritschy, D. Benke, and H. Möhler (1993) Neuron specific expression of $GABA_A$-receptor subtypes: differential association of the α1- and α3-subunits with serotonergic and GABAergic neurons. Neuroscience *54:*881–892.

Greferath, U., J.H. Brandstätter, H. Wässle, J. Kirsch, J. Kuhse, and U. Grünert (1994a) Differential expression of glycine receptor subunits in the retina of the rat: a study using immunocytochemistry and in situ hybridization. Visual Neurosci. *11:*721–729.

Greferath, U., U. Grünert, F. Müller, and H. Wässle (1994b) Localization of $GABA_A$ receptors in the rabbit retina. Cell Tissue Res. *276:*295–307.

Greferath, U., U. Grünert, J.-M. Fritschy, A. Stephenson, H. Möhler, and H. Wässle (1995) $GABA_A$ receptor subunits have differential distributions in the rat retina: in situ hybridization and immunocytochemistry. J. Comp. Neurol. *353*:553–571.

Greferath, U., F. Müller, H. Wässle, B. Shivers, and P.H. Seeburg (1993) Localization of $GABA_A$ receptors in the rat retina. Visual Neurosci. *10*:551–561.

Grünert, U., and H. Wässle (1993) Immunocytochemical localization of glycine receptors in the mammalian retina. J. Comp. Neurol. *335*:523–537.

Grünert, U., and H. Wässle (1996) Glycine receptors in the rod pathway of the macaque monkey retina. Visual Neurosci. *13*:101–115.

Hall, Z.W., and J.R. Sanes (1993) Synaptic structure and development—the neuromuscular junction. Cell *72*:99–121.

Hartveit, E., J.H. Brandstätter, M. Sassoè-Pognetto, D.J. Laurie, P.H. Seeburg, and H. Wässle (1994) Localization and developmental expression of the NMDA receptor subunit NR2A in the mammalian retina. J. Comp. Neurol. *348*:570–582.

Horsburgh, G.M., and A.J. Sefton (1987) Cellular degeneration and synaptogenesis in the developing retina of the rat. J. Comp. Neurol. *263*:553–566.

Jacob, M.H., and D.K. Berg (1983) The ultrastructural localization of α-bungarotoxin binding sites in relation to synapses on chick ciliary ganglion neurons. J. Neurosci. *3*:260–271.

Jacob, M.H., D.K. Berg, and J.M. Lindstrom (1984) Shared antigenic determinant between the electrophorus acetylcholine receptor and a synaptic component on chicken ciliary ganglion neurons. Proc. Natl. Acad. Sci. USA *81*:3223–3227.

Kapfhammer, J.P., F. Christ, and M. Schwab (1994) The expression of GAP-43 and synaptophysin in the developing rat retina. Dev. Brain Res. *80*:251–260.

Killisch, I., C.G. Dotti, D.J. Laurie, H. Lüddens, and P.H. Seeburg (1991) Expression patterns of $GABA_A$ receptor subtypes in developing hippocampal neurons. Neuron *7*:927–936.

Kirsch, J., and H. Betz (1993) Widespread expression of gephyrin, a putative glycine receptor-tubulin linker protein, in rat brain. Brain Res. *621*:301–310.

Kirsch, J., and H. Betz (1995) The postsynaptic localization of the glycine receptor associated protein gephyrin is regulated by the cytosckeleton. J. Neurosci. *15*:4148–4156.

Kirsch, J., J. Kuhse, and H. Betz (1995) Targeting of glycine receptor subunits to gephyrin-rich domains in transfected human embryonic kidney cells. Mol. Cell. Neurosci. *6*:450–461.

Kirsch, J., D. Langosch, P. Prior, U.Z. Littauer, B. Schmitt, and H. Betz (1991) The 93-kDa glycine receptor-associated protein binds to tubulin. J. Biol. Chem. *266*:22242–22245.

Kirsch, J., M.-L. Malosio, I. Wolters, and H. Betz (1993a) Distribution of gephyrin transcripts in the adult and developing rat brain. Eur. J. Neurosci. *5*:1109–1117.

Kirsch, J., I. Wolters, A. Triller, and H. Betz (1993b) Gephyrin antisense oligonucleotides prevent glycine receptor clustering in spinal neurons. Nature *366*:745–748.

Koulen, P., M. Sassoè-Pognetto, U. Grünert, and H. Wässle (1996) Selective clustering of $GABA_A$- and glycine-receptors in the mammalian retina. J. Neurosci. *16*:2127–2140.

Lau, K.C., K.-F. So, and D. Tay (1992) APV prevents the elimination of transient dendritic spines on a population of retinal ganglion cells. Brain Res. *595*:171–174.

Marksitzer, R., D. Benke, J.-M. Fritschy, A. Trzeciak, B. Bannwarth, and H. Möhler (1993) $GABA_A$-receptors: drug binding profile and distribution of receptors containing the α2-subunit in situ. J. Recept. Res. *13*:467–477.

Martin, L.J., C.D. Blackstone, A.I. Levey, L.R. Huganir, and D.L. Price (1993) AMPA glutamate receptor subunits are differentially distributed in rat brain. Neuroscience *53*:327–358.

Maslim, J., and J. Stone (1986) Synaptogenesis in the retina of the cat. Brain Res. *373*:35–48.

Maslim, J., and J. Stone (1988) Time course of stratification of the dendritic fields of ganglion cells in the retina of the cat. Dev. Brain Res. *44*:87–93.

McKernan, R.M., P. Cox, N.P. Gillard, and P. Whiting (1991) Differential expression of $GABA_A$ receptor α-subunits in rat brain during development. FEBS Lett. *286*:44–46.

McMahan, U.J., S.E. Horton, M.J. Werle, L.S. Honig, S. Kröger, M.A. Ruess, and G. Escher (1992) Agrin isoforms and their role in synaptogenesis. Curr. Opin. Cell Biol. *4*:869–874.

Messersmith, E.K., and D.A. Redburn (1993) The role of GABA during development of the outer retina in the rabbit. Neurochem. Res. *18*:463–470.

Meyer, G., J. Kirsch, H. Betz, and D. Langosch (1995) Identification of a gephyrin binding motif on the glycine receptor β subunit. Neuron *15*:563–572.

Molnár, E., A. Baude, S.A. Richmond, P.B. Patel, P. Somogyi, and R.A.J. McIlhinney (1993) Biochemical and immunocytochemical characterization of antipeptide antibodies to a cloned GluR1 glutamate receptor subunit: cellular and subcellular distribution in the rat forebrain. Neuroscience *53*:307–326.

Morest, D. K. (1970) The pattern of neurogenesis in the retina of the rat. Z. Anat. Entwickl.-Gesch. *131*:45–67.

Nastuk, M. A., and J.R. Fallon (1993) Agrin and the molecular choreography of synapse formation. Trends Neurosci. *16*:72–76.

Nicola, M.-A., C.-M. Becker, and A. Triller (1992) Development of glycine receptor alpha subunit in cultivated rat spinal neurons: an immunocytochemical study. Neurosci. Lett. *138*:173–178.

Nishimura, Y., and P. Rakic (1985) Development of the Rhesus monkey retina. I. Emergence of the inner plexiform layer and its synapses. J. Comp. Neurol. *241*:420–434.

Nishimura, Y., and P. Rakic (1987) Development of the Rhesus monkey retina. II. A three-dimensional analysis of the sequences of synaptic combinations in the inner plexiform layer. J. Comp. Neurol. *262*:290–313.

Nomura, A., R. Shigemoto, Y. Nakamura, N. Okamoto, N. Mizuno, and S. Nakanishi (1994) Developmentally regulated postsynaptic localization of a metabotropic glutamate receptor in rat rod bipolar cells. Cell *77*:361–369.

Nusser, Z., J.D.B. Roberts, A. Baude, J.G. Richards, and P. Somogyi (1995) Relative densities of synaptic and extrasynaptic $GABA_A$ receptors on cerebellar granule cells as determined by a quantitative immunogold method. J. Neurosci. *15*:2948–2960.

Perez-Velazques, J.L., and K.L. Angelides (1993) Assembly of $GABA_A$ receptor subunits determines sorting and localization in polarized cells. Nature *361*:457–460.

Petralia, R.S., and R.J. Wenthold (1992) Light and electron immunocytochemical localization of AMPA-selective glutamate receptors in rat brain. J. Comp. Neurol. *318*:329–354.

Petralia, R.S., Y.-X. Wang, and R.J. Wenthold (1994a) The NMDA receptor subunits NR2A and NR2B show histological and ultrastructural localization patterns similar to those of NR1. J. Neurosci. *14*:6102–6120.

Petralia, R.S., N. Yokotani, and R.J. Wenthold (1994b) Light and electron microscope distribution of the NMDA receptor subunit NMDAR1 in the rat nervous system using a selective anti-peptide antibody. J. Neurosci. *14*:667–696.

Pfeiffer, F., R. Simler, G. Grenningloh, and H. Betz (1984) Monoclonal antibodies and peptides mapping reveal structural similarities between the subunits of the glycine receptor of rat spinal cord. Proc. Natl. Acad. Sci. USA *81*:7224–7227.

Pourcho, R.G., and M.T. Owczarzak (1991) Glycine receptor immunoreactivity is localized at amacrine synapses in cat retina. Visual Neurosci. *7*:611–618.

Ramoa, A.S., G. Campbell, and C.J. Shatz (1988) Dendritic growth and remodeling of cat retinal ganglion cells during fetal and postnatal development. J. Neurosci. *8*:4239–4261.

Richards, J.C., P. Schoch, P. Haring, B. Takacs, and H. Möhler (1987) Resolving $GABA_A$/benzodiazepine receptors: cellular and subcellular localization in the CNS with monoclonal antibodies. J. Neurosci. *7*:1866–1886.

Robinson, S.R. (1991) Development of the mammalian retina. In B. Dreher and S.R. Robinson (eds.): Neuroanatomy of the Visual Pathways and Their Development. London: Macmillan, pp. 69–128.

Sargent, P.B., and D.Z. Pang (1989) Acetylcholine receptor-like molecules are found in both synaptic and extrasynaptic clusters on the surface of neurons in the frog cardiac ganglion. J. Neurosci. *9*:1062–1072.

Sassoè-Pognetto, M., A. Feigenspan, J. Bormann, and H. Wässle (1996) Synaptic organization of an organotypic slice culture of the mammalian retina. Visual Neurosci. *13*:759–771.

Sassoè-Pognetto, M., J. Kirsch, U. Grünert, U. Greferath, J.-M. Fritschy, H. Möhler, H. Betz, and H. Wässle (1995) Colocalization of gephyrin and $GABA_A$- receptor subunits in the rat retina. J. Comp. Neurol. *357*:1–14.

Sassoè-Pognetto, M., H. Wässle, and U. Grünert (1994) Glycinergic synapses in the rod pathway of the rat retina: cone bipolar cells express the α1 subunit of the glycine receptor. J. Neurosci. *14*:5131–5146.

Schmitt, B., P. Knaus, C.-M. Becker, and H. Betz (1987) The M 93,000 polypeptide of the postsynaptic glycine receptor complex is a peripheral membrane protein. Biochemistry *26*:805–811.

Schröder, S., W. Hoch, C.-M. Becker, G. Grenningloh, and H. Betz (1991) Mapping of antigenic epitopes on the α1 subunit of the inhibitory glycine receptor. Biochemistry *30:*42–47.

Seitanidou, T., A.-M. Nicola, A. Triller, and H. Korn (1992) Partial glycinergic denervation induces transient changes in the distribution of a glycine receptor-associated protein in a central neuron. J. Neurosci. *12:*116–131.

Soltesz, I., J.D.B. Roberts, H. Takagi, J.G. Richards, H. Möhler, and P. Somogyi (1990) Synaptic and nonsynaptic localization of benzodiazepine/$GABA_A$ receptor/Cl^- channel complex using monoclonal antibodies in the dorsal lateral geniculate nucleus of the cat. Eur. J. Neurosci. *2:*414–429.

Somogyi, P., H. Takagi, J.G. Richards, and H. Möhler (1989) Subcellular localization of benzodiazepine/$GABA_A$ receptors in the cerebellum of rat, cat, and monkey using monoclonal antibodies. J. Neurosci. *9:*2197–2209.

Takahashi, T., A. Momiyama, K. Hirai, F. Hishinuma, and H. Akagi (1992) Functional correlation of fetal and adult forms of glycine receptors with developmental changes in inhibitory synaptic receptor channels. Neuron *9:*1155–1161.

Todd, A.J., R.C. Spike, D. Chong, and M. Neilson (1995) The relationship between glycine and gephyrin in synapses of the rat spinal cord. Eur. J. Neurosci. *7:*1–11.

Triller, A., F. Cluzeaud, F. Pfeiffer, H. Betz, and H. Korn (1985) Distribution of glycine receptors at central synapses: an immunoelectron microscopy study. J. Cell Biol. *101:*683–688.

Vardi, N., and P. Sterling (1994) Subcellular localization of $GABA_A$ receptors on bipolar cells in macaque and in human retina. Vision Res. *34:*1235–1246.

Vernallis, A.B., W.G. Conroy, and D.K. Berg (1993) Neurons assemble acetylcholine receptors with as many as three kinds of subunits while maintaining subunit segregation among receptor subtypes. Neuron *10:*451–464.

Wässle, H. (1988) Dendritic maturation of retinal ganglion cells. Trends Neurosci. *11:*87–89.

Wässle, H., and B.B. Boycott (1991) Functional architecture of the mammalian retina. Physiol. Rev. *71:*447–480.

Weidman, T.A., and T. Kuwabara (1968) Postnatal development of the rat retina. Arch. Ophthalmol. *79:*470–484.

Wong, R.O.L., R.M.Yamawaki, and C.J. Shatz (1992) Synaptic contacts and the transient dendritic spines of developing retinal ganglion cells. Eur. J. Neurosci. *4:*1387–1397.

Yamasaki, E.N., and A.S. Ramoa (1993) Dendritic remodelling of retinal ganglion cells during development of the rat. J. Comp. Neurol. *329:* 277–289.