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Colocalization of GABA, Glycine, and their Receptors at Synapses in the Rat Spinal Cord

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To determine whether GABA and glycine can act as cotransmitters at synapses in the rat spinal cord, we have compared the ultrastructural distribution of $GABA_A$-receptor β_3 subunit with that of the glycine receptor-associated protein gephyrin and combined this with postembedding detection of GABA and glycine. We also used a dual-immunofluorescence method to confirm that gephyrin was associated with the glycine-receptor α_1 subunit throughout the cord. $GABA_A$ β_3-subunit immunoreactivity was restricted primarily to synapses, and at a majority of these synapses the presynaptic axon was GABA-immunoreactive. Many synapses showed both $GABA_A$ β_3 and gephyrin immunoreactivity, and at most of these synapses GABA and glycine were enriched in the presynaptic axon. These results strongly support the idea that cotransmission by GABA and glycine occurs in the spinal cord.

Key words: $GABA_A$ receptor; glycine receptor; gephyrin; transmitter colocalization; immunocytochemistry; electron microscopy

GABA and glycine, the major inhibitory neurotransmitters in the spinal cord, act on ligand-gated chloride channels: $GABA_A$ and glycine receptors. The glycine receptor consists of α (ligand-binding) and β (structural) subunits together with gephyrin, a peripheral membrane protein that anchors the receptors at synapses by linking them to underlying microtubules (Kirsch et al., 1991, 1993). The main adult form of the α subunit (α_1) apparently is restricted to glycinergic synapses; however, the β subunit is found in many areas in which glycinergic transmission is believed not to occur (Malosio et al., 1991; Sato et al., 1991; Kirsch and Betz, 1993). The distribution of gephyrin is complex: in certain regions, such as spinal ventral horn, it is colocalized with the glycine-receptor α_1 subunit (Triller et al., 1987), whereas in many parts of the central nervous system it occurs without the α_1 subunit (Kirsch and Betz, 1993; Sassoè-Pognetto et al., 1995). $GABA_A$-receptor subunits have been divided based on sequence homology into four main classes—α, β, γ, and δ—and it is believed that naturally occurring $GABA_A$ receptors contain α, β, and γ subunits (Sigel et al., 1990; Verdoorn et al., 1990; Sieghart, 1992; Amin and Weiss, 1993). At least four different α subunits are present in rat spinal cord; however, in the case of β and γ subunits, a single type (β_3, γ_2) is predominant and is expressed by most, if not all, spinal neurons (Persohn et al., 1991, 1992; Wisden et al., 1991; Ma et al., 1993).

Many spinal neurons are inhibited by GABA and glycine (Curtis et al., 1968), and certain types of evoked inhibition can be blocked by both strychnine and bicuculline (Game and Lodge, 1975; Cullheim and Kellerth, 1981; Rudomin et al., 1990; Schneider and Fyffe, 1992; Yoshimura and Nishi, 1995), which indicates that inhibition is mediated through $GABA_A$ and glycine receptors. Because many boutons in the spinal cord contain both GABA and glycine (Örnung et al., 1994; Taal and Holstege, 1994; Todd et al., 1995), these compounds may act as cotransmitters. However, the presence of GABA and glycine in axon terminals does not prove that they both function as transmitters at synapses formed by these axons. For example, GABA and glycine coexist in cerebellar Golgi cells (Ottersen et al., 1988), but there is doubt that glycine functions as a transmitter in this situation (Luque et al., 1995). In addition, the two compounds coexist at axo-axonic synapses in spinal cord (Todd et al., 1995), although glycine is believed not to be involved in presynaptic inhibition.

To examine the possibility that GABA and glycine act as cotransmitters, we used an antibody directed against $GABA_A$-receptor β_3 subunit and a monoclonal antibody against gephyrin (which we found to be colocalized with the glycine-receptor α_1 subunit throughout the cord) to search for ultrastructural evidence that $GABA_A$ and glycine receptors coexist at synapses in rat spinal cord. We also combined this approach with postembedding immunocytochemistry to look for the presence of GABA and glycine at such synapses.

MATERIALS AND METHODS

Preparation of antibody against $GABA_A$-receptor β_3 subunit

The β_3 antibody was raised in rabbit against a fusion protein consisting of maltose-binding protein and amino acids 345–408 of the rat β_3 subunit, part of the large intracellular loop between putative transmembrane domains M3 and M4 (Vector pmal C2; New England Biolabs, Beverly, MA). Antibodies were isolated by chromatography on a fusion protein consisting of glutathione S-transferase, amino acids 345–408 of the β_3 subunit, and seven histidines (Vector pGEX-4T-1; Pharmacia, Vienna, Austria). The procedure was otherwise identical to that described previously (Mossier et al., 1994; Tögel et al., 1994). The antibody identified two proteins with M_r values of 53 and 56 kDa in Western blots of affinity-purified $GABA_A$ receptors. In addition, in preliminary experiments this antibody was found to precipitate recombinant $GABA_A$ receptors efficiently from extracts of human embryonic kidney cells transfected with $\alpha_1\beta_3\gamma_2$ subunits. The antibody did not precipitate $\alpha_1\beta_1\gamma_2$ receptors, and the amount of $\alpha_1\beta_2\gamma_2$ receptor precipitated was ~6% of that precipitated from extracts of $\alpha_1\beta_3\gamma_2$-transfected cells (K. Fuchs and W. Sieghart, unpublished data).

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Immunocytochemistry

Tissue preparation. Eight albino Swiss rats (either sex; 220–390 gm) were anesthetized deeply and fixed by vascular perfusion. For light-microscopic detection of $GABA_A$-receptor β_3 subunit, three rats were perfused with 4% formaldehyde. For electron-microscopic detection of the β_3 subunit, alone or in combination with gephyrin, two rats were perfused with 4% formaldehyde/0.05% glutaraldehyde and three rats were perfused with 1% formaldehyde/1% glutaraldehyde. Lumbar spinal cord segments were removed, stored for 2–4 hr in the same fixative, and then cut into 60 μm transverse sections on a Vibratome. Detection of glycine-receptor α_1 subunit and gephyrin was performed on 7 μm cryostat sections of lumbar spinal cord obtained from an additional two rats (male; 350 and 380 gm) that had been decapitated.

Pre-embedding immunocytochemistry. For light-microscopic detection of $GABA_A$-receptor β_3 subunit, sections from the rats fixed with 4% formaldehyde were incubated overnight in β_3 antibody (0.11–0.9 μg/ml) and reacted by the avidin–biotin complex (ABC) method with nickel-intensified diaminobenzidine (DAB) as chromogen. For electron microscopy of β_3 immunoreactivity, sections from the three rats perfused with 1% formaldehyde/1% glutaraldehyde were incubated overnight in β_3 antibody (0.9 μg/ml), reacted by the ABC method with DAB as chromogen, post-fixed in osmium tetroxide, dehydrated, and flat-embedded in Durcupan. From each rat, ultrathin sections were cut from blocks that included superficial dorsal horn (laminae I–III), deep dorsal horn (laminae IV–V), and part of the ventral horn (including lamina IX).

For simultaneous ultrastructural visualization of β_3 and gephyrin immunoreactivity, double labeling was performed using the method of Chan et al. (1990). Vibratome sections from four of the rats perfused with formaldehyde/glutaraldehyde fixatives were incubated overnight in a mixture of β_3 antibody (0.9 μg/ml) and antibody 7a (diluted 1:5000; Boehringer Mannheim, Mannheim, Germany), followed by biotinylated anti-rabbit IgG (1:200; Vector, Peterborough, UK) and anti-mouse IgG coated with 1 nm gold particles (1:200; British Biocell, Cardiff, UK). The gold particles (representing gephyrin immunoreactivity) were intensified with silver (IntenSE kit, Amersham, Arlington Heights, IL), and β_3-immunoreactivity then was revealed with the ABC technique and DAB. The sections were osmicated, dehydrated, and embedded in Durcupan. Ultrathin sections were obtained from superficial and deep parts of the dorsal horn and from the ventral horn.

An absorption control for $GABA_A$-receptor β_3-subunit immunostaining was performed by incubating sections of spinal cord fixed with 1% glutaraldehyde/1% formaldehyde in β_3 antibody (0.9 μg/ml) to which fusion protein (1 μg/ml glutathione S-transferase, amino acids 345–408 of the β_3 subunit, and seven histidines) had been added 2 hr previously. This abolished immunostaining. Controls for the dual-labeling pre-embedding reaction involved omitting one of the two primary antibodies.

Postembedding immunogold method. To examine the relationship between the $GABA_A$-receptor β_3 subunit and GABA, ribbons of ultrathin sections were cut from material that had been reacted with the β_3 antibody by the pre-embedding method (see above). From each of the three animals, ultrathin sections from the superficial and deep parts of the dorsal horn and from the ventral horn were studied. In each case, a single ultrathin (reference) section was mounted onto a copper-mesh grid and stained with lead citrate, and the two succeeding sections were collected onto a Formvar-coated, single-slot nickel grid and reacted with antiserum to GABA by a modification of the method of Holstege (1991). The grid was incubated for 1 hr with PBS (containing 0.1% Triton X-100 and 1% bovine serum albumin) and overnight with the same solution containing antiserum to GABA (diluted 1:2000) (Pow and Crook, 1993). Then it was rinsed and incubated with goat anti-rabbit IgG coated with 10 nm gold particles (diluted 1:25; British Biocell). From each of the nine reference sections, 10 β_3-immunoreactive synapses and 5 asymmetrical synapses were selected, and these then were located and photographed on the two corresponding serial sections reacted with GABA antiserum. The background level of gold-particle density was taken as the average density over the axons presynaptic at the asymmetrical synapses (which were presumed not to be GABAergic). Axons presynaptic at β_3-immunoreactive synapses were counted as GABA-immunoreactive if the density of gold particles over them exceeded the background level by at least fourfold on both sections.

Detection of GABA- and glycine-like immunoreactivity was performed on material that had been reacted with antibodies to both the $GABA_A$-receptor β_3 subunit and gephyrin (see above) from two of the rats fixed with 1% formaldehyde/1% glutaraldehyde. From each rat, one block of deep dorsal horn and one block of ventral horn were used. Ribbons of five ultrathin sections were cut from these blocks and mounted onto grids in serial order. The third (reference) section was mounted onto a copper-mesh grid and stained with lead citrate, and the other sections were collected onto Formvar-coated, single-slot nickel grids. The grid with the first two sections of the ribbon was reacted with antiserum to GABA as described above, and the grid with the fourth and fifth sections was reacted in the same way, but with antiserum to glycine (1:1000) (Pow and Crook, 1993). Forty synapses showing both β_3 and gephyrin immunoreactivity were selected from the reference sections (10 from each block); they then were examined and photographed on both of the sections reacted with GABA antiserum, and both sections reacted with glycine antiserum. GABA immunoreactivity in the presynaptic axons was defined as above, whereas axons were considered to be glycine-immunoreactive if the gold-particle density exceeded the background level (over 5 axons that formed asymmetrical synapses on the same grid) by at least twofold on both sections. We have found that this value provides a suitable cut-off point for distinguishing axonal boutons in the spinal cord that are enriched with glycine from those that are not (Todd et al., 1995). We have shown previously that immunostaining with the GABA and glycine antisera can be blocked selectively by pretreatment with a conjugate of the corresponding amino acid but not with a conjugate of the other amino acid (see Fig. 5 of Todd et al., 1995). The antibodies also have been applied to test sections and found to show no cross-reactivity with the inappropriate amino acid (glycine or GABA) or with a variety of other amino acids, including taurine, glutamate, glutamine, or aspartate (Pow, 1994; Pow and Robinson, 1994). The GABA and glycine antisera were donated by Dr. D. V. Pow (University of Queensland).

Immunofluorescent detection of monoclonal antibodies 2b and 7a. To determine the relationship between gephyrin and the glycine-receptor α_1 subunit, a modification of the method of Triller et al. (1987) was used. Fifteen unfixed cryostat sections of spinal cord taken from two rats were mounted onto gelatinized slides, incubated in monoclonal antibody 2b (diluted 1:50 or 1:100; donated by H. Betz, Max Planck Institute for Brain Research, Frankfurt, Germany), rinsed, and fixed briefly in 4% formaldehyde. They were treated with a Fab′ fragment of goat anti-mouse IgG conjugated to lissamine rhodamine (1:100; Jackson Immunoresearch, West Grove, PA), and then with unconjugated Fab′ fragment of goat anti-mouse IgG (1:20; Jackson) in an attempt to saturate binding to monoclonal antibody 2b. They then were incubated in monoclonal antibody 7a (1:100) and, finally, with horse anti-mouse IgG conjugated to fluorescein (1:50; Vector). Sections were viewed with conventional epifluorescence and with a Bio-Rad MRC 1024 confocal laser scanning microscope equipped with a krypton–argon laser (Hercules, CA). For confocal microscopy, sections were scanned sequentially with the 488 and 568 nm laser lines to prevent bleed-through fluorescence. Control sections were treated in the same way, except that one of the primary antibodies was omitted, and additional sections were incubated only with antibody 2b or 7a and the corresponding fluorescent secondary antibody.

RESULTS

$GABA_A$-receptor β_3-subunit immunoreactivity

In sections of lumbar spinal cord incubated with antibody to $GABA_A$-receptor β_3 subunit, immunoreactivity was present in all parts of the spinal gray matter but varied in intensity in different areas (Fig. 1*A*). Immunostaining was most dense in laminae I and II and was present at moderate levels in a band including laminae III and IV, the medial aspect of the deep dorsal horn, and extending to the area around the central canal, as well as in the motor nuclei of the ventral horn. In the lateral part of the deep dorsal horn and the remainder of the ventral horn, immunostaining was less dense. At high magnification, punctate immunoreactivity was found in all parts of the gray matter, but a few immunoreactive cell bodies were present in laminae III and IV (Fig. 1*B*), and strongly immunoreactive dendrites were common in laminae II and III. In some cases, dendrites could be followed from the immunoreactive cell bodies. Elsewhere in the spinal gray matter, extensive staining of dendritic shafts was extremely rare,

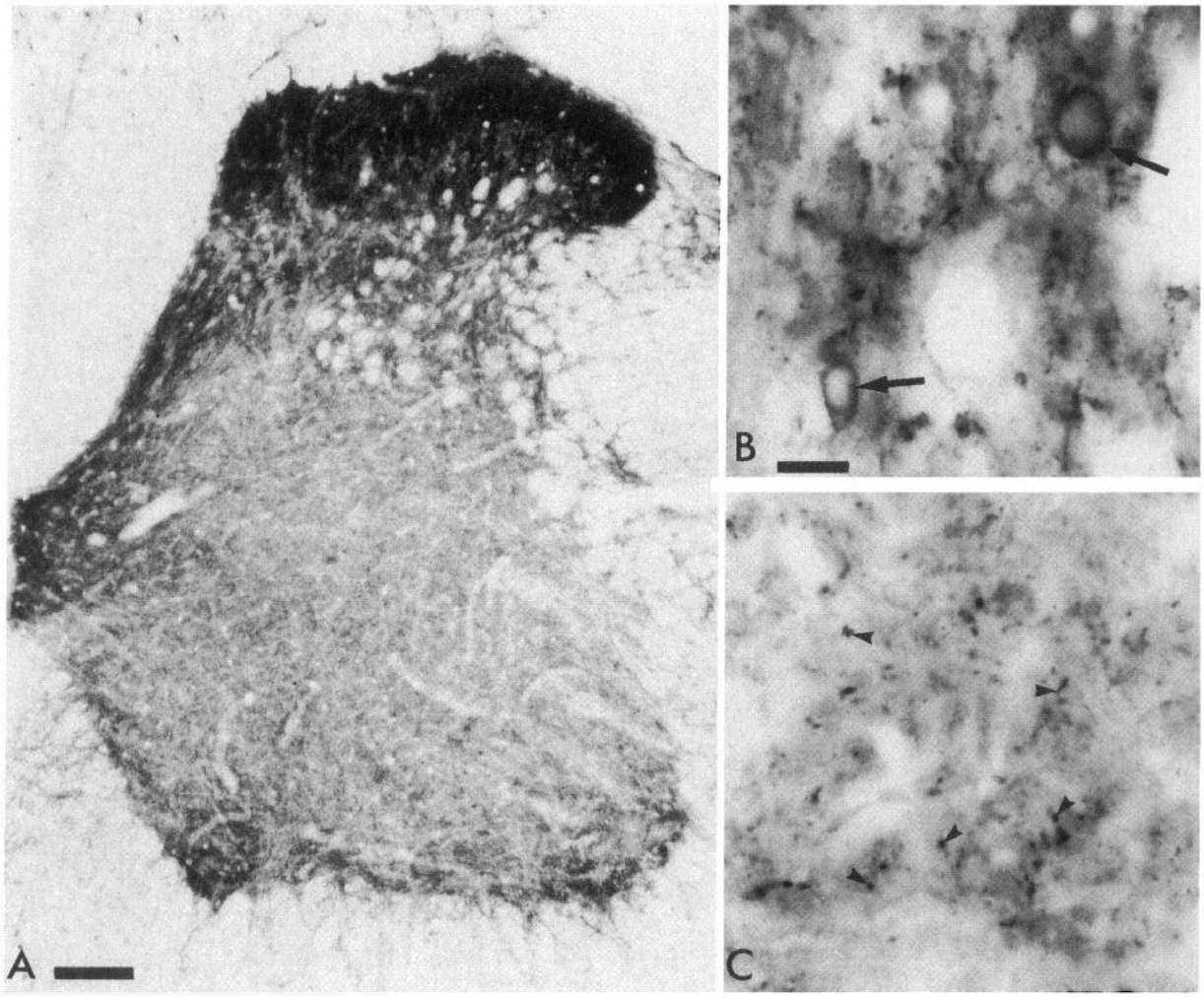

Figure 1. The light-microscopic appearance of immunostaining with antibody to $GABA_A$-receptor β_3 subunit in the spinal cord. *A*, Immunoreactivity is present throughout the gray matter but is most dense in the superficial dorsal horn and moderate in the medial part of the deep dorsal horn, the area around the central canal, and in the motor nuclei of the ventral horn. *B*, Part of lamina III at high magnification. Two immunoreactive cell bodies (*arrows*) are visible. *C*, Punctate immunostaining in lamina IX. Some of the puncta are indicated with *arrowheads*. Scale bars: *A*, 100 μm; *B*, *C*, 10 μm.

and the immunoreactivity was almost exclusively punctate (Fig. 1*C*).

With electron microscopy, most of the immunoreactive puncta were found to correspond to synapses. Reaction product was present on the internal aspect of the plasma membrane of the postsynaptic element and showed limited extension into the adjacent cytoplasm (Figs. 2–4). There usually was little spread of reaction product away from the active site, so nearby synapses were unlabeled (Figs. 2, 4). As suggested from light-microscopic observations, some cell bodies and many dendrites in laminae II–IV of the dorsal horn showed more extensive filling with reaction product.

Because immunostaining with this antibody tolerated fixation with 1% glutaraldehyde, we were able to carry out postembedding immunocytochemistry with antiserum to GABA on ultrathin sections, to determine whether the axons presynaptic at β_3-immunoreactive synapses were GABAergic. Ninety randomly selected β_3-immunoreactive synapses were examined (10 in the ventral horn and 10 in both superficial and deep parts of the dorsal horn, each in 3 rats), and at 84 of these (93%) the presynaptic element was GABA-immunoreactive (Fig. 2).

$GABA_A$-receptor β_3-subunit and gephyrin immunoreactivity

To study the relationship between $GABA_A$ and glycine receptors at the ultrastructural level, we used a double-label pre-embedding immunocytochemical technique to reveal $GABA_A$-receptor β_3 subunit with the ABC method; silver-intensified immunogold was used to reveal gephyrin. In ultrathin sections cut from this material, the two types of reaction product could be distinguished easily (Figs. 3, 4). Each type showed a similar distribution to that seen in sections processed with the corresponding antibody alone, and omission of either primary antibody abolished the associated type of immunostaining. In each area examined (the ventral horn and superficial and deep parts of the dorsal horn), many synapses possessed only silver particles (corresponding to gephyrin) or DAB (corresponding to $GABA_A$-receptor β_3 subunit), whereas other synapses were double-labeled (Figs. 3, 4). At double-labeled synapses, the two types of reaction product could be either intermingled (Figs. 3*A*, 4*A*) or partially or completely segregated. In the latter case, the reaction products often were side-by-side, but a common arrangement was for the DAB to be concentrated at

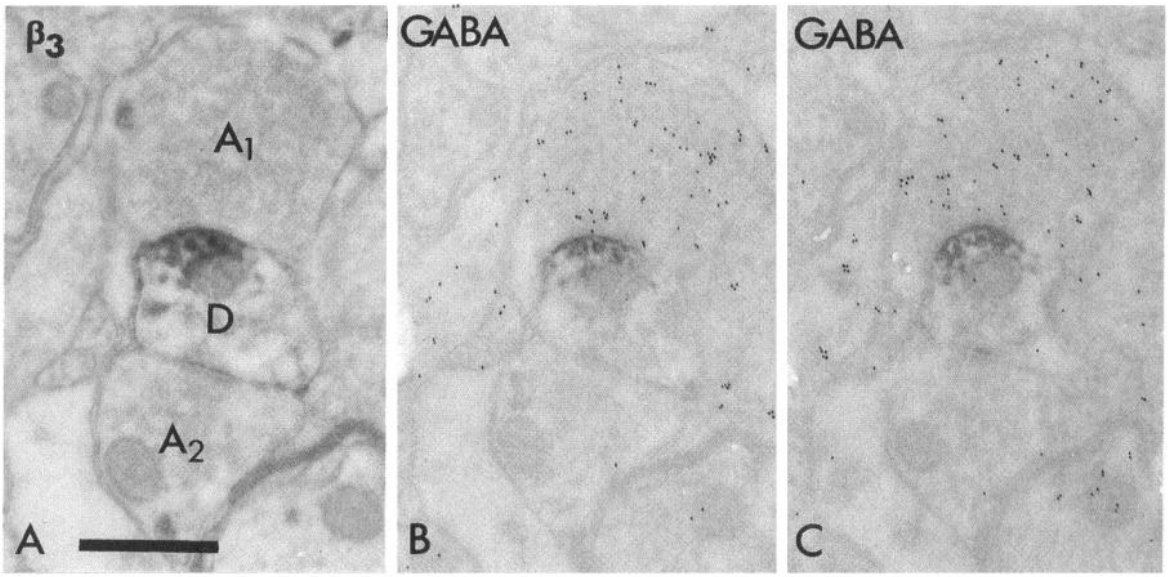

Figure 2. $GABA_A$-receptor β_3 subunit-immunoreactive synapse in lamina III and its relationship to GABA. *A*, Ultrathin section showing a dendrite (*D*) receiving two synapses from axons (A_1 and A_2). The synapse formed by A_1 is β_3-immunoreactive, but there is little spread of reaction product, so the asymmetrical synapse formed by A_2 is unstained. *B*, *C*, Equivalent region in two serial sections that were reacted with GABA antiserum by the postembedding method. A_1 is labeled heavily with gold particles, whereas A_2 is not. Scale bar, 0.5 μm.

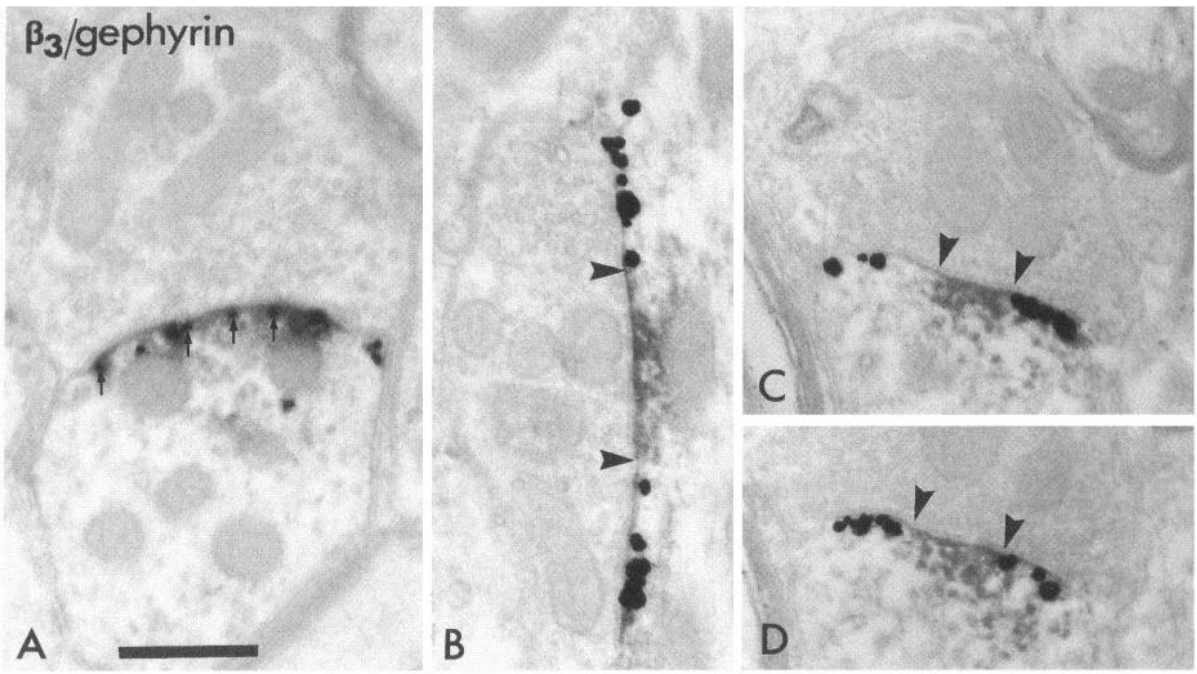

Figure 3. Colocalization of $GABA_A$-receptor β_3 and gephyrin immunoreactivity at three synapses in the ventral horn. In each case, the gephyrin immunoreactivity is represented by silver particles and the β_3 immunoreactivity is represented by diffuse (DAB) reaction product. *A*, Synapse at which the two types of immunoreactivity are intermingled. *Arrows* indicate some of the silver particles. *B*, Synapse at which β_3 immunoreactivity (between *arrowheads*) is located centrally and is surrounded by regions with gephyrin immunoreactivity. *C*, *D*, Two consecutive ultrathin sections through another synapse with a central region of β_3 immunoreactivity (between *arrowheads*) and peripheral gephyrin immunoreactivity. Scale bar, 0.5 μm.

the central part of the active site with silver particles present in clusters on either side (Fig. 3*B–D*). Because heavy deposition of silver particles could obscure the DAB precipitate, it was not always possible to determine whether particular synapses or areas within synapses were double-labeled.

Because of the likelihood of false-negative results (e.g., attributable to incomplete penetration of antibodies), we did not attempt to quantify single- and double-labeled synapses. However, in the deep part of the dorsal horn and in the ventral horn, the density of synapses at which $GABA_A$-receptor β_3-subunit immunoreactivity was present was much lower than that of gephyrin-immunoreactive synapses, so only a small minority of gephyrin-immunoreactive synapses were double-labeled. Approximately half of the β_3-immunoreactive synapses in these areas were also

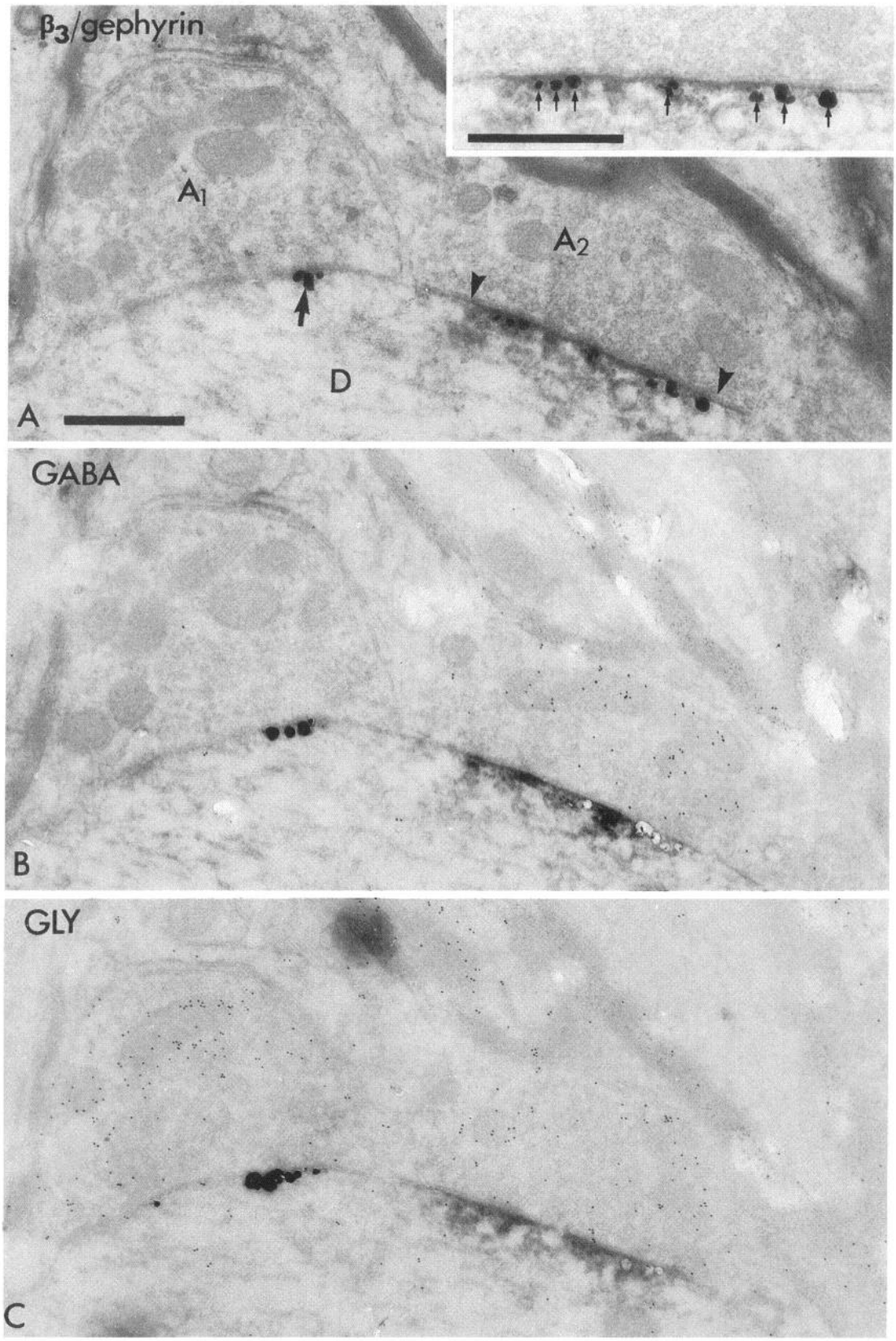

Figure 4. GABA and glycine immunoreactivity at a synapse in the deep dorsal horn that possessed both $GABA_A$-receptor β_3 and gephyrin immunoreactivity. *A*, Dendrite (*D*) receives synapses from two axons, A_1 and A_2. The synapse involving A_1 is gephyrin-immunoreactive only (*arrow*), whereas that involving A_2 (between *arrowheads*) has both gephyrin and β_3 immunoreactivity. The *inset* shows this synapse at higher magnification to reveal the silver particles more clearly (*arrows*). *B*, *C*, The preceding and subsequent ultrathin sections reacted with antiserum to GABA and glycine (*GLY*), respectively. Axon A_2 shows both GABA and glycine immunoreactivity, whereas A_1 is immunoreactive only with glycine antiserum. Scale bar, 0.5 μm.

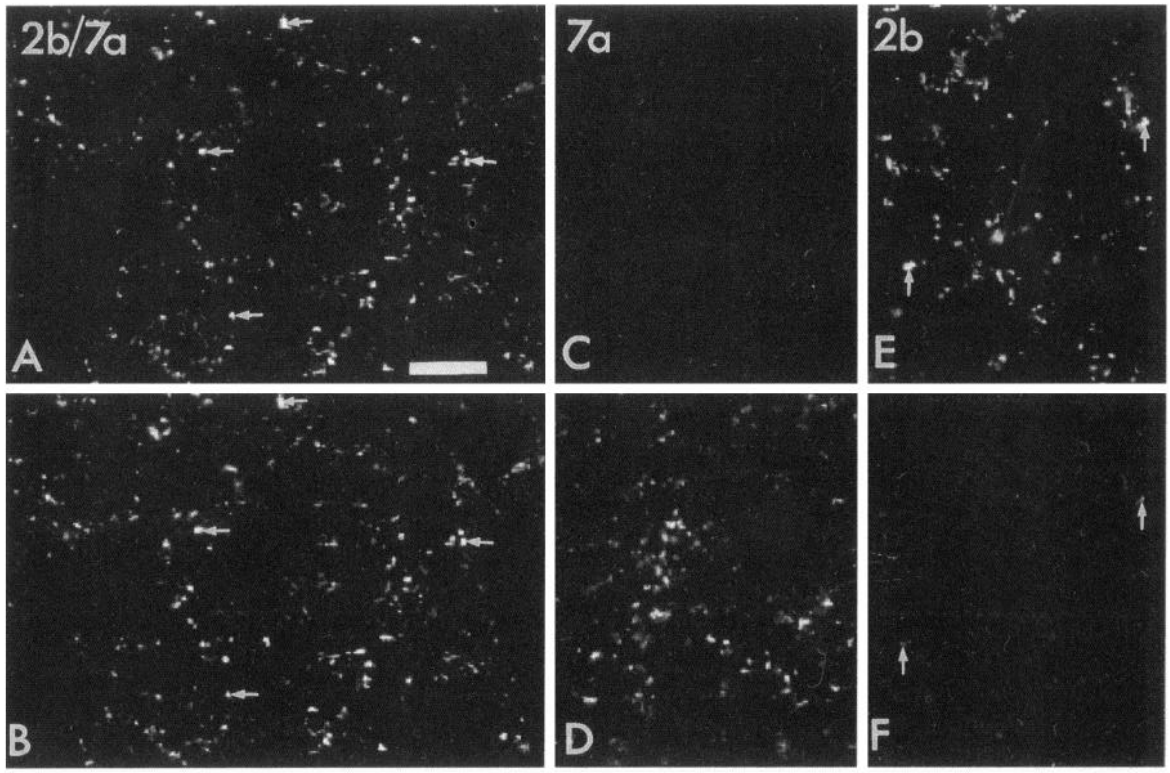

Figure 5. Pairs of confocal images of a section reacted with monoclonal antibodies 2b and 7a (*A, B*) or control sections reacted only with antibody 7a (*C, D*) or 2b (*E, F*). The sections were scanned with the 488 and 586 nm lines of the laser sequentially to avoid bleed-through fluorescence. In each case, the upper picture (*A, C, E*) was obtained with the 568 nm line to reveal lissamine rhodamine (corresponding to immunostaining with antibody 2b), and the lower picture (*B, D, F*) was obtained with the 488 nm line for fluorescein (corresponding to antibody 7a). *A, B*, Part of lamina IV of the dorsal horn and virtually all of the profiles that are immunoreactive with 7a (seen in *B*) are also immunoreactive with 2b (*A*). Some of these profiles are indicated with *arrows*. In a control section, omission of antibody 2b caused a complete loss of rhodamine fluorescence (*C*), whereas strong fluorescein staining was still present (*D*). This indicates that the rhodamine fluorescence was attributable entirely to antibody 2b. When antibody 7a was omitted, very weak residual staining with fluorescein was observed, so the brightest profiles in *E* are just visible in *F* (*arrows*). Scale bar, 10 μm.

gephyrin-immunoreactive. In the superficial part of the dorsal horn, the DAB reaction product (corresponding to β_3 immunoreactivity) often extended beyond active sites and, therefore, it was more difficult to assess the relative frequency of single- and double-labeled synapses.

To determine whether GABA and glycine were colocalized in axons that formed synapses possessing both $GABA_A$-receptor β_3 subunit and gephyrin, we selected 40 such synapses and examined them on serial sections reacted with antibodies to GABA and glycine. At 32 of these synapses, the presynaptic axon was enriched significantly with both GABA- and glycine-like immunoreactivity (Fig. 4), and at an additional 7 synapses, the axon was either GABA- or glycine-immunoreactive.

Gephyrin and glycine-receptor α_1-subunit immunoreactivity

Because there are differences in the relationship between gephyrin and the glycine-receptor α_1 subunit in different parts of the nervous system, we tested the reliability of gephyrin as a marker for synapses that possess glycine receptors in the spinal cord. A dual-immunofluorescence method was used to compare the distribution of staining with two monoclonal antibodies: 2b (which recognizes the α_1 subunit; revealed with lissamine rhodamine) and 7a (which recognizes gephyrin; revealed with fluorescein). In sections treated with both antibodies, virtually all of the profiles that were stained with fluorescein also were stained with lissamine rhodamine (and vice versa), although the relative intensity of the two types of fluorescence varied from profile to profile (Fig. 5*A,B*). This pattern of colocalization was found in all laminae of the spinal gray matter and in the lateral and ventral columns of the white matter (where immunoreactive puncta are occasionally present). Omission of antibody 2b caused the complete absence of rhodamine fluorescence (Fig. 5*C,D*), indicating that this fluorescence was attributable exclusively to glycine-receptor α_1-subunit immunoreactivity. However, when antibody 7a was omitted, very weak fluorescein staining still was observed (Fig. 5*E,F*). This was not attributable to bleed-through fluorescence, because it was observed only when fluorescein-labeled secondary antibody was present and presumably was caused by incomplete blocking of antibody 2b before the second immunofluorescent reaction.

DISCUSSION

$GABA_A$ receptors in the spinal cord

The antibody used here was raised against part of the β_3 subunit that shows little homology with β_1 or β_2 subunits (Ymer et al., 1989). It selectively precipitates recombinant receptors containing β_3 subunit, and we have found that in cerebellum it caused immunostaining only in the granule cell layer (A. J. Todd, R. C. Spike, and W. Sieghart, unpublished observations), which is consistent with the pattern of β_3-subunit expression (Persohn et al., 1992). The immunostaining that we observed, therefore, presumably represents the $GABA_A$-receptor β_3 subunit. The laminar distribution of β_3 immunoreactivity closely matches that of glu-

tamic acid decarboxylase (GAD) (McLaughlin et al., 1975) and, because β_3-immunoreactivity was associated with synapses involving GABA-immunoreactive axons, it appears that this subunit is present at GABAergic synapses throughout the spinal cord. Because of limitations of the pre-embedding method, we could not determine whether β_3 subunit was present at all GABAergic synapses.

Early studies with antibodies against various $GABA_A$-receptor subunits (Richards et al., 1987; Somogyi et al., 1989; Zimprich et al., 1991; Fritschy et al., 1992) reported extensive labeling of neurons, with staining of much of the cell membrane; this was taken as evidence of relatively high levels of extrasynaptic $GABA_A$ receptor (Somogyi et al., 1989). More recent studies with postembedding immunocytochemistry have indicated that in cerebellum and dentate gyrus, α_1 and $\beta_{2/3}$ subunits are highly concentrated at GABAergic synapses, with extrasynaptic labeling present at lower levels (Nusser et al., 1995a,b). The discrepancy between these results was believed to reflect limited access of antibodies recognizing extracellular epitopes to synaptic clefts with the pre-embedding method, whereas these sites would be accessible on ultrathin sections. It was suggested that, although there are extrasynaptic $GABA_A$ receptors on these neurons, these receptors are present at much lower concentrations than those found at GABAergic synapses.

The distribution of β subunits in rat spinal cord has been reported by Richards et al. (1987) with monoclonal antibody bd17, which recognizes an extracellular epitope on both β_2 and β_3 subunits. They found diffuse granular immunostaining in superficial dorsal horn (as reported here) but extensive staining of cell bodies and dendritic trees of neurons elsewhere in the gray matter. Although the difference between the extensive immunostaining of neuronal membranes reported with bd17 and the punctate distribution observed with β_3 antibody in this study could be attributable to the presence of the β_2 subunit, it is unlikely because only a few spinal neurons express this subunit (Persohn et al., 1991, 1992; Wisden et al., 1991; Ma et al., 1993). A more likely explanation is that the β_3 subunit is highly concentrated at GABAergic synapses and, because the antibody we used recognizes an intracellular epitope, it has unrestricted access, causing predominantly synaptic labeling. Although extrasynaptic β_3 subunits are present on many spinal neurons (and are detected by antibody bd17), their density presumably is below the threshold of detection of our immunocytochemical method. The β_3-immunoreactive cell bodies and dendrites that we observed in laminae II–IV apparently are exceptions to this. We have observed similar cell bodies and dendrites in these laminae that were strongly immunostained with antibody bd17 (A. J. Todd and R. C. Spike, unpublished observations). Presumably, these cells have much higher concentrations of extrasynaptic $GABA_A$-receptor β_3 subunit than other spinal neurons.

There have been few immunocytochemical studies of other $GABA_A$-receptor subunits in rat spinal cord. Bohlhalter et al. (1994) used an antibody directed against an extracellular part of the α_1 subunit and reported widespread immunostaining of somatic and dendritic membranes of certain neurons, although confocal microscopy revealed the presence of "hotspots" that frequently were apposed to GAD-immunoreactive profiles, suggesting that these hotspots represented accumulations of α_1 subunit at GABAergic synapses. Sur et al. (1995) have reported recently that immunostaining in ventral horn with an antibody recognizing an intracellular epitope of the γ_2 subunit was restricted primarily to synapses.

Immunocytochemical markers for glycine receptors in the spinal cord

Various monoclonal antibodies have been used for immunocytochemical detection of glycine receptors (Pfeiffer et al., 1984). Antibody 2b is specific for the glycine-receptor α_1 subunit and, therefore, provides the most reliable way of identifying receptors at presumed glycinergic synapses (Kirsch and Betz, 1993). Unfortunately, immunostaining with antibody 2b is extremely sensitive to fixation, and we were able to obtain satisfactory immunostaining only by delaying fixation until after incubation in primary antibody. Immunostaining with β_3 antibody was not observed under these conditions and, therefore, we could not compare directly the distribution of glycine-receptor α_1 subunit with that of $GABA_A$-receptor β_3 subunit.

Immunostaining with antibody 7a (which recognizes gephyrin) is resistant to fixation, and this antibody can be used in conditions that allow postembedding detection of GABA and glycine. There is evidence already that gephyrin is restricted to synapses that possess glycine receptors in the spinal cord. The laminar distribution of gephyrin closely matches that of glycine-immunoreactive boutons (van den Pol and Gores, 1988; Mitchell et al., 1993), and we have shown that glycine is enriched in most axons at gephyrin-immunoreactive synapses (Todd et al., 1995). In addition, Triller et al. (1987) reported a perfect match between immunofluorescence with antibodies 2b and 7a in ventral horn. Because we found that virtually all gephyrin-immunoreactive structures throughout the spinal cord also were labeled with antibody 2b, it appears that in spinal cord gephyrin is restricted to synapses at which glycine-receptor α_1 subunit is present.

Colocalization of $GABA_A$ and glycine receptors

The present results suggest that at certain synapses in spinal cord $GABA_A$ and glycine receptors are both present on the postsynaptic membrane, whereas GABA and glycine are enriched in the presynaptic axon. They confirm and extend previous reports demonstrating that GAD- or GABA-containing axons can be presynaptic at gephyrin-immunoreactive synapses (Triller et al., 1987; Mitchell et al., 1993) and that hotspots of $GABA_A$-receptor α_1-subunit immunoreactivity coexist with gephyrin and are adjacent to GAD-immunoreactive profiles (Bohlhalter et al., 1994). However, this apparently is the first study to provide ultrastructural evidence that two classical transmitters and their receptors are present at the same synapse, and it strongly supports the idea that GABA and glycine can act as cotransmitters within the spinal cord.

We frequently observed synapses labeled with only gephyrin or $GABA_A$-receptor β_3 subunit immunoreactivity, which suggests that some synapses have glycine receptors, some have $GABA_A$ receptors, and others possess both receptors. This is consistent with pharmacological results showing that inhibition evoked in spinal neurons may be antagonized by strychnine, bicuculline, or both. Studies with antagonists have indicated that there are two components of inhibition of spinal neurons: a fast phase involving glycine receptors and a slower phase mediated by $GABA_A$ receptors (Game and Lodge, 1975; Baba et al., 1994; Yoshimura and Nishi, 1995), and synapses with different receptor combinations, therefore, presumably will generate IPSPs with different time courses.

Although most of the axons presynaptic at gephyrin-immunoreactive and $GABA_A$-receptor β_3-immunoreactive synapses were enriched with both GABA and glycine, some were not; and at all but one of these synapses, the axon was either GABA- or glycine-immunoreactive. We also found that a few axons at β_3-immunoreactive synapses were not GABA-immunoreactive, and we

have reported previously that some axons presynaptic at gephyrin-immunoreactive synapses were not glycine-immunoreactive (Todd et al., 1995). This may indicate that at certain synapses there is a mismatch between transmitter and receptor; however, it is possible that in axons that use both GABA and glycine, one of the transmitters is present at a relatively low concentration that we are unable to detect with our postembedding method.

An unexpected finding in the present study was that when gephyrin and the $GABA_A$-receptor β_3 subunit both were present at synapses, they often were separated partially or completely (*Fig. 3B–D*). This is unlikely to be attributable to suppression of one type of reaction, because the particulate marker was deposited first and should not have impeded binding of ABC complex or subsequent deposition of DAB. We cannot be certain that the distribution of gephyrin exactly parallels that of the glycine receptor, and it is difficult to assess the spread of the DAB reaction product but, nonetheless, these results suggest that $GABA_A$ and glycine receptors are concentrated at different parts of the active site at some synapses at which they are both present. Nusser et al. (1994) have shown that there is separation of ionotropic and metabotropic glutamate receptors at synapses in cerebellum, and the present findings provide additional evidence that the distribution of receptors at synapses may not be homogeneous.

Sassoè-Pognetto et al. (1995) recently demonstrated that in retina gephyrin is colocalized with $GABA_A$ receptors at synapses that lack glycine receptors. They suggested that gephyrin is involved in aggregation of $GABA_A$ receptors at these synapses, which is consistent with the finding that $GABA_A$ receptors can bind to tubulin (Item and Sieghart, 1994). However, the frequent separation of gephyrin and $GABA_A$-receptor β_3 subunit that we observed makes it unlikely that gephyrin is involved in anchoring $GABA_A$ receptors at synapses in spinal cord.

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