GABAergic Innervation Organizes Synaptic and Extrasynaptic $GABA_A$ Receptor Clustering in Cultured Hippocampal Neurons

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We have studied the effects of GABAergic innervation on the clustering of $GABA_A$ receptors ($GABA_A$Rs) in cultured hippocampal neurons. In the absence of GABAergic innervation, pyramidal cells form small (0.36 ± 0.01 μm diameter) $GABA_A$R clusters at their surface in the dendrites and soma. When receiving GABAergic innervation from glutamic acid decarboxylase-containing interneurons, pyramidal cells form large (1.62 ± 0.08 μm breadth) $GABA_A$R clusters at GABAergic synapses. This is accompanied by a disappearance of the small $GABA_A$R clusters in the local area surrounding each GABAergic synapse. Although the large synaptic $GABA_A$R clusters of any neuron contained all $GABA_A$R subunits and isoforms expressed by that neuron, the small clusters not localized at GABAergic synapses showed significant heterogeneity in subunit and isoform composition. Another difference between large GABAergic and small non-GABAergic $GABA_A$R clusters was that a significant proportion of the latter was juxtaposed to postsynaptic markers of glutamatergic synapses such as PSD-95 and AMPA receptor GluR1 subunit. The densities of both the glutamate receptor-associated and non-associated small $GABA_A$R clusters were decreased in areas surrounding GABAergic synapses. However, no effect on the density or distribution of glutamate receptor clusters was observed. The results suggest that there are local signals generated at GABAergic synapses that induce both assembly of large synaptic $GABA_A$R clusters at the synapse and disappearance of the small $GABA_A$R clusters in the surrounding area. In the absence of GABAergic innervation, weaker $GABA_A$R-clustering signals, generated at glutamatergic synapses, induce the formation of small postsynaptic $GABA_A$R clusters that remain juxtaposed to glutamate receptors at glutamatergic synapses.

Key words: $GABA_A$ receptor; subunit isoform; synaptogenesis; GABA; synapse formation; hippocampus; neuron culture; glutamate receptor; gephyrin; clustering

Low-density hippocampal cultures, in combination with fluorescence immunocytochemistry, have proven very useful for studying the clustering of $GABA_A$ receptors ($GABA_A$Rs) in individual GABAergic synapses (Killisch et al., 1991; Craig et al., 1996). Observations from hippocampal and spinal cultures have revealed the colocalization of postsynaptic $GABA_A$R clusters (Craig et al., 1994; Levi et al., 1999) with presynaptic GABAergic boutons that contain glutamic acid decarboxylase (GAD). In addition, cultured neurons showed $GABA_A$R clusters that did not colocalize with GAD boutons (Kannenberg et al., 1999; Levi et al., 1999; Scotti and Reuter, 2001).

Despite these aforementioned studies, there has not been a systematic study on the characteristics of the GAD-related and GAD-independent $GABA_A$R clusters. Moreover, in these studies, the possible heterogeneity of the $GABA_A$ subunit or isoform composition in the receptor clusters has not been addressed. This is an important issue in light of observations that in the intact brain and retina some GABAergic synapses and puncta show selectivity for certain α subunit-isoform-containing $GABA_A$Rs (Fritschy et al., 1992; Koulen et al., 1996; Nusser et al., 1996a; Fletcher et al., 1998; Nyiri et al., 2001).

In the present study, we have used low-density hippocampal cultures in combination with triple-label immunofluorescence to examine (1) the organizing effects that the presynaptic GABAergic innervation exerts on synaptic and extrasynaptic $GABA_A$R clustering in hippocampal pyramidal neurons, (2) the $GABA_A$R subunit and isoform expression in individual cells in culture, (3) whether there is selectivity in the subunit and isoform composition in the various synaptic and extrasynaptic $GABA_A$R clusters, and (4) the relationship between $GABA_A$R clusters and glutamate receptor clusters.

Received Aug. 1, 2001; revised Oct. 16, 2001; accepted Nov. 9, 2001.

This work was supported by National Institute of Neurological Disorders and Stroke Grants NS38752 and NS39287.

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MATERIALS AND METHODS

Antibodies. The primary antibodies, guinea pig anti-α_1 [1–15 amino acids (aa)], rabbit anti-α_1 (1–15 aa), rabbit anti-α_2 (417–423 aa), rabbit anti-α_3 (1–13 aa), and rabbit anti-γ_2 (1–15 aa), were raised and affinity purified in our laboratory against synthetic peptides made to unique extracellular epitopes (N terminus for α_1, α_3 and γ_2, and C terminus for α_2) of rat $GABA_A$R subunits (Miralles et al., 1999). The monoclonal mouse anti-$\beta_{2/3}$ (62–3G1) was raised in our laboratory to affinity-purified $GABA_A$R (De Blas et al., 1988; Vitorica et al., 1988). This antibody recognizes an extracellular N-terminus epitope that is common to β_2 and β_3 subunits but is not present in β_1 (Ewert et al., 1992). Antibodies to fusion proteins of the intracellular loops of β_1, β_2, and β_3 were also raised in rabbits in our laboratory and affinity purified with purified intracellular loop of the respective isoform (Moreno et al., 1994; Li and De Blas, 1997). Subunit and isoform-specific antibodies made in several species in our laboratory have allowed us to study colocalization by triple-label immunofluorescence (see below). All antibodies to $GABA_A$R subunits used in this study have been thoroughly characterized, and their specificities have been determined elsewhere (De Blas et al., 1988; Vitorica et al., 1988; Moreno et al., 1994; Miralles et al., 1999). Specificity tests of $GABA_A$R antibodies included ELISA, immunoblotting, light microscopy immunocytochemistry, displacement of immunoreactivity in these assays by specific peptides, and subunit-specific staining in host-transfected cell lines. The specificity of some antibodies was also tested for the absence of immunoreactivity in knock-out mouse mutants. The monoclonal mouse anti-gephyrin (mAb 7a) was purchased from Cedarlane (Accurate Chemical and Scientific Corp., Westbury, NY). Rabbit anti-GluR1 was from

Chemicon (Temecula, CA), mouse monoclonal anti-PSD-95 was from Upstate Biotechnology (Lake Placid, NY), and mouse monoclonal anti-SV2 was a gift of Dr. Kathleen M. Buckley (Harvard Medical School). Sheep anti-GAD (gift of I. Kopin), GAD 65-specific mouse monoclonal GAD6 (Developmental Studies Hybridoma Bank, University of Iowa), affinity-purified rabbit anti-GABA Transporter-1 (GAT-1) from DiaSorin (Stillwater, MN), and affinity-purified rabbit anti-synaptic vesicle GABA Transporter (VGAT) from Alpha Diagnostics International (San Antonio, TX) were used for identifying interneurons and GABAergic presynaptic processes. For the exo-endocytotic assay, a rabbit antibody to the lumenal N terminus (1–23 aa) of synaptotagmin-I (Syt-N) from StressGen Biotechnologies (Victoria, B.C., Canada) was used.

Low-density and micro-island hippocampal cultures. Hippocampal cultures were prepared as described by Banker and Goslin (1998). Briefly, embryonic day 18 Wistar rat pup hippocampi were dissected in HBSS, followed by treatment with 0.25% trypsin (Sigma, St. Louis, MO) and trituration using a fire-polished Pasteur pipette. Dissociated cells were centrifuged in HBSS for 2 min at 1500 rpm, and the pellet was resuspended in plating medium [10% horse serum (Invitrogen) in DMEM with 0.6% glucose and 26 mM $NaHCO_3$]. The suspended cells were plated at a density of 5,000–10,000 cells per 18-mm-diameter circular coverslip treated with poly-L-lysine (Sigma). These cultures contained 90–95% pyramidal cells and 5–10% interneurons (Benson et al., 1994). At the aforementioned plating density, pyramidal cells receive limited GABAergic innervation. The coverslips with cell suspension media were then placed in 5% CO_2 at 37°C for 3–4 hr to allow settling and attachment of cells. The coverslips with attached cells were then placed upside down in 60-mm-diameter Petri dishes containing a glia-conditioned medium and placed in a 5% CO_2 atmosphere at 37°C. After 2–3 d, a final concentration of 5×10^{-6} M cytosine arabinoside was added for 16 hr. Cultures were maintained by replacement of one-half volume of fresh N2-supplemented DMEM every 3–5 d for 19–22 d.

For micro-island cultures, cells were prepared as above and plated on 18 mm coverslips that had been prepared by a modified method described previously by Segal (1991). The coverslips were coated with 0.2% Agarose, dried overnight under UV light, then sprayed with a misted solution of 1% poly-L-lysine in 1 M borate buffer, pH 8.5, and dried again. This was finally followed by overnight incubation with 10% horse serum (Invitrogen) in DMEM with 0.6% glucose and 26 mM $NaHCO_3$ before hippocampal culturing. The maintenance schedule of micro-island cultures was identical to that described above for other cultures.

Glial-conditioned medium was prepared as described by Banker and Goslin (1998). Briefly, astroglia were prepared by tryptic dissociation of postnatal day 0 (P0) rat cortex and plated at a density of 300,000 cells/5 ml of DMEM with 0.6% D-glucose, 26 mM $NaHCO_3$, and 10% horse serum on poly-L-lysine-coated 35 mm Petri dishes. Glial cultures were maintained until they reached 80–100% confluence (10–14 d). Two days before hippocampal culture, media was fully exchanged with DMEM containing N2 supplement (Invitrogen), 1% D-glucose, 0.1% ovalbumin, 1 mM sodium pyruvate, and 26 mM sodium bicarbonate.

Fluorescence immunocytochemistry. Triple-label immunofluorescence detection of $GABA_A$R subunit isoforms, gephyrin, and GAD was conducted by fixing 19–22 d *in vitro* (DIV) cultured hippocampal neurons in a PBS solution containing 4% paraformaldehyde and 4% sucrose for 15 min at room temperature followed by permeabilization with 0.25% Triton X-100 in PBS. Nonspecific antibody labeling was minimized by treatment with 5% donkey serum in PBS for 30 min at room temperature. Primary antibodies were diluted in 0.25% Triton X-100 PBS and then applied to coverslips, followed by incubation for 2 hr at room temperature. To ensure the specificity of the various $GABA_A$R subunit isoform antibodies in the triple-label fluorescence immunocytochemistry, control conditions were examined in which each primary anti-$GABA_A$R subunit antibody was incubated for 30 min with 20 μg/ml of the corresponding peptide sequence, before incubation with cultures. Specificity was also demonstrated from the data presented in which some receptor clusters were labeled with some subunit isoform-specific antibodies but not others. After incubation with primary antibodies, cultures were twice washed for 5 min with PBS, followed by application of secondary antibodies raised in donkey and conjugated to Texas Red, FITC, or aminomethylcoumarin fluorophores (1:150 dilution in 0.25% Triton X-100 PBS; Jackson Immunochemicals) for 1 hr at room temperature. The coverslips were again twice washed with PBS for 5 min each and mounted using Prolong anti-fade mounting solution (Molecular Probes, Eugene, OR). For surface labeling of receptor subunits, cultures were fixed as above, followed by antibody incubations and washes without Triton X-100.

Exo-endocytotic assay. An exo-endocytotic assay was performed for labeling the recycling synaptic vesicles in living neurons according to a modification of a previously described method (Matteoli et al., 1992; Kraszewski et al., 1995; Bacci et al., 2001). Briefly, 3- and 4-week-old neuronal cultures were incubated with a rabbit antibody to the N-terminal domain of Syt-N in N-2-supplemented DMEM in a 5% CO_2 atmosphere or 15 mM HEPES-DMEM, pH 7.2, for 1 hr at 37°C. After incubation with the primary antibody, coverslips containing the cultured neurons were washed twice for 5 min with the same medium at 37°C. The neurons were fixed with PBS containing 4% paraformaldehyde and 4% sucrose for 15 min at room temperature followed by permeabilization with 0.25% Triton X-100 in PBS and incubation with sheep anti-GAD, and guinea pig anti-γ_2 or mouse anti-gephyrin, for triple-label immunofluorescence, as described above. To ensure that Syt-N labeling at 37°C was caused by the exposure of the synaptotagmin N-terminal epitope by exo-endocytotic activity, neurons were incubated with the Syt-N antibody at 4°C in N-2-supplemented 15 mM HEPES-DMEM, pH 7.2. Coverslips were washed twice for 5 min at 4°C with the same medium, then fixed and incubated with guinea pig anti-γ_2 and sheep anti-GAD antibodies, as indicated above.

Image acquisition and analysis. Images were collected using a 60× pan-fluor objective on a Nikon Eclipse T300 microscope with a Sensys KAF 1401E CCD camera, driven by IPLab 3.0 (Scanalytics, Fairfax, VA) acquisition software. Image files were then processed and merged for color colocalization figures using PhotoShop 4.01 (Adobe). Control slides in which one or more primary antibodies were omitted showed no spillover in the other two fluorescence channels. Random drift of the fluorescence signal of the sample between channels was controlled by alignment of all channels using triple-labeled fluorescent microspheres (0.1 and 0.4 μm diameter; Molecular Probes). Quantification of colocalized signal was performed by normalizing intensity data between fluorophore channels followed by the subtraction of background fluorescence signal seen in the dendrites. The two or three color channel images to be compared were merged, and $GABA_A$R clusters were counted over a 50 μm section of dendritic shaft and compared for colocalization or juxtaposition to other clusters. To determine the mean and SE for each condition, a minimum of 15 measurements were made of randomly selected dendrites from pyramidal neurons that showed limited GABAergic innervation in different areas of the coverslip. For experiments requiring matched dendrites within the same neuron, two dendrites were selected on the basis of dendrite thickness, one with GABAergic innervation and one without. Quantitation of the density of large and small clusters along 50 μm segments was performed as above.

Measurements of cluster size, area, and average fluorescence intensity were performed using IPLabs 3.0 software. Twelve-bit images (4096 grayscale intensity levels) were segmented, on the basis of fluorescence intensity levels, to create a binary mask that maximized the number of clusters for analysis. For comparisons of small and large clusters, data were collected from different areas of the same neuron to eliminate bias between neuronal samples.

RESULTS

Two types of $GABA_A$R clusters are present in hippocampal neurons that receive GABAergic innervation: GABAergic innervation induces the formation of large $GABA_A$R clusters located at GABAergic synapses; small $GABA_A$R clusters are not associated with GABAergic innervation

The expression and clustering of $GABA_A$R subunits and gephyrin in relationship to GABAergic innervation were examined in 19–23 DIV cultured neurons by using triple-label immunofluorescence with combinations of $GABA_A$R subunit isoform-specific antibodies in conjunction with an anti-gephyrin antibody and antibodies to GABAergic presynaptic markers such as GAD, VGAT, and GAT-1. We have included gephyrin in this study because it has been shown that in cultured hippocampal and spinal motor neurons gephyrin forms clusters that frequently colocalize with $GABA_A$R clusters in GABAergic synapses (Craig et al., 1996; Levi et al., 1999). Gephyrin also colocalizes with

$GABA_A$Rs in the intact brain, retina, and spinal cord (Levi et al., 1999; Fischer et al., 2000; Sassoe-Pognetto et al., 2000). Additionally, it has been proposed that gephyrin is involved in the postsynaptic clustering of $GABA_A$Rs (Essrich et al., 1998; Kneussel et al., 1999), although no direct binding of $GABA_A$Rs to gephyrin has been demonstrated and other proteins might also be involved in $GABA_A$R clustering (Knuesel et al., 1999, 2001; Wang et al., 1999; Fischer et al., 2000; Kneussel et al., 2000, 2001).

Two types of receptor clusters were observed with the various *GABA*$_A$R subunit-specific antibodies used in this study, such as α_1, $\beta_{2/3}$, γ_2 (Fig. 1A,*D*,*G*,*H*,*J*,*K*), α_2, α_3 (Fig. 2*B*,*E*), β_1, and β_2 (Fig. 3*A*,*D*): (1) large $GABA_A$R clusters [1.62 ± 0.08 (SEM) μm breadth (range = 0.65–5.5 μm); 1.14 ± 0.09 μm^2 area (range = 0.15–7.83 μm^2); n = 136 clusters] that colocalized with GAD-containing boutons (Fig. 1*A–C*; Figs. 1*D–L*, 2*A–F*, 3*A–F*, *arrows*) at GABAergic synapses and (2) smaller $GABA_A$R clusters [0.36 ± 0.01 μm diameter (range = 0.2–0.65 μm); 0.09 ± 0.01 μm^2 area (range = 0.02–0.26 μm^2); n = 197 clusters] that did not colocalize with the GAD-containing boutons (Figs. 1*D–L*, 2*A–F*, 3*A–F*, *filled arrowheads*). In addition to a greater size of the $GABA_A$R clusters colocalizing with GABAergic contacts, there was a greater average fluorescence intensity (1565 ± 21 intensity units per pixel; n = 136) when compared with extrasynaptic clusters present in other areas of the same neuron (1178 ± 9 intensity units per pixel; n = 197 clusters; $p < 0.001$), indicating that the large clusters have a higher receptor density than the smaller clusters. Often, the largest postsynaptic $GABA_A$R clusters of pyramidal neurons were composed of several smaller clusters (Fig. 1*D*).

The GAD-containing endings also contained the synaptic vesicle GABA transporter VGAT (Fig. 4*A–C*) and the presynaptic membrane GABA transporter GAT-1 (data not shown). Thus, the existence of a complete set of presynaptic and postsynaptic GABAergic elements plus the demonstrated existence of functional GABAergic synapses in these cultures, as shown by electrophysiological techniques (Segal and Barker, 1984; Jensen et al., 1999), indicated that the observed GABAergic innervation of pyramidal cells by the axonal endings from interneurons established functional synapses at the points where the presynaptic and postsynaptic elements concentrated and converged. We further demonstrated that this is the case by showing that the studied GABAergic contacts have exo-endocytotic activity of synaptic vesicles. For this purpose we have used an antibody (Syt-N) to the lumenal N-terminus domain of the synaptic vesicle protein synaptotagmin (Fig. 4*E*). In this assay (Matteoli et al., 1992; Bacci et al., 2001) at 37°C, only the functional presynaptic terminals undergoing exo-endocytosis of synaptic vesicles became labeled with the Syt-N antibody. This labeling does not occur when the cultures are exposed to the antibody at 4°C, a temperature at which synaptic vesicle fusion with the presynaptic membrane did not occur (Fig. 4*H*). Additionally, when the antibody recognizing the cytoplasmic protein gephyrin was incubated together with the Syt-N antibody at 37°C, there was labeling of active synapses by the Syt-N antibodies without detectable labeling of gephyrin clusters (data not shown), indicating that cytoplasmic antigens that do not become exposed to the cell surface are not immunolabeled. Therefore, Figure 4, D–F, shows that labeling of recycling synaptic vesicles occurs in the GAD-containing terminals and colocalizes with the large postsynaptic $GABA_A$R clusters.

Almost every neuron examined (175 of 177) expressed clusters of both the γ_2 subunit-containing $GABA_A$R and gephyrin (Fig. 1*A–F*). Moreover, 94.7% of all γ_2 subunit-containing $GABA_A$R clusters colocalized with gephyrin clusters of identical size and shape, and 90.0% of gephyrin clusters colocalized with γ_2 (Fig. 1*D*,*E*; see Table 2). The size, distribution, and density of clusters per cell varied depending on the neuron type and total amount of innervation present. The two types of clusters were found in pyramidal cells and interneurons, although pyramidal neurons typically had a higher density of clusters than did GAD-positive interneurons.

Surface labeling of $GABA_A$R clusters under nonpermeabilizing conditions with antibodies raised against extracellular N-terminus epitopes of α_1, α_3, $\beta_{2/3}$, and γ_2 showed that both the synaptic clusters and the small clusters were surface expressed. Under the same nonpermeabilizing conditions, antibodies to the intracellular proteins gephyrin and GAD or to the cytoplasmic epitopes of $GABA_A$R showed no immunolabeling. Intracellular proteins and epitopes were labeled only after the fixed cells were permeabilized with 0.25% Triton X-100. These experiments showed that in the nonpermeabilizing conditions only the external $GABA_A$R epitopes were accessible to the antibodies. Therefore, the immunolabeling of the small $GABA_A$R clusters obtained under nonpermeabilizing conditions indicated that these $GABA_A$R clusters (that were not associated with GABAergic synapses) were located at the cell surface and not in trafficking internal vesicles.

We have found that small $GABA_A$R clusters and gephyrin clusters are already present at 3.5 DIV (the earliest time studied) within 2–4% of neurons. All cells that had clusters of $GABA_A$R also had colocalized gephyrin clusters, and vice versa. At this early time point, GAD expression was very low, and therefore we could not determine whether any of the clusters were associated with GABAergic innervation. Nevertheless, immunolabeling with synaptic vesicle markers synaptophysin and SV2 suggested that $GABA_A$R clusters were frequently localized to sites of presynaptic contacts (data not shown). This result and the observed presence of $GABA_A$R clusters in single-cell cultures of glutamate neurons apposed to autaptic glutamate containing terminals shown by Rao et al. (2000) and confirmed in our laboratory (data not shown) indicate that small $GABA_A$R clusters can form in the absence of any GABAergic innervation, although they are frequently associated with other presynaptic contacts.

Individual hippocampal pyramidal cells and interneurons in culture form clusters of $GABA_A$Rs containing various $GABA_A$R subunits and isoforms

Mammalian brain $GABA_A$Rs are pentameric proteins composed of combinations of various subunit classes and isoforms (α_{1-6}, β_{1-3}, γ_{1-3}, δ, ϵ, and θ) and known splice variants (i.e., γ_2 long and γ_2 short forms) (for review, see Barnard et al., 1998; Mehta and Ticku, 1999; Whiting et al., 1999). The most common $GABA_A$R subunit combination found in the brain contains two α subunits, two β subunits, and one γ subunit (Im et al., 1995; Chang et al., 1996; Li and De Blas, 1997; Jechlinger et al., 1998; Farrar et al., 1999), although combinations of two α, one β, and two γ subunits also occur in the brain (Backus et al., 1993; Khan et al., 1994a,b, 1996).

We have investigated the possible heterogeneity of $GABA_A$R subunit isoform expression in both the pyramidal cells and interneurons. We observed that although most pyramidal cells and interneurons expressed the γ_2 subunit (98.9%) and $\beta_{2/3}$ subunits (99.1%), the α subunit isoforms were not expressed in all cells. Thus, α_1-containing clusters were present in 69.6% of pyramidal and 73.5% of interneurons, α_2 clusters were present in 95.4% of

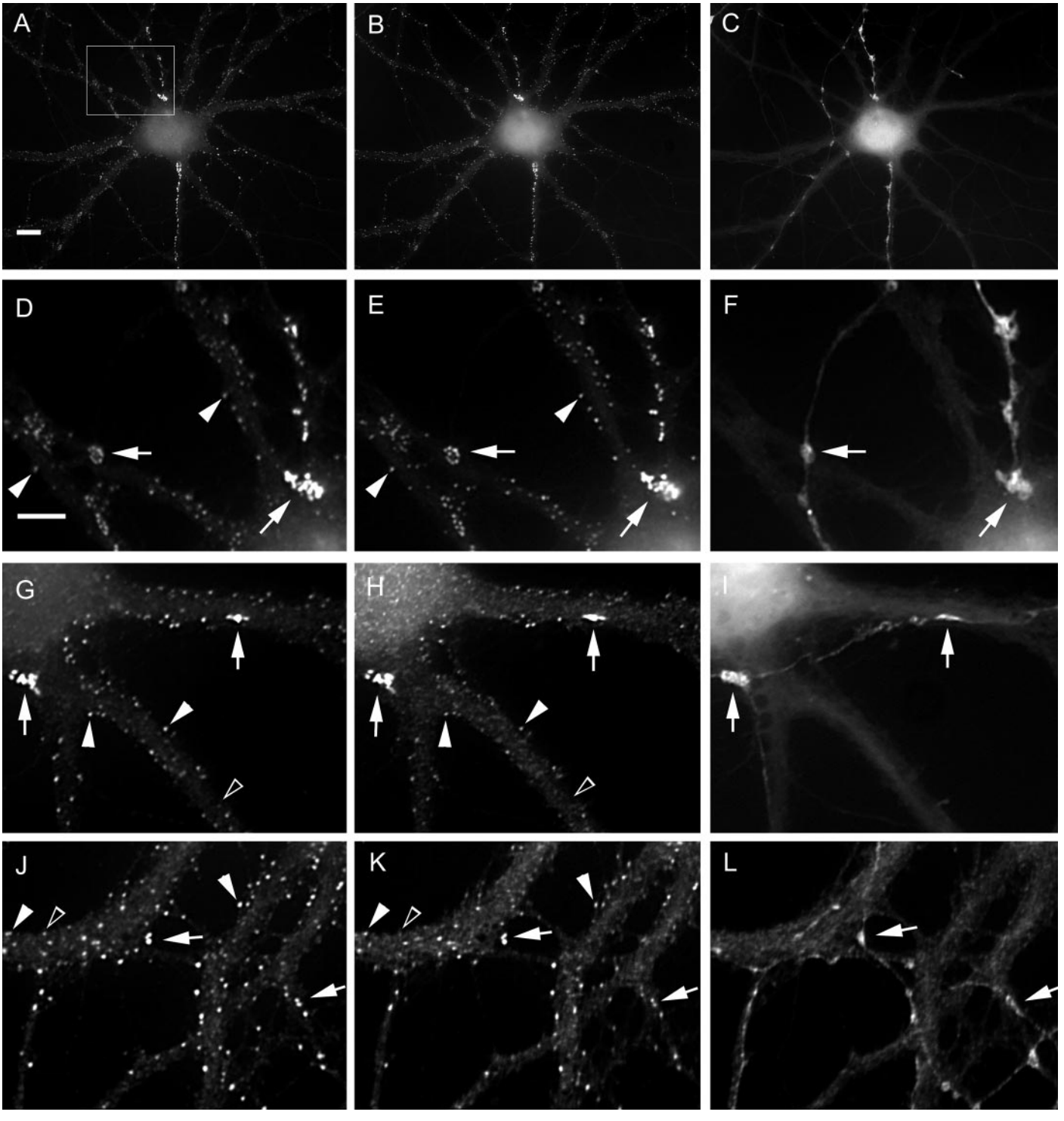

Figure 1. GABAergic innervation induces the formation of large $GABA_AR$ clusters at GABAergic synapses. Smaller $GABA_AR$ clusters are also present outside GABAergic synapses. Hippocampal neurons after 19–22 d in culture were immunolabeled with the rabbit anti-$GABA_A$ receptor subunit γ_2 (*A*, *D*, *G*, *J*), mouse monoclonal anti-gephyrin (*B*, *E*), sheep anti-GAD (*C*, *F*, *I*, *L*), guinea pig anti-α_1 (*H*), or mouse monoclonal anti-$\beta_{2/3}$ (*K*). *D–F* show at high magnification the fields in *A–C* corresponding to the *boxed area* in *A*, respectively. Large clusters of $GABA_A$ receptors (*D*, *G*, *H*, *J*, *K*, *arrows*) and gephyrin (*E*, *arrows*) colocalize with GAD-positive boutons (*F*, *I*, *L*, *arrows*), whereas small clusters of $GABA_ARs$ (*D*, *G*, *H*, *J*, *K*, *filled arrowheads*) colocalize with small clusters of gephyrin (*E*, *filled arrowheads*) but not with GAD boutons. A number of small $GABA_AR$ clusters not colocalizing with GAD contained only one of the two subunit classes (*G*, *H*, *J*, *K*, *empty arrowheads*). Scale bar (shown in *A*): *A–C*, 10 μm; (shown in *D*): *D–L*, 5 μm.

pyramidal neurons and 91.0% of interneurons, and α_3 was present in 53.5% of pyramidal neurons and 65.8% of interneurons. Often, more than one α subunit isoform was expressed by the same neuron (Table 1).

It is worth noting that the intensity level of fluorescent signal in the receptor clusters for a particular subunit varied within and between pyramidal cells and interneurons. Thus, many of the interneurons shared very high expression levels of α_1 and β_2,

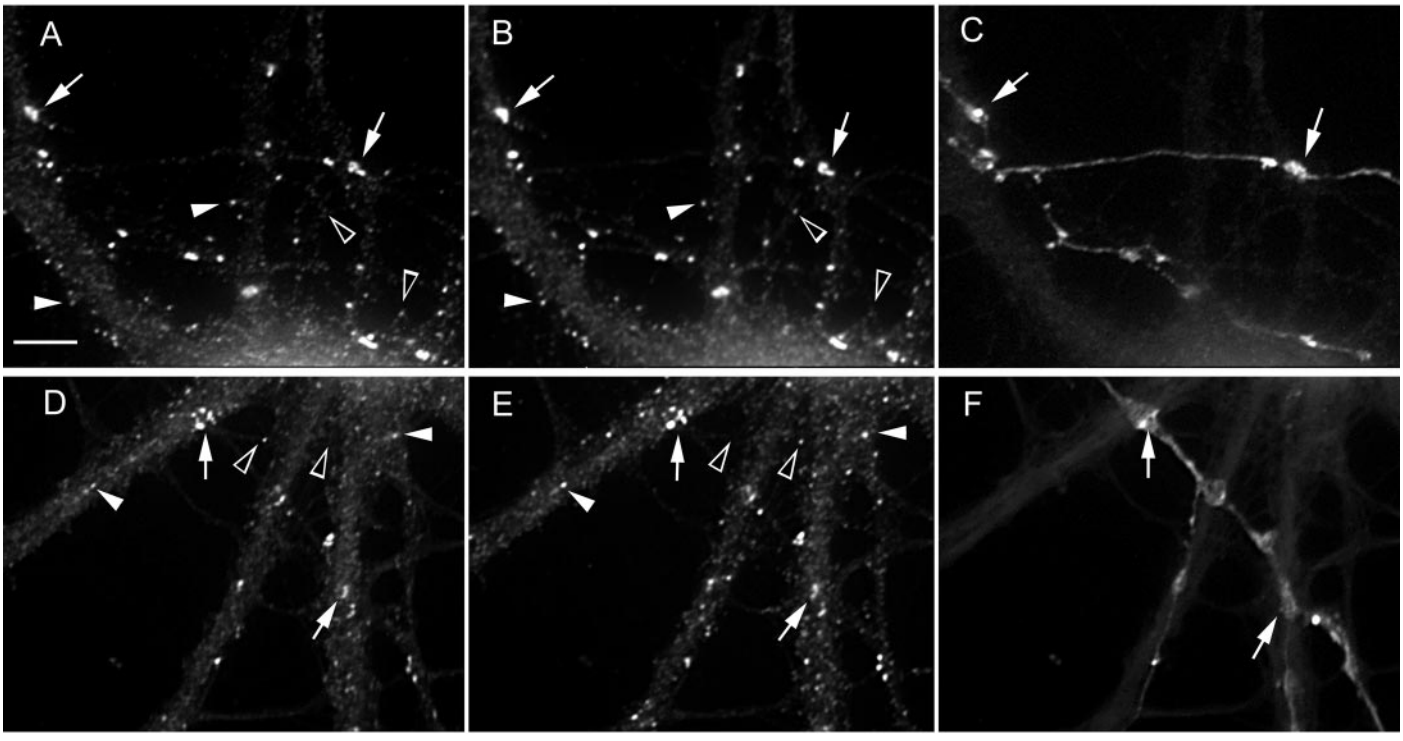

Figure 2. Various α subunit isoforms of the $GABA_AR$ colocalize in all large GABAergic synaptic clusters, but not in all smaller non-GABAergic clusters. Hippocampal pyramidal neurons were triple labeled with the $GABA_A$ subunit isoform-specific antibodies guinea pig anti-α_1 (*A*, *D*), rabbit anti-α_2 (*B*), or rabbit anti-α_3 (*E*) in conjunction with sheep anti-GAD (*C*, *F*). All of the larger GAD-colocalizing $GABA_AR$ clusters (*A*, *B*, *D*, *E*, *arrows*) and many smaller clusters (*A*, *B*, *D*, *E*, *filled arrowheads*) that do not colocalize GAD show the presence of the two α subunit isoforms. However, a significant population of the smaller clusters contained only one of the two α−subunit isoforms (*A*, *B*, *D*, *E*, *empty arrowheads*). Neurons were cultured for 19 d. Scale bar (shown in *A*): 5 μm.

consistent with our previous observations in the intact hippocampus (Miralles et al., 1999). The α_1 subunit was also highly expressed by a subset of pyramidal neurons. The α_3 subunit also showed a degree of variability in expression, with stronger signal levels seen in some interneurons and pyramidal neurons. Less variability was found in the expression of the α_2, β_1, $\beta_{2/3}$, or γ_2 subunits or gephyrin, because they were fully expressed by most pyramidal cells and interneurons (Table 1) (data not shown). By using single-cell PCR, others have shown that individual hippocampal neurons frequently express several α and β subunit isoform mRNAs (Brooks-Kayal et al., 1998). The aforementioned data demonstrate that this isoform heterogeneity in individual hippocampal neurons is also observed at the protein level. It is also worth mentioning that with the exception of α_3, the other studied subunits are highly expressed in the intact hippocampus (Fritschy and Möhler, 1995; Sperk et al., 1997; Miralles et al., 1999; Christie et al., 2002). However, in the intact hippocampus, the α_3 subunit is expressed at very low levels, as shown with various antibodies, including the one used in the present study. Therefore, the cultured hippocampal neurons show upregulation of the α_3 subunit expression.

The immunofluorescence labeling of the $GABA_AR$ clusters with subunit-specific antibodies represents the labeling of the complete and fully assembled $GABA_AR$ pentamers. (1) All the subunits that are necessary to form complete receptors (i.e., α, β, and γ) colocalize in the synaptic and extrasynaptic clusters (Figs. 1–3). (2) It has been shown that individual subunits or incomplete $GABA_ARs$ are retained within the endoplasmic reticulum and are quickly degraded (Connolly et al., 1996; Taylor et al., 1999). (3) Only fully assembled receptor pentamers containing α and β or α, β, and γ subunits reach the cell surface (Connolly et al., 1996, 1999; Gorrie et al., 1997). In the absence of other subunits, β_3 can form homopentamers that can be transported to the cell surface (Wooltorton et al., 1997; Taylor et al., 1999). However, this is unlikely to occur in the hippocampal cultures because various α isoforms and the γ_2 subunit are also coexpressed by these cells. (4) The $GABA_AR$ clusters studied in the present communication are localized at the cell surface, because they are labeled by antibodies to external epitopes in nonpermeabilized cells as shown above, and (5) the data in the literature show that these cultured neurons express benzodiazepine-sensitive functional $GABA_ARs$ (Segal et al., 1984; Jensen et al., 1999). The latter require the formation of pentamers and the presence of α, β, and γ_2 subunits. The α and β subunits are necessary for GABA binding, whereas the α and γ_2 subunits are necessary for benzodiazepine binding.

There is no segregation of $GABA_AR$ subunit isoforms to individual GABAergic synaptic clusters; however, there is partial segregation of $GABA_AR$ subunit isoforms in the small $GABA_AR$ clusters found outside GABAergic synapses

We have investigated the possibility that $GABA_ARs$ with different subunit composition might be targeted to different synapses as has been reported with the α_2 subunit in the intact hippocampus (Nusser et al., 1996a; Nyiri et al., 2001). For this purpose, we examined the colocalization of various $GABA_AR$ subunits and isoforms in the receptor clusters of the cells that express two

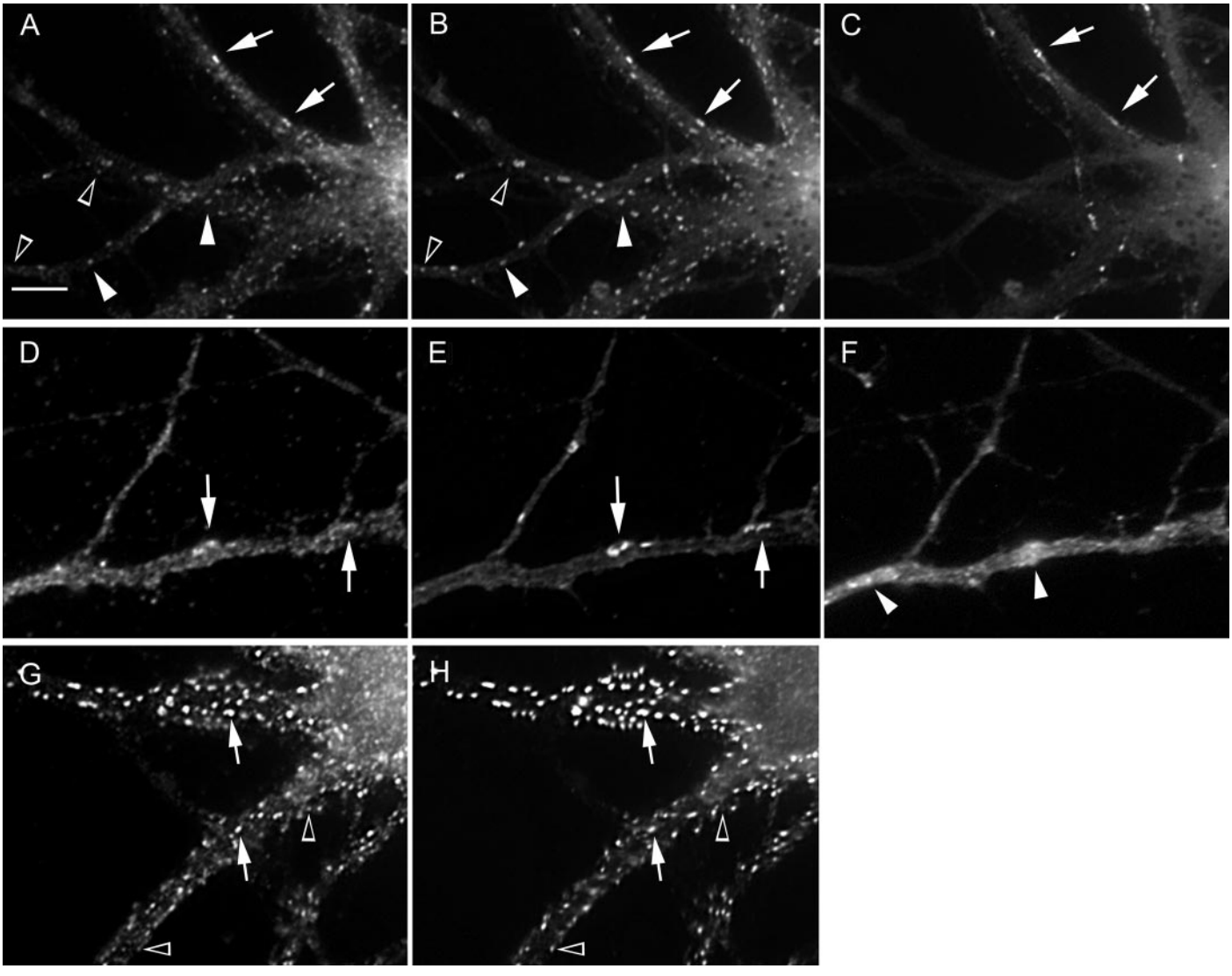

Figure 3. Various β subunit isoforms of the $GABA_AR$ colocalize in all large GABAergic synaptic clusters and most small non-GABAergic clusters. Cultured hippocampal neurons were triple labeled with the $GABA_AR$ subunit isoform-specific antibodies rabbit anti-β_1 (*A*), mouse monoclonal (62–3G1) anti-$\beta_{2/3}$ (*B*, *E*, *H*), rabbit anti-β_2 (*D*), and rabbit anti-β_3 (*G*) in conjunction with sheep anti-GAD (*C*, *F*). All of the larger GAD-colocalizing $GABA_AR$ subunit isoform clusters (*A*, *B*, *D*, *E*, *arrows*) and many smaller clusters of β_1-IL and $\beta_{2/3}$ (*A*, *B*, *filled arrowheads*) or β_3 and $\beta_{2/3}$ (*G*, *H*, *arrows*) that do not colocalize GAD show colocalization of the two β-subunit isoforms. β_2 expression was observed only within GAD-positive interneurons (*F*, *filled arrowheads*). Some small $GABA_AR$ clusters contained only one of the two β-subunit isoforms examined (*A*, *B*, *G*, *H*, *empty arrowheads*). Neurons shown in *A–F* were cultured for 19 d; neuron in *G* and *H* was cultured for 28 d. *A–C*, *G*, and *H* show the processes of pyramidal neurons; *D–F* show the processes of an interneuron. Scale bar (shown in *A*): 5 μm.

isoforms. We observed complete colocalization of the α_1 and α_2 (Fig. 2*A–C*, *arrows*), α_1 and α_3 (Fig. 2*D–F*, *arrows*), β_1 and $\beta_{2/3}$ (Fig. 3*A–C*, *arrows*), and α_1 and γ_2 (Fig. 1*G–I*, *arrows*) subunit isoforms in all (100%) the large GABAergic synaptic clusters examined (i.e., colocalizing with GAD boutons) (Table 2). Therefore, in these cultures we have found no evidence for the segregation of receptors containing different isoforms of α or β subunits to different GABAergic synapses on the same pyramidal cell.

Regarding the small $GABA_AR$ clusters not associated with GAD, Figures 1–3 and Table 2 show that there was a high degree of colocalization of the various $GABA_AR$ subunits and isoforms with each other and with gephyrin. However, within the same cell there is also a significant proportion of small clusters that show one or the other isoform but not both (Figs. 1*G*,*H*,*J*,*K*; 2*A*,*B*,*D*,*E*; 3*A*,*B*,*G*,*H*, *empty arrowheads*). This result contrasts with the complete (100%) colocalization of all the studied subunits and isoforms found within the large clusters at GABAergic synapses. Thus, in neurons that express both isoforms, α_1 and α_2 colocalize in 58.4% of all the small clusters, whereas the remaining 41.6% of the small clusters had only α_1 or α_2 but not both. For α_1 versus α_3, 52.7% of all small clusters contained both isoforms, and the remaining 47.3% had only α_1 or α_3 (Table 2). These results showed that in pyramidal neurons that expressed two α subunit isoforms, 25.5–30.8% of the small clusters contained only one of the two. Therefore, in some of the small extrasynaptic $GABA_AR$ clusters, there is partial segregation of the $GABA_ARs$ that contain different α subunit isoforms. This observation is consistent with the notion that the pyramidal cells express a population of $GABA_ARs$ in which the two α subunits present in the pentamer

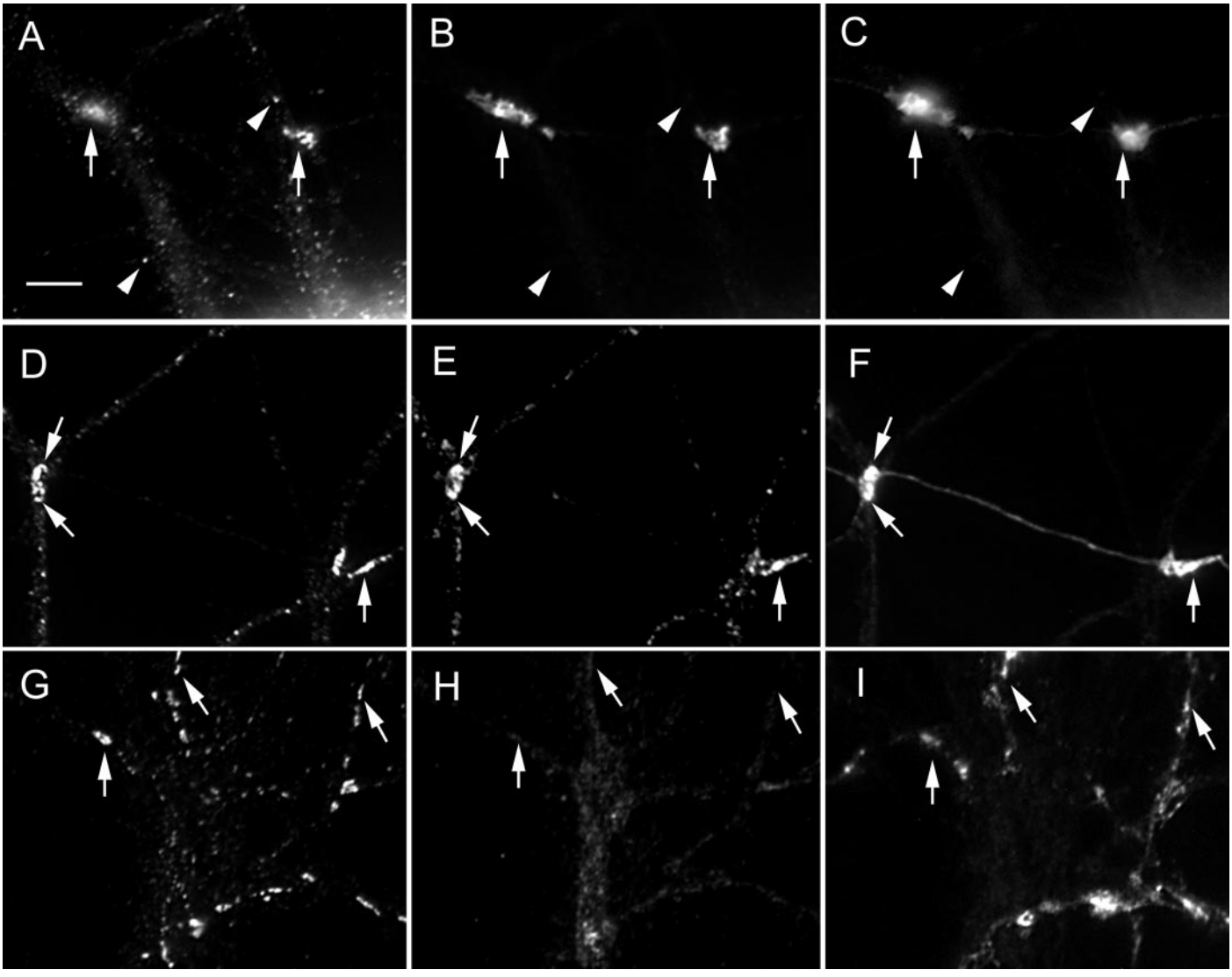

Figure 4. Large clusters of $GABA_AR$ accumulate postsynaptically to active GABAergic presynaptic boutons and show exo-endocytosis of synaptic vesicles. Hippocampal cultures were triple labeled with guinea pig anti-γ_2 subunit (*A*), rabbit anti-VGAT (*B*), and sheep anti-GAD (*C*) antibodies. VGAT and GAD concentrate presynaptically in the interneuronal varicosities (*A*, *B*, *arrows*) contacting a pyramidal neuron and colocalize with large postsynaptic clusters of the γ_2 subunit-containing $GABA_ARs$ (*arrows*) but do not colocalize with the small clusters of $GABA_ARs$ (*A–C*, *filled arrowheads*). In live cell labeling conditions for the synaptic vesicle exo-endocytotic assay (*D–I*), active synapses were labeled with the Syt-N anti-synaptotagmin antibody at 37°C (*E*, *arrows*). However, when live cells were incubated at 4°C (to prevent exocytosis and endocytosis), no labeling of synaptotagmin with the Syt-N antibody was observed (*H*, *arrows*). After live cell labeling with the Syt-N antibodies, cells were fixed and incubated with guinea pig anti-γ_2 subunit and sheep anti-GAD to reveal the localization of large $GABA_AR$ clusters (*D*, *G*, *arrows*) and GAD-containing presynaptic terminals (*F*, *I*, *arrows*). Note that the Syt-N labeling of active synapses occurs in GAD-containing terminals and colocalizes with large $GABA_AR$ clusters (*D*, *E*, *F*, *arrows*). Neurons in *A–C* were cultured for 21 d, and neurons in *D–I* were cultured for 28 d. Scale bar (shown in *A*): 5 μm.

are of the same isoform. Nevertheless, these cells are also very likely to have pentamers that contain two different α subunit isoforms (McKernan et al., 1991; Khan et al., 1994a, 1996; Araujo et al., 1996, 1999; Jechlinger et al., 1998; Sigel and Baur, 2000). We do not know whether the small clusters that show colocalization of α_1 and α_2 or α_1 and α_3 have receptors that contain both isoforms or a mixture of receptors containing only one type of isoform. Immunocytochemistry lacks the resolution to address this issue.

The colocalization of α_1 with γ_2 in the small clusters was higher than that of α_1 with either α_2 or α_3. Thus, α_1 with γ_2 colocalized in 68.7% of all small non-GABAergic clusters (Fig. 1*G*,*H*, *filled arrowheads*), with only 14.1% of the small α_1 receptor clusters not colocalized with γ_2. The highest level of colocalization was observed between γ_2 and gephyrin (Table 2), in which 86.0% of all small non-GABAergic clusters had both γ_2 subunit and gephyrin. In this case, 94.7% of all γ_2 clusters had gephyrin, and 90.0% of the gephyrin clusters had γ_2 (Table 2). Some of the gephyrin clusters that did not have γ_2 might have γ_3 (Baer et al., 1999), because pyramidal neurons also express this subunit (Wisden et al., 1992).

We also examined the aggregated colocalization of the α_1, α_2, and α_3 (α_{1-3}) subunits with $\beta_{2/3}$ and gephyrin in the small $GABA_AR$ clusters not associated with GABAergic synapses. We found that in 64.8 and 66.2% of all small clusters, α_{1-3} subunits were colocalized with $\beta_{2/3}$ and gephyrin, respectively (Table 2).

Table 1. Expression of α subunit isoforms by hippocampal neurons in culture

Pyramidal neurons expressing		Sample size: number of neurons (cultures)	Interneurons expressing		Sample size: number of neurons (cultures)
α_1	69.6 ± 2.5%	573 (4)	α_1	73.5 ± 2.9%	212 (6)
α_2	95.4 ± 2.4%	630 (3)	α_2	91.0 ± 2.2%	84 (3)
α_3	53.5 ± 13.8%	799 (3)	α_3	65.8 ± 1.9%	117 (3)
α_1 and α_2	70.9%	285 (2)	α_1 and α_2	75.0%	68 (2)
α_1 and α_3	36.7%	417 (2)	α_1 and α_3	46.5%	99 (2)

Hippocampal neurons were cultured for 19–22 d, followed by immunolabeling with guinea pig anti-α_1, sheep anti-GAD, and rabbit anti-α_2 or rabbit anti-α_3 (see Materials and Methods). Interneurons were identified by GAD labeling. Values represent the percentage ± SEM of observed neurons that express clusters for a particular α subunit isoform or the percentage of total neurons expressing clusters of the two subunit isoforms in the same cell. Values in parentheses are the number of cultures sampled for each subunit or subunit combination.

Table 2. Colocalization of $GABA_AR$ subunit isoforms in GABAergic synaptic and extrasynaptic clusters in cultured pyramidal neurons

	% Colocalization						
	GABAergic synaptic clusters	Non-GABAergic clusters					
Subunit isoform	(1)	(2)	(3)	(3)	Number of clusters	Number of dendrites	Number of neurons (4)
α_1 versus α_2	100	58.4 ± 6.4	α_1: 73.1 ± 2.2	α_2: 74.5 ± 2.3	1705	27	10
α_1 versus α_3	100	52.7 ± 4.5	α_1: 69.2 ± 2.2	α_3: 73.0 ± 2.1	1623	29	11
α_1 versus γ_2	100	68.7 ± 5.3	α_1: 85.9 ± 1.1	γ_2: 77.9 ± 1.9	1702	24	8
α_{1-3} versus $\beta_{2/3}$ (5)	100	64.8 ± 6.9	α_{1-3}: 78.9 ± 2.0	$\beta_{2/3}$: 77.4 ± 2.7	1136	17	6
α_{1-3} versus gephyrin (5)	100	66.2 ± 8.0	α_{1-3}: 83.8 ± 1.8	gephyrin: 75.6 ± 1.8	1699	21	7
γ_2 versus gephyrin	100	86.0 ± 7.8	γ_2: 94.7 ± 0.8	gephyrin: 90.0 ± 1.1	1477	22	8

(1) GABAergic synapses are identified by the colocalization of presynaptic GAD-positive terminal and $GABA_AR$ clusters in the postsynaptic area.

(2) Non-GABAergic clusters are the $GABA_AR$ clusters not colocalizing with GAD-containing terminals. Values (mean ± SEM) reflect the percentage of clusters that contain both subunit isoforms (i.e., α_1 and α_2) with respect to the total number of clusters containing at least one of the two subunits (i.e., α_1 or α_2 or both).

(3) Values represent the percentage of clusters that contains the subunit isoform that also contains a second isoform (i.e., 73.1% of all α_1-containing clusters also contain the α_2 subunit and 74.5% of all α_2-containing clusters also contain the α_1 subunit).

(4) Number of neurons from two independent cultures examined for each subunit or pair (three to five neurons per culture).

(5) α_{1-3} refers to clusters labeled after the application of a mixture of the three rabbit anti-α subunit antibodies (α_1, α_2, and α_3).

We also found that 78.9% of the small clusters that contained at least one of the three α subunits also had $\beta_{2/3}$. The α_{1-3} clusters that did not colocalize with $\beta_{2/3}$ clusters might contain β_1, which is also expressed by pyramidal cells of the hippocampus (see below) (Wisden et al., 1992). Table 2 also shows that 83.8% of clusters that contained at least one of the three α subunit isoforms examined colocalized with gephyrin clusters. Thus, the gephyrin clusters that did not show α_{1-3} immunoreactivity might have α_4 or α_5 subunit-containing receptors, because pyramidal neurons also express α_4 and α_5 (Wisden et al., 1992). The aforementioned results support the intimate relationship between the clustering of gephyrin and the clustering of $GABA_AR$s that contain γ_2 (Essrich et al., 1998; Kneussel et al., 1999). It is also worth noting that 5.3% of the small γ_2 clusters and 16.2% of α_{1-3} clusters did not contain gephyrin, which also supports the existence of a gephyrin-independent $GABA_AR$ clustering mechanism that operates within a small percentage of $GABA_AR$ clusters (Kneussel et al., 2001), as well as the existence of some gephyrin clusters that might not contain $GABA_AR$ clusters (Levi et al., 1999). The colocalization of the β_1 or β_3 subunits with the $\beta_{2/3}$ subunits was also compared in pyramidal neurons (Fig. 3*A*,*B*,*G*,*H*). We also found that large synaptic clusters at all GABAergic synapses contained all the β subunit isoforms expressed by that neuron. These β subunit isoforms also colocalized within the majority of the small extrasynaptic clusters (Fig. 3*A*,*B*, *filled arrowheads*), but not all (Fig. 3*A*,*B*,*G*,*H*, *empty arrowheads*).

GABAergic innervation not only induces an increase in the size of the $GABA_AR$ clusters at the GABAergic synapse, but it also leads to the disappearance of the small $GABA_AR$ clusters in the local area surrounding the GABAergic synapse

We have shown that GABAergic presynaptic inputs induce the formation of large postsynaptic $GABA_AR$ clusters at the contact sites (Figs. 1*A*,*D*, 5*A*). We have also observed that this effect is accompanied by a reduction in the density of the small $GABA_AR$ clusters in dendrites that receive GABAergic innervation. We examined 50-μm-long dendrite segments, each innervated by 4–10 GAD-containing boutons, and found that there was an average of 60.6 ± 5.7% reduction ($p < 0.001$; $n = 17$) in the density of small non-GABAergic synaptic $GABA_AR$ clusters, when compared with noninnervated dendrite segments of the same neurons. Thus innervated dendrites had 27.2 ± 4.3 $GABA_AR$ clusters (mean ± SEM) in 50 μm length, whereas noninnervated dendrites had 72.2 ± 7.8 clusters in 50 μm length. We also determined that this reduction in the density of small $GABA_AR$ clusters occurs locally in the proximity of individual GABAergic synapses. Figure 5*A* shows that there was a significant decrease in the density of small $GABA_AR$ clusters in the first 0–5 μm segment of dendritic length adjacent to a GABAergic synapse (1.4 ± 0.7 clusters/10 μm^2; $p < 0.005$), as well as in the 5–10 μm segment from the synapse (1.8 ± 0.4 clusters/10 μm^2; $p < 0.01$) and 10–15 μm segment from the synapse (3.3 ± 0.3 clusters/10 μm^2; $p < 0.05$) compared with a control average in noninner-

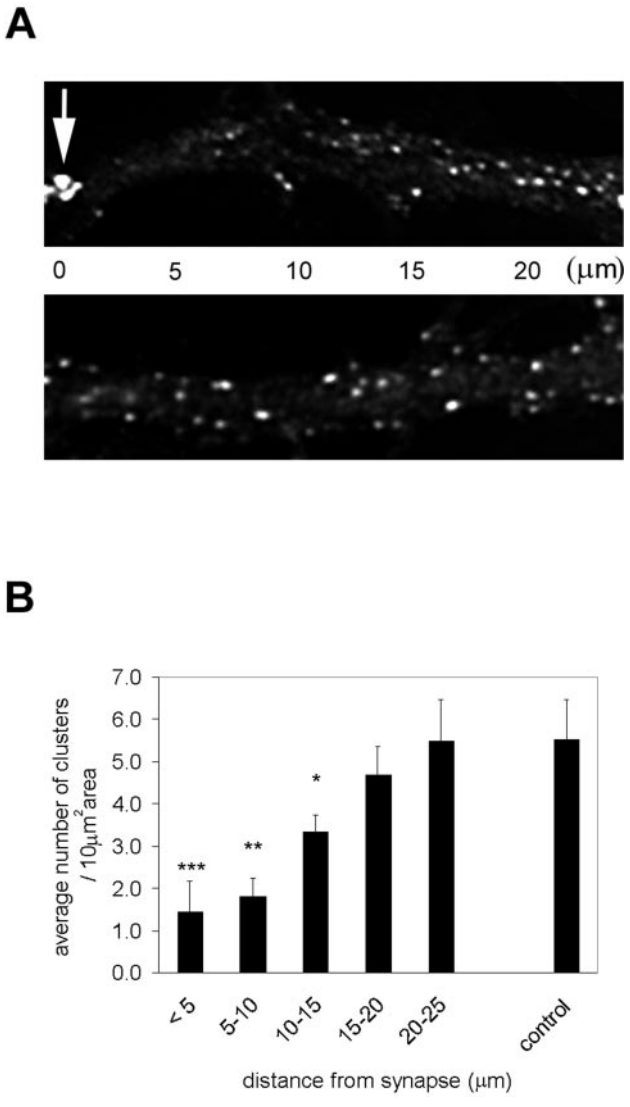

Figure 5. GABAergic innervation induces a reduction in the density of small clusters in dendritic areas adjacent to GABAergic synapses. *A*, Dendrite segments (25 μm long) from two dendrites from the same pyramidal neuron, one receiving GABAergic innervation (*top panel*) and another dendrite not receiving GABAergic innervation (*bottom panel*). $GABA_A$R clusters were visualized with a rabbit anti-γ_2 antibody. Note the presence of a large $GABA_A$R cluster (*arrow* in *top panel*) at the GABAergic synapse (identified by the colocalization of a GAD-containing bouton), and the lower density of small $GABA_A$R clusters in the adjacent area (noticeable up to 15 μm distance). *B*, The graph shows that the reduction of the density of small clusters is significant up to 15 μm from the synapse. Beyond 15 μm, the density of the clusters is similar to that of dendrites not receiving GABAergic innervation (*control*). Quantification of the average cluster density around the synapse was done in five 5 μm zones distal to the site of a GABAergic synapse and compared with non-GABAergic innervated dendritic areas of the same neuron in 22-d-old cultures. ($^{***}p < 0.005$, $^{**}p < 0.01$, $^{*}p < 0.05$; $n = 6$ matched dendrite pairs).

vated dendrites of 5.5 ± 1.1 clusters/10 μm^2 (Fig. 5*B*). Beyond 15 μm, there was no significant difference in the density of small $GABA_A$R clusters compared with control (15–20 μm segment: 4.7 ± 0.4/10 μm^2, $p = 0.23$; 20–25 μm segment: 5.5 ± 0.7/10 μm^2, $p = 0.49$).

We have also compared the density of small $GABA_A$R and gephyrin clusters in dendrites of single pyramidal neurons in micro-island cultures with those of dendrites from pyramidal cells in mixed cultures that receive limited GABAergic innervation from interneurons. There was no significant difference in the size or density of the small $GABA_A$R or gephyrin clusters between the dendrites of single pyramidal cells (that receive no GABAergic innervation anywhere) and the dendrites that receive no GABAergic innervation in pyramidal cells that receive GABAergic innervation in other dendrites (data not shown). These observations indicate that (1) the formation of small $GABA_A$R clusters is not dependent on GABAergic innervation and (2) GABAergic innervation produces a reduction in the density of small clusters. Moreover, this phenomenon is a local effect that is restricted to the neighborhood of the GABAergic synapse. Therefore, the formation of large $GABA_A$R clusters occurs exactly at the synaptic site, whereas the disappearance of the smaller clusters is a gradient effect extending to an average distance of 15–20 μm from the GABAergic synapse.

A population of small $GABA_A$R clusters associate with glutamatergic synapses in pyramidal neurons receiving both glutamatergic and GABAergic innervation

Recently, Rao et al. (2000) reported that microcultures of isolated glutamatergic pyramidal neurons, where no GAD-containing synapses were present, showed mismatched $GABA_A$R clusters localized postsynaptically to synaptic vesicle-containing terminals, presumably containing glutamate. We tested whether this was an anomalous situation that occurs only in the total absence of GABAergic innervation or if this also occurs in pyramidal cells that receive both GABAergic innervation from interneurons and glutamatergic innervation from themselves (autapses) and other pyramidal cells. We examined whether $GABA_A$R clusters are associated with the postsynaptic glutamate receptor markers PSD-95 (for NMDA receptors) (Fig. 6*A–C*) and GluR1 (for AMPA receptors) (Fig. 6*D–F*). We found that 33.2% of the small non-GABAergic $GABA_A$R clusters (19.5 ± 2.2 of 58.7 ± 7.3 total γ_2 clusters/50 μm length; $n = 14$ matched dendrites) were juxtaposed to PSD-95 clusters (Fig. 6*A*,*B*, *filled arrowheads*). Similarly, the association of $GABA_A$R $\beta_{2/3}$ or gephyrin clusters with AMPA receptor subunit GluR1 was also found (Fig. 6*D*,*E*, *filled arrowheads*). Although most of the large and small $GABA_A$R and gephyrin clusters were localized to the dendritic shaft, the association of the small $GABA_A$R or gephyrin clusters with GluR1 was particularly evident at the level of the dendritic spines, which were enriched in GluR1 immunoreactivity (Fig. 6*D*,*E*, *arrowheads*). We have also found that most (74.2 ± 9.1%) of the small $GABA_A$R clusters that did not colocalize with GAD-positive boutons were associated with the synaptic vesicle marker SV2 (Fig. 6*G–I*, *filled arrowheads*). Taken together, these results suggest that in pyramidal cells that receive GABAergic innervation, at least one-third of the small (non-GABAergic) $GABA_A$R clusters are associated with glutamatergic synapses. In addition, ~25% of small receptor clusters colocalized with neither SV2 nor GAD, suggesting that $GABA_A$R can also form small $GABA_A$ clusters in the absence of any GABAergic or glutamatergic synaptic contact (Fig. 6*G*,*H*, *empty arrowheads*).

GABAergic innervation induces the local disappearance of small $GABA_A$R clusters, including the ones associated with glutamatergic synapses, without affecting the organization of glutamatergic receptor clusters

The aforementioned local effect of GABAergic innervation on the disappearance of small $GABA_A$R clusters also applies to the

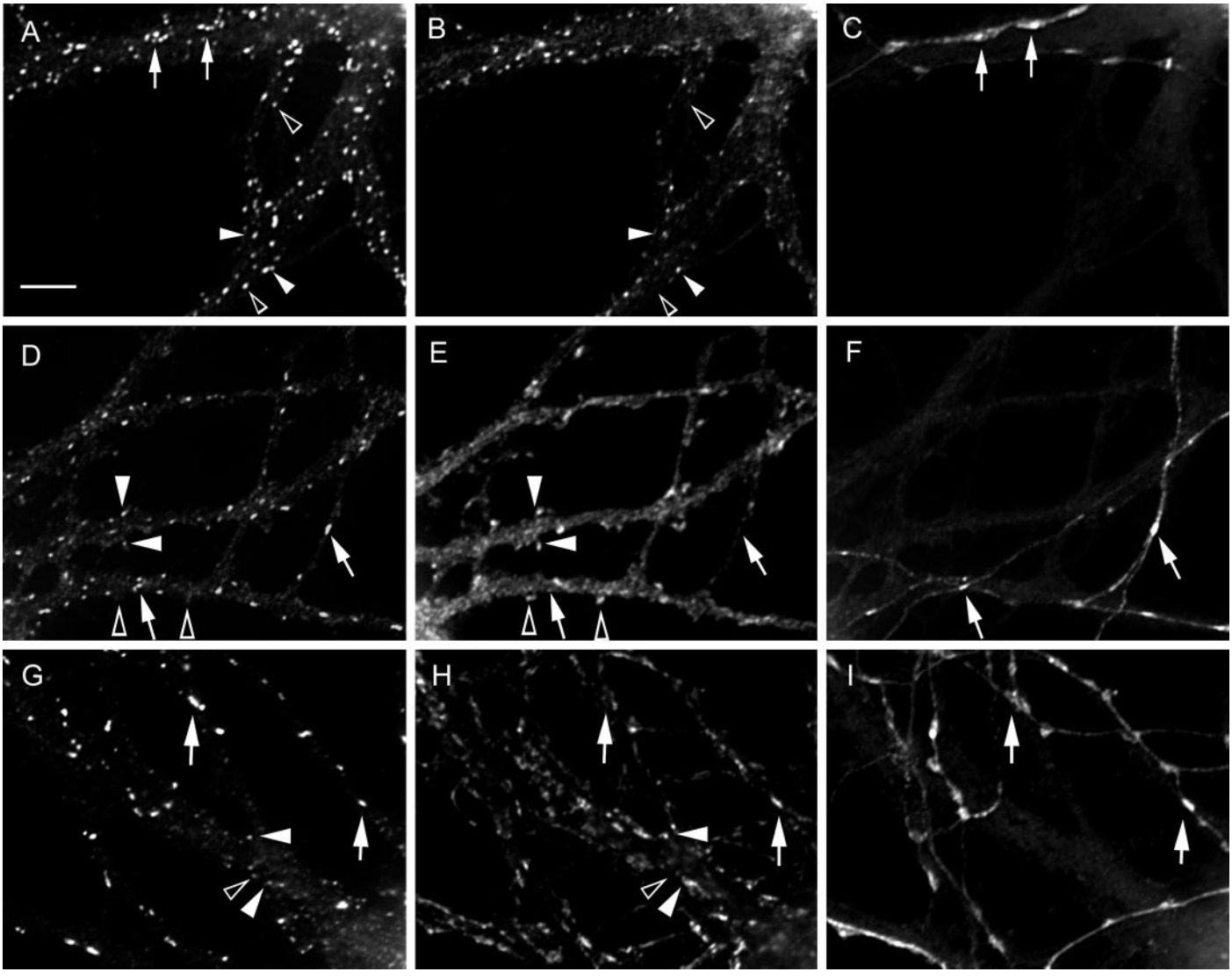

Figure 6. A population of small $GABA_AR$ clusters are associated with glutamatergic synaptic markers. GABAergic innervation reduces the local density of small $GABA_AR$ clusters that associate with glutamatergic synapses within pyramidal neurons. Hippocampal neurons were labeled with rabbit anti-γ_2 (*A*), mouse monoclonal anti-PSD-95 (*B*), mouse monoclonal anti-$\beta_{2/3}$ (*D*), rabbit anti-GluR1 (*E*), guinea pig anti-α_1 (*G*), and mouse monoclonal SV2 (*H*) in conjunction with GAD (*C*, *F*, *I*). The large $GABA_AR$ clusters colocalized with GAD and SV2 in GABAergic synapses (*A–I*, *arrows*). Some small $GABA_A$ subunit clusters that were not associated with GAD were associated with PSD-95 (*A*, *B*, *filled arrowheads*), GluR1 (*D*, *E*, *filled arrowheads*), and SV2 (*G*, *H*, *filled arrowheads*). Some small $GABA_AR$ clusters were not associated with glutamatergic markers (*A*, *B*, *G*, *H*, *empty arrowheads*). A comparison between processes of the same neuron (the *two lower horizontal dendrites* in *D–F*) shows that the association between small $GABA_AR$ clusters and GluR1 clusters in dendritic spines observed in the top dendrite that does not receive GABAergic innervation (*D*, *E*, *filled arrowheads*) is not seen in the GluR1-containing spines of the bottom dendrite that receives GABAergic innervation (*D*, *E*, *empty arrowheads*). Scale bar (shown in *A*): 5 μm.

small $GABA_AR$ clusters associated with glutamatergic synapses. After GABAergic innervation, the density of both the small $GABA_AR$ clusters associated with PSD-95 clusters and the ones not associated was decreased in the same proportion around the GABAergic synapse. This is also illustrated in Figure 6*D–F*, which shows the association of $GABA_AR$ clusters with GluR1 in two dendrites of the same pyramidal cell, one with GABAergic innervation (Fig. 6*D–F*, *empty arrowheads*) and another one without (Fig. 6*D–F*, *filled arrowheads*). In the absence of local GABAergic innervation (Fig. 6*D–F*, *top dendrites*), the $GABA_AR$ clusters associate with the GluR1 clusters, as clearly seen on dendritic spines (6*D–F*, *filled arrowheads*). However, in the dendrite that receives GABAergic innervation (*bottom dendrite*), the $GABA_AR$ clusters are predominantly colocalized with the GAD-containing processes (Fig. 6*D–F*, *arrows*) instead of associating with the GluR1 clusters of dendritic spines (Fig. 6*D–F*, *empty arrowheads*).

This organizational effect was specific for $GABA_AR$ clustering because GABAergic innervation did not affect the density of PSD-95 or GluR1 clusters in the neighborhood of GABAergic synapses. There was no significant difference in the density or organization of PSD-95 clusters present in dendrites of the same pyramidal neuron that received GABAergic innervation (50.9 ± 5.0 clusters/50 μm dendrite length) with those that did not (47.9 ± 5.3 clusters/50 μm dendrite length; $p = 0.34$; $n = 14$ dendrites).

We have also found that only 4.7 + 1.4% of the PSD-95 clusters are associated with GABAergic synapses containing GAD and large synaptic $GABA_AR$ clusters. Even in this situation, there was

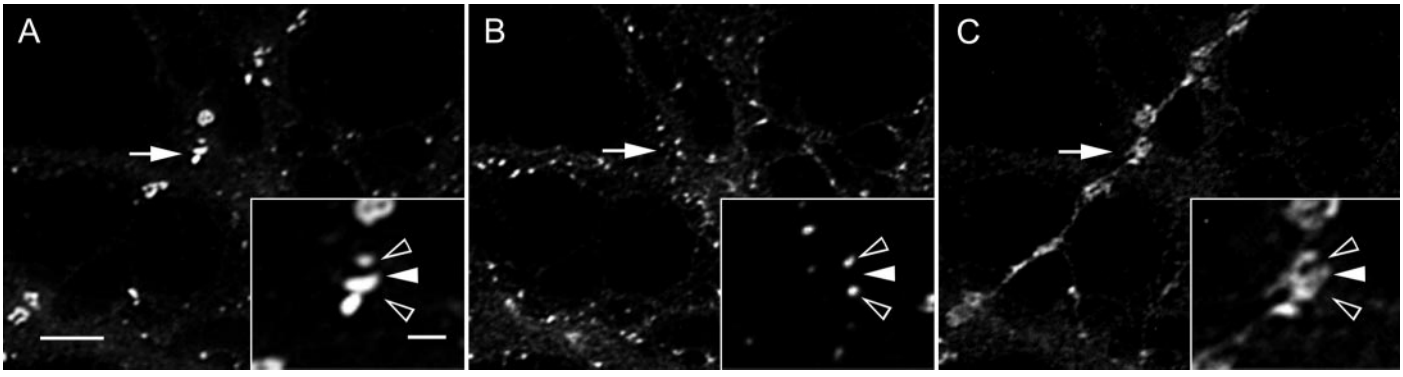

Figure 7. Clusters of glutamatergic postsynaptic density protein PSD-95 can be apposed to presynaptic and postsynaptic GABAergic synaptic markers, but they remain segregated from the GABAergic markers. Hippocampal cultures were labeled with rabbit anti-γ_2 (*A*), mouse monoclonal anti-PSD-95 (*B*), and sheep anti-GAD (*C*). Large clusters of $GABA_AR$ γ_2 subunit colocalize with GAD-positive presynaptic boutons (*arrows*). A single GABAergic synapse (*A–C*, *arrows*) has been magnified in the *insets* to show detail. Note colocalization of GAD terminal and the $GABA_AR$ clusters and how the presynaptic GAD and postsynaptic $GABA_AR$ clusters "avoid" the PSD-95 clusters (*empty arrowheads*) by taking a digitated shape, preserving the segregation of the two postsynaptic receptor clusters. Neurons were cultured for 21 d. Scale bar (shown in *A*): 5 μm; *inset* scale bar, 1 μm.

juxtaposition of the PSD-95 clusters with $GABA_AR$ clusters at GABAergic synapses rather than true colocalization. This segregation is shown in Figure 7, where the large postsynaptic $GABA_AR$ clusters follow the pattern of the GABAergic presynaptic terminal, both avoiding overlap with the postsynaptic glutamatergic receptor clusters (Fig. 7, *insets*, *empty arrowheads*) by taking a digitated shape rather than the normal circular or elongated form.

DISCUSSION

It has been reported previously, using similar types of hippocampal cultures, that $GABA_ARs$ clusters were localized at GABA synapses but not outside GABAergic synapses (Craig et al., 1994, 1996). Moreover, most studies on $GABA_AR$ clustering do not distinguish between the two types of clusters and treat $GABA_AR$ clustering as a single phenomenon. In this communication, we report that $GABA_ARs$ not only form large clusters at GABAergic synapses, but they also form small clusters outside GABAergic synapses. These small $GABA_AR$ clusters are localized at the neuronal surface, which excludes the possibility of them being intracellular trafficking receptors en route to GABAergic synaptic sites.

Our data also show that GABAergic innervation induces both the formation of large $GABA_AR$ clusters at GABAergic synapses and the disappearance of small clusters in the surrounding area. There is a gradient effect in which the disappearance of small clusters is strongest in areas immediately adjacent to GABAergic synapses and extending to 15–20 μm distance from the synapse. The greater the amount of GABAergic innervation that a pyramidal dendrite received, the higher the density of large synaptic $GABA_AR$ clusters and the lower the density of small $GABA_AR$ clusters that the dendrite showed.

Although it appears that large $GABA_AR$ clusters are formed by recruitment of smaller extrasynaptic clusters (Figs. 1–7), it is unlikely that such large aggregates of receptors, gephyrin, and presumably other proteins could laterally diffuse in the membrane. Instead, we favor a mechanistic process of disassembly and assembly of the clusters. Recruitment of $GABA_ARs$ to GABAergic synapses might be caused by a clustering-inducing signal generated at GABAergic synapses that induces or stabilizes the assembly of laterally mobile individual receptors into large synaptic clusters. This mechanism would also favor disassembly of small clusters present in surrounding areas by a mass action phenomenon. Meier et al. (2001) showed that glycine receptors can laterally diffuse in the membrane and be trapped into gephyrin-containing glycine receptor clusters. Nevertheless, the disappearance of small $GABA_AR$ clusters from areas surrounding GABAergic synapses could also be actively induced by a small cluster disassembly-inducing signal. For example, presynaptic terminals of the neuromuscular junction release agrin, which induces clustering of nicotinic acetylcholine receptor (nAchR) at synapses, and also provide a nerve-derived dispersal factor that disassembles extrasynaptic nAchR clusters (Lin et al., 2001). These hypothesized mechanisms do not preclude incorporation of $GABA_AR$ into clusters from internal or subsynaptic pools. Others have also reported innervation-dependent accumulation of $GABA_AR$ clusters at GABAergic synapses in cultured neurons by using immunofluorescence microscopy techniques (Craig et al., 1994; Levi et al., 1999). To the best of our knowledge, however, this is the first time that the effect of innervation on both the large synaptic $GABA_AR$ clusters and the small non-GABAergic clusters and their possible relationship has been studied.

Rao et al. (2000) reported recently that in the absence of GABAergic innervation, isolated hippocampal pyramidal cells in culture form many $GABA_AR$ clusters that were mismatched to presynaptic glutamate-containing terminals. One could argue that this is the result of an abnormal situation in which pyramidal neurons were devoid of GABAergic innervation. However, in our cultures (in which pyramidal neurons receive both GABAergic and glutamatergic innervation), a significant population of small $GABA_AR$ also associated with glutamatergic synaptic markers (GluR1, PSD-95, and synaptic vesicle marker SV2, but not GAD). This association was highest in dendrites or dendritic regions not receiving GABAergic innervation.

It is also worth noting that small $GABA_AR$ clusters associated with glutamatergic synapses were juxtaposed to GluR1 receptor clusters or PSD-95 clusters, indicating that $GABA_AR$ clusters and glutamate receptor clusters did not readily mix with each other, even if they were associated with the same glutamate-containing presynaptic terminal. These synapses contained segregated $GABA_ARs$ and glutamate receptors in postsynaptic microdomains. In this respect, our results also differed from those ob-

tained with single-cell cultures (Rao et al., 2000). They found that PSD and GluR1 receptor clusters were well separated from $GABA_A$R clusters, proposing that either $GABA_A$Rs or glutamatergic receptors (but not both) cluster in front of a single presynaptic glutamatergic terminal. Avoidance of mixing of $GABA_A$R and glutamate receptor clusters occurred not only at glutamatergic synapses but it also occurred at GABAergic synapses (Fig. 7*A–C*).

We have also investigated the possible heterogeneity of $GABA_A$R subunit composition in both large $GABA_A$R clusters at GABAergic synapses and in small receptor clusters located outside GABAergic synapses. We found that 100% of large synaptic $GABA_A$R clusters contained all $GABA_A$R subunit isoforms and classes expressed by that particular neuron. However, two populations of small $GABA_A$R clusters were found in the same neuron: 53–59% of small clusters in neurons expressing pairs of α subunit isoforms (Table 2) showed colocalization of the two isoforms, whereas the remainder of small clusters had receptors containing only one of the two isoforms. This heterogeneity in subunit isoform composition of small $GABA_A$R clusters suggests the existence of some selectivity in clustering or trafficking of $GABA_A$R into the small clusters.

EM immunogold studies of GABAergic synapses onto pyramidal neurons of the CA1 region of the intact hippocampus have shown a differential distribution of $GABA_A$Rs containing α1 or α2 subunits in synapses made by basket cells innervating the soma and proximal dendrites of pyramidal cells (Nyiri et al., 2001). The α_2 subunit concentrates postsynaptically to terminals of parvalbumin-negative basket cells and in axo-axonic synapses on the axon initial segment of hippocampal CA1 pyramidal cells (Nusser et al., 1996a). Others have also presented data at the light microscopy level consistent with differential distribution of $GABA_A$R α_1, α_2, and α_3 subunit isoforms in various synapses (Fritschy et al., 1992; Koulen, 1996; Fletcher et al., 1998). However, it is not entirely proven that the receptor puncta observed with the light microscope correspond to synapses. As indicated above, in our cultures, we found differential distribution of the α subunit isoforms in a proportion of the small clusters but not in large receptor clusters at GABAergic synapses (i.e., all GABAergic synapses had both α_1 and α_2). This is therefore different from results obtained with EM immunogold in the intact hippocampus. This difference might be attributable to selective loss of GABAergic synaptic heterogeneity in the cultures, resulting from a loss of cell types and inputs with respect to the intact brain. Some selective population of interneurons that induce and/or hinder postsynaptic accumulation of the α_2-containing $GABA_A$R in pyramidal cells might not survive the culture conditions. Interestingly, our cultures have interneurons expressing calbindin or calretinin but not parvalbumin (data not shown).

Thus we hypothesize that (1) in the intact hippocampus, GABAergic inputs from different sources have unidentified signals that are involved in targeting of postsynaptic $GABA_A$Rs containing specific α subunit isoforms to specific synapses; (2) in the absence of the normal and heterogeneous GABAergic innervation and with limited GABAergic input, the various types of $GABA_A$Rs containing specific subunit isoforms are pulled together to the existing GABAergic synapses; and (3) in the absence of local GABAergic innervation, $GABA_A$Rs form small clusters, some of which localize at glutamatergic synapses where they remain juxtaposed to (but not displacing) glutamate receptor clusters.

It seems that GABA itself is not the signal that induces the formation of small clusters, because the latter can form in isolated glutamatergic cells, nor does activity seem to be necessary for the formation of $GABA_A$R clusters (Craig et al., 1994; Craig, 1998), although levels of $GABA_A$R expression are affected by neuronal activity (Penschuck et al., 1999). Formation of large $GABA_A$R clusters at GABAergic synapses could be attributable to the presynaptic release of a molecule with a function equivalent to agrin, for nAchR clustering (Lupa and Caldwell, 1991; Lin et al., 2001) or Narp, as proposed for AMPA receptor clustering (O'Brien et al., 1999). Alternatively, direct interaction of membrane molecules from the GABAergic presynaptic terminal with membrane molecules of pyramidal neurons could trigger accumulations of large GABAergic synaptic clusters. Candidate molecules for organizing the presynaptic and postsynaptic glutamate synapse machinery at contact points are ephrins and Eph receptors (Bruckner and Klein, 1998; Torres et al., 1998), cadherins and protocadherins (Shapiro et al., 1995; Benson and Tanaka, 1998; Wu and Maniatis, 1999; Tanaka et al., 2000), and neurexins–neuroligins (Scheiffele et al., 2000). In addition, there is evidence that N-cadherins and their β-catenin partners accumulate at GABAergic synapses early in development (Benson and Tanaka, 1998). The large number of genes and alternatively spliced variants described for some of the aforementioned molecules makes them candidate molecules for also organizing the clustering of $GABA_A$Rs at GABAergic synapses. Some molecules that trigger glutamate receptor clustering at glutamatergic synapses might also trigger the formation of small $GABA_A$R postsynaptic clusters that remain juxtaposed to glutamate receptor clusters. Our results obtained with these cultures may have a bearing on processes operating in the brain, because in the cerebellum the localization of α_6, γ_2, and $\beta_{2/3}$ $GABA_A$R subunits is not restricted to GABAergic synapses. They are also present in some glutamatergic synapses (Nusser et al., 1996b, 1998). These $GABA_A$Rs could participate in the regulation of glutamatergic synaptic excitability, perhaps by binding GABA that has spilled over from neighboring GABAergic synapses. Nevertheless, co-release of glutamate and GABA in some synapses has been reported (Walker et al., 2001).

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