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Segregation of Different $GABA_A$ Receptors to Synaptic and Extrasynaptic Membranes of Cerebellar Granule Cells

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Two types of $GABA_A$ receptor-mediated inhibition (phasic and tonic) have been described in cerebellar granule cells, although these cells receive GABAergic input only from a single cell type, the Golgi cell. In adult rats, granule cells express six $GABA_A$ receptor subunits abundantly (α1, α6, β2, β3, γ2, and δ), which are coassembled into at least four to six distinct $GABA_A$ receptor subtypes. We tested whether a differential distribution of $GABA_A$ receptors on the surface of granule cells could play a role in the different forms of inhibition, assuming that phasic inhibition originates from the activation of synaptic receptors, whereas tonic inhibition is provided mainly by extrasynaptic receptors. The α1, α6, β2/3, and γ2 subunits have been found by immunogold localizations to be concentrated in GABAergic Golgi synapses and also are present in the extrasynaptic membrane at a lower concentration. In contrast, immunoparticles for the δ subunit could not be detected in synaptic junctions, although they were abundantly present in the extrasynaptic dendritic and somatic membranes. Gold particles for the α6, γ2, and β2/3, but not the α1 and δ, subunits also were concentrated in some glutamatergic mossy fiber synapses, where their colocalization with AMPA-type glutamate receptors was demonstrated. The exclusive extrasynaptic presence of the δ subunit-containing receptors, together with their kinetic properties, suggests that tonic inhibition could be mediated mainly by *extrasynaptic* $\alpha_6\beta_{2/3}\delta$ receptors, whereas phasic inhibition is attributable to the activation of *synaptic* $\alpha_1\beta_{2/3}\gamma_2$, $\alpha_6\beta_{2/3}\gamma_2$, and $\alpha_1\alpha_6\beta_{2/3}\gamma_2$ receptors.

Key words: neurotransmission; cerebellum; inhibition; synapse; ion channel; immunocytochemistry

In most brain regions several distinct types of GABAergic interneuron evolved to fulfill complex functional requirements, such as setting the threshold for activation (Andersen et al., 1963), synchronizing sub- and suprathreshold oscillations (Cobb et al., 1995; Whittington et al., 1995; Jefferys et al., 1996), preventing the active backpropagation of fast action potentials in the dendrites (Buzsáki et al., 1996; Tsubokawa and Ross, 1996), inhibiting Ca^{2+} electrogenesis in the dendrites (Midtgaard, 1992; Miles et al., 1996), or shunting excitatory synaptic inputs (Qian and Sejnowski, 1990; Staley and Mody, 1992). It also has been demonstrated that most of these cells exert their influence on the postsynaptic cells via $GABA_A$ receptors (Buhl et al., 1994; Miles et al., 1996). Other cell types in the CNS, such as cerebellar granule cells, receive GABAergic input from a single source only (Mugnaini and Oertel, 1985). Nevertheless, GABA also may serve several functional roles for these cells, because two distinct types of $GABA_A$ receptor-mediated inhibition recently have been described (Brickley et al., 1996; Wall and Usowicz, 1997). GABA modulates granule cell excitability phasically via discrete postsynaptic currents that result from the synchronous opening of 18–32 synaptic $GABA_A$ receptors and tonically via the persistent opening of several $GABA_A$ receptor channels (Brickley et al., 1996). We have tested the hypothesis that subcellular segregation of distinct $GABA_A$ receptor subtypes may underlie the different forms of inhibition, assuming that the phasic inhibition is attributable to the activation of *synaptic* $GABA_A$ receptors and that the tonic inhibition originates mainly from the activation of *extrasynaptic* receptors (Brickley et al., 1996; Wall and Usowicz, 1997). High-resolution immunogold localization was used at the electron microscopic level with antibodies selective for the α1, β2/3, γ2, and δ subunits of the $GABA_A$ receptor.

MATERIALS AND METHODS

Preparation of animals and tissue. Four adult mice (~40 gm; Black6) and two rats (120–200 gm; Wistar) were anesthetized with Sagatal (pentobarbitone sodium, 220 mg/kg, i.p.) and perfused through the heart with 0.9% saline, followed by a fixative containing 4% paraformaldehyde, 0.05% glutaraldehyde, and ~0.2% picric acid dissolved in 0.1 M phosphate buffer (PB), pH 7.4, for 10–17 min. After perfusion the brains were removed; blocks from the vermis of the cerebellar cortex were cut out and either were post-fixed in the same fixative for 2 hr or were washed in several changes of PB.

Antibodies. Rabbit polyclonal antibody (code number P16) was raised to a synthetic peptide corresponding to residues 1–9 of the rat α1 subunit. Antibody specificity has been described earlier (Zezula et al., 1991). Immunoreactions with affinity-purified P16 antibody were performed at a final protein concentration of 1.6 μg/ml.

Mouse monoclonal antibody (code number bd17; Haring et al., 1985), recognizing the β2 and β3 subunits of the $GABA_A$ receptors, was used at a protein concentration of 40 μg/ml for postembedding reactions.

Guinea pig polyclonal antibody [code number γ2(1–29)] was raised to a synthetic peptide corresponding to residues 1–29 of the γ2 subunit of the $GABA_A$ receptor, as described earlier (Benke et al., 1996; Somogyi

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et al., 1996). The antibody was used at a final protein concentration of 1 μg/ml.

Rabbit polyclonal antibody [code number δ(1–44)R5] was raised to maltose binding protein–δ-(1–44)-7His fusion protein and was purified by affinity chromatography on a column containing the corresponding glutathione *S*-transferase–δ(1–44)-7His fusion protein (Jones et al., 1997; Sperk et al., 1997). Antibody δ(1–44)R5 strongly reacted with a 51 kDa protein on Western blot and revealed a weak band at 119 kDa. Antibody δ(1–44)R5 was used at final protein concentrations of 1 and 1.7 μg/ml for pre- and postembedding reactions, respectively.

The production and characterization of rabbit antiserum to glutamate (code number Glu13) have been described previously (Ottersen and Storm-Mathisen, 1984). The antiserum was used in a final dilution of 1:200.

Polyclonal antibody (GluR2/3/4c) to a C-terminal peptide common to the GluR2, GluR3, and GluR4c subunits of the AMPA-type glutamate receptor was used at the final protein concentration of 0.7 μg/ml for postembedding reactions. The characterization of antibody GluR2/3/4c has been described earlier (Wenthold et al., 1992).

Controls. Selective labeling, resembling that obtained with the specific antibodies, could not be detected when the primary antibodies either were omitted or were replaced by 5% normal serum of the species of the primary antibody. No immunostaining for the δ subunit could be detected in δ subunit-deficient mice (animals kindly provided by Dr. G. Homanics), demonstrating that all of the staining observed in control sections with our antibody δ(1–44)R5 is attributable to the reaction with the δ subunit. For double- and triple-labeling experiments, the specificity of the secondary antibodies was tested as follows. Separate sections were reacted for the α1 (rabbit antibody), β2/3 (mouse antibody), and γ2 (guinea pig antibody) subunits. After incubation in primary antibodies, the same sections were incubated with inappropriate secondary antibodies; after a reaction with a rabbit antibody to the α1 subunit, goat anti-mouse or goat anti-guinea pig secondary antibodies were applied. No labeling could be detected in such incubations, demonstrating the specificity of the secondary antibodies.

Preembedding immunocytochemistry. Normal goat serum (20%) was used in 50 mM Tris–HCl, pH 7.4, containing 0.9% NaCl (TBS) as the blocking solution, for 1 hr, followed by the incubation with purified primary antibody δ(1–44)R5 diluted in TBS containing 2% normal goat serum and 0.05% Triton X-100 overnight. After washing, the sections were incubated for 90 min in either biotinylated (diluted 1:50 in TBS; Vector Laboratories, Peterborough, UK) or 1.4-nm gold-coupled goat anti-rabbit IgG (diluted 1:100 in TBS; Nanogold, Nanoprobes, Stony Brook, NY). The sections for peroxidase reaction were incubated in avidin–biotinylated horseradish peroxidase complex (1:100 dilution in TBS) for 2 hr before peroxidase enzyme reaction was performed with 3,3′-diaminobenzidine tetrahydrochloride as chromogen and H_2O_2 as oxidant. Gold particles (1.4 nm) were silver-enhanced with the HQ Silver kit, as described by the manufacturer (Nanoprobes) for 8–15 min. Then the sections were processed routinely for electron microscopic examination.

Freeze substitution and Lowicryl embedding. The same procedure was used as described earlier (Nusser et al., 1995a). After perfusion, blocks of tissue were washed in PB, followed by Vibratome sectioning (500 μm thickness) and washing in PB overnight. The sections were cryoprotected in 1 M sucrose solution in PB for 2 hr before being slammed to a copper block cooled in liquid N_2. Freeze substitution with methanol took place in a Reichert CS auto machine (Leica AG, Austria) at −80°C, followed by embedding in Lowicryl HM 20 (Chemische Werke Lowi GMBH, Germany) at −50°C.

Postembedding immunocytochemistry on electron microscopic sections. A similar method was used as described earlier (Matsubara et al., 1996) and will be referred to as a double-sided reaction because the antibodies have access to both sides of the sections. Reactions were performed on 70-nm-thick sections of slam-frozen, freeze-substituted, Lowicryl-embedded cerebellar cortex. They were picked up on gold grids (400 mesh) that had been coated with coat-quick "G" medium (Daido Sangyo Company, Japan) to prevent the detachment of the sections during processing. Then the sections were treated with a saturated solution of NaOH in 100% ethanol for ~3 sec. After being washed, the sections were incubated in 0.1% sodium borohydrate and 50 mM glycine in TBS containing 0.1% Triton X-100 (TBST) for 10 min. Human serum albumin (HSA; 2% in TBST) was used for blocking for 30 min, followed by an incubation with the primary antibodies (diluted in TBST containing 2% HSA) overnight.

For single-labeling experiments, only one primary antibody was used on a given section. After several washes the sections were incubated in the appropriate secondary antibodies (goat anti-rabbit, goat anti-guinea pig, and goat anti-mouse IgGs coupled to 10-nm gold particles; Nanoprobes) diluted (1:180) in TBST containing 2% HSA and 5 mg/ml polyethyleneglycol.

For double-labeling experiments, a mixture of antibodies δ(1–44)R5 and bd17 was applied overnight, followed by several washes and an incubation in a mixture of goat anti-rabbit IgGs coupled to either 18-nm (dilution 1:200; Jackson ImmunoResearch, West Grove, PA) or 20-nm gold particles (dilution 1:50; BioClinical Services, Cardiff, UK) and goat anti-mouse IgGs coupled to 10-nm gold particles (dilution 1:180; Nanoprobes; same buffers as above).

Double-labeling experiments for the β2/3 subunits and glutamate were performed as follows: a mixture of antibodies bd17 and Glu13 was applied overnight, followed by several washes and an incubation in a mixture of goat anti-mouse IgGs coupled to 10-nm gold particles (dilution 1:180; Nanoprobes) and goat anti-rabbit IgGs coupled to 18-nm gold particles (dilution 1:200; Jackson ImmunoResearch; same buffers as above).

Double-labeling experiments for AMPA and $GABA_A$ receptor subunits were performed as follows: a mixture of antibodies bd17 and GluR2/3/4c was applied overnight, followed by several washes and an incubation in a mixture of goat anti-mouse IgGs coupled to 10-nm gold particles (dilution 1:180; Nanoprobes) and goat anti-rabbit IgGs coupled to either 18-nm (dilution 1:200; Jackson ImmunoResearch) or 20-nm (dilution 1:50; BioClinical Services; same buffers as above) gold particles.

For triple-labeling experiments the sections were incubated in a mixture of antibodies P16, bd17, and γ2(1–29) overnight, followed by several washes and an incubation in a mixture of goat anti-rabbit IgGs coupled to 20-nm gold particles (dilution 1:50; BioClinical Services), goat anti-mouse IgGs coupled to 5-nm gold particles (dilution 1:50; BioClinical Services), and goat anti-guinea pig IgGs coupled to 10-nm gold particles (dilution 1:180; Nanoprobes; same buffers as above).

Incubations in secondary antibodies were followed by washing in ultra pure water. Then the sections were contrasted with saturated aqueous uranyl acetate, followed by lead citrate.

Quantification of immunoreactive δ subunits on the extrasynaptic somatic and dendritic membranes was done in a similar way to that described in Nusser et al. (1995b). Briefly, glomeruli were selected randomly from a well preserved strip of an ultrathin section and were photographed and printed at a magnification of 53,000×. Granule cell bodies also were photographed randomly around the glomeruli. Somatic membranes were included in the measurements only if they were directly apposed to other granule cell somatic membranes. The length of the sectioned extrasynaptic plasma membranes was measured with a digitizing tablet (Ranforly MicroSystems, UK), and gold particles were counted within 30 nm lateral to the plasma membrane on both sides. Measurements were not corrected for background labeling because the latter was very low.

RESULTS

The δ subunit is present on extrasynaptic somatic and dendritic membranes

The regional and cellular distribution of immunoreactivity provided by antibody δ(1–44)R5 in rat and mouse CNSs was very similar to that of δ subunit mRNA (Persohn et al., 1992) and to immunoreactivity obtained with other δ subunit-selective antibodies (Fritschy and Mohler, 1995; Sperk et al., 1997). The lack of immunostaining with antibody δ(1–44)R5 in δ subunit-deficient mouse brain confirmed the specificity of the immunolabeling provided by our antibody. Here we describe the subcellular distribution of immunoreactive δ subunits in rat and mouse cerebellar granule cells only, because the most intense staining has been found in this cell type. No difference was seen in the distribution of $GABA_A$ receptor subunits between rat CNS and mouse CNS; therefore, the species will not be stated specifically in the following part of the paper. Immunogold localizations at the electron microscopic level allowed us to reveal the precise location of receptors at both synaptic and extrasynaptic sites with a resolution of 15–30 nm (Baude et al., 1993, 1995; Nusser et al., 1995a,b,

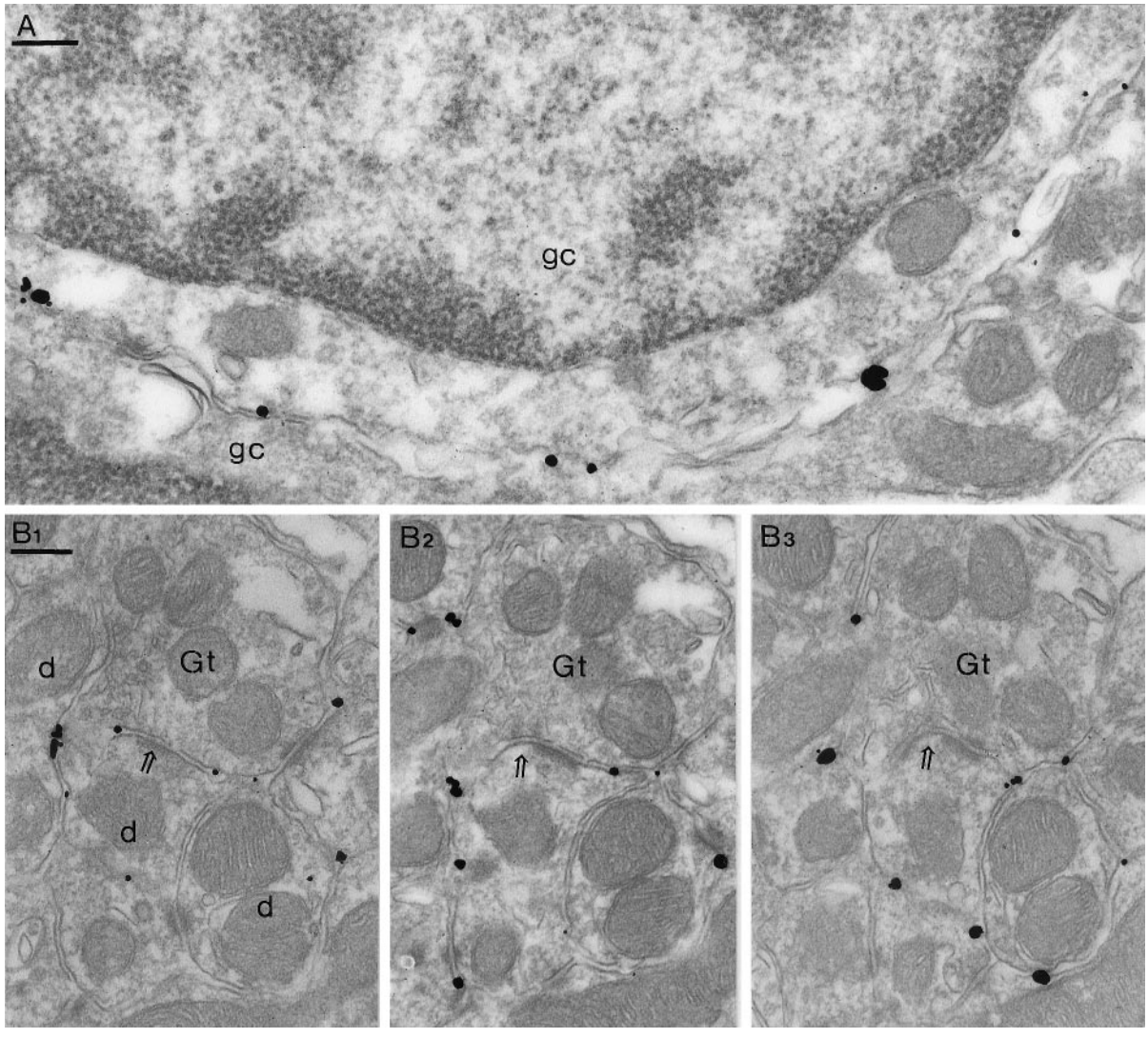

Figure 1. Distribution of immunoreactivity for the δ subunit of the $GABA_A$ receptor in the granule cell layer of mouse cerebellum as revealed by a preembedding, silver-intensified immunogold reaction. *A*, Immunoparticles are present along the nonsynaptic somatic membrane of granule cells (*gc*). B_1–B_3, Serial ultrathin sections of a glomerulus showing that a synapse (*open arrow*) between a Golgi cell terminal (*Gt*) and a granule cell dendrite (*d*) is immunonegative for the δ subunit, although particles are present at the extrasynaptic dendritic membrane. Note that, when the membranes are cut at right angle (e.g., in B_1), most of the particles are seen at the external face of the plasma membrane corresponding to the extracellular location of epitope(s) recognized by the antibody δ(1–44)R5. Scale bars, 0.2 μm.

1997; Matsubara et al., 1996; Popratiloff et al., 1996; Shigemoto et al., 1996; Landsend et al., 1997).

Preembedding immunogold reactions with silver-intensified 1.4-nm gold particles revealed that the majority of immunoparticles for the δ subunit was associated with the extrasynaptic somatic (Fig. 1*A*) and dendritic (Fig. 1*B*) membranes of granule cells. The labeling of somata and dendrites was consistent through serial ultrathin sections (Fig. $1B_1$–B_3). Using this method, we could not detect immunoparticles in synaptic junctions either between glutamatergic mossy fiber terminals and granule cell dendrites or between GABAergic Golgi cell terminals and granule cell dendrites (Fig. 1*B*). The lack of labeling was also consistent through serial sections (Fig. $1B_1$–B_3). Immunoparticles were not associated with somatodendritic synaptic or extrasynaptic membranes in the molecular layer, in agreement with the restricted expression of the δ subunit in granule cells. The lack of synaptic labeling is not surprising with the preembedding immunogold method, because in previous studies synaptic enrichment of immunoparticles for ionotropic glutamate and other $GABA_A$ receptor subunits (e.g., α1, α6, and β2/3) could not be detected with this method, but a postembedding immunogold method revealed their enrichment in hippocampal and cerebellar synapses (Baude et al., 1995; Nusser et al., 1995a,b, 1996b). To overcome this technical limitation, we have applied postembedding immunogold localization of the δ subunit on freeze-substituted, Lowicryl resin-embedded cerebellar tissue.

Lack of synaptic labeling for the δ subunit

With a postembedding immunogold method, gold particles for the δ subunit were present almost exclusively on the extrasynaptic somatic (Fig. 2*A*) and dendritic (Fig. 2*B*) membranes of granule cells, in agreement with the preembedding localization. We detected a somewhat higher (57%) immunoparticle density on extrasynaptic dendritic membranes [rat1: 2.8 ± 0.9 gold/μm (mean ± SD), $n = 2$ areas, 218 gold; rat2: 1.4 ± 0.5 gold/μm, $n = 3$ areas, 94 gold] than on somatic membranes (rat1: 1.7 ± 0.6

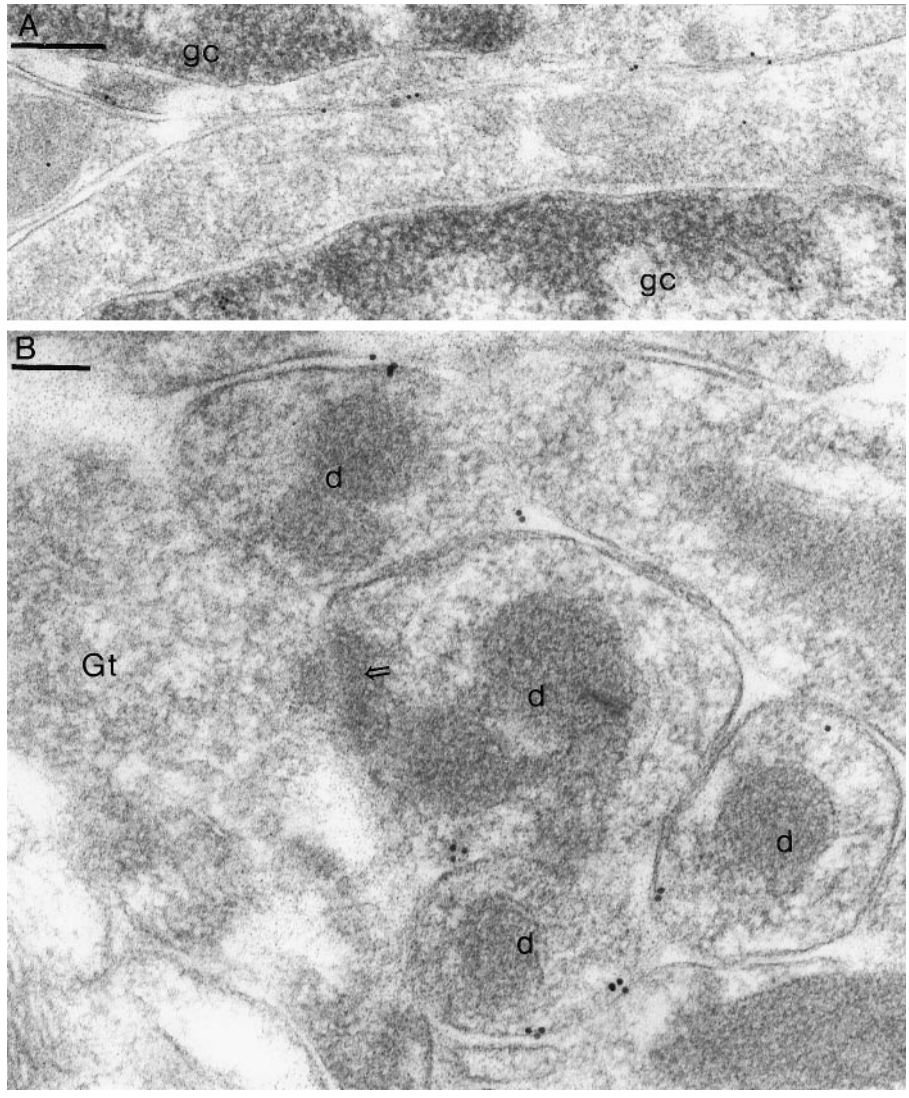

Figure 2. Immunoreactivity for the δ subunit in the granule cell layer of rat cerebellum as revealed by a postembedding immunogold technique (10-nm gold). *A*, Immunoparticles are present along the extrasynaptic somatic membrane of granule cells (*gc*), including areas in which two cells are directly apposed. *B*, Immunogold particles are associated with the extrasynaptic membranes of granule cell dendrites (*d*), but no particles are seen in a synapse (*open arrow*) made by a Golgi cell terminal (*Gt*) and a granule cell dendrite. Scale bars: *A*, 0.2 μm; *B*, 0.1 μm.

gold/μm, $n = 4$ areas, 92 gold; rat2: 0.9 ± 0.2 gold/μm, $n = 3$ areas, 26 gold). This is in line with our previous results, showing lower immunoparticle densities for the α1 and β2/3 subunits on the somatic compartment (Nusser et al., 1995b). The absolute values for the δ subunit are five- to 10-fold higher than those in our previous study, demonstrating a higher sensitivity of the currently used method and possible differences in labeling efficiencies of the different antibodies.

Occasionally, gold particles could be detected intracellularly in association with the endoplasmic reticulum (ER) and the Golgi apparatus (Fig. 3*D*). Surprisingly, symmetrical synapses made by GABAergic Golgi cell terminals with granule cell dendrites were immunonegative for the δ subunit, although extrasynaptic membranes were immunopositive (Fig. 2*B*). To exclude the possibilities that the lack of labeling was a consequence of an inaccessibility of synaptic receptors to the antibodies or that the immunoreactivity of synaptic receptors was selectively lost during processing, we performed double-labeling experiments for the β2/3 and δ subunits with two different sizes of gold particles. Although extrasynaptic dendritic (Fig. 3*A–C*) and somatic (Fig. 3*D*) membranes of granule cells were outlined by gold particles for both subunits, Golgi synapses were immunopositive only for the β2/3 subunits (Fig. 3*A–C*). Labeling for the δ subunit was not just unspecifically associated with extrasynaptic membranes, because neither synaptic nor extrasynaptic membranes in the molecular layer showed any labeling for the δ subunit. However, symmetrical synapses on Purkinje cells and interneurons in the molecular layer showed a selective labeling for the β2/3 subunits (Fig. 3*E*). Synapses between glutamatergic mossy fiber terminals and granule cell dendrites or between parallel fiber terminals and Purkinje cell spines were also immunonegative for the δ subunit.

The α6, β2/3, and γ2, but not the α1 and δ, subunits are concentrated in excitatory mossy fiber to granule cell synapses

We have reported previously an enrichment of immunoparticles for the α6 subunit of the $GABA_A$ receptor in excitatory mossy fiber to granule cell synapses (Nusser et al., 1996b). To determine whether this distribution is unique for the α6 subunit or whether other subunits of the $GABA_A$ receptor also may be present in these excitatory synapses, we reexamined the previously reported distribution of the α1, β2/3, and γ2 subunits (Nusser et al., 1995b; Somogyi et al., 1996) in a double-sided reaction (Matsubara et al., 1996) that, for these antibodies, has a higher sensitivity than the method we applied previously. Gold particles for both the β2/3 (Fig. 4*A,B*) and γ2 (Fig. 5*A,B*) subunits also were present in some

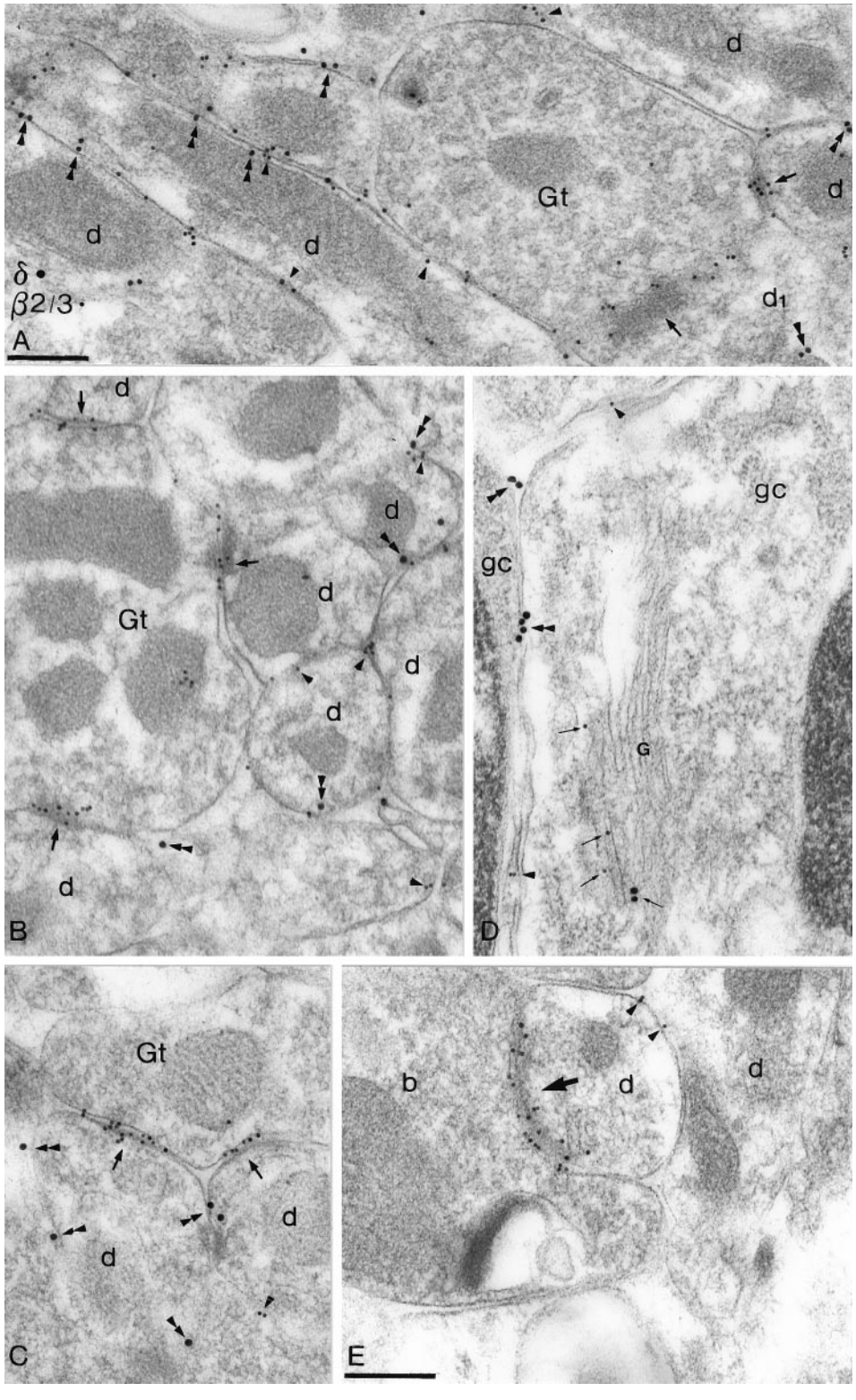

Figure 3. Electron micrographs showing double labeling for the β2/3 (10 nm particles) and the δ (*A*, 18 nm; *B–E*, 20 nm particles) subunits. Postembedding immunogold reactions are shown for rat (*A* and *E*) and mouse (*B–D*) cerebella. *A–C*, Synapses (*arrows*) made by Golgi cell terminals (*Gt*) with granule cell dendrites (*d*) are not labeled for the δ subunit, although the enrichment of immunoparticles for the β2/3 subunits shows that receptor immunoreactivity is well preserved in these GABAergic synapses. In addition, the presence of immunoparticles for the δ subunit (*double arrowheads*) at the extrasynaptic dendritic membranes demonstrates that the method is sensitive enough to visualize this subunit. Note that immunoparticles for the β2/3 subunits also are associated with the extrasynaptic dendritic membranes (e.g., *single arrowheads*). *D*, Immunoparticles for both the β2/3 (*arrowheads*) and the δ (*double arrowheads*) subunits are present at the somatic membrane of granule cells (*gc*). A Golgi apparatus (*G*) shows immunoreactivity for both of these subunits (*small arrows*). *E*, In the molecular layer symmetrical synapses (*arrow*) on interneuron dendrites (*d*) or on Purkinje cells and extrasynaptic (*arrowheads*) membranes showed immunoreactivity for the β2/3 subunits, but never for the δ subunit. *b*, Bouton. *A–D* have the same magnification; scale bars, 0.2 μm.

asymmetrical mossy synapses when they were localized with the double-sided method. In these reactions the density of gold particles in Golgi synapses and on the extrasynaptic membranes was higher than that obtained in our previous reactions (Nusser et al., 1995b; Somogyi et al., 1996). In agreement with our previous observation on the α6 subunit, not every mossy synapse contained a detectable level of β2/3 and γ2 subunits (Figs. 4*B*, 5*B*), which may indicate a heterogeneity of mossy fiber to granule cell synapses. The density of immunoparticles for both of these subunits was somewhat lower in mossy synapses than in GABAergic Golgi synapses (e.g., Fig. 4*B*), but it was higher than on the extrasynaptic membrane. To test whether mossy terminals making $GABA_A$ receptor-immunopositive asymmetrical synapses are glutamatergic, as described earlier (Somogyi et al., 1986), we

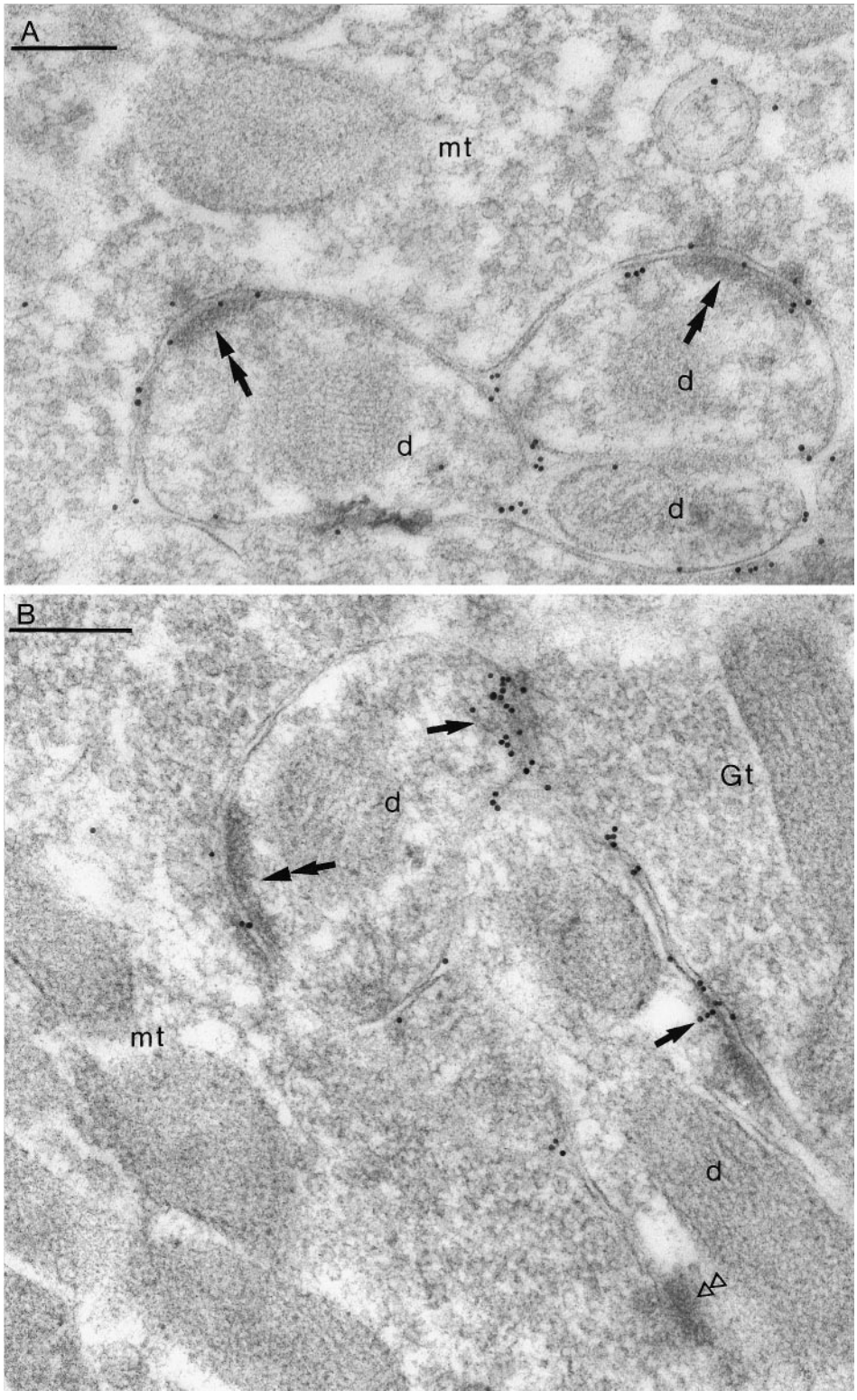

Figure 4. Immunoreactive β2/3 subunits are present in both GABAergic and glutamatergic synapses on granule cells. Postembedding immunogold reactions are shown on mouse cerebellum with 10-nm gold particles. *A*, *B*, Gold particles are present in asymmetrical synapses (*double arrows*) between mossy fiber terminals (*mt*) and granule cell dendrites (*d*) and in synapses (*arrows* in *B*) made by a Golgi cell terminal (*Gt*) with granule cell dendrites. One of the asymmetrical synapses (*double open triangles*) is immunonegative for these subunits. Usually a higher density of immunoparticles is found in Golgi synapses (*single arrows*) than in mossy fiber to granule cell synapses (synapses in *B*). Scale bars, 0.2 μm.

performed double-labeling experiments for the β2/3 subunits and glutamate. A high density of immunoparticles for glutamate was found in mossy terminals making synapses immunopositive for the β2/3 subunits, suggesting that these terminals use glutamate as a neurotransmitter (result not shown). It remains to be determined whether glutamate is the only neurotransmitter in these terminals or whether other neuroactive substances are released also (e.g., GABA, β-alanine, γ-hydroxybutyrate, or taurine). Furthermore, we have tested whether the asymmetrical synapses immunopositive for the $GABA_A$ receptor subunits contain AMPA-type ionotropic glutamate receptors. Double-labeling experiments for the β2/3 subunits of the $GABA_A$ receptor and GluR2/3/4c subunits of the AMPA-type glutamate receptor (Fig. 6) revealed that some of the asymmetrical synapses made by mossy fiber terminals with granule cell dendrites were immunopositive for both $GABA_A$ and AMPA receptors.

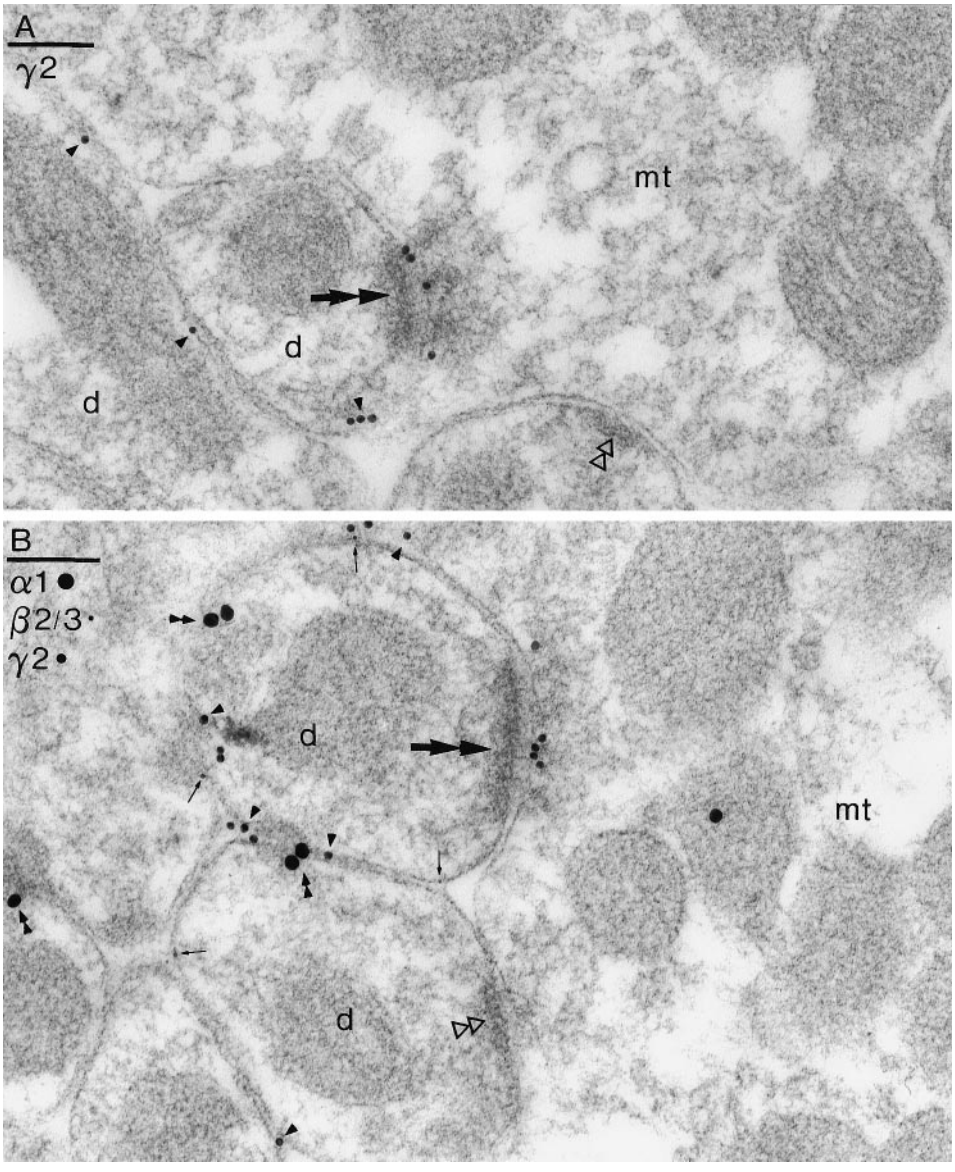

Figure 5. Immunoreactive γ2 subunits are present in mossy fiber to granule cell synapses. Postembedding immunogold reactions are shown on Lowicryl resin-embedded rat cerebellum. *A*, An asymmetrical synapse (*double arrows*) made by a mossy fiber terminal (*mt*) with a granule cell dendrite (*d*) is immunopositive for the γ2 subunit. Particles also are present on the extrasynaptic dendritic membranes (*arrowheads*). *B*, A triple-labeling experiment shows the presence of gold particles for the γ2 subunit in an asymmetrical synapse (*double arrow*) made by a mossy fiber terminal with a granule cell dendrite (*d*) and the presence of the α1 (*double arrowheads*), β2/3 (*small arrows*), and γ2 (*arrowheads*) subunits on the extrasynaptic membranes. In this example the immunoparticles for the β2/3 subunits are not detected in mossy synapses. One of the asymmetrical mossy synapses (*double open triangles*) is immunonegative for all of the subunits. Scale bars, 0.1 μm.

No enrichment of gold particles for the α1 subunit could be detected in glutamatergic mossy synapses even if the more sensitive double-sided reaction was applied, although a higher density of particles was observed in Golgi synapses and on the extrasynaptic membranes than that obtained in our previous reactions (Nusser et al., 1995b). Triple-labeling experiments for the α1, β2/3, and γ2 subunits with three different sizes of gold particles showed that, even if the β2/3 or γ2 subunits were present in mossy synapses, the α1 subunit was present only on the extrasynaptic membranes (see Fig. 5*B*) and in Golgi synapses (Fig. 7). The colocalization of these three subunits was found in many Golgi synapses (Fig. 7*A,B*), similar to that reported earlier (Somogyi et al., 1996).

DISCUSSION

We have demonstrated that distinct $GABA_A$ receptor subtypes are segregated to synaptic and extrasynaptic membranes of cerebellar granule cells (Fig. 8). Such a subcellular segregation may allow a differential activation of distinct receptor subtypes that, together with dissimilar kinetic properties, will have diverse functional consequences on the behavior of the cell on the release of GABA. The δ subunit-containing $GABA_A$ receptors are present only extrasynaptically, have high affinity for GABA (Saxena and Macdonald, 1996), and do not desensitize on the prolonged presence of agonist (Saxena and Macdonald, 1994); therefore, they are well suited to mediate tonic inhibition, which originates from the persistent activation of $GABA_A$ receptors (Brickley et al., 1996). The enrichment of the α1, α6, β2/3, and γ2 subunits in GABAergic Golgi synapses, very likely resulting in $GABA_A$ receptors with $\alpha_1\beta_{2/3}\gamma_2$, $\alpha_6\beta_{2/3}\gamma_2$, and $\alpha_1\alpha_6\beta_{2/3}\gamma_2$ subunit composition (Caruncho and Costa, 1994; Khan et al., 1994, 1996; Quirk et al., 1994; Pollard et al., 1995; Jones et al., 1997), indicates that phasic inhibition is mediated by these receptors. Furthermore, we have demonstrated that not only the α6 (Nusser et al., 1996b) but also the β2/3 and γ2 subunits are concentrated in some glutamatergic mossy fiber to granule cells synapses, suggesting that $\alpha_6\beta_{2/3}\gamma_2$ receptors may have functional roles in these excitatory synapses.

Possible functional consequences of the subcellular segregation of distinct $GABA_A$ receptors

Although cerebellar granule cells receive GABAergic input on their distal dendrites from a single cell type only, they express six

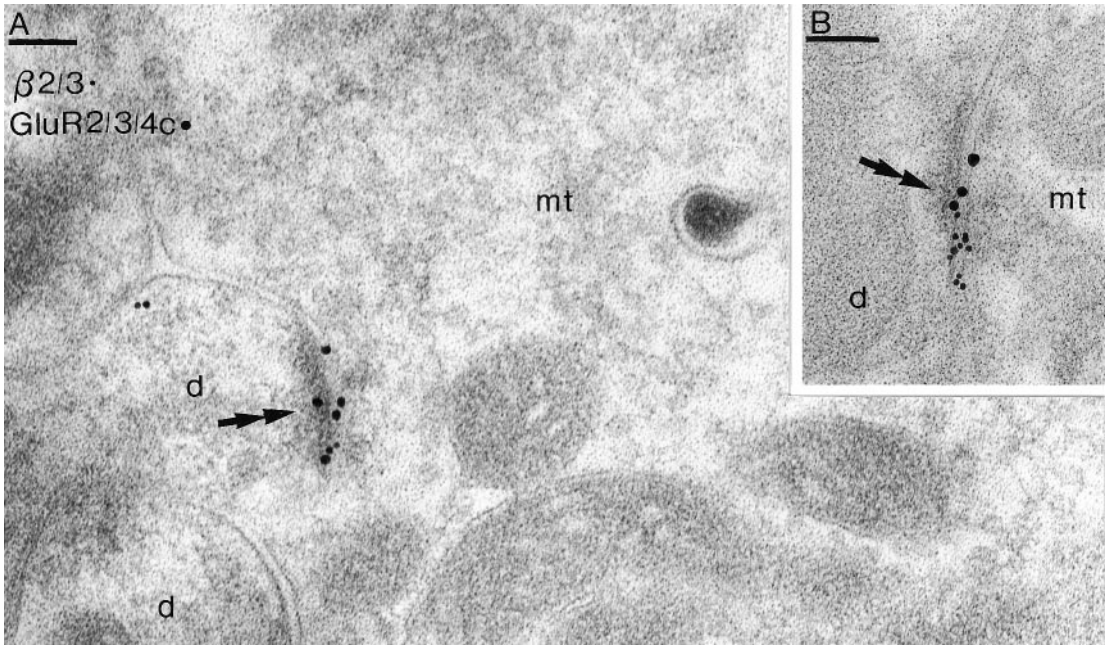

Figure 6. Synaptic colocalization of $GABA_A$ (antibody to the β2/3 subunits; 10-nm gold) and AMPA-type glutamate receptors (antibody to the GluR2/3/4c subunits; 20-nm gold). Asymmetrical synapses (*double arrows*) between mossy fiber terminals (*mt*) and granule cell dendrites (*d*) are immunopositive for both the $GABA_A$ receptor and the AMPA-type glutamate receptor. Postembedding reactions are shown in rat (*A*) and mouse (*B*) cerebella. Scale bars, 0.1 μm.

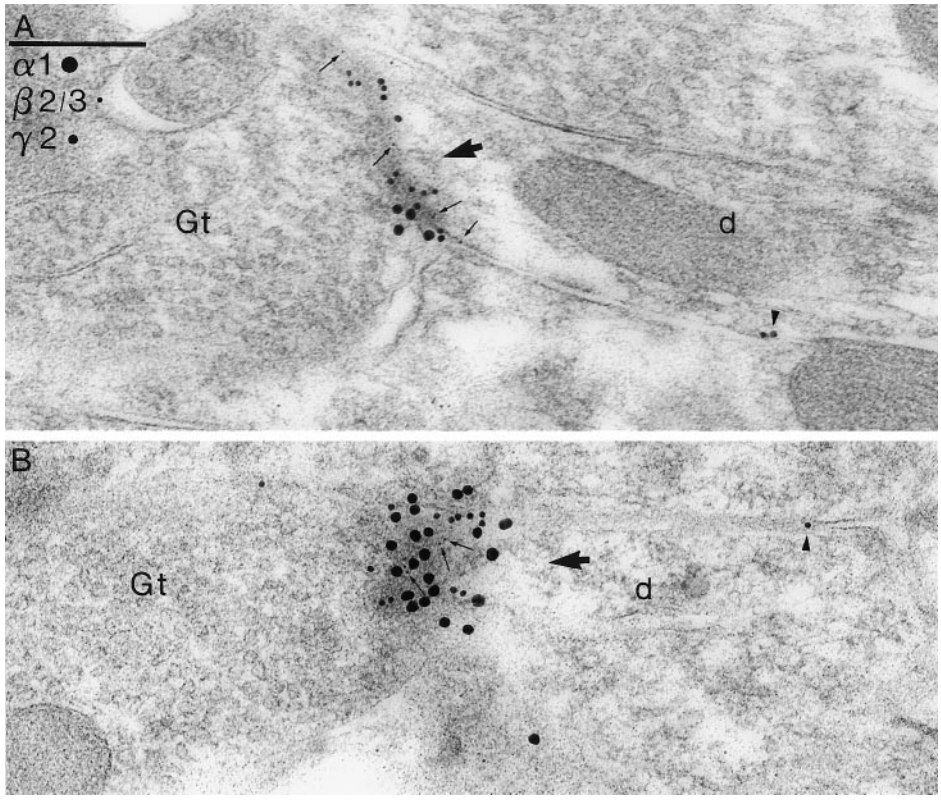

Figure 7. Colocalization of the α1 (20-nm gold), β2/3 (5-nm gold), and γ2 (10-nm gold) subunits of the $GABA_A$ receptor in Golgi synapses of the mouse cerebellum. *A*, *B*, Labeling for each subunit is present in synapses (*large arrows*) between Golgi cell terminals (*Gt*) and granule cell dendrites (*d*). *Small arrows* point to 5-nm particles, indicating immunoreactive β2/3 subunits. Some extrasynaptic particles are shown by *arrowheads*. The synapse in *B* is cut tangentially; thus the receptor immunoreactivity is shown *en face*. *A* and *B* have the same magnification. Scale bar, 0.2 μm.

$GABA_A$ receptor subunits abundantly (Laurie et al., 1992; Persohn et al., 1992), which are coassembled into at least four to six $GABA_A$ receptor subtypes (Sieghart, 1995; McKernan and Whiting, 1996; Jones et al., 1997). We have demonstrated a great degree of segregation of distinct $GABA_A$ receptor subtypes on the surface of granule cells (Fig. 8). The δ subunit-containing receptors ($\alpha_6\beta_{2/3}\delta$ receptors; Caruncho and Costa, 1994; Quirk et al., 1994; Jones et al., 1997) are present exclusively at the nonsynaptic membranes. The α1 subunit ($\alpha_1\beta_{2/3}\gamma_2$ receptors; Caruncho and Costa, 1994; Quirk et al., 1994; Jones et al., 1997) is

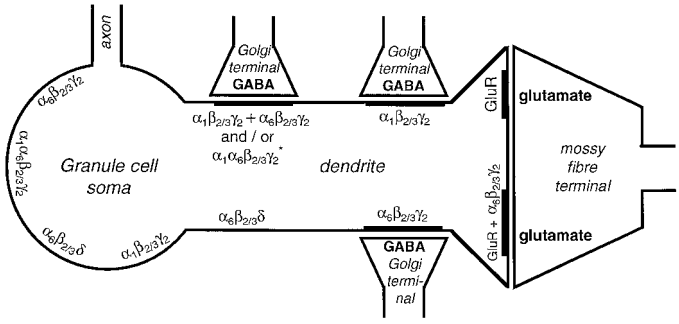

Figure 8. Schematic representation of the differential distribution of $GABA_A$ receptor subtypes on cerebellar granule cells, assuming that every receptor subtype is expressed by a single cell. The $\alpha6$, $\beta2/3$, and $\gamma2$ subunits ($\alpha_6\beta_{2/3}\gamma_2$ receptors) are present in Golgi synapses, on the extrasynaptic membranes, and in some of the mossy fiber to granule cell synapses. Immunoreactive δ subunits ($\alpha_6\beta_{2/3}\delta$ receptors) are found only on the extrasynaptic somatic and dendritic membranes. Immunoreactivity for the $\alpha1$ subunit ($\alpha_1\beta_{2/3}\gamma_2$ receptors) is found in some Golgi cell to granule cell synapses and on the extrasynaptic membranes. *The $\alpha1$ and $\alpha6$ subunits are found colocalized in some Golgi synapses, suggesting either that some of these synapses contain both $\alpha_1\beta_{2/3}\gamma_2$ and $\alpha_6\beta_{2/3}\gamma_2$ receptors or that a receptor population with $\alpha_1\alpha_6\beta_{2/3}\gamma_2$ subunit composition exists (see Discussion) in these synapses and in the extrasynaptic membranes. The $\beta2/3$ subunits were found colocalized with AMPA-type glutamate receptors (*GluR*) in some mossy fiber to granule cell synapses, but others were labeled only for AMPA receptors. Some of the data are from Nusser et al. (1995b, 1996b) and Jones et al. (1997).

concentrated in Golgi synapses and is present in a low concentration on the extrasynaptic membranes (Nusser et al., 1995b). The $\alpha6$, $\beta2/3$, and $\gamma2$ subunits ($\alpha_6\beta_{2/3}\gamma_2$ receptors; Caruncho and Costa, 1994; Khan et al., 1994; Quirk et al., 1994; Pollard et al., 1995; Jones et al., 1997) are present in some GABAergic Golgi synapses, on the extrasynaptic membranes, and in some of the mossy fiber to granule cell synapses. The $\alpha1$ and $\alpha6$ subunits are found colocalized in some Golgi synapses (Nusser et al., 1996b), suggesting either that some of these synapses contain both $\alpha_1\beta_{2/3}\gamma_2$ and $\alpha_6\beta_{2/3}\gamma_2$ receptors or that a receptor population with $\alpha_1\alpha_6\beta_{2/3}\gamma_2$ subunit composition exists in these synapses (see Fig. 8; Pollard et al., 1995; Khan et al., 1996). Taking these data together, we conclude that the δ subunit-containing receptors ($\alpha_6\beta_{2/3}\delta$) are present exclusively on nonsynaptic membranes, that GABAergic Golgi synapses are heterogeneous with respect to their $GABA_A$ receptor content, and that only one receptor subtype ($\alpha_6\beta_{2/3}\gamma_2$) is present in glutamatergic mossy synapses. Receptors containing both $\alpha1$ and δ subunits have not been reported in cerebellar granule cells. If such receptors exist, they may be located in extrasynaptic membranes also, because both of these subunits are present abundantly on nonsynaptic membranes.

Kinetic and pharmacological properties of $GABA_A$ receptors depend on the subunit composition (Pritchett et al., 1989; Verdoorn et al., 1990; Angelotti and Macdonald, 1993; Macdonald and Olsen, 1994; Sieghart, 1995). Several studies examined these properties of native and recombinant receptors to identify functional fingerprints of different $GABA_A$ receptor subtypes (Puia et al., 1994; Saxena and Macdonald, 1994, 1996; Brickley et al., 1995; Kaneda et al., 1995; Tia et al., 1996a,b). It has been shown that the affinity of $\alpha_1\beta_3\gamma_{2L}$ receptor for GABA (EC_{50} = 13 μM) is ~50-fold lower than that of the $\alpha_6\beta_3\delta$ receptor (EC_{50} = 0.27 μM), the $\alpha_6\beta_3\gamma_{2L}$ receptor having an intermediate affinity (EC_{50} = 1.9 μM; Saxena and Macdonald, 1996). In addition, δ subunit-containing receptors do not desensitize on the prolonged presence of GABA (Saxena and Macdonald, 1994). This is in contrast to $\alpha_1\beta_x\gamma_{2L}$ receptors, which desensitize rapidly with a time constant (τ = ~10 msec; Tia et al., 1996b) somewhat slower than that of the decay of synaptic currents in granule cells (τ = 5–8 msec; Puia et al., 1994; Brickley et al., 1996; Tia et al., 1996a). The $\alpha_6\beta_2\gamma_2$ receptors have a very slow desensitization rate (Tia et al., 1996b). Additional differences between δ and $\gamma2$ subunit-containing receptors include a smaller single channel conductance (22 vs 30 pS for $\alpha_1\beta_1\delta$ and $\alpha_1\beta_1\gamma_{2L}$ receptors, respectively) and a much longer open time (400 vs 5 msec for $\alpha_1\beta_1\delta$ and $\alpha_1\beta_1\gamma_{2L}$ receptors, respectively) of the δ subunit-containing channels (Saxena and Macdonald, 1994). In summary, the $\alpha_6\beta_{2/3}\delta$ receptors have a high affinity for GABA, do not desensitize on the persistent presence of GABA, and have a very long open time. These properties, taken together with the exclusive presence of the $\alpha_6\beta_{2/3}\delta$ receptors on the nonsynaptic plasma membrane of granule cells, indicate that tonic inhibition is mediated mainly by the persistent activation of these receptors by GABA that is present in the extracellular space of glomeruli. The contribution of $\alpha_6\beta_{2/3}\gamma_2$ and $\alpha_1\beta_{2/3}\gamma_2$ receptors to tonic inhibition is probably less, because these receptors have a lower affinity for GABA, they show a more pronounced desensitization, and they also have much shorter open times than the δ subunit-containing receptors. However, these properties suit phasic inhibition because these receptors are concentrated in synaptic junctions, where a high concentration of GABA is present only for a very short period (Maconochie et al., 1994; Jones and Westbrook, 1995; Clements, 1996). Hence, it is likely that phasic inhibition of granule cells is attributable to the transient activation of synaptic $\alpha_6\beta_{2/3}\gamma_2$ and/or $\alpha_1\beta_{2/3}\gamma_2$ receptors. Although the functional role of the two different forms of inhibition is not understood very well, we suggest that tonic inhibition may regulate the passive membrane properties of granule cells (e.g., membrane time constant and input resistance) to influence the time window for synaptic integration (Gabbiani et al., 1994; Hausser and Clark, 1997), whereas phasic inhibition may modify the firing pattern of these cells (Hausser and Clark, 1997). Whether δ subunit-containing receptors are excluded from synaptic junctions of other cell types and whether a tonic form of inhibition is characteristic for every δ subunit expressing cell type remain to be determined.

The previously described enrichment of the $\alpha6$ subunit in glutamatergic mossy fiber to granule cell synapses raised the possibility that this subunit, at this location, may not form functional GABA-gated Cl^- channels (Nusser et al., 1996b), because only this subunit could be detected in these excitatory synapses. Here we have demonstrated that the $\beta2/3$ and $\gamma2$, but not the $\alpha1$ and δ, subunits also are present in some of the mossy fiber synapses. The $\alpha6$, $\beta2/3$, and $\gamma2$ subunits can form functional pentameric $GABA_A$ receptors (Saxena and Macdonald, 1996; Tia et al., 1996b), which indeed occur in the cerebellum *in vivo* (Khan et al., 1994; Quirk et al., 1994; Pollard et al., 1995). Hence it is very likely that the $\alpha6$, $\beta2/3$, and $\gamma2$ subunits form functional receptors in glutamatergic mossy synapses that colocalize with functional AMPA-type glutamate receptors. However, the way in which these $GABA_A$ receptors are activated remains unknown.

Differential targeting of neurotransmitter receptor subtypes on the surface of nerve cells

Most nerve cells in the CNS express a large variety of GABA and glutamate receptor subtypes, which may enable them to respond in a differential manner to the release of the same transmitter. It is important for our understanding of synaptic operation to determine whether every expressed receptor subtype has the same

distribution on the surface of a nerve cell. Immunogold localization of receptors at the electron microscopic level allows us to address this question, because with this method subcellular compartments (e.g., synapses, nonsynaptic plasma membrane, Golgi apparatus, et cetera) can be identified easily, and receptors are labeled with nondiffusible markers (gold particles) with a resolution of 15–30 nm, allowing most immunoparticles to be allocated to certain subcellular compartments. Furthermore, reacting the surface of a resin-embedded electron microscopic section (postembedding reactions) makes quantitative comparisons possible between different tissue elements, because they have similar access to the antibodies.

It has been shown previously that the α1 and α2 subunits of the $GABA_A$ receptor are targeted differentially to GABAergic synapses on hippocampal pyramidal cells (Nusser et al., 1996a). The α1, α2, and α3 subunits also have a nonoverlapping distribution on the surface of retinal α ganglion cells (Koulen et al., 1996). Furthermore, it also has been reported that AMPA-type, NMDA-type, δ glutamate receptors, and the metabotropic glutamate receptor 1α are targeted selectively to a subset of glutamatergic synapses on fusiform cells of the dorsal cochlear nucleus, CA3 pyramidal cells of the hippocampus, and Purkinje cells of the cerebellum (Fritschy et al., 1997; Landsend et al., 1997; Rubio and Wenthold, 1997).

The mechanism by which subcellular segregation of receptors is achieved is as yet unknown. Three possible processes have been suggested previously (Davis et al., 1987; Craig et al., 1994; Racca et al., 1997). In the first one, the receptors are added to the somatic and dendritic plasma membrane nonselectively; they move by lateral diffusion before they are trapped at synaptic sites. In the second process, receptors are packed into different intracellular transport vesicles that move intracellularly and fuse only at the appropriate synaptic sites. According to the third scheme, mRNAs for neurotransmitter receptors are targeted to postsynaptic domains, where receptor proteins are translated and subsequently are inserted in the synaptic membrane. The lack of prominent intracellular labeling for $GABA_A$ receptor subunits in proximal and distal dendrites, although they are present in the ER and Golgi apparatus together with the high density of extrasynaptic receptors (Somogyi et al., 1989; Fritschy and Mohler, 1995; Nusser et al., 1995a,b; Koulen et al., 1996; this study), supports the first scheme. A subsynaptic matrix of receptor-associated proteins (Kannenberg et al., 1997) may play an important role in trapping and anchoring certain receptor subtypes (for review, see Froehner, 1993; Kirsch et al., 1996; Sheng, 1997). We suggest that such subsynaptic matrices may be selective for certain receptor subtypes and may not exist for other ones. Accordingly, $\alpha_1\beta_{2/3}\gamma_2$ receptors, selectively excluded from mossy synapses, may not be able to combine with the anchoring proteins for $\alpha_6\beta_{2/3}\gamma_2$ receptors. Similarly, $\alpha_6\beta_{2/3}\delta$ receptors may not associate with anchoring proteins for either the $\alpha_1\beta_{2/3}\gamma_2$ or the $\alpha_6\beta_{2/3}\gamma_2$ receptors because δ subunit-containing receptors are not present in synaptic junctions.

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