The α6 Subunit of the $GABA_A$ Receptor Is Concentrated in Both Inhibitory and Excitatory Synapses on Cerebellar Granule Cells

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Although three distinct subunits seem to be sufficient to form a functional pentameric $GABA_A$ receptor channel, cerebellar granule cells express mRNA for nine subunits. They receive GABAergic input from a relatively homogeneous population of Golgi cells. It is not known whether all subunits are distributed similarly on the surface of granule cells or whether some of them have differential subcellular distribution resulting in distinct types of synaptic and/or extrasynaptic channels. Antibodies to different parts of the α6 and α1 subunits of the $GABA_A$ receptor and electron microscopic immunogold localization were used to determine the precise subcellular distribution of these subunits in relation to specific synaptic inputs. Both subunits were present in the extrasynaptic dendritic and somatic membranes at lower densities than in synaptic junctions. The α6 and α1 subunits were colocalized in many GABAergic Golgi synapses, demonstrating that both subunits are involved in synaptic transmission in the same synapse. Synapses immunopositive for only one of the α subunits were also found. The α6, but not the α1, subunit was also concentrated in glutamatergic mossy fiber synapses, indicating that the α6 subunit may have several roles depending on its different locations. The results demonstrate a partially differential synaptic targeting of two distinct $GABA_A$ receptor subunits on the surface of the same type of neuron.

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

Cerebellar granule cells receive GABAergic input from a largely homogeneous population of Golgi cells at a restricted location on their distal dendrites. They express mRNA for nine subunits of the $GABA_A$ receptor (Laurie et al., 1992; Persohn et al., 1992). Immunoprecipitation and immunoaffinity purification of native cerebellar $GABA_A$ receptors have revealed several possible subunit combinations (Duggan et al., 1991; McKernan et al., 1991; Endo and Olsen, 1993; Mertens et al., 1993; Pollard et al., 1993; Khan et al., 1994; Mathews et al., 1994; Quirk et al., 1994; Togel et al., 1994). Because only three different subunits are required to form a pentameric functional channel (Sigel et al., 1990; Verdoorn et al., 1990; Angelotti and Macdonald, 1993; Nayeem et al., 1994), the expression of numerous $GABA_A$ receptor channels, differing in their subunit composition, raises at least two possible scenarios regarding their distribution. First, every subunit may be distributed in a similar manner on the surface of granule cells, resulting in identical types of $GABA_A$ receptor channels at synaptic and extrasynaptic sites. This hypothesis is supported by the finding that GABA pulses to nucleated somatic patches, which lack synapses, elicited GABA currents with similar kinetics to spontaneous inhibitory postsynaptic currents (sIPSCs) (Puia et al., 1994). Second, different subunits may have dissimilar subcellular locations on the surface of granule cells, resulting in distinct synaptic and extrasynaptic $GABA_A$ receptors. The latter idea has already been suggested for the α1 versus α6 subunits using immunoperoxidase localization (Baude et al., 1992). However, the immunoperoxidase technique has several limitations: namely, the peroxidase reaction end-product diffuses from the site of the antibody and, as a result, the precise origin of the reaction cannot be determined. Furthermore, quantitative differences cannot be assessed in the label intensity between synaptic and extrasynaptic sites. These problems are overcome by immunogold localization, which provides a quantifiable and nondiffusible label with a resolution of ~20 nm (Triller et al., 1985; Hansen et al., 1991; Baude et al., 1993; Caruncho et al., 1993; Fujimoto, 1993; Nusser et al., 1994, 1995a,b).

We have reported the relative densities of immunoreactive α1 and β2/3 subunits in synapses between Golgi cell terminals and granule cell dendrites using a quantitative immunogold method (Nusser et al., 1995b). Approximately 180- to 230-fold higher density of immunolabel could be found at synaptic sites than on the somatic membrane for the α1 and β2/3 subunits. Furthermore, the immunolabeling density for the α1 subunit suggested two populations of Golgi synapses, having either a high or a low concentration of α1 subunit. It is possible that synapses having low levels of the α1 subunit contain more α6 subunit, the other α subunit abundantly expressed by granule cells. Cloned α6βxγ2 receptors, in contrast to α1βxγ2 receptors, have low affinity for benzodiazepines but bind the partial inverse agonist Ro15-4513 with high affinity (Sieghart et al., 1987; Luddens et al., 1990; Quirk et al., 1994). Thus, it was of particular interest to establish the distribution of the α6 subunit in relation to synapses immunoreactive for the α1 subunit. We used immunogold localization and three subunit-specific antibodies, recognizing distinct parts of the

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α6 subunit, to define the distribution of the α6 subunit on the surface of cerebellar granule cells.

MATERIALS AND METHODS

Preparation of animals and tissue

Nine female Wistar rats (120–200 gm) were anesthetized with Sagatal (pentobarbitone sodium, 220 mg/kg, i.p.) and perfused through the heart with 0.9% saline followed by the fixative containing 4% *p*-formaldehyde, 0.05% glutaraldehyde, and ~0.2% picric acid dissolved in 0.1 M phosphate buffer (PB), pH 7.4, for 7–15 min (Somogyi et al., 1989). After perfusion, the brains were removed and blocks from the vermis of the cerebellar cortex were cut out and washed in several changes of 0.1 M PB.

Antibodies

Three antibodies against different parts of the α6 subunit of the $GABA_A$ receptor were used for immunocytochemistry.

(1) Affinity-purified polyclonal antibody (R54XV) was raised against a synthetic peptide corresponding to residues 1–15 of the amino acid sequence of the bovine α6 subunit with an additional C-terminal cysteine (Thompson et al., 1992). Antibody R54XV was *preadsorbed* to aldehyde-fixed forebrain sections before immunoblotting and immunocytochemistry.

Adsorption of antibody R54XV to fixed forebrain. Three female Wistar rats were perfused through the heart as described above; 70- to 100-μm-thick forebrain sections were cut with a vibratome, and the sections were incubated in blocking solution consisting of 20% normal goat serum containing Tris-buffered saline (TBS), pH 7.4, for 1–2 hr before incubation in primary antibody R54XV at a final concentration of 1.1–5.3 μg of protein/ml. After adsorption for 2–3 hr, the antibody solution was directly applied for pre- and postembedding immunohistochemistry and for immunoblotting.

Brain membrane preparation and immunoblotting. Four brain regions, the cerebral cortex, cerebellum, hippocampus, and brainstem, were dissected from adult rat brain, and membrane fractions were prepared according to the method of Duggan et al. (1991). Protein concentrations were determined by the Lowry method (Lowry et al., 1951). Immunoblotting was carried out as described by Pollard et al. (1993) using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 10% polyacrylamide minislab gels under reducing conditions, with a final antibody concentration of 2.5 μg/ml and the enhanced chemiluminescence (ECL) detection method. The ECL Western blotting system, Hyperfilm-ECL, and horseradish peroxidase-linked secondary antibody were from Amersham (Bucks, UK). Anti-α1(413–429) $GABA_A$ receptor subunit antibody (Pollard et al., 1993) was used only for immunoblotting.

(2) Rabbit affinity-purified polyclonal antibody (P24) was raised against a synthetic peptide corresponding to residues 429–434 of the amino acid sequence of rat α6 subunit.

Brain membrane preparation and immunoblotting. Membranes from adult rat cerebellum were subjected to SDS-PAGE (Sieghart et al., 1987). Proteins were transferred to nitrocellulose and probed with 5 μg/ml polyclonal antipeptide antibody P24 in the presence or absence of 50 μg/ml corresponding peptide α6(429–434). Bound antibodies were detected with biotinylated goat anti-rabbit IgG (Gibco, Gaithersburg, MD), streptavidin–alkaline phosphatase conjugate (Amersham), and an alkaline phosphatase conjugate substrate kit. The experiment was performed three times with similar results.

(3) Rabbit affinity-purified polyclonal antibody α6(313–395) was raised against the putative cytoplasmic loop polypeptide of the α6 subunit as described previously (Baude et al., 1992; Quirk et al., 1994). This antibody was able to precipitate ~60% of cerebellar [^{3}H]muscimol and 55% of the [^{3}H]Ro15-4513 binding sites from detergent-solubilized extracts. Western blot analysis revealed one band with M_r 58 kDa in the cerebellum but not in cortex, hippocampus, or striatum (Quirk et al., 1994).

In addition, affinity-purified rabbit polyclonal antibodies to the α1 subunit were used (Zezula et al., 1991; Zimprich et al., 1991). The antiserum (P16) was raised to a synthetic peptide corresponding to residues 1–9 of the rat α1 subunit. This antiserum recognizes a single protein with M_r 51 kDa in immunoblots of rat brain membranes or of affinity-purified $GABA_A$ receptors (Zezula et al., 1991). A band with identical mobility was photolabeled by [^{3}H]flunitrazepam. The purified antibodies used in immunoaffinity purification retained between 25 and 40% of specific [^{3}H]flunitrazepam binding from rat brain (Zezula et al., 1991).

Antibody specificity for the polyclonal antibody to GABA (no. 9) has been described previously (Hodgson et al., 1985).

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 rabbit serum, or after antibodies R54XV and P24 were preadsorbed with the corresponding peptides (50 μg of peptide/ml). Using the rabbit polyclonal antibody to GABA (Hodgson et al., 1985), no plasma membrane labeling was observed with our method, indicating that the labeling observed on the plasma membrane was attributable to the anti-receptor antibodies P24, preadsorbed R54XV, α6(313–395), and P16. Antibodies P24, α6(313–395), and preadsorbed antibody R54XV selectively stained the cerebellar granule cell layer, demonstrating that the protein(s) recognized by these antibodies is expressed at high concentrations by cerebellar granule cells. Using antibodies P24 and preadsorbed R54XV under postembedding conditions, no immunolabeling was observed at symmetrical or asymmetrical synapses in the cerebellar molecular layer or in the hippocampal CA3 area. None of our antibodies recognizes glutamate receptor (GluR) subunits, which are known to be expressed by cerebellar granule cells when they are expressed in COS-7 cells. Although the GluRD subunit could not be tested in transfected cells, this subunit is expressed strongly in the cerebellar molecular layer (Sato et al., 1993), where none of our α6 subunit antibodies labeled any structure at light or electron microscopic levels.

Immunostaining of COS-7 cells transfected with GluR

COS-7 cells were grown in Dulbecco's modified Eagle's medium containing fetal calf serum supplemented with penicillin/streptomycin (100 mg/ml) and sodium pyruvate (1 mM). Cells were transfected with DNA for GluRB, NR1A, NR2A, NR2B, and NR2C subunits at 0.9–1.5 μg of DNA/25 cm^2 flask using *O*-(diethylaminoethyl)-dextran as described by Seed and Aruffo (1987). Cells were cultured on glass coverslips, and all subsequent procedures were performed on the coverslip-grown monolayer cells. The cells were washed twice in 0.1 M PB before fixation in 4% *p*-formaldehyde for 10 min. After several washes, the cells were permeabilized in 0.3% Triton X-100 containing PB for 5 min. The coverslips were then washed in TBS, pH 7.4, before blocking in 20% normal goat serum containing TBS for 30 min. The coverslips were then transferred to a moist chamber and overlaid with the primary antibody solution (1–5 μg of protein/ml). The incubation was performed at room temperature for 2 hr followed by three washes in TBS. The sections were then incubated in a solution of horseradish peroxidase-conjugated swine anti-rabbit IgG for 1 hr at room temperature. Peroxidase reaction was carried out as described below. The cells were then dehydrated and mounted in a synthetic medium (XAM; BDH Chemicals, Poole, UK) for light microscopy. The success of transfection was ascertained by immunostaining the COS-7 cells with specific antibodies to GluR subunits.

Pre-embedding immunohistochemistry

Normal goat serum was used in TBS as the blocking solution for 1 hr, and then the purified primary antibodies were used at a final protein concentration of 1.1, 1.7, and 5 μg/ml for preadsorbed R54XV, P24, and α6(313–395), respectively. After washing, the sections were incubated for 90 min in either biotinylated (Vector, Peterborough, UK) or 1.4 nm gold-coupled goat anti-rabbit IgG (Nanogold, Nanoprobes, Stony Brook, NY). The sections for peroxidase reaction were incubated in avidin-biotinylated horseradish peroxidase complex (diluted 1:100 in TBS) for 2 hr before peroxidase enzyme reaction was carried out with diaminobenzidine as chromogen and H_2O_2 as oxidant. Gold particles (1.4 nm) were silver-enhanced with an HQ Silver kit as described by the manufacturer (Nanoprobes) for 10–15 min. Sections were then processed routinely for electron microscopic examination (Somogyi et al., 1989).

Freeze substitution and Lowicryl embedding

The same procedure was used as described previously (Baude et al., 1993; Nusser et al., 1995a). Briefly, after perfusion blocks of tissue were washed in 0.1 M PB followed by vibratome sectioning (500 μm thickness) and washing in 0.1 M PB overnight. The sections were placed into 1 M sucrose solution in 0.1 M PB for 2 hr for cryoprotection before slamming, freeze-substitution, and embedding in Lowicryl HM 20 (Chemie GmbH, Deisenhofen, Germany).

Postembedding immunocytochemistry on electron microscopic sections

Postembedding immunocytochemistry was carried out on 70-nm-thick sections of slam-frozen, freeze-substituted, Lowicryl-embedded cerebel-

lar cortex from four blocks of three animals. The sections were picked up on pioloform-coated nickel grids (100 mesh). Next they were incubated on drops of blocking solution for 30 min and then incubated on drops of primary antibodies overnight at room temperature. The blocking solution, which also was used for diluting the primary and secondary antibodies, consisted of 0.1 M phosphate-buffered saline (PBS), pH 7.4, containing 0.8% ovalbumin (Sigma, St. Louis, MO), 0.1% cold-water fish skin gelatin (Sigma), and 5% fetal calf serum. Immunostaining was carried out using affinity-purified polyclonal antibodies P24, P16, and preadsorbed R54XV at final concentrations of 5.2, 7, and 5.3 μg of protein/ml, respectively. Antiserum to GABA (no. 9) was used at a final dilution of 1:2500. After incubation in primary antibody, sections were washed and incubated on drops of goat anti-rabbit IgG coupled to 1.4 nm gold (diluted 1:100, Nanoprobes) for 2 hr at room temperature. After several washes, sections were fixed in a 2% glutaraldehyde solution, dissolved in 0.1 M PB for 2 min, and then transferred to drops of ultrapure water before silver enhancement in the dark with an HQ Silver kit (Nanoprobes) for 4–5.5 min. After further washing in ultrapure water, the sections were contrasted with saturated aqueous uranyl acetate and then lead citrate.

Identification of subcellular profiles in the cerebellar granule cell layer

The electron microscopic appearance of the slam-frozen, freeze-substituted, Lowicryl-embedded tissue is different from that of osmium-treated, epoxy resin-embedded material. The criteria used to identify the subcellular profiles were as follows.

(1) Golgi cell terminals: immunopositive for GABA; mainly located at the periphery of the glomeruli; smaller size compared with mossy terminals; vesicles are generally visible; making synaptic contacts with granule cell dendrites.

(2) Mossy fiber terminals: immunonegative for GABA; large number of mitochondria in the center; vesicles are generally visible; large size; mainly located in the middle of the glomeruli; establishing asymmetrical (type I) synapses with granule cell dendrites and, occasionally, with dendrites of other neurons.

(3) Granule cell dendrites: immunonegative for GABA; having ovoid or spheroid shape with diameters between 0.5 and 1 μm on average; no synaptic vesicles; may have puncta adherentia between them.

(4) Granule cell body: immunonegative for GABA; oval or round soma 5–8 μm in diameter; little cytoplasm.

RESULTS

Antibodies

The subcellular distribution of the α6 subunit of the $GABA_A$ receptor on cerebellar granule cells has been revealed by pre- and postembedding immunogold methods using three affinity-purified polyclonal antibodies against distinct parts of the subunit.

The experiments described below were performed at the School of Pharmacy, London. Antibody R54XV was raised against a 16-amino-acid-long peptide (Thompson et al., 1992) corresponding to the N-terminal end of the α6 subunit and was prepared in an animal not reported previously. In agreement with previous observations using antiserum from a different animal, it was found that antibody R54XV recognized an M_r 58 kDa polypeptide in cerebellar membranes. A polypeptide in the same position could be detected after immunoaffinity purification of the receptor and also could be photolabeled by [^{3}H]Ro15-4513 (Pollard et al., 1993; Thompson and Stephenson, 1994). Similar proteins were undetectable in either cerebral cortical or hippocampal membranes (Fig. 1*A*). Weak immunoreactivity at the M_r 58 kDa position was seen in membranes prepared from brainstem with extended film exposure times (data not shown). This band probably corresponds to the $GABA_A$ receptor α6 subunit expressed by granule cells in the cochlear nuclei (Varecka et al., 1994). Additional bands reactive with this antibody were found in all four brain regions tested. After preadsorption of the antibody to aldehyde-fixed forebrain sections, the additional immunoreactive bands disappeared (Fig. 1*A*). Some immunoreactivity was still detectable, however, at the interface of the stacking and running gels (compare Fig. 1*A*, *lanes 7–10*), which may correspond to higher-M_r aggregated proteins. Antibody R54XV was used for both pre- and postembedding immunocytochemistry.

The experiments described below were performed at the Department of Biochemical Psychiatry, Vienna. Antibody P24 was raised against a synthetic peptide corresponding to residues 429–434 of the C-terminal end of the protein. Immunoblot analysis with antibody P24 of cerebellar membranes revealed a major immunoreactive band with M_r 57 kDa (Fig. 1*B*). The immunolabeling of the M_r 57 kDa protein could be prevented by the preadsorption of the antibody with the corresponding peptide (Fig. 1*B*). The M_r 57 kDa protein could be photolabeled by [^{3}H]Ro15-4513 (Togel et al., 1994). Antibody P24 also was used for both pre- and postembedding immunohistochemistry.

Antibody α6(313–395) was raised against the putative cytoplasmic sequence between the third and fourth transmembrane domains (Baude et al., 1992; Quirk et al., 1994) and was suitable only for pre-embedding immunohistochemistry.

Distribution of immunoreactivity for the α6 subunit of the $GABA_A$ receptor as detected by light microscopy

Antibodies α6(313–395), P24, and purified R54XV selectively stained the cerebellar granule cell layer (Fig. 2*A–C*), as reported previously using antibody α6(313–395) (Baude et al., 1992) or an antibody similar to R54XV but originating from another animal (Thompson et al., 1992; Turner et al., 1994). The molecular layer and the white matter showed no immunoreactivity (Fig. 2*A–C*). No immunoreactivity could be observed in the forebrain using any of our antibodies. However, immunoreactive cells were found in the cochlear nuclei (data not shown), which is in line with the strong mRNA expression in cochlear granule cells (Varecka et al., 1994).

Pre-embedding immunogold localization of the α6 subunit of the $GABA_A$ receptor

Strong immunostaining of synapses made by Golgi cell terminals with granule cell dendrites has been reported using electron microscopic immunoperoxidase localization with antibody α6(313–395) (Baude et al., 1992). In this study, we applied immunogold localization of the α6 subunit. The irregularly shaped silver particles produced by silver enhancement of the 1 nm gold particles coupled to secondary antibody were always associated with granule cell plasma membranes (Fig. 2*D–F*). The extrasynaptic dendritic and somatic membrane showed strong immunoreactivity for the α6 subunit with all of our antibodies (Fig. 2*D–F*). Using antibodies to the C- and N-terminal ends of the protein, immunoparticles were located at the external face of the plasma membrane (Fig. 2*F*), corresponding to the predicted extracellular positions of the epitopes recognized by these antibodies. However, using antibody α6(313–395) against the putative cytoplasmic polypeptide, particles were always located at the internal face of the somatic and dendritic membranes (Fig. 2*D,E*), corresponding to the intracellular location of the epitope(s). Asymmetrical synapses between mossy fiber terminals and granule cell dendrites were always immunonegative with the pre-embedding method (Fig. 2*D–F*). Synaptic junctions made by GABAergic Golgi cell terminals with granule cell dendrites were also

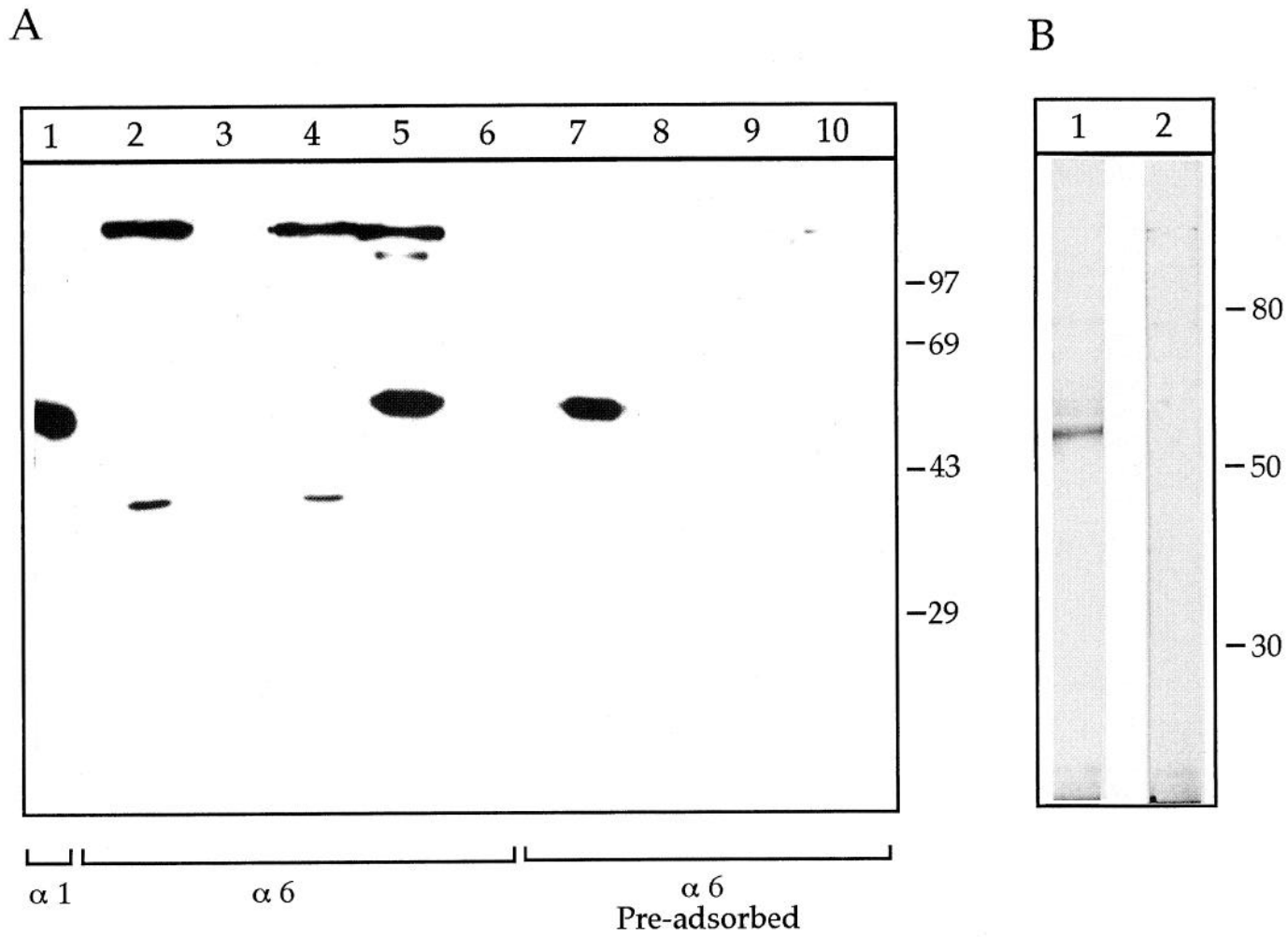


Figure 1. Demonstration of the specificity of antibody R54XV preadsorbed to fixed forebrain sections (*A*) and antibody P24 (*B*). The positions of prestained protein standards (in kDa) are shown on the *right*. *A*, Membranes were prepared from four brain regions of adult rat and used as antigens (25 μg of protein/gel lane) in immunoblots using the ECL detection method. The antibodies used were affinity-purified R54XV before (*lanes 2–6*) or after (*lanes 7–10*) adsorption to fixed forebrain sections and anti-α1(413–429) affinity-purified antibody at a final concentration of 2.5 μg of protein/ml. Gel lanes are as follows: *1, 6*, benzodiazepine affinity-purified $GABA_A$ receptor (10 μl); *2, 10*, cerebral cortical membranes; *3, 9*, brainstem membranes; *4, 8*, hippocampal membranes; *5, 7*, cerebellar membranes. Antibody R54XV recognized an M_r 58 kDa protein in cerebellar membranes only (*lanes 5* and *7*). Additional bands were also found. After adsorption of antibody R54XV to fixed forebrain sections, most of the additional immunoreactivity disappeared. *B*, Immunoblots of proteins from rat cerebellar membranes, probed with 5 μg/ml polyclonal antibody P24, in the absence (*lane 1*) or the presence (*lane 2*) of 50 μg/ml corresponding peptide α6(429–434). Antibody P24 predominantly labeled an M_r 57 kDa protein. Labeling of the M_r 57 kDa protein was prevented in the presence of the peptide α6(429–434).

immunonegative for the α6 subunit (Fig. 2*D,F*) as well as for the α1 and β2/3 subunits using *pre-embedding* immunogold localization (Nusser et al., 1995b). However, when the α1 and β2/3 subunits were localized with the postembedding method, Golgi synapses were strongly and selectively immunopositive for these subunits (Nusser et al., 1995b). Therefore, postembedding immunogold reactions were carried out on Lowicryl-embedded cerebellum for the α6 subunit.

Postembedding localization of the α6 subunit of the $GABA_A$ receptor

Immunoreactions were carried out on slam-frozen, freeze-substituted, and Lowicryl-embedded cerebellum. The electron microscopic appearance of a Lowicryl-embedded tissue differs from that of osmium-treated, epoxy resin-embedded tissue, but cellular profiles such as granule cell soma, granule cell dendrites, mossy fiber, and Golgi cell terminals can be identified by anatomical criteria (see Materials and Methods). Immunoreactions for GABA were also carried out on sections serial to the receptor-reacted sections to identify GABAergic terminals (Fig. 4*B*).

An enrichment of immunoparticles for the α6 subunit was observed in synaptic junctions between Golgi cell terminals and granule cell dendrites using antibodies to both the C-terminal (Figs. 3, 5) and the N-terminal domains of the subunit (Figs. 3, 4). Particles could be present along the entire length of the synaptic specialization with an abrupt decrease in labeling outside the synapses (Figs. 3–5). No specific immunosignal could be obtained using antibody α6(313–395) under postembedding conditions because of either the lower antibody sensitivity or the inaccessibility of the epitope(s) recognized by this antibody. The postembedding method also confirmed the extrasynaptic location of the α6 subunit on dendritic and somatic membranes, but the density of labeling was much lower than at synapses (Figs. 3–5).

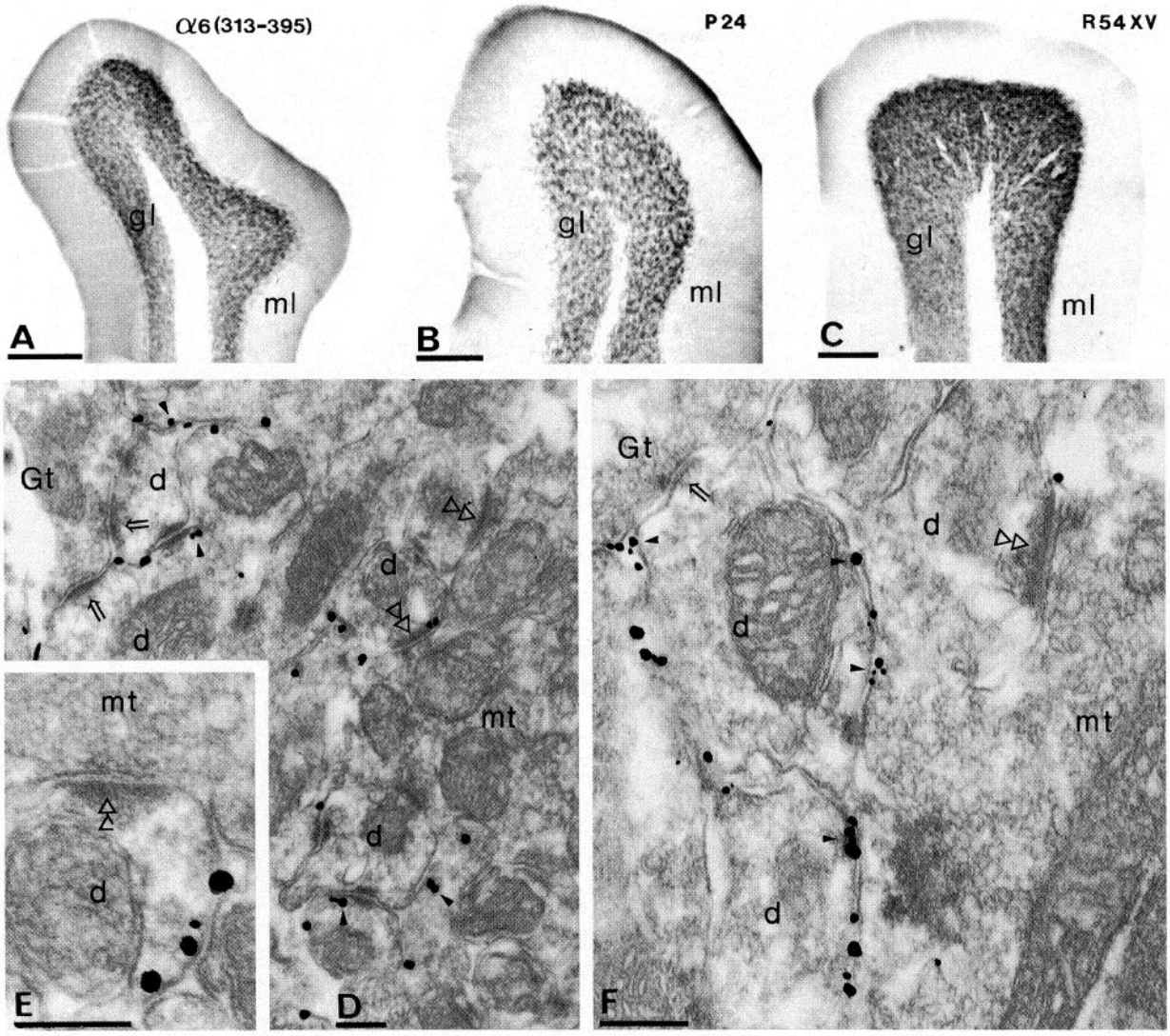
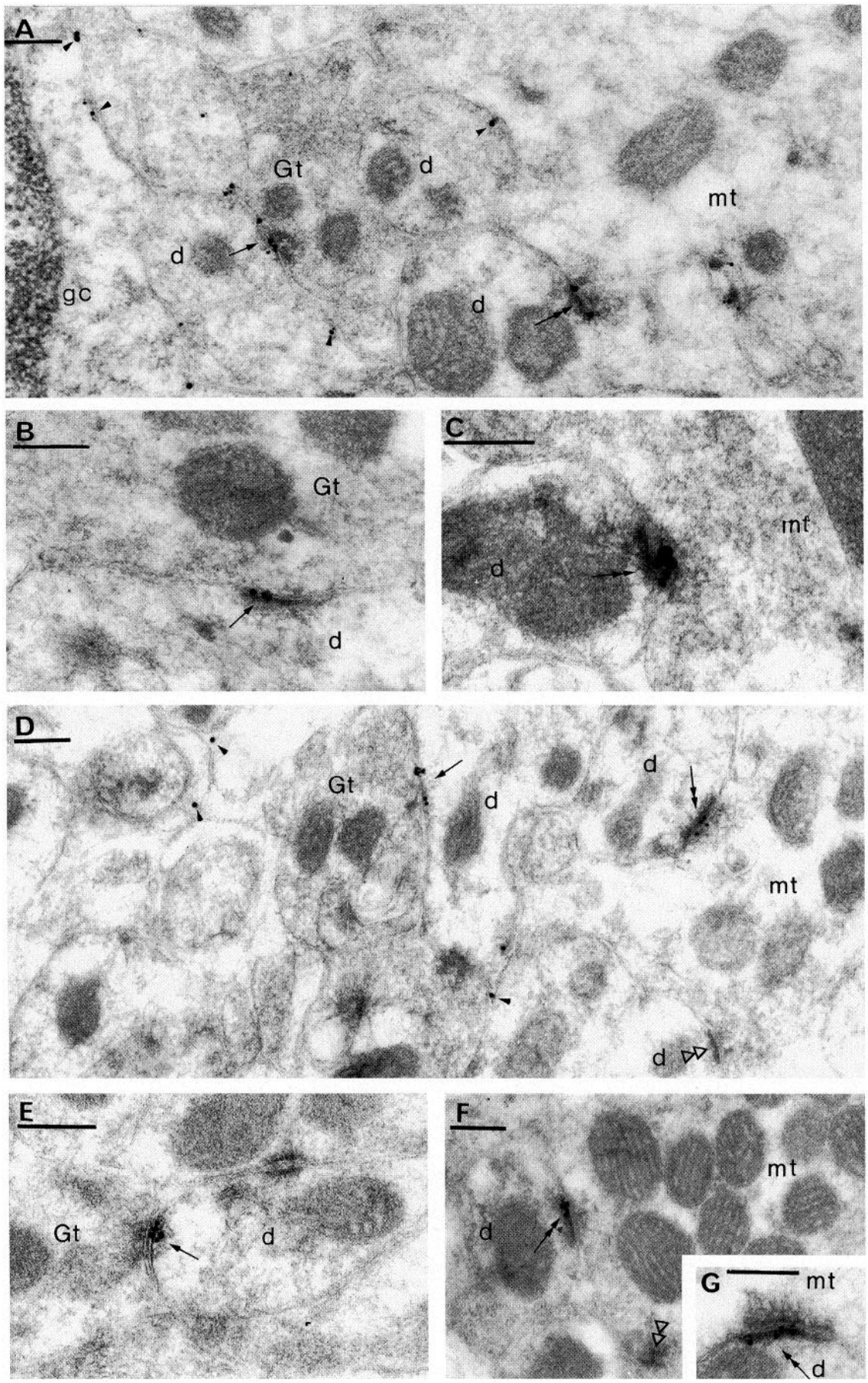


Figure 2. Distribution of immunoreactivity for the α6 subunit of the $GABA_A$ receptor in the cerebellar cortex as revealed by either light microscopy (*A–C*) or electron microscopy (*D–F*) using polyclonal antibody α6(313–395) (*A, D, E*); antibody P24 to the C-terminal end of the protein (*B*) and purified antibody R54XV to the N-terminal end of the subunit (*C, F*). *A–C*, The granule cell layer (*gl*) shows similarly strong immunoreactivity, particularly concentrated to the glomeruli, using any of our antibodies. The molecular layer (*ml*) and the white matter are immunonegative. *D–F*, Using the pre-embedding immunogold method, immunoparticles were present along the extrasynaptic dendritic membrane (*d*) of granule cells. Synapses between presumed Golgi cell terminals (*Gt*) and granule cell dendrites (*d*) are always immunonegative with this method (*open arrows* in *D* and *F*), as are synapses (*open double triangles* in *D–F*) made by mossy fiber terminals (*mt*). Particles are located at the internal face (*D, E*) or at the external face (*F*) of the plasma membrane corresponding to the intra- or extracellular location of epitopes recognized by antibodies α6(313–395) and R54XV, respectively. Scale bars: *A–C*, 50 μm; *D–F*, 0.2 μm.

Comparison of the pre- and postembedding immunogold localization of receptors

When colloidal gold particles are used to visualize the immunosignal, particles >5 nm in diameter are easily detectable in the electron microscope, but immunoglobulins coupled to these large particles do not penetrate easily into the tissue. Therefore, in the present study secondary antibodies coupled to 1.4 nm gold particles were used to facilitate the penetration of immunoreagents in the pre-embedding method. Silver intensification of the gold particles was carried out to make the particle size detectable in the electron microscope. In the pre-embedding methods (both peroxidase and immunogold), using antibodies recognizing epitope(s) on either the intra- or the extracellular side of the membrane, the immunosignal does not penetrate through the lipid bilayer without detergent treatment. Therefore, the pre-embedding methods are reliable to determine the location of the epitope(s) in relation to the two faces of the plasma membrane (Baude et al., 1995; Nusser et al., 1995a,b). However, in the postembedding method the surface of the ultrathin section is reacted and the particles can be displaced from the epitope(s) by ~20 nm, which is greater than the width of the membrane. Therefore, the postembedding immunogold method is not reliable to determine the location of the epitope(s) in relation to the two faces of the plasma membrane.

Synaptic receptors could not be detected using the pre-embedding immunogold method with several antibodies against the α1, α6, and β2/3 subunits of the $GABA_A$ receptor (Nusser et al., 1995a,b) (this work) or against the GluRA and GluRB/C subunits of the glutamate receptor (Baude et al., 1995). However,


A
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when postembedding immunogold localization was applied on Lowicryl-embedded materials for the same subunits with the same antibodies, immunoparticles for ionotropic $GABA_A$ and glutamate receptors were found to be concentrated in the main body of the synaptic junctions (Nusser et al., 1994, 1995a,b; Baude et al., 1995) (this work). The false negative results on synaptic junctions, obtained with the pre-embedding immunogold method, may be explained by the inaccessibility of epitopes for antibodies in the synaptic cleft and in the dense protein network of the postsynaptic density using aldehyde-fixed brain tissues without strong detergent treatment. In the postembedding method, the surface of the electron microscopic section is in direct contact with the antibodies; therefore, there is no difference between the sectioned and exposed synaptic or extrasynaptic membranes in their access to antibodies, making the synaptic localization possible.

Colocalization of the α1 and α6 subunits in Golgi synapses

To characterize further the α-subunit content of α1-subunit-immunopositive Golgi synapses (Nusser et al., 1995b), we applied immunogold labeling for the α1 and α6 subunits on consecutive electron microscopic sections of rat cerebellum. Numerous Golgi synapses were found to be immunopositive for both the α1 and the α6 subunits using antibodies P16 and R54XV (Fig. 4*C,D*) or antibodies P16 and P24 (Fig. 5).

In addition to Golgi synapses having both α1 and α6 subunits, synapses were observed being immunopositive for the α1 but immunonegative for the α6 subunit (Fig. 5) or vice versa (data not shown). Immunonegativity of a certain synapse can be attributable either to a genuine absence of receptors or to technical limitations, such as the lack of preservation of receptor antigenicity and/or the variation of immunoreactivity within the embedded block of tissue, thus resulting in uneven signal intensity within the section. Furthermore, the inaccessibility of receptors in tangentially cut synapses that do not reach the surface of the sections may also cause false immunonegativity. Nevertheless, immunonegative synapses were present in well immunoreacted areas surrounded by positive synapses within a few micrometers (Fig. 5). The antigenicity of receptors generally was well preserved in the synapses that were immunonegative for one or the other subunit, because in serial sections of the same synapse immunoreactivity for another $GABA_A$ receptor subunit could be demonstrated under identical conditions (Fig. 5).

Enrichment of immunoreactive α6 subunits in mossy synapses

Surprisingly, immunoreactivity for the α6 subunit was also observed in asymmetrical synapses between the glutamatergic mossy fiber terminals and granule cell dendrites (Figs. 3–5). Regardless of whether antibodies to the C-terminal (Figs. 3, 5) or the N-terminal (Fig. 3) regions of the subunit were used for postembedding localization, an enrichment of immunoparticles was detected in many mossy synapses. The labeling intensity of immunopositive mossy synapses (3.21 ± 1.34 particles/synapse; $n = 62$) was comparable with that of Golgi synapses (3.68 ± 1.32 particles/synapse; $n = 38$). Interestingly, not every mossy synapse showed immunoreactivity within a well reacted area (Fig. 3; see above for criterion). Identical results were obtained in a total of four blocks from three 6- to 9-week-old rats. Immunopositive mossy synapses were found in lobuli IXb and X in the same block and in three blocks from other parts of the vermis of the cerebellar cortex.

DISCUSSION

The α6 subunit of the $GABA_A$ receptor is also present at extrasynaptic sites

Using electron microscopic immunoperoxidase localization, it was found that the α6 subunit of the $GABA_A$ receptor is present mainly, if not exclusively, in synapses between cerebellar Golgi cell terminals and granule cell dendrites (Baude et al., 1992). Although immunoperoxidase localization of transmembrane proteins is a sensitive method, it has several disadvantages (see introductory remarks) compared with the more recently introduced immunogold technique. The present results, using immunogold localization, confirm the concentration of the α6 subunit in Golgi synaptic junctions and, in addition, reveal its widespread extrasynaptic distribution similar to that reported for the α1 and β2/3 subunits (de Blas et al., 1988; Somogyi et al., 1989; Nusser et al., 1995b).

Cloned $GABA_A$ receptors containing only the α1 as α subunit have high affinity for benzodiazepines (Pritchett et al., 1989), whereas receptors with the α6 subunit have low affinity for benzodiazepines but high affinity for the partial inverse agonist Ro15-4513 (Luddens et al., 1990). One report has shown that α6 subunit-containing receptors contain a single type of α subunit that has low affinity for benzodiazepines (Quirk et al., 1994). However, other immunoprecipitation studies suggest that the α1 and α6 subunits sometimes coexist in the same receptor complex (Pollard et al., 1993; Khan et al., 1994). These receptors have low affinity for benzodiazepines (Khan et al., 1994). Thus, potentially several populations of pharmacologically distinct $GABA_A$ receptors may be present on the extrasynaptic plasma membrane of granule cells. Indeed, Puia et al. (1994) reported the presence of diazepam-sensitive and -insensitive $GABA_A$ receptors at both synaptic and extrasynaptic sites in 14- to 18-d-old rats. The presence of more than one type of $GABA_A$ receptor on the extrasynaptic somatic membrane is supported further by outside-out patch-clamp recordings revealing two or three different $GABA_A$ receptor channels (Kaneda et al., 1994; Brickley et al., 1995b).

Colocalization of the α1 and α6 subunits in GABAergic Golgi synapses

The subunit composition of synaptic $GABA_A$ receptors is uncertain in cells that express more than three different subunits. The use of subunit-specific antibodies has shown the presence of two distinct α subunits in native $GABA_A$ receptors (Duggan et al., 1991; Endo and Olsen, 1993; Mertens et al., 1993; Pollard

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Figure 3. Electron photomicrographs showing immunoreactivity for the α6 subunit in the cerebellar glomeruli as demonstrated by postembedding immunogold localization using polyclonal antibodies to the N-terminal (*A–C*) or C-terminal (*D–G*) end of the protein. An enrichment of immunoparticles is detected in synapses made by both Golgi cell terminals (*Gt; arrows* in *A, B, D,* and *E*) and some mossy fiber terminals (*mt; double arrows* in *A, C, D, F,* and *G*) with granule cell dendrites (*d*). Golgi terminals are always located at the periphery of glomeruli near the somata of granule cells (*gc* in *A*). Immunonegative mossy synapses are also found in the same glomeruli (*double open triangles* in *D* and *F*). Note that immunoparticles can be present at any site along the entire length of synaptic junctions. Particles are also present on the extrasynaptic somatic and dendritic membranes (*arrowheads* in *A* and *D*). Scale bars, 0.2 μm.

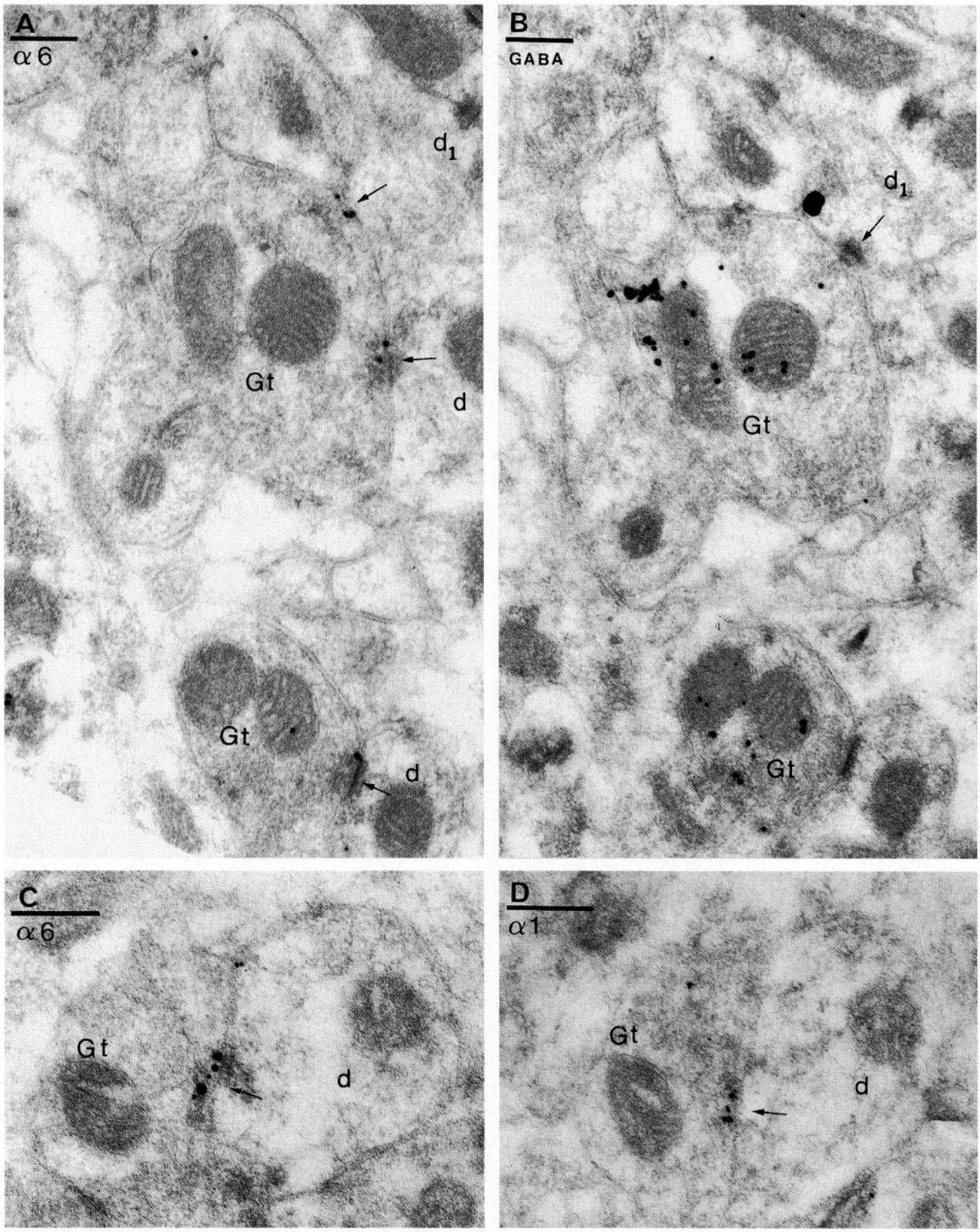

Figure 4. Electron micrographs showing immunoreactivity for the α6 (*A* and *C*; N-terminal antibody) or α1 subunits (*D*; antibody P16) of the $GABA_A$ receptor and for GABA (*B*) on serial (*A* to *B*; *C* to *D*) ultrathin sections of rat cerebellum. Postembedding reactions. *A, B*, Immunoparticles for the receptors are located in synaptic junctions (*arrows* in *A*) between GABA-immunopositive (note the enrichment of immunoparticles in *B*) Golgi cell terminals (*Gt*) and granule cell dendrites (*d*). Three immunoparticles are located between a Golgi cell terminal and a granule cell dendrite (d_1) at a site where the synaptic junction is not evident. However, on the next serial section (*B*) the synaptic specialization is clearly visible. *C, D*, Electron microscopic demonstration of the colocalization of the α6 (*C*) and the α1 (*D*) subunits of the $GABA_A$ receptor in a synaptic junction (*arrows* in *C* and *D*) made by a Golgi cell terminal (*Gt*) with a granule cell dendrite (*d*). Scale bars, 0.2 μm.

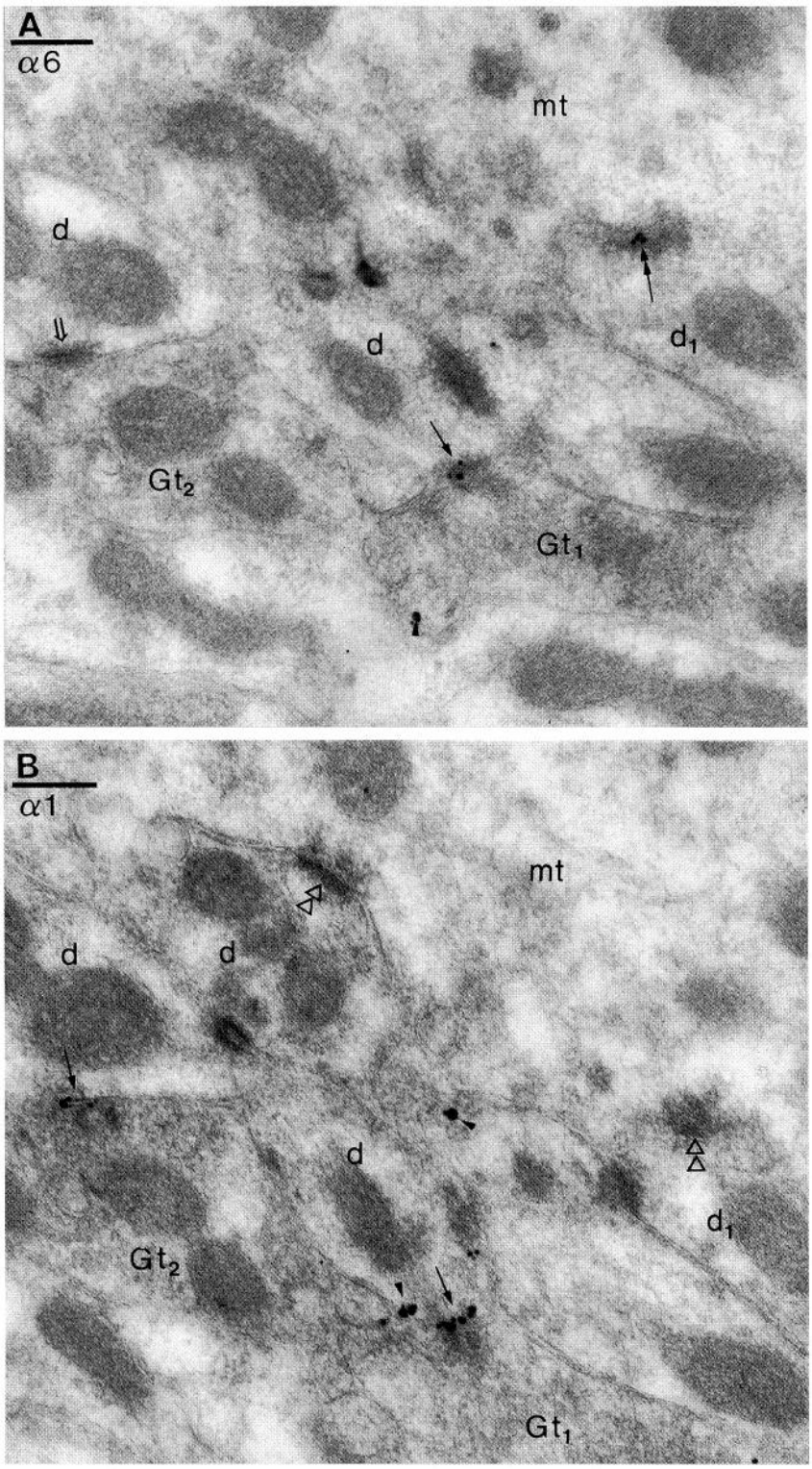

Figure 5. Serial ultrathin sections reacted for either the α6 (*A*; C-terminal antibody) or the α1 (*B*; antibody P16) subunit of the $GABA_A$ receptor. A synapse (*arrow*) made by a Golgi cell terminal (Gt_1) with a granule cell dendrite (*d*) shows immunoreactivity for both the α6 and the α1 subunits. Another synaptic junction between a Golgi cell terminal (Gt_2) and a granule cell dendrite is immunopositive for the α1 (*arrow* in *B*) but immunonegative for the α6 subunit (*open arrow* in *A*). Mossy synapses are immunonegative for the α1 subunit (*mt*; *double open triangles* in *B*), but one of them is immunopositive for the α6 subunit (on d_1; *double arrow* in *A*). Immunoparticles are also present at extrasynaptic sites (*arrowheads*). Scale bars, 0.2 μm.

et al., 1993; Khan et al., 1994); however, the lack of coexistence of different α subunits has also been reported (McKernan et al., 1991; Quirk et al., 1994). In these studies, the receptors may derive from synaptic and extrasynaptic plasma membranes as well as from the intracellular pools. The amount of extrasynaptic receptors is not negligible, because we have estimated previously that the total population of extrasynaptic receptors on granule cells exceeds the synaptic population severalfold (Nusser et al., 1995b).

Two α subunits that produce pharmacologically distinct $GABA_A$ receptors when expressed separately, α1 and α2, have been colocalized in patches on the surface of neurons in the globus pallidus using immunofluorescence double labeling (Fritschy et al., 1994). The α1 and α6 subunits have been colocalized in patches on the surface of cultured cerebellar granule cells, but the relationship of the patches to synapses is unknown in such cultures (Caruncho and Costa, 1994). The present colocalization of the α1 and α6 subunits in the *same*

Golgi cell synapse does not resolve if the two polypeptides are coassembled to form one $GABA_A$ receptor—they segregate to separate channels, or both possibilities occur. However, our results suggest that the two pharmacologically distinct synaptic $GABA_A$ receptor channels (Puia et al., 1994) correspond to $GABA_A$ receptors containing only $\alpha 1$ as α subunit and to $\alpha 6$ subunit-containing receptors.

Heterogeneity of Golgi synapses according to their α subunit content

Quantitative measurements of $GABA_A$ receptor immunoreactivity in Golgi synapses revealed one synapse population for the $\beta 2/3$ subunits and two populations for the $\alpha 1$ subunit of the $GABA_A$ receptor. A possible explanation for the existence of two Golgi synapse populations with respect to their $\alpha 1$ subunit content is that synapses having lower $\alpha 1$ subunit immunoreactivity contain higher concentrations of the $\alpha 6$ subunit (Nusser et al., 1995b). Although numerous Golgi synapses were immunopositive for both the $\alpha 1$ and $\alpha 6$ subunits, many synapses were found to be immunopositive for only one of these α subunits. This result indicates a heterogeneity of Golgi cell to granule cell synapses with a variable $\alpha 1$ to $\alpha 6$ subunit ratio. Spontaneous IPSCs in cerebellar granule cells decay with a fast and a slow component (Brickley et al., 1995a). These components could be modified differentially by diazepam, indicating that they may correspond to the activation of two $GABA_A$ receptor populations (Puia et al., 1994). The proportion of the fast versus slow decay components of individual IPSCs varied within granule cells (Puia et al., 1994), which is in agreement with our results predicting different ratios of the $\alpha 1$ to $\alpha 6$ subunit in individual Golgi synapses. The ratio of different $GABA_A$ receptor channels in GABAergic synapses may be modified according to the activity of the pre- or postsynaptic cells.

Enriched immunoreactivity for the $\alpha 6$ subunit in excitatory mossy synapses

A vast majority of mossy fiber terminals use glutamate as a transmitter (Somogyi et al., 1986); therefore, the presence of the $GABA_A$ receptor $\alpha 6$ subunit in mossy synaptic junctions in addition to glutamate receptors (Silver et al., 1992; Nusser et al., 1994) is unexpected. Cross-reactivity with an unknown glutamatergic synapse-specific protein cannot be excluded using immunocytochemistry, but it is unlikely that both antibodies, each with a different specificity, would cross-react with the same unrelated protein(s) that is enriched exclusively in mossy fiber synapses. A possible cross-reactivity with GluR subunits is unlikely, because all of our antibodies selectively stained the cerebellar granule cell layer; therefore, the cross-reacting GluR should be restricted to the granule cell layer, and such receptors have not been described. These antibodies did not label glutamatergic synapses in the molecular layer of the cerebellum or in various layers of the hippocampus using immunogold localization. Furthermore, the possible cross-reactivity with GluR subunits, expressed by granule cells (Sato et al., 1993; Akazawa et al., 1994), can be excluded by the lack of immunoreactivity on GluR-expressing cells with our $\alpha 6$ subunit antibodies. False positive labeling of mossy synapses from the extrasynaptic plasma membrane can be excluded because the labeling intensity of the mossy synapse is higher than that of the nonsynaptic membrane. Furthermore, we used a nondiffusible marker with a maximum of 20 nm displacement from the epitope, a distance much smaller than the half-length of the synaptic specialization. Identical results were obtained in four different areas of the cerebellar cortex, indicating that mossy fiber terminals establishing synapses immunopositive for the $\alpha 6$ subunit of the $GABA_A$ receptor originate from diverse sources.

The functional significance of the presence of the $GABA_A$ receptor $\alpha 6$ subunit in mossy fiber synapses is unknown, but several possibilities can be envisaged.

(1) The $\alpha 6$ subunit in mossy synapses may be in a different form than that in the Golgi cell synapses. Short ($\alpha 6S$) and long ($\alpha 6L$) spliced variants of the $\alpha 6$ subunit have been reported (Korpi et al., 1994). Recombinant receptors, resulting from the coexpression of $\alpha 6S$ with $\beta 2$ and $\gamma 2$ subunits, failed to bind [^{3}H]muscimol or [^{3}H]Ro15-4513 and did not pass significant Cl^- current (Korpi et al., 1994). It is possible that the short version of the $\alpha 6$ subunit, which should be recognized by both of our antibodies, is present in mossy synapses, resulting in nonfunctional channels even in combination with other subunits.

(2) The $\alpha 6$ subunits may form functional channels in mossy synapses, probably in combination with other subunits, but the endogenous ligands of these receptors are not released from mossy fiber terminals. Nevertheless, the receptors could be activated by GABA released from Golgi terminals and diffusing subsequently into mossy synapses. Such a distant action of GABA would be similar to that in the hippocampus, where GABA can act on presynaptic $GABA_B$ receptors on excitatory terminals some distance from its release site (Isaacson et al., 1993).

(3) The $\alpha 6$ subunits in mossy synapses may form functional chloride channels that are activated by the synaptic release of endogenous ligands from mossy fiber terminals. Neurotransmitter GABA is synthesized mainly by glutamic acid decarboxylase (GAD) in nerve terminals. However, there is evidence of alternative pathways producing GABA from γ-hydroxybutyrate (Baxter, 1976) or from L-ornithine (Yoneda et al., 1982). A vast majority of mossy fiber terminals are immunonegative for both GAD and GABA (Somogyi et al., 1985, 1986; Ottersen et al., 1988). This does not exclude the possibility that GABA is present in mossy fiber terminals at a concentration undetectable by immunocytochemistry. Furthermore, a few GABA-immunopositive mossy fiber terminals have been reported to originate from the deep cerebellar nuclei in cat (Hamori and Takacs, 1989) but, even if they were present in rat, their paucity cannot explain the present results. In addition to GABA, β-alanine, γ-hydroxybutyrate, and taurine have been suggested as endogenous ligands of Cl^- channels (Snead and Nichols, 1987; Horikoshi et al., 1988). Although their distribution in the cerebellar glomeruli is unknown, they are potential candidates to act as ligands for the $\alpha 6$ subunit-containing $GABA_A$ receptors in mossy fiber synapses.

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