Relative Densities of Synaptic and Extrasynaptic GABA$_A$ Receptors on Cerebellar Granule Cells As Determined by a Quantitative Immunogold Method

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Ion channels gated by the inhibitory neurotransmitter γ-aminobutyric acid (GABA) are thought to be located in synaptic junctions, but they have also been found throughout the somatodendritic membrane of neurons independent of synapses. To test whether synaptic junctions are enriched in GABA$_A$ receptors, and to determine the relative densities of synaptic and extrasynaptic receptors, the α1 and β2/3 subunits of the GABA$_A$ receptor were localized on cerebellar granule cells using a postembedding immunogold method in cats. Immunoparticle density for the α1 and β2/3 subunits was approximately 230 and 180 times more concentrated, respectively, in the synaptic junction made by GABAergic Golgi cell terminals with granule cell dendrites than on the extrasynaptic somatic membrane. Quantification of immunoreactivity revealed one synapse population for the β2/3, but appeared to show two populations for the α1 subunit immunoreactivity. The concentration of these subunits on somatic membrane was significantly lower than on the extrasynaptic dendritic membrane. Synaptic junctions with glutamatergic mossy fiber terminals were immunonegative. The results demonstrate that granule cells receiving GABAergic synapses at a restricted location on their distal dendrites exhibit a highly compartmentalized distribution of GABA$_A$ receptor in their plasma membrane.

[Key words: ion channels, GABA$_A$ receptor, synapse, cerebellum, immunocytochemistry, inhibition]

The fast onset and rise time of GABA$_A$ receptor-mediated inhibitory postsynaptic currents indicate that synaptically activated GABA-gated chloride channels are close to the GABA release site (Edwards et al., 1990; Konnerth et al., 1990; Otis and Mody, 1992; Llano and Gerschenfeld, 1993; De Koninck and Mody, 1994; Puia et al., 1994; Soltesz and Mody, 1994). A quantum of GABA molecules has been calculated to activate approximately 10–60 channels during synaptic transmission (Edwards et al., 1990; De Koninck and Mody, 1994; Soltesz and Mody, 1994). Neurotransmitter receptors are thought to be located in

the anatomically defined synaptic junction. However, when GABA$_A$ receptors were localized at the electron microscopic level they were found throughout the neuronal surface, independent of synaptic contacts (Richards et al., 1987; De Blas et al., 1988; Meinecke et al., 1989; Somogyi, 1989; Somogyi et al., 1989; Yazulla et al., 1989; Soltesz et al., 1990; Waldvogel et al., 1990; Hansen et al., 1991; Greferath et al., 1993; Spreafico et al., 1993a). The extrasynaptic location of receptors has been confirmed by outside-out patch-clamp recordings of single GABA-gated chloride channels isolated from the soma and dendrites of a variety of neurons (Cull-Candy and Ogden, 1985; Edwards et al., 1990; Sakmann, 1992; Llano and Gerschenfeld, 1993; Kaneda et al., 1994; Macdonald and Olsen, 1994; Puia et al., 1994).

In theory, electron microscopic immunocytochemistry should provide the resolution for the precise localization of neurotransmitter receptors, and GABA$_A$ receptors have been localized in the CNS. Although in addition to extrasynaptic sites, synaptic receptor localization has also been reported (Richards et al., 1987; De Blas et al., 1988; Meinecke et al., 1989; Somogyi, 1989; Somogyi et al., 1989; Yazulla et al., 1989; Soltesz et al., 1990, Waldvogel et al., 1990; Greferath et al., 1993; Spreafico et al., 1993a), all studies in the brain used immunoperoxidase methods employing a diffusible marker which provides poor resolution and cannot be easily quantified. The results reported with the immunoperoxidase method did not indicate an enrichment of GABA$_A$ receptor immunoreactivity in synaptic junctions. Furthermore, the diffusion of immunoperoxidase end-product may result in a secondary deposition of label, that was originally produced at extrasynaptic sites, in synaptic location. For example, the metabotropic glutamate receptor was found to be preferentially located at perisynaptic plasma membrane using a particulate immunogold marker (Baude et al., 1993; Nusser et al., 1994), but when it was localized using the diffusible peroxidase product the whole synaptic specialization appeared labeled (Martin et al., 1992; Gorcs et al., 1993). This example also shows that different neurotransmitter receptors, apparently activated by transmitter from the same nerve terminal, may be localized in a restricted area of the postsynaptic membrane. Thus, due to the above mentioned technical limitations, the precise distribution of GABA$_A$ receptors has remained uncertain.

The postembedding immunogold method offers several advantages for the localization of transmitter receptors. First, it provides a nondiffusible label with a resolution of about 20 nm which is suitable to define receptors in the synaptic junction

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(Triller et al., 1985, 1986; Hansen et al., 1991; Baude et al., 1993; Nusser et al., 1994, 1995). Second, the particulate marker can be quantified (Fujimoto, 1993; Nusser et al., 1994). Third, since the surface of the electron microscopic sections are directly in contact with the antibodies, there is no difference between different tissue elements in their access to antibodies, making quantitative comparisons possible. Therefore, we applied the postembedding method to study how $GABA_A$ receptors were distributed on the neuronal surface and to what degree receptors were expressed at different densities in relation to synaptic junctions. The cerebellar cortex was chosen for this study because the origin and the position of GABAergic synapses are well known and both the pre- and postsynaptic elements can be easily identified. Cerebellar granule cells, the main focus of this study, form a relatively homogeneous neuronal population with a simple synaptic organization. They are activated by glutamatergic mossy fibers and receive GABA from local Golgi cells only on their distal dendrites (Ottersen and Storm-Mathisen, 1984; Mugnaini and Oertel, 1985; Ottersen et al., 1988). However, they express $GABA_A$ receptor protein on their extrasynaptic somatic, dendritic, and synaptic membranes (Somogyi et al., 1989), making quantitative comparisons of different compartments possible.

Materials and Methods

Preparation of tissue. Two adult female cats (2 kg body weight) were anesthetized intramuscularly with 0.6 ml of a mixture containing one part Rompun (20 mg/ml xylasin) and two parts Vetalar (100 mg/ml ketamine). The cats were perfused through the heart, first with cold artificial cerebrospinal fluid for 1 min, then with ice-cold fixative containing 4% paraformaldehyde, 0.05% glutaraldehyde, and ~0.2% picric acid dissolved in 0.1 M phosphate buffer (PB, pH = 7.4) for 7 min (Somogyi et al., 1989). The brain was removed, and small blocks from the cerebellum were cut out and washed well. Then the blocks for pre-embedding immunocytochemical experiments were immersed in 10% sucrose solution in 0.1 M PB for 1 hr, followed by 20% sucrose until they sank. To facilitate the penetration of reagents, the blocks were frozen in liquid N_2 then thawed in 0.1 M PB, and 70 μm thick sections were cut on a Vibratome.

Antibodies and controls. Antibody specificity has already been described for monoclonal antibodies bd-24 and bd-17 (Haring et al., 1985; Schoch et al., 1985), for the polyclonal antibody to GABA, No. 9 (Hodgson et al., 1985), and for the polyclonal antibody against synapsin I (Naito and Ueda, 1981).

Method specificity was tested as follows. Immunoreactivity could not be detected when the antibodies (bd-24 and bd-17 affinity-purified monoclonal antibodies, GABA No. 9 and synapsin polyclonal antibodies) were either omitted or replaced by 5% normal mouse or rabbit serum. Using another monoclonal antibody, raised to somatostatin (Vincent et al., 1985), no plasma membrane labeling was observed with our methods, demonstrating that the labeling observed at the plasma membrane is due to the anti-receptor antibodies (bd-24 and bd-17).

Pre-embedding immunocytochemistry. Normal rabbit serum was used in Tris-buffered saline (pH = 7.4) as blocking solution for 1 hr, then the purified primary antibodies were used at a protein concentration of 0.04 μg/ml and 6.6 μg/ml for bd-24 and bd-17, respectively. After washing, the sections were incubated for 90 min in goat anti-mouse IgG coupled to 1.4 nm gold (Nanogold, Nanoprobes Inc., Stony Brook, NY). Gold particles were silver enhanced with HQ Silver kit as described by the manufacturer (Nanoprobes Inc.). Sections were then routinely processed for electron microscopic examination (Somogyi et al., 1989; Baude et al., 1993).

Freeze substitution and Lowicryl embedding. A similar method was used as reported earlier (Baude et al., 1993). Briefly, after perfusion the blocks were washed in 0.1 M PB followed by Vibratome sectioning (500 μm) and washing with 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 they were slammed to a polished copper block cooled with liquid N_2 (Reichert MM80 E). Small blocks were trimmed from the cryofixed tissue and transferred to a Leica CS Auto at −80°C where freeze-sub-

stitution proceeded as follows: methanol at −80°C for 36 hr; the temperature was increased by 10°C/hr to −50°C and all the following steps were conducted at this temperature; methanol/Lowicryl HM20 (Chemische Werke Lowi GMBH and Co.) 1:1 90 min, 1:2 90 min; neat Lowicryl 90 min; fresh Lowicryl overnight; after embedding, polymerization in fresh Lowicryl under UV light for 48 hr.

Postembedding immunohistochemistry. Ultrathin sections (90 nm) from Lowicryl-embedded blocks were incubated on drops of blocking solution for 30 min. The blocking solution consisted of 0.1 M phosphate-buffered saline containing 0.8% ovalbumin, 0.1% cold-water fish skin gelatin (Sigma, Pool, UK), and 5% fetal calf serum. The primary and secondary antibodies were also made up in this solution. Two methods were used: (1) double immunostaining for receptors (antibodies diluted 0.26–0.52 and 10–20 μg protein/ml for the monoclonal antibodies bd-24 and bd-17, respectively) and for GABA (antiserum diluted 1:3000–1:4000) on the same ultrathin section; and (2) alternating serial sections incubated either for receptor or GABA or for receptor or synapsin on separate grids.

Sections were incubated in primary antibodies overnight at room temperature. After washing, the sections were incubated for 90 min with a mixture of goat anti-mouse IgG coupled to 1.4 nm gold (Nanogold, Nanoprobes Inc.) for receptors and goat anti-rabbit IgG coupled to 15 nm gold (BioClinical Services Ltd., Cardiff, UK) for GABA or synapsin. Sections were washed in 0.1 M PB then transferred to 2% glutaraldehyde in PB for 2 min. After washing with ultrapure water, the 1.4 nm gold–containing sections were silver enhanced with HQ silver kit (Nanoprobes) for 4 min. Standard uranyl acetate and lead staining were used.

Measurements of $GABA_A$ receptor immunoreactivity following postembedding immunogold localization. Measurements were taken either from sections double immunostained for $GABA_A$ receptor and GABA, or from serial immunostained sections for $GABA_A$ receptor or either GABA or synapsin on different grids. The preservation of tissue and immunoreactivity was uneven within the slam-frozen blocks of tissue; therefore, we defined a reproducible method of sampling. We scanned systematically the well preserved strip until the first GABAergic Golgi cell terminal was observed in a glomerulus, and determined whether there was a synaptic junction between this Golgi cell terminal and a granule cell dendrite. Next we determined whether the glomerulus was in an area immunoreactive for receptor. An arbitrary criterion at least two gold particles in a synaptic junction was used as the lower cut of value for accepting the area as immunoreactive. This synapse was placed in the middle of the photograph and a 4 × 3 μm area was analyzed at a final magnification of 46,600×. If there was no granule cell somatic membrane present in the field of view, the somatic membrane of the nearest granule cell to our synapse was photographed. The profiles within this rectangle were analyzed. Four different profiles were distinguished on the basis of their morphology, localization, size, and GABA and synapsin reactivity. The appearance of the slam-frozen, freeze-substituted, Lowicryl-embedded tissue is different from osmium-treated, epoxy resin–embedded tissue. The criteria used to differentiate the cellular profiles was as follows. (1) Golgi terminals: immunoreactive for GABA and synapsin; mainly located at the periphery of the glomeruli; smaller size compared to mossy terminals; vesicles are generally visible. (2) Mossy terminals: immunonegative for GABA but immunopositive for synapsin; large number of mitochondria; vesicles are generally visible; large size; mainly located in the middle of the glomeruli. (3) Granule cell dendrites: GABA and synapsin immunonegative; no synaptic vesicles; may have puncta adherentia between them. (4) Granule cell body: GABA immunonegative; oval or round soma of 5–8 μm diameter; little cytoplasm; densely packed.

The length of the sectioned plasma membrane of granule cells was measured using a digitizing tablet and the MACSTEREOLOGY computer program (Ranforly MicroSystems, UK).

The small immunometal particles were grouped in three categories and counted. If a gold particle was not further than 10 nm (approximately 0.5 mm on the micrographs; the "effective plasma membrane width") from the membrane we considered it to be associated with the plasma membrane. If it was further away we considered it to represent background, nonspecific distribution of immunometal particles. All of these nonspecific particles were counted and the density per nm² was determined. The area of section available for immunoreaction and occupied by the "effective width of granule cell plasma membrane" was calculated by multiplying the total measured length by 10 nm. To obtain
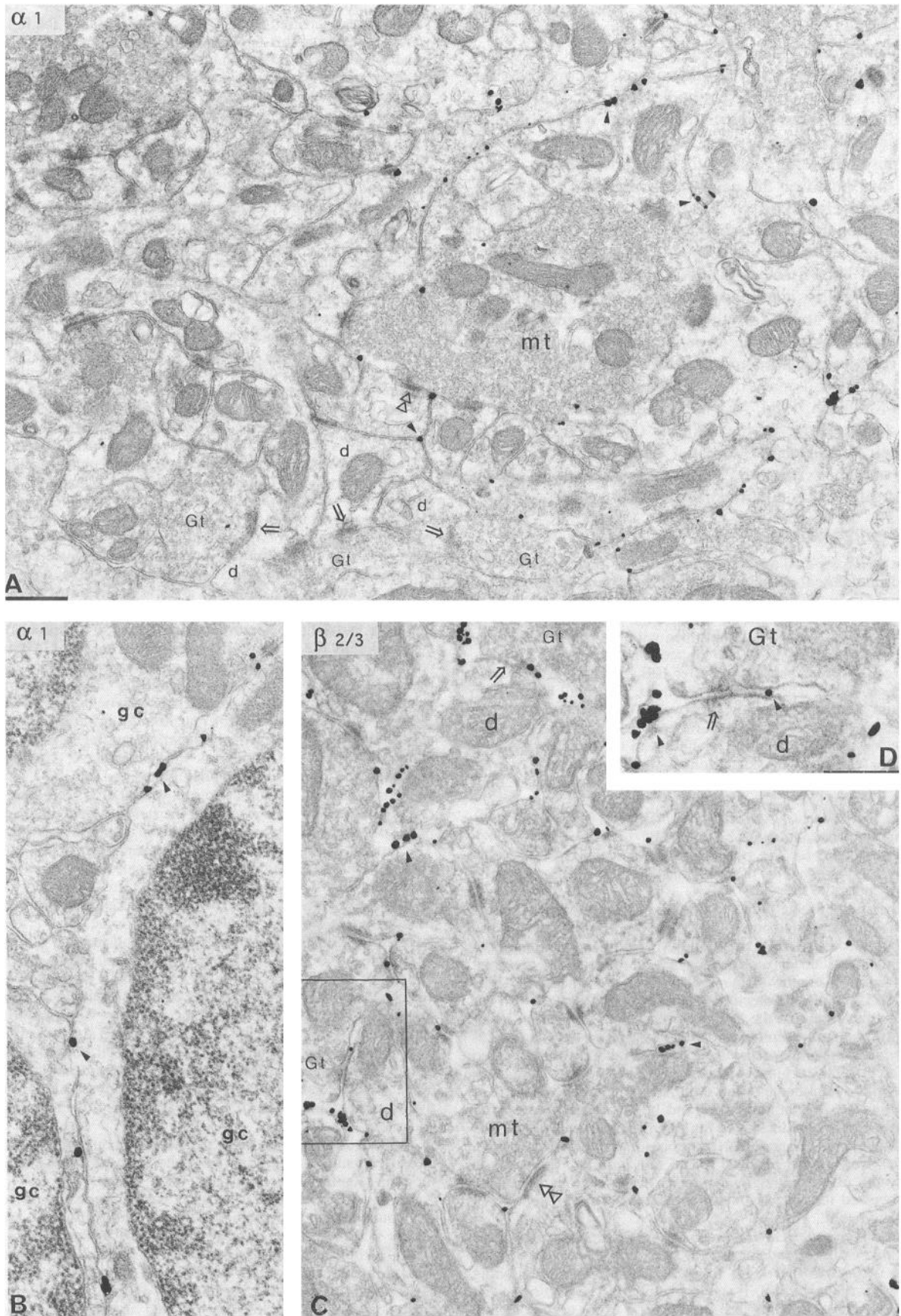
Figure 1. Electron micrographs showing immunoreactivity for the α1 (*A, B*) and β 2/3 (*C, D*) subunits of the GABA_A receptor as demonstrated with a *pre-embedding* silver-intensified immunogold technique in the cerebellar granule cell layer. Immunoparticles are located at the external face
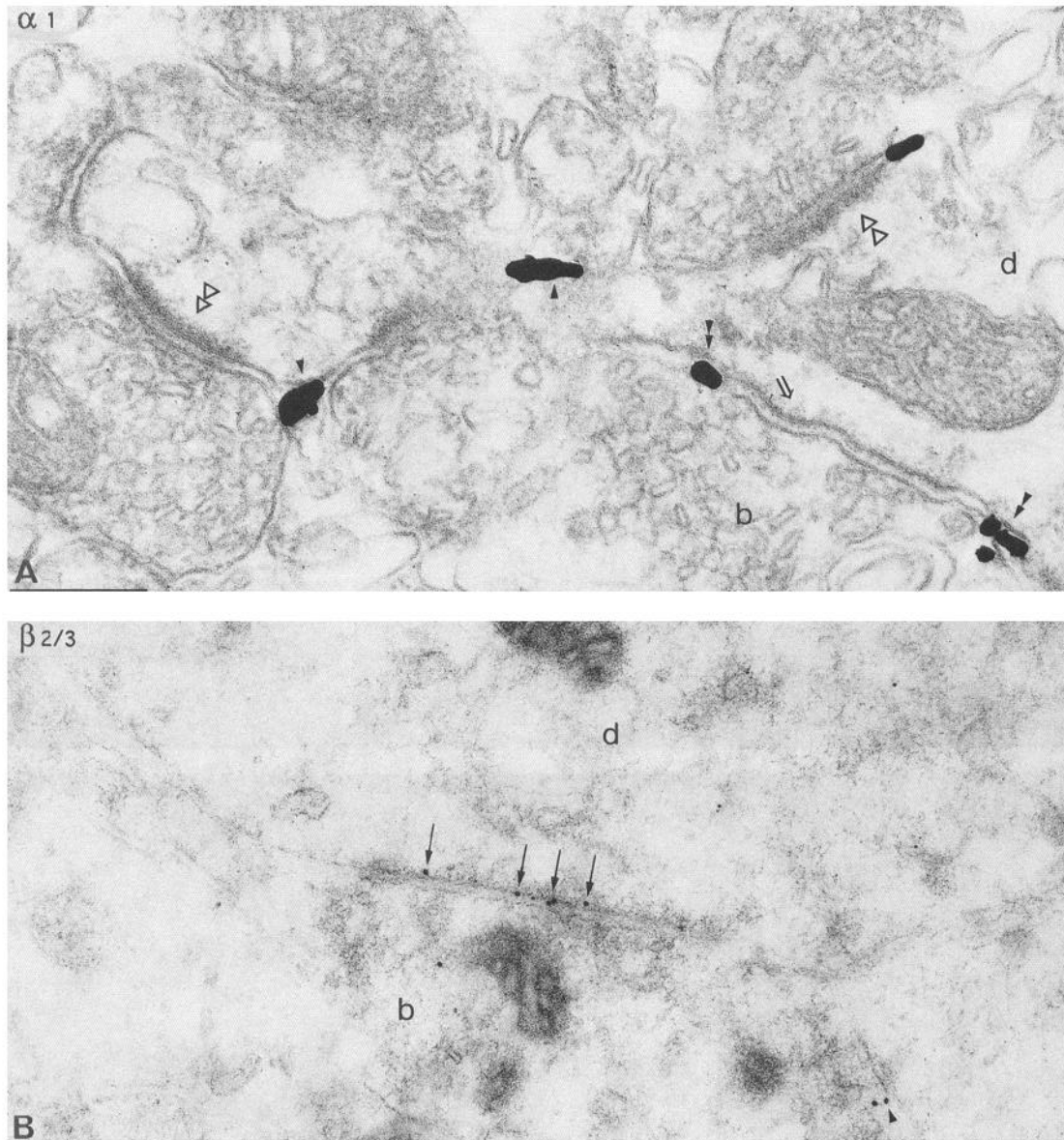
Figure 2. Immunoreactivity for the α1 (*A*) and β2/3 (*B*) subunits in the molecular layer of cat cerebellum as demonstrated by either *pre-embedding* (*A*, epoxy resin) or *postembedding* techniques (*B*, freeze-substituted Lowicryl-embedded material). *A*, Particles are not present in synapses (*double triangles*), including the type 2 synapse (e.g., *open arrow*) between a presumed stellate cell terminal (*b*) and an interneuron dendrite (*d*). Immunoparticles are often located at the edge of type 2 synapses (*double arrowheads*) as well as at the extrasynaptic membrane (*arrowheads*). *B*, The postembedding reaction produces a row of silver-intensified gold particles (*arrows*) on the postsynaptic plasma membrane at a synaptic junction established by a bouton (*b*) with a Purkinje cell dendrite (*d*). Some immunoparticles are also present at the extrasynaptic plasma membrane (*arrowhead*). Scale bars, 0.2 μm.

gold density corrected for "background reaction," the above density was reduced by the density of the nonspecifically distributed gold. At each GABAergic synapse we measured the length of synaptic junction and counted the number of gold particles to calculate the synaptic immunoparticle density. We defined the width of the "effective synaptic plasma membrane" as 10 nm.

Results

Antibodies

Monoclonal antibody bd-24 was shown to react only with the α1 subunit (Ewert et al., 1990) which is abundantly expressed

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of the extrasynaptic dendritic membrane of granule cells (e.g., *arrowheads* in *A, C, D*) corresponding to the location of epitopes recognized by monoclonal antibodies bd-24 and bd-17. Synapses between presumed Golgi terminals (*Gt*) and granule cell dendrites (*d*) are always immunonegative with this method for both subunits (*open arrows* in *A, C, D*), as are synapses between mossy terminals (*mt*) and granule cell dendrites (*double triangles* in *A, C*). Immunoparticles are also present along the somatic plasma membrane of granule cells (*gc*), including areas where two cells are directly apposed (*arrowheads* in *B*). Scale bars: *A–C*, 0.5 μm; *D*, 0.2 μm.
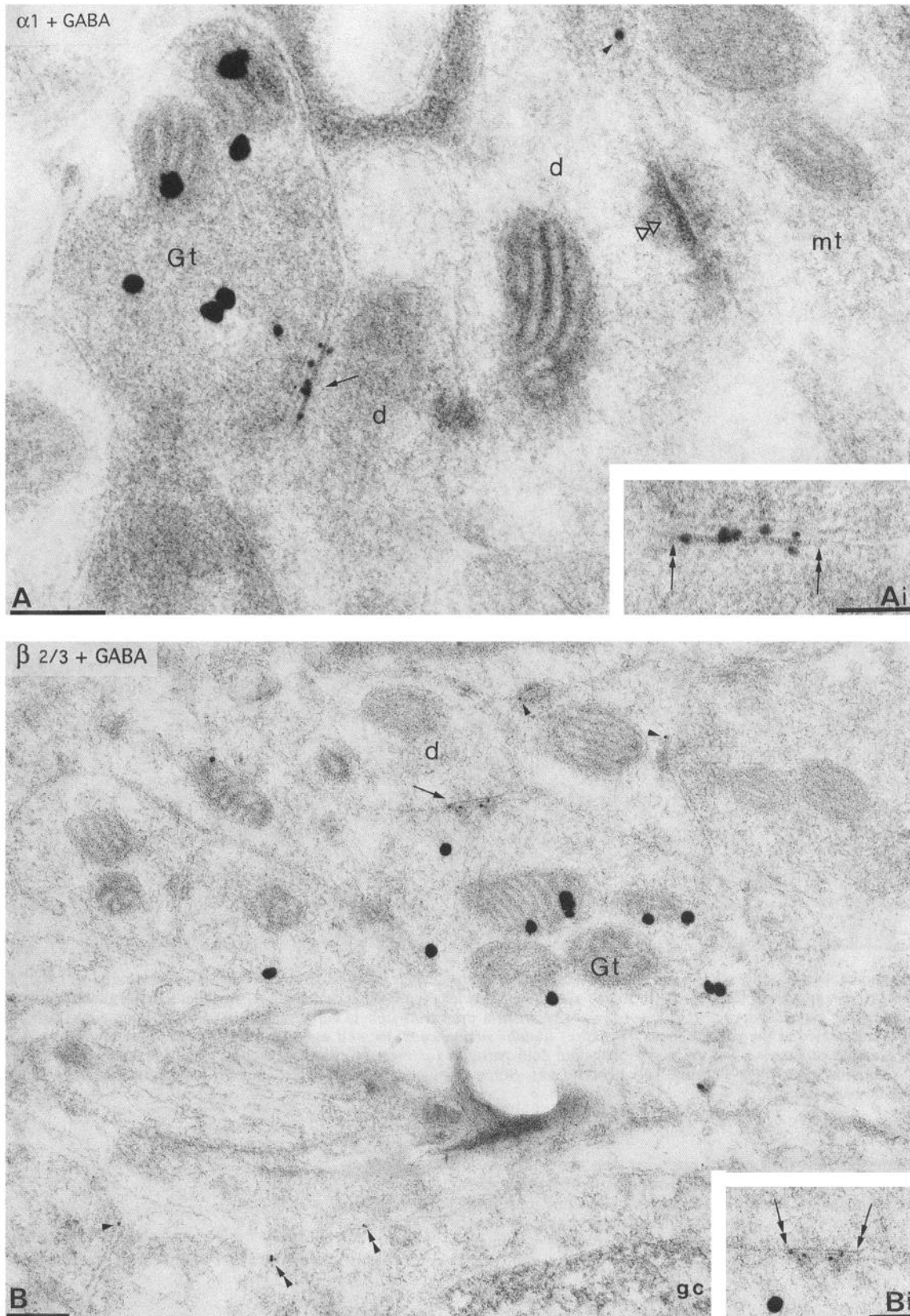
Figure 3. Electron micrographs of freeze-substituted, *Lowicryl-embedded* ultrathin sections demonstrating immunoreactivity for the α1 (*A, Ai*) or β2/3 (*B, Bi*) subunits of GABA_A receptor (small particles, silver-intensified 1 nm gold) and for GABA (large particles, silver-intensified 15 nm gold). The enrichment of large particles shows GABA immunoreactivity of Golgi cell terminals (*Gt*). The synaptic junctions (*arrow*) are covered by small particles demonstrating enrichment of receptor immunoreactivity. Note the presence of receptor immunoparticles on the somatic (*B, double arrowheads*) and extrasynaptic dendritic membrane (*arrowheads*) of granule cells (*gc*) and the immunonegative synapse (*double triangles*) estab-

by granule cells. Antibody bd-17 reacts with both the β2 and β3 subunits. Since granule cells express mRNA for both subunits (Laurie et al., 1992; Persohn et al., 1992), immunoreactivity is referred to as representing β2/3 subunits. These subunits are highly homologous and it has not been possible to develop immunohistochemically usable antibodies that would differentiate between them.

Pre-embedding immunogold localization of GABA_A receptors

The use of the immunogold method confirmed some of the results obtained earlier with an immunoperoxidase method (De Blas et al., 1988; Meinecke et al., 1989; Somogyi et al., 1989). However, the absence of signal diffusion in the immunogold method makes the localization more accurate. Immunoparticles were restricted to the extracellular face of the somatic and dendritic membranes (Figs. 1A–C, 2A) for both antibodies. The size of the particles is about the same as the width of the extracellular space, thus a particle touches both apposed plasma membranes. Therefore, the location of the epitope can be allocated to the process of a particular cell type when both apposed membranes belong to the same population, for example, granule cell dendrites. The putative epitope recognized by antibody bd-24 was predicted to be in the N-terminal part of the polypeptide (Ewert et al., 1990), which is at the extracellular face of the plasma membrane. Thus the immunogold localization of the epitope is in agreement with the predicted transmembrane conformation of the polypeptide and also demonstrates that antibody bd-17, for which the epitope is unknown, also recognizes an extracellular epitope.

Granule cells showed the strongest immunoreactivity for the studied subunits of GABA_A receptor in the cerebellum with immunoparticles located along the extrasynaptic dendritic membrane (Fig. 1A,C). Immunoreactivity was also present at the somatic membrane including areas where two somata were directly apposed (Fig. 1B), providing evidence that the receptor is in the granule cell membrane. Immunoreactivity was never observed in the asymmetrical synaptic junctions between the glutamatergic mossy fiber terminals and granule cell dendrites (Fig. 1A,C). Surprisingly, using the pre-embedding immunogold method, immunoparticles were also absent from the synaptic junctions established by Golgi cell terminals with granule cell dendrites (Fig. 1A,C). An absence of immunoparticles was also observed in the symmetrical synapses between stellate or basket cell terminals and dendrites in the molecular layer (e.g., Fig. 2A). The lack of immunoreactivity for the GABA_A receptor in the anatomically defined GABAergic synaptic junctions could be genuine or due to technical limitations, such as the inability of the immunoreagents to penetrate into the synaptic cleft, or the cleft material masking the epitopes. To decide between these possibilities, postembedding reactions were carried out on Lowicryl-embedded tissue.

Postembedding immunogold localization of GABA_A receptors

Using the postembedding method, electron microscopic ultrathin sections are floated onto antibody solutions, exposing directly the entire cut length of the membrane to the antibody, including the synaptic junctional membranes. No immunocytochemical la-

beling could be obtained in epoxy resin–embedded tissue. Therefore, slam-frozen, freeze-substituted, Lowicryl-embedded material was used that has been useful for the high-resolution localization of glutamate receptors (Baude et al., 1993; Nusser et al., 1994). However, fine structural details in this material are less well defined than in osmium-treated, epoxy resin–embedded tissue. Thus, in order to identify cellular profiles, and in particular Golgi cell terminals, GABA or synapsin immunolabeling were carried out on either the same (Figs. 3A,B; 4A,B) or serial (Fig. 4C) sections to those which were reacted for the GABA_A receptor. Using double immunostaining experiments on the same ultrathin sections, large particles (silver-intensified 15 nm gold) indicate the immunoreactivity for GABA, whereas small particles (silver-intensified 1 nm gold) represent the immunoreactivity for the GABA_A receptor subunits (Figs. 3A,B; 4A,B). Synaptic junctions were identified on the basis of the rigid apposition of the membranes of Golgi terminals and granule cell dendrites (Figs. 3, 4), the usually narrow but distinct postsynaptic membrane specialization (Figs. 3, 4), and, when present, the presynaptic dense material (Figs. 3B, 4A,C).

In contrast to the result of the pre-embedding reactions, an enrichment of immunoparticles was detected for both the α1 and β2/3 subunits in the synaptic junctions established by the GABAergic Golgi cell terminals and granule cell dendrites in Lowicryl-embedded tissue (Figs. 3, 4). The overwhelming majority of Golgi synapses were strongly immunopositive for the β2/3 subunits (Figs. 3B, 5). Most of these synapses were also labeled with immunoparticles for the α1 subunit (Figs. 3A, 4A–C, 5), but sometimes an absence of the α1 subunit immunoreactivity was also observed (Figs. 4B, 5). Immunoparticles for the GABA_A receptor were located along the postsynaptic specialization in the main body of Golgi synapses (Figs. 3, 4), frequently in one or more clusters (Fig. 4B).

A similar result was obtained in the molecular layer. Synapses between terminals and Purkinje cell or interneuron dendrites showed strong immunoreactivity for the GABA_A receptor using the postembedding method (Fig. 2B).

Postembedding immunogold reactions have also confirmed the extrasynaptic location of the immunoreactivity for the α1 and β2/3 subunits on the somatic and dendritic membrane of granule cells (Figs. 3, 4). Immunoparticles for the β2/3 subunits (Fig. 3B) were more frequent at the extrasynaptic dendritic membrane than for the α1 subunit (Figs. 3A, 4A–C). The synapses between mossy fiber terminals and granule cell dendrites were always immunonegative for these GABA_A receptor subunits.

Relative immunoparticle densities on different compartments of the surface of granule cells

The immunogold reaction under postembedding conditions provided an opportunity to carry out a quantitative evaluation of immunoreactivity. Immunoparticle densities were calculated on different parts of the surface of cerebellar granule cells. Data were obtained from four different blocks (4/8, 3/8, 1a/10, 1b/10) of two animals (cats 21/4 and 1/6). Immunoreactions on different specimens and on separate occasions resulted in somewhat different overall density of particles. Therefore, relative

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lished by a mossy terminal (mt). The insets (Ai, Bi) show the demarcation of the edge of synaptic junctions (double arrows) based on the electron-dense postsynaptic membrane specialization. Scale bars: A and B, 0.2 μm; Ai and Bi, 0.1 μm.

α1 + GABA

α1 + GABA

α1
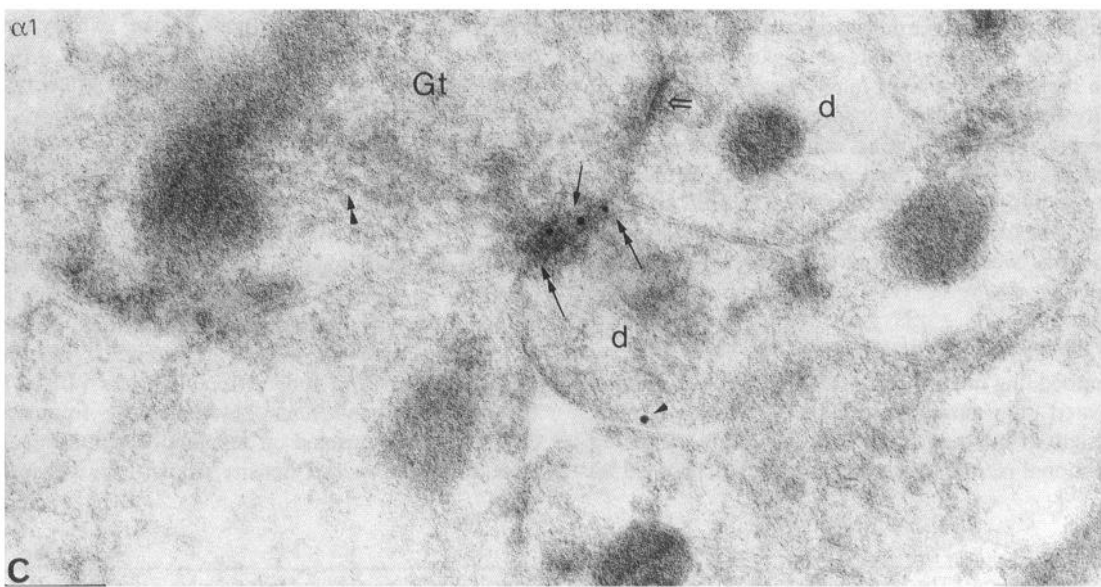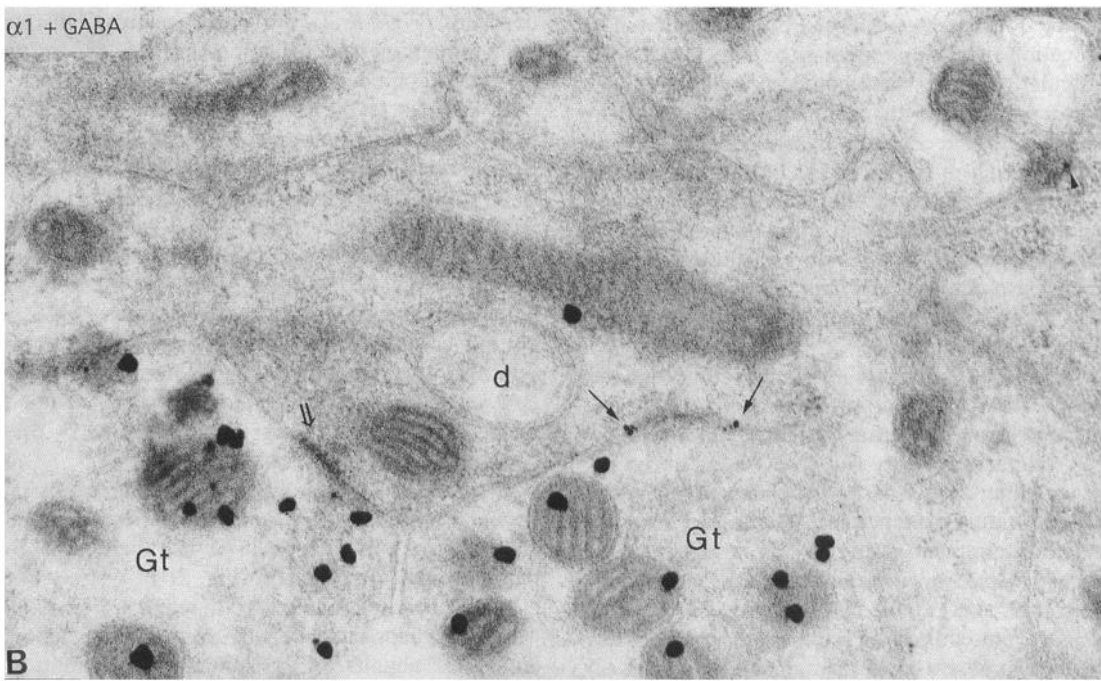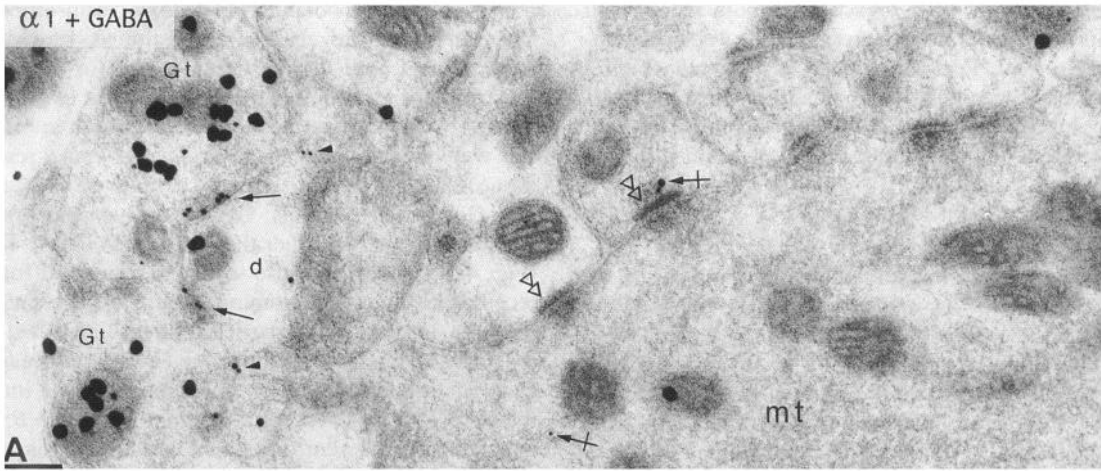
immunoparticle densities on somatic, extrasynaptic dendritic, and synaptic membranes *were calculated by comparing densities obtained from the same ultrathin sections.* The relative immunoparticle density for the β2/3 subunits in the synapses established by Golgi cell terminals was 54.68 (10.38) [mean (SD)] times higher than in the extrasynaptic dendritic membrane (Table 1). For the α1 subunit, synaptic immunoparticle density was 127.24 (39.59) times higher than in the extrasynaptic dendritic membrane. The above result indicates a relative density approximately twice as high for the α1 subunit in the Golgi synapses as for the β2/3 subunits (Table 1). The length of the measured extrasynaptic dendritic membranes was 155.25 (52.18) times longer than the length of Golgi synaptic membranes in the pictures, but dendrites were measured only in the glomeruli. Immunoparticle densities on the extrasynaptic dendritic membrane were 1.80 (0.11) and 3.28 (1.02) times higher for the α1 and β2/3 subunits, respectively, than on the somatic membrane (Table 1). The ratios of immunoparticle density on the four separately examined compartments of the surface of granule cells, namely, the membrane postsynaptic to the *mossy* fiber terminals (MS), membrane of *granule* cell body (BG), extrasynaptic *dendritic* membrane of *granule* cell (DG), and postsynaptic membrane to the *Golgi* cell terminals (GS), were approximately MS:BG:DG:GS = 0:1:2:230 and MS:BG:DG:GS = 0:1:3.3:180 for the α1 and β2/3 subunits, respectively.

Are Golgi cell synapses homogeneous according to their α1 and β2/3 subunits' immunoreactivity?

Measuring every Golgi cell synapse on our micrographs, synaptic immunoparticle density for the β2/3 subunits was calculated as being 18.37 (9.12) and 19.55 (9.50) particle/μm membrane in cats 12/4 and 1/6, respectively. This corresponds to 3.2 immunoparticles per Golgi cell synapse [average length, 0.17 (0.02) μm]. The frequency distribution of synapses immunoreactive for the β2/3 subunits was normal in both animals (Fig. 5*B*), indicating the existence of one population of Golgi synapses (Fig. 5*B*).

Immunoparticle density for the α1 subunit was 16.81 (10.85) and 18.28 (9.47) particle/μm membrane in cats 12/4 and 1/6, respectively, when every Golgi cell synapse was included (Fig. 5*A*). However, the frequency distributions of the α1 subunit immunoreactive synapses, provided by GABA-positive terminals, significantly differed from normal (Fig. 5*A*), and a high proportion of immunonegative synapses was observed. This suggests the possibility of two populations of GABAergic synapses in cerebellar glomeruli with regard to α1 subunit concentration. One of the two populations may have a lower density or no α1 subunit (see Fig. 4 for immunonegative synapses). When synapses having more than one immunoparticle for the α1 subunit were tested for heterogeneity the population was not significantly different from normal (Table 1), indicating that they formed one population. Therefore, in the calculations of relative immunoreactivity signals between synaptic versus nonsynaptic

plasma membranes, we used for comparison the synapse population showing a higher density of immunoparticles [Table 1, mean 22.26 (6.11) and 21.92 (6.49) particle/μm membrane in cats 12/4 and 1/6, respectively].

Discussion

Synaptic enrichment of GABA$_A$ receptors

Postsynaptic neurotransmitter receptors in the brain are thought to be located at high concentration in the junctional plasma membrane opposite to the transmitter release site. The results of this study demonstrate that immunoreactivity for the ionotropic GABA$_A$ receptor is highly concentrated in the main body of the GABAergic synaptic junction and there is a sharp decrease in labeling density at the edge of the synaptic specialization. Assuming that the antibodies used in this study recognize receptors at different locations in the membrane with equal probability, the differences in labeling density probably reflect differences in receptor/channel densities.

Prior to the present high-resolution localization of receptors, the best evidence for the close association of transmitter release and receptor location has been provided by the fast onset and rise time of IPSCs evoked by spontaneously released transmitter in preparations that maintain the organization of brain tissue (Edwards et al., 1990; Konnerth et al., 1990; Otis and Mody, 1992; Llano and Gerschenfeld, 1993; De Koninck and Mody, 1994; Puia et al., 1994; Soltesz and Mody, 1994). At synaptic sites, 10–60 GABA$_A$ receptor channels have been suggested to be activated by a single packet of transmitter (Edwards et al., 1990; De Koninck and Mody, 1994; Soltesz and Mody, 1994), suggesting a synaptic enrichment of the receptors. However, outside-out patch-clamp recording of single receptor channels obtained from somatic and dendritic membrane of different neurons demonstrated GABA$_A$ receptors at extrasynaptic sites having pharmacological and kinetic parameters similar to those predicted for synaptic receptors (Cull-Candy and Ogden, 1985; Edwards et al., 1990; Sakmann, 1992; Llano and Gerschenfeld, 1993; Kaneda et al., 1994; Macdonald and Olsen, 1994; Puia et al., 1994). The extrasynaptic presence of receptors has been confirmed by immunocytochemical localization of GABA$_A$ receptor protein at extrasynaptic membranes using immunoperoxidase methods (Richards et al., 1987; De Blas et al., 1988; Meinecke et al., 1989; Somogyi, 1989; Somogyi et al., 1989; Yazulla et al., 1989; Soltesz et al., 1990; Waldvogel et al., 1990; Greferath et al., 1993; Spreafico et al., 1993a). The latter studies could not show an enrichment of receptor in the synaptic junction due to the limitations of the technique.

Further uncertainty into the precise location of receptors was introduced by the unexpected exclusion of the metabotropic glutamate receptor from the main body of the synaptic junction. Using a high-resolution particulate immunomarker it was found that the metabotropic receptor was concentrated at the edge of the synaptic specialization, still within easy reach of transmitter, but nevertheless largely outside the anatomically defined syn-

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Figure 4. Immunoreactivity for the α1 subunit of the GABA$_A$ receptor (small particles, silver enhanced 1 nm gold) and for GABA (large particles, silver enhanced 15 nm gold) in Golgi cell terminals. *Postembedding* reactions. Granule cell dendrites (*d*) and mossy fiber terminals (*mt*) are immunonegative for GABA. A high density of α1 subunit immunoreactivity is observed in the majority of GABAergic synapses (*arrows*) but not in the synapses between a mossy terminal and granule cell dendrites (*double triangles*). Small gold particles are also present at the extrasynaptic dendritic membrane (*arrowheads*), and some background particles (e.g., *crossed arrow*) are also detected. Some Golgi synapses are immunonegative (*open arrows*). Sometimes immunoparticles are observed in clusters in the synaptic junction (e.g., *arrows* in *B*). The demarcation of the edge of synaptic junctions (e.g., *double arrows* in *C*) is based on the electron-dense membrane specializations; the identification of Golgi terminals (*Gt*) is based on the presence of synaptic vesicles (e.g., *double arrowhead* in *C*) and GABA immunoreactivity. Scale bars, 0.2 μm.

Synaptic GABA$_A$ receptor α1 subunit

■ Cat 21/4, mean (SD)=16.81 (10.85), n=38
□ Cat 1/6, mean (SD)=18.28 (9.47), n=23

No. of synapses

0 5 10 15 20 25 30 35

Immunoparticle density (No./μm)

Synaptic GABA$_A$ receptor β2/3 subunits

■ Cat 21/4, mean (SD)=18.37 (9.12), n=28
□ Cat 1/6, mean (SD)=19.55 (9.50), n=18

No. of synapses

0 5 10 15 20 25 30 35

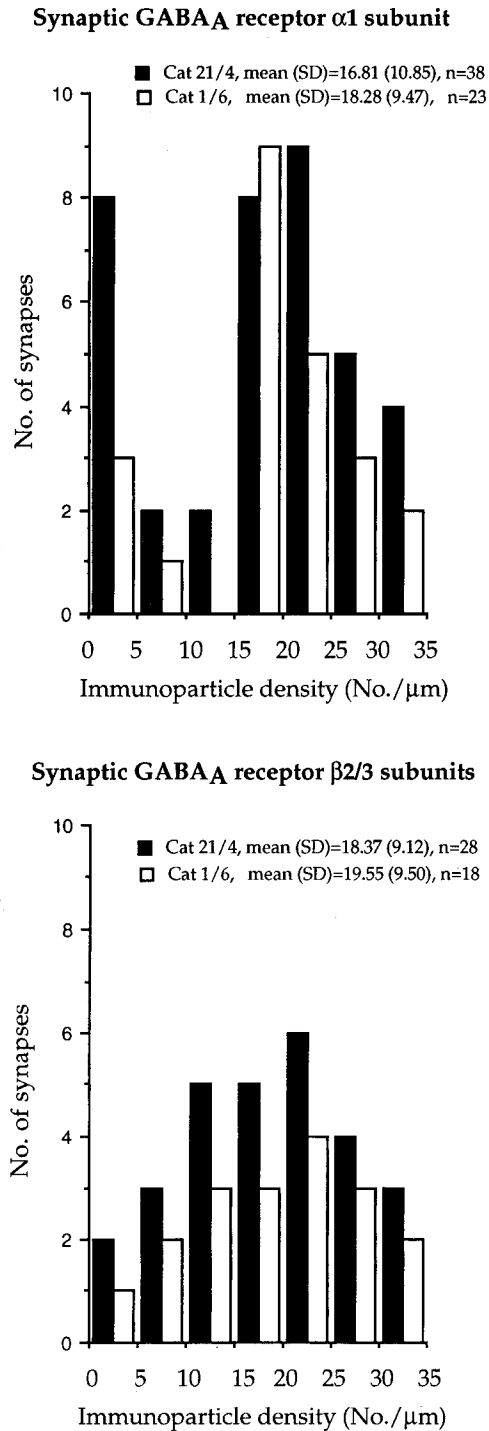Immunoparticle density (No./μm)

Figure 5. Quantitative distribution of synapses established by Golgi cell terminals and granule cell dendrites according to their α1 (A) and β2/3 (B) subunits' immunoreactivity. A, The distribution of Golgi synapses with regard to α1 subunit immunoreactivity is different from normal distribution (χ^2 test, $\chi^2 = 17.46$, $p < 0.002$), indicating two populations of synapses. The distribution in the two animals is not different (Mann–Whitney test, $Z = -0.11$, $p = 0.91$). B, The frequency distribution of the β2/3 subunits immunoreactive Golgi synapses is *normal* in both animals (χ^2 test: cat 21/4, $\chi^2 = 0.115$, $p = 0.862$; cat 1/6, $\chi^2 = 0.096$, $p = 0.997$), indicating one population of Golgi synapses for β2/3 subunits immunoreactivity.

aptic junction (Baude et al., 1993; Nusser et al., 1994). The present finding of the ionotropic GABA$_A$ receptor concentrated in the main body of the GABAergic synapses demonstrates that the perisynaptic location may be peculiar for the G-protein-coupled receptors. Indeed another fast, ionotropic amino acid receptor, the α-amino-3-hydroxy-5-methylisoxazole-4-propionate-type glutamate receptor, is also highly concentrated in the postsynaptic membrane specialization, as revealed by the post-embedding immunogold method (Nusser et al., 1994).

Immunoparticles for the GABA$_A$ receptor were often distributed in one or more groups along the main body of the postsynaptic membrane, indicating a possible clustering of receptors. There is a sharp decrease in immunoparticle density at the edge of the synaptic specialization for both the α and the β subunits of the GABA$_A$ receptor. Using confocal immunofluorescent mapping, both GABA$_A$ and glycine receptors have been found to be concentrated in patches having sharp borders on the surface of central neurons (Becker et al., 1991; Triller et al., 1991; Nicola et al., 1992; Bohlhalter et al., 1994). The concentrated placement of receptors with a sharp decrease in channel density at the edge of the synaptic specialization ensures that diffusion of transmitter from the release site to the synaptically activated receptor channels does not prolong the fast activation of postsynaptic response. Another factor that may contribute to the abrupt decrease of receptor protein at the edge of the anatomically defined synaptic junction is that the edge of the synapse may be occupied preferentially by other membrane proteins such as G-protein-coupled receptors and the voltage-gated ion channels that they modulate (Baude et al., 1993; Nusser et al., 1994).

Compartmentalized distribution of the α1 and β2/3 subunits on the surface of cerebellar granule cells

In addition to the high concentration of the α1 and β2/3 subunits of the GABA$_A$ receptor in the GABAergic synaptic junction, consistent levels of nonsynaptic labeling on the same cells have also been found. In contrast, no GABA$_A$ receptors could be detected, with our antibodies and at the sensitivity level of our method, in mossy fiber synapses, demonstrating the ability of the cell to exclude this protein from glutamatergic synapses. The density of receptors also seems to decrease in the nonsynaptic plasma membrane the further the membrane is from the GABA release site. Thus, the membrane of the granule cell body, which does not receive any synaptic contact, showed significantly lower immunolabeling than the nonsynaptic dendritic membrane in the glomeruli. We did not try to measure separately proximal versus distal dendritic membrane due to the convoluted course of granule cell dendrites, which in any case are very short compared to the dendrites of other central neurons. In the absence of GABAergic innervation, cultured granule cells show a relatively even distribution of GABA$_A$ receptors on their dendritic and somatic plasma membranes, although a high concentration of labeling has been reported in spots (Hansen et al., 1991). Furthermore, it has been shown that GABA and the GABA$_A$ receptor agonist 4,5,6,7-tetrahydroisoxazolo [5,4-c]pyridin-3-ol enhance the morphological development of the cells (Hansen et al., 1984). They also promote expression of low-affinity GABA$_A$ receptors (Meier et al., 1984; Belhage et al., 1986; Kim et al., 1993) and the density of immunocytochemically detected GABA$_A$ receptors (Hansen et al., 1991) in cultured cerebellar granule cells. It is possible that *in situ* in the brain, the GABAergic nerve terminal, similar to the cholinergic endplate in the neuromuscular junction (Fambrough, 1979; Laufer and Changeux,

Table 1. Absolute and relative immunoparticle densities for the α1 and β2/3 subunits of the GABA$_A$ receptor on the somatic, extrasynaptic dendritic, and GABAergic synaptic membranes of granule cells

Subunit	Animals	Blocks	Immunoparticle density (particle/μm membrane)			Syn./dend. ratio	Syn./som. ratio	Dend./som. ratio
			Somatic	Dendritic mean (SD) [n+]	Synaptic mean (SD) [n]			
α1	21/4	4/8	0.108	0.209 (0.071) [11]	23.59 (6.17) [13]	114.59	218.43	1.93
		3/8	0.103	0.190 (0.080) [10]	21.35 (5.89) [15]	112.36	207.28	1.84
	1/6	1a/10	0.055	0.096 (0.034) [5]	17.80 (2.59) [6]	185.41	323.64	1.74
		1b/10	0.144	0.241 (0.128) [10]	23.29 (7.10) [13]	96.63	161.74	1.67
Mean (SD)			0.103 (0.036)*	0.184 (0.062)*	21.51 (2.66)	127.24 (39.59)	227.77 (68.45)	1.80 (0.11)
β2/3	21/4	4/8	0.108	0.368 (0.065) [5]	20.75 (9.56) [10]	56.38	192.12	3.40
		3/8	0.181	0.327 (0.208) [10]	17.04 (8.86) [18]	52.11	94.14	1.80
	1/6	1a/10	0.086	0.356 0.053 [5]	24.08 (3.56) [7]	67.64	280	4.12
		1b/10	0.103	0.391 0.175 [5]	16.66 (11.06) [11]	42.60	161.74	3.79
Mean (SD)			0.120 (0.042)**	0.361 (0.026)**	19.63 (3.49)	54.68 (10.38)	182 (77.1)	3.28 (1.02)

Immunoparticle density in the synaptic junction (n, number of synapses) was significantly higher (Student t test, $p \ll 0.001$) than on the extrasynaptic dendritic membrane. Synapses appeared heterogeneous for α1 subunit immunoreactivity (Fig. 5, all synapses); therefore, only synapses with more than one particle are included in the table for calculating the synaptic density of α1 immunoparticles. These synapses formed a homogeneous population (distribution not different from normal, $\chi^2 = 2.06$, $p = 0.36$). Immunoparticle density for both the α1 (*, $p < 0.01$, Student t test) and the β2/3 (**, $p < 0.005$, Student t test) subunits was significantly lower on somatic membrane than on the extrasynaptic dendritic membrane, paired comparisons giving ratios of 1.80 (0.11) and 3.28 (1.02) for the α1 and the β2/3 subunits, respectively. Each dendritic measurement was obtained from the total glomerular dendritic membrane length in an electron micrograph (n+, number of micrographs). Immunoparticle density on the somatic membrane was calculated by dividing the total number of particles by the sum of the measured somatic membrane length [69 (34) μm/block] from several micrographs obtained from each block.

1989), is responsible for increasing and maintaining a high receptor concentration close to the release site in the GABAergic synaptic junction.

It is not yet known how the measured immunoparticle density is related to receptor channel density. If a saturation of immunocytochemical signal occurs at high channel density, for example in the synaptic membrane, then the approximately 125 (α1 subunit, higher density synapse population) and 55 (β2/3 subunits, all synapses) times enrichment of signal in the synaptic junction relative to nonsynaptic dendritic membrane would be an underestimate of real channel density differences. The relative particle densities at sites labeled at low level are probably more representative of channel density differences. In any case, the absolute number of extrasynaptic receptors could be higher than all the receptors in synaptic junctions. Only a small fraction of the granule cell membrane is covered by GABAergic synapses. Taking into account the measured synaptic and nonsynaptic dendritic plasma membrane ratio in the glomeruli (although not all extrasynaptic reactivity could be measured and the value is an underestimate), it can be calculated that at least three and 1.25 times more β2/3 and α1 subunits exist, respectively, at extrasynaptic glomerular sites than at synaptic sites. These values are further increased by adding the nonglomerular dendritic and somatic extrasynaptic receptors.

The possible functional role of extrasynaptic receptors at sites remote from GABAergic synapses has been discussed earlier (Somogyi et al., 1989). A recent electrophysiological study on cerebellar granule cells demonstrated that GABA-evoked currents in outside-out nucleated patches, presumably corresponding to the somatic membrane and therefore containing only extrasynaptic receptors, and spontaneous IPSCs, evoked synaptically, had similar kinetic and pharmacological properties (Puia et al., 1994). On the other hand, GABA exogenously applied to granule cells in the dentate gyrus activates GABA$_A$ receptor channels with kinetics dissimilar to those at the synapse (De Koninck and Mody, 1994). Thus, it is still uncertain whether extrasynaptic receptors are functionally similar to synaptic receptors.

Two populations of Golgi synapses for α1 subunit immunoreactivity

Cerebellar granule cells express mRNA for the α1, α4, α5, α6, β1, β2, β3, γ2, and δ subunits of the GABA$_A$ receptor, as detected by in situ hybridization (Laurie et al., 1992; Persohn et al., 1992). Immunocytochemical studies have demonstrated the α1, α3, α6, β2/3, γ2, and δ subunits in the granule cell layer and the enrichment of immunoreactivity in the glomeruli indicates that the receptor may be located in the dendritic membrane (Richards et al., 1987; De Blas et al, 1988; Meinecke et al., 1989; Somogyi et al., 1989; Benke et al., 1991b,c; Zimprich et al., 1991; Baude et al., 1992; Thompson et al., 1992; Turner et al., 1994). Immunoprecipitation studies show that not all the subunits are combined into the same channel assembly (Duggan and Stephenson, 1990; Benke et al., 1991a; Duggan et al., 1991; Luddens et al., 1991; McKernan et al., 1991; Zezula et al., 1991; Endo and Olsen, 1993; Mertens et al., 1993; Pollard et al., 1993; Khan et al., 1994; Quirk et al., 1994; Togel et al., 1994). Furthermore, the kinetic properties of channels vary according to subunit composition (Pritchett et al., 1989; Sigel et al., 1990; Verdoorn et al., 1990; Angelotti and Macdonald, 1993; Mathews et al., 1994). Since individual granule cells receive GABA in a largely homogeneous population of synapses on their distal dendrites mainly from Golgi cells, the multiple channel types could all be located in the same synapses. This is supported by recent results showing that granule cells respond to synaptically released GABA with at least two different current components which are differentially modulated by diazepam, indicating the existence of different receptor channels (Puia et al., 1994).

Quantification of Golgi cell synapses for α1 subunit immunoreactivity indicates the possible presence of two populations of synapses with different levels of this subunit, but one population for the β subunits. All GABA$_A$ receptor channels in vivo

are thought to contain β subunits (Schofield et al., 1987; Sigel et al., 1990; Verdoorn et al., 1990; Amin and Weiss, 1993; Angelotti and Macdonald, 1993). Granule cells express mRNA for the β2 and β3 subunits at high levels, but only moderate expression for the β1 subunit has been detected using *in situ* hybridization (Laurie et al., 1992; Persohn et al., 1992). Indeed, the granule cell layer appears to be immunonegative for the β1 subunit, demonstrating that either cerebellar granule cells do not express β1 subunit protein or express it at a very low level (Z. Nusser, W. Sieghart, and P. Somogyi, unpublished observations). Monoclonal antibody bd-17 used in our study recognizes epitopes on both β2 and β3 subunits (Ewert et al., 1990). Therefore, it is assumed that the overwhelming majority of GABA_A receptor channels are recognized by antibody bd-17 on granule cells. Because only one population of Golgi synapse was found according to β2/3 subunit immunoreactivity, it is unlikely that the existence of two populations for the α1 subunit is a result of the presence of synapses with different numbers of GABA receptor channels. A more likely explanation is that those Golgi synapses having lower α1 subunit immunoreactivity may contain higher concentrations of receptor channels in which the α1 subunit is replaced by another α subunit, probably the α6 subunit, known to be present in granule cells at high levels (Baude et al., 1992; Laurie et al., 1992; Persohn et al., 1992; Thompson et al., 1992). Immunoreactivity for the α6 subunit has been shown in synapses between Golgi cell terminals and granule cell dendrites of rat (Baude et al., 1992). Unfortunately, our antibody recognizes epitopes on the α6 subunit of the rat but not on that of the cat (Baude et al., 1992) and, conversely, the antibody to the α1 subunit does not recognize this subunit in rat (Ewert et al., 1990). Therefore, we were unable to colocalize these two α subunits or to calculate relative densities on different compartments of the surface of granule cells in the same species.

In conclusion, it has been demonstrated that the postembedding immunogold method is suitable for the measurement of relative neurotransmitter receptor densities in different parts of the same cell and it should also be possible to compare different cell types. The expression of GABA_A receptor and its availability to ligands on the cell surface changes rapidly in the brain upon the influence of sensory stimuli, environmental factors, neurodegenerative disorders, and exposure of the animal to drugs (Hendry et al., 1990; Morrow et al., 1992; Li et al., 1993; Mhatre et al., 1993; Mody, 1993; Montpied et al., 1993; Spreafico et al., 1993b). The present approach offers a sensitive and high-resolution method to define the cellular and subcellular sites for these changes with a resolution of about 20 nm.

Note added to the proof: After the acceptance of this article it came to our attention that Caruncho and his colleagues (Caruncho et al., 1993; Caruncho and Costa, 1994) reported concentration of the α1, α6, β2/3, γ2 and δ subunits of the GABA_A receptor in patches on the somata of cultured cerebellar granule cells. The existence of synapses or the relationship of the receptor clusters to synapses on cultured granule cells in unknown.

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