Bergmann Glia GABA_A Receptors Concentrate on the Glial Processes That Wrap Inhibitory Synapses

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We studied the cellular and subcellular distribution of GABA_A receptors in the Bergmann glia and Purkinje cells in the molecular layer of the cerebellum by using electron microscopy postembedding immunogold techniques. Gold particles corresponding to $\alpha 2$ and $\gamma 1$ immunoreactivity were localized in Bergmann glia processes that wrapped Purkinje cell somata, dendritic shafts, and some dendritic spines. The gold particles were mainly located on the glial plasma membrane or intracellularly but near the plasma membrane. The density of gold particles corresponding to $\alpha 2$ and $\gamma 1$ GABA_A receptor subunits was 4.3-fold higher in the glial processes wrapping Purkinje cell somata than in the glial processes wrapping Purkinje cell dendritic spines. Moreover, the Bergmann glia GABA_A receptors were often located in close proximity to the type II GABAergic synapses made by the basket cell axons on Purkinje cell so-

mata. These GABAergic synapses were enriched in neuronal GABA_A receptors containing $\alpha 1$ and $\beta 2/3$ subunits. Unexpectedly, 2.8% of the Purkinje cell dendritic spines also showed immunoreactivity for the neuronal $\alpha 1$ or $\beta 2/3$ subunits, which were located on the spine in type I synapses or extrasynaptically. Double-labeling immunogold experiments showed that ~50% of the dendritic spines that were immunolabeled with the neuronal GABA_A receptors were wrapped by Bergmann glia processes containing glial GABA_A receptors. These results are consistent with a role of the Bergmann glial GABA_A receptors in sensing GABAergic synaptic function.

Key words: GABA_A receptor; Bergmann glia; astrocyte; synapse; Purkinje cell; neuron; glia; immunogold; electron microscopy; immunocytochemistry; cerebellum

GABA_A receptors (GABA_AR) are highly expressed in neurons and to a much lesser extent in glial cells (Blankenfeld and Kettenmann, 1992; Bovolin et al., 1992; Laurie et al., 1992; Persohn et al., 1992). GABA_AR have been electrophysiologically detected in cultured astrocytes from hippocampus (Blankenfeld and Kettenmann, 1992; Fraser et al., 1994), cerebral cortex (Bormann and Kettenmann, 1988; Blankenfeld et al., 1991), and cerebellum (Bovolin et al., 1992) and in astrocytes from hippocampal slices (Kang et al., 1998) and Bergmann glia from cerebellar slices (Müller et al., 1994). Nevertheless, the functional role of the glial GABA_AR is not known.

Bergmann glia are cerebellar astroglial cells that have their soma in the Purkinje cell layer and extend fibers across the molecular layer to the pial membrane. Their processes wrap somata, dendrites, and dendritic spines of the Purkinje cells and their excitatory and inhibitory synapses (Palay and Chan-Palay, 1974). Bergmann glia express GABA and glutamate transporters that are involved in the clearance of GABA and glutamate from synapses (Chaudhry et al., 1995; Conti et al., 1999). Bergmann glia and other astrocytes also express GABA_B receptors (GABA_BR) and various glutamate, acetylcholine, and P2X receptors (Ortega et al., 1991; Blankenfeld and Kettenmann, 1992; Fraser et al., 1994; Kang et al., 1998; Berthele et al., 2001;

Lino et al., 2001; Rubio and Soto, 2001; Sharma and Vijayaraghavan, 2001; Araque et al., 2002).

It has been proposed that GABA_AR expressed by Bergmann glia and other astrocytes are functionally related to GABAergic synaptic transmission (Blankenfeld and Kettenmann, 1992; Fraser et al., 1995; Kang et al., 1998; Bekar et al., 1999) and/or synapse formation and stabilization (Blankenfeld and Kettenmann, 1992; Matsutani and Yamamoto, 1997, 1998). Nevertheless, Bergmann glia GABA_AR could also be involved in nonsynaptic functions. Bergmann glia fibers are organized in parallel palisades, which might play a fundamental role in directing and/or maintaining the geometrical organization of the molecular layer of the cerebellum (De Blas, 1984; De Blas and Cherwinski, 1985). In developing cerebellum, Bergmann glia fibers guide the migrating granule cells between the external and internal granule cell layers (Mugnaini and Forstronen, 1967; Rakic, 1971).

In situ hybridization and light microscopy immunocytochemistry studies have shown that Bergman glia express $\alpha 2$ and $\gamma 1$ GABA_AR subunits (Wisden et al., 1989; Laurie et al., 1992; Persohn et al., 1992; Müller et al., 1994; Khan et al., 1996; Miralles et al., 1999). Nevertheless, there are no studies on the subcellular localization of these subunits at the electron microscopy level.

In this communication, we report, by using electron microscopy postembedding immunogold techniques, the localization of the Bergmann glia GABA_AR in relationship to the localization of the type II and type I synapses that Purkinje cells receive in their cell bodies and dendritic spines, respectively. We also report the presence of neuronal GABA_AR in some type I synapses of Purkinje cell dendritic spines and glial GABA_AR in the Bergmann glia processes that wrap these synapses. Glial GABA_AR were identified with anti- $\gamma 1$ and anti- $\alpha 2$ antibodies. Neuronal

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GABA_AR in Purkinje cell somata and dendritic spines were identified with anti- α 1 and/or anti- β 2/3 antibodies.

MATERIALS AND METHODS

All of the animal protocols have met the approval of the Institutional Animal Care and Use Committee and followed the National Institutes of Health guidelines.

Antibodies

The primary antibodies, rabbit or guinea pig anti- α 1 [amino acids (aa) 1–15], rabbit anti- α 2 (aa 417–423), and rabbit anti- γ 1 (aa 359–370), were raised and affinity purified in our laboratory. They were antibodies to synthetic peptides made to unique epitopes (extracellular N terminus for α 1 QPSQDELKDNTTVFT; extracellular C terminus for α 2 PVLGVSP; and large intracellular loop for γ 1 SMPQGEDDYGYQ) of the rat GABA_AR subunits (Khan et al., 1996; Miralles et al., 1999). The mouse monoclonal antibody (mAb) anti- β 2/3 (62-3G1) was raised in our laboratory to affinity-purified GABA_AR (De Blas et al., 1988; Vitorica et al., 1988). This antibody recognizes an extracellular N-terminus epitope that is common to β 2 and β 3 subunits but is not present in β 1 (Ewert et al., 1992). All antibodies to GABA_AR subunits used in this study have been thoroughly characterized, and their specificities were determined previously (De Blas et al., 1988; Vitorica et al., 1988; Moreno et al., 1994; De Blas, 1996; Miralles et al., 1999; Christie et al., 2002a,b). Specificity tests of GABA_AR antibodies included ELISA, immunoblotting, immunoprecipitation, light microscopy and electron microscopy immunocytochemistry, displacement of immunoreactivity in these assays by specific peptides, and subunit-specific staining in host-transfected cell lines.

Light microscopy immunocytochemistry

The procedure has been described previously (De Blas, 1984; De Blas et al., 1988; Moreno et al., 1994). Briefly, two 60-d-old Sprague Dawley rats were deeply anesthetized (8 mg/kg xylazine, 2 mg/kg acepromazine maleate, and 80 mg/kg ketamine HCl, i.p.) and transcardially perfused with fixative consisting of 0.01 M periodate–0.075 M lysine–4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 (PB). The brains were frozen and sliced in parasagittal sections (25 μ m thick) with a freezing microtome. Slices were incubated overnight at 4°C with affinity-purified rabbit anti- α 2 or rabbit anti- γ 1 in 0.1 M phosphate buffer, pH 7.4, with 0.3% Triton X-100. The sections were processed using an avidin–biotin–peroxidase system (Vectastain Elite; Novocastra, Burlingame, CA). Peroxidase reaction was performed with 3–3' diaminobenzidine tetrahydrochloride in the presence of cobalt chloride and nickel ammonium sulfate as chromogens and hydrogen peroxide as oxidant. Controls were performed by either omitting the primary antibody or incubating the primary antibody with the corresponding peptide (Miralles et al., 1999).

Electron microscopy immunocytochemistry

Tissue preparation, freeze substitution, and embedding in Lowicryl resin. Five adult (ranging from 35 to 70 d old) Sprague Dawley rats were anesthetized as described above, and each one was perfused with 60 ml of Ringer's solution [pH 6.9, room temperature (RT), for 1 min]. Three rats were perfused with 800 ml of fixative A (4% paraformaldehyde, 0.05% glutaraldehyde, and 0.2% picric acid in PB), and two rats were perfused with fixative B (4% paraformaldehyde and 0.5% glutaraldehyde in PB). For each antibody, the two fixatives gave similar immunolabeling. Fixative B has higher concentration of glutaraldehyde and yielded better preservation of the tissue morphology. Therefore, the two rats (35 and 63 d old) treated with fixative B were used for quantitative analysis and illustrations. No differences in qualitative or quantitative immunolabeling were observed in the studied age range. The fixed brains were washed in PB, and 300- to 500- μ m-thick sections were cut with a vibratome and immersed in PB (with or without 4% sucrose) at 4°C. Cryoprotection of the tissue was done by sequential incubations in 10, 20, and 30% glycerol or in 1 and 2 M sucrose in PB for 1 hr in each step and overnight in the last one, all at 4°C. Tissue sections were plunge-frozen in liquid propane cooled by liquid nitrogen (–190°C). A Leica (Vienna, Austria) AFS freeze substitution instrument was used for tissue embedding. The samples were immersed in 1.5% uranyl acetate in anhydrous methanol (–90°C, 30 hr), and the temperature was raised to –45°C. The samples were washed in anhydrous methanol, infiltrated with Lowicryl HM20 resin (Polysciences, Warrington, PA), and polymerized with UV light (–45 to 0°C) during 72 hr.

Postembedding immunogold. Seventy- to 80-nm-thick (gold interference color) sections were collected on 400-mesh gold-gilded nickel grids,

which were coated previously with the Quick pen (Electron Microscopy Sciences, Fort Washington, PA). A double-sided immunoreaction procedure (Matsubara et al., 1996) was used. It included the following (all at RT): (1) etching of the sections with a saturated solution of sodium ethanolate, (2) incubation with 0.1% sodium borohydride and 50 mM glycine in TBST (Tris-buffered saline plus 0.1% Triton X-100) for 10 min, (3) incubation with 2% normal goat serum (NGS) in TBST for 30 min, (4) incubation with affinity-purified primary antibodies in TBST containing 1% NGS, overnight, and (5) incubation with species-specific colloidal gold-coupled secondary antibody diluted 1:20–1:40 in TBST with 1% NGS and 5 mg/ml polyethylene glycol for 2 hr. The secondary antibodies were spun down at 2000 rpm before use to eliminate aggregated gold particles. After 15–24 hr, the tissue sections were counterstained with 1% uranyl acetate for 10 min and 0.3% lead citrate for 4 min, both at RT.

For single-labeling experiments, only a primary antibody was used: guinea pig anti- α 1, rabbit anti- α 2, or rabbit anti- γ 1. After incubation with the primary antibody and washes, a goat-anti rabbit IgG coupled to 6-nm-diameter (Jackson ImmunoResearch, West Grove, PA) gold particle or a goat-anti guinea pig IgG coupled to 12-nm-diameter gold particle (ICN, Costa Mesa, CA) were used. For double-labeling experiments, the sections were incubated with a mixture of primary mouse mAb 62-3G1 to β 2/3 and rabbit anti- α 1, or a mixture of mAb 62-3G1 to β 2/3 and rabbit anti- α 2. After several washes, the grids containing the tissue sections were incubated with a mixture of goat anti-rabbit IgG coupled to 6- or 18-nm-diameter gold particle (Jackson ImmunoResearch) and goat anti-mouse IgG coupled to 10 nm gold particle (ICN). The ultrathin sections were observed in a Philips 300 transmission electron microscope at 80 kV. Controls included omission of the primary antibody. Nonspecific binding (i.e., gold particles associated with nuclei and mitochondria) was minimized by testing various concentrations of NGS and various dilutions of the primary antibodies and choosing the best combinations.

Quantification of immunolabeling

Cellular and subcellular neuronal and glial structures were identified according to Palay and Chan-Palay (1974). Lowicryl blocks from five rats were used for qualitative analysis. Quantification was done with sections from two embedded cerebellar blocks (from different rats) for γ 1, three blocks (from the same two rats) for α 2, and one block from one rat for α 2 and β 2/3 double labeling. For quantification, the secondary antibodies were coupled to 6-nm-diameter gold particles for α 2 and γ 1 and 10 nm for β 2/3. For quantification of gold particles in the cell somata region, we selected the largest Purkinje cell somata present in the grid. For each Purkinje cell, all of the somata plasma membrane was scanned, and all areas in contact with Bergmann glia were photographed. For quantification of gold particles in dendritic spines, photographs of randomly selected dendritic spines were taken along different depths of the molecular layer (from the basal portion of the molecular layer proximal to the apical half of the Purkinje cell somata to the pial surface of the molecular layer). The criteria for dendritic spine selection were as follows: (1) that they had synapses, (2) that they had a well preserved plasma membrane, and (3) that the membrane of the wrapping Bergmann glia was well preserved. For quantification, all photographic film negatives were taken at 26,900 $\times$ and printed at 67,250 $\times$. A total of 457 photographs from two rats were used for the quantification of α 2 and γ 1 immunolabeling. For the α 2 subunit, 73 photographs of Purkinje cell somata and 183 photographs of Purkinje cell dendritic spines were used. For the γ 1 subunit, 61 and 140 photographs of Purkinje cell somata and Purkinje cell dendritic spines were used, respectively.

Film negatives were scanned and digitized, and area measurements were made with NIH Image 6.1 software. Positive labeling was scored when at least two gold particles were found near each other aiming to minimize nonspecific labeling, although it most likely led to underestimating the amount of labeling. For quantification, we only considered clearly identifiable glial processes that wrapped Purkinje neuron somata, dendrites, or dendritic spines. For quantitative comparison of labeling of Bergmann glia processes wrapping the Purkinje cell somata versus those wrapping dendritic spines, we counted the gold particles that were present on both the Bergmann glia plasma membrane and the intracellular submembranous glial cytoplasm located 0.1 μ m beneath the membrane. That is, we counted gold particles in 0.1- μ m-wide “rectangles” of Bergmann glia, with their length being the glial plasma membrane in contact with the Purkinje cell. Thus, the density units are given in number of gold particles per square micrometer instead of per length. We chose 0.1- μ m-wide because that was the minimum thickness of the

Figure 1. Light microscopy immunocytochemistry of $\alpha 2$ (*A*, *C*) and $\gamma 1$ (*B*, *D*) subunits of the GABA_A receptor in the cerebellar cortex. The immunolabeling for both subunits concentrates in the Bergmann glia cell bodies that are located in the Purkinje cell layer (*PKL*) and the Bergmann glia fibers that are located in the molecular layer (*ML*). There is no labeling in the granule cell layer (*GL*). Scale bars, 50 μ m.

Bergmann glia processes that wrap Purkinje cell dendritic spines, whereas the processes that wrap the Purkinje cell somata are thicker. In this way, we compared particles that were on the membrane or near the membrane in both somata and spines. For quantification of gold particles in the Bergmann glia processes that wrap Purkinje cell somata, each independent measurement was taken on an uninterrupted segment of Bergmann glia membrane that was in contact with the Purkinje somata. If a picture showed interruption of this contact (i.e., by a synaptic terminal or any other neuronal process), then two independent measurements of gold particles were taken, one for each continuous segment of Bergmann glia membrane.

In addition to the experimental controls, specificity of the $\gamma 1$ and $\alpha 2$ immunolabeling of Bergmann glia is supported by the following: (1) as indicated below, the density of gold particles corresponding to $\gamma 1$ and $\alpha 2$ immunolabeling was 28.1- and 5.1-fold higher, respectively, in the wrapping Bergmann glia processes than in the adjacent Purkinje cell somata; (2) the similar subcellular distribution in the Bergmann glia of the immunogold particles for two different antibodies to two different GABA_AR subunits ($\alpha 2$ and $\gamma 1$); and (3) the excellent agreement between our EM immunogold data and the light microscopy immunocytochemistry and *in situ* hybridization data from various laboratories regarding the expression of these subunits in Bergmann glia, as shown and discussed below.

RESULTS

Light microscopy immunocytochemistry and EM postembedding immunogold show that, in the cerebellum, $\alpha 2$ and $\gamma 1$ GABA_A receptor subunits are mainly expressed by Bergmann glia

Light microscopy immunocytochemistry revealed that, in the rat cerebellum, the GABA_A receptor subunits $\alpha 2$ (Fig. 1*A,C*) and $\gamma 1$

(Fig. 1*B,D*) are expressed in the molecular layer. The granule cell layer shows little or no immunoreactivity. Both antibodies showed similar distribution and intensity of the immunoperoxidase staining. In the molecular layer, both antibodies showed a staining pattern corresponding to the Bergmann glia. It included the Bergmann glia small cell bodies, which are located in or next to the Purkinje cell layer, and the Bergmann glia fibers, which expand vertically across the molecular layer. No staining of neuronal elements that are present in this layer, such as stellate or basket cells, was observed. Faint immunolabeling was found in the somata of Purkinje cells. It is not clear whether the faint immunolabeling (or a fraction of it) of the Purkinje cell somata is specific, because the latter show very faint labeling in control experiments in which the primary antibodies were omitted or the primary antibody was incubated with the peptide. It is worth noting that the two antibodies showed (1) stronger immunoreactivity of the molecular layer proximal to the Purkinje cell layer in which GABAergic synapses concentrate and (2) a gradient of decreasing immunolabeling across the molecular layer from proximal to the Purkinje cell layer to the pial surface. The localization of $\alpha 2$ and $\gamma 1$ on the Bergmann glia agrees with previous light microscopy immunocytochemistry studies in the cerebellum on $\alpha 2$ (Müller et al., 1994; Miralles et al., 1999) and $\gamma 1$ (Khan et al., 1996; Pirker et al., 2000) and with *in situ* hybridization studies (Wisden et al., 1989; Laurie et al., 1992; Persohn et al., 1992).

Figure 2. The $\alpha 2$ (*A, C*) and $\gamma 1$ (*B, D*) subunits of the GABA_AR are localized on the plasma membrane or intracellularly but near the plasma membrane of the Bergmann glia processes wrapping the Purkinje cell somata. In *A*, the *top arrow* points to gold particles associated with the Bergmann glia (*Bg*) plasma membrane. The *bottom arrow* points to gold particles, which seem to be associated with the Bergmann glia plasma membrane that is facing a Purkinje cell somata (*PKsm*). In *B*, the *arrow* shows gold particles on or closely associated with the plasma membrane of a Bergmann glia process facing both a Purkinje somata and a presynaptic terminal (*T*) making a type II synapse on the Purkinje cell somata. In *C*, the *arrow* points to four gold particles, three of which are located intracellularly and one of which is located on the Bergmann glia plasma membrane. In *D*, the *arrow* points to gold particles located near the Bergmann glia plasma membrane that are facing both a Purkinje cell somata and a presumed presynaptic terminal. Gold particle size, 6 nm in diameter. Scale bar, 0.15 μ m.

It should be noted that the intensity of the immunoreaction for the Bergmann glia GABA_AR is considerably lower than that of the neuronal GABA_AR in cerebellum, such as $\alpha 1$, $\beta 2/3$, and $\gamma 2$ (data not shown) (Fritschy and Mohler, 1995; Miralles et al., 1999; Pirker et al., 2000). This is also consistent with the electrophysiology data with Bergmann glia and astrocytes, showing that the density of GABA_AR in these glial cells is considerably smaller than in neurons (Blankenfeld and Kettenmann, 1992; Müller et al., 1994). Light microscopy immunocytochemistry does not have the necessary resolution to reveal the subcellular localization of these glial receptors. For this purpose, we used EM postembedding immunogold techniques.

The EM postembedding immunogold data also showed that $\gamma 1$ and $\alpha 2$ are exclusively or predominantly expressed by Bergmann glia. Thus, the average density of $\gamma 1$ gold particles (per square micrometer) in the two studied rats was 28.1-fold higher in the wrapping Bergman glia processes than in the adjacent Purkinje cells (mean $\pm$ SEM; 1.54 ± 0.68 vs 0.05 ± 0.04 ; $p < 0.05$; $n = 61$). The average density of the $\alpha 2$ gold particles (per square micrometer) in three different embedding blocks from two different animals was 5.2-fold higher in Bergmann glia than in Purkinje cells (3.42 ± 0.39 vs 0.66 ± 0.07 ; $p < 0.001$; $n = 74$), which

suggests that $\alpha 2$ might be expressed at low levels in Purkinje cells. These cells predominantly express the $\alpha 1$ isoform (Laurie et al., 1992; Persohn et al., 1992; Fritschy and Mohler, 1995; Miralles et al., 1999; Pirker et al., 2000). As indicated above, light microscopy showed that the $\alpha 2$ and $\gamma 1$ immunolabeling intensity signals are relatively low compared with that of the neuronal GABA_AR subunits. The immunolabeling signal is further weakened in EM immunocytochemistry. Therefore, this study deals with relatively low labeling signal.

The $\alpha 2$ and $\gamma 1$ subunits of the GABA_AR are localized on the plasma membrane, or near the plasma membrane, of the Bergmann glia processes that wrap the Purkinje cell somata, dendritic shafts, and dendritic spines

EM postembedding immunogold with anti- $\alpha 2$ and anti- $\gamma 1$ antibodies revealed that the two GABA_A receptor subunits showed similar subcellular distribution. Gold particles corresponding to $\alpha 2$ and $\gamma 1$ were located in Bergmann glia process that wrapped the Purkinje cell somata (Fig. 2), dendritic shafts (Fig. 3), and dendritic spines (Fig. 4). Gold particles were located on the Bergmann glia plasma membrane (Figs. 2*A–C*, 3*B,C*, 4) or intra-

Figure 3. The $\alpha 2$ (*A, C*) and $\gamma 1$ (*B, D*) subunits of the GABA_A receptor are localized in the Bergmann glia processes that wrap the Purkinje cell dendrites shafts. EM postembedding immunogold. Arrows show that the gold particles are located on the plasma membrane or intracellularly but near the plasma membrane of the Bergmann glia processes (*Bg*) that face the shafts of the Purkinje cell dendrites (*PKd*). Their cisternal stacks and/or their connection with the Purkinje cell soma identified Purkinje cell dendrites. Gold particle size, 6 nm in diameter. Scale bar, 0.15 μ m.

cellularly but often near the plasma membrane (Figs. 2*C,D*, 3*A,D*, 4*A,D*). In these examples, the labeling of the Bergmann glia was apposed to the Purkinje cells. Sometimes, the labeling was found in Bergmann glia membranes distant from the Purkinje cell (Fig. 2*A*). In Bergmann glia processes surrounding dendritic spines, we found gold particles located in the Bergmann glia membrane apposed to type I presumably glutamatergic synapses (Fig. 4*C,D*). We considered the labeling to be specific when two or more gold particles were close to each other. This is not to suggest that single gold particle labeling is not specific; it is just to indicate that, in our analysis, we studied labeling that has the highest probability of being specific, and therefore it underestimates the quantitative labeling values.

Quantitative analysis revealed that ~40% of gold particles for $\alpha 2$ and $\gamma 1$ were associated with Bergmann glia plasma membranes, and 60% were intracellular. A large percentage (83% for $\alpha 2$ and 89% for $\gamma 1$) of the intracellular particles were located close to the Bergmann glia plasma membrane (<0.1 μ m from the plasma membrane). Nevertheless, the estimate of the amount of gold particles associated with Bergmann glia plasma membrane was difficult because of the poorer preservation of the Bergmann glia membranes when compared with that of the neuronal membranes. Thus, the 40% value for the gold particles associated with the Bergmann glia plasma membrane may be underestimated. Moreover, the intracellular gold particles that are located near the plasma membrane might be labeling receptors located on the membrane of intracellular vesicles. These membranes could con-

tain trafficking receptors in their way in or out of the membrane, or they could be a reservoir of membrane receptors.

The Bergmann glia $\alpha 2$ and $\gamma 1$ GABA_A receptor subunits concentrate but are not exclusively localized in the vicinity of GABAergic synapses

Purkinje neurons receive a large number of GABAergic synapses from basket cell axon terminals on the somata and proximal dendritic shafts. Lower density of GABAergic innervation from stellate cells is received throughout the Purkinje cell dendritic tree. Purkinje cell dendritic spines seldom receive GABAergic innervation. Therefore, to test the hypothesis that Bergmann glia GABA_AR are functionally related to neuronal GABAergic synapses, we investigated whether the glial GABA_AR were spatially associated with GABAergic synapses and whether they concentrate around the Purkinje cell somata. We found that many $\alpha 2$ and $\gamma 1$ related gold particles that were present in the Bergmann glia processes that wrap Purkinje cell somata were facing (Fig. 2*B,D*) or were near (Fig. 2*C*) type II synapses.

There were also gold particles in processes not clearly associated with synapses (Fig. 2*A*). Nevertheless, there might be inhibitory synapses in the neighborhood although outside the section plane. Only serial reconstruction could determine whether that were the case.

We also found $\alpha 2$ and $\gamma 1$ immunolabeling in Bergmann glia processes that wrapped dendritic spines and that received type I synapses (Fig. 4). Quantification indicated that 3.6 and 4.4% of

Figure 4. The $\alpha 2$ (*A*, *C*) and $\gamma 1$ (*B*, *D*) subunits of the GABA_A receptor are localized in Bergmann glia processes that wrap Purkinje cell dendritic spines. All of the Purkinje cell dendritic spines (*PKsp*) have a type I excitatory synaptic contact from a parallel fiber (*PF*). In *A*, the *top* arrow points to gold particles located on the plasma membrane of a Bergmann glia (*Bg*) process, and the *bottom* arrow points to gold particles located in the same process but intracellularly. In *B*, gold particles (*arrow*) are associated with the plasma membrane of a Bergmann glia process. In *C* and *D*, the gold particles concentrate on a region of the Bergmann glia processes that is adjacent to type I synapses. Gold particle size, 6 nm in diameter. Scale bar, 0.09 μ m.

Table 1. Bergmann glia GABA_AR concentrate in the processes that wrap Purkinje cell somata over the processes that wrap Purkinje cell dendritic spines

Rat cerebellum	EM block	GABA _A R subunit	Gold particle density in Bg processes that wrap		Density ratio in Bg processes wrapping somata/spines
			PK somata	PK spines	
1	1a	$\alpha 2$	4.44 ± 1.57 ($n = 30$)	1.02 ± 0.72 ($n = 48$)	4.4
1	1b	$\alpha 2$	4.20 ± 1.63 ($n = 16$)*	1.01 ± 0.72 ($n = 62$)	4.2
2	2a	$\alpha 2$	4.80 ± 1.46 ($n = 28$)*	1.07 ± 0.60 ($n = 73$)	4.5
1	1a	$\gamma 1$	1.50 ± 0.67 ($n = 34$)*	0.46 ± 0.26 ($n = 74$)	3.2
2	2a	$\gamma 1$	2.16 ± 1.36 ($n = 27$)*	0.41 ± 0.30 ($n = 65$)	5.3

Values (mean $\pm$ SEM) are density of gold particles (number per square micrometer) in Bergmann glia (*Bg*) processes that wrap Purkinje cell (*PK*) somata versus processes that wrap *PK* dendritic spines. In the processes wrapping the Purkinje cell somata, *n* indicates the number of samples measured, taken from 12 different somata. The total surface analyzed was 26.1 μ m². In the processes wrapping Purkinje dendritic spines, *n* is the number of dendritic spines analyzed. A total number of 322 spines were analyzed, corresponding to 38.9 μ m². *Indicates that, for an individual experiment, the differences in gold particle density are significant in processes wrapping somata versus dendritic spines ($p < 0.05$ by Student's *t* test). In a paired *t* test combining the five experiments, $p = 0.007$. In a Wilcoxon's signed *t* test, $p = 0.031$.

the Bergmann glia processes that wrapped dendritic spines showed immunolabeling for $\gamma 1$ and $\alpha 2$, respectively.

We also quantified and compared the density of gold particles (number of gold particles per square micrometer) corresponding to $\alpha 2$ and $\gamma 1$ in the Bergmann glia processes that wrap Purkinje cell somata versus the processes that wrap dendritic spines. Table 1 shows that the density of gold particles for $\alpha 2$ and $\gamma 1$ was significantly higher (4.4 times higher for $\alpha 2$ and 4.1 times higher for $\gamma 1$) in the Bergmann glia processes that wrap the somata than in the processes that wrap dendritic spines. In rat 2, for example, the density of gold particles for the $\alpha 2$ subunit was 4.8 and 1.07 particles per square micrometer in the Bergmann glia processes wrapping Purkinje cell somata and dendritic spines, respectively. For the $\gamma 1$ subunit (in tissue from the same rat and the same block; for details, see Table 1), these values were 2.16 and 0.41 particles per square micrometer, respectively. Synapses on the

Purkinje cell somata are GABAergic. Thus, the quantitative and qualitative data show that the Bergmann glia GABA_AR concentrate on the processes that wrap the Purkinje cell somata and that these glial receptors are spatially associated with GABAergic synapses. These results are consistent with the hypothesis that the Bergmann glia GABA_AR play a role in GABAergic synaptic transmission. The results are also consistent with the light microscopy immunocytochemistry data (Fig. 1), which show a gradient of immunolabeling for Bergmann glia GABA_AR across the molecular layer, being highest proximal to the Purkinje cell layer.

Some type I synapses of Purkinje cell dendritic spines contain neuronal GABA_AR, and the Bergmann glia processes surrounding these synapses frequently contain glial GABA_A receptors

We showed above the presence of glial GABA_AR in some Bergmann glia processes wrapping type I synapses on Purkinje cell dendritic spines. Others have shown the presence of neuronal GABA_AR in type I synapses made by mossy fibers on cerebellar granule cells (Nusser et al., 1996, 1998). Therefore, we studied whether type I synapses on Purkinje cell dendritic spines contain GABA_AR and, if so, whether the Bergmann glia processes that wrap these synapses contain glial GABA_AR.

Neuronal GABA_AR immunoreactivity was detected with three different antibodies (rabbit anti- α 1, guinea pig anti- α 1, and mouse mAb 62-3G1 to β 2/3), which gave similar results. The α 1, β 2, and β 3 GABA_AR subunits are expressed by Purkinje cells but not by Bergmann glia (Somogyi et al., 1989; Laurie et al., 1992; Persohn et al., 1992; Moreno et al., 1994; Fritschy and Mohler, 1995; Miralles et al., 1999; Pirker et al., 2000). Double-labeling immunogold experiments with anti- α 1 and anti- β 2/3 antibodies show (Fig. 5) that, as expected, most of the neuronal GABA_AR α 1 and β 2/3 subunits colocalized at high density on the type II GABAergic synapses of Purkinje cell somata and dendritic shafts. The synaptic localization of these subunits on type II synapses on Purkinje cells is in agreement with Nusser and Somogyi's (1997) findings.

In addition, we also found neuronal GABA_AR containing α 1 and β 2/3 subunits in some type I synapses that Purkinje cells receive in their dendritic spines (Fig. 6A–D) from presynaptic parallel fiber (Fig. 6B–F) and climbing fiber (Fig. 6A) terminals. Some of the neuronal GABA_AR in dendritic spines were located on the plasma membrane but extrasynaptically (Fig. 6E,F). Of the 767 Purkinje cell dendritic spines analyzed, 2.8% showed β 2/3 immunoreactivity.

We checked whether there was a spatial relationship between the Purkinje cell dendritic spines that contained neuronal GABA_AR and the Bergmann glia processes that wrapped these spines and contained glial GABA_AR. As indicated above, 3.6 and 4.4% of the Bergmann glia processes that wrap dendritic spines show immunolabeling for γ 1 and α 2, respectively. Therefore, if there is no spatial relationship, the probability of finding glial and neuronal GABA_AR in the glial process and their corresponding wrapped dendritic spines, respectively, would be extremely small (<0.16%). To address this question, we did double-labeling EM postembedding immunogold experiments with a rabbit anti- α 2 antibody (for the Bergmann glia GABA_A receptor) and a mouse anti- β 2/3 mAb (for the neuronal GABA_A receptor). These experiments not only confirmed the presence of neuronal GABA_AR in some asymmetrical type I synapses between parallel fibers and Purkinje cell dendritic spines, but they also showed that the Bergmann glia processes that wrapped these labeled synapses and

Figure 5. Neuronal GABA_AR containing α 1 and β 2/3 subunits colocalize in the type II synapses present on the shafts of Purkinje cell dendrites. Double-labeling postembedding immunogold with rabbit anti- α 1 and mouse mAb anti- β 2/3. Each panel shows a presynaptic terminal (*T*) making a type II synapse on a dendritic shaft of a Purkinje cell. The larger gold particles (18-nm-diameter) are for the α 1 subunit (arrows), and the smaller ones (10-nm-diameter) are for β 2/3 (arrowheads). Scale bar, 0.15 μ m.

the corresponding dendritic spines frequently had glial GABA_AR (Fig. 7A–C). Approximately 50% of the dendritic spines that showed anti- β 2/3 labeling of type I synapses also had wrapping Bergmann glia processes showing anti- α 2 immunolabeling. Thus, there is spatial association of glial and neuronal GABA_AR not only at the type II inhibitory synapses on the Purkinje cell somata but also in the type I excitatory synapses at the Purkinje cell dendritic spines.

DISCUSSION

The main findings of this communication are as follows. (1) Bergmann glia express GABA_AR containing α 2 and γ 1 subunits that concentrate on the glial processes that wrap Purkinje cell somata, which are heavily innervated by inhibitory GABAergic type II synapses from basket cell axons. The Bergmann glia

Figure 6. Neuronal GABA_AR are present in the Purkinje cell dendritic spines both at type I synapses and extrasynaptically. Postembedding EM immunogold with guinea pig anti- $\alpha 1$ (*A*, *C*; 12-nm-diameter gold particles), rabbit anti- $\alpha 1$ (*E*; 18-nm-diameter gold particles), or mouse mAb anti- $\beta 2/3$ (*B*, *D*, *F*; 10-nm-diameter gold particles). The gold particles are located at the periphery of the synapse (*A*) or are distributed along the synapse (*B–D*). Some particles are extrasynaptically localized on the dendritic spine plasma membrane (*E*, *F*). *Bg*, Bergmann glia; *CF*, climbing fiber; *PF*, parallel fiber; *PKsp*, Purkinje cell dendritic spine. Scale bar, 0.18 μ m.

GABA_AR are frequently localized adjacent to these GABAergic type II synapses. As expected, strong immunogold labeling for neuronal $\alpha 1$ and $\beta 2/3$ subunit-containing GABA_AR was found on the axo-somatic type II GABAergic synapses. (2) Unexpectedly, 2.8% of the Purkinje cell dendritic spines showed immunolabeling for the neuronal GABA_AR (containing $\alpha 1$ and $\beta 2/3$ subunits), frequently localizing at type I presumably glutamatergic synapses. (3) Purkinje cell dendritic spines and their type I synapses containing neuronal GABA_AR (with $\alpha 1$ and $\beta 2/3$ subunits) were wrapped by Bergmann glia processes that frequently contained glial GABA_AR (with $\alpha 2$ and $\gamma 1$ subunits). Therefore, there is a correlation between the location of Bergmann glia GABA_AR and the location of GABAergic synapses and neuronal GABA_AR.

This is consistent with the hypothesis that Bergmann glia GABA_AR detect synaptically released GABA.

The postembedding immunogold technique allows reliable localization of membrane proteins at the nanometer level. However, some degree of spatial accuracy error is still present (Kellenberger and Hayat, 1991). For this reason, some gold particles localized on the Bergmann glia plasma membrane could correspond to receptors localized at the contacting Purkinje cell plasma membrane. This is unlikely because the aforementioned *in situ* hybridization and light microscopy immunocytochemistry studies have shown that $\alpha 2$ and $\gamma 1$ are expressed by Bergmann glia and not by Purkinje cells. Occasionally, individual gold particles corresponding to $\alpha 2$ or $\gamma 1$ were found in the Purkinje cell

Figure 7. Neuronal GABA_AR are present in type I glutamatergic synapses between parallel fibers and Purkinje cell dendritic spines. The Bergmann glia processes that wrap these spines also have glial GABA_AR. Double-labeling EM postembedding immunogold using rabbit anti- $\alpha 2$ (6-nm-diameter gold particles) and mouse mAb anti- $\beta 2/3$ (10-nm-diameter gold particles). *Arrowheads* show that neuronal $\beta 2/3$ GABA_AR subunits are present at type I synapses between presynaptic parallel fibers (PF) and postsynaptic Purkinje cell dendritic spines (PKSp). *Arrows* show that the $\alpha 2$ GABA_AR subunits are present in neighboring Bergmann glia processes (Bg) that wrap the Purkinje cell dendritic spines. Scale bar, 0.15 μ m.

cytoplasm, quite distant from the Purkinje cell plasma membrane. This labeling was considered nonspecific (see Materials and Methods). Similar rationale was applied for assigning the location of $\alpha 1$ and $\beta 2/3$ to the Purkinje cell membrane rather than to the membrane of the wrapping Bergmann glia process. Light microscopy and electron microscopy experiments have shown that these subunits are present in Purkinje cell dendrites but not in Bergmann glia (Somogyi et al., 1989; Hansen et al., 1991; Fritschy and Mohler, 1995; Miralles et al., 1999). Moreover, in our studies, gold particles corresponding to $\alpha 1$ and $\beta 2/3$ were in most cases clearly localized on Purkinje cell plasma membranes, frequently at the synaptic cleft, in which no Bergmann glia membranes were present.

Astrocytes release neurotransmitters and other substances that modulate synaptic transmission (Haydon, 2001; Smit et al., 2001; Ullian et al., 2001). Some of these substances might be released in response to activation of glial GABA_AR. Activation of glial GABA_AR depolarizes the glial membrane and induces an efflux of Cl^- ions. Thus, glial GABA_AR might be involved in maintaining the extracellular Cl^- concentration at the synapse (Blankenfeld and Kettenmann, 1992; Fraser et al., 1995). GABA_AR might also be involved in maintaining the extracellular pH and K^+ synaptic homeostasis (Fraser et al., 1995; Bekar et al., 1999). In addition to GABA_AR, Bergmann glia express mRNA for GABA_{B1} receptor (Berthele et al., 2001). In the hippocampus, the activation of astrocytic GABA_BR, and to a lesser extent GABA_AR, results in the potentiation of inhibitory synaptic transmission (Kang et al., 1998).

Bergmann glia GABA_AR that are adjacent to GABAergic synapses might sense synaptically released GABA, whereas the glial GABA_AR distant from GABAergic synapses and the ones adjacent to the glutamatergic type I synapses in dendritic spines might sense GABA spillover from more distant GABAergic synapses. GABA spillover in the cerebellar glomerulus is able to activate $\alpha 6$ subunit-containing GABA_AR within a 2.5 μ m radius (Rossi and Hamann, 1998). GABA spillover also occurs in the hippocampus (Isaacson et al., 1993). GABA spillover could be sensed not only by the Bergmann glia GABA_AR but also by the postsynaptic neuronal GABA_AR that are present on the dendritic spines localizing either in type I, presumably glutamatergic, synapses (Figs. 6A–D, 7A–C) or extrasynaptically (Fig. 6E,F). It has also been shown that GABA_{B1a/b} and GABA_{B2} receptor subunits are found not only postsynaptically in type II synapses but also presynaptically and postsynaptically in type I synapses and around these glutamatergic synapses (Gonchar et al., 2001; Kulik et al., 2002). Moreover, Bergmann glia also express GABA_BR (Berthele et al., 2001). Therefore, spilled over GABA would activate the presynaptically and postsynaptic GABA_AR and GABA_BR of the glutamatergic synapses at Purkinje cell dendritic spines and the GABA_AR and GABA_BR of the Bergmann glia processes that wrap these synapses.

Another possible functional role for postsynaptic GABA_AR in Purkinje cell dendritic spines is that these GABAergic neurons might release GABA from the dendritic spines into the synaptic cleft of these glutamatergic synapses activating the postsynaptic GABA_AR and therefore resulting in the efficient inhibition of these glutamatergic synapses. The dendritic synaptic release of GABA would further inhibit the glutamatergic synapse by activating the presynaptic GABA_BR present in these synapses blocking glutamate release, among other actions. Such mechanism has been documented in the neocortex (Zilberter et al., 1999).

The presence of GABA_AR containing $\alpha 6$, $\gamma 2$, and $\beta 2/3$ (but not

$\alpha 1$) subunits in cerebellar granule cell type I glutamatergic synapses from mossy fibers has been reported previously (Nusser et al., 1996, 1998). Note that, in contrast, GABA_AR containing the $\alpha 1$ subunit are present on the type I synapses at Purkinje cell dendritic spines. Therefore, the presence of GABA_AR in type I glutamatergic synapses might be a more common event than initially suspected. Moreover, we and others have found, by using hippocampal cultures in combination with light microscopy immunofluorescence techniques, that, in the absence of GABAergic synapses, GABA_AR can form postsynaptic clusters apposed to presynaptic glutamatergic terminals (Rao et al., 2000; Christie et al., 2002a,b).

Another possibility is that some of the type I synapses at Purkinje cell dendritic spines are GABAergic rather than glutamatergic. Thus, some axon terminals of stellate, basket, and Purkinje cell recurrent collaterals form type I synapses on Purkinje cell dendritic spines (Mugnaini, 1972; Palay and Chan-Palay, 1974). The existence of some GABAergic terminals making synapses on dendritic spines has also been described in the cerebral cortex (Beaulieu and Somogyi, 1990) and hippocampus (Fifková et al., 1992). It is also possible that the same Purkinje cell dendritic spine has both GABAergic and glutamatergic synapses.

Bergmann glia GABA_AR might also be involved in other roles. It has been shown that synapse formation and maintenance in the molecular layer of the cerebellum depend on the wrapping of the Purkinje cells and synapses by the Bergmann glia processes. These processes allow or disallow direct synaptic contacts among neurons. Bergmann glia AMPA receptors seem to be involved in this phenomenon (Lino et al., 2001), as well as Bergmann glia GABA_AR (Blankenfeld and Kettenmann, 1992). GABA released by neurons cocultured with astrocytes induced increased complexity of the astrocytic processes. This phenomenon results from the activation of glial GABA_AR (Matsutani and Yamamoto, 1997). The expression of Bergmann glia GABA_AR is higher in the early developmental stages of the cerebellum than in the adult (Müller et al., 1994). This could be related to the enhanced wrapping and unwrapping activity of the Bergmann glia at a time of extensive neuronal migration (particularly granule cells that vertically migrate over the Bergmann glia) and synapse formation and remodeling.

GABA_AR are heteropentamers composed of combinations of various subunit classes and isoforms ($\alpha 1$ – $\alpha 6$, $\beta 1$ – $\beta 3$, $\gamma 1$ – $\gamma 3$, δ , ϵ , and θ). The most abundant combination contains α , β , and γ subunits, although some contain α , β , and δ or just α and β subunits (for review, see Barnard et al., 1998; Mehta and Ticku, 1999; Whiting et al., 1999). Bergmann glia express $\alpha 2$, $\beta 1$, and $\gamma 1$ subunits, as shown by *in situ* hybridization (Laurie et al., 1992; Persohn et al., 1992) and immunocytochemical studies for $\alpha 2$ (Müller et al., 1994; Miralles et al., 1999; this study) and $\gamma 1$ (Khan et al., 1996; Pirker et al., 2000; this study). Bergmann glia GABA_AR are insensitive to benzodiazepine agonists and antagonists (Müller et al., 1994). This pharmacology is consistent with the presence of the $\gamma 1$ subunit in combination with α and β subunits (Ymer et al., 1990) (for review, see De Blas, 1996) in the Bergmann glia GABA_AR. Quantitative immunoprecipitation studies have shown that ~8–16% of the cerebellar GABA_AR contain $\gamma 1$ (Quirk et al., 1994; Khan et al., 1996), that they also contain $\alpha 2$ (Quirk et al., 1994), and that they do not show high-affinity binding for benzodiazepine receptor agonists, antagonists, or inverse agonists (Quirk et al., 1994; Khan et al., 1996). These results, together with the similar subcellular distribution of $\alpha 2$ and $\gamma 1$ reported in this communication, indicate that both

subunits are components of the Bergmann glia GABA_AR. These receptors must also contain the $\beta 1$ subunit because (1) this is the only β subunit isoform expressed in Bergmann glia (Persohn et al., 1992) and other astrocytes (Gu et al., 1992; Rosier et al., 1993) and (2) the GABA_AR must contain at least a β subunit for GABA and muscimol to bind to the receptor (Amin and Weiss, 1993; Smith and Olsen, 1995). Therefore, most of the Bergman glia GABA_A receptor pentamers contain $\alpha 2$, $\beta 1$, and $\gamma 1$ subunits.

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