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The $\gamma 2$ Subunit of the $GABA_A$ Receptor is Concentrated in Synaptic Junctions Containing the $\alpha 1$ and $\beta 2/3$ Subunits in Hippocampus, Cerebellum and Globus Pallidus

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Summary—The $\gamma 2$ subunit is necessary for the expression of the full benzodiazepine pharmacology of $GABA_A$ receptors and is one of the major subunits in the brain. In order to determine the location of channels containing the $\gamma 2$ subunit in relation to GABA-releasing terminals on the surface of neurons, a new polyclonal antipeptide antiserum was developed to the $\gamma 2$ subunit and used in high resolution, postembedding, immunoelectron-microscopic procedures. Dual immunogold labelling of the same section for two subunits, and up to three sections of the same synapse reacted for different subunits, were used to characterize the subunit composition of synaptic receptors. The $\gamma 2$ subunit was present in type 2, "symmetrical" synapses in each of the brain areas studied, with the exception of the granule cell layer of the cerebellum. The $\gamma 2$ subunit was frequently co-localized in the same synaptic junction with the $\alpha 1$ and $\beta 2/3$ subunits. The immunolabelling of synapses was coincident with the junctional membrane specialization of the active zone. Immunolabelling for the receptor often occurred in multiple clusters in the synapses. In the hippocampus, the $\gamma 2$ subunit was present in basket cell synapses on the somata and proximal dendrites and in axo-axonic cell synapses on the axon initial segment of pyramidal and granule cells. Some synapses on the dendrites of GABAergic interneurones were densely labelled for the $\gamma 2$, $\alpha 1$ and $\beta 2/3$ subunits. In the cerebellum, the $\gamma 2$ subunit was present in both distal and proximal Purkinje cell dendritic synapses established by stellate and basket cells, respectively. On the soma of Purkinje cells, basket cell synapses were only weakly labelled. Synapses on interneuron dendrites were more densely labelled for the $\gamma 2$, $\alpha 1$ and $\beta 2/3$ subunits than synapses on Purkinje or granule cells. Although immunoperoxidase and immunofluorescence methods show an abundance of the $\gamma 2$ subunit in granule cells, the labelling of Golgi synapses was much weaker with the immunogold method than that of the other cell types. In the globus pallidus, many type 2 synapses were labelled for the $\gamma 2$ subunit together with $\alpha 1$ and $\beta 2/3$ subunits. The results show that $\gamma 2$ subunit-containing receptor channels are highly concentrated in GABAergic synapses that also contain the $\alpha 1$ and $\beta 2/3$ subunits. Channels containing the $\gamma 2$ subunit are expressed in synapses on functionally distinct domains of the same neuron receiving GABA from different presynaptic sources. There are quantitative differences in the density of $GABA_A$ receptors at synapses on different cell types in the same brain area. Copyright © 1996 Elsevier Science Ltd

Keywords—$GABA_A$ receptor, subunit composition, benzodiazepine receptor, electron microscopy, immunocytochemistry, hippocampus, pyramidal cell, cerebellum, globus pallidus, inhibition.

The $\gamma 2$ subunit of the $GABA_A$ receptor is required for normal channel conductance and the formation of benzodiazepine sites *in vitro* and *in vivo* (Pritchett *et al.*, 1989; Sigel *et al.*, 1990; Moss *et al.*, 1991; Wafford *et al.*, 1991; Gunther *et al.*, 1995). This subunit has been estimated to contribute to at least 84% of receptors (Duggan *et al.*, 1992; Benke *et al.*, 1996; McKernan and Whiting, 1996), making it one of the most abundant subunits. Immunoprecipitation studies suggest that the $\gamma 2$ subunit can be co-assembled with the $\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 5$, $\alpha 6$, $\beta 2$, $\beta 3$, $\gamma 3$ and δ subunits (Duggan and Stephenson, 1990; Benke *et al.*, 1991a, 1991c, 1991d, 1996; Duggan *et al.*,

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1991; McKernan *et al.*, 1991; Mertens *et al.*, 1993; Pollard *et al.*, 1993; Khan *et al.*, 1994a, 1994b; Quirk *et al.*, 1994a, 1994b; for reviews, see Mohler *et al.*, 1995; Sieghart, 1995; Stephenson, 1995), but the receptor stoichiometry in the brain has not been established. The solubilized receptors studied in immunopurification experiments may be derived from receptors extracted from the synaptic junctions, the extrasynaptic plasma membrane or from the intracellular pool. The extrasynaptic pool is significant and in most areas of the central nervous system, light microscopic immunocytochemistry has revealed a continuous diffuse immunolabelling of cell bodies and processes for $GABA_A$ receptors (Richards *et al.*, 1987; de Blas *et al.*, 1988; Houser *et al.*, 1988; Somogyi *et al.*, 1989; Soltesz *et al.*, 1990; Waldvogel *et al.*, 1990; Fritschy *et al.*, 1992, 1994; Gao *et al.*, 1993; Greferath *et al.*, 1993; Spreafico *et al.*, 1993; Fritschy and Mohler, 1995). Indeed, it has been calculated that, on cerebellar granule cells, the extrasynaptic plasma membrane receptor pool may exceed the synaptic pool several-fold (Nusser *et al.*, 1995b). Furthermore, different cell types in the same brain area may contain distinct complements of subunits (Laurie *et al.*, 1992; Persohn *et al.*, 1992; Wisden *et al.*, 1992; Gao *et al.*, 1993, 1995; Fritschy and Mohler, 1995). Recent reports also suggest that, even within the same cell, some channels, containing different subunits, may be targeted selectively to distinct sites in the plasma membrane (Koulen *et al.*, 1996; Nusser *et al.*, 1996a, 1996b), and receptors can be clustered on the cell surface, even in the absence of GABAergic innervation (Caruncho *et al.*, 1993). Accordingly, establishing whether channels incorporating particular subunits participate in synaptic transmission on defined domains of neurons requires the visualization of synaptic junctions and the localization of receptors with high resolution. One of the most direct methods satisfying these requirements is the application of subunit-specific antibodies and postembedding immunocytochemistry using particulate immunomarkers, such as gold particles (Baude *et al.*, 1993).

The present study was undertaken in order to determine the location of the $\gamma 2$ subunit in relation to other abundant subunits on the surface of neurons in the central nervous system. We were particularly interested as to whether there was any selectivity in the targeting of the $\gamma 2$ subunit to synapses on particular domains of the plasma membrane of cell types that are known to receive GABA from several distinct inputs. In addition, the use of particulate immunomarkers and the postembedding method, that exposes the sections uniformly to the antibody, provided an opportunity to compare different types of cells and synapses in the same area of the brain.

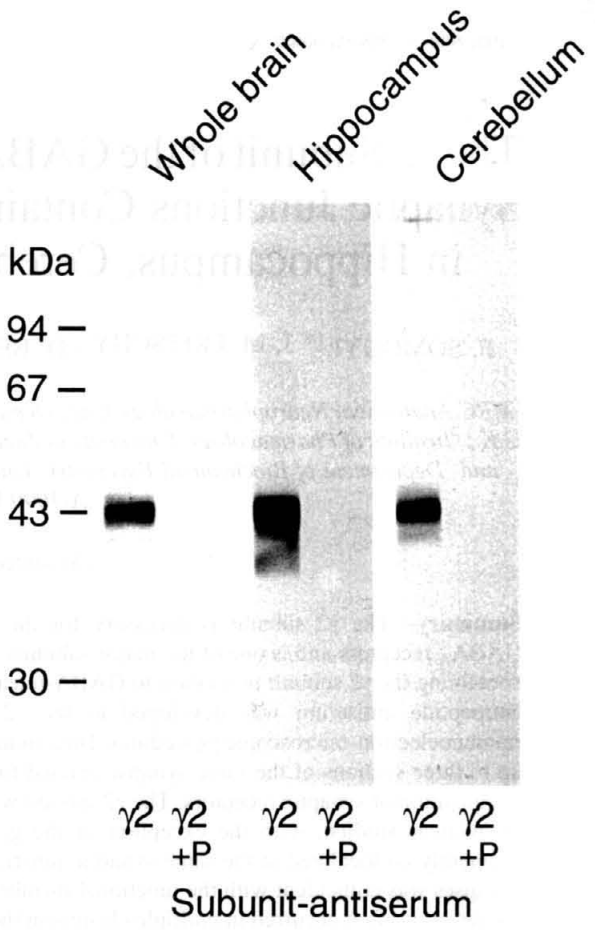

Fig. 1. Immunochemical characterization of antibodies to the $\gamma 2$ subunit. Crude membrane fractions isolated from adult rat brain (whole brain), hippocampus or cerebellum were subjected to Western blotting with affinity purified antibodies to the $\gamma 2$-subunit (3 μg/ml). In each sample, a broad band centred around 43 kDa, that corresponds to the $\gamma 2$ subunit, was detected. The specificity of the immunoreaction was assessed by preincubation with 10 μg/ml of peptide antigen ($\gamma 2$+P). The position of molecular weight markers is indicated on the left.

Fig. 2. Photomicrographs of sections processed for immunoperoxidase staining depicting the distribution of immunoreactivity for the $\gamma 2$ subunit in three brain regions where it is abundantly expressed (see Fig. 3 for a comparison with images from confocal microscopy). (A) In the dentate gyrus and the CA3 region of the hippocampus, immunoreactivity is predominantly in the dendritic layers, whereas the cell body layers contain less staining; a few interneurons appear more strongly labelled (arrowheads). (B) In the cerebellum, immunoreactivity appears most intense in the glomeruli of the granule cell layer (bottom) and is moderate in the molecular layer, where most of the staining is apparent on the soma and dendrites of interneurons (triangles). There is a light labelling around the cell body and on the initial part of the dendrites of Purkinje cells (arrowheads). (C) In the globus pallidus, the immunoreactivity decorates individual neurons and their dendrites, which appear like a dense network of intensely stained processes; the boundary with the much less labelled neostriatum is visible on the left side of the panel. Scale bars: 100 μm.

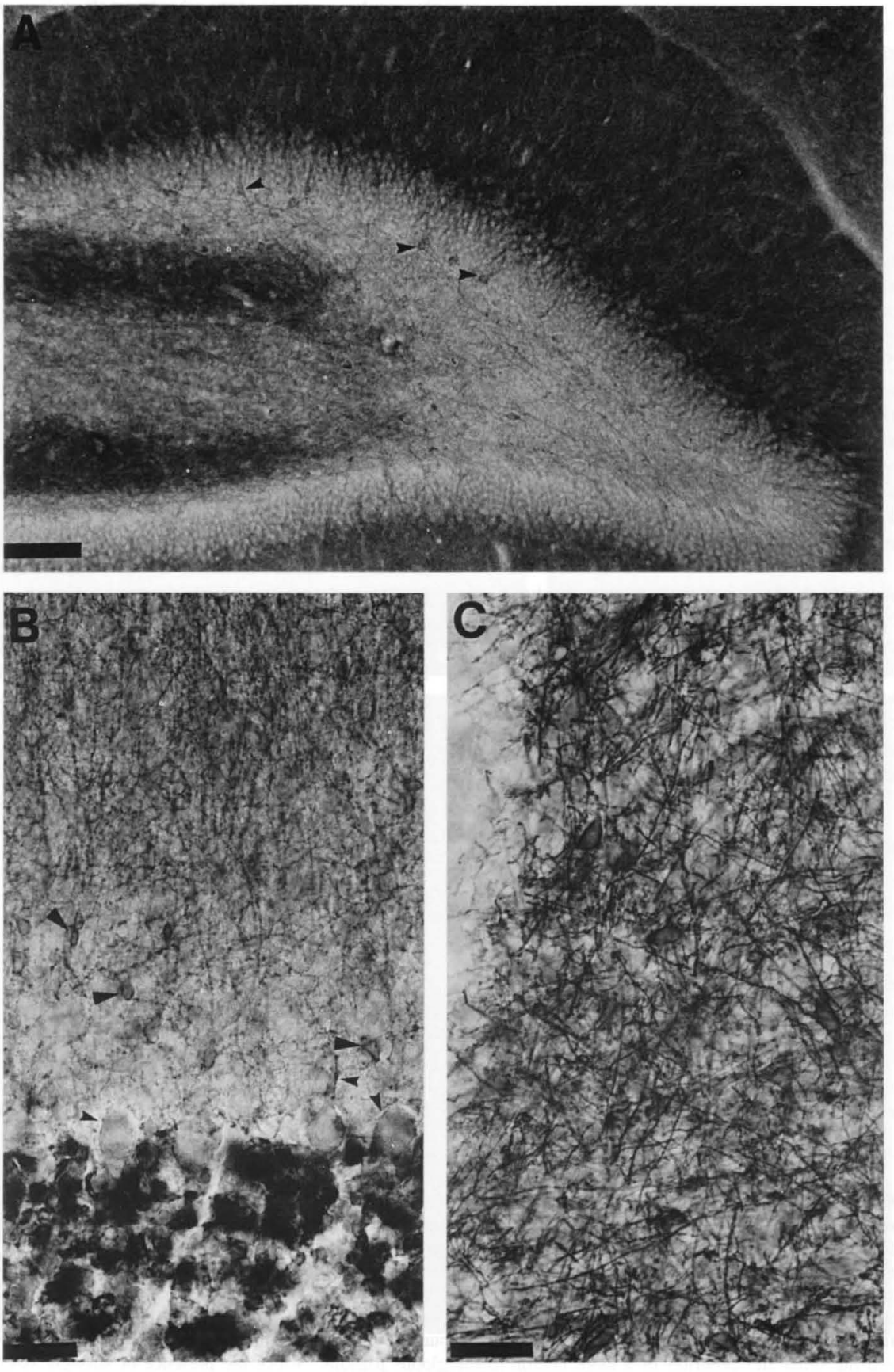

Fig. 2—*legend opposite.*

METHODS

Light microscopy

Immunohistochemistry was performed as described in Bohlhalter *et al.* (1996). Four rats were deeply anaesthetized with pentobarbital (50 mg/kg, i.p.) and perfused with 4% paraformaldehyde and ~0.2% picric acid in phosphate buffer. The brain was removed immediately after the perfusion and postfixed for 4 hr in the same fixative at 4°C. Thereafter, the tissue was processed with a modified antigen-retrieval protocol aimed at optimizing the signal-to-noise ratio in the subsequent immunohistochemical staining. Blocks of tissue were preincubated overnight at room temperature in 0.1 M sodium citrate buffer, pH 4.5, and irradiated with microwaves (95°C, 120 sec) at full power (650 W) in the same buffer. The tissue was then stored for 3 hr in phosphate-buffered saline (PBS) containing 10% dimethyl sulfoxide for cryoprotection. Free-floating sections cut from frozen blocks with a sliding microtome were incubated overnight with affinity-purified subunit-specific antibodies recognizing the γ2-subunit (1.25–2.5μg/ml, in Tris–saline containing 0.2% triton X-100 and 2% normal serum).

One series of sections was processed for avidin–biotin immunoperoxidase staining (Vectastain Elite kits), as described in the manufacturer's instructions, using diaminobenzidine hydrochloride as chromogen. Additional sections were processed for single and double immunofluorescence labelling using the antibodies to the γ2 subunit and a rabbit anti-α1 subunit antiserum in combination with secondary antibodies coupled to DTAF and Cy3 (Fritschy and Mohler, 1995).

Antibodies

The antiserum to the γ2 subunit was raised in guinea-pigs and affinity-purified as described in Benke *et al.* (1991c, 1996). Briefly, a synthetic peptide corresponding to the N-terminal 1–29 amino acids of the rat γ2 subunit was synthesized, purified and coupled through an additional C-terminal cysteine to keyhole limpet hemocyanin using *n*-succinimidyl 3-maleimidoproprionate as a cross-linker. Guinea-pigs were injected intradermally with the antigen emulsified 1:1 in Freund's complete adjuvant. Due to the shared N-terminal domain, the antibody is expected to recognize both the short and the long splice variants of the γ2 subunit (Kofuji *et al.*, 1991). Postembedding immunoreactions with affinity-purified antibodies were carried out at a final protein concentration of 10–25 μg/ml.

A rabbit antiserum to the α1 subunit was prepared and characterized as described earlier (Benke *et al.*, 1991b, 1991c), and it was used in the light microscopic studies. A rabbit polyclonal antiserum (code No. P16) was raised to a synthetic peptide corresponding to residues 1–9 of the rat α1 subunit. Antibody specificity has been described earlier (Zezula *et al.*, 1991). Postembedding immunoreactions with affinity-purified P16 antibody were carried out at a final protein concentration of 1.4–7 μg/ml.

Antibody specificity has been described for monoclonal antibody bd-17 (Haring *et al.*, 1985; Schoch *et al.*, 1985) that has been shown to react with both the β2 and β3 subunits (Ewert *et al.*, 1990). Therefore, the immunoreactivity detected by this antibody is referred to as β2/3. Antibodies were diluted to 10–20 μg protein/ml.

A commercial monoclonal antibody (diluted 1:10, Novocastra Lab., Newcastle upon Tyne, U.K.) to glial fibrillary acidic protein (GFAP) was used in control experiments (data not shown). A polyclonal antibody (kindly donated by Drs H. Ohishi and R. Shigemoto, Kyoto University), raised in guinea-pig and recognizing the metabotropic glutamate receptors type 2 and 3, was used in control experiments (data not shown).

An affinity-purified polyclonal antibody, raised in rabbit to a peptide sequence corresponding to residues 1117–1130 and recognizing the metabotropic glutamate receptor type 1α (Baude *et al.*, 1993), was used in control experiments (see Fig. 4).

Western blotting

Membranes from adult rat brain were prepared as described by Mertens *et al.* (1993) and stored at −30°C until use. Aliquots of the washed membranes (~20 μg protein/lane) were subjected to SDS–polyacrylamide gel electrophoresis (PAGE) and Western blotting, as described previously (Benke *et al.*, 1991a, 1991c; Mertens *et al.*, 1993), using the antibodies to the γ2-subunit. Immunoreactivity was detected by the luminescence

Fig. 3. Digital video images from confocal scanning laser microscopy illustrating the cellular distribution of immunoreactivity for the γ2 subunit in the dentate gyrus (A), cerebellum (B) and globus pallidus [(C) and (D)]. In these images, signals from immunofluorescence appear white. (A) Immunolabelling is apparent around the cell body of granule cells (e.g. arrowheads) and is intense in the molecular layer; labelled neurons and processes are also evident in the hilus (lower right corner). (B) In the cerebellar cortex, the strongest immunolabelling is around interneurons in the molecular layer (triangles) and around some granule cells (arrowheads) as well as in the glomeruli, but it is relatively weak around Purkinje cell somata (arrows). (C)–(D) Double-immunofluorescence labelling for the γ2 subunit (C) and α1-subunit (D), illustrating the nearly identical distribution of the two subunits on the soma and dendrites of a large neuron in the globus pallidus. Notice the presence of strongly labelled spots irregularly distributed along the cell surface (arrowheads). These images were obtained by superimposition of eight confocal images spaced by 0.5 μm. The top and the bottom of the soma of the large neuron are not entirely included in the series and hence appear partially unlabelled. Scale bars: 25 μm.

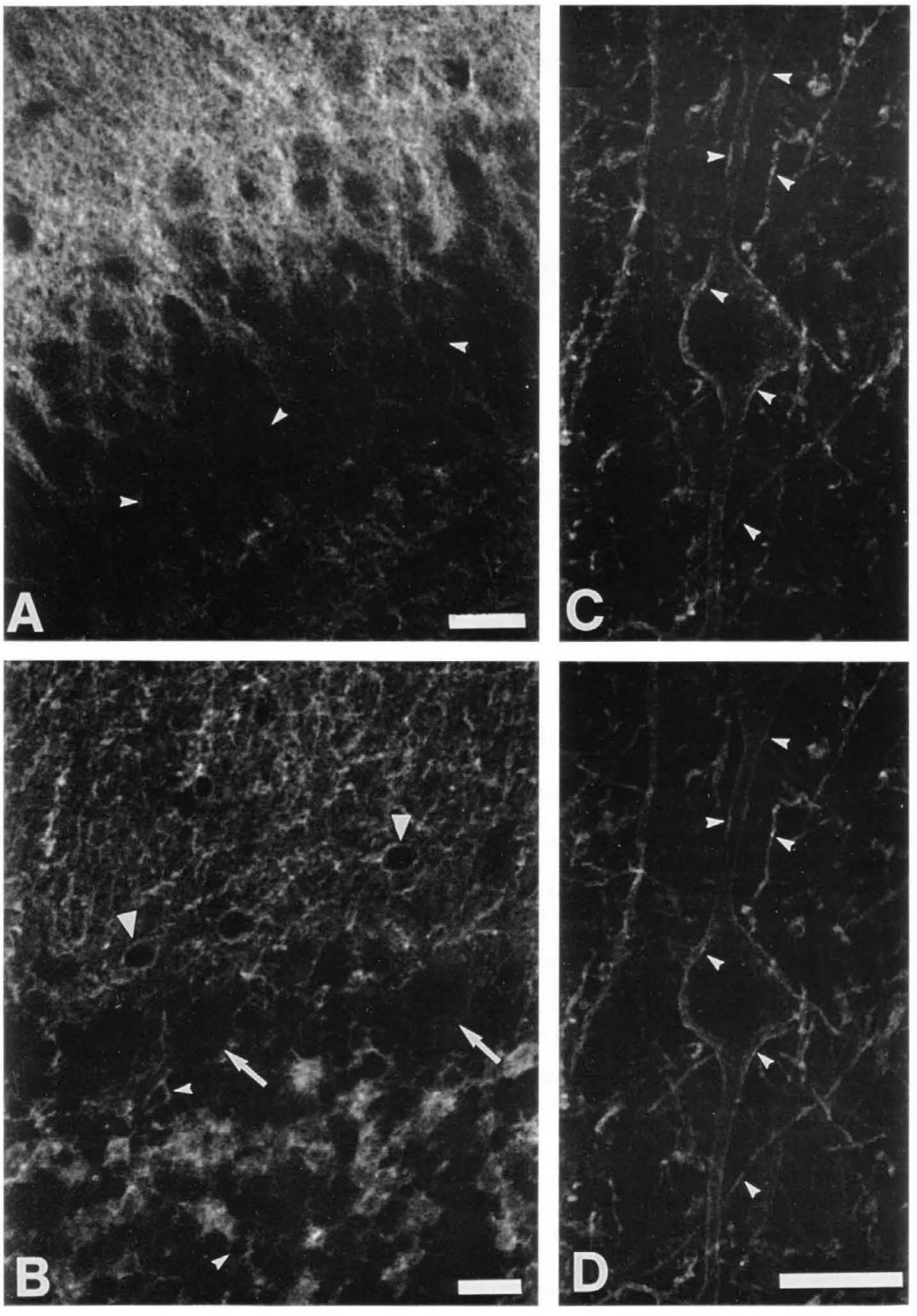

Fig. 3—*legend opposite.*

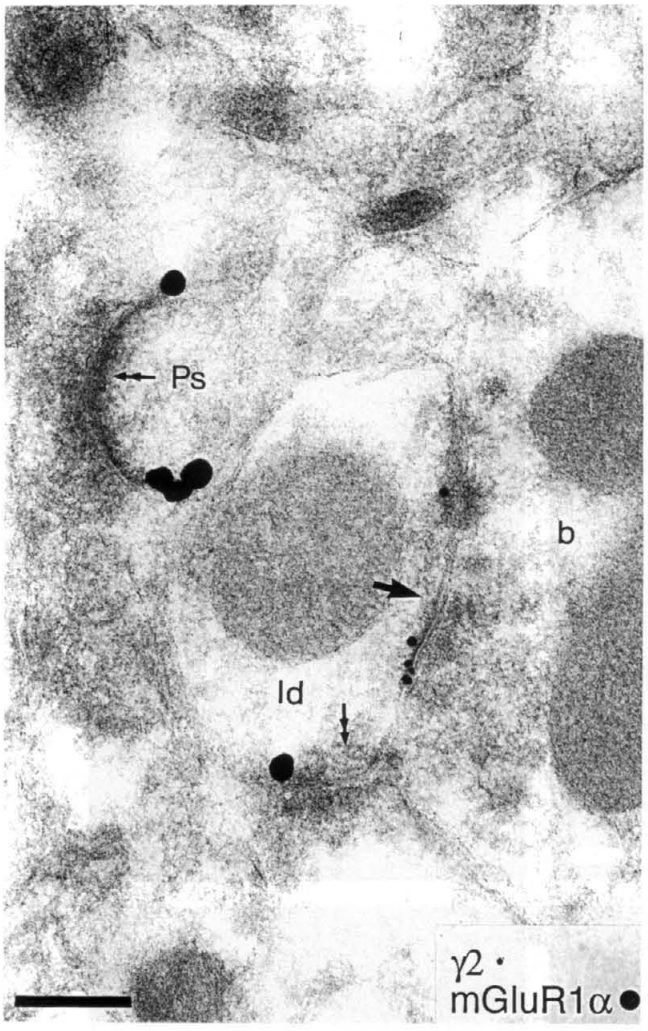

Fig. 4. Double immunogold labelling control experiment. Electron micrograph of the molecular layer in the cerebellar cortex following double immunolabelling for the metabotropic glutamate receptor type 1α (rabbit antibody, 10 nm gold label) and the γ2 subunit of the $GABA_A$ receptor (guinea-pig antibody, 1.4 nm gold label). Silver-intensified postembedding reaction, as in all subsequent figures. Immunoreactivity for the γ2 subunit is in a type 2 synaptic junction (arrow) made by a bouton (b) with an interneuron dendrite (Id). In contrast, labelling for mGluR1α is in a perisynaptic position at type 1 synapses (double arrows) both on the dendrite and on a Purkinje cell spine (Ps). The lack of co-localization of the two different size immunolabels for receptors that are in distinct domains of the plasma membrane demonstrates the reliability of the method for double labelling of GABAergic synapses. Scale bar: 0.2 μm.

method (ECL, Amersham, Bucks, U.K.) according to the instructions of the manufacturer. The specificity of the γ2-subunit antiserum was established by competition with the peptide used for immunization (10 μg/ml). The antiserum displays no cross-reactivity with either the γ1 or γ3 subunits, and it recognizes recombinant receptors containing the γ2-subunit (Benke *et al.*, 1996). The affinity-purified antibodies labelled a broad band, centred around 43 kDa and corresponding to the γ2 subunit, in blots of crude membrane fractions isolated from either adult rat brain (whole brain), or hippocampus, or cerebellum (Fig. 1). The immunoreaction was eliminated by preincubation with the peptide antigen (Fig. 1).

Preparation of animals and tissues for methacrylate embedding

Four adult Wistar rats were anaesthetized with Sagatal (pentobarbitone sodium, 220 mg/kg i.p.) and perfused through the heart with saline followed by fixative containing 4% paraformaldehyde, 0.05–0.1% glutaraldehyde and ~0.2% picric acid for 13–25 min. After perfusion, the brains were removed, and blocks from the dorsal hippocampus, cerebellum and globus pallidus/neostriatum were cut out and washed in several changes of 0.1 M phosphate buffer (PB).

Freeze substitution and low-temperature embedding in Lowicryl resin

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

Postembedding immunocytochemistry on ultrathin sections

Postembedding immunocytochemistry was carried out on 70- to 80-nm-thick sections of slam-frozen, freeze-substituted, Lowicryl-embedded material from four rats. The sections were picked up on pioloform-coated nickel grids. They were then incubated on drops of blocking solution for 30 min, followed by incubation on drops of primary antibodies overnight or for 2 days. When two subunits were localized on the same section, the primary antibodies from different species were mixed. The blocking solution, which was also used for diluting the primary and secondary antibodies, consisted of 0.1 M phosphate-buffered saline (pH 7.4) containing 0.8% ovalbumin (Sigma, St Louis, MO), 0.1% cold water fish skin gelatine (Sigma), and 5% foetal calf serum. After incubation in primary antibody, sections were washed and incubated on drops of goat anti-rabbit-, goat anti-mouse- or goat anti-guinea-pig IgG coupled to 1.4 nm gold (diluted 1:100, Nanoprobes Inc. Stony Brook, NY) or in goat anti-rabbit IgG adsorbed to 5 or 10 nm colloidal gold (diluted 1:30 or 1:60, Nanoprobes Inc.) for 2 hr at room temperature. When two subunits were localized on the same section, the two appropriate secondary antibodies were mixed. Following several washes, sections were fixed in a 2% glutaraldehyde solution for 2 min and then transferred to drops of ultrapure water prior to silver enhancement in the dark with an HQ Silver kit for 4–

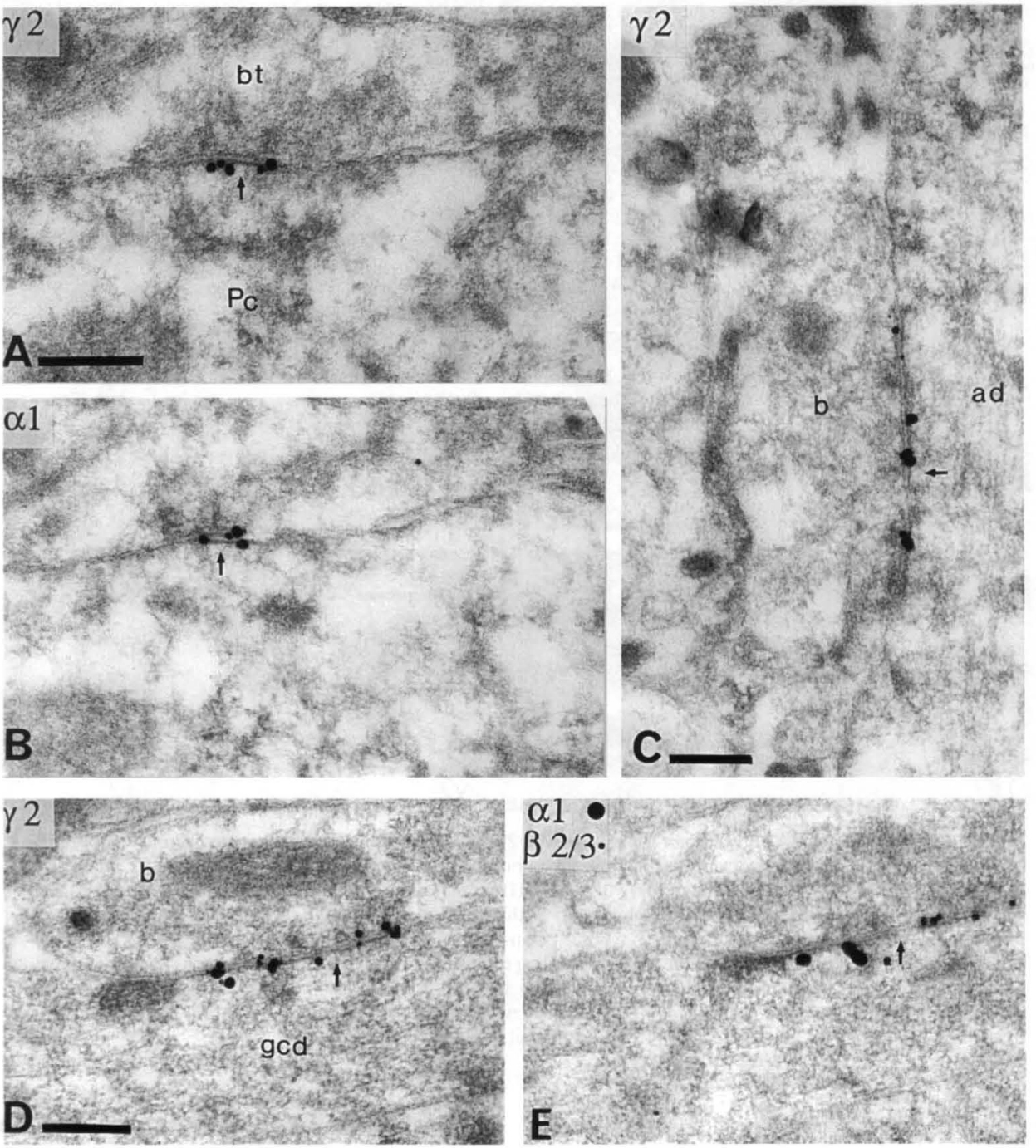

Fig. 5. Synaptic co-localization of the $\gamma 2$, $\alpha 1$ and $\beta 2/3$ subunits on hippocampal pyramidal cells [(A)–(C)] and a dentate granule cell dendrite [(D) and (E)]. Electron micrographs of serial sections [(A) and (B); (D) and (E)] reacted for a single subunit or for two subunits (E) as indicated. (A) and (B) A basket cell terminal (bt) makes an immunopositive synapse (arrow) on a pyramidal cell body (Pc). (C) An apical dendrite (ad) labelled for the $\gamma 2$ subunit alone is the postsynaptic target of a bouton (b). (D) and (E) All three subunits are highly enriched in a synaptic junction (arrow) on a dendrite that has the characteristics of granule cells. Silver-intensified postembedding immunoreaction using 1.4-nm gold label, except for the $\alpha 1$ subunit in (E) (5-nm gold label). Note the clustered distribution of labelling. Serial sections: same magnification. Scale bars: 0.2 μm.

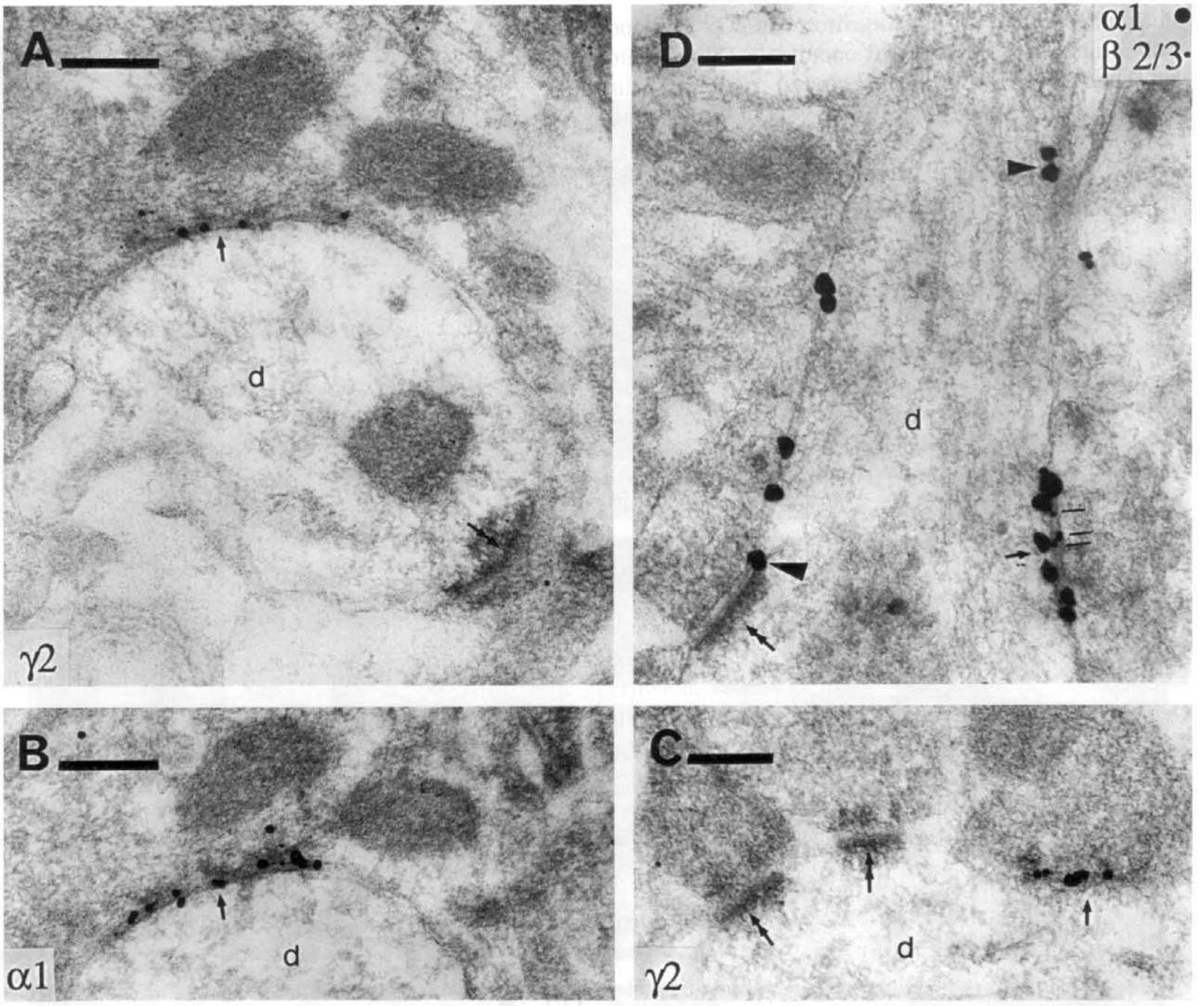

Fig. 6. Enrichment of $GABA_A$ receptor subunits in type 2 synaptic junctions (arrows) on interneuron dendrites (d) in the CA1 area of the hippocampus. Electron micrographs of serial [(A) and (B)] or individual [(C) and (D)] sections reacted for a single subunit or for two subunits (D) as indicated. The $\alpha 1$ subunit is shown here in the same synaptic specialization as either the $\gamma 2$ subunit (A) or the $\beta 2/3$ subunits [bars in (D)]. Interneuron dendrites are identified from the frequently occurring type 1 synapses (double arrows) on the dendritic shafts. Note the additional extrasynaptic location of the immunolabelling for the $\alpha 1$ subunit (e.g. triangles) which can occur around type 1 synapses (double arrow) that here are unlikely to receive GABA from the terminal giving rise to the junction. Silver-intensified postembedding immunoreaction using 1.4-nm gold label except for the $\alpha 1$ subunit in (D) (10-nm gold label). Scale bars: 0.2 μm.

5 min. After further washing in ultra pure water, the sections were contrasted with saturated aqueous uranyl acetate and then with lead citrate.

Controls

Controls for specificity in the light microscopic material included the omission of the affinity-purified antibodies, their replacement by non-immune guinea-pig or rabbit IgG of approximately the same concentration, and pre-absorption of the antibody with the antigen (5 μg/ml).

In the incubations for electron microscopy, selective labelling, resembling that obtained with the specific antibodies, could not be detected when the primary antibodies were either omitted or replaced by normal serum. Using polyclonal antibodies to synapsin (Naito and Ueda, 1981), no plasma membrane labelling was observed with our methods, demonstrating that the labelling observed on the plasma membrane is due to the anti-receptor antibodies.

For the double labelling experiments, a set of different antibodies raised in the same species as the three primary antibodies to $GABA_A$ receptor subunits was used as controls. The monoclonal antibody to the $\beta 2/3$ subunits was replaced with a commercial anti-GFAP antibody. The rabbit antibody to the $\alpha 1$ subunit was replaced with

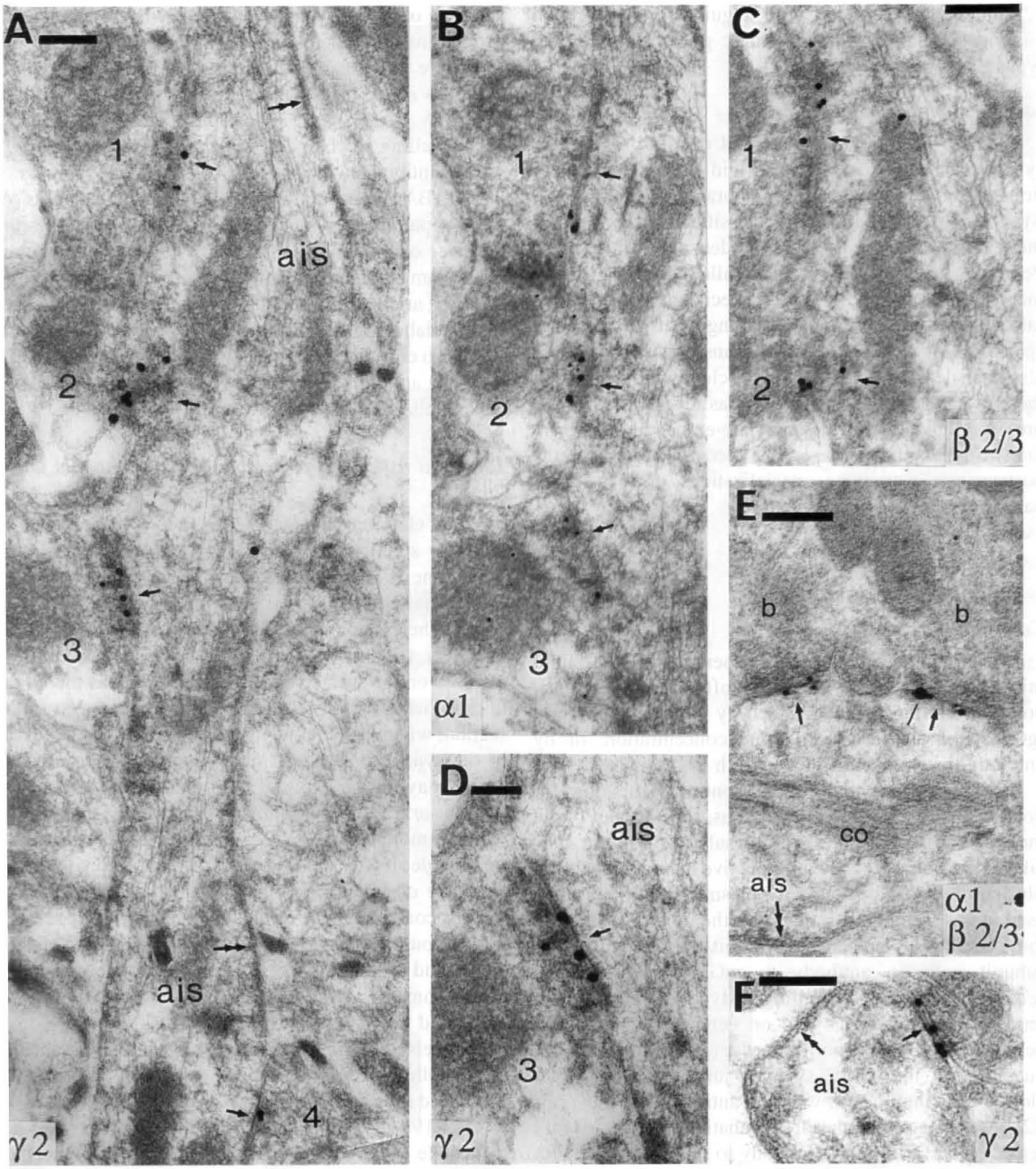

Fig. 7. Subunit composition of $GABA_A$ receptors in axo-axonic synapses on the axon initial segments (ais) of a CA1 pyramidal cell [(A)–(D)] and dentate granule cells [(E) and (F)]. Initial segments are identified by the electron dense membrane undercoating (double arrows) and/or the cisternal organelle (co). (A)–(D) All synaptic junctions (arrows) made by presynaptic boutons (1–4) are immunolabelled for the $\gamma 2$ subunit. In serial sections, three of the synapses are shown to be labelled for the $\alpha 1$ subunit and two for the $\beta 2/3$ subunits. The synapses are cut tangentially, which increases the probability of labelling, and can be tilted in the electron microscope to show the location of the immunoparticles in the synaptic cleft [e.g. bouton 3, arrow in (D)]. (E) The $\alpha 1$ subunit (bar) is shown in the same synaptic specialization as the $\beta 2/3$ subunits. (F) A synaptic junction on a granule cell axon initial segment is labelled only over the synaptic cleft. Silver-intensified postembedding immunoreactions using 1.4-nm gold label, except for the $\alpha 1$ subunit in (E) (5-nm gold label). (A) and (B): same magnification. Scale bars: (A)–(C), (E), (F): 0.2 μm; (D): 0.1 μm.

an antibody to mGluR1α. The guinea-pig antibody to the γ2 subunit was replaced with a guinea-pig polyclonal antibody recognizing metabotropic glutamate receptors types 2 and 3.

Identification of synaptic junctions

Synaptic junctions are identified on the basis of the synaptic vesicle accumulation, rigid membrane appositions and/or post-synaptic membrane specializations. Synaptic junctions have been classified on the basis of the extent of the post-synaptic density (Gray, 1959; Peters *et al.*, 1991) as type 1 (also called "asymmetrical", meaning extensive postsynaptic specialization) or type 2 (also called "symmetrical", meaning small postsynaptic specialization). Although some transitional forms have also been described, most synapses clearly fall into one of the two categories in the three areas investigated in the present study. In the methacrylate-embedded material, the two types of synapse can be recognized because the postsynaptic membrane specializations of type 1 synapses become very conspicuous.

RESULTS

Control experiments

No immunolabelling was observed in the light microscopic material when the affinity-purified antibodies were omitted or replaced by non-immune rabbit IgG of approximately the same concentration, or by antibody that was preincubated with the antigen.

In the electron microscopic control incubation for double labelling of the same sections, replacement of the monoclonal antibody to the β2/3 subunits with an anti-GFAP antibody resulted in selective labelling of glial fibrillary bundles, but never the plasma membrane, when used alone or in combination with the rabbit antibody to the α1 subunit. Replacing the rabbit antibody to the α1 subunit with an antibody to mGluR1α resulted in selective perisynaptic labelling of type 1 synapses both on Purkinje cell spines and on cerebellar interneuron dendrites (Fig. 4) as reported earlier (Baude *et al.*, 1993), but never of the type 2 synaptic junctions, when used alone or in combination with the antibodies to the γ2 or β2/3 subunits. A control incubation performed by replacing the guinea-pig antibody to the γ2 subunit with a guinea-pig polyclonal antibody recognizing metabotropic glutamate receptors types 2 and 3 did not result in labelling of type 2 synaptic junctions when used alone or in combination with the rabbit antibody to the α1 subunit. Therefore, we conclude that labelling of type 2 synaptic junctions is due to the specific primary antibodies raised to $GABA_A$ receptor (β2/3) or to the peptides (α1, γ2). The dual labelling of the same synaptic junctions with two sets of antibodies, recognizing two different subunits of the $GABA_A$ receptor and visualized by two sets of immunoparticles having different sizes, demonstrates the presence of immunoreactivity for two different subunits in the same synaptic junction and is not due to a cross-labelling artefact of the method. In most cases, when dual immunolabelling of single sections was used the results were also corroborated by incubating serial sections, each with one antibody only. The two experimental strategies gave similar results.

Technical aspects of postembedding immunogold labelling

When electron microscopic sections are incubated on antibody solution, the whole cut surface of the plasma membrane is uniformly exposed to the reagents, which means that comparisons can be made between different parts of the membrane of the same cell and different cells. However, the antibodies probably only reach the epitopes exposed on the surface of the section, whereas the image of the synapse is formed from the whole thickness of the section, which is about 60–80 nm. Therefore, some parts of the synaptic junctional membrane that appear in the image may not be exposed on the incubated side of the section surface. Furthermore, tangentially cut membranes result in more exposed epitopes than membranes cut at a right angle. It was apparent that the most heavy labelling could be observed on tangentially cut synapses. These factors, contributing to variations in labelling, were taken into account when drawing conclusions in the present study, and although observations were made over large populations of synapses, only a few cases can be illustrated for limitations on space.

Two sets of two different secondary antibodies labelled either with 1.4- or 5-nm, or 1.4- or 10-nm gold particles were used on single sections, as described earlier (Nusser *et al.*, 1994, 1995b). The 1.4-nm particles are not detectable with conventional transmission electron microscopy; therefore, silver intensification was used to

Fig. 8. Subunit composition of $GABA_A$ receptors in synapses (arrows) on cerebellar Purkinje cells. Purkinje cell dendrites (Pd) are identified by their emergence from the cell body, and/or size and the presence of cisternal stacks [e.g. in (A)–(C)]. (A)–(C) Serial sections. Two synaptic junctions (arrows) made by stellate cell terminals in the outer molecular layer are immunolabelled for the γ2 and α1 subunits; the one to the left is also labelled for the β2/3 subunits. (D) A presumed basket cell terminal in the lower molecular layer bridges two Purkinje dendrites. The immunolabelled synapse is clearly seen at the lower dendrite, and the upper one is just beginning to appear. (E) A rare labelled synaptic junction formed by a basket terminal on the cell body (Pc). (F) and (G) The α1 subunit is shown in the same basket (F) and stellate (G) cell synaptic specialization as the γ2 and β2/3 subunits (bars). Silver-intensified postembedding immunoreaction using 1.4-nm gold label, except for the α1 subunit in (B) and (C) (10-nm gold label), (G) and (F) (5-nm gold label). (A)–(C): same magnification. Scale bars: 0.2 μm.

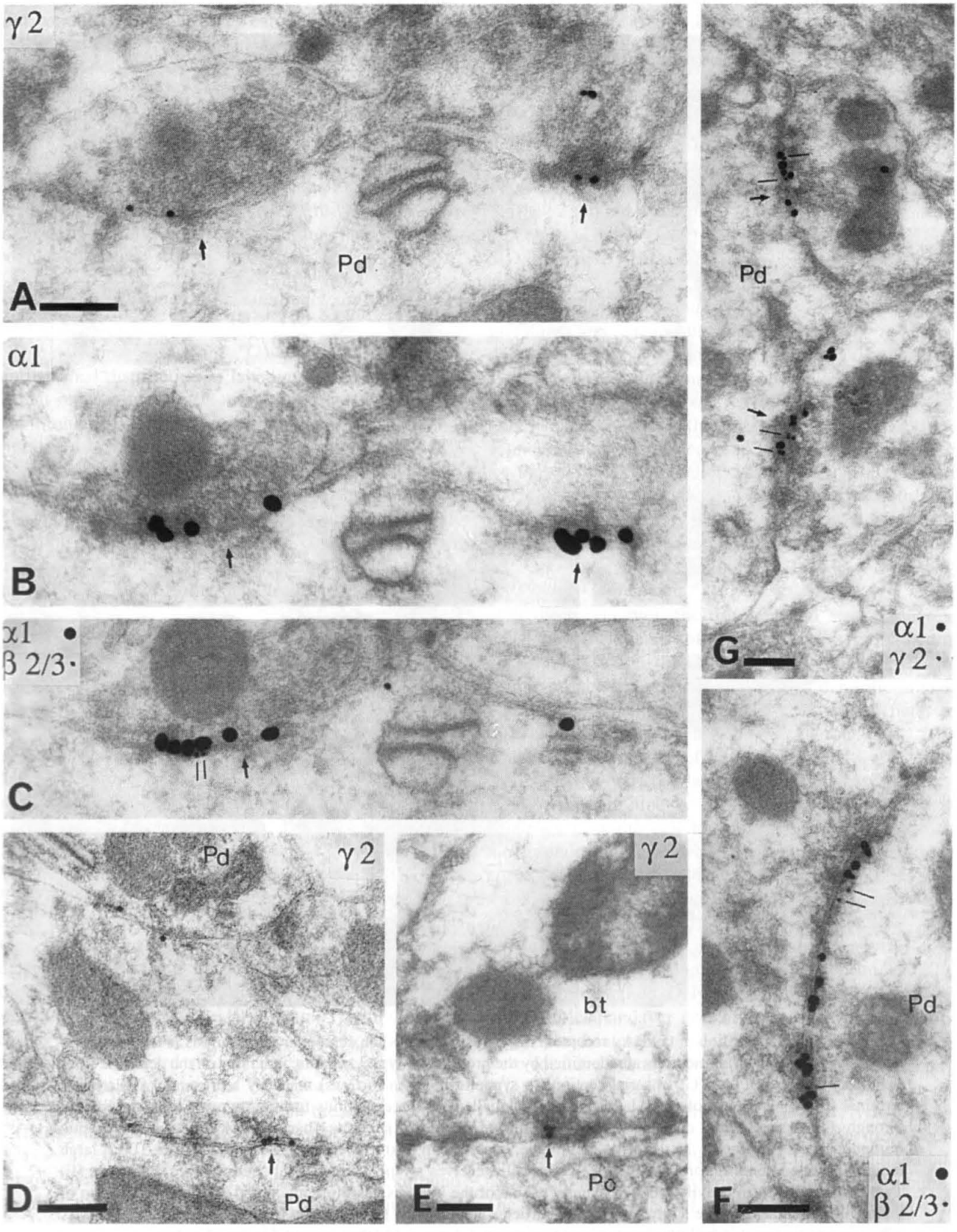

Fig. 8—*legend opposite.*

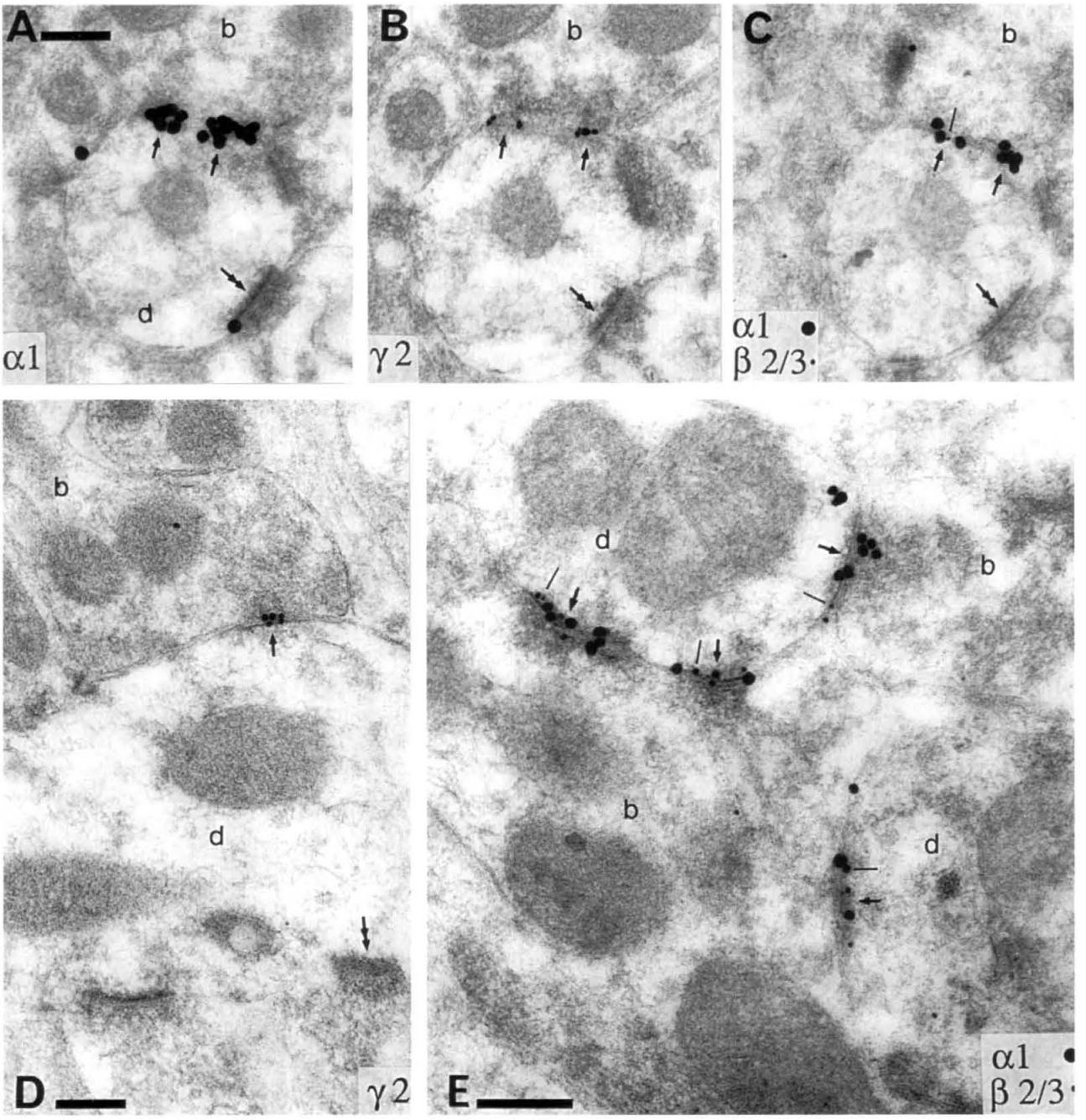

Fig. 9. Subunit composition of $GABA_A$ receptors in synapses (arrows) on cerebellar interneurons in the molecular layer. Dendrites (d) of interneurons are identified by the presence of type 1 synaptic junctions on the dendritic shaft (e.g. double arrows). (A)–(C) In serial sections, a synaptic junction (arrows) made by a presumed stellate cell terminal (b) in the outer molecular layer is immunolabelled for three subunits forming two clusters consistently through the series. Only one particle is visible for the $\beta 2/3$ subunits (bar). In (A), two extrasynaptic immunoparticles appear on the dendritic membrane, one of them next to the type 1 synaptic junction. (D) A large radially oriented dendrite, probably from a Golgi cell, receives a type 2 synapse labelled for the $\gamma 2$ subunit. (E) Two boutons (b) form heavily labelled synapses. One of the boutons has three junctional specializations, two of them with the same dendrite. Only some of the particles labelling the $\beta 2/3$ subunits (bars) are indicated for clarity. Silver-intensified postembedding immunoreaction using 1.4-nm gold label, except for the $\alpha 1$ subunit in (A) and (C) (10-nm gold label), and in (E) (5-nm gold label). (A)–(C): same magnification. Scale bars: 0.2 μm.

increase particle sizes. Because final particle size is variable and critically depends on the silver intensification conditions, particle sizes can only be compared on sections placed on the same electron microscopic grid. There was never any overlap between the particle sizes resulting from the use of 1.4- and 10-nm particles, but because of their large size, the latter may mask the presence of the smaller particles. Therefore, the 5-nm particles were also used in combination with the 1.4-nm particles, but in this case, in some incubations which were not included in the present study, there was an overlap between the particle sizes resulting from the two populations.

Regional distribution of immunoreactivity for the $\gamma 2$ subunit

The new antibodies to the $\gamma 2$ subunit resulted in a very similar areal distribution of labelling in the rodent brain to that reported earlier (Gutierrez *et al.*, 1994; Fritschy and Mohler, 1995). A moderate to intense $\gamma 2$ subunit immunoreactivity was detected throughout the brain. The globus pallidus, hippocampal formation and cerebellum were among the most intensely stained areas. The cellular distribution of the reaction product was markedly different in these three regions, as seen in both light and confocal laser scanning microscopy (Figs 2 and 3); therefore they were further analysed on the cellular and subcellular levels.

Hippocampus

In the hippocampus and dentate gyrus [Figs 2(A) and 3(A)] the staining was very light in the cell body layers, but intense and diffuse in dendritic layers. The immunolabelling was more intense in the innermost region of the dentate molecular layer, and this was particularly evident in the confocal images. In addition, isolated cells with a morphology of interneurons were distinctly and strongly labelled throughout the hippocampal formation [Fig. 2(A)] as described earlier for other subunits (Gao and Fritschy, 1994; Fritschy and Mohler, 1995; Nusser *et al.*, 1995a). However, in contrast to interneurons, in the cell body layers, the labelling could not be unequivocally allocated to individual principal cells in the light microscopic material since dendrites and axon initial segments, innervated by GABAergic terminals, are known to pass among the somata. Therefore, electron microscopy was used to test the presence of the $\gamma 2$ subunit in synaptic junctions on the somata and other parts of the cells in the CA1 area and the dentate gyrus.

The application of the new affinity-purified antibody in the postembedding protocol resulted in a clear signal in synaptic junctions with very little, if any, background labelling. Only type 2 (symmetrical) synaptic junctions were labelled (Figs 5, 6 and 7). The postsynaptic targets of $\gamma 2$ subunit positive synapses were somata [Fig. 5(A, B)], apical and basal dendrites of pyramidal cells [Fig. 5(C)], the dendrites of granule cells [Fig. 5(D, E)], the dendrites of interneurons (Fig. 6) and axon initial segments (Fig. 7) of both pyramidal and granule cells. In the CA1 area, the dendrites of pyramidal cells and interneurons are easily differentiated because the latter receive numerous type 1 synapses (Fig. 6) which are absent from the former. In the dentate gyrus, granule cell dendritic shafts may receive occasional type 1 synapses, but these are more numerous on interneuron dendrites. Axon initial segments are recognized on the basis of the electron-dense membrane undercoating and the presence of cisternal organelles (Fig. 7). Synapses immunolabelled for the $\gamma 2$ subunit were found to contain the $\alpha 1$ [Figs 5(A, B; D, E), 6(A, B) and 7(A–D)] and $\beta 2/3$ subunits [Figs 5(D, E) and 7(C)]. There were no obvious regions on the surface of postsynaptic cells lacking the $\gamma 2$ subunit in synapses, although immunopositive synapses in stratum radiatum and lacunosum moleculare on pyramidal dendrites were very rare. The most heavily labelled synapses were found on interneuron dendrites for all three subunits. There were many synapses which were only labelled for one of the subunits, but negative results cannot be taken as evidence for the absence of the subunit as the different antibodies have a different affinity. Therefore, different labelling intensity between antibodies is not necessarily related to the relative abundance of the respective subunits.

Cerebellar cortex

In the cerebellum, the different cell types could be recognized on the basis of their immunoreactivity for the $\gamma 2$ subunit. In the molecular layer, interneurons were distinctly labelled on their somata and dendritic arbors, whereas the labelling of Purkinje cells was difficult to ascertain among the prominent interneurons [Figs 2(B) and 3(B)]. The somata of Purkinje neurons were lightly surrounded by varicose labelling, but the possibility that this was partly due to the presence of interneuron dendrites near the cell bodies cannot be excluded. In the granule cell layer, intense staining was observed around some granule cell somata [Figs 2(B) and 3(B)], as well as in glomeruli, which appeared uniformly and densely labelled [Fig. 2(B)].

The immunolabelling of synaptic junctions could be established through the application of the postembedding immunogold method. Only type 2 synapses were labelled in the molecular layer on Purkinje cell dendritic shafts (Fig. 8) and interneuron dendrites (Fig. 9). The former were identified usually from the presence of characteristic cisternal stacks [Fig. 8(A–D)], their large size or on the basis of their origin from the cell body. Synapses on Purkinje cell dendrites were labelled close to the cell body as well as near the pial surface, indicating that both basket and stellate cell synapses contain the $\gamma 2$ subunit. The synapses on the cell body provided by basket cell axons were only rarely and lightly labelled [Fig. 8(E)]. The $\gamma 2$ subunit was frequently co-localized with the $\alpha 1$ subunit and the $\beta 2/3$ subunits [Fig. 8(A–C, G)].

Interneuron dendritic synapses were usually much more heavily labelled for all subunits than synapses on

Purkinje cells. Because of the heavy labelling, the clustered distribution of the labelling was very apparent in single synaptic specializations [Fig. 9(A–C)], and the same presynaptic terminals could also provide more than one synaptic junction to the same postsynaptic dendrite [Fig. 9(E)].

In the granule cell layer, synaptic labelling for the $\gamma 2$ subunit was much more difficult to find than in the molecular layer. It is likely that the strong immunoreactivity detected at the light microscopic level results from the high volume density of dendritic membranes abundant in extrasynaptic receptors (Nusser *et al.*, 1995b), which were rarely detected with the postembedding method that is less sensitive than the preembedding methods. The occasional synapses that were immunopositive for the $\gamma 2$ subunit were also immunopositive for the $\alpha 1$ and/or the $\beta 2/3$ subunits (Fig. 10). The antibody to the $\alpha 1$ subunit labelled many Golgi cell to granule cell synapses, having either thin (type 2) or thick (type 1) postsynaptic membrane specialization (Fig. 10) consistently from section to section, and it also revealed the abundant extrasynaptic receptors both on the dendritic and on the somatic membranes, as reported earlier (Nusser *et al.*, 1995b). The mossy terminal synapses, some of which are labelled for the $\alpha 6$ subunit (Nusser *et al.*, 1996b), were not labelled for any of the subunits studied in the present report.

Globus pallidus

A network of very intensely stained dendrites and somata was observed [Fig. 2(C)], and the morphology of individual neurons could be analysed in great detail by confocal scanning laser microscopy [Fig. 3(C)]. The soma and dendrites were distinctly outlined by immunolabelling for the $\gamma 2$ subunit, with local variations in intensity suggesting receptor aggregates. Double-labelling with the $\alpha 1$-subunit antiserum revealed a nearly identical distribution of the $\alpha 1$- and $\gamma 2$-subunits [Fig. 3(C–D)], suggesting their co-localization in similar ratio throughout the neuronal surface.

The synaptic enrichment and co-localization of both $\alpha 1$ and $\gamma 2$ subunits with the $\beta 2/3$ subunits were revealed by postembedding immunogold labelling (Fig. 11). Synapses on both small and large diameter dendrites were immunopositive, and extrasynaptic receptor labelling was evident, particularly for the $\alpha 1$ subunit [Fig. 11 (A, F)]. The clustered distribution of immunoreactivity in the active zone was best seen in tangentially cut synapses [Fig. 11 (C)]. As in the other areas, often only the edges of the synaptic active zones were labelled [Fig. 11 (H)].

DISCUSSION

Immunolabelling in relation to the synaptic active zone

The synaptic active zone, which is thought to be the site of vesicular transmitter release and action, is recognized in electron microscopic specimens on the basis of the rigid pre- and postsynaptic membrane apposition, the cleft material, the postsynaptic membrane specialization and the presynaptic grid consisting of dense projections (see, for example, Fig. 4). The present study confirms previous reports for other subunits of the $GABA_A$ receptor (Nusser *et al.*, 1995a, 1995b, 1996b; Nusser and Somogyi, 1996; Todd *et al.*, 1996), demonstrating that the $\gamma 2$ subunit is also highly enriched in the active zone, with a sharp fall in the density of immunolabelling at the edge of the membrane specialization. The presence of a defined area of high receptor density at the transmitter release site underlies the reproducibility of synaptic current evoked in the postsynaptic cell from release event to release event. It was also noted that, in all three areas of the brain studied here, the receptor labelling of the active zone appeared clustered in some synapses. This does not appear to be a technical artefact since it was observed with three different primary and three different secondary antibodies. The significance of this structured distribution of receptors is unknown and remains to be analysed quantitatively.

In some immunopositive synapses, the labelling was present only at the edge of the membrane specialization; a similar distribution is apparent in previously published illustrations (Nusser *et al.*, 1995a, 1995b, 1996b) for other subunits. Since the postembedding method has a limited sensitivity due to the restriction of the antibodies to the section surface, it is likely that such a labelling represents a visualization of only the highest density of receptors rather than the absence of receptor from the middle of the active zone. Since desensitization may play a role in GABAergic synaptic transmission (Jones and Westbrook, 1996), the possible increase of receptor density towards the edge of the synapse in some synaptic

Fig. 10. Synaptic concentration and co-localization of the $\gamma 2$ and $\alpha 1$ subunits in synapses (arrows) on dendrites (d) of cerebellar granule cells in the glomeruli. (A) and (B) and (C)–(E) are serial sections of the same synaptic junctions, respectively. (A) and (B) Two synapses made by Golgi terminals (Gt1, Gt2) are immunolabelled for the $\gamma 2$ subunit. The synapse made by Gt1 is labelled strongly also for the $\alpha 1$ subunit. The synapse made by Gt2 is not labelled for the $\alpha 1$ subunit, probably because it did not reach the incubated surface of the section. Two additional labelled synapses (open triangles) appear in the section shown in (B), which also shows extensive extrasynaptic labelling on dendrites [see also (E)] and a nearby granule cell soma (gc). (C)–(E) Serial sections of a synaptic junction labelled for three subunits. Note consistent labelling for the $\alpha 1$ subunit and the labelling of the edge of the synaptic junction for the $\gamma 2$ subunit. Silver-intensified postembedding immunoreaction using 1.4-nm gold label for the $\gamma 2$ and $\beta 2/3$ [bar in (E)] subunits and 10-nm gold label for the $\alpha 1$ subunit. (A) and (B), and (C) and (D): same magnification, respectively. Scale bars: 0.2 μm.

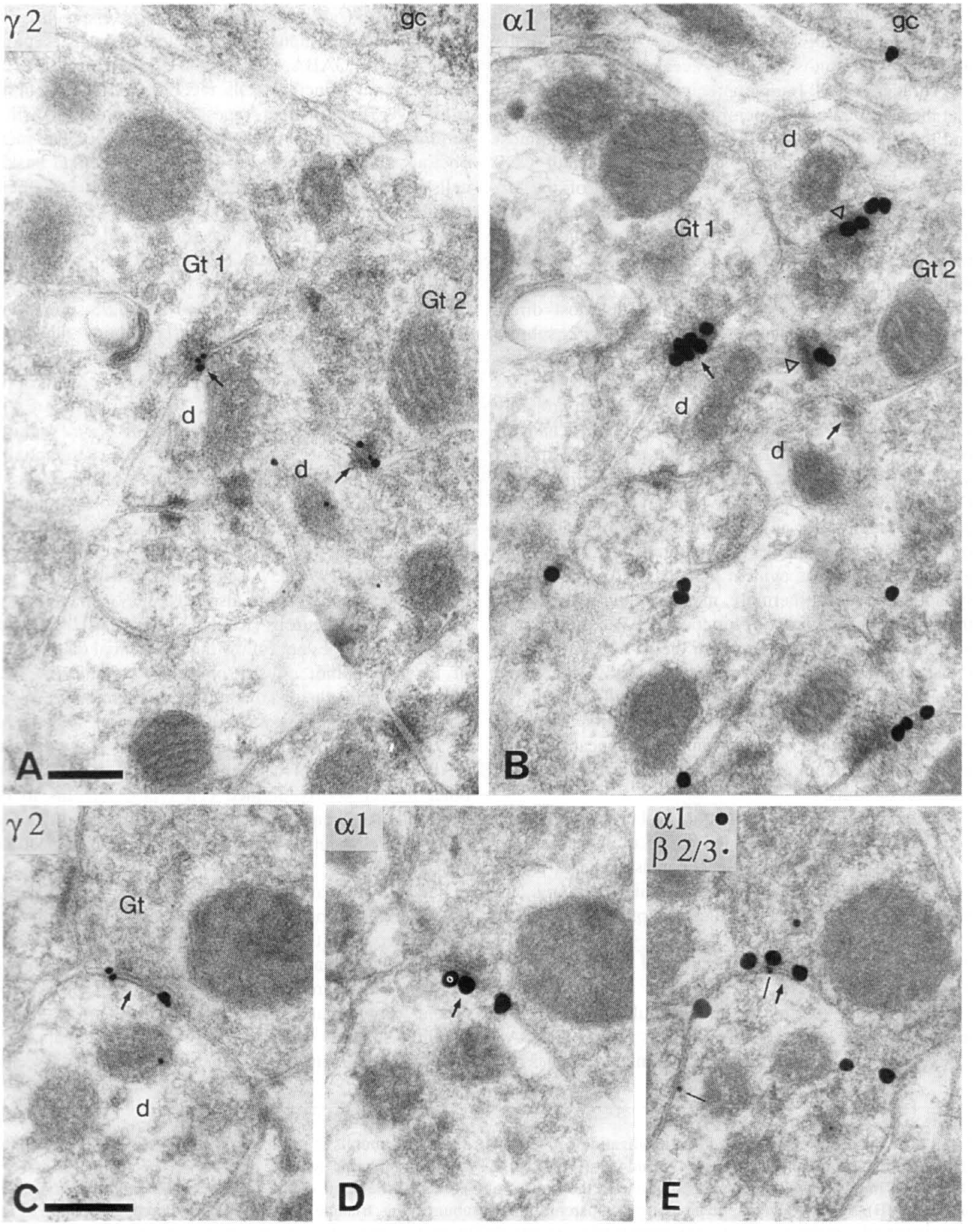

Fig. 10—*legend opposite.*

junctions may provide a mechanism for the fine tuning of postsynaptic responses.

Co-localization of the γ2 subunit with other subunits

The regional co-localization of the γ2 subunit with the α1 and β2/3 subunits has been revealed earlier (Fritschy and Mohler, 1995). Immunoprecipitation studies suggest that the γ2 subunit is frequently co-assembled with the α1 and the β2 subunits, and this triplet represents the most abundant $GABA_A$-receptor subtype in the adult CNS (Benke *et al.*, 1994, 1996). The presence of the γ2-subunit has also been reported in channels containing the α2, α3, α5, α6, β3, γ3, δ subunits (see Introduction for references). The high proportion of receptors containing the γ2 subunit suggests that at least some of them could be derived from the synaptic pool. The most direct evidence for the synaptic occurrence of γ2 subunit containing channels was provided so far by the potentiation of synaptic responses by benzodiazepine receptor agonists (De Koninck and Mody, 1994), which probably can exert their full agonist effect only on channels containing the γ2 subunit (Pritchett *et al.*, 1989; Sigel *et al.*, 1990; Moss *et al.*, 1991; Wafford *et al.*, 1991; Gunther *et al.*, 1995). Since the same individual synapses could be labelled for at least three of the most abundant subunits in the hippocampus, cerebellar cortex and globus pallidus, it is evident that they all participate in synaptic events. Furthermore, most synaptic junctions on the axon initial segment of hippocampal pyramidal cells have been shown to contain both the α1 and α2 subunits (Nusser *et al.*, 1996a). The present study shows that many synapses on the axon initial segments labelled for the α1 subunit also contain the γ2 and β2/3 subunits. Thus, the synapses established by axo-axonic cells appear to be the first which contain at least four different $GABA_A$ receptor subunits.

The resolution of the immunogold labelling (~20 nm) does not allow any conclusion to be drawn about the subunit composition of single channels; instead, it is a very direct method to determine which subunits are allocated by the cell to particular synaptic inputs.

Subunit composition of distinct sets of synapses on the same cell type

Hippocampal pyramidal and granule cells express mRNA for at least 10–11 subunits of the $GABA_A$ receptor, but only three different subunits are necessary to form a fully functional pentameric (Nayeem *et al.*, 1994) channel. Even if more than three different subunits were co-assembled, it would be very likely that the same cell would be able to produce receptor channels with distinct subunit composition. Are these distinct receptors targeted to all GABAergic synaptic sites on a single cell? Hippocampal principal cells receive GABAergic input from over five distinct types of interneurons, which subdivide the surface of the postsynaptic cell. The most obvious division is between the axo-axonic and basket cells innervating, respectively, the axon initial segment and the soma/proximal dendrites (Somogyi *et al.*, 1983) and acting through $GABA_A$ receptors (Buhl *et al.*, 1994). The γ2 subunit is present in both the axo-axonic and basket cell synapses, supporting the previous conclusion based on the distribution of the α1 and β2/3 subunits in the dentate gyrus, that channels containing the same subunits can be targeted to functionally distinct synaptic sites receiving GABA from different sets of presynaptic cells (Nusser *et al.*, 1995a). In contrast, a recent quantitative study of the distribution of the α2 subunit demonstrated that some channels are preferentially targeted to a particular synaptic input. It has been found that the axo-axonic synapses were much more frequently labelled for the α2 subunit than the synapses on the somata/proximal dendrites of CA1 pyramidal cells (Nusser *et al.*, 1996a). A segregated distribution of the α1, α2 and α3 subunits has also been demonstrated in the retina on single neurons (Koulen *et al.*, 1996). Although the γ2 subunit does not follow the differential distribution of the α2 subunit on the proximal synaptic sites of pyramidal cells, the distal dendritic domain, that receives GABAergic input from several interneurons, such as the bistratified cell (Buhl *et al.*, 1994), the somatostatin/GABA-containing alvear cells (Gulyas *et al.*, 1993) and other as yet poorly defined cell types, remains to be analysed in a manner that allows the precise identification of the presynaptic terminal in γ2 immunopositive synapses.

Purkinje cells also receive GABAergic input from at least two major sources: the basket and stellate cells, the latter innervating the more distal dendritic domain. Some basket cell terminals can be recognized on the basis of the characteristic neurofilament content, which appears to be absent from stellate cell terminals (Peters *et al.*, 1991). All three subunits studied here are present in synapses on both proximal and distal dendrites on Purkinje cells,

Fig. 11. Synaptic concentration and co-localization of the γ2, α1 and β2/3 subunits in synapses (arrows) on dendrites (d) in the globus pallidus. (A) and (B), (C) and (D), and (E)–(G) are serial sections of the same synaptic junctions, respectively. (A) and (B) Of the two immunolabelled synapses, the one to the right is almost out of the plane of the section and is labelled by only one particle in (B). Some extrasynaptic labelling is apparent for both subunits (e.g. triangle). (C) and (D) Clustered distribution of the immunoparticles in the postsynaptic membrane of a tangentially cut small dendrite. (E)–(G) Serial sections of a synaptic junction are labelled for the γ2, β2/3 and α1 subunits, the latter also appearing along the extrasynaptic membrane (triangles). (H) Only the edges of a synaptic junction are immunolabelled for the γ2 subunit. Silver-intensified postembedding immunoreaction using 1.4-nm gold label, except for the α1 subunit in (A) and (G) (5-nm gold label). (A) and (B), (C) and (D), and (E)–(G): same magnification, respectively. Scale bars: 0.2 μm.

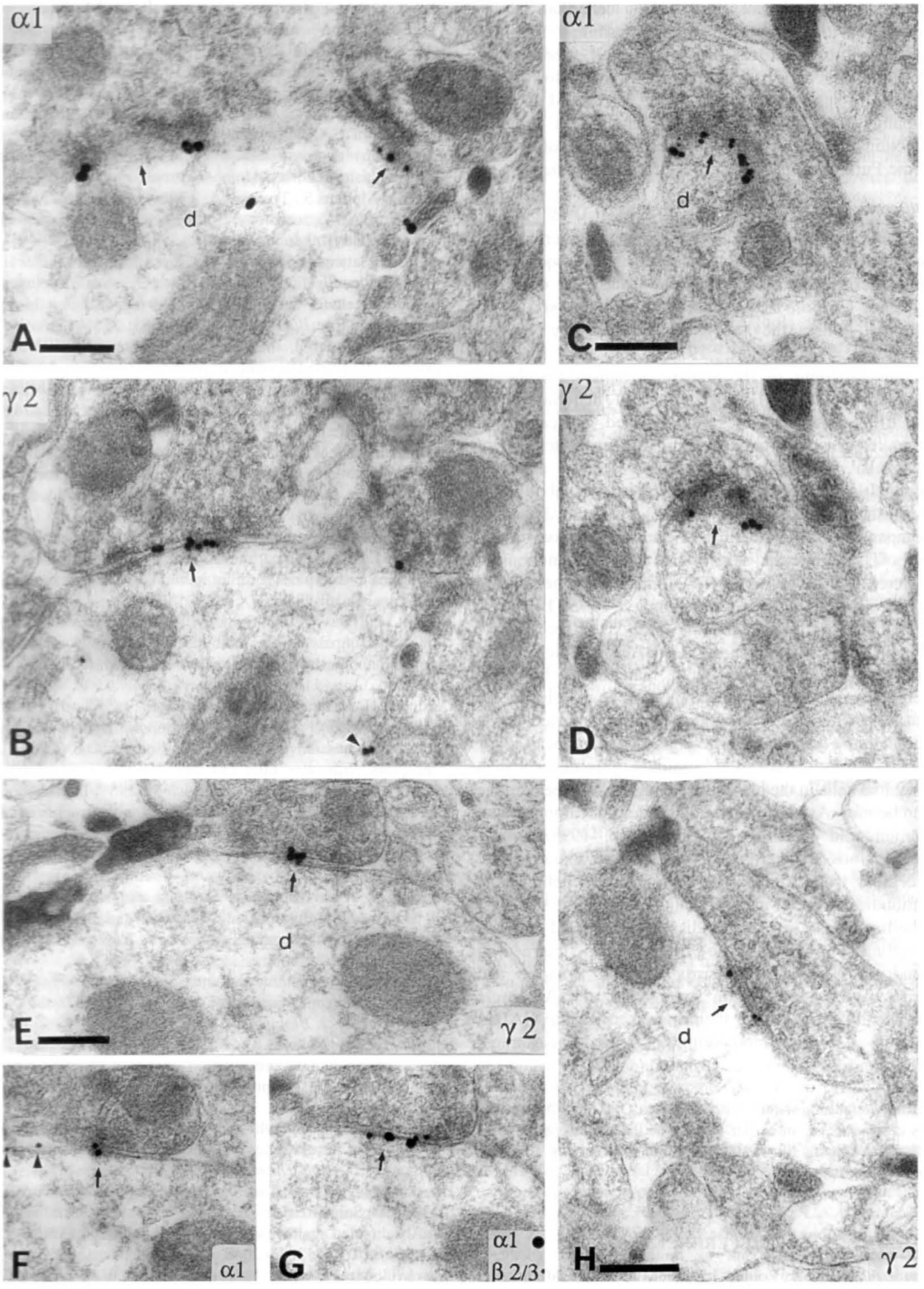

Fig. 11—*legend opposite.*

providing a further example of a cell that targets receptors with the same subunit composition to synapses innervated by different presynaptic cell types. Since Purkinje cells appear to express mRNA only for the $\alpha 1$, $\beta 2$ and $\gamma 2$ subunits (Persohn *et al.*, 1992), and all three subunits were found to be concentrated in synaptic junctions, the complete subunit composition of at least some Purkinje cell synapses has been established.

Differences in labelling intensity between synapse types

The axons of cerebellar basket cells innervate the proximal dendrites of Purkinje cells and also provide thick perisomatic axons forming occasional chemical synapses *en route* to the pinceau around the axon initial segment, where they very rarely make any synapse (Somogyi and Hamori, 1976). The low level of immunolabelling of the somatic synapses for the $\gamma 2$ subunit of the $GABA_A$ receptor, in material where dendritic synapses were well labelled, indicates that somatic synapses may contain a lower density of channels. Differences in receptor density are also probably responsible for the much stronger labelling of synapses on interneurons in the molecular layer, as compared with the labelling of Purkinje cells or granule cells. The differential labelling of synaptic junctions of different cell types in the cerebellum has also been noted for the $\alpha 1$ subunit (Nusser and Somogyi, 1996). However, the intense continuous labelling for $GABA_A$ receptors of the plasma membrane of GABAergic interneurons apparent in light microscopic material in both the cerebellum and the hippocampus is not merely due to the higher density of synaptic receptors detected in the cerebellum, because interneurons do not appear to be innervated by GABAergic terminals more densely than principal cells in the hippocampus or Purkinje cells in the cerebellum. As shown earlier in the hippocampus using immunogold labelling (Nusser *et al.*, 1995a), these interneurons have a much higher density of extrasynaptic receptors than principal cells. In the cerebellum, the antibodies to the $\alpha 1$ subunit, which provide the strongest labelling of the three antibodies, also revealed frequent labelling of extrasynaptic receptors on interneuron dendrites. The higher density of both synaptic and extrasynaptic receptors on some types of GABAergic interneurons may render them more sensitive to GABA, as well as to drugs modulating GABAergic neurotransmission.

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REFERENCES

Baude A., Nusser Z., Roberts J. D. B., Mulvihill E., McIlhinney R. A. J. and Somogyi P. (1993) The metabotropic glutamate receptor (mGluR1α) is concentrated at perisynaptic membrane of neuronal subpopulations as detected by immunogold reaction. *Neuron* **11:** 771–787.

Benke D., Cicin-Sain A., Mertens S. and Mohler H. (1991a) Immunochemical identification of the $\alpha 1$- and $\alpha 3$-subunits of the $GABA_A$-receptor in rat brain. *J. Rec. Res.* **11:** 407–424.

Benke D., Mertens S. and Mohler H. (1991b) Ubiquitous presence of $GABA_A$ receptors containing the $\alpha 1$-subunit in rat brain demonstrated by immunoprecipitation and immunohistochemistry. *Molec. Neuropharmac.* **1:** 103–110.

Benke D., Mertens S., Trzeciak A., Gillessen D. and Mohler H. (1991c) $GABA_A$ receptors display association of $\gamma 2$-subunit with $\alpha 1$- and $\beta 2/3$-subunits. *J. Biol. Chem.* **266:** 4478–4483.

Benke D., Mertens S., Trzeciak A., Gillessen D. and Mohler H. (1991d) Identification and immunohistochemical mapping of $GABA_A$ receptor subtypes containing the δ-subunit in rat brain. *Fedn Eur. Biochem. Socs Lett.* **283:** 145–149.

Benke D., Fritschy J. -M., Trzeciak A., Bannwarth W. and Mohler H. (1994) Distribution, prevalence, and drug binding profile of γ-aminobutyric acid type A receptor subtypes differing in the β-subunit variant. *J. Biol. Chem.* **269:** 27100–27107.

Benke D., Honer M., Michel C. and Mohler H. (1996) $GABA_A$-receptor subtypes differentiated by their γ-subunit variants: prevalence, pharmacology and subunit architecture. *Neuropharmacology* **35:** 1413–1422.

Bohlhalter S., Weinmann O., Mohler H. and Fritschy J.- M. (1996) Laminar compartmentalization of $GABA_A$-receptor subtypes in the spinal cord: an immunohistochemical study. *J. Neurosci.* **16:** 283–297.

Buhl E. H., Halasy K. and Somogyi P. (1994) Diverse sources of hippocampal unitary inhibitory postsynaptic potentials and the number of synaptic release sites. *Nature* **368:** 823–828.

Caruncho H. J., Puia G., Slobodyansky E., da Silva P. P. and Costa E. (1993) Freeze-fracture immunocytochemical study of the expression of native and recombinant $GABA_A$ receptors. *Brain Res.* **603:** 234–242.

de Blas A. L., Vitorica J. and Friedrich P. (1988) Localization of the $GABA_A$ receptor in the rat brain with a monoclonal antibody to the 57,000 M_r peptide of the $GABA_A$ receptor/benzodiazepine receptor/Cl^- channel complex. *J. Neurosci.* **8:** 602–614.

De Koninck Y. and Mody I. (1994) Noise analysis of miniature IPSCs in adult rat brain slices: Properties and modulation of synaptic $GABA_A$ receptor channels. *J. Neurophysiol.* **71:** 1318–1335.

Duggan M. J., Pollard S. and Stephenson A. (1991) Immunoaffinity purification of $GABA_A$ receptor α-subunit iso-oligomers. *J. Biol. Chem.* **266:** 24778–24784.

Duggan M. J., Pollard S. and Stephenson F. A. (1992) Quantitative immunoprecipitation studies with anti-γ-aminobutyric $acid_A$ receptor $\gamma 2$ 1–15 Cys antibodies. *J. Neurochem.* **58:** 72–77.

Duggan M. J. and Stephenson F. A. (1990) Biochemical evidence for the existence of γ-aminobutyrate$_A$ receptor iso-oligomers. *J. Biol. Chem.* **265:** 3831–3835.

Ewert M., Shivers B. D., Luddens H., Mohler H. and Seeburg P. H. (1990) Subunit selectivity and epitope characterization of mAbs directed against the $GABA_A$/benzodiazepine receptor. *J. Cell Biol.* **110:** 2043–2048.

Fritschy J.-M., Benke D., Mertens S., Oertel W. H., Bachi T. and Mohler H. (1992) Five subtypes of type A γ-aminobutyric acid receptors identified in neurons by double

and triple immunofluorescence staining with subunit-specific antibodies. *Proc. natn. Acad. Sci. U.S.A.* **89:** 6726–6730.

Fritschy J.-M. and Mohler H. (1995) $GABA_A$-receptor heterogeneity in the adult rat brain: differential regional and cellular distribution of seven major subunits. *J. Comp. Neurol.* **359:** 154–194.

Fritschy J.-M., Paysan J., Enna A. and Mohler H. (1994) Switch in the expression of rat $GABA_A$-receptor subtypes during postnatal development: An immunohistochemical study. *J. Neurosci.* **14:** 5302–5324.

Gao B. and Fritschy J.-M. (1994) Selective allocation of $GABA_A$ receptors containing the $\alpha 1$ subunit to neurochemically distinct subpopulations of rat hippocampal interneurons. *Eur. J. Neurosci.* **6:** 837–853.

Gao B., Fritschy J.-M., Benke D. and Mohler H. (1993) Neuron-specific expression of $GABA_A$-receptor subtypes: Differential association of the $\alpha 1$- and $\alpha 3$-subunit with serotonergic and GABAergic neurons. *Neuroscience* **54:** 881–892.

Gao B., Hornung J. P. and Fritschy J.-M. (1995) Identification of distinct GABA(A)-receptor subtypes in cholinergic and parvalbumin-positive neurons of the rat and marmoset medial septum–diagonal band complex. *Neuroscience* **65:** 101–117.

Gray E. G. (1959) Axo-somatic and axo-dendritic synapses of the cerebral cortex: an electron microscope study. *J. Anat.* **93:** 420–433.

Greferath U., Muller F., Wassle H., Shivers B. and Seeburg P. (1993) Localization of $GABA_A$ receptors in the rat retina. *Visual Neurosci.* **10:** 551–561.

Gutierrez A., Khan Z. U. and De Blas A. L. (1994) Immunocytochemical localization of γ_2 short and γ_2 long subunits of the $GABA_A$ receptor in the rat brain. *J. Neurosci.* **14:** 7168–7179.

Gulyas A. I., Miles R., Sik A., Toth K., Tamamaki N. and Freund T. F. (1993) Hippocampal pyramidal cells excite inhibitory neurons through a single release site. *Nature* **366:** 683–687.

Gunther U., Benson J., Benke D., Fritschy J. -M., Reyes G., Knoflach F., Crestani F., Aguzzi A., Arigoni M., Lang Y., Bluethmann H., Mohler H. and Luscher B. (1995) Benzodiazepine-insensitive mice generated by targeted disruption of the $\gamma 2$ subunit gene of γ-aminobutyric acid type A receptors. *Proc. Natn. Acad. Sci. U.S.A.* **92:** 7749–7753.

Haring P., Stahli C., Schoch P., Takacs B., Staehelin T. and Mohler H. (1985) Monoclonal antibodies reveal structural homogeneity of γ-aminobutyric acid/benzodiazepine receptors in different brain areas. *Proc. Natn. Acad. Sci. U.S.A.* **82:** 4837–4841.

Houser C. R., Olsen R. W., Richard J. G. and Mohler H. (1988) Immunohistochemical localization of benzodiazepine/$GABA_A$ receptors in the human hippocampal formation. *J. Neurosci.* **8:** 1370–1383.

Jones M. V. and Westbrook G. L. (1996) The impact of receptor desensitization on fast synaptic transmission. *Trends Neurosci.* **19:** 96–101.

Khan Z. U., Gutierrez A. and de Blas A. L. (1994a) Short and long form $\gamma 2$ subunits of the $GABA_A$/benzodiazepine receptors. *J. Neurochem.* **63:** 1466–1476.

Khan Z. U., Gutierrez A. and de Blas A. L. (1994b) The subunit composition of a $GABA_A$/benzodiazepine receptor from rat cerebellum. *J. Neurochem.* **63:** 371–374.

Kofuji P., Wang J. B., Moss S. J., Huganir R. L. and Burt D. R. (1991) Generation of two forms of the γ-aminobutyric $acid_A$ receptor $\gamma 2$-subunit in mice by alternative splicing. *J. Neurochem.* **56:** 713–715.

Koulen P., Sassoe Pognetto M., Grunert U. and Wassle H. (1996) Selective clustering of $GABA_A$ and glycine receptors in the mammalian retina. *J. Neurosci.* **16:** 2127–2140.

Laurie D. J., Seeburg P. H. and Wisden W. (1992) The distribution of 13 $GABA_A$ receptor subunit mRNAs in the rat brain. II. Olfactory bulb and cerebellum. *J. Neurosci.* **12:** 1063–1076.

McKernan R. M., Quirk K., Prince R., Cox P. A., Gillard N. P., Ragan C. I. and Whiting P. (1991) $GABA_A$ receptor subtypes immunopurified from rat brain with a subunit-specific antibodies have unique pharmacological properties. *Neuron* **7:** 667–676.

McKernan R. M. and Whiting P. (1996) Which $GABA_A$ receptor subtypes really occur in the brain? *Trends Neurosci.* **19:** 139–143.

Mertens S., Benke D. and Mohler H. (1993) $GABA_A$ receptor populations with novel subunit combinations and drug binding profiles identified in brain by $\alpha 5$- and δ-subunit-specific immunopurification. *J. Biol. Chem.* **268:** 5965–5973.

Mohler H., Benke D., Benson J., Luscher B. and Fritschy J. M. (1995) $GABA_A$-receptor subtypes *in vivo*: cellular localization, pharmacology and regulation. In: *$GABA_A$ Receptors and Anxiety. From Neurobiology to Treatment* (Biggio G., Sanna E., Serra M. and Costa E., Eds), pp. 41–56. Raven Press, New York.

Moss S. J., Ravindran A., Mei L., Wang J. B., Kofuji P., Huganir R. L. and Burt D. R. (1991) Chracterization of recombinant $GABA_A$ receptors produced in transfected cells from murine α_1, β_1, and γ_2 subunit cDNAs. *Neurosci. Lett.* **123:** 265–268.

Naito S. and Ueda T. (1981) Affinity-purified anti-protein I antibody. *J. Biol. Chem.* **256:** 10657–10663.

Nayeem N., Green T. P., Martin I. L. and Barnard E. A. (1994) Quaternary structure of the native $GABA_A$ receptor determined by electron microscopic image analysis. *J. Neurochem.* **62:** 815–818.

Nusser Z., Mulvihill E., Streit P. and Somogyi P. (1994) Subsynaptic segregation of metabotropic and ionotropic glutamate receptors as revealed by immunogold localization. *Neuroscience* **61:** 421–427.

Nusser Z., Roberts J. D. B., Baude A., Richards J. G., Sieghart W. and Somogyi P. (1995a) Immunocytochemical localisation of the $\alpha 1$ and $\beta 2/3$ subunits of the $GABA_A$ receptor in relation to specific GABAergic synapses in the dentate gyrus. *Eur. J. Neurosci.* **7:** 630–646.

Nusser Z., Roberts J. D. B., Baude A., Richards G. and Somogyi P. (1995b) Relative densities of synaptic and extrasynaptic $GABA_A$ receptors on cerebellar granule cells as determined by a quantitative immunogold method. *J. Neurosci.* **15:** 2948–2960.

Nusser Z., Sieghart W., Benke D., Fritschy J.-M. and Somogyi P. (1996a) Differential synaptic localization of two major γ-aminobutyric acid type A receptor α subunits on hippocampal pyramidal cells. *Proc. Natn. Acad. Sci. U.S.A.* in press.

Nusser Z., Sieghart W., Stephenson F. A. and Somogyi P. (1996b) The $\alpha 6$ subunit of the $GABA_A$ receptor is concentrated in both inhibitory and excitatory synapses on cerebellar granule cells. *J. Neurosci.* **16:** 103–114.

Nusser Z. and Somogyi P. (1996) Compartmentalised distribu-

tion of $GABA_A$ and glutamate receptors in relation to transmitter release sites on the surface of cerebellar neurones. *Progr. Brain Res.* in press.

Persohn E., Malherbe P. and Richards J. G. (1992) Comparative molecular neuroanatomy of cloned $GABA_A$ receptor subunits in the rat CNS. *J. Comp. Neurol.* **326:** 193–216.

Peters A., Palay S. L., Webster H. (1991) *The Fine Structure of the Nervous System.* Oxford University Press, New York.

Pollard S., Duggan M. J. and Stephenson F. A. (1993) Further evidence for the existence of a subunit heterogeneity within discrete γ-aminobutyric $acid_A$ receptor subpopulations. *J. Biol. Chem.* **268:** 3753–3757.

Pritchett D. B., Sontheimer H., Shivers B. D., Ymer S., Kettenmann H., Schofield P. R. and Seeburg P. H. (1989) Importance of a novel $GABA_A$ receptor subunit for benzodiazepine pharmacology. *Nature* **338:** 582–585.

Quirk K., Gillard N. P., Ragan C. I., Whiting P. J. and McKernan R. M. (1994a) γ-aminobutyric acid type A receptors in the rat brain can contain both $\gamma 2$ and $\gamma 3$ subunits, but $\gamma 1$ does not exist in combination with another γ subunit. *Molec. Pharmac.* **45:** 1061–1070.

Quirk K., Gillard N. P., Ragan C. I., Whiting P. J. and McKernan R. M. (1994b) Model of subunit composition of $GABA_A$ receptor subtypes expressed in rat cerebellum with respect to their α and γ/δ subunits. *J. Biol. Chem.* **269:** 16020–16028.

Richards J. G., Schoch P., Haring P., Takacs B. and Mohler H. (1987) Resolving $GABA_A$/benzodiazepine receptors: cellular and subcellular localization in the CNS with monoclonal antibodies. *J. Neurosci.* **7:** 1866–1886.

Schoch P., Richards J. G., Haring P., Takacs B., Stahli C., Staehelin T., Haefely W. and Mohler H. (1985) Colocalization of $GABA_A$ receptors and benzodiazepine receptors in the brain shown by monoclonal antibodies. *Nature* **314:** 168–171.

Sieghart W. (1995) Structure and pharmacology of γ-aminobutyric $acid_A$ receptor subtypes. *Pharmac. Rev.* **47:** 181–234.

Sigel E., Baur R., Trube G., Mohler H. and Malherbe P. (1990) The effect of subunit composition of rat brain $GABA_A$ receptors on channel function. *Neuron* **5:** 703–711.

Soltesz I., Roberts J. D. B., Takagi H., Richards J. G., Mohler H. and Somogyi P. (1990) Synaptic and nonsynaptic localization of benzodiazepine/$GABA_A$ receptor/Cl^- channel complex using monoclonal antibodies in the dorsal lateral geniculate nucleus of the cat. *Eur. J. Neurosci.* **2:** 414–429.

Somogyi P. and Hamori J. (1976) A quantitative electron microscopic study of the Purkinje cell axon initial segment. *Neuroscience* **1:** 361–366.

Somogyi P., Nunzi M. G., Gorio A. and Smith A. D. (1983) A new type of specific interneuron in the monkey hippocampus forming synapses exclusively with the axon initial segments of pyramidal cells. *Brain Res.* **259:** 137–142.

Somogyi P., Takagi H., Richards J. G. and Mohler H. (1989) Subcellular localization of benzodiazepine/$GABA_A$ receptors in the cerebellum of rat, cat, and monkey using monoclonal antibodies. *J. Neurosci.* **9:** 2197–2209.

Spreafico R., De Biasi S., Amadeo A. and De Blas A. L. (1993) $GABA_A$-receptor immunoreactivity in the rat dorsal thalamus: an ultrastructural investigation. *Neurosci. Lett.* **158:** 232–236.

Stephenson F. A. (1995) The $GABA_A$ receptors. *Biochem. J.* **310:** 1–9.

Todd A. J., Watt C., Spike R. C. and Sieghart W. (1996) Colocalization of GABA, glycine, and their receptors at synapses in the rat spinal cord. *J. Neurosci.* **16:** 974–982.

Wafford K. A., Burnett D. M., Leidenheimer N. J., Burt D. R., Wang J. B., Kofuji P., Dunwiddie T. V., Harris R. A. and Sikela J. M. (1991) Ethanol sensitivity of the $GABA_A$ receptor expressed in *Xenopus* oocytes requires 8 amino acids contained in the $\gamma 2L$ subunit. *Neuron* **7:** 27–33.

Waldvogel H. J., Faull R. L. M., Jansen K. L. R., Dragunow M., Richards J. G., Mohler H. and Streit P. (1990) GABA, GABA receptors and benzodiazepine receptors in the human spinal cord: an autoradiographic and immunohistochemical study at the light and electron microscopic levels. *Neuroscience* **39:** 361–385.

Wisden W., Laurie D. J., Monyer H. and Seeburg P. H. (1992) The distribution of 13 $GABA_A$ receptor subunit mRNAs in the rat brain. I. Telencephalon, diencephalon, mesencephalon. *J. Neurosci.* **12:** 1040–1062.

Zezula J., Fuchs K. and Sieghart W. (1991) Separation of $\alpha 1$, $\alpha 2$ and $\alpha 3$ subunits of the $GABA_A$-benzodiazepine receptor complex by immunoaffinity chromatography. *Brain Res.* **563:** 325–328.