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DIFFERENTIAL SUBCELLULAR DISTRIBUTION OF THE α6 SUBUNIT VERSUS THE α1 AND β2/3 SUBUNITS OF THE $GABA_A$/BENZODIAZEPINE RECEPTOR COMPLEX IN GRANULE CELLS OF THE CEREBELLAR CORTEX

A. BAUDE,* J.-M. SEQUIER,† R. M. MCKERNAN,† K. R. OLIVIER† and P. SOMOGYI*

*Medical Research Council, Anatomical Neuropharmacology Unit, Oxford University, Mansfield Road, Oxford OX1 3TH, U.K.

†Department of Biochemistry, Neuroscience Research Centre, Merck Sharp and Dohme Research Laboratories, Eastwick Road, Harlow CM20 2QR, U.K.

The distribution of the α6 subunit of the $GABA_A$ receptor has been established in rat cerebellum and compared to the distribution of the α1 (cat) and the β2/3 (rat, cat) subunits, using immunocytochemistry. The synapses established by Golgi cell terminals on the dendrites of granule cells were immunoreactive for the α6, α1 and β2/3 subunits in virtually all glomeruli, indicating that two variants (α1 and α6) of the same subunit are co-localized at the same synapses. The somatic membranes of the granule cells, which receive no synapses, were immunopositive for the α1 and β2/3 subunits, but not for the α6 subunit. Thus, the α1 and the β2/3 subunits are located at both synaptic and extrasynaptic sites, but the α6 subunit is detectable only at synaptic sites.

The fast synaptic action of GABA is mediated by the $GABA_A$/benzodiazepine receptor complex, a multi-subunit ligand-gated chloride channel which also constitutes a site of action for neuroactive drugs such as benzodiazepines and barbiturates. Several lines of evidence suggest that the $GABA_A$ receptor has a pentameric structure consisting of different subunits (for review see Refs 3, 20, 22). Molecular cloning has revealed the existence of at least five groups of subunits in the central nervous system, α, β, γ, δ and ρ, each group including several variants, giving more than sixteen different subunits.[5,16,30] As is evident from *in vitro* co-expression studies, the various subunits confer different, and often highly specific pharmacological properties to the $GABA_A$ receptor (e.g. see Refs 12, 15, 25, 26). This predicts that the action of GABA and the effect of drugs on GABAergic transmission in the brain will vary depending on the subunit composition of the receptor complex at specific sites.

Very little is known about the composition of the $GABA_A$ receptor at particular sites *in situ* in the brain, although the pharmacological heterogeneity of the receptors has been known (for review, see Refs 22, 35). There is evidence that receptor complexes with different subunit composition probably exist *in vivo*,[1,6,14,19] but whether they are expressed on the surface of the cell and at which sites remains open to question. The distribution of some subunits of the receptor complex in the plasma membrane has been studied at a few sites for a limited number of subunits using immunocytochemistry.[2,4,28,36,38,47] It is now clear from *in situ* hybridization studies that the same cell can express more than the five subunits necessary for a channel, implying that more than one channel type might be expressed on the surface of cells. Because multiple copies of the same subunit variant may occur in a single receptor complex, a large number of channel types may exist.[14] It remains to be established how the different subunits are distributed on the cell's surface, particularly in relation to specific synaptic input.

The present study addresses this question in the cerebellum, which is a favoured area for studying the expression of the $GABA_A$ receptor, due to the easy recognition of different cell types.[7,27] *In situ* hybridization showed that different neuronal and glial cells express various subunits to a different degree or not at all (e.g. see Refs 13, 43). Here we focus on granule cells which express α1, α6, β1, β2, β3, γ2, δ and possibly also α4 and γ3 subunits.[13,15,17,26,31–33,42,43,46] Immunohistochemical localization also suggested the presence of α3 subunit,[47] but this has not been confirmed by *in situ* hybridization.[43] The granule cell of the cerebellum provides an excellent opportunity to study the organization of the different subunits, and their possible physiological role because this cell receives GABAergic input almost exclusively from

Abbreviations: ABC, avidin biotinylated horseradish peroxidase complex; SSC, standard saline citrate; SDS–PAGE, sodium dodecyl sulphate/polyacrylamide gel electrophoresis.

only one source, the Golgi cells. A minor GABAergic projection from the deep cerebellar nuclei might influence some granule cells.[10] Previous immunohistochemical studies using monoclonal antibodies[29,41] showed that immunoreactivity for the $GABA_A$ receptor is highly concentrated in the granule cell layer of the cerebellar cortex.[4,28,38] Subsequent testing of antibodies on cell lines expressing individual subunits showed that antibody bd-24 selectively reacts with the $\alpha 1$ subunit and bd-17 reacts with both the $\beta 2$ and $\beta 3$ subunits which show strong homology.[8] High-resolution electron microscopy showed that both the $\alpha 1$ and the $\beta 2/3$ subunits were detected in the synapses established by Golgi cell terminals with granule cell dendrites, but immunoreactivity was also present at non-synaptic sites throughout the surface of the cell including the somatic membrane which does not receive synapses.[38] Granule cells also express the $\alpha 6$ subunit[15,43] which, when co-expressed with $\beta 2$ and $\gamma 2$ subunits *in vitro*, forms a receptor complex which is insensitive to benzodiazepine agonists, but binds with high affinity the partial inverse agonist Ro15-4513.[15] In the present study we investigated the subcellular distribution of the $\alpha 6$ subunit using an antiserum raised against a polypeptide constituting the predicted second intracellular loop of the transmembrane protein.[15]

In situ *hybridization*

The distribution of mRNA coding for the $\alpha 6$ subunit of the $GABA_A$ receptor in the rat brain was mapped first using a radioactively labelled antisense oligonucleotide and *in situ* hybridization. A very intense hybridization signal was restricted to the granular layer of the cerebellar cortex (Fig. 1A), and confined to the cell bodies of the granule cells. The transcript was not detected in other structures of the rat brain (Fig. 1A), confirming the study of Luddens *et al.*[15] who used a probe to a different part of the mRNA. When a sense probe was used, no hybridization signal was detected.

Comparison of immunoreactivity for the $\alpha 6$, $\alpha 1$ *and* $\beta 2/3$ *subunits*

The intensity of immunoreactivity for the $\alpha 6$ subunit was strongly dependent on the fixation

Fig. 1. Distribution of the $\alpha 6$ subunit of the $GABA_A$ receptor in the rat brain. (A) The $\alpha 6$ mRNA is restricted to the granule cell layer of the cerebellar cortex, as demonstrated by a film autoradiogram using *in situ* hybridization. Scale bar = 2 mm. A 51-mer oligonucleotide was used, containing a 42-mer portion complementary to the rat cDNA sequence (nucleotides 1020–1062 according to Luddens *et al.*,[15] and having at its 3′ end a 9-mer sequence complementary to the 3′ end of an oligo-dT primer. After annealing, the probe was labelled with [^{35}S]α-dATP, using Klenow polymerase and purified on a Sephadex G50 column. Fresh, frozen sections of rat brain were fixed in 4% paraformaldehyde dissolved in 0.01 M phosphate-buffered saline for 40 min, rinsed in the same buffer and dehydrated in ethanol. Sections were then hybridized overnight at 42°C in 50 μl hybridization solution {50% formamide, 4 × SSC (standard saline citrate) [1 × SSC is 0.15 M NaCl, 0.015 M Na-citrate], 2 mg/ml bovine serum albumin, 10% dextran sulphate, 1 mg/ml tRNA, 1 mg/ml polyA and 10 mM dithiothreitol} containing radioactive probe (around 10^7 c.p.m./ml). A similarly labelled sense probe was also prepared and applied. Sections were washed first for 15 min at room temperature in a solution containing 50% formamide and 1 × SSC, then 40 min at 40°C in 1 × SSC and 1 h in 1 × SSC at room temperature. After dehydration, the sections were exposed for one week to Hyperfilm β-max (Amersham) and then dipped in K5 emulsion (Ilford), diluted 1:1 with water, and exposed for several weeks. The sections were counterstained with Methylene Blue after developing. (B) Immunoreactivity for the $\alpha 6$ subunit demonstrated with a polyclonal antiserum. The granule cell layer (gl) shows strong immunoreactivity. The molecular layer (ml) and the white matter (wm) are immunonegative. Silver-intensified immunoperoxidase reaction. Scale bar = 100 μm. Antisera were raised in rabbits to polypeptide produced by bacteria. Expression of the $\alpha 6$ subunit putative cytoplasmic loop protein was performed essentially as described earlier.[19] The region encoding Ser_{313}-Thr_{395} was produced by *E. coli* (BL21 DE3 strain) after transformation with an expression vector (pRSET) containing a PCR product obtained from a rat whole brain cDNA using oligonucleotide primers designed to encompass this portion of the putative cytoplasmic loop. The expressed polypeptide was recovered from the insoluble fraction after homogenization and purified by preparative sodium dodecyl sulphate/polyacrylamide gel electrophoresis (SDS–PAGE). The purified polypeptide (1 mg/ml) was emulsified with Freunds complete adjuvant (1:1) and rabbits were immunized with 100-μg aliquots subcutaneously, and boosted at monthly intervals with 50 μg of polypeptide emulsified with Freunds incomplete adjuvant. Rabbits were bled seven days after each boost and the serum was tested for its ability to immunoprecipitate [^{3}H]muscimol binding (Quirk *et al.*, unpublished observations). Antibodies were then purified using the bacterially expressed protein immobilized on nitrocellulose. Bacterial lysates were separated by 15% SDS–PAGE and lightly stained with Coomassie Blue in water (without methanol). The band corresponding to the expressed $\alpha 6$ polypeptide was excised and blotted onto nitrocellulose, blocked with 5% milk powder in Tris-buffered saline [10 mM Tris–HCl (pH 7.4), 150 mM NaCl] overnight, and cut into small strips. Antiserum was diluted with the same buffer (1:1) and incubated with the nitrocellulose strips for 3 h at room temperature and washed extensively. Antibodies were eluted with 2 × 1 ml of 10 mM citrate buffer, pH 2.5 and immediately neutralized with 1 M Tris base. The protein concentration was measured by absorbance at 280 nm and the antibodies concentrated by ultrafiltration to 1 mg/ml. Antibodies to the $\alpha 6$ subunit were able to immunoprecipitate around 60% of cerebellar [^{3}H]muscimol binding and 50% of [^{3}H]Ro15-4513 binding (Quirk *et al.*, unpublished observations). A Western blot analysis revealed one band at 58,000 mol. wt in cerebellum only (Quirk *et al.*, unpublished observations). Immunocytochemical procedure is described further in Fig. 2.

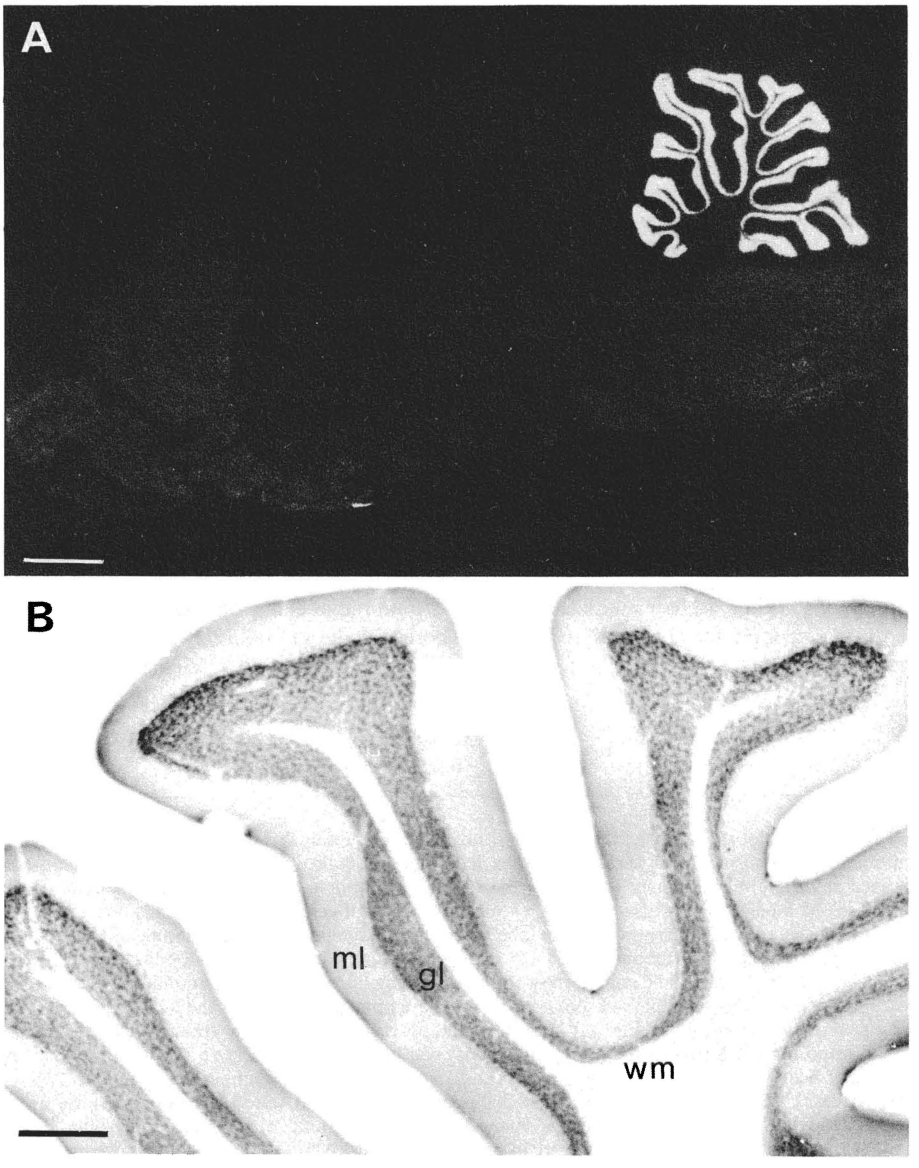

Fig. 1

procedure, and was more sensitive to particular conditions than immunoreactivity for the other subunits. Increasing concentration of glutaraldehyde (over 0.05%), longer duration of fixation (over 10 min) or higher temperature (best results were obtained with ice-cold fixative) reduced the immunoreactivity probably by altering the epitope(s). The antiserum specific for the $\alpha 6$ subunit was applied to sections of rat and cat brain, but immunoreactivity could only be observed in rat tissue. The lack of immunoreactivity in the cat may indicate that the antiserum does not cross-react with cat protein, although the serum was raised to a large polypeptide containing several possible epitopes. However, there is no evidence yet that the $\alpha 6$ subunit is expressed in cat cerebellum. Antibody bd-17 specific for the $\beta 2/3$ subunits gave similar results in both species; antibody bd-24 specific for the $\alpha 1$ subunit reacted only with cat tissue. It has been shown that both antibodies bd-17 and bd-24 react with human, monkey, bovine and cat protein, but only antibody bd-17 recognizes the rat protein.[11,28,38] Antibody bd-24 lacks reactivity with the rat polypeptide due to the absence of a leucine at position 4 as compared to the bovine polypeptide.[8]

Immunoreactivity for the $\alpha 6$ subunit was restricted to the granule cell layer of the cerebellar cortex (Fig. 1B) in the rat brain, in agreement with the *in situ* hybridization results. No systematic differences were seen between the different folia in the intensity of the

reaction. Reactivity for the α6 subunit was exclusively localized to the glomeruli (Fig. 2B, E), which were also strongly labelled with monoclonal antibodies to the β2/3 (Fig. 2A, D) and α1 (Fig. 2C, F) subunits. A pale centre, representing the unstained mossy fibre terminal, was often surrounded by a heavily labelled ring corresponding to the granule cell dendrites. The somata of granule cells were also immunopositive for subunits β2/3 and α1, but immunonegative for the α6 subunit. Granule cell somata are often in direct membrane contact with each other, and the boundaries could be seen delineated by the immunoperoxidase end-product for the β2/3 and α1 subunits (Fig. 2D, F).

Immunoreactivity could not be seen in Golgi cell bodies for any of the antibodies. No immunoreactivity could be observed in the molecular layer, in the white matter (Figs 1B, 2B), or in the deep cerebellar nuclei for the α6 subunit. Immunoreactivity for both α1 and β2/3 subunits was present in the molecular layer; the somata of Purkinje, stellate and basket cells were labelled more strongly for the α1 than for the β2/3 subunit (Fig. 2A, C).

Subcellular distribution of receptor immunoreactivity

Immunoreactivity for the α6 subunit was present in virtually all glomeruli, as revealed by electron microscopy (Fig. 3A). The strongest reaction end-product was located at the intracellular face of the postsynaptic plasma membrane of granule cell dendrites, at the synaptic junctions established by presynaptic terminals of Golgi cells (Fig. 3B). The end-product diffused from the synaptic membrane into the cytoplasm of the dendrite secondarily covering all intracellular organelles. Some dendrites were so heavily filled that the reaction end-product also covered the puncta adherentia, established between granule cell dendrites, and the postsynaptic density of the mossy terminals. However, in less densely reacting dendrites from the gradual decrease of reaction end-product density it was clear that the immunoreactivity originated at or near the Golgi cell synapses and not the mossy fibre synapses (Fig. 3B).

Immunolabelling obtained with monoclonal antibodies to the α1 and β2/3 subunits was similar to that described previously.[38] Virtually all glomeruli were

Fig. 2. Distribution of the β2/3 (A, D), α6 (B, E) and α1 (C, F) subunits of the $GABA_A$ receptor revealed by immunocytochemistry in the cerebellar cortex fixed under identical conditions. For all antibodies in both species the granule cell layer (gl) shows the strongest immunoreactivity. Immunoreactivity can be observed in the molecular layer (ml) for the α1 subunit in the cat cerebellum where Purkinje cells (thick arrow) and basket cells (thin arrows) are also labelled. Higher magnification of the granule cell layer shows that the glomeruli (e.g. thick arrows, D–F) are strongly immunoreactive for all subunits. The glomeruli appear as a "rosette" with a pale core corresponding to mossy fibre terminals surrounded by the labelled dendrites of granule cells. Granule cell somata (asterisks) are immunopositive for the β2/3 and α1 subunits along the plasma membrane of the cell bodies, including regions where two granule cells are directly apposed (opposing thin arrows). No immunoreactivity can be observed for the α6 subunit in the granule cell somata. Scale bars = 50 μm A–C; 10 μM (D–F). For immunocytochemical experiments, Wistar/Charles River, U.K., female rats ($n = 11$, 150–200 g) and one male cat were used. Animals were deeply anaesthetized with Pentobarbitone sodium (150 mg/kg, i.p.) for rats and with a mixture of ketamine and xylazine (5:1, 0.6 ml/kg, i.m., supplemented to abolish all reflexes) for the cat. Animals were perfused transcardially with saline for 1 min, followed by fixatives (ice-cold or at room temperature) containing paraformaldehyde (2 or 4%), glutaraldehyde (0.025–0.1%), and picric acid (~0.2%) made up in 0.1 M phosphate buffer (pH 7.2–7.4), for 10–30 min. After perfusion, the cerebellum was removed, cut in 3-mm blocks which were extensively washed in phosphate buffer and put in 20% sucrose in phosphate buffer until the blocks sank. The blocks were frozen in liquid nitrogen. Sagittal and frontal sections were obtained either with a Vibratome (70–100 μm thick) or with a cryostat (20–40 μm thick). Immunocytochemistry was performed using the avidin biotinylated horseradish peroxidase complex (ABC) method. After blocking with normal serum (1 h at room temperature), sections were incubated for one to two days at 4°C in the primary antibodies. Antiserum against the α6 subunit was diluted at 1:100 to 1:200 final dilution. For the detection of α1 and β2/3 subunits, we used undiluted or 1:2 diluted hybridoma supernatants, containing monoclonal antibodies bd-24 or bd-17. The antibody bd-17 recognizes epitopes on both β2 and β3 subunits, and antibody bd-24 recognizes the α1 subunit.[8,11,29] Biotinylated anti-rabbit or anti-mouse IgG (1:50, Vector) and finally ABC (1:100, Vector) were applied on sections. All washes were done in Tris-buffered saline (50 mM, pH 7.4) and all immunoreagents, except the ABC, were diluted in the same buffer containing 1% of normal serum. Triton X-100 (0.3%) was used in all the antibody solutions and washing solutions. 3-3′-Diaminobenzidine 4 HCl (0.05%) was used as chromogen. Some sections were treated for silver intensification of the peroxidase reaction.[9,40] Sections were mounted on gelatine coated slides, dehydrated and embedded in DePeX. Control experiments for α6 immunocytochemistry were performed as follows: replacement of the α6 antiserum by preimmune serum (diluted 1:100) resulted in a homogeneous and non-selective peroxidase labelling; incubation of sections with the α6 antiserum (10 μg protein/ml) preabsorbed for 12 h with the polypeptide used as antigen (25 μg polypeptide/ml) resulted in the disappearance of labelling confirming method specificity. For the monoclonal antibodies, replacement of the hybridoma supernatant containing monoclonal antibodies, with one that was not used to grow cells resulted in the absence of peroxidase reaction, showing method specificity.

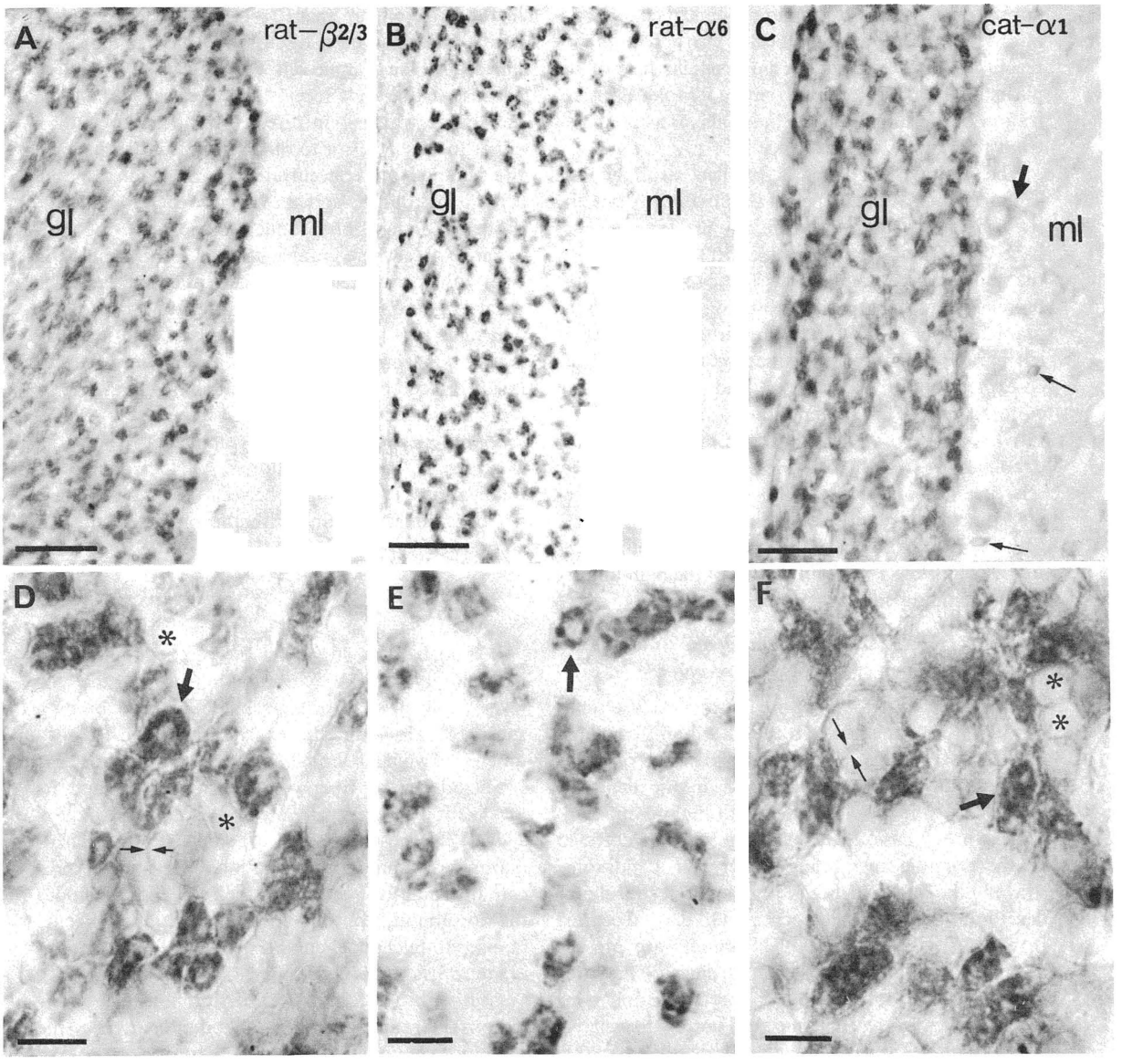

Fig. 2

also immunoreactive and immunoreactivity was present at the extracellular face of the granule cell plasma membrane, demonstrating extracellular epitopes. The reaction was present all around the granule cell dendrites including the synaptic cleft of the Golgi cell terminals. The granule cell somatic membrane was also immunopositive for both the α1 and β2/3 subunits. As previously described, peroxidase end-product was present between the membranes of neighbouring granule cell bodies, although they received no synapses.[38] This was in contrast to peroxidase reaction end-product observed after using antiserum to the α6 subunit. In the latter case only rarely were small patches of peroxidase reaction product detected in the somata of granule cells. This labelling was present in all layers and in all cells and was a result of background staining in the avidin biotinylated horseradish peroxidase complex (ABC) procedure. Immunoreactivity could not be observed in any axon terminal, Golgi or glial cells for any of the subunits studied here.

Distribution of immunoreactivity for the α6 subunit in comparison with in situ *hybridization and receptor binding*

Using an antiserum specific for the α6 subunit of the $GABA_A$ receptor, the subunit is found restricted to the dendrites of the granule cells. This correlates well with the expression of the mRNA coding for the α6 subunit in the granule cells.[13,15] The selective localization of immunoreactivity to granule cells of the cerebellum also confirms the specificity of the

antiserum suggested by immunochemical characterization. Furthermore, the localization of immunoreaction end-product to the intracellular face of the plasma membrane confirms the topographical organization of the subunit in the lipid bilayer as predicted from the amino acid sequence.[15,30]

The granule cell layer is labelled strongly with [^{3}H]muscimol,[24] showing high-affinity GABA binding sites, but few benzodiazepine agonist binding sites have been found (for review, see Ref. 21). However, the partial inverse agonist [^{3}H]Ro15-4513, in the presence of diazepam, shows binding restricted to the granular layer of the cerebellar cortex.[34] This apparent paradox has been explained by results from the co-expression of the $\alpha 6$, $\beta 2$ and $\gamma 2$ subunits in cultured cells. This subunit composition produced a $GABA_A$ receptor which binds with high affinity muscimol, as well as Ro15-4513 and the structurally related antagonist Ro15-1788, but not other benzodiazepine agonists or β-carbolines.[15] Taken together with the restricted localization of the $\alpha 6$ subunit, this suggests that the $\alpha 6$ subunit is part of the high-affinity GABA binding site on granule cells.

Different subcellular distribution for the $\alpha 6$ versus $\alpha 1$ and $\beta 2/3$ subunits of the $GABA_A$ receptor

From the distribution of the peroxidase end-product it is concluded that the $\alpha 6$ subunit is mainly located at synapses, although the presence of lower amounts at non-synaptic receptor in the dendritic membrane cannot be excluded with the present technique. In any case, the $\alpha 6$ subunit was not detected in the somatic membrane under our conditions, showing the first evidence of a differential subcellular distribution of subunits of the $GABA_A$ receptor. In contrast, the $\alpha 1$ and $\beta 2/3$ subunits are present both at synaptic and extrasynaptic sites.[38] The latter two subunits have been found at synaptic and non-synaptic sites in the thalamus and cortex as well,[36,37] indicating that the granule cells are not unique in this respect. The possibility that the apparent widespread localization of the $\alpha 1$ and $\beta 2/3$ subunits is due to technical factors connected to the extracellular epitopes, e.g. the spread of the peroxidase end-product in the extracellular space from synaptic to extrasynaptic sites, has been discussed earlier, and is very unlikely.[38] The granule cell was chosen for this study because it does not receive synapses on its somatic membrane which is in direct membrane apposition either to astrocytes or to other granule cells. Since all the synapses are concentrated in the glomeruli well separated from the somata it is not easy to envisage diffusion of reaction product to the somatic membrane to sites where granule cell bodies are contacting each other over tens of square micrometres. The presence of extrasynaptic receptor/channel complexes which are able to bind GABA and pass chloride ions, is also confirmed by whole-cell patch-clamp recordings from cerebellar slices, which show chloride currents with fast rise-time in response to brief (5–10 ms) ionophoretic pulses of GABA applied to the soma (Farrant M. and Cull-Cundy S. G., personal communication). It remains to be seen whether the channel behaviour is in line with the predicted subunit composition.

Composition of $GABA_A$ receptor complex in granule cells

Ligand binding studies in the cerebellum showed high-affinity GABA receptors in the granule cell layer, but few benzodiazepine agonist binding sites.[21,24,44] However, the expression of the $\alpha 1$ subunit[17,31,33] (which is thought to be responsible for type I benzodiazepine receptor pharmacology[25]) and its localization in the granule cell plasma membrane,[38] suggest the presence of at least some sites for benzodiazepines. Immunoprecipitation experiments indicate that a native receptor in the cerebellum includes the $\alpha 6$ subunit,[15] but the $\alpha 6$ subunit probably occurs in a separate channel complex from the one containing the $\alpha 1$ subunit (Quirk K., Gillard N., Ragan C. I., Whiting P., McKernan R. M., unpublished observations). Granule cells also abundantly express the δ and $\gamma 2$ subunits. Immunoreactivity for the δ subunit seems to be concentrated in the glomeruli,[2] suggesting colocalization with the $\alpha 6$ subunit. Immunoprecipitation results indeed reveal that both $\alpha 6$ and δ subunits exist in a receptor complex which

Fig. 3. Electron micrographs showing immunoreactivity for the $\alpha 6$ subunit in granule cells of the cerebellar cortex in rat. Immunoreactivity is only present in the dendrites (asterisks in A) of the granule cells (gc). The immunoperoxidase end-product is most dense at the synaptic junctions (thin arrows in B) formed with Golgi cell terminals (Gt). The weaker diffuse reaction end-product in the dendrites is interpreted as originating mainly from synaptic plasma membranes. One of the dendrites (asterisk in B) receives an immunopositive synapse from a Golgi terminal (thin arrow) and an immunonegative synapse from a mossy fibre terminal (mf). Immunoreactivity for the $\alpha 6$ subunit is absent from a granule cell soma (gc) and a glial cell (Gl). Scale bars = 0.5 μm (A); 0.2 μm (B). Immunocytochemistry was performed as described in Fig. 2 with the difference that no Triton X-100 was used. After 3,3′-diaminobenzidine reaction, the sections were treated with 1% OsO_4 in 0.1 M phosphate buffer for 1 h, washed in distilled water and placed for 1 h in uranyl acetate (1% in distilled water). After dehydration in graded ethanol and propylene oxide, the sections were infiltrated with epoxy resin (DURCUPAN ACM, Fluka) overnight and flat-embedded on glass slides for light microscopic assessment. Areas of interest were re-embedded for electron microscopic sectioning. A total of 13 blocks, including all layers of the cerebellar cortex were studied for the $\alpha 6$ subunit. The ultrathin sections were not stained. Three blocks for the $\beta 2/3$ subunit and two blocks for the $\alpha 1$ subunit were also studied according to the same procedure.

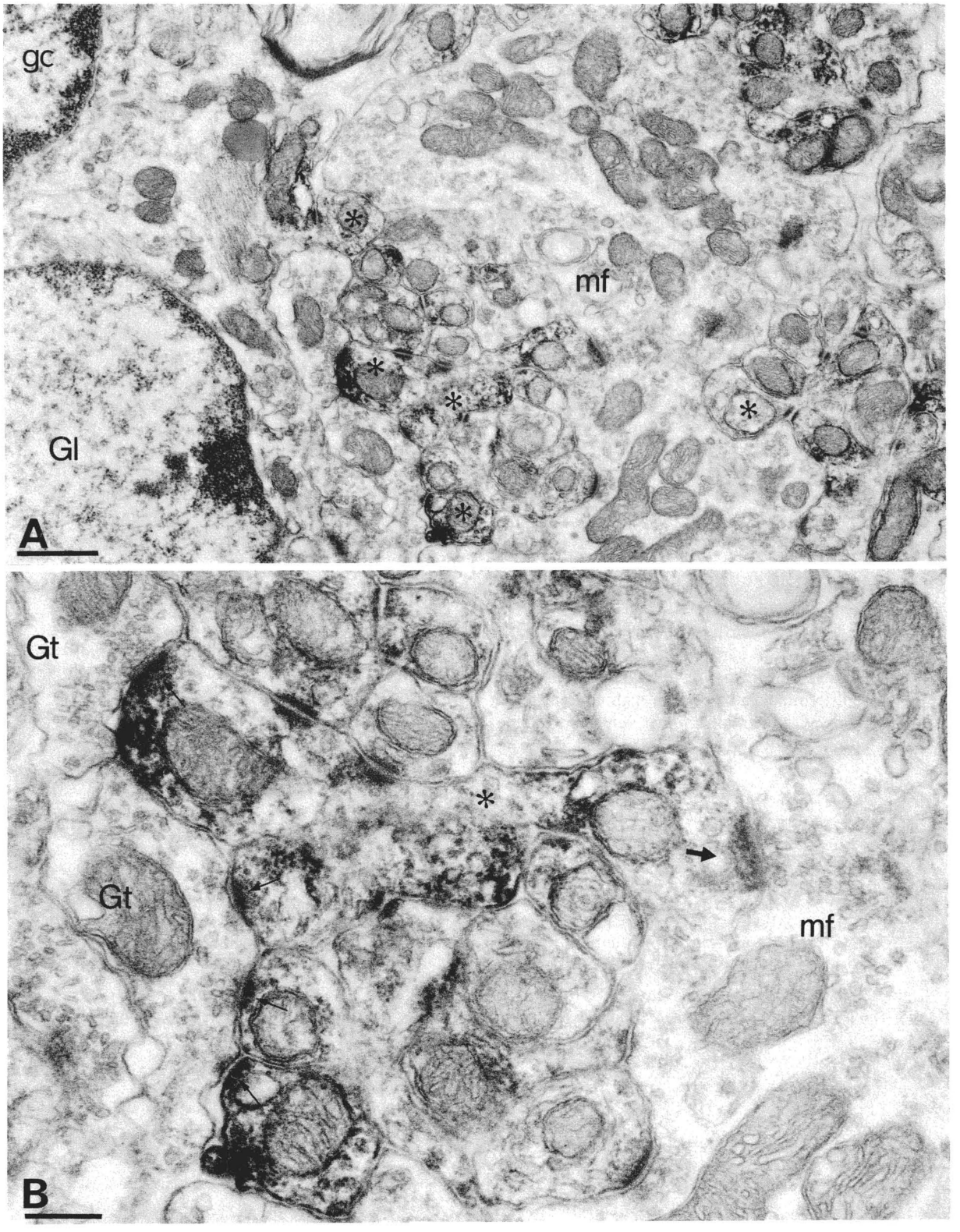

Fig. 3

binds [^{3}H]muscimol, but not benzodiazepines (Quirk *et al.*, unpublished observations). Immunoprecipitation data for other α subunits also suggest that they participate in different receptor/channel complexes,[1,6,19] but the presence of several different variants in a single receptor complex has also been reported.[14] There is no reason why these two possibilities should be mutually exclusive for all areas of the brain. Whatever the case, our results in the cerebellum suggest that, at least in the somatic membrane, receptors exist without the α6 subunit.

The immunolabelling of virtually all glomeruli for all three subunits shows that granule cells are a homogeneous population of neurons with respect to their $GABA_A$ receptor composition, although clearly further studies are needed on the other subunits that they are known to express (see Introduction for Refs). We show here that the α6 subunit is part of the synaptic receptor complex in the granule cell membrane. The same site, i.e. the synapse with the Golgi cell, also includes the β2/3 subunits in the rat. Unfortunately, since the antibody to the α1 subunit does not cross react with the rat receptor, we could not test its presence at the synapses of the rat. Nevertheless, granule cells in the rat express the α1 subunit abundantly[31] and in homology with the cat, it is very likely to be present at the Golgi cell synapses of the rat as well. Consequently, GABA released at the same synapse probably activates different receptors that include either the α1 or the α6 subunits, the latter unique to this synapse.

Another special feature of this synapse is that the Golgi cell terminals also contain high concentration of not only GABA, but another inhibitory amino acid, glycine.[23] Glycine is released in a Ca^{2+}-dependent manner from cerebellar slices,[39] but no strychnine binding sites have been found in the granule cell layer,[45] demonstrating that the glycine released form Golgi cells does not act on strychnine-sensitive glycine receptors coupled to chloride channels. However, mRNAs coding for the α3 and β subunits of the glycine receptor complex are abundant in granule cells.[18] It would be interesting to test the effect of glycine on various combinations of $GABA_A$ receptor subunits known to be present in granule cells, and also under conditions expressed together with the glycine receptor α3 and β subunits.

Most cells in the CNS receive GABA from more than one source and the surface of neurons is spatially subdivided amongst the different inputs (e.g. see Ref. 37). The multiplicity of the $GABA_A$ receptor may reflect, at the molecular level, this structural design and may support distinct pharmacological actions of GABA at separate inputs. However, most of the granule cells of the cerebellar cortex receive GABAergic input only from the Golgi cells, and still they express more than one type of receptor complex, with differential distribution on the surface of the cell. Consequently, the response of the cell to drugs and endogenous agonists may change according to the dynamic distribution of the different channel types of the $GABA_A$ receptor.

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REFERENCES

1. Benke D., Cicin-Sain A., Mertens S. and Mohler H. (1991) Immunochemical identification of the α1- and α3-subunits of the $GABA_A$ receptor in rat brain. *J. Recept. Res.* **11,** 407–424.
2. Benke D., Mertens S., Trzeciak A., Gillessen D. and Mohler H. (1991) Identification and immunohistochemical mapping of $GABA_A$ receptor subtypes containing the γ-subunit in rat brain. *Fedn Eur. biochem. Soc. Lett.* **283,** 145–149.
3. Betz H. (1990) Ligand-gated ion channels in the brain: the amino acid receptor superfamily. *Neuron* **5,** 383–392.
4. 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 Mr peptide of the $GABA_A$ receptor/benzodiazepine receptor/Cl^- channel complex. *J. Neurosci.* **8,** 602–614.
5. Doble A. and Martin I. L. (1992) Multiple benzodiazepine receptors: no reason for anxiety. *Trends pharmac. Sci.* **13,** 76–81.
6. Duggan M. J. and Stephenson F. A. (1990) Bicohemical evidence for the existence of γ-aminobutyrate$_A$ receptor iso-oligomers. *J. biol. Chem.* **265,** 3831–3835.
7. Eccles J. C., Ito M. and Szentagothai J. (1967) *The Cerebellum as a Neuronal Machine.* Springer, Berlin.
8. 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.
9. Gallyas F., Gorcs T. and Merchenthaler I. (1982) High grade intensification of the end product of DAB reaction demonstrating peroxidase activity. *J. Histochem. Cytochem.* **30,** 183–184.
10. Hamori J. and Takacs J. (1989) Two types of GABA-containing axon terminals in cerebellar glomeruli of cat: an immunogold study. *Expl Brain Res.* **74,** 471–479.
11. 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.
12. Knoflach F., Backus K. H., Giller T., Malherbe P., Pflimlin P., Mohler H. and Trube G. (1992) Pharmacological and electrophysiological properties of recombinant $GABA_A$ receptors comprising the α3, β1 and γ2 subunits. *Eur. J. Neurosci.* **4,** 1–9.

13. 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.
14. Luddens H., Killisch I. and Seeburg P. H. (1991) More than one alpha variant may exist in a $GABA_A$/benzodiazepine receptor complex. *J. Recept. Res.* **11,** 535–551.
15. Luddens H., Pritchett D. B., Kohler M., Killisch I., Keinanen K., Monyer H., Sprengel R. and Seeburg P. H. (1990) Cerebellar $GABA_A$ receptor selective for a behavioural alcohol antagonist. *Nature* **346,** 648–651.
16. Luddens H. and Wisden W. (1991) Function and pharmacology of multiple $GABA_A$ receptor subunits. *Trends Pharmac. Sci.* **12,** 49–51.
17. Malherbe P., Sigel E., Baur R., Persohn E., Richards J. G. and Mohler H. (1990) Functional characteristics and sites of gene expression of the $\alpha 1,\beta 1,\gamma 2$-isoform of the rat $GABA_A$ receptor. *J. Neurosci.* **10,** 2330–2337.
18. Malosio M. L., Marqueze-Pouey B., Kuhse J. and Betz H. (1991) Widespread expression of glycine receptor subunit mRNAs in the adult and developing rat brain. *Eur. molec. Biol. Org. J.* **10,** 2401–2409.
19. 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 α subunit-specific antibodies have unique pharmacological properties. *Neuron* **7,** 667–676.
20. Olsen R. W., Bureau M., Khrestchatisky M., MacLennan A. J., Chiang M. Y., Tobin A. J., Xu W., Jackson M., Sternini C. and Brecha N. (1990) Isolation of pharmacologically distinct GABA-benzodiazepine receptors by protein chemistry and molecular cloning. *Adv. biochem. Psychopharmac.* **46,** 35–49.
21. Olsen R. W., McCabe R. T. and Wamsley J. K. (1990) $GABA_A$ receptor subtypes: autoradiographic comparison of GABA, benzodiazepine, and convulsant binding sites in the rat central nervous system. *J. chem. Neuroanat.* **3,** 59–76.
22. Olsen R. W. and Tobin A. J. (1990) Molecular biology of $GABA_A$ receptors. *Fedn Am. Socs exp. Biol. J.* **4,** 1469–1480.
23. Ottersen O. P., Storm-Mathisen J. and Somogyi P. (1988) Colocalization of glycine-like and GABA-like immunoreactivities in Golgi cell terminals in the rat cerebellum: a postembedding light and electron microscopic study. *Brain Res.* **450,** 342–353.
24. Palacios J. M., Young W. S. and Kuhar M. J. (1980) Autoradiographic localization of γ aminobutyric acid (GABA) receptors in rat cerebellum. *Proc. natn. Acad. Sci. U.S.A.* **77,** 670–679.
25. Pritchett D. B., Luddens H. and Seeburg P. H. (1989) Type I and type II $GABA_A$-benzodiazepine receptors produced in transfected cells. *Science* **245,** 1389–1392.
26. 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.
27. Ramon-y-Cajal S. (1955) *Histologie du Systeme Nerveux de L'Homme et des Vertebres*, Vol. 2, pp. 1–106. Inst. Ramon y Cajal, Madrid.
28. 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.
29. Schoch P., Richards J. G., Haring P., Takacs B., Stahli C., Staehelin T., Haefely W. and Mohler H. (1985) Co-localization of $GABA_A$ receptors and benzodiazepine receptors in the brain shown by monoclonal antibodies. *Nature* **314,** 168–171.
30. Schofield P. R., Darlison M. G., Fujita N., Burt D. R., Stephenson F. A., Rodriguez H., Rhee L. M., Ramachandran J., Reale V., Glencorse T. A., Seeburg P. H. and Barnard E. A. (1987) Sequence and functional expression of the $GABA_A$ receptor shows a ligand-gated receptor super-family. *Nature* **328,** 221–227.
31. Sequier J. M., Richards J. G., Malherbe P., Price G. W., Mathews S. and Mohler H. (1988) Mapping of brain areas containing RNA homologous to cDNAs encoding the α and β subunits of the rat $GABA_A$ γ-aminobutyrate receptor. *Proc. natn. Acad. Sci. U.S.A.* **85,** 7815–7819.
32. Shivers B. D., Killisch I., Sprengel R., Sontheimer H., Kohler M., Schofield P. R. and Seeburg P. H. (1989) Two novel $GABA_A$ receptor subunits exist in distinct neuronal subpopulations. *Neuron* **3,** 327–337.
33. Siegel R. E. (1988) The mRNAs encoding $GABA_A$/benzodiazepine receptor subunits are localized in different cell populations of the bovine cerebellum. *Neuron* **1,** 579–584.
34. Sieghart W., Eichinger A., Richards J. G. and Mohler H. (1987) Photoaffinity labeling of benzodiazepine receptor proteins with the partial inverse agonist [^{3}H]Ro 15-4513: a biochemical and autoradiographic study. *J. Neurochem.* **48,** 46–52.
35. Sivilotti L. and Nistri A. (1991) GABA receptor mechanisms in the central nervous system. *Prog. Neurobiol.* **36,** 35–92.
36. 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.
37. Somogyi P. (1989) Synaptic organisation of GABAergic neurons and $GABA_A$ receptors in the lateral geniculate nucleus and visual cortex. In *Neural Mechanisms of Visual Perception. Proceedings of the Retina Research Foundation Symposia* (eds Lam D. K.-T. and Gilbert C. D.), pp. 35–62. Portfolio, The Woodlands, Texas.
38. 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.
39. Toggenburger G., Wiklund L., Henke H. and Cuenod M. (1983) Release of endogenous and accumulated amino acids from slices of normal and climbing fiber-deprived rat cerebellar slices. *J. Neurochem.* **41,** 1606–1613.
40. van den Pol A. N. and Gorcs T. (1988) Glycine and glycine receptor immunoreactivity in brain and spinal cord. *J. Neurosci.* **8,** 472–492.
41. Vitorica J., Park D., Chin G. and de Blas A. L. (1988) Monoclonal antibodies and conventional antisera to the $GABA_A$ receptor/benzodiazepine receptor/Cl^- channel complex. *J. Neurosci.* **8,** 615–622.
42. Wilson-Shaw D., Robinson M., Gambarana C., Siegel R. E. and Sikela J. M. (1991) A novel γ subunit of the $GABA_A$ receptor identified using the polymerase chain reaction. *Fedn Eur. biochem. Socs Lett.* **284,** 211–215.
43. 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.
44. Young W. S., Niehoff D. L., Kuhar M. J., Beer B. and Lippa A. S. (1981) Multiple benzodiazepine receptor localization by light microscopic radiohistochemistry. *J. pharmac. exp. Ther.* **216,** 425–436.
45. Zarbin M. A., Wamsley J. K. and Kuhar M. J. (1981) Glycine receptor: light microscopic autoradiographic localization with [^{3}H]strychnine. *J. Neurosci.* **1,** 532–547.

46. Zhang J. H., Sato M. and Tohyama M. (1991) Region-specific expression of the mRNAs encoding β subunits (β1, β2, and β3) of $GABA_A$ receptor in the rat brain. *J. comp. Neurol.* **303,** 637–657.
47. Zimprich F., Zezula J., Sieghart W. and Lassmann H. (1991) Immunohistochemical localization of the α1, α2 and α3 subunit of the $GABA_A$ receptor in the rat brain. *Neurosci. Lett.* **127,** 125–128.

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