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Research report

Cellular and sub-cellular localisation of $GABA_{B1}$ and $GABA_{B2}$ receptor proteins in the rat cerebellum

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Abstract

Following the recent discovery that $GABA_B$ receptors expressed in cell lines are only functional when both $GABA_{B1}$ and $GABA_{B2}$ are expressed, the present study reports on the development of polyclonal antisera specific for carboxyl-terminal portions of the two related $GABA_B$ receptor components respectively. Western blotting indicated the specificity of affinity-purified antibodies for native or recombinant expressed $GABA_{BR1}$ and $GABA_{BR2}$, with no cross-reactivity, both antisera detecting the heterodimer in rat cerebellar membranes. Immunohistochemistry revealed a distinct distribution of both receptor proteins in rat cerebellum. $GABA_{B1}$ immunoreactivity was primarily located in the granule cell layer and Purkinje cells, with discrete immuno-positive cell bodies being present in the molecular layer. $GABA_{B2}$ staining revealed intense immunoreactivity in the molecular layer, with weaker staining in the granule cell layer. Purkinje cell bodies were less intensely immuno-positive for $GABA_{B2}$. Co-localisation of both receptor proteins was observed using double immunofluorescence techniques, consistent with the notion that both proteins are required for the formation of functional $GABA_B$ receptors in vivo. Immunofluorescence also indicated that $GABA_B$ receptors did not co-localise with glial fibrillary acid protein, confirming a neuronal localisation for $GABA_B$ receptors. Electron microscopic analysis of the molecular layer revealed that the distribution of immunolabelling for both $GABA_{B1}$ and $GABA_{B2}$ was mainly located on the membrane of Purkinje cell dendrites and spines and in parallel fibre terminals.

Theme: Neurotransmitters, modulators, transporters and receptors

Topic: GABA receptors

Keywords: $GABA_B$; Receptor; Distribution; Electron microscopy; Antibody

1. Introduction

Metabotropic γ-aminobutyric acid-B ($GABA_B$) receptors, modulate synaptic transmission by activating inwardly rectifying potassium channels (Kir3), inhibiting adenylyl cyclase and voltage-gated calcium channels through pertussis toxin sensitive G_i and G_o proteins [26]. $GABA_B$ receptors may be implicated in many neurological disorders including anxiety, epilepsy, depression and spasticity, and may also be involved in the regulation of a variety of functions including appetite, learning, mood, neuroprotection and pain [21].

The first $GABA_B$ receptor clone was identified using an expression cloning technique and screening with a labelled antagonist. This clone was termed $GABA_{B1}$ (GB_1) [17]. It was established that GB_1 is a member of the 7-transmembrane G-protein coupled receptor family, and two splice variants, (GB_{1a} and GB_{1b}), which differ only at the N-terminus were reported [17]. Cell lines heterologously expressing the cloned cDNA encoding GB1 protein exhibited high affinity antagonist binding sites, but produced little of the functional activity demonstrated by native $GABA_B$ receptors in the brain [17]. These confounds were explained by the observation that GB_1 does not localise to the plasma membrane in transfected cells, but remains in

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the endoplasmic reticulum [9]. This, together with data showing differences in agonist binding potencies between native and recombinant $GABA_B$ receptors, suggested that additional proteins might be required to direct GB_1 receptor protein to the cell surface, as in the case of calcitonin receptor-like receptor and odorant receptors [12,25].

Homology-based expressed sequence tags screening led to the discovery of an additional gene that encoded a related $GABA_B$ protein, $GABA_{B2}$ (GB_2), which is also a seven transmembrane domain protein [15,18,20,23,27,36]. Co-expression of $GB_{1a/1b}$ and GB_2 produced a functional heterodimeric $GABA_B$ receptor which would activate K^+ channels, showed enhanced agonist and antagonist binding, elicited a decrease in forskolin-stimulated adenosine 2, 5 monophosphate (cAMP) production and was immunoprecipitated as a heterodimer [15,18,20,23,27,36]. Furthermore, yeast two-hybrid studies also showed that the heterodimer was formed through an interaction at the C terminal tail [27,36], and the heterodimer is apparently assembled by parallel coiled-coil α-helices [16].

In view of these findings, the present study reports the development of antisera specific for GB_1 and GB_2 using unique peptide sequences specific to these proteins. These affinity-purified antisera were used to study the concentration of these proteins in the cerebellum of the rat brain. The cerebellum contains amongst the highest densities of $GABA_B$ binding sites in the brain [5,7,38], and its relatively well studied neuronal circuitry make it a particularly useful region for the study of $GABA_B$ receptors. GABAergic transmission is also known to be the sole output from the cerebellar cortex, arising from Purkinje cells, which project to the deep cerebellar nuclei. Earlier receptor autoradiography studies using lesioned rats or mutant mice suggest that $GABA_B$ receptors are found in the cerebellum and have furthermore attempted to elucidate the localisation of these receptors to specific cell types [1,7,14,33,37].

The aim of the present study was to perform a detailed immunohistochemical study of $GABA_B$ receptor distribution in the rat cerebellum, using specific antisera. Fluorescence immunohistochemistry was used to demonstrate the co-localisation of GB_1 and GB_2 in the same cells, which would be expected, if they form functional heterodimers and to confirm their association with purely neuronal cell types. Immunogold pre-embedding immunohistochemistry visualised by electron microscopy was used to study the localisation of the GB_1 and GB_2 proteins in sub-cellular compartments.

2. Materials and methods

2.1. Tissue preparation

Adult male Wistar rats (Babraham Institute colony) were anaesthetised with pentobarbitone sodium (Sagatal, Rhone Merieux, Dublin); and fixation carried out by intracardiac perfusion with 4% neutral buffered paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (PBS). The brain and spinal cord were excised and left overnight in 4% PFA and then stored at 4°C for 2–3 days in 30% sucrose/PBS for cryoprotection. Environmental conditions for housing of the rats, and all procedures that were performed on them, were in accordance with the Animals (Scientific Procedures) Act 1986 and in accordance with the European Communities Council Directive (80/609/EEC).

2.2. Antisera production

Antisera were raised to short synthetic peptides, specific for GB_1 (NH_2-DGSRVHLLYK-COOH; residues 952–961 of GB_{1a}) and GB_2 (NH_2-VPPSFRVMVSGL-COOH; residues 930–941). Both sequences are located at the extreme end of the C termini. The peptides were coupled to tuberculin protein derivative (PPD), and antisera raised by immunising sheep (GB_1) or New Zealand white rabbits (GB_2).

2.3. Antisera purification

The GBR antibodies were purified from crude antiserum by immunoaffinity chromatography according to standard methodology [30]. Briefly, 4 mg of peptide was immobilised by covalent coupling to an insoluble agarose Sulpholink coupling gel (Pierce and Warriner, Chester, UK). The peptide-coupled agarose matrix was then transferred into a glass Econo-column (Biorad, Hemel Hempstead, Hertfordshire, UK) and 4 ml of the crude antiserum was applied and circulated for 48 h at 4°C. The column was washed with 0.1 M PBS and bound antibodies were eluted with 100 mM glycine (pH 2.5). The eluate was immediately neutralised with 1 M Tris (pH 8.0) to physiological pH and then concentrated using a Minicom B-15 concentrator (Amicon, Gloucester, UK) The concentrated antiserum was resuspended in storage buffer (0.1 M PBS, 2 mg/ml BSA). Aliquots were stored at −70°C until further use.

2.4. Western blotting

Immunoblot analyses were performed to validate the specificity of the two antisera for GB_1 and GB_2, as described previously [30]. Membranes were prepared from HEK293T cells transfected with GB_{1a} and GB_2, GB_{1b} and GB_2 or GB_2 alone, and from fresh rat cerebellum, by homogenisation of samples in 10 volumes of ice-cold buffer (50 mM HEPES, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, pH 7.4) and centrifugation at 2000 rpm for 30 min at 4°C to remove cell debris and nuclei. Supernatants were collected and centrifuged for a further 10 min at

18 300 rpm. The resulting pellets were resuspended in buffer and centrifuged again to three washes. Finally, the pellets were resuspended in buffer and protein content determined using bicinchoninic acid, according to the method of Smith et al. [32].

Extracted proteins were denatured with sodium dodecyl sulphate (SDS) and β-mercaptoethanol, and samples were loaded and separated by SDS–polyacrylamide gel electrophoresis (PAGE), then transferred to nitrocellulose membrane. These were blocked with 5% milk/Tris-buffered saline (TBST) prior to a 1-h incubation with GB_1 or GB_2 antiserum in 5% milk/TBST. Following extensive washes, membranes were incubated with peroxidase-labelled secondary antibodies for 1 h and extensively washed before ECL detection (Amersham).

2.5. Immunohistochemistry

Free-floating 30-μm sections were prepared using a freezing sledge microtome, and treated with 20% methanol and 5% H_2O_2 in distilled H_2O to reduce endogenous peroxidase activity. Following washes (3×10 min) in 0.1 M PBS, the sections were incubated for 1 h in 1% normal goat serum (GB_2) or 1% rabbit serum (GB_1) in buffer A (0.1 M PBS/0.3% Triton X-100). Sections were then incubated for 48 h at 4°C in affinity-purified antibody at dilutions of 1:800 (GB_2) and 1:4000 (GB_1) in buffer A.

Following washes, sections were incubated at room temperature for 2 h in secondary antibody (1:200, biotinylated goat anti-rabbit IgG (GB_2) or biotinylated rabbit anti-sheep (GB_1)) (Vector Laboratories, Peterborough, UK). After washes in 0.1 M PBS, sections were incubated for 45 min in preformed avidin–biotin–horseradish peroxidase complex (ABC reagent, Vector Laboratories). Bound antibodies were detected with 0.5 mg/ml 3,3′-diaminobenzidine tetrachloride (DAB) and 0.03% H_2O_2 (Vector Laboratories). Sections were mounted from distilled water onto charged microscopic slides (Superfrost Plus; BDH-Merck, Poole, UK), and air-dried before being coverslipped with DePex mounting medium (BDH-Merck).

Controls for antibody specificity included substitution of the primary antibody with 0.1 M PBS, substitution of primary antibody with pre-immune serum or incubation with antiserum pre-absorbed with the appropriate peptide against which the antiserum was raised (overnight at 4°C, 100 μM/ml) prior to use.

2.6. Double immunofluorescence

Free-floating sections were treated with 1% normal donkey serum (Jackson ImmunoResearch) for 1 h and incubated overnight at room temperature with 1:2000 affinity-purified antibody GB_1 in buffer A. Following washes, they were incubated for 2 h in 1:100 fluoroscein isothiocyanate (FITC)-conjugated donkey anti-sheep IgG (Jackson ImmunoResearch). After further washes, sections were incubated in normal goat serum for 1 h, then left overnight in 1:400 affinity-purified GB_2 antiserum in buffer A. GB_2 antiserum was detected with Texas Red conjugated anti-rabbit IgG (Vector Laboratories).

2.7. Electron microscopy

Tissue for electron microscopic analysis of the distribution of immunolabelling for $GABA_B$ receptors was obtained from Sprague–Dawley rats (Charles River, Margate, Kent, UK; 200–250 g). They were deeply anaesthetised with sodium pentobarbitone (60 mg/kg, i.p.) and then perfused transcardially with 50–100 ml of 0.9% NaCl followed by 250 ml of fixative consisting of 3% paraformaldehyde with 0.1% glutaraldehyde in 0.1 M phosphate buffer (PB, pH 7.4), and then with about 100 ml of phosphate-buffered saline (PBS, 0.01 M phosphate, pH 7.4). The brain was quickly removed and sections from the cerebellum and other regions were cut on a vibrating microtome at about 70 μm and collected in PBS. In order to enhance the penetration of the immunoreagents procedures, the sections were equilibrated in a cryoprotectant solution (PB 0.05 M (pH 7.4), containing 25% sucrose and 10% glycerol) and freeze-thawed by freezing in isopentane (BDH Chemicals, Letterworth, Leicestershire) in liquid nitrogen, and then in liquid nitrogen and thawing in PBS [35]. The sections were pre-incubated in 2% human serum albumin (HSA) in PBS for 30 min at room temperature. Immunolabelling was carried out by the ABC method essentially as described for the frozen sections and by the pre-embedding immunogold method as previously described [2,3,39]. Briefly, the sections were incubated in primary antibody solutions as described above. After washing (2×PBS, 2×PBS supplemented with 0.5% bovine serum albumin and 0.1% gelatin (PBS-BSA)), they were incubated in goat anti-rabbit (for $GABA_BR2$) or rabbit anti-sheep IgGs (for $GABA_BR1$) conjugated to colloidal gold (1.4 nm diameter; Nanoprobes, Stony Brook, USA; 1:100 in PBS-BSA) for 2 h at room temperature. The sections were then washed (2×PBS-BSA, 2×PBS) and post-fixed in 1% glutaraldehyde in PBS for 10 min. After washing (2×PBS; 2×sodium acetate buffer, 0.1 M, pH 7.0), the colloidal gold labelling was intensified using a silver enhancement kit (HQ silver, Nanoprobes) for 3–5 min at room temperature in the dark. The sections were finally washed in acetate buffer and then in phosphate buffer.

The sections were post-fixed in osmium tetroxide (1% in PB 0.1 M, pH 7.4) for 25 min for the DAB-reacted sections or 10 min for the immunogold-reacted sections at room temperature. After washing (3×PB), they were dehydrated in an ascending series of dilutions of ethanol. Uranyl acetate (1%) was included in 70% ethanol. They were then treated with propylene oxide (2×10 min) and equilibrated in resin overnight (Durcupan ACM, Fluka,

Gillingham, Dorset, UK), mounted on glass slides and cured at 60°C for 48 h. The sections were first examined in the light microscope. Areas of interest were photographed and were cut out from the slide and glued to blank cylinders of resin. Serial ultrathin sections were cut on a Reichert Ultracut E and collected on piloform-coated single slot grids. The sections were stained with lead citrate and examined in a Philips CM10 electron microscope.

3. Results

3.1. Western blotting

The specificity of the GB_1 and GB_2 antisera was verified by western blotting of membranes from recombinant cell lines and native rat cerebellar tissue. The GB_2 antiserum revealed a discrete band of approximately 120 kDa in GB_2 alone, GB_2/GB_{1a}, and GB_2/GB_{1b} cell line membranes, and cerebellar membranes, whereas the GB_1 antiserum produced bands of approximately 130 kDa and 100 kDa in GB_2/GB_{1a} and GB_2/GB_{1b} cell lines, respectively (Fig. 1). The GB_1 antiserum did not produce any bands in the GB_2-alone lane, indicating the lack of cross-reactivity between antisera. No positives were detected in membranes prepared from mock transfected HEK 293T cells and competition of the antisera with the corresponding peptide and or omission of the primary antibody abolished all specific bands.

3.2. Immunohistochemistry

Immunohistochemical control experiments confirmed the specificity of the antisera. Staining was absent when GB_1 antiserum was pre-incubated with the peptide against which it was raised. Similar results were obtained when the primary antiserum was omitted, and following substitution of the primary antiserum with pre-immune serum. Corresponding controls for the GB_2 antiserum also produced no staining.

GB_1 and GB_2 immunoreactivities displayed a laminar distribution in the cerebellar cortex. (Fig. 2). The granule cell layer contained GB_1 immunoreactive granule and Golgi cell bodies (Fig. 2A). Granule cells were also visualised with the GB_2 antiserum. Purkinje cells, evident as a discrete monolayer of cells between the granule cell and molecular layers, were immunopositive for both proteins, though less intensely so for GB_2 (Fig. 2B). The molecular layer possessed very intense neuropil staining for both GB_1 and GB_2 protein, although this was most evident for GB_2. GB_1 antisera also stained cells in the molecular layer, which are presumably basket and stellate cells (Fig. 2A).

Double immunofluorescence in the cerebellum demonstrated the co-localisation of GB_1 and GB_2 protein. (Fig. 3). GB_1 (green) and GB_2 (red) immunoreactivities were shown to coexist (yellow) (3C) in granule cells, Purkinje cells and particularly brightly in the neuropil of the molecular layer.

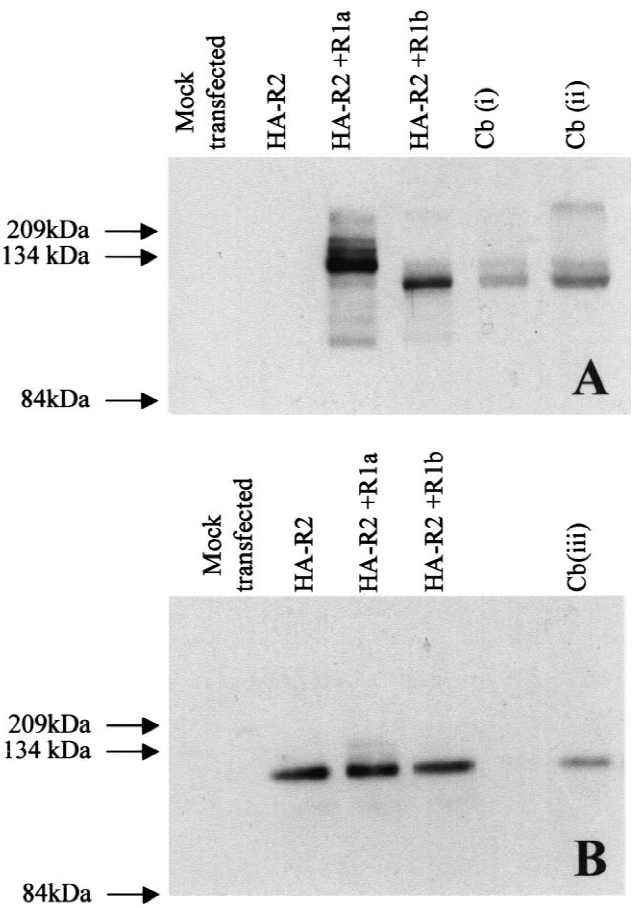

Fig. 1. Western blot analysis showing the specificity of the GB_1 and GB_2 antibodies. GB_1 antibody (A) showing bands of 130 and 100 kDa for GB_{1a} and GB_{1b}, respectively, in cell lines (5 μg) and (cb) cerebellum (i) 10 μg (ii) 20 μg membrane preparations, whereas antiserum for GB_2 (B) revealed 120 kDa bands each 5 μg cell line and cerebellum (iii) 5 μg membrane preparation.

3.3. Immunogold pre-embedding immunohistochemistry

Consistent with the observations in the fluorescence-labelled sections, immunolabelling by the peroxidase and the immunogold methods resulted in strong labelling of the cerebellum. The labelling for the GB_2 was more robust than the labelling for GB_1 but the pattern of labelling was very similar. The most prominent labelling for both the GB_1 and GB_2 antibodies occurred in the molecular layer. Purkinje cell bodies were also labelled and there was weak labelling of the granular cell layer. Electron microscopic analysis of immunogold labelled sections (Fig. 4) revealed, consistent with previous observations [13,18] that most of the immunolabelling in the molecular layer was associated with Purkinje cell dendrites and spines. The majority of the

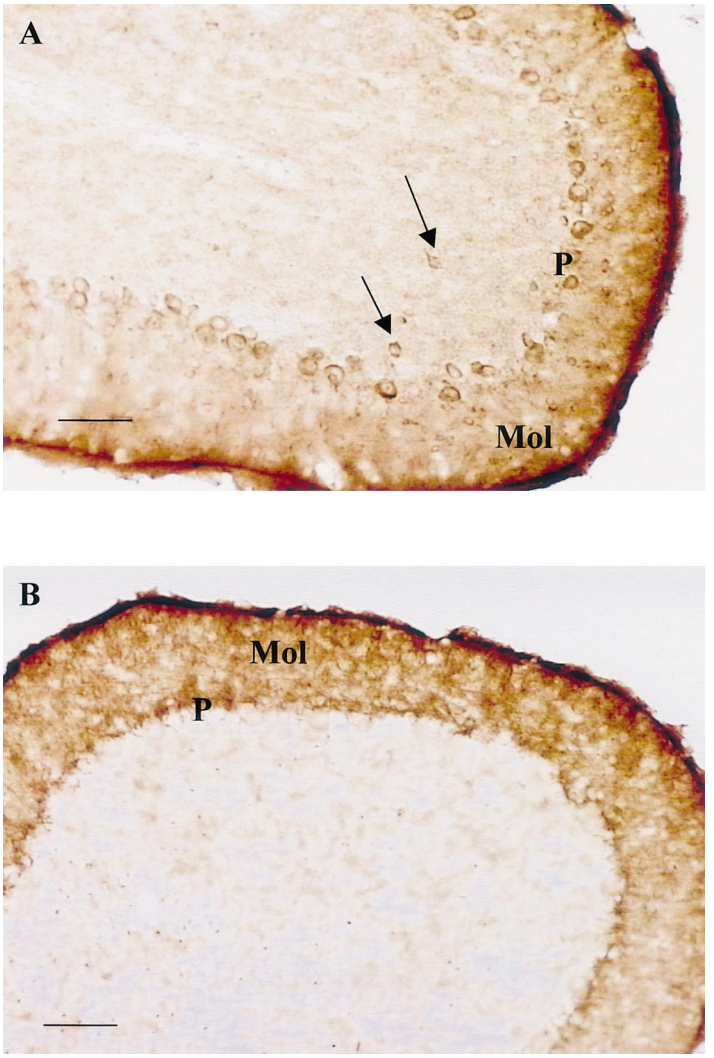

Fig. 2. Localisation of GB_1 (A) and GB_2 (B) displayed using immunohistochemistry GB_1 showed distinct staining in this region in particular the Purkinje cell bodies (P). The molecular layer (mol) also reveals some staining particularly in cells resembling stellate and basket cells, whilst in the granule layer (Gr) some granule cells are visible as are some large Golgi cells (indicated by arrowheads). GB_2 (B) reveals staining that is strongest in the molecular layer (mol) with moderate staining of Purkinje cell bodies (p) and granule cells. A and B are at the same magnification; Scales bars=100 μm.

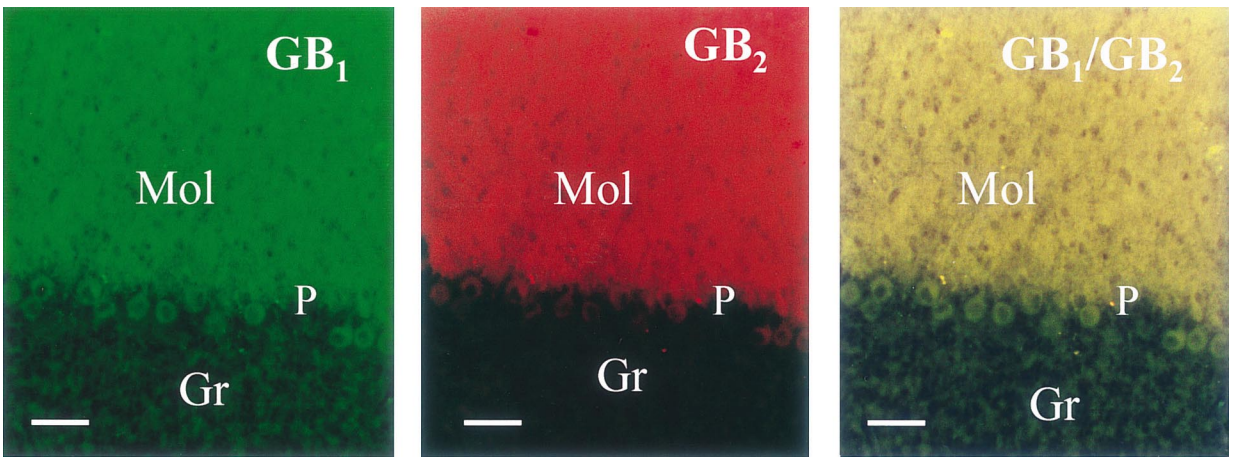

Fig. 3. Immunofluorescent labelling of GB_1 (green) and GB_2 (red). Double labelling (yellow) shows the co-localisation of GB_1 and GB_2 in the cerebellum. Scale bars=100 μm.

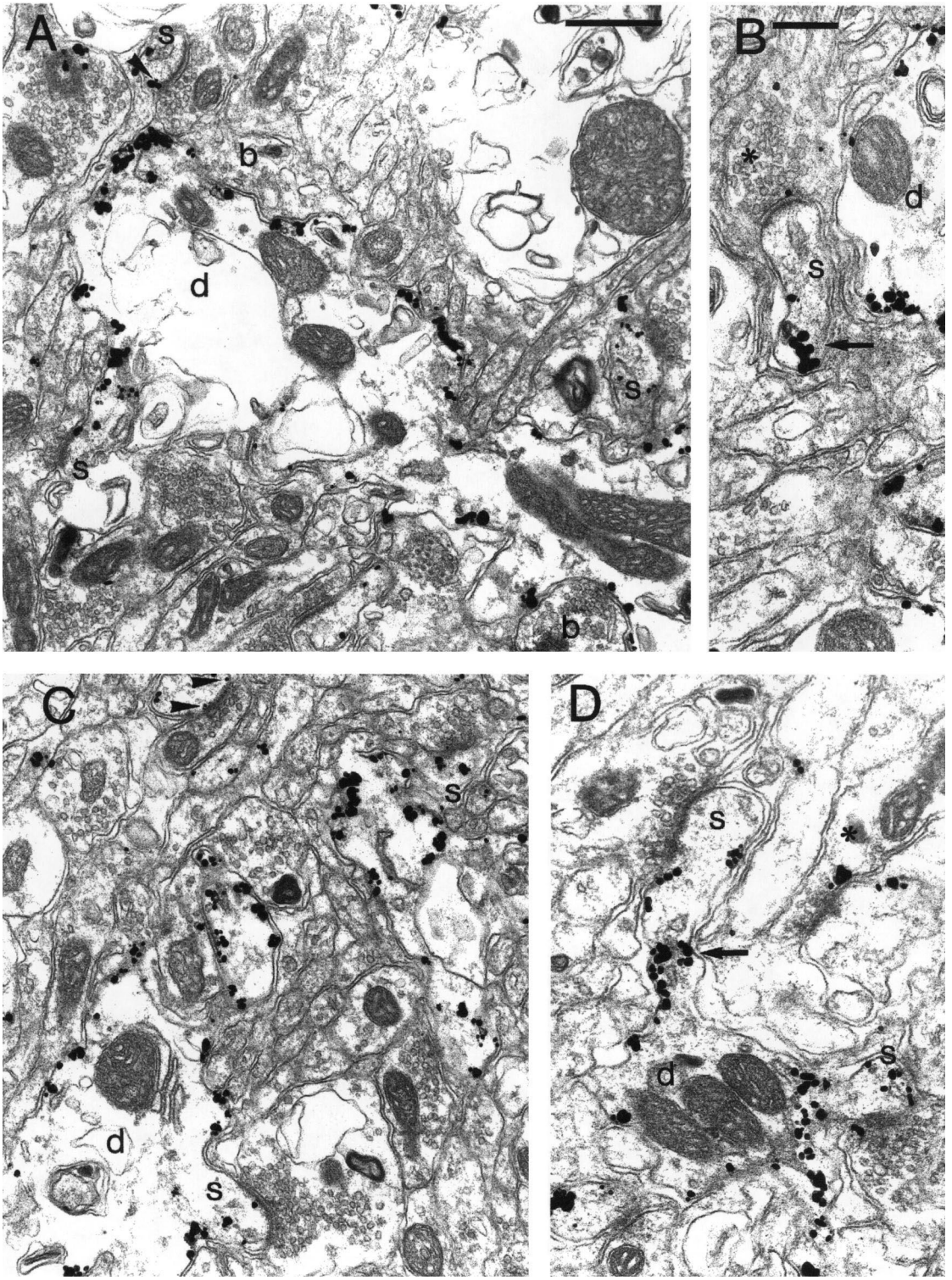

Fig. 4. Ultrastructural localisation of immunolabelling for GB_1 (A,B) and GB_2 (C,D) in the molecular layer of the cerebellum revealed by the pre-embedding immunogold method. (A,B) Immunolabelling for GB_1 is largely associated with the cell membrane of Purkinje cell dendrites (d) and spines (some indicated by s); labelling also occurs in terminals formed by parallel fibres (some indicated by asterisks). The immunolabelling is mostly at extrasynaptic sites, but sometimes occurs at the edge of postsynaptic densities (arrowhead in the spine at the upper left of A). Intracellular labelling is often clustered on the spiny apparatus (arrow in B). The Purkinje cell dendrite in A is apposed by two boutons (b) which are probably derived from stellate or basket cells. Although not synaptic at this level, immunogold particles are associated with the membrane to which they are apposed. (C,D) Immunolabelling for GB_2 shows a very similar distribution to that of GB_1. The most prominent is of the plasma membrane of Purkinje cell dendrites (d) and spines (s). The labelling is largely at extrasynaptic sites although sometimes occurs at the edge of postsynaptic densities (arrowheads in the spine at the upper left of B). labelling is also with spiny apparatus of some spines (arrow in d) and some parallel fibre terminals (asterisk in D) Scales: A and C are at the same magnification, bar in A=0.5 μm; B and D are at the same magnification, bar in B=0.25 μm.

immunoparticles were located on the internal surface of the plasma membrane where they occurred as single particles or clumps of several immunogold particles. They were located at non-synaptic sites although occasionally in spines (Fig. 4A,C), labelling occurred adjacent to the post-synaptic density associated with the parallel fibre terminals. Occasionally labelling was associated with the membrane of the dendritic shaft that was apposed by axonal boutons that were probably derived from stellate cells or basket cells. Intracellular labelling was associated with cisterns of endoplasmic reticulum both in dendrites and the Purkinje cell perikarya. The spiny apparatus of Purkinje cell spines were often strongly labelled (Fig. 4B,D). Axon terminals forming asymmetric synapses with Purkinje cell spines, which were presumably terminals of parallel fibres, were also sometimes labelled with both antibodies (Fig. 4B,D).

4. Discussion

This study describes the generation of GB_1 and GB_2 specific polyclonal antisera. Western blotting generated bands of the appropriate sizes for GB_1 (both a and b splice variants) and GB_2, and detected the recombinant receptors expressed in HEK293 cells without cross-reactivity between GB_1 and GB_2. Immunohistochemical controls also demonstrated the specificity of the antisera for the peptides against which the antisera were raised.

In a recent study, low levels of GB_1 mRNA was observed in the cerebellar molecular layer [4], however in previous autoradiographic studies this area has been reported a region rich in $GABA_B$ receptors [5,7,37]. This was corroborated by immunohistochemical data using an antibody raised against, the $GABA_B$ agonist baclofen [24], and more recently by GB_1 immunohistochemistry [22,31]. $GABA_B$ receptors in this region are thought to exist on parallel fibre and climbing fibre terminals, and Purkinje cell dendrites [6]. The hyperpolarising action of baclofen on Purkinje cells has been long established [10], and Purkinje cell dendrite $GABA_B$ receptors have been postulated to be postsynaptic in nature [34].

Furthermore, mapping studies of GB_{1a} and GB_{1b} mRNAs in the cerebellum had already suggested a postsynaptic localisation of GB_{1b} on the dendrites of the Purkinje cells and a presynaptic localisation of GB_{1a} on parallel fibre terminals [4], whilst GB_2 receptor localisation was suggested at both sites [18]. The present findings support and extend these studies. Thus GB_1 and GB_2 immunolabelling was observed at both presynaptic sites (presumably parallel fibre terminals) and at postsynaptic sites on purkinje cell perikarya, dendrites and spines. However, the present study suggests lower levels of GB_1 immunoreactivity than GB_2. This may reflect differences in protein trafficking rates out of the soma into processes, or may imply the existence of further $GABA_B$ receptor proteins capable of forming part of a heterodimer.

In agreement with previous findings from in situ hybridisation and immunohistochemical studies Purkinje cell bodies were distinctly labelled by the GB_1 antiserum [22]. GB_2 immunoreactivity was less intense over Purkinje cell bodies, and more intense in the molecular layer, the converse of that seen with the GB_1 antiserum. This also differs from mRNA studies which show high GB_2 in the Purkinje cell bodies [11,18], though this could be due to transportation of the proteins to the Purkinje dendrites in the molecular layer. These findings are consistent with the suggestion that there is a difference in dendritic trafficking rates between GB_1 and GB_2 proteins.

Though a recent report by Fritschy and colleagues has shown high levels of GB_{1a} in the granule layer [13] in agreement with a recent GB_2in situ hybridisation study [8], all other studies have reported low levels of $GABA_B$ mRNA and $GABA_B$ binding in this layer [4,5,7]. Likewise, our study shows diffuse granule cell immunoreactivity for both GB_1 and GB_2, and also GB_1 immunoreactivity in Golgi cells, which concurs with recent protein distribution studies [22]. This discrepancy may explained by production of GB_2 in the granule cells and then its transportation at various rates to the granule cells axons, i.e. parallel fibres, which has been shown to be rich in GB_2 protein. Golgi cells were not visualised with the GB_2 antiserum; which may indicate that these cells make only low levels of this receptor protein or it may reflect the existence of further $GABA_B$ receptor proteins or trafficking proteins yet undiscovered.

The co-localisation of GB_1 and GB_2 in cerebellar neurones supports the notion that GB_1 and GB_2 are required to form a functional $GABA_B$ receptor.

The electron microscopic findings confirm and extend previous ultrastructural observations of GB_1 [13,18] and GB_2 [18] in the rat cerebellum. Thus, Purkinje cell dendrites and spines were strongly immunoreactive for both subtypes of the $GABA_B$ receptor and this labelling. Most of the immunolabelling was localised on the plasma membrane mainly at extrasynaptic sites although it was sometimes closely apposed to the postsynaptic density of the parallel fibre terminal synapses, as was noticed in a previous study [19]. The function of receptors localised at non-synaptic sites remains an issue for debate but is commonly the case for metabotropic receptors of various types [2,28,29,39]. In addition to the membrane labelling we also observed, as did Kulik and colleagues [19], intracellular labelling, most prominently of the spiny apparatus of purkinje cell spines. This immunolabelling may represent a receptor in the process of synthesis, transport or recycling or an intracellular receptor pool. For both GB_1 and GB_2, we also observed pre-synaptic labelling in the terminals of parallel fibres in agreement with an earlier study [19]. Only a subpopulation were labelled in our sections but this probably represents an underestimate

of the true distribution because of the low expression of the receptor in the terminals and because the labelling was not examined in serial sections. Whatever the situation, these receptors are in a position to mediate GABAergic control or modulation of the release glutamate from parallel fibre terminals [2,13,18,28,29,39]. This similar distribution of GB_1 and GB_2 labelling at the light and electron microscopic level supports previous studies [18] and is consistent with the formation of functional heterodimers.

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