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$GABA_B$ receptor isoforms GBR1a and GBR1b, appear to be associated with pre- and post-synaptic elements respectively in rat and human cerebellum

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1 Metabotropic γ-aminobutyric acid (GABA) receptors, $GABA_B$, are coupled through G-proteins to K^+ and Ca^{2+} channels in neuronal membranes. Cloning of the $GABA_B$ receptor has not uncovered receptor subtypes, but demonstrated two isoforms, designated GBR1a and GBR1b, which differ in their N terminal regions. In the rodent cerebellum $GABA_B$ receptors are localized to a greater extent in the molecular layer, and are reported to exist on granule cell parallel fibre terminals and Purkinje cell (PC) dendrites, which may represent pre- and post-synaptic receptors.

2 The objective of this study was to localize the mRNA splice variants, GBR1a and GBR1b for $GABA_B$ receptors in rat cerebellum, for comparison with the localization in human cerebellum using *in situ* hybridization.

3 Receptor autoradiography was performed utilizing [^{3}H]-CGP62349 to localize $GABA_B$ receptors in rat and human cerebellum. Radioactively labelled oligonucleotide probes were used to localize GBR1a and GBR1b, and by dipping slides in photographic emulsion, silver grain images were obtained for quantification at the cellular level.

4 Binding of 0.5 nM [^{3}H]-CGP62349 demonstrated significantly higher binding to $GABA_B$ receptors in the molecular layer than the granule cell (GC) layer of rat cerebellum (molecular layer binding $200 \pm 11\%$ of GC layer; $P < 0.0001$). GBR1a mRNA expression was found to be predominantly in the GC layer (PC layer grains $6 \pm 6\%$ of GC layer grains; $P < 0.05$), and GBR1b expression predominantly in PCs (PC layer grains $818 \pm 14\%$ of GC layer grains; $P < 0.0001$).

5 The differential distribution of GBR1a and GBR1b mRNA splice variants for $GABA_B$ receptors suggests a possible association of GBR1a and GBR1b with pre- and post-synaptic elements respectively.

Keywords: $GABA_B$ receptors; cerebellum; *in situ* hybridization; human tissue; [^{3}H]-CGP62349; splice variant

Abbreviations: GC, granule cell; Mol, molecular layer; PC, Purkinje cell

Introduction

Metabotropic receptors for the neurotransmitter γ-aminobutyric acid (GABA), i.e. $GABA_B$ receptors, are coupled through G-proteins to K^+ and Ca^{2+} channels in neuronal membranes (see Bettler *et al*., 1998). Association with a particular channel type may be, at least in part, determined by their anatomical location (Lüscher *et al*., 1997). It is well established that $GABA_B$ receptor agonists increase neuronal K^+ conductance to produce membrane hyperpolarization, an effect which may be post-synaptic, whereas the same agonists acting *via* pre-synaptic receptors on nerve terminals reduce Ca^{2+} conductance to decrease the evoked release of neurotransmitter (Andrade *et al*., 1986; Takahashi *et al*., 1998). Much attention has focused on the nature of the receptors at these two locations with speculation that they may be pharmacologically distinct (Dutar & Nicoll, 1988; Pitler & Alger, 1994; Deisz *et al*., 1997). Furthermore, studies on pre-synaptic receptors in synaptosomes have indicated that multiple $GABA_B$ receptors may exist even at the single location (Bonanno & Raiteri, 1993; Bonanno *et al*., 1997). However, definitive evidence from pharmacological studies in a variety of tissues is still lacking due primarily to the lack of receptor ligands able to select for potential receptor subtypes.

Cloning of the $GABA_B$ receptor by Kaupmann *et al*. (1997) has so far failed to substantiate the existence of multiple receptor subtypes, but has provided evidence for two isoforms, GBR1a and GBR1b, which differ only by the presence of extra residues at the N terminal in GBR1a. We and others have previously described the distribution of $GABA_B$ receptors in rat and human brain using *in vitro* receptor autoradiography (Bowery *et al*., 1987; Albin & Gilman, 1990; Chu *et al*., 1990). Among the brain regions which showed a discrete distribution of $GABA_B$ sites was the molecular layer of the cerebellum. Biochemical studies in cerebellar tissue suggest that these $GABA_B$ receptors may be located on granule cell (GC) parallel fibre terminals and Purkinje cell (PC) dendrites within the molecular layer (Bowery *et al*., 1983; Wojcik & Neff, 1984). In the study by Kaupmann *et al*. (1997), it was apparent that mRNA for GBR1 in rat cerebellum is not located in the molecular layer, but within the GC and PC layers. In the present study we have examined this more closely by focusing on the locations of the mRNA for each of the two receptor isoforms in rat and human cerebellum using an *in situ* hybridization technique. The data obtained suggest that GBR1a may be associated with pre-synaptic receptors on the GCs whilst GBR1b is responsible for the post-synaptic

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receptors on the PC dendrites. These results have, in part, been presented in abstract form (Billinton *et al*., 1998).

Methods

Tissue preparation

Male Wistar rats (Bantin & Kingman, Hull, U.K.; 250 g) were sacrificed, and their brains frozen at −45°C in isopentane on dry ice. A human cerebellar sample (male; 91 years old; *post mortem* interval 24 h; cause of death, cardiac failure) was obtained *post mortem* and frozen between two brass plates at −70°C. Both tissues were sectioned at 10 μm in a cryostat and sections mounted on charged microscope slides (Superfrost Plus, BDH, U.K.), which were either stored at −80°C until autoradiography assay or fixed with 4% paraformaldehyde in ice cold phosphate buffered saline (PBS, pH 7.2) for 5 min, washed twice in fresh PBS (1 min each), dehydrated using increasing concentrations of ethanol and stored in 95% ethanol at 4°C until *in situ* hybridization assay.

Receptor autoradiography

Sections were thawed, then pre-incubated (20 min then 60 min) in fresh assay buffer (TRIS/HCl (50 mM), pH 7.4, $CaCl_2$ (2.5 mM)) before incubation for 60 min at 25°C with 0.5 nM [^{3}H]-CGP62349 (85 Ci mmol^{-1}, Bittiger *et al*., 1996). Non-specific binding was determined in the presence of 10 μM CGP54626A (Bittiger *et al*., 1992). Slides were washed twice for 1 min in assay buffer at 25°C, dipped briefly in distilled water, air dried and apposed to [^{3}H]-sensitive Hyperfilm (Amersham, U.K.) along with [^{3}H]-impregnated plastic standard strips (Amersham, U.K.) for 3 weeks at room temperature.

Oligonucleotide sequences

Oligonucleotides for rat GBR1 sequences were designed using the sequences deposited in the Genebank by Kaupmann *et al*. (1997) and FASTA homology searches within the GCG sequence analysis program. Oligonucleotides chosen had no significant homology to other known mRNA sequences. Oligonucleotides for GBR1pan (i.e. recognizing both splice variants) and GBR1a were designed to common parts of the rat and human sequences, however GBR1b required different oligonucleotides for rat and human tissues. Antisense oligonucleotides were synthesised by Alta Bioscience, University of Birmingham, U.K., and the sequences used were as follows: GBR1pan, 5′-CAT TCC CCT CGG GTG ATC AGC CTG CGC ATC TTG GGC ACA AAG AGC ACA AC-3′, corresponding to nucleotides 2545–2594; GBR1a, 5′-CAA ATA AGA CTT GGA GCA GAT TCG GAC ACA GCG GCT GGG TGT TGC CAT AT-3′, corresponding to nucleotides 263–312; GBR1b (rat), 5′-GTG AGG CCG CGG GAG ATG AGG GGA GTG AGA-3′, corresponding to nucleotides 88–117 and GBR1b (human), 5′-CTG TTG GGG AGC GTT AGG AGC TCA GGG GGG ACA CTT TTC CTG GGG AGG GC-3′.

In situ *hybridization*

Sections were removed from ethanol and allowed to dry. Oligonucleotides were labelled with ^{35}S-dATP (Dupont-NEN, Brussels, Belgium) using a 3′-terminal deoxynucleotidyl transferase enzyme kit (Boehringer-Mannheim) and diluted to a concentration of 1×10^7 d.p.m. ml^{-1} in hybridization buffer (containing: 50% formamide, 4 × SSC (standard saline citrate (mM): sodium chloride 300, sodium citrate 0.3), sodium phosphate (25 mM), sodium pyrophosphate (10 mM), 5 × Denhardt's solution, 200 μg ml^{-1} fish sperm, 100 μg ml^{-1} polyadenylic acid and 10% dextran sulphate). Hybridization was performed in a humid chamber overnight at 42°C (~16 h) with each slide covered with a parafilm coverslip. Sections were then washed in 1 × SSC for 2 × 30 min at 55°C, rinsed in 1 × SSC and 0.1 × SSC (1 min each at room temperature), dehydrated using increasing concentrations of ethanol, and air dried before being apposed to [^{3}H]-sensitive film for 14 days. Non-specific hybridization was demonstrated in the presence of 100 fold excess unlabelled oligonucleotide. Following film exposure the slides were dipped in Ilford K5 emulsion (6 g in 9 ml of 2% glycerol in distilled water at 43°C), which was allowed to set on a cold plate before storage in a light-proof box at 4°C for 28 days. The emulsion was developed in D-19 (Kodak) and fixed in Unifix (Kodak). Sections were immediately stained with 0.1% methylene blue and dehydrated before mounting coverslips using DEPEX mounting medium.

Data analysis

[^{3}H]-CGP62349 binding images were analysed on an MCID M4 image analysis system (Imaging Research Inc., Ontario, Canada), and optical density converted to fmol mg^{-1} of bound ligand using the image generated by the [^{3}H]-

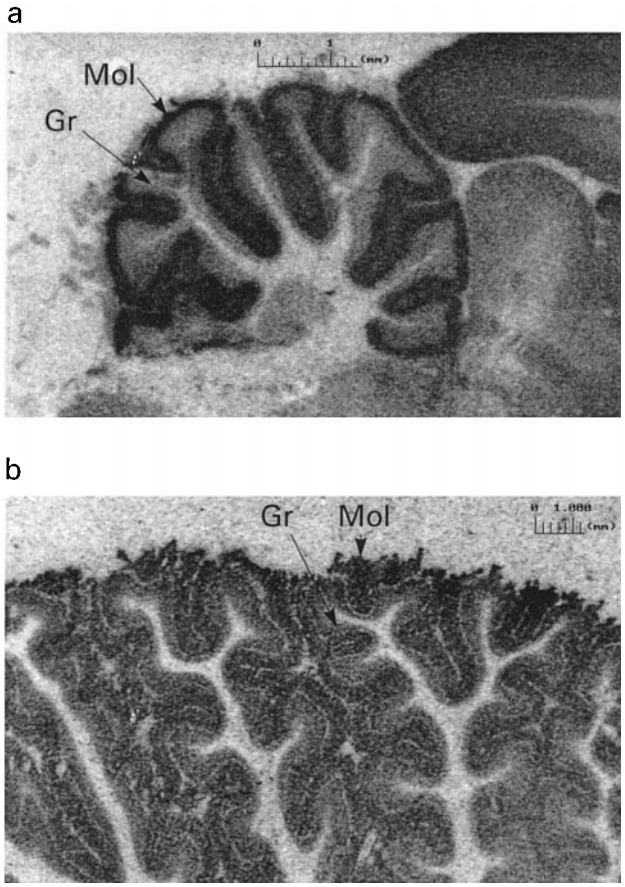

Figure 1 Autoradiographic images depicting total binding of 0.5 mM [^{3}H]-CGP62349 in 10 μm sections of (a) rat cerebellum, and (b) human cerebellum. Non specific binding was equivalent to film background. Abbreviations: Gr, granule cell layer; Mol, molecular layer.

impregnated plastic standard strips. Total binding was assessed in three sections per rat, and four sections from the human sample.

Silver grains generated by emulsion dipping of *in situ* hybridization sections were quantified using the MCID M4 grain counting protocol. Grains over 50 PCs, 50 squares of C layer and 50 cells in the molecular layer were counted. Three sections from three rat cerebella, and four sections from one human cerebellar sample were analysed. Background grain levels were determined using sections hybridized in the presence of 100 fold excess of oligonucleotide. Statistical analysis was performed using Prism (GraphPad Software, San Diego, CA, U.S.A.) and utilized Student's unpaired *t*-test (two-tailed). Data are quoted as $\pm$s.e.mean where applicable.

Results

[^{3}H]-CGP62349 autoradiography

In sections from rat cerebellum, the highest levels of 0.5 nM [^{3}H]-CGP62349 binding were evident in the molecular layer (185.8 ± 10.4 fmol mg^{-1}), with a significantly lower level observed in the GC layer (90.5 ± 3.7 fmol mg^{-1}; $P<0.0001$; Figure 1). The same pattern was observed in human tissue, with expression levels of 96.6 fmol mg^{-1} and 43.5 fmol mg^{-1} in molecular and GC layers respectively. Non-specific binding was equivalent to film background.

Distribution of GBR1pan in cerebellar cortex

The GBR1pan oligonucleotide, recognizing both splice variants, showed significantly greater labelling over the PC layer (1085 ± 29 grains 1000 μm^{-2}), than over the GC layer (575 ± 45 grains 1000 μm^{-2}; $P<0.05$), with a level virtually equivalent to background over the molecular layer in rat cerebellum (Figure 2a and b). The same distribution was also

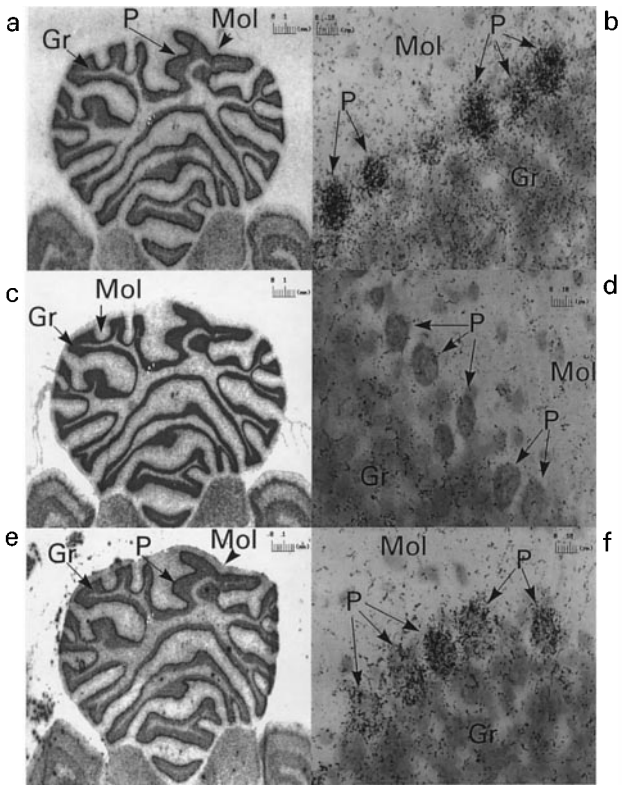

Figure 2 Autoradiographic images at macro- and microscopic levels depicting *in situ* hybridization of ^{35}S-dATP labelled oligonucleotides specific for GABA$_B$ splice variants in 10 μm sections of rat cerebellum, in (a) and (b) GBR1pan, (c) and (d) GBR1a and (e) and (f) GBR1b. Abbreviations: Gr, granule cell layer; Mol, molecular layer; P, Purkinje cell.

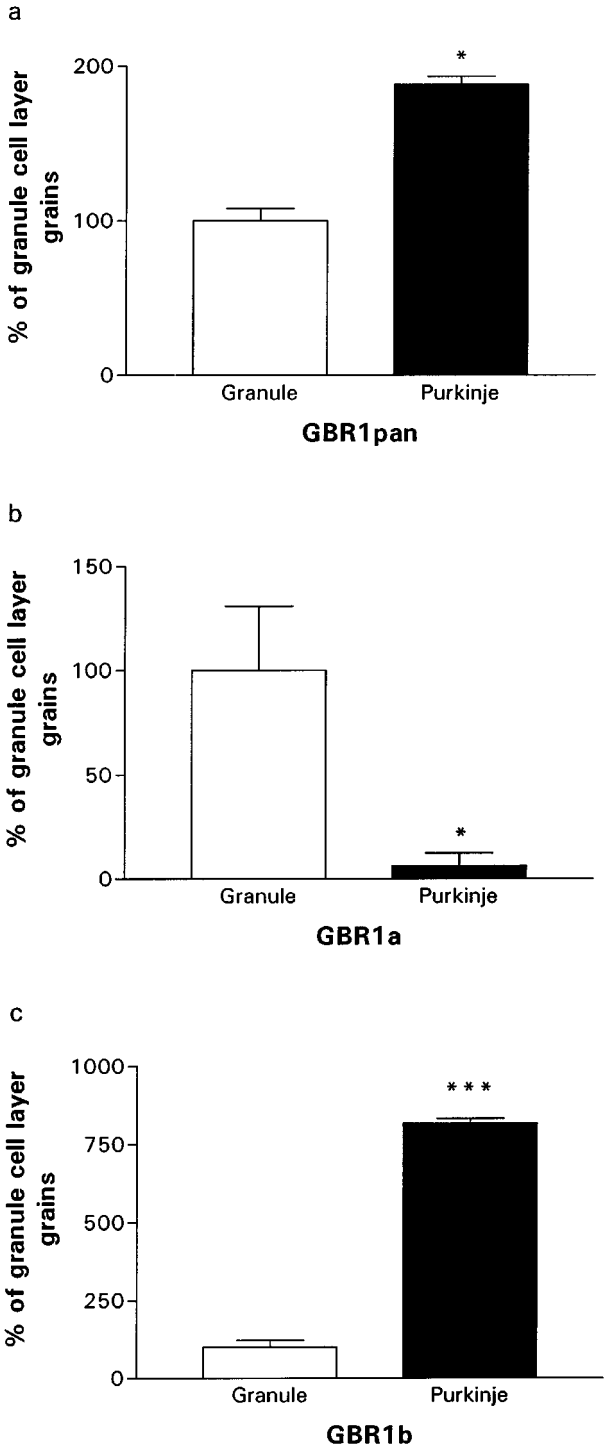

Figure 3 Mean number of grains counted over Purkinje cells (filled bars) and granule cell layer (open bars) in rat cerebellum expressed as a percentage of grains counted in the granule cell layer (mean $\pm$ s.e.mean, $n=3$) for (a) GRB1pan, (b) GBR1a and (c) GBR1b. Statistical analysis used Student's *t*-test (two tailed) where * represents $P<0.05$; *** represents $P<0.0001$.

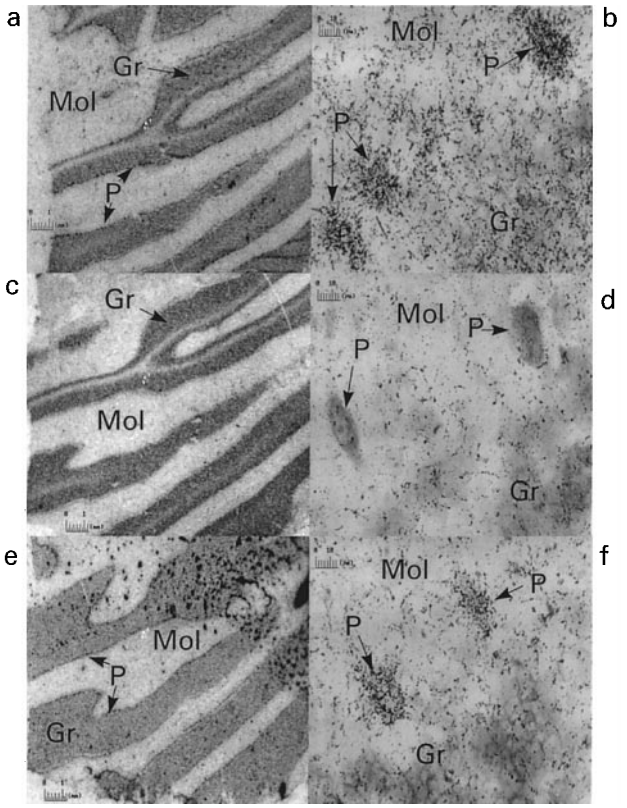

Figure 4 Autoradiographic images at macro- and microscopic levels depicting *in situ* hybridization of ^{35}S-dATP labelled oligonucleotides specific for GABA$_B$ splice variants in 10 μm sections of human cerebellar cortex, in (a) and (b) GBR1pan, (c) and (d) GBR1a and (e) and (f) GBR1b. The black areas at the top right of (e) represent artefact. Abbreviations: Gr, granule cell layer; Mol, molecular layer; P, Purkinje cell.

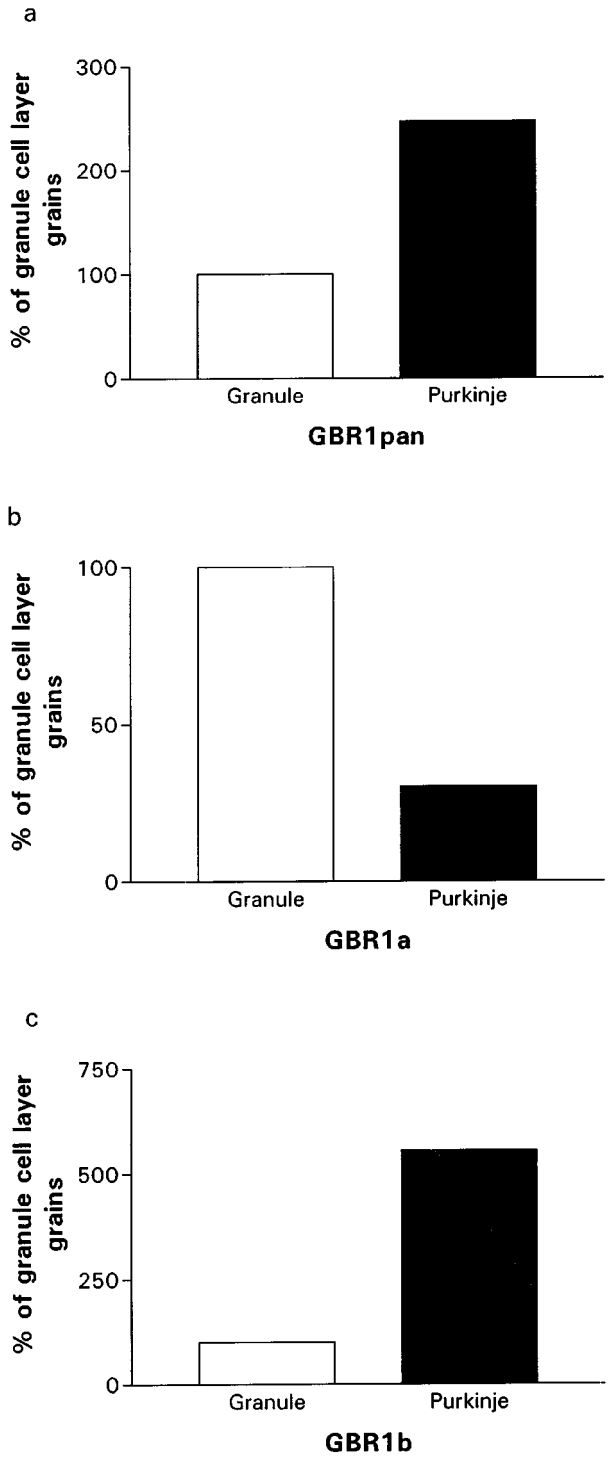

Figure 5 Number of grains counted over Purkinje cells (filled bars) and granule cell layer (open bars) of human cerebellum expressed as a percentage of grains counted in the granule cell layer ($n=1$) for (a) GBR1pan, (b) GBR1a and (c) GBR1b.

seen in the equivalent layers of the human cerebellum (Figure 4a and b) and was confirmed by grain counts (PC – 1254 grains 1000 μm^{-2}, GC layer – 507 grains 1000 μm^{-2}; Figure 5a), though the PCs appeared to be more diffusely located between the GC and molecular layers. Examination of the emulsion dipped slides confirmed this finding (Figure 4b).

Distribution of GBR1a and GBR1b splice variants in cerebellar cortex

GBR1a and 1b transcripts showed a distinct difference in distribution over the PC and GC layers, with GBR1a dominating the GC layer, and GBR1b being the most abundant splice variant in the PCs. This difference was evident in both rat and human cerebellar tissues (Rat – Figure 2c, d, e and f and human – Figure 4c, d, e and f). These visual differences were confirmed when grain counting was performed (Figures 3 and 5). Analysis of data from rat cerebellum demonstrated that for GBR1a, the PC layer possessed significantly less grains than the GC layer (6 ± 6% of the number of grains over the GC layer, $P<0.05$). The converse was true of GBR1b, which showed a significantly higher number of grains due to GBR1b over the PC layer rather than over the GC layer (818 ± 14% of the number of GC layer grains, $P<0.0001$). An identical pattern was observed in the human cerebellum, (GBR1a PCs – 30% of GC layer; GBR1b PCs – 555% of GC layer) though statistical analysis was not performed ($n=1$; see Figure 5). Expression levels of both GBR1a and GBR1b in the molecular layer were equivalent to background in the human cerebellum, and in the rat cerebellum they were just above background (GBR1a 12 ± 12 grains 1000 μm^{-2}; GBR1b 67 ± 17 grains 1000 μm^{-2}; and see Figures 2 and 4).

Discussion

In this study we have confirmed, using a high affinity antagonist radioligand, previous reports that the molecular layer of the cerebellar cortex possesses the majority of GABA$_B$

receptors present in this brain structure in both rats and humans (Wilkin *et al*., 1981; Bowery *et al*., 1987; Albin & Gilman, 1990; Chu *et al*., 1990). These studies could not, however, ascertain the cellular location of GABA$_B$ receptors in the cerebellum. Turgeon & Albin (1993), attempted to clarify the situation using three types of cerebellar lesion: stumbler mice (reduced numbers of PCs and GCs); methyl azoxymethanol (MAM) injected rats (reduced GCs and interneurones) and 3-acetylpyridine (3-AP) (destruction of the inferior olive and climbing fibre input to cerebellum), and examining cerebellar GABA$_B$ receptors using receptor autoradiography. Their data show that PC loss induces the largest deficit in GABA$_B$ binding in the molecular layer, neither MAM nor 3-AP lesioned rats showed a significant decrease. This does not rule out the existence of GABA$_B$ receptors on parallel and climbing fibre terminals, however it does support the notion that GABA$_B$ receptors are located on the PC dendrites (Bowery *et al*., 1983), in the molecular layer, as demonstrated in this study.

The non-splice variant specific GBR1pan oligonucleotide generated images indicate a high level of GBR1 mRNA expression in PCs, with moderate expression in GCs, the same regional distribution of GBR1 mRNA in rat cerebellum as demonstrated with riboprobes by Kaupmann *et al*. (1997). This finding validates the use of oligonucleotides rather than riboprobes, and provides confirmation of the specificity of the GBR1pan oligonucleotide in this study. The same difference in expression between PCs and GCs was also found to occur in human cerebellum, implying at least a similar role for GABA$_B$ receptors in human cerebellar neurotransmission.

The high level of expression of GBR1a in GCs shown in this study suggests that GBR1a mRNA may be responsible for the GABA$_B$ protein reported to be present on GC parallel fibre terminals, which are located in the molecular layer (Wojcik & Neff, 1984). It is also possible that some of this expression is due to Golgi cells present in the GC layer, although GC expression would be the greater, simply due to the large number of cells. A low level of expression of GBR1a was seen in PCs (Figures 3 and 5), which may account for pre-synaptic GABA$_B$ receptors reported to be located on the terminals of PC axons in the deep cerebellar nuclei (DCN; Mouginot & Gähwiler, 1996). These pre-synaptic GABA$_B$ receptors have not however been shown to be activated by endogenous GABA (Morishita & Sastry, 1995).

A proportion of GABA$_B$ receptors in the molecular layer of the cerebellum have been shown to be located on PC dendrites (Bowery *et al*., 1983; Turgeon & Albin, 1993). The hyperpolarizing action of baclofen on PCs is well established (Curtis *et al*., 1974), and more recently these dendritic GABA$_B$ receptors have been shown to be post-synaptic in nature (Vigot & Batini, 1997). Therefore, the high level of expression of the splice variant GBR1b in PCs, may suggest that GBR1b encodes a post-synaptic GABA$_B$ protein expressed on PC dendrites. The weak expression of GBR1b in the GC layer could be accounted for by regulation of GC firing by Golgi cells, and hence a post-synaptic GABA$_B$ receptor presence on granule cell bodies. Alternatively, GBR1b may be located, in part, over non-neuronal elements, as there is evidence for the existence of GABA$_B$ receptors on cultured astrocytes (Hösli & Hösli, 1990).

Low levels of expression of both GBR1a and GBR1b mRNA in the molecular layer may be explained by the presence of GABAergic interneurones such as basket and stellate cells, which are present in this layer. Their role is to regulate the excitability of PCs, and interneurone-interneurone connections are also possible. If GBR1a is indeed associated with a pre-synaptic role in this brain region, it may encode these receptors regulating the release of GABA from interneurone terminals. Following this logic, interneurone-interneurone innervation could account for post-synaptic GABA$_B$ receptors, and hence the expression of GBR1b.

The postulated separation of pre- and post-synaptic GABA$_B$ effects has possible therapeutic implications, particularly as the GBR1a/GBR1b mRNA separation was evident in human as well as rat tissue. Obviously, selective compounds would need to be developed to utilize any differences in synaptic location, and this could generate far reaching possibilities. Selective post-synaptic antagonists would be potentially beneficial in absence epilepsy, where the GABA$_B$ mediated IPSP activates low-threshold Ca^{2+} channels, which leads to burst firing and oscillatory behaviour in thalamic neurones (Crunelli & Leresche, 1991; Snead, 1995). Regulation of neurotransmitter release at nerve terminals by pre-synaptic GABA$_B$ receptors is complicated by the existence of GABA$_B$ auto- and hetero-receptors (Bonanno & Raiteri, 1993). GABA$_B$ hetero-receptor antagonists could have potential benefits in convulsive epilepsy, depression and pain.

In summary, this study has demonstrated a clear separation of GBR1a and GBR1b splice variant mRNA distribution in rat and human cerebellar cortex. This may have implications towards the association of GBR1a with a putative pre-synaptic GABA$_B$ protein present on GC parallel fibre terminals, and GBR1b with a putative post-synaptic GABA$_B$ protein present on PC dendrites. However, at present the evidence for this is purely anatomical, and experiments are underway in our laboratory to functionally characterize GBR1a and GBR1b expression in cerebellar neurones. The association of GBR1a and GBR1b with pre- and post-synaptic elements would be further resolved by the development of splice variant-specific antibodies, along with subtype specific ligands, which will undoubtedly be vital steps in the evolution of GABA$_B$ receptor pharmacology, and in the validation of GABA$_B$ receptors as a potential therapeutic target in the future.

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