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Biochemical Pharmacology 65 (2003) 1103–1113
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Pharmacological characterisation of a cell line expressing $GABA_{B1b}$ and $GABA_{B2}$ receptor subunits

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Received 25 September 2002; accepted 28 November 2002

Abstract

The γ-aminobutyric acid ($GABA_B$) receptor has been shown to be a heterodimer consisting of two receptor subunits, $GABA_{B1}$ and $GABA_{B2}$. We have stably co-expressed these two subunits in a CHO cell line, characterised its pharmacology and compared it to the native receptor in rat brain membranes. Radioligand binding using [^{3}H]CGP54626A demonstrated a similar rank order of potency between recombinant and native receptors: CGP62349 > CGP54626A > SCH 50911 > 3-aminopropylphosphinicacid (3-APPA) > GABA > baclofen > saclofen > phaclofen. However, differences were observed in the affinity of agonists, which were higher at the native receptor, suggesting that in the recombinant system a large number of the receptors were in the low agonist affinity state. In contrast, [^{35}S]GTPγS binding studies did not show any differences between recombinant and native receptors with the full agonists GABA and 3-APPA. Measurement of cAMP accumulation in the cells revealed a degree of endogenous coupling of the receptors to G-proteins. This is most likely to be due to the high expression levels of receptors (B_{max} = 22.5 ± 2.5 pmol/mg protein) in this experimental system. There was no evidence of $GABA_{B2}$ receptors, when expressed alone, binding [^{3}H]CGP54626A, [^{3}H]GABA, [^{3}H]3-APPA nor of GABA having any effect on basal [^{35}S]GTPγS binding or cAMP levels.

Keywords: GABA; Baclofen; $GABA_B$ receptor; Radioligand binding; [^{35}S]GTPγS binding; cAMP

1. Introduction

GABA is the major inhibitory neurotransmitter in the mammalian CNS. GABA mediates its effects through ligand-gated chloride channels ($GABA_{A/C}$ receptors) to produce fast synaptic inhibition (reviewed by [1]) and metabotropic $GABA_B$ receptors to produce slow, prolonged inhibitory signals [2–4]. $GABA_B$ receptors are thought to be involved in a number of physiological and disease processes, including nociception, cognition, epilepsy, depression and drug addiction [3,5].

The $GABA_B$ receptor was first identified pharmacologically as a bicuculline-insensitive binding site with affinity for both baclofen and GABA [6–8]. $GABA_B$ receptor activation inhibits adenylyl cyclase activity and causes prolonged synaptic inhibition through restriction of presynaptic calcium channel activity and activation of postsynaptic potassium channels [3,5,9,10]. These effects are inhibited by pertussis toxin suggesting the involvement of G_i and G_o proteins, although selective antisense oligonucleotide studies suggest a greater role for the G_o protein [9]. Native receptors have been shown to be activated by baclofen [6] and selectively antagonised by phaclofen and saclofen [11]. The discovery of more potent and selective antagonists [12–14] and the subsequent radiolabelling of two of these compounds, [^{125}I]CGP64213 and [^{125}I]CGP71872, enabled the expression cloning of two $GABA_{B1}$ receptor splice variants, $GABA_{B1a}$ and $GABA_{B1b}$ [15]. Although this receptor displayed many of the antago-

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Abbreviations: GABA, γ-aminobutyric acid; 3-APPA, 3-aminopropylphosphinic acid.

0006-2952/03/$ – see front matter
doi:10.1016/S0006-2952(02)01658-1

nist binding characteristics of the native $GABA_B$ receptor, it did not couple efficiently to adenylyl cyclase or potassium channels [15]. Further studies suggested that the lack of functional coupling to a wide variety of effector pathways was due to a lack of cell surface expression of the $GABA_{B1a}$ and $GABA_{B1b}$ proteins [16,17]. A $GABA_{B2}$ subunit, with 35% sequence homology and 54% sequence similarity to the $GABA_{B1}$ subunits, was subsequently identified and studies demonstrated the functional requirement of heterodimerisation between the $GABA_{B1}$ and $GABA_{B2}$ receptor subunits (reviewed in [18]). The heterodimerisation of $GABA_{B1}$ and $GABA_{B2}$ receptor subunits occurs, at least in part, by interactions between homologous α-helical coiled-coil domains present in the intracellular C-termini of both $GABA_{B1}$ and $GABA_{B2}$ [19–21]. *In situ* hybridisation, immunohistochemical and autoradiographic studies have shown that the $GABA_B$ receptors are distributed throughout the brain. Intriguingly, there are many reports of differential expression patterns, not only between the $GABA_{B1}$ splice [22–24] but also between the $GABA_{B1}$ and $GABA_{B2}$ receptor subunits [25,26] where, for example, in the striatum $GABA_{B2}$ mRNA and protein are barely detectable but there are abundant levels of the $GABA_{B1}$ receptor subunits. These observations, together with previous studies [27,28] have led to speculation of the existence of pharmacologically distinct receptor subtypes. However, recent reports on a $GABA_{B1}$ receptor knockout demonstrate that this subunit is essential for all $GABA_B$ receptor-mediated mechanisms and cast doubt on the existence of non-$GABA_{B1}$-based subtypes [29,30].

We have generated a Chinese Hamster Ovary (CHO) cell line stably expressing the $GABA_{B1b}$ and $GABA_{B2}$ receptor subunits which will be used both in experiments to further our understanding of $GABA_B$ receptor biology and particularly in the drug discovery process to identify novel compounds acting at these receptors. In this study, we extend previously reported data describing the ligand binding characteristics of recombinant heterodimeric $GABA_B$ receptors [31] to focus on the functional characterisation of a recombinant cell line $GABA_B$ receptor subunits and compare its pharmacology to that of native $GABA_B$ receptors in rat cerebellar membranes. A preliminary account of the data presented here has been published in abstract form [32].

2. Materials and methods

2.1. Materials

GABA, (*R*,*S*)-baclofen, GTPγS and GDP were purchased from Sigma. [*S*-(*R**,*R**)]-[3-[[1-(3,4-Dichlorophenyl)ethyl]amino]-2-hydroxypropyl]-(cyclohexylmethyl)phosphinic acid (CGP54626A), saclofen and phaclofen were purchased from Tocris-Cookson. 3-APPA, SKF-97530 and CGP27492 and 3-[1-(*R*)-[[2(*S*)-hydroxy-3-[hydroxy[(4-methoxyphenylmethyl)]phosphinyl]propyl]amino]-ethyl]-benzoic acid (CGP62349) were manufactured by GlaxoSmithKline. All cell culture reagents were from Life Technologies. HEPES, EDTA, Tris–HCl and all other buffer constituents were purchased from Merck-BDH or Sigma. [^{3}H]CGP-54626A (40 Ci/mmol) was purchased from Tocris-Cookson and [^{35}S]GTPγS (1123 Ci/mmol) and wheatgerm agglutinin SPA beads were purchased from Amersham-Pharmacia. Cyclic AMP (cAMP) flashplate assays (SMP 004) were from NEN Life Science Products.

2.2. Generation of a stable cell line

CHO DG44 cells, maintained in complete medium, comprising alpha MEM with nucleosides/ribonucleosides plus 10% foetal bovine serum and 2 mM L-glutamine, were transfected using Lipofectamine Plus, according to the manufacturers guidelines, with equivalent amounts of human $GABA_{B1b}$-*myc* and $GABA_{B2}$ cDNA in pcDNA3.1-neo and pcDNA3.1-hygro (InVitrogen), respectively.

Forty-eight hour post-transfection, cells were placed into complete medium containing the selection agents geneticin, 800 μg/mL, and hygromycin, 400 μg/mL. After 10-day selection the polyclonal cultures were detached using versene (1:5000), spun at 140 *g* and re-suspended in MEM without phenol red with 3% foetal clone II (Hyclone) containing FITC-labelled anti-c-*myc* monoclonal antibody, 0.2 μg/10^5 cells (Santa Cruz). Cells were incubated on ice for 60 min, washed twice in the same medium, re-suspended and filtered (50 μm filcon; Becton Dickinson). Dead cells were excluded using propidium iodide. Single cells were sorted into 96-well plates by flow cytometry using an FACS Vantage with ACDU (Becton Dickinson). Twenty-one clones were selected, expanded and screened by [^{3}H]CGP54626A radioligand binding (as described later). This led to the selection of one clone, named 1E4. This selected cell line was maintained in complete medium plus selection agents and passaged twice weekly. A $GABA_{B2}$ cell line was generated as described earlier, except that a mass culture was used. All cell culture and transfection reagents were purchased from Life Technologies.

2.3. Preparation of cell membranes

To prepare membranes for radioligand binding, cells were homogenised (3% v/v) in 50 mM Tris–HCl (pH 7.4; 4°) and centrifuged at 48,000 *g* for 10 min at 4°. The supernatant was discarded and the pellet re-suspended in (3% v/v) in 50 mM Tris–HCl (pH 7.4; 4°) and homogenised. The centrifugation, re-suspension and re-homogenisation processes were repeated twice more. Membrane preparations were then aliquoted and frozen at −80°.

To prepare membranes for [^{35}S]GTPγS binding assays, cells were homogenised (3% v/v) in 20 mM HEPES, 10 mM EDTA (pH 7.4; 4°), and centrifuged at 48,000 *g* for 20 min at 4°. The supernatant was discarded and the pellet re-homogenised in (3% v/v) 20 mM HEPES,

0.1 mM EDTA (pH 7.4; 4°). The pellet was re-centrifuged as described earlier, the supernatant discarded and the pellet re-suspended in (10% v/v) 20 mM HEPES, 0.1 mM EDTA (pH 7.4; 4°) and re-homogenised before being aliquoted and frozen at −80°.

For rat brain membranes, cerebella from male Sprague–Dawley rats were removed and homogenised, using an Ultra Turrax homogeniser, in 30 mM Tris–HCl, 0.5 mM EDTA buffer (containing Complete-TM protease inhibitor cocktail tablets, Boehringer) and then centrifuged at 30,000 *g* for 20 min. Pellets were washed, homogenised and centrifuged a further three times followed by an incubation of the homogenate at 37° for 30 min to reduce the levels of endogenous GABA by enzymatic degradation. Three more washing steps were performed before the pellet was finally re-suspended in 30 mM Tris–HCl and stored at −80° until required.

2.4. Radioligand binding assays

Cell membranes (approximately 10 μg protein/well) were incubated with [^{3}H]CGP54626A in a 50 mM Tris–HCl, 2.5 mM $MgCl_2$ (pH 7.4) buffer for 45 min at 23°. Non-specific binding was measured in the presence of 1 mM GABA. Incubations were terminated by filtration through GF/B filters, pre-soaked in 0.3% polyethyleneimine (PEI), and washed with 6 mL of ice-cold 50 mM Tris–HCl, 2.5 mM $MgCl_2$ (pH 7.4) buffer using a 48-well Brandel cell harvester. Radioactivity was determined by liquid scintillation spectrometry using a Canberra-Packard 2700 liquid scintillation counter.

In competition binding experiments, 10 concentrations of the competing ligands were used with a final [^{3}H]CGP-54626A concentration of 3 nM. In saturation binding studies eight concentrations of [^{3}H]CGP54626A were used (final concentrations of approximately 0.2–45 nM rat cerebellum membranes or 0.2–79 nM 1E4 cell membranes).

Cell membranes (approximately 50 μg protein/well) were incubated with 10 nM [^{3}H]GABA or [^{3}H]3-APPA for 25 min at room temperature. Non-specific binding was determined using 1 mM GABA. Experiments were terminated by centrifugation at 20,000 *g*. The supernatant was aspirated off and the pellet was washed briefly in cold buffer and then dissolved in 0.5 mL of soluene (Canberra-Packard), the contents were than transferred to a scintillation vial containing 4 mL of Ultima Gold MV and the tritium content estimated by liquid scintillation spectrometry using a Canberra-Packard 2700 liquid scintillation counter.

2.5. [^{35}S]GTPγS binding assays

Cell membranes expressing recombinant $GABA_{B1b}$ and $GABA_{B2}$ receptor subunits or $GABA_{B2}$ receptor subunits alone were re-constituted in a 20 mM HEPES buffer (pH 7.4) containing 3 mM $MgCl_2$ and 100 mM NaCl, and homogenised to produce a solution containing approximately 1×10^6 cells/mL (40 μg protein/well). Cell membranes were incubated with 10 μM GDP, and either increasing concentrations of agonist alone or antagonist in the presence of 100 μM GABA. Following a 30-min pre-incubation at 30° in a 20 mM HEPES buffer (pH 7.4) containing 3 mM $MgCl_2$ and 100 mM NaCl, 0.1 nM [^{35}S]GTPγS was added to each well and incubated for a further 30 min at 30°. Non-specific binding was determined in the presence of 20 μM GTPγS. The incubation was terminated by filtration through GF/B filter plates on a 96-well Brandel cell harvester and the membranes were washed with 3 mL of ice-cold HEPES buffer (pH 7.4) containing 3 mM $MgCl_2$, left to dry before 40 μL of Microscint 20 was added to each well and counted on a Canberra-Packard TopCount system.

Membranes from rat cerebella (10 μg protein/well) were diluted to 0.083 mg/mL in assay buffer (20 mM HEPES, 100 mM NaCl, 10 mM $MgCl_2$, pH 7.4) supplemented with saponin (10 mg/L) and pre-incubated with 40 μM GDP together with either increasing concentrations of agonist alone or antagonist in the presence of 30 μM GABA. [^{35}S]GTPγS at 0.3 nM was added (total volume of 100 μL) and binding was allowed to proceed at room temperature for 30 min. Non-specific binding was determined by the inclusion of 0.6 mM GTPγS. Wheatgerm agglutinin SPA beads (Amersham-Pharmacia) (0.5 mg) in 25 μL assay buffer were added and incubated at room temperature for 30 min with agitation. Plates were centrifuged at 1500 *g* for 5 min and bound [^{35}S]GTPγS was determined by scintillation counting on a Wallac 1450 microbeta Trilux scintillation counter.

2.6. cAMP accumulation assays

cAMP levels in cells were determined by radioimmunoasay (NEN, SMP004) following the manufacturer's guidelines. In brief, cells were washed once with Ca^{2+}-free PBS, scraped up in the same buffer and centrifuged at 400 *g* for 5 min at 21°. The supernatant was discarded and the pellet re-suspended in manufacturer's stimulation buffer and 50,000 cells were added to the appropriate wells of the NEN flashplates together with 10 μM forskolin and increasing concentration of agonist. Apparent pK_B values were determined for the antagonists in the presence of 10 μM forskolin, 1 μM GABA plus increasing concentration of antagonist. Plates were incubated for 15 min at 37°, before the addition of the manufacturer's detection mixture containing [^{125}I]cAMP-tracer (0.16 μCi/mL) to the wells. Plates were covered and left for 12 hr prior to counting on a Packard TopCount.

2.7. Bradford protein assay

Protein concentrations were determined using the Bradford assay method (Bio-Rad protein assay kit) using BSA as a standard.

2.8. Data analysis

In saturation binding studies, K_D and B_{max} values were calculated using Radlig and LIGAND [33,34]. The concentration of drug inhibiting specific radioligand binding by 50% (IC_{50}) was determined by iterative curve fitting [35] pK_i values (the negative $\log_{10}$ of the molar K_i) for receptor binding were then calculated from the IC_{50} values as described by Cheng and Prusoff [36] using the K_D values determined in the saturation binding studies.

[^{35}S]GTPγS binding and cAMP accumulation data were generated in duplicate within each experiment. Each experiment was carried out at least three times. Curve fitting of the mean data was generated by a four parameter logistic equation, using GRAFIT. The pEC_{50} were calculated from the determined EC_{50} values (the negative $\log_{10}$ of the molar EC_{50}). Apparent pK_B determinations were made using the Gaddum equation, using concentration ratios calculated from the EC_{50} from each individual curve.

3. Results

3.1. Radioligand binding studies

Saturation analysis of [^{3}H]CGP54626A binding to CHO cells stably transfected with the $GABA_{B1b}$ and $GABA_{B2}$ subunits (1E4 cell line) revealed a single binding site (Fig. 1A) with a K_D of 4.2 ± 0.8 nM and a B_{max} of 22.5 ± 2.5 pmol/mg protein. No specific binding of any of the radioligands used was found in cells transfected with only the $GABA_{B2}$ subunit (data not shown). At a radioligand concentration of 3 nM, the concentration used in the competition binding experiments, specific binding represented $91 \pm 0.2\%$ of total binding. Fig. 1B shows representative saturation curves and Scatchard analyses of specific [^{3}H]CGP54626A binding to rat cerebellum membranes, this revealed a single binding site with a K_D of 2.3 ± 0.4 nM and a B_{max} of 1.95 ± 0.2 pmol/mg protein. At a radioligand concentration of 3 nM, specific binding represented $62.2 \pm 3.8\%$ of total binding.

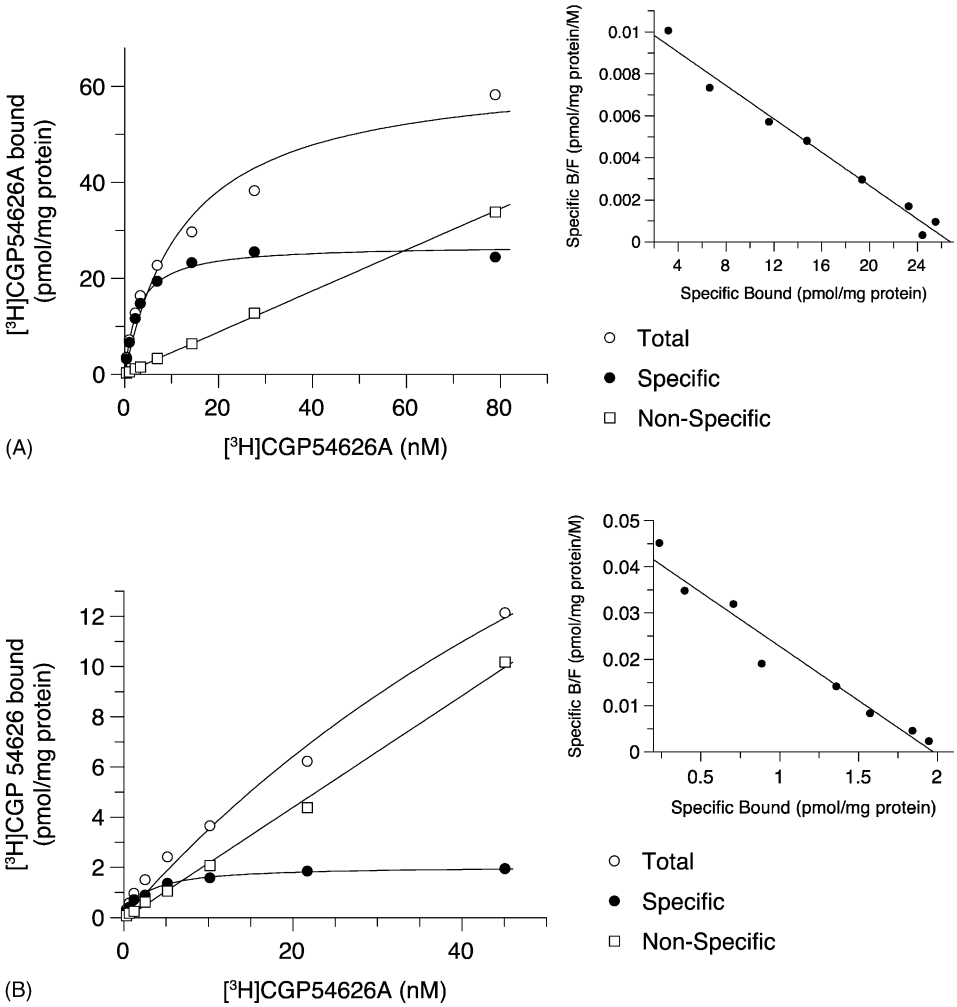

Fig. 1. Saturation and Scatchard analysis (insets) of [^{3}H]CGP54626A binding to (A) cells co-expressing the $GABA_{B1b}$ and $GABA_{B2}$ receptor subunits and (B) rat cerebellar membranes. Membranes were incubated with 0.2–79 nM (cells) or 0.2–45 nM (tissue) [^{3}H]CGP54626A for 45 min at 37°. Non-specific binding was determined in the presence of 1 mM GABA. The data shown are from one of four experiments, each performed in triplicate.

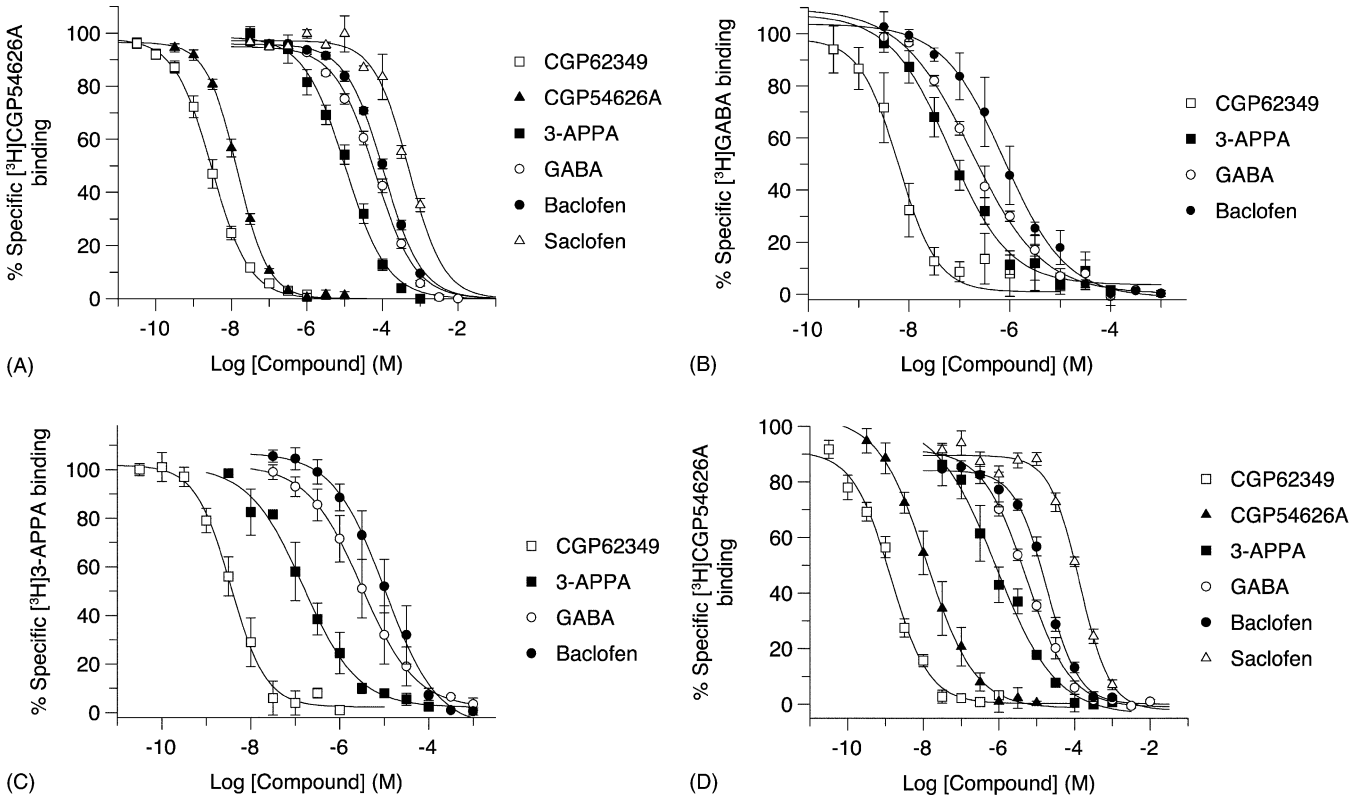

Fig. 2. Pharmacological profile of (A) [^{3}H]CGP54626A, (B) [^{3}H]GABA, (C) [^{3}H]3-APPA binding to cells co-expressing the $GABA_{B1b}$ and $GABA_{B2}$ receptor subunits and (D) [^{3}H]CGP54626A binding to rat cerebellar membranes. Competition experiments with a selection of agonists and antagonists are shown, curves have been constrained to zero where there were not sufficient data to clearly define the bottom of the curve. Data points represent the means ± SEM of three to seven independent experiments. Mean pK_i and SEM for all compounds tested are given in Table 1.

Competition binding analysis was used to characterise the binding properties of the 1E4 cell line, and to compare pK_i values of numerous $GABA_B$ agonists and antagonists with those obtained in rat cerebellum membranes (Fig. 2, Table 1). A comparison of the affinity of previously characterised $GABA_B$ agonists and antagonists to compete with [^{3}H]CGP54626A, [^{3}H]GABA and [^{3}H]3-APPA binding to cells expressing the $GABA_{B1b}$ and $GABA_{B2}$ subunits is shown in Fig. 2A–C and Table 1. Only a selection of the full complement of compounds was used in the profile of [^{3}H]GABA and [^{3}H]3-APPA binding. The rank order of affinity to inhibit [^{3}H]CGP54626A binding was: CGP62349 > CGP54626A > SCH 50911 > 3-APPA > GABA > baclofen > saclofen > phaclofen. This rank order of potency was the same in both [^{3}H]GABA and [^{3}H]3-APPA binding, allowing for the omission of selective compounds. However, the affinity of agonists when using these radioligands are considerably higher than those obtained for the same compounds when [^{3}H]CGP54626A was used as a radioligand.

The same compounds were used to profile the radioligand binding of [^{3}H]CGP54626A in native tissue. The pK_i rank order of potency at the rat cerebellum membranes was: CGP62349 > CGP54626A > 3-APPA > SCH 50911 >

Table 1
Pharmacological profile of [^{3}H]CGP54626A in rat cerebellum membranes and [^{3}H]CGP54626A, [^{3}H]GABA and [^{3}H]3-APPA in the 1E4 cell line

Compounds	[^{3}H]CGP54626A rat cerebellum membranes	[^{3}H]CGP54626A 1E4 cell line	[^{3}H]GABA 1E4 cell line	[^{3}H]3-APPA 1E4 cell line
GABA	5.71 ± 0.05	4.47 ± 0.09	6.69 ± 0.11	5.71 ± 0.38
Baclofen	5.12 ± 0.07	4.28 ± 0.05	6.25 ± 0.25	5.14 ± 0.44
3-APPA	6.44 ± 0.11	5.23 ± 0.08	7.25 ± 0.18	7.02 ± 0.11
CGP62349	9.31 ± 0.04	8.86 ± 0.11	8.50 ± 0.14	8.56 ± 0.13
CGP54626A	8.35 ± 0.19	8.15 ± 0.06	n.d.	n.d.
SCH 50911	6.32 ± 0.10	5.97 ± 0.03	n.d.	n.d.
Saclofen	4.35 ± 0.05	3.70 ± 0.06	n.d.	n.d.
Phaclofen	<3.5	<3.5	n.d	n.d.

Data are means ± SEM from three to seven experiments.

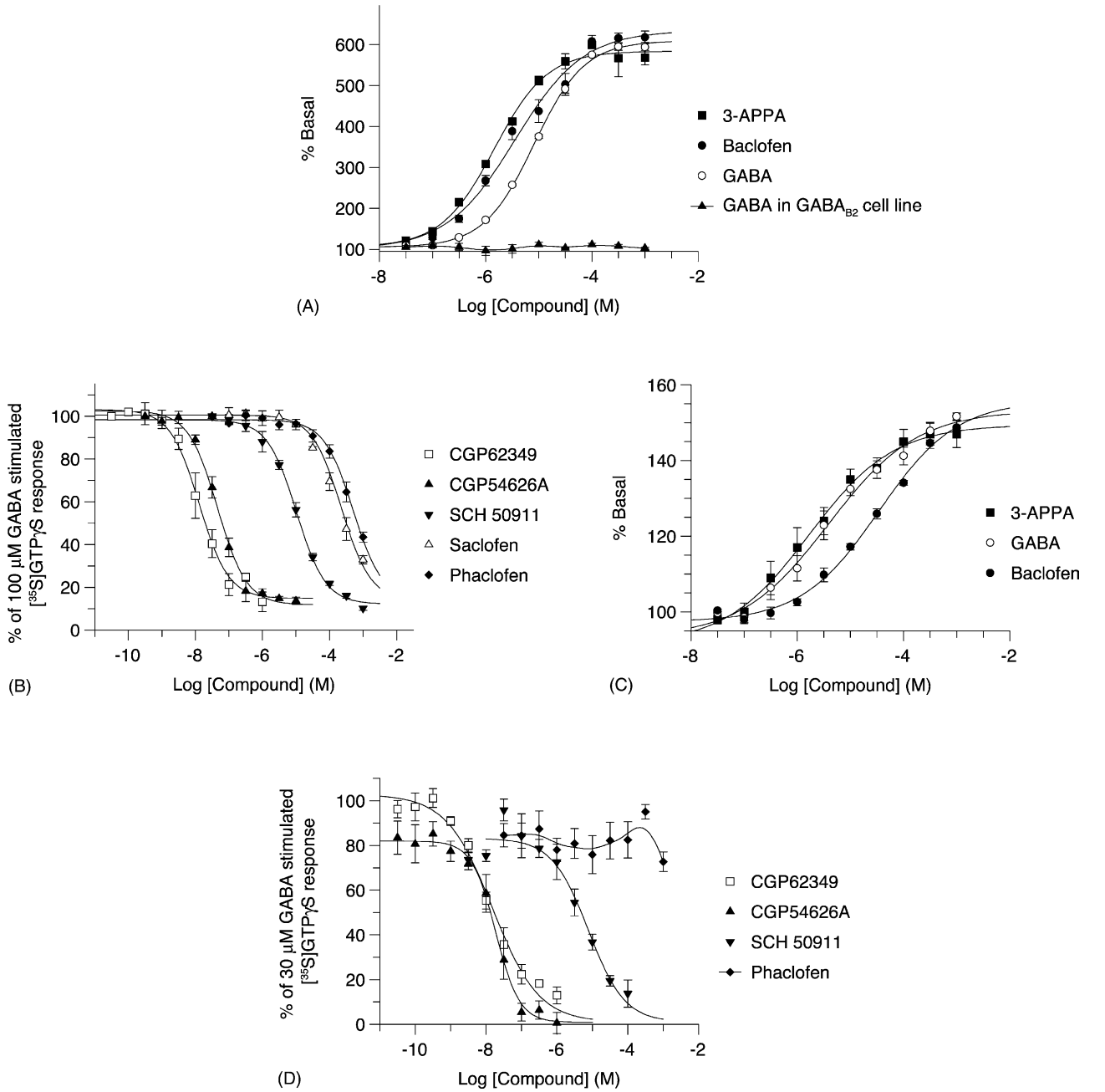

Fig. 3. [^{35}S]GTPγS binding. (A) GABA, baclofen and 3-APPA all stimulated [^{35}S]GTPγS binding in cells co-expressing the $GABA_{B1b}$ and $GABA_{B2}$ receptor subunits and cells expressing the $GABA_{B2}$ receptor subunit alone, specific basal binding was 5555 ± 788 cpm. (B) A range of antagonists inhibited 100 µM GABA-stimulated [^{35}S]GTPγS binding in the 1E4 cells. (C) Similar experiments were performed using rat cerebellar membranes incubated with agonists, specific basal binding was 6990 ± 480 cpm and (D) antagonists, in the presence of 30 µM GABA. Data points represent the means ± SEM of three to six independent experiments. Mean pEC_{50} and apparent pK_B values are given in Table 2.

GABA > baclofen > saclofen > phaclofen. Antagonist pK_i values were similar to those obtained in the cell line (Table 1). However, agonist potencies differed by approximately 1 log unit.

3.2. [^{35}S]GTPγS binding

In the CHO cells stably transfected with the $GABA_{B1b}$ and $GABA_{B2}$ subunits, GABA, baclofen and 3-APPA all produced a robust, concentration-dependent stimulation of [^{35}S]GTPγS binding, with maximal stimulation of approximately 600% of basal levels (Fig. 3A). No agonist stimulation of [^{35}S]GTPγS binding was seen in cells transfected with the $GABA_{B2}$ subunit alone (Fig. 3A). The rank order of potency in this functional binding assay was: 3-APPA > baclofen > GABA; the pEC_{50} values for these compounds are shown in Table 2. The profile of antagonists in this assay was established by comparing the inhibition of a submaximal (100 µM) concentration of GABA elicited by a range of compounds (Fig. 3B). The rank order of

Table 2
Pharmacological profile of the effects of agonists and antagonists (presence of 100 or 30 μM GABA) on [^{35}S]GTPγs binding in the 1E4 cell line and rat cerebellum tissue

Compounds	pEC_{50} 1E4 cell line	pEC_{50} rat cerebellum tissue	Apparent pK_B 1E4 cell line	Apparent pK_B rat cerebellum tissue
GABA	5.09 ± 0.01	5.55 ± 0.22	–	–
Baclofen	5.62 ± 0.21	4.47 ± 0.11	–	–
3-APPA	5.81 ± 0.15	6.00 ± 0.35	–	–
CGP62349	–	–	8.49 ± 0.06	8.99 ± 0.03
CGP54626A	–	–	7.83 ± 0.05	8.79 ± 0.07
SCH 50911	–	–	5.48 ± 0.09	6.36 ± 0.19
Saclofen	–	–	4.23 ± 0.08	<3.5
Phaclofen	–	–	3.82 ± 0.09	<3.5

Data are means ± SEM from three to seven experiments.

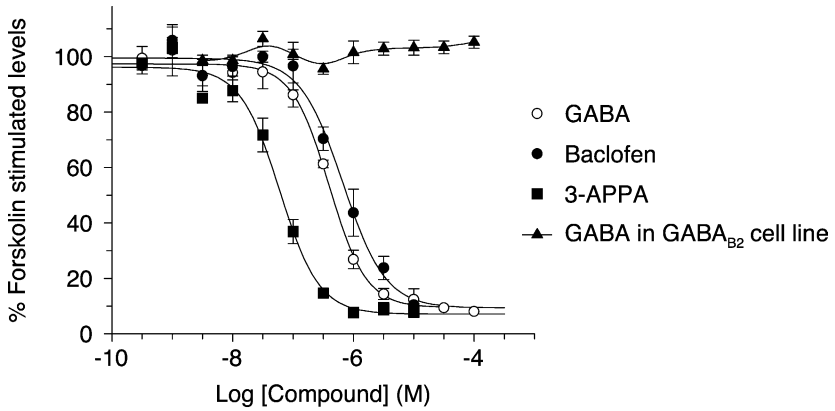

Fig. 4. Agonist profile of the inhibition of forskolin-stimulated cAMP levels in cells co-expressing the $GABA_{B1b}$ and $GABA_{B2}$ receptor subunits and cells expressing the $GABA_{B2}$ receptor subunit alone. Ten micromoles forskolin stimulated cAMP levels to 705 ± 64 pmol/mL from basal cAMP levels of 22.3 ± 2 pmol/mL. Data points represent the means ± SEM for 6–9 independent experiments, each performed in duplicate. Mean pEC_{50} values are given in Table 3.

potency was: CGP62349 > CGP54626A > SCH 50911 > phaclofen > saclofen (Table 2). At native $GABA_B$ receptors, in rat cerebellum membranes, 3-APPA, GABA and baclofen were all full agonists (Fig. 3C, Table 2). The rank order of potency for antagonists was unchanged except that neither phaclofen nor saclofen were able to completely inhibit the 30 μM GABA-stimulated [^{35}S]GTPγS binding (Fig. 3D, Table 2). Similar results were obtained for rat cerebral cortex membranes (data not shown).

3.3. cAMP accumulation assays

Forskolin (10 μM) stimulated cAMP levels, in the cells stably expressing the $GABA_{B1b}$ and $GABA_{B2}$ subunits, to 3160% of basal levels. GABA, baclofen and 3-APPA completely inhibited the forskolin-stimulated adenylyl cyclase activity in a concentration-dependent manner with pIC_{50} values of 6.37 ± 0.08, 6.12 ± 0.11 and 7.13 ± 0.07, respectively (Fig. 4). These responses were pertussis toxin sensitive, confirming the preferential coupling of these receptors through $G_{i/o}$ in this experimental system (data not shown). Neither GABA nor baclofen had any effect on either basal or forskolin-stimulated cAMP levels in cells transfected with the $GABA_{B2}$ subunit alone (Fig. 4). When a $GABA_B$ antagonist (CGP62349), was added to 1E4 cells together with 10 μM forskolin, an augmentation of the forskolin-stimulated adenylyl cyclase activity was observed (data not shown). The effects of antagonists were investigated using an EC_{70} concentration of GABA (1 μM) together with 10 μM forskolin. An attenuation of the GABA inhibition was observed (Fig. 5). The antagonists exhibited the expected rank order of potency: CGP62349 > CGP54626A > SCH50911 > phaclofen = saclofen, apparent pK_B values are shown in Table 3. Again, saclofen and phaclofen appeared inactive. As with the administration

Table 3
Pharmacological profile of the effects of agonists and antagonists (presence of 1 μM GABA) on the forskolin cAMP stimulation

Compounds	pEC_{50} 1E4 cell line	Apparent pK_B 1E4 cell line
GABA	6.37 ± 0.08	–
Baclofen	6.12 ± 0.11	–
3-APPA	7.13 ± 0.07	
CGP62349	–	8.48 ± 0.37
CGP54626A	–	7.13 ± 0.11
SCH 50911	–	5.08 ± 0.12
Saclofen	–	<3
Phaclofen	–	<3

Data are means ± SEM from 3 to 9 experiments.

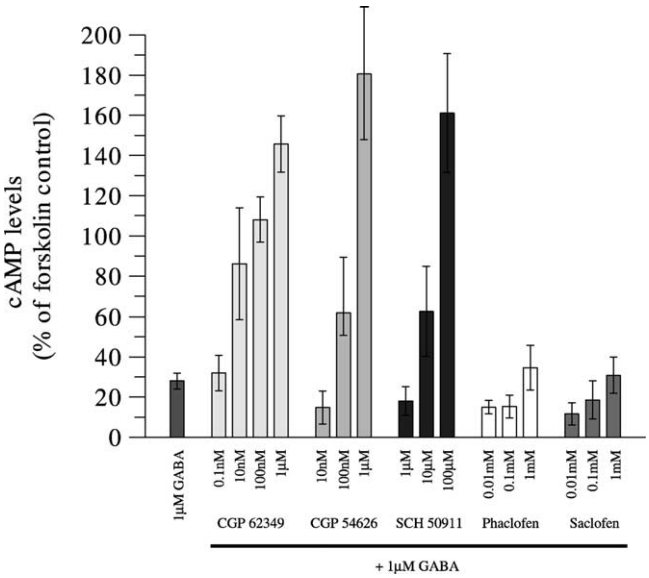

Fig. 5. Comparison of agonist and antagonist effect on the forskolin-stimulated cAMP levels in cells co-expressing the $GABA_{B1b}$ and $GABA_{B2}$ receptor subunits. The antagonists, CGP62349, CGP54626A and SCH 50911, not only reversed the EC_{70} (1 μM) effects of GABA but also, at the highest concentrations, potentiated the cAMP levels above the forskolin controls. This effect was not seen with phaclofen or saclofen. Data points represent the means ± SEM for 4–9 independent experiments, each performed in duplicate. Mean pEC_{50} and apparent pK_B values are expressed in Table 3.

of CGP62349 in the absence of 1 μM GABA an augmentation of the cAMP levels above the 100% forskolin levels was observed for CGP62349, CGP54626A and SCH 50911 (Fig. 5).

4. Discussion

We have previously described further two other stable cell lines in which the calcium sensing properties of the $GABA_B$ receptor were investigated [39]. We also reported that the ligand binding characteristics of each of the major isoforms of recombinantly expressed $GABA_B$ receptors were identical [31]. However, these studies were not extended to complete a thorough comparison with native receptors. The present study describes the pharmacological characterisation of a CHO DG44 cell line stably expressing the $GABA_{B1b}$ and $GABA_{B2}$ receptor subunits. Previous studies have also described cell lines generated with chimeric G-proteins which couple to phospholipase C and measured either phosphoinositide hydrolysis [37] or increases in intracellular Ca^{2+} [38].

The use of the new cell line reported herein has permitted a functional evaluation of native $GABA_B$ receptor pharmacology with recombinantly expressed receptors and includes comparative radioligand and [^{35}S]GTPγS binding studies. The importance of such a comparison between native and recombinant receptor pharmacology is particularly relevant in this receptor subclass, where two distinct subunits need to dimerise in order for receptors to reach the cell surface [16,19–21] and to function correctly [18]. The necessity for a detailed comparison is increased given the possible differences in regulation, expression and dimerisation between recombinant and native systems.

Saturation binding studies with the high affinity antagonist radioligand [^{3}H]CGP54626A [40] gave K_D values of 4.2 and 2.3 nM for recombinant and native receptors, similar to previously published figures [31,40]. In the competition binding assays with the recombinant receptors, using the high affinity $GABA_B$ receptor antagonist [^{3}H]CGP54626A or the agonists [^{3}H]GABA or [^{3}H]3-APPA, the rank order of potency of the $GABA_B$ agonists and antagonists was identical. However, the affinities of the agonists were increased by approximately 15–25-fold in the binding assays using [^{3}H]GABA or [^{3}H]3-APPA whereas the affinity of the antagonist, CGP62349, was unaffected. These differences can be explained by the existence of high and low agonist affinity states of the recombinant receptor as predicted in the ternary complex model of ligand–receptor–G-protein interactions (reviewed by [41,42]). This model predicts that all receptors will bind antagonists with the same affinity, whereas the affinity of agonists is dependent on the coupling of receptors to G-proteins, where in the case of $GABA_B$ receptors this requires dimerisation. In the present cell line, the receptor expression levels, as measured by [^{3}H]CGP54626A binding, are extremely high (B_{max} 22.5 pmol/mg protein) and consequently it is possible that the endogenous G-proteins, relative to receptor subunits, are likely to be limiting, resulting in a large number of receptors present in the low agonist affinity state.

It has also been demonstrated that heterodimeric $GABA_B$ receptors have a higher affinity for agonists ligands compared with monomeric $GABA_{B1b}$ subunits [18] whereas antagonist affinity is unaffected. An alternative explanation could be that in this particular recombinant cell line there may be an excess of monomeric $GABA_{B1b}$ subunits and hence when labelling receptors with agonist radioligands affinities for competing agonists would be higher when compared to using antagonist radioligands.

Competition binding experiments at the native receptor in rat cerebellar membranes yielded agonist pK_i values lower than those generated in the recombinant cell line using [^{3}H]GABA (see Table 1). This possibly indicates that not all the native $GABA_B$ receptors in the rat cerebellum are in the high affinity state or that not all exist as functional heterodimers. It could also be explained due to the presence of endogenous GABA in the membranes derived from rat cerebellum.

This interpretation is further corroborated by the [^{35}S]GTPγS binding data (a measure of functional heterodimeric receptors), where the pEC_{50} values of GABA and 3-APPA are virtually identical in both the cell line and in the native tissue (see Table 2). Furthermore, potencies are similar to the binding pK_i values generated using agonist

radioligands and affinities at the native receptors. This would be expected in a functional assay given that only the pool of heterodimeric receptors is contributing the $GABA_B$ receptor-mediated effect. Despite the differences highlighted earlier and with the exception of baclofen in the [^{35}S]GTPγS binding assay, the rank order of potency of all the compounds investigated was identical across the two techniques at both recombinant and native receptors. It is worth noting that in our hands both saclofen and phaclofen had much lower potencies than some previously published observations [11], but in agreement with our previous studies using another $GABA_B$ cell line [37].

The attenuation of forskolin-stimulated cAMP accumulation by the $GABA_B$ agonists showed the same rank order of potency as that demonstrated in the [^{3}H]CGP54626A competition binding studies, namely: 3-APPA > GABA > baclofen. Due to receptor reserve and the potential amplification in the second messenger pathway it is not surprising that the pEC_{50} values obtained for these agonists differed by up to two orders of magnitude when compared to the binding pK_i values for the antagonist radioligand. However, when the pEC_{50} values were compared to the pK_i values obtained for [^{3}H]GABA competition binding experiments the results obtained were almost identical further supporting the high agonist affinity state of coupled receptors. The pEC_{50} values from the present study in the $GABA_B$ cell line are analogous to previously published values in rat cortical slices [43].

An augmentation of maximal forskolin-stimulated cAMP accumulation in the cell line was detected in the presence of the $GABA_B$ antagonist CGP62349 and in the absence of agonist. Furthermore, this was also observed once the antagonists CGP54626A and SCH 50911 had reversed the GABA-attenuated decrease in forskolin-stimulated cAMP accumulation. This is most likely to be a consequence of endogenous coupling of the receptor G-protein to adenylyl cyclase, resulting in a basal amount of inhibitory tone in the system. An alternative explanation could be receptor activation by endogenous GABA, however, we failed to detect any GABA in the cell culture media used (data not shown). Receptor occupancy by the antagonist uncouples this complex allowing the forskolin present to stimulate the released adenylate cyclase causing an augmentation of the previously maximal effect. This is possible due to the use of a supramaximal concentration of forskolin being applied to the cells as a stimulator of cAMP formation. The antagonists reversed the effects of an EC_{70} concentration of GABA with the rank order of potency for CGP62349, CGP54626A and SCH 50911 identical to that seen in competition binding studies and [^{35}S]GTPγS binding studies. Data obtained for both saclofen and phaclofen continued to highlight their reduced potency as antagonists at $GABA_B$ receptors; both antagonised the GABA attenuation to such a minor extent that no apparent pK_B could be calculated ($pK_B < 3$). Constitutive activity of numerous other receptors expressed in recombinant systems has previously been shown [44–46]. According to the allosteric ternary complex model all receptors undergo an activating conformational change which is not only induced by ligand binding, but can also occur spontaneously [47]. Hence, all receptors possess some degree of constitutive activity, depending on the ease with which they attain the active conformation in the absence of agonist and the propensity of the activated receptor to bind the G-protein. This model also appears to apply to the present, more complicated, system where not only the receptors subunits need to dimerise but recent data from our group has shown that the $GABA_{B1}$ subunit binds agonists but only the $GABA_{B2}$ subunit interacts with the G-protein [48]. It is worth noting that, despite the high levels of receptor expression in the present system, endogenous coupling was only detected in the cAMP assays and not in [^{35}S]GTPγS binding assays (data not shown) suggesting that a relatively small number of the receptors were endogenously coupled. [^{35}S]GTPγS binding also is a direct measure of receptor activation compared with cAMP second messenger assays which capture receptor activation further down the signalling cascade and is more likely to be influenced by signal amplification.

Two early reports suggested that $GABA_{B2}$ subunits expressed alone were capable of producing a $GABA_B$ agonist attenuation of forskolin-stimulated cAMP accumulation [49,50]. The data we have presented herein does not support this hypothesis. In cells where $GABA_{B2}$ receptor subunits are expressed alone, $GABA_B$ agonists had no effect on forskolin-stimulated cAMP accumulation, failed to cause detectable stimulation of [^{35}S]GTPγS binding and did not alter specific binding of [^{3}H]CGP54626A, [^{3}H]GABA or [^{3}H]3-APPA. This adds to the growing body of evidence indicating that the $GABA_{B2}$ receptor subunit cannot function as a receptor for GABA in its own right [30,31].

In conclusion, we have characterised a CHO cell line stably expressing the $GABA_{B1b}$ and $GABA_{B2}$ receptor subunits. The expressed receptors show both binding and functional characteristics similar to those of the native receptor. This particular heterodimeric $GABA_B$ receptor also displays many of the functional characteristics of other G-protein-coupled receptors in its ability to assume high and low affinity states and exhibit constitutive activity. Crucially, this recombinant line can be used not only for the screening of new $GABA_B$ ligands but also in experiments to further our understanding of $GABA_B$ receptor biology.

References

[1] Mehta AK, Ticku MK. An update on $GABA_A$ receptors. Brain Res Rev 1999;29:196–217.
[2] Bowery NG. $GABA_B$ receptor pharmacology. Annu Rev Pharmacol Toxicol 1993;33:109–47.
[3] Couve A, Moss SJ, Pangalos MN. $GABA_B$ receptors: a new paradigm in G protein signaling. Mol Cell Neurosci 2000;16:296–312.
[4] Mott DD, Lewis DV. The pharmacology and function of central $GABA_B$ receptors. Int Rev Neurobiol 1994;36:97–223.

[5] Bettler B, Kaupmann K, Bowery NG. $GABA_B$ receptors: drugs meet clones. Curr Opin Neurobiol 1998;8:345–50.
[6] Bowery NG, Hill DR, Hudson AL, Doble A, Middlemiss DN, Shaw J, Turnbull M. (−) Baclofen decreases neurotransmitter release in the mammalian CNS by an action at a novel GABA receptor. Nature 1980;283:92–4.
[7] Hill DR, Bowery NG. [^{3}H]-Baclofen and [^{3}H]-GABA bind to bicuculline-insensitive GABA B sites in rat brain. Nature 1981;290:149–52.
[8] Bowery NG, Hill DR, Hudson AL. Characteristics of $GABA_B$ receptor binding sites on rat whole brain synaptic membranes. Br J Pharmacol 1983;78:191–206.
[9] Kerr DI, Ong J. $GABA_B$ receptors. Pharmacol Ther 1995;67:187–246.
[10] Bowery NG, Enna SJ. Gamma-aminobutyric acid(B) receptors: first of the functional metabotropic heterodimers. J Pharmacol Exp Ther 2000;292:2–7.
[11] Kerr DI, Ong J, Prager RH, Gynther BD, Curtis DR. Phaclofen: a peripheral and central baclofen antagonist. Brain Res 1987;405:150–4.
[12] Olpe HR, Karlsson G, Pozza MF, Brugger F, Steinmann M, Van Riezen H, Fagg G, Hall RG, Froestl W, Bittiger H. CGP 35348: a centrally active blocker of $GABA_B$ receptors. Eur J Pharmacol 1990;187:27–38.
[13] Froestl W, Bettler B, Bittiger H, Heid J, Kaupmann K, Mickel SJ, Strub D. Ligands for the isolation of GABA(B) receptors. Neuropharmacology 1999;38:1641–6.
[14] Bittiger H, Froestl W, Mickel S, Olpe HR. $GABA_B$ receptor antagonists: from synthesis to therapeutic applications. Trends Pharmacol Sci 1993;14:391–4.
[15] Kaupmann K, Huggel K, Heid J, Flor PJ, Bischoff S, Mickel SJ, McMaster G, Angst C, Bittiger H, Froestl W, Bettler B. Expression cloning of GABA(B) receptors uncovers similarity to metabotropic glutamate receptors. Nature 1997;386:239–46.
[16] Couve A, Filippov AK, Connolly CN, Bettler B, Brown DA, Moss SJ. Intracellular retention of recombinant $GABA_B$ receptors. J Biol Chem 1998;273:26361–7.
[17] White JH, Wise A, Main MJ, Green A, Fraser NJ, Disney GH, Barnes AA, Emson P, Foord SM, Marshall FH. Heterodimerization is required for the formation of a functional $GABA_B$ receptor. Nature 1998;396:679–82.
[18] Marshall FH, Jones KA, Kaupmann K, Bettler B. GABAB receptors—the first of the heterodimers. Trends Pharmacol Sci 1999;20:396–9.
[19] Margeta-Mitrovic M, Jan YN, Jan LY. A trafficking checkpoint controls $GABA_B$ receptor heterodimerization. Neuron 2000;27:97–106.
[20] Calver AR, Robbins MJ, Cosio C, Rice SQ, Babbs AJ, Hirst WD, Boyfield I, Wood MD, Russell RB, Price GW, Couve A, Moss SJ, Pangalos MN. The C-terminal domains of the $GABA_B$ receptor subunits mediate intracellular trafficking but are not required for receptor signaling. J Neurosci 2001;21:1203–10.
[21] Pagano A, Rovelli G, Mosbacher J, Lohmann T, Duthey B, Stauffer D, Ristig D, Schuler V, Meigel I, Lampert C, Stein T, Prezeau L, Blahos J, Pin J, Froestl W, Kuhn R, Heid J, Kaupmann K, Bettler B. C-terminal interaction is essential for surface trafficking but not for heteromeric assembly of GABA(b) receptors. J Neurosci 2001;21:1189–202.
[22] Bischoff S, Leonhard S, Reymann N, Schuler V, Shigemoto R, Kaupmann K, Bettler B. Spatial distribution of GABA(B)R1 receptor mRNA and binding sites in the rat brain. J Comp Neurol 1999;412:1–16.
[23] Margeta-Mitrovic M, Mitrovic I, Riley RC, Jan LY, Basbaum AI. Immunohistochemical localization of $GABA_B$ receptors in the rat central nervous system. J Comp Neurol 1999;405:299–321.
[24] Fritschy JM, Meskenaite V, Weinmann O, Honer M, Benke D, Mohler H. $GABA_B$-receptor splice variants GB1a and GB1b in rat brain: developmental regulation, cellular distribution and extrasynaptic localization. Eur J Neurosci 1999;11:761–8.
[25] Calver AR, Medhurst AD, Robbins MJ, Charles KJ, Evans ML, Harrison DC, Stammers M, Hughes SA, Hervieu G, Couve A, Moss SJ, Middlemiss DN, Pangalos MN. The expression of GABA(B1) and GABA(B2) receptor subunits in the CNS differs from that in peripheral tissues. Neuroscience 2000;100:155–70.
[26] Ng TK, Yung KK. Differential expression of GABA(B)R1 and GABA(B)R2 receptor immunoreactivity in neurochemically identified neurons of the rat neostriatum. J Comp Neurol 2001;433:458–70.
[27] Bonanno G, Raiteri M. Multiple $GABA_B$ receptors. Trends Pharmacol Sci 1993;14:259–61.
[28] Yu B, Yamada K, Gallagher JP. $GABA_B$ auto- versus hetero-receptor sensitivity: implications for novel pharmacotherapy. Neuropharmacology 1999;38:1805–9.
[29] Prosser HM, Gill CH, Hirst WD, Grau E, Robbins M, Calver A, Soffin EM, Farmer CE, Lanneau C, Gray J, Schenck E, Warmerdam BS, Clapham C, Reavill C, Rogers DC, Stean T, Upton N, Humphreys K, Randall A, Geppert M, Davies CH, Pangalos MN. Epileptogenesis and enhanced prepulse inhibition in $GABA_{B1}$-deficient mice. Mol Cell Neurosci 2001;17:1059–70.
[30] Schuler V, Luscher C, Blanchet C, Klix N, Sansig G, Klebs K, Schmutz M, Heid J, Gentry C, Urban L, Fox A, Spooren W, Jaton A, Vigouret J, Pozza M, Kelly PH, Mosbacher J, Froestl W, Kaslin E, Korn R, Bischoff S, Kaupmann K, van der PH, Bettler B. Epilepsy, hyperalgesia, impaired memory, and loss of pre- and postsynaptic $GABA_B$ responses in mice lacking $GABA_{B(1)}$. Neuron 2001;31:47–58.
[31] Green A, Walls S, Wise A, Green RH, Martin AK, Marshall FH. Characterization of [^{3}H]-CGP54626A binding to heterodimeric $GABA_B$ receptors stably expressed in mammalian cells. Br J Pharmacol 2000;131:1766–74.
[32] Hirst WD, Rice SQJ, Minton JAL, Calver AR, Pangalos MN, Jenkins O, Price GW. Characterisation of a CHO cell line stably co-expressing $GABA_{B1b}$ and $GABA_{B2}$ receptors. Br J Pharmacol 2000;129:80P.
[33] McPherson GA. KELL: a collection of radioligand binding analysis programs. Cambridge, UK: Biosoft; 1997.
[34] Munson PJ, Rodbard D. LIGAND: a versatile computerized approach for characterization of ligand-binding systems. Anal Biochem 1980;107:220–39.
[35] Bowen WP, Jerman JC. Nonlinear regression using spreadsheets. Trends Pharmacol Sci 1995;16:413–7.
[36] Cheng YC, Prusoff WH. Relationship between inhibition constant (K_i) and the concentration of inhibitor which causes 50% inhibition (IC_{50}) of an enzymatic reaction. Biochem Pharmacol 1973;92:881–94.
[37] Brauner-Osborne H, Krogsgaard-Larsen P. Functional pharmacology of cloned heterodimeric GABAB receptors expressed in mammalian cells. Br J Pharmacol 1999;128:1370–4.
[38] Wood MD, Murkitt KL, Rice SQ, Testa T, Punia PK, Stammers M, Jenkins O, Elshourbagy NA, Shabon U, Taylor SJ, Gager TL, Minton J, Hirst WD, Price GW, Pangalos M. The human $GABA_{B1b}$ and $GABA_{B2}$ heterodimeric recombinant receptor shows low sensitivity to phaclofen and saclofen. Br J Pharmacol 2000;131:1050–4.
[39] Wise A, Green A, Main MJ, Wilson R, Fraser N, Marshall FH. Calcium sensing properties of the $GABA_B$ receptor. Neuropharmacology 1999;38:1647–56.
[40] Bittiger H, Reymann N, Froestl W, Mickel SJ. [^{3}H]CGP54626A: a potent antagonist radioligand for $GABA_B$ receptors. Pharmacol Commun 1992;2:23.
[41] Kenakin T. Agonist-receptor efficacy. I. Mechanisms of efficacy and receptor promiscuity. Trends Pharmacol Sci 1995;16:188–92.
[42] Kenakin T. The classification of seven transmembrane receptors in recombinant expression systems. Pharmacol Rev 1995;48:413–63.
[43] Knight AR, Bowery NG. The pharmacology of adenylyl cyclase modulation by GABAB receptors in rat brain slices. Neuropharmacology 1996;35:703–12.
[44] Pauwels PJ, Wurch T. Review: amino acid domains involved in constitutive activation of G-protein-coupled receptors. Mol Neurobiol 1998;17:109–35.
[45] Samama P, Cotecchia S, Costa T, Lefkowitz RJ. A mutation-induced activated state of the beta 2-adrenergic receptor. Extending the ternary complex model. J Biol Chem 1993;268:4625–36.

[46] Westphal RS, Sanders-Bush E. Reciprocal binding properties of 5-hydroxytryptamine type 2C receptor agonists and inverse agonists. Mol Pharmacol 1994;46:937–42.
[47] Lefkowitz RJ, Cotecchia S, Samama P, Costa T. Constitutive activity of receptors coupled to guanine nucleotide regulatory proteins. Trends Pharmacol Sci 1993;14:303–7.
[48] Robbins MJ, Calver AR, Filippov AK, Hirst WD, Russell RB, Wood MD, Nasir S, Couve A, Moss SJ, Pangalos MN. $GABA_{B2}$ is essential for G protein coupling of the $GABA_B$ receptor heterodimer. J Neurosci 2001;21(20):8043–52.
[49] Kuner R, Kohr G, Grunewald S, Eisenhardt G, Bach A, Kornau HC. Role of heteromer formation in $GABA_B$ receptor function. Science 1999;283:74–7.
[50] Martin SC, Russek SJ, Farb DH. Molecular identification of the human GABABR2: cell surface expression and coupling to adenylyl cyclase in the absence of GABABR1. Mol Cell Neurosci 1999;13:180–91.