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Correspondence and request for materials should be addressed to K.A.J. (e-mail: kjones@synapticcorp. com).
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Heterodimerization is required for the formation of a functional $GABA_B$ receptor

Julia H. White*, Alan Wise*, Martin J. Main*, Andrew Green*, Neil J. Fraser*, Graham H. Disney*, Ashley A. Barnes*, Piers Emson†, Steven M. Foord* & Fiona H. Marshall*

** Receptor Systems, Molecular Pharmacology Unit, GlaxoWellcome, Medicines Research Centre, Gunnels Wood Road, Stevenage, Hertfordshire SG1 2NY, UK*
† Department of Neurobiology, Babraham Institute, Babraham, Cambridge CB2 4AT, UK

GABA (γ-aminobutyric acid) is the main inhibitory neurotransmitter in the mammalian central nervous system, where it exerts its effects through ionotropic ($GABA_{A/C}$) receptors to produce fast synaptic inhibition and metabotropic ($GABA_B$) receptors to produce slow, prolonged inhibitory signals. The gene encoding a $GABA_B$ receptor ($GABA_BR1$) has been cloned[1]; however, when expressed in mammalian cells this receptor is retained as an immature glycoprotein on intracellular membranes[2] and exhibits low affinity for agonists compared with the endogenous receptor on brain membranes. Here we report the cloning of a complementary DNA encoding a new subtype of the $GABA_B$ receptor ($GABA_BR2$), which we identified by mining expressed-sequence-tag databases. Yeast two-hybrid screening showed that this new $GABA_BR2$-receptor subtype forms heterodimers with $GABA_BR1$ through an interaction at their intracellular carboxy-terminal tails. Upon expression with $GABA_BR2$ in HEK293T cells, $GABA_BR1$ is terminally glycosylated and expressed at the cell surface. Co-expression of the two receptors produces a fully functional $GABA_B$ receptor at the cell surface; this receptor binds GABA with a high affinity equivalent to that of the endogenous brain receptor. These results indicate that, *in vivo*, functional brain $GABA_B$ receptors may be heterodimers composed of $GABA_BR1$ and $GABA_BR2$.

Using a combination of bioinformatics and conventional molecular biology techniques, we cloned human homologues of the two alternatively spliced rat cDNAs encoding $GABA_BR1a$ and $GABA_BR1b$, which differ in their amino termini. The human homologues are 98% identical to the respective rat cDNAs. Database searches with the human $GABA_BR1a$ cDNA sequence identified several expressed sequence tags (ESTs) which show homology to the human $GABA_BR1$ sequence. Each of these ESTs encodes a part of the same receptor and, using this information, a new receptor subtype, which we have called R2, was assembled. Like R1, the R2 receptor is a seven-transmembrane-domain protein with a signal sequence and an extended extracellular N terminus, but it also possesses an unusually long intracellular C-

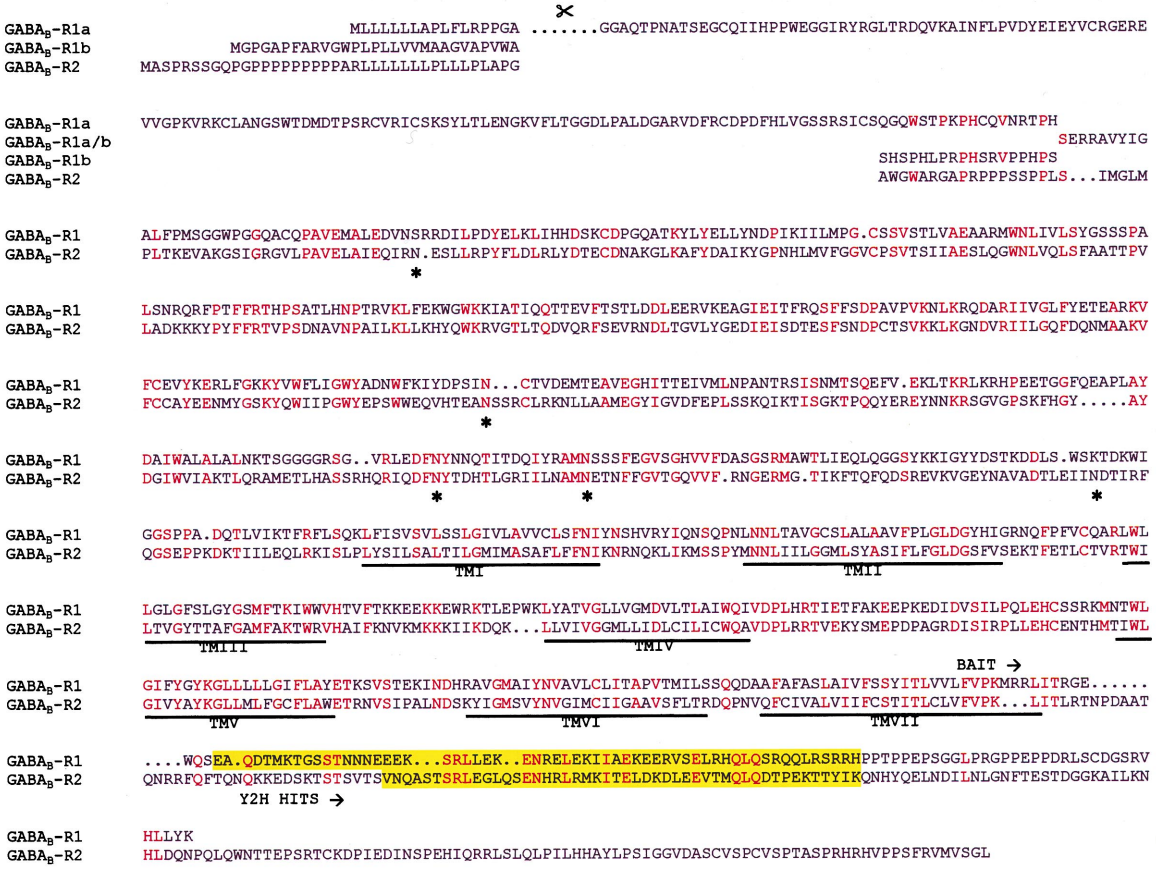

Figure 1 Amino-acid sequences of human $GABA_BR1a$, $GABA_BR1b$ and $GABA_BR2$, aligned for comparison. The cleavage point of putative signal sequences (scissors), transmembrane (TM) domains and yeast two-hybrid 'bait' and hits (Y2H hits) are shown. Motif searches identified a coiled-coiled domain in the C terminus of both receptors (yellow) and five putative *N*-linked glycosylation sites (asterisks).

terminal tail (Fig. 1). At the protein level the two receptors are 35% identical and 54% similar.

Myc- or haemagglutinin (HA)-tagged and untagged constructs for human R1a, R1b or R2 were transiently transfected into HEK293T cells. We showed that each receptor is highly expressed by using gel electrophoresis of whole-cell membranes followed by western blotting. However, in assays of both [^{35}S]GTP-γS-binding and cyclic AMP production, we were unable to detect any functional activation of the cloned receptors by GABA or other $GABA_B$-selective agonists (data not shown), indicating that the receptors do not form a functional $GABA_B$ receptor when expressed in mammalian cells.

A single transmembrane protein, receptor-activity-modifying protein 1 (RAMP1), is required for the trafficking, terminal glycosylation and expression of the calcitonin-receptor-like receptor as a functional receptor for calcitonin-gene-related peptides[3]. In addition, many receptors interact with synaptic 'anchoring proteins'[4,5]. We and others[2], therefore, proposed that an accessory protein may be necessary for expression of maturely glycosylated $GABA_BR1$ at the cell surface. To investigate this possibility, we used the yeast two-hybrid system to search for proteins that interact with human $GABA_BR1$. We used the intracellular C terminus of $GABA_BR1$ as the 'bait' as it contains a 'coiled-coil' domain; such domains mediate several protein–protein interactions[6]. Screening of a human brain cDNA library (total of 4.3×10^6 cDNAs) produced several positive hits. A common hit was the C terminus of human $GABA_BR2$, which contains a coiled-coil domain similar to that seen in $GABA_BR1$ (Fig. 1). Further experiments in yeast confirmed that the interaction between the $GABA_BR1$ and $GABA_BR2$ C termini is specific, and showed that neither receptor will form homodimers (Fig. 2a). These results indicate that the two $GABA_B$ receptors may heterodimerize through an interaction between their intracellular C-terminal tails, possibly through a conserved coiled-coil domain.

To test this hypothesis directly we carried out co-immunoprecipitation experiments in HEK293T cells expressing Myc-tagged $GABA_BR1b$ and HA-tagged $GABA_BR2$ (Fig. 2b). Immunoprecipitation of $GABA_BR1b$ from detergent-solubilized cell fractions with anti-Myc antisera led to detection of HA–$GABA_BR2$ within immune complexes (Fig. 2b, lane 3). Conversely, Myc–$GABA_BR1b$ was detected on a western blot following precipitation of $GABA_BR2$ with anti-HA antisera (Fig. 2b, lane 6). These data are compelling evidence for a physical association between $GABA_BR1b$ and $GABA_BR2$ following their expression in mammalian cells. Results from preliminary *in situ* hybridization studies indicate that, in the human brain, the alternative transcripts $GABA_BR1a$ and $GABA_BR1b$ each co-localize with the $GABA_BR2$ transcript in the cortex and

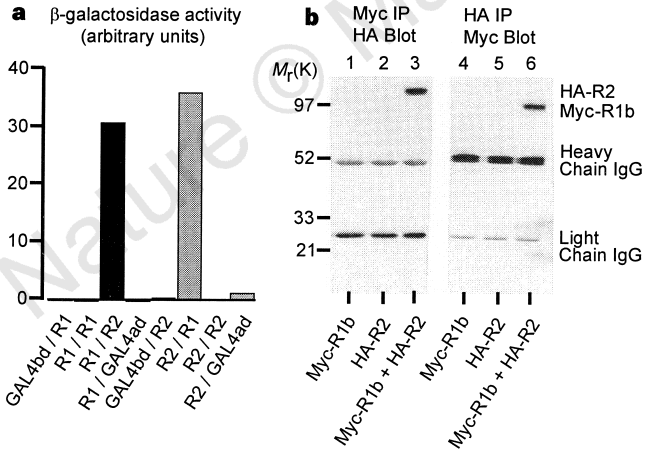

Figure 2 Interaction of $GABA_BR1$ and $GABA_BR2$. **a**, β-Galactosidase activity was measured in yeast strain Y190 expressing $GABA_BR1$ or $GABA_BR2$ C termini, either with empty vector or with each other in all combinations. Of each pair of proteins expressed, the first is the fusion with the Gal4 binding domain and the second is the Gal4ad fusion. (Gal4ad is Gal4 activation domain.) GAL4bd and GAL4ad denote empty-vector controls. β-Galactosidase activity is quantified relative to cell numbers. **b**, Co-immunoprecipitation studies. HEK293T cells were transfected with Myc–$GABA_BR1b$ (lanes 1, 4), HA–$GABA_BR2$ (lanes 2, 5) or both receptors (lanes 3, 6), collected, lysed and immunoprecipitated (IP) using anti-Myc (lanes 1–3) or anti-HA (lanes 4–6) antisera. Immune complexes were subjected to SDS–PAGE and transferred to nitrocellulose, and Myc- or HA-tagged receptors were identified by immunoblotting with either anti-HA (lanes 1–3) or anti-Myc (lanes 4–6) antisera. In cells co-expressing Myc–$GABA_BR1b$ and HA–$GABA_BR2$, the two receptors immunoprecipitated together, as indicated by immunoblotting with anti-HA (lane 3) or anti-Myc (lane 6) antisera.

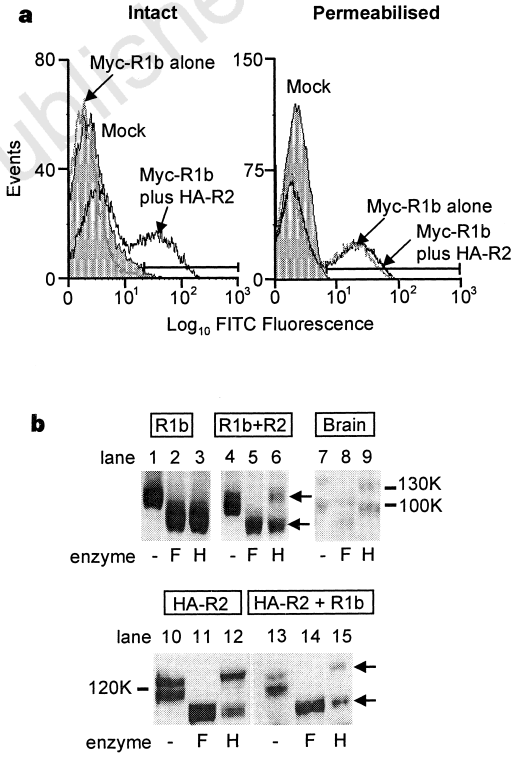

Figure 3 $GABA_BR2$ enables cell-surface expression of $GABA_BR1b$ as a mature glycoprotein. **a**, Flow-cytometry analysis. In mock-transfected cells (shaded) and cells expressing Myc–$GABA_BR1b$ alone, no anti-Myc FITC fluorescence was detected (marker indicates fluorescence measured over background) unless cells were permeabilized. Co-expression of HA–$GABA_BR2$ with Myc–$GABA_BR1b$ led to surface expression of Myc–$GABA_BR1b$ in 20% of cells sampled. **b**, Glycosylation status was assessed following treatment of membranes from HEK293T cells, transfected with $GABA_BR1b$ (lanes 1–3), HA–$GABA_BR2$ (lanes 10–12) or combinations of $GABA_BR1b$, $GABA_BR2$ and HA–$GABA_BR2$ (lanes 4–6 and 13–15), with vehicle (–), endoglycosidase F or endoglycosidase H. Parallel experiments were carried out using rat brain membranes (lanes 7–9). Samples were resolved by SDS–PAGE and immunoblotted with antiserum 501 (lanes 1–9) or anti-HA antiserum (lanes 10–15). $GABA_BR1$ appears as a mature, endoglycosidase-H-resistant glycoprotein (top arrow, lane 6) only after co-expression with $GABA_BR2$. In contrast, endogenous rat $GABA_BR1$ (distinct bands at 130K ($GABA_BR1a$) and 100K ($GABA_BR1b$)) are expressed in an endoglycosidase-H-resistant form (lane 9). $GABA_BR2$ runs as a doublet at 120K, a component of which is endoglycosidase-H-resistant (top arrow). Top arrows represent terminally glycosylated forms of $GABA_BR1$ and $GABA_BR2$ in lanes 6 and 15, whereas bottom arrows represent core-glycosylated forms of $GABA_BR1$ and $GABA_BR2$.

hippocampus (data not shown), implying that a similar interaction occurs *in vivo*.

Further analysis of $GABA_BR1$ expressed in HEK293T cells showed that the receptor is retained as an immature glycoprotein on intracellular membranes (Fig. 3a; Fig. 3b, lanes 1–3), presumably explaining why we saw no responses of $GABA_BR1$ to GABA. We therefore proposed that co-expression of $GABA_BR1$ with $GABA_BR2$ may promote expression of $GABA_BR1$ at the cell surface and enable the $GABA_BR1/GABA_BR2$ heterodimer to function as a $GABA_B$ receptor. We used flow-cytometry analysis to study the cellular distribution of immunotagged $GABA_B$ receptors expressed in HEK293T cells (Fig. 3a). In intact cells expressing Myc–$GABA_BR1b$ alone, no cell-surface anti-Myc immunoreactivity was detected. In contrast, immunoreactivity towards Myc was detected in 35% of permeabilized cells, reflecting intracellular expression of $GABA_BR1b$. However, when $GABA_BR2$ was co-transfected with Myc–$GABA_BR1b$, 20% of intact cells showed cell-surface anti-Myc immunoreactivity. As the transfection efficiency in these experiments was ~35% (estimated from total expression in permeabilized cells), these data indicate that, in the presence of $GABA_BR2$, $GABA_BR1b$ is efficiently moved to the cell membrane.

Endoglycosidases F and H can be used to differentiate between immature, core-glycosylated proteins and terminally glycosylated proteins that have passed through the Golgi apparatus[3,7,8]. On a western blot, HEK293T cell membranes expressing $GABA_BR1b$ produced a single distinct band of relative molecular mass 100,000 (M_r 100K; Fig. 3b, lane 1). This band was reduced to 80K following treatment with endoglycosidase F or H (Fig. 3b, lanes 2, 3), indicating that the expressed protein may be core-glycosylated but lacks terminal glycosylation. In contrast, following co-expression of $GABA_BR1b$ and $GABA_BR2$ in HEK293T cells, a component of the $GABA_BR1$ immunoreactivity was resistant to endoglycosidase H (Fig. 3b, lane 6), indicating that a significant fraction of the $GABA_BR1$ receptor might now be mature glycoprotein. $GABA_BR2$ (which runs as a doublet at ~120K) was expressed at the cell surface and an endoglycosidase-H-insensitive band was observed even in the absence of $GABA_BR1$ (Fig. 3b, lane 12). Co-expression of $GABA_BR1$ had no effect on the glycosylation status of $GABA_BR2$ (Fig. 3b, lane 13–15).

We investigated whether the $GABA_BR1/GABA_BR2$ heterodimer behaves in a similar manner to the endogenous rat brain receptor when expressed in HEK293T cells. Expression of $GABA_BR1$ alone, but not $GABA_BR2$ alone, produced high levels of specific binding of the $GABA_B$ antagonist ^{3}H-labelled CGP54626. However, as reported for binding of ^{125}I-labelled CGP64213 (a structural analogue of ^{3}H-labelled CGP54626)[1], agonist inhibition curves were shifted about 30-fold to the right (half-maximal inhibitory concentration (IC_{50}) = 28.8 μM) compared with binding to rat brain membranes (IC_{50} = 1.1 μM; Fig. 4a). Transfection of an increasing ratio of $GABA_BR2$ to $GABA_BR1$ led to a progressive leftwards shift in the GABA competition curve (data not shown). At a ratio (0.25 μg $GABA_BR1$:1 μg $GABA_BR2$ DNA) that gave equal expression of the two receptor proteins, as determined by western blotting, an IC_{50} of 2.1 μM was recorded; this is comparable to the IC_{50} of 1.1 μM determined with rat brain membranes (Fig. 4a). Co-expression of $GABA_BR1a$ and $GABA_BR2$ (in combination with exogenous $G\alpha_{o1}$, a G-protein α-subunit) in HEK293T cells also resulted in robust, agonist-dependent stimulation of [^{35}S]GTP-γS binding (Fig. 4b). This was concentration-dependent, with a half-maximal effector concentration (EC_{50}; 95 ± 11 μM) similar to that seen in rat brain membranes (59 ± 4 μM). Similar results were obtained from these cells using inhibition of forskolin-evoked cyclic AMP as a measurement (38 ± 9% inhibition, EC_{50} = 3.7 ± 1 μM; data not shown). Finally, we co-expressed $GABA_BR1a$ and $GABA_BR2$ in *Xenopus* oocytes to study coupling of $GABA_B$ receptors to the G-protein-regulated potassium channels GIRK1 and GIRK4 (ref. 9). As seen in our other functional experiments, there were no responses to GABA in oocytes expressing either receptor in isolation (Fig. 4c; n = 7 for $GABA_BR1a$ and n = 8 for $GABA_BR2$). However, in 21 out of 21 oocytes co-expressing $GABA_BR1a$ and $GABA_BR2$, application of 100 μM GABA or 1 mM baclofen activated a large inward current (343 ± 76 nA for GABA; Fig. 4c), which gave a current–voltage curve consistent with activation of the GIRK1/GIRK4 heteromer (Fig. 4d). In all these experiments, similar results were obtained with the $GABA_BR1a$ and $GABA_BR1b$ splice variants.

Our results indicate that the human $GABA_B$ receptor is composed of two distinct, seven-transmembrane-domain receptor subunits, which exist either as a heterodimer or possibly as a larger oligomer. These data are, to our knowledge, the first evidence for heterodimerization of a seven-transmembrane-domain receptor. Co-expression of $GABA_BR1$ and $GABA_BR2$ appears to be a prerequisite for maturation and transport of $GABA_BR1$ to the plasma membrane and results in high-affinity GABA binding and G-protein activation.

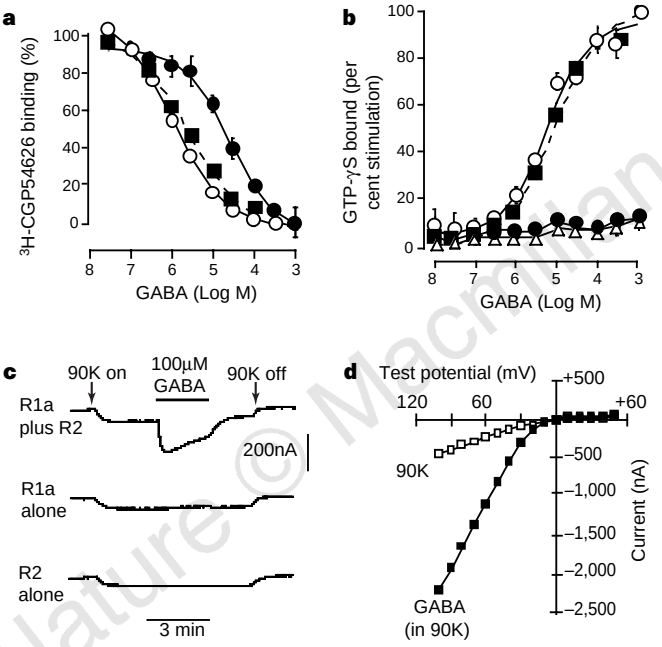

Figure 4 Ligand binding and functional studies. ^{3}H-labelled CGP54626 competition binding and [^{35}S]GTP-γS binding in membranes from HEK293T cells expressing $GABA_BR1b$ (filled circles), $GABA_BR2$ (triangles), $GABA_BR1b$ plus $GABA_BR2$ (squares, dashed line), or in rat brain membranes (open circles). **a**, A high level of specific binding of ^{3}H-labelled CGP54626 was recorded in membranes expressing $GABA_BR1b$; binding was displaced in a concentration-dependent manner by GABA. Transfection of $GABA_BR2$ (which did not itself bind ^{3}H-labelled CGP54626) with $GABA_BR1$ led to a leftwards shift in the GABA-displacement curve to a position similar to that in rat brain membranes. **b**, Stimulation by GABA of [^{35}S]GTP-γS binding. Data are normalized to the peak response to GABA. No GABA-stimulated [^{35}S]GTP-γS binding was recorded in $GABA_BR1b$- or $GABA_BR2$-expressing membranes; however, GABA stimulated a dose-dependent increase in [^{35}S]GTP-γS binding both in rat brain and in cells expressing $GABA_BR1b$ plus $GABA_BR2$. In all functional assays, identical results were obtained with the $GABA_BR1a$ and $GABA_BR1b$ splice variants. **c**, **d**, Oocyte data. Sample currents (**c**, top panel) and a current–voltage curve (**d**) are shown for individual oocytes expressing $GABA_BR1a$ plus $GABA_BR2$ in combination with the potassium channels GIRK1 and GIRK4. Switching from low (ND96) to high (**c**, 90K; **d**, open squares) potassium solution led to an inward shift in holding current, indicating expression of the GIRK1 and GIRK4 potassium channels. Subsequent addition of 100 μM GABA (filled squares in **d**) activated a large inward current through GIRK1/GIRK4.

Homodimerization of receptors has been reported for several seven-transmembrane-domain receptors[10], including the δ-opioid[11], β_2-adrenergic[12] and metabotropic glutamate (mGlu) receptors[13]. In the case of mGlu receptors, which are related to $GABA_BR1$ (ref. 1) and $GABA_BR2$, it has been suggested that homodimerization occurs through disulphide bridges in the N-terminal extracellular domains[13]. However, there are no cysteine residues in the corresponding regions of $GABA_BR1$ or $GABA_BR2$ and we believe that interaction occurs instead between the intracellular C termini of the two $GABA_B$ receptors, probably though conserved coiled-coil domains, although other regions within the receptors might interact. The finding that the $GABA_B$ receptor is a heterodimer containing $GABA_BR1$ and $GABA_BR2$ demonstrates a new mechanism of receptor behaviour and will further our understanding of the role of these receptors in the nervous system. Furthermore, the ability to reconstitute functional $GABA_B$ receptors in recombinant systems will allow the development of new drugs at an important therapeutic target. □

Methods

Molecular biology. We used a bioinformatic approach to identify human ESTs showing homology to the rat $GABA_BR1$ receptor. These ESTs were aligned to provide coding sequences for human $GABA_BR1a$ and $GABA_BR1b$ (ESTs X90542, X90543, AA317417, D80024, AA348199, AA628887, T07518 and T06711) and for a putative $GABA_B$-receptor subtype, $GABA_BR2$. The predicted open reading frame of $GABA_BR1a$ and $GABA_BR1b$ was amplified by a combination of reverse transcription with polymerase chain reaction (RT-PCR) using human cerebellum poly(A)$^+$ RNA (Clontech) and rapid amplification of cDNA ends (RACE)-PCR against Marathon-Ready human cerebellum cDNA (Clontech), and was cloned into vector pcDNA3.1 (Invitrogen). The entire open reading frame of $GABA_BR2$ could be represented by the human ESTs H14151, T07621, AA324303 and Z43654. Restriction fragments and PCR products generated from these clones were used to clone the full-length coding sequence into pcDNA3.1. For epitope-tagging of receptors, cDNAs encoding Myc (used with monoclonal antibody 9E10) and HA (used with monoclonal antibody 12CA5) epitopes were fused in-frame to the 5′ end of cDNAs encoding $GABA_BR1b$ and $GABA_BR2$. In each case the native signal sequence was removed and replaced with that for CD33 (ref. 14) (Myc–$GABA_BR1b$) or T8 (HA–$GABA_BR1b$, HA–$GABA_BR2$). All modified sequences were compared to 'native' sequences in the assays described and behaved in an identical manner.

Yeast two-hybrid studies. Yeast (*Saccharomyces cerevisiae* strain Y190)[15] expressing a fusion protein containing a Gal4 binding domain and the $GABA_BR1$ C terminus were selected and transformed with a human brain 'Matchmaker' cDNA library (HL4004H, Clontech). Enough independent cDNAs were transformed to give a threefold representation of the library. Interacting clones were selected by growth under 20 mM 3-amino-1,2,4-triazole selection, followed by production of β-galactosidase as determined by a freeze-fracture assay. Plasmid DNA was recovered from yeast cells, transformed into *Escherichia coli* and sequenced.

Cell biology. HEK293T cells were maintained in DMEM medium containing 10% fetal calf serum and 2 mM glutamine, grown to 60–80% confluency in 60-mm dishes, transfected with cDNA using lipofectamine reagent (10 μl, Life Technologies; total DNA = 3 μg) and collected 48–72 h after transfection. Binding and glycosylation studies were done using plasma-membrane-containing P2 particulate fractions as described[2]. Competition binding assays were done using displacement by GABA of the $GABA_B$ antagonist ^{3}H-labelled CGP54626 (ref. 16) (Tocris Cookson). Experiments were done in 50 mM Tris-HCl (pH 7.4) containing 2.5 mM Ca^{2+} and 40 μM isoguvacine, and nonspecific binding was determined using 1 mM GABA. Bound ligand was recovered using a Brandel 48-well harvester and measured by liquid scintillation counting. [^{35}S]GTP-γS-binding assays were done at room temperature in 96-well format as described[17]. Bound [^{35}S]GTP-γS was determined by scintillation counting. In glycosylation studies, enzymatic removal of asparagine-linked (*N*-linked) carbohydrate moieties with endoglycosidases F and H was done essentially according to the manufacturer's instructions (Boehringer). $GABA_B$-receptor glycosylation status was studied following SDS–PAGE/immunoblotting of samples and receptors were visualized by immunoblotting with antiserum 501, a polyclonal antibody raised against a synthetic peptide corresponding to the C-terminal 15 amino acids of $GABA_BR1$, or an anti-HA antisera.

For co-immunoprecipitation experiments, HEK293T cells were collected and homogenized (in lysis buffer: 50 mM Tris-HCl, 150 mM NaCl, 1% (*v/v*) Nonidet P40, 0.5% (*w/w*) sodium deoxycholate, pH 7.5, supplemented with 'Complete' protease inhibitor cocktail tablets (1 tablet per 25 ml, Boehringer)) and precleared supernatant was collected by incubation with 50 μl protein A–agarose (Boehringer). Immunoprecipitation with either anti-HA or anti-Myc antisera was allowed to proceed for 1 h at 4 °C and capture of immune complexes was progressed overnight following the addition of 50 μl protein A–agarose. Immune complexes were collected by microcentrifugation, released from protein A–agarose and analysed by SDS–PAGE followed by immunoblotting.

Flow-cytometry analysis was done using intact HEK293T cells, which were incubated with primary antibodies (9E10 or 12CA5) for 15 min at room temperature followed by secondary antibody (sheep anti-mouse Fab_2 coupled with fluorescein isothiocyanate (FITC)) for a similar period of time. For permeabilized cells, a Fix and Perm kit (Caltag) was used. Cells were analysed on a Coulter Elite flow cytometer set up to detect FITC fluorescence; 30,000 cells were analysed for each sample.

Oocytes. Capped *in vitro*-transcribed RNA (20–50 ng $GABA_BR1a$; $GABA_BR2$; GIRK1 and GIRK4 per oocyte) was injected into stage V–VI defolliculated oocytes[18] and two microelectrode voltage clamp recordings were made 3–7 days after RNA injection from a holding potential of −60 mV. Oocytes were superfused with ND96 solution (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM HEPES, pH 7.5, at 25 °C) at a flow rate of 2 ml min^{-1}. To facilitate the recording of GIRK1/GIRK4 potassium currents, the extracellular solution was switched to a high-potassium solution (90 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM HEPES). Recording electrodes had a resistance of 0.5–1.0 MΩ when filled with 3 M KCl. GABA was applied by addition to the superfusate.

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Correspondence and requests for materials should be addressed to F.H.M. (e-mail: fhm27375@glaxowellcome.co.uk). The cDNA sequences for human $GABA_BR1a$, $GABA_BR1b$ and $GABA_BR2$ have been submitted to EMBL under the accession numbers AJ012185, AJ012186 and AJ012188, respectively.