Structural mechanism of ligand activation in human GABAB receptor

Yong Geng¹, Martin Bush¹, Lidia Mosyak¹, Feng Wang¹ & Qing R. Fan¹,²

Human GABAB (γ-aminobutyric acid class B) receptor is a G-protein-coupled receptor central to inhibitory neurotransmission in the brain. It functions as an obligatory heterodimer of the subunits GBR1 and GBR2. Here we present the crystal structures of a heterodimeric complex between the extracellular domains of GBR1 and GBR2 in the apo, agonist–bound and antagonist–bound forms. The apo and antagonist–bound structures represent the resting state of the receptor; the agonist–bound complex corresponds to the active state. Both subunits adopt an open conformation at rest, and only GBR1 closes on agonist–induced receptor activation. The agonists and antagonists are anchored in the interdomain crevice of GBR1 by an overlapping set of residues. An antagonist confines GBR1 to the open conformation of the inactive state, whereas an agonist induces its domain closure for activation. Our data reveal a unique activation mechanism for GABAB receptor that involves the formation of a novel heterodimer interface between subunits.

GABA is the predominant inhibitory neurotransmitter in the central nervous system. Metabotropic GABAB receptor is a G-protein-coupled receptor that mediates slow and prolonged synaptic inhibition through G0 or G1 protein. Presynaptic GABAB receptor suppresses neurotransmitter release, and postsynaptic GABAB receptor causes hyperpolarization of neurons. Malfunction of GABAB receptor can lead to various neurological disorders, including spasticity and epilepsy. Baclofen, a selective GABAB agonist, is used clinically to treat muscle spasticity associated with multiple sclerosis, cerebral palsy and spinal cord injury.

GABAB receptor belongs to the distinct class-C family of G-protein-coupled receptors. Ligand binding to these receptors takes place within a large extracellular Venus flytrap (VFT) module that has sequence homology to bacterial periplasmic amino-acid-binding proteins. Unlike metabotropic glutamate receptors (mGluRs) and extracellular calcium-sensing receptor, which function as disulphide-tethered homodimers, GABAB and taste receptors act as heterodimers.

GABAB receptor functions as a heterodimeric assembly of the subunits GBR1 and GBR2. GBR2 facilitates cell surface expression of GBR1 by masking an endoplasmic reticulum retention signal of GBR1. Although GBR2 does not bind any known GABA ligand, its ectodomain directly interacts with the GBR1 ectodomain to enhance agonist affinity and is required for receptor activation.

Most of the current knowledge about the structures of class-C G-protein-coupled receptors derives from homodimeric mGluRs. The ectodomain structures of three mGluR subtypes have been determined with and without ligand. Here we assembled a stable heterodimeric complex of GBR1b and GBR2, in which the protomers adopt an open conformation when compared with the known structures of mGluRs. The ligand-binding subunit, GBR1b, can oscillate between open and closed conformations, in which the compact closed conformation is associated with agonist binding. In contrast, the non-ligand-binding subunit, GBR2, has nearly identical conformations with and without dimer partner GBR1b.

Despite similarities, the GBR1b and GBR2 structures have different interdomain arrangements, consistent with their disparate ligand-binding characteristics. In the crystal structure of apo-GBR1b:GBR2, both subunits adopt an open conformation when compared with the known structures of mGluRs. In the crystal structure of apo-GBR1b:GBR2, both subunits adopt an open conformation when compared with the known structures of mGluRs. The crystal structures of apo-GBR1b:GBR2, both subunits adopt an open conformation when compared with the known structures of mGluRs.
Figure 1 | Crystal structures of the GBR1bVFT:GBR2VFT complex. a, Apo structure; b, CGP54626ANT-bound structure; c, (R)-baclofenAGO-bound structure. Each complex is shown in two views related by a 90° rotation about the vertical axis. Front view (left panel) is shown as a ribbon diagram; side view (right panel) is presented as a molecular surface. GBR1b VFT and GBR2VFT are coloured blue and green, respectively. The observed carbohydrates are shown as ball-and-stick models in grey. Disulphide bridges are in magenta. The ligands are displayed as space-filling models.

( Supplementary Fig. 4). The ligand-binding cleft of GBR1bVFT stays open with each bound antagonist. In addition, GBR2VFT remains wide open with an empty interdomain cleft. This open–open configuration of the apo and antagonist-bound structures corresponds to the resting (or inactive) state of the heterodimeric receptor.

Agonist binding causes large conformational changes within the heterodimeric complex. First, both the agonists GABA and (R)-baclofenAGO induce domain closure of GBR1bVFT, as previously predicted42 (Fig. 2a). When the LB1 domains of apo and agonist-bound GBR1bVFT are superimposed, their LB2 domains can be related by a 29° rotation about a nearly horizontal interdomain axis. Because the rotational axis has a slight vertical offset, this transformation also brings the LB2 domain of GBR1bVFT into close contact with the LB2 domain of GBR2VFT to form a large heterodimer interface unique to the active state.

Second, GBR2VFT remains open in the agonist-bound state, consistent with our previous prediction that GBR2VFT has a constitutively open conformation39. Nevertheless, the LB2 domain of GBR2VFT undergoes a twist motion of 9° around a nearly vertical axis, and moves towards the LB2 domain of GBR1bVFT to form new heterodimeric contacts (Fig. 2b).

Finally, the substantial rearrangement of the LB2 domains from the apo to the agonist-bound state shortens the distance between the C termini of the two subunits from 45 to 32 Å (Fig. 2c, d and Supplementary Fig. 5). This decrease in the separation between membrane-proximal LB2 domains may lead to changes in the relative orientation of the transmembrane domains. In summary, both agonist-bound GBR1bVFT:GBR2VFT complexes adopt a closed–open structural arrangement, which corresponds to the active state of the receptor (Supplementary Fig. 4).

Common subunit–subunit interactions

In both the resting and active states, GBR1bVFT and GBR2VFT interact through their LB1 domains (Supplementary Figs 6 and 7). In the apo
and antagonist-bound structures, the subunit association is exclusively facilitated by this LB1–LB1 contact. The heterodimer buries more than 1,400 Å² of solvent-accessible surface area and has exceptionally high interfacial shape correlation (Supplementary Table 2).

The LB1–LB1 interaction is mediated by the B and C helices of both subunits (Fig. 3a). The heterodimer interface can be divided into three regions (Fig. 3b). Site I is located at the centre of the interface, and it is flanked, one on either side, by sites II and III.

Site I consists of a central hydrophobic patch surrounded by hydrogen bonds. The heterodimer contacts within this site are highly conserved in all of the GBR1bVFT:GBR2VFT structures. In particular, it features three deeply buried tyrosine residues (Tyr 113 and Tyr 117 of GBR1bVFT and Tyr 118 of GBR2VFT) that are critical for heterodimer interaction and receptor activation24. These tyrosine residues participate in aromatic stacking interactions, and form interfacial hydrogen bonds. Together with the adjacent lysine and tryptophan residues, they are responsible for the majority of hydrophobic contacts at the LB1–LB1 heterodimer interface.

Interactions at site II are mostly hydrogen bonds, and include a universal salt bridge (GBR1bVFT-R141:GBR2VFT-D109) as well as a conserved hydrogen bond (GBR1bVFT-E138:GBR2VFT-N110). Site III consists predominantly of water-mediated contacts, and is the most variable part of the LB1–LB1 interface.

**Agonist-induced heterodimer interface**

Agonist binding induces the formation of an additional heterodimer interface between the LB2 domains of the GBR1bVFT and GBR2VFT subunits (Supplementary Fig. 7). This is consistent with our calorimetry measurements showing that GBR2VFT has higher affinity for agonist-bound than antagonist-bound GBR1bVFT (ref. 26). The LB2–LB2 interface buries more than 1,300 Å² of solvent-accessible surface area, has poor shape complementarity and is dominated by polar interactions (Supplementary Table 2).

The LB2–LB2 interaction is mediated by two strand–loop–helix motifs from each LB2 domain (Fig. 3c). Neighbouring strands f and g are part of the central β-sheet in LB2, and helices F and G flank the β-sheet. The heterodimer contacts consist primarily of hydrogen bonds, some of which are mediated by water molecules. The interface can be divided into three adjacent areas (Fig. 3d). Sites IV and V each features a large cluster of hydrogen bonds, and site VI mostly consists of isolated contacts. The GBR2VFT Residue Asn 213 is located at the intersection of sites IV and V, and it bridges the hydrogen-bond networks within these two regions. In addition, a minor LB2–LB1 contact involving helix D of GBR2VFT is formed at the edge of site IV.

To confirm the importance of the LB2–LB2 heterodimer interface to receptor activation, we carried out alanine-scanning mutagenesis of the interfacial residues. We identified several polar residues from each subunit that are critical to agonist-dependent G protein activity (Supplementary Fig. 7). These include the GBR1bVFT residues Thr 198, Glu 201 and Ser 225, and the GBR2VFT residues Asp 204, Gln 206, Asn 213 and Ser 233. All of these residues are engaged in multiple interfacial hydrogen bonds at the LB2–LB2 interface. This reliance on hydrophilic interactions to form a distinct subunit interface in the active state allows the receptor to dissociate readily on returning to its resting state. Previous studies have also shown that introduction of a large N-linked glycan into the LB2 domain of either GABA B subunit inhibits agonist-induced receptor activation25.

**Ligand recognition**

All of the antagonists are derivatives of GABA, and have the general structure of a γ-amino acid. The receptor–antagonist interactions are mediated largely by hydrogen bonds (Fig. 4a, b and Supplementary Fig. 8). First, each antagonist is anchored at the crevice of GBR1bVFT by two sets of hydrogen bonds. The α-carboxylate group at one end forms hydrogen bonds with the LB1 residues Ser 130 and Ser 153, and the γ-amino group at the other end is hydrogen-bonded to His 170 and Glu 349. Second, Trp 65 makes van der Waals contacts with all of the antagonists. Third, the β-hydroxyl substituent of CGP54626 ANT and (S)-2-OH-saclofen ANT makes additional hydrogen bonds with the receptor that are specific to these antagonists. Finally, all of the antagonists except SCH50911 ANT and (R)-phaclofen ANT participate in water-mediated interaction with Ser 131. These extensive contacts indicate that the LB1 domain is primarily responsible for anchoring antagonist.

In contrast, the interaction between the LB2 domain and bound antagonist is sparse and varies among the different antagonists (Supplementary Fig. 8). Only two antagonists, CGP54626 ANT and SCH50911 ANT, directly contact Trp 278 of LB2 through a large γ-substituent. As a result of this additional LB2 interaction, both compounds have higher binding affinity to GABA B receptor than the other antagonists reported here1. This suggests that the LB2 domain has an auxiliary role in antagonist recognition, and enhances the potency of selective antagonists.

GABA B receptor recognizes both the agonists GABA and (R)-baclofen AGO. In essentially the same manner (Fig. 4c, d and Supplementary Fig. 9). (R)-baclofen AGO is a derivative of GABA, and contains a...
A GABA subunit contains an $\alpha$-cyclohexyl group and a $\gamma$-dichlorophenyl group. The adverse interactions of these moieties with Tyr 250 and Trp 278 would be expected to prevent the LB1 and LB2 domains from approaching each other (Fig. 4e). Similarly, each of the other antagonists CGP46381 ANT, CGP35348 ANT and SCH50911 ANT has a bulky substituent at either the $\alpha$-position or the $\gamma$-position to block GBR1bVFT domain closure (Supplementary Figs 8 and 9). Although the antagonists (S)-2-OH-saclofen ANT and (R)-phaclofen ANT are structurally analogous to the agonist (R)-baclofen AGO, their $\alpha$-acid motifs assume a tetrahedral coordination geometry that is incompatible with the active-state conformation of Tyr 250 (Fig. 4f). Furthermore, the $\alpha$-substituents push the $\beta$-chlorophenyl ring towards the $\gamma$-amino end of each antagonist, thereby generating potential steric interactions with Ile 276 and Trp 278 to prevent GBR1bVFT domain closure.

All of the residues at the ligand-binding site are conserved within GBR1 sequences across different species (Supplementary Fig. 11). Some of the ligand-binding residues, including Ser 130, Gly 151, Ser 153 and Glu 349 of GBR1b, have been implicated by previous studies.21,26,44–46.

The LB1 residues are required for both agonist and antagonist recognition. We found that the Trp65Ala substitution caused substantial loss of ligand binding and receptor function (Fig. 4g, h). The His170Ala mutation essentially abolished antagonist binding, and lowered the maximum agonist-induced [35S]GTP$\gamma$S binding to half that of wild-type level (Fig. 4g, h). These data indicate that both Trp 65 and His 170 are indispensable for ligand recognition.

The LB2 residues are essential for agonist binding. First, the Trp278Ala mutant retained the ability to bind the antagonist [3H]CGP54626 ANT, although with decreased potency (Fig. 4g). This is consistent with the auxiliary role of Trp 278 in antagonist recognition. However, this mutation is detrimental to receptor activation, because it not only reduced the maximum GABA-dependent [35S]GTP$\gamma$S binding, but also increased the half-maximum effective concentration (EC$_{50}$) of GABA by more than 500-fold (Fig. 4h). Second, the Tyr250Ala mutation had no effect on antagonist binding, in agreement with our structural observations (Fig. 4g). However, it decreased the agonist response, and increased the EC$_{50}$ of GABA by more than 100-fold (Fig. 4h). These data indicate that both Tyr 250 and Trp 278 are critical to agonist recognition.

**Implications for receptor activation**

Structural comparison indicates that the concept of major intersubunit relocation that holds for the activation of mGluRs cannot be applied to GABAR$_{s}$ receptor. The extracellular domains of these receptors share a common mode of dimerization through their LB1 domains (Supplementary...
Figure 5 | Constitutive activity of disulphide-tethered GBR1b:GBR2 heterodimer. a, Position of cysteine mutations (spheres) at the LB2–LB2 heterodimer interface of (R)-baclofen_AGG-GBR1b_VFT:GBR2_VFT. b, Western-blot analysis of membranes from cells expressing different combinations of wild-type (WT) and mutant GABA\(_B\) receptor subunits (GBR1b-Thr198Cys, abbreviated as T198C; GBR2-Gln206Cys, abbreviated as Q206C). The samples were assayed in the presence of 10 \(\mu\)M GABA under reducing (+DTT) and non-reducing (−DTT) conditions. The double cysteine mutant (Thr198Cys Gln206Cys) was also analysed in the absence of ligand. GBR1b and GBR2 were detected by anti-Flag and anti-HA antibodies, respectively. Arrow 1, GBR1b–GBR2 heterodimer; arrows 2 and 4, GBR2 monomer; arrows 3 and 5, GBR1b monomer. c, GABA-stimulated, dose-dependent \(^{35}\text{S}\)GTP\(_\gamma\)S binding in membranes from cells expressing wild-type or various cysteine mutant receptors in the presence or absence of DTT. Data points represent average ± s.e.m. of triplicate measurements.

Methods summary

The extracellular VFT module of human GBR1b and GBR2 were co-secreted from baculovirus-infected insect cells, and purified by anti-Flag M2 antibody affinity chromatography followed by size-exclusion chromatography. Apo and antagonist-bound GBR1b:VFT:GBR2:VFT complexes were crystallized in the P\(_2\)\(_1\)2\(_1\)2 space group. All of the structures were solved by molecular replacement. Radioligand binding and agonist-stimulated \(^{35}\text{S}\)GTP\(_\gamma\)S binding were measured using HEK293 cells co-transfected with complementary DNAs encoding full-length GBR1b and GBR2.

Received 8 January; accepted 30 September 2013.

1. Betler, B., Kaumann, K., Mosbacher, J. & Gassmann, M. Molecular structure and physiological functions of GABA(B) receptors. Physiol. Rev. 84, 835–867 (2004).
Kunishima, N. et al. Structural basis of glutamate recognition by a dimeric metabotropic glutamate receptor. *Nature* **407**, 971–977 (2000).

Tsuchiya, D., Kunishima, N., Kamiya, N., Jingami, H. & Morikawa, K. Structural views of the ligand-binding cores of a metabotropic glutamate receptor complexed with an agonist and both glutamate and Gd(III). *Proc. Natl Acad. Sci. USA* **99**, 2660–2665 (2002).

Muto, T., Tsuchiya, D., Morikawa, K. & Jingami, H. Structures of the extracellular regions of the group II/III metabotropic glutamate receptors. *Proc. Natl Acad. Sci. USA* **104**, 3759–3764 (2007).

van den Akker, F. et al. Structure of the dimerized hormone-binding domain of a guanylyl-cyclase-coupled receptor. *Nature* **406**, 101–104 (2000).

He, X., Chow, D., Martick, M. M. & Garcia, K. C. AllostERIC activation of a spring-loaded natriuretic peptide receptor dimer by hormone. *Science* **293**, 1657–1662 (2001).

Jin, R. et al. Crystal structure and association behaviour of the GluR2 amino-terminal domain. *EMBO J.* **28**, 1812–1823 (2009).

Karacas, E., Simorowski, N. & Furukawa, H. Structure of the zinc-bound amino-terminal domain of the NMDA receptor NR2B subunit. *EMBO J.* **28**, 3910–3920 (2009).

Kumar, J., Schuck, P., Jin, R. & Mayer, M. L. The N-terminal domain of GluR6-subtype glutamate receptor ion channels. *Nature Struct. Mol. Biol.* **16**, 631–638 (2009).

Sack, J. S., Saper, M. A. & Quiocho, F. A. Periplasmic binding protein structure and function. *Annu. Rev. Biochem.* **78**, 71–99 (2009).

Kniazeit, J. et al. Binding of the dimeric GABA(B) receptor to its active state. *J. Neurosci.* **24**, 370–377 (2004).

Rondard, P. et al. Functioning of the dimeric GABA(B) receptor extracellular domain revealed by glycan wedge scanning. *EMBO J.* **27**, 1331–1332 (2008).

Galvez, T. et al. Mutagenesis and modeling of the GABA(B) receptor extracellular domain support a venus flytrap mechanism for ligand binding. *J. Biol. Chem.* **274**, 13362–13369 (1999).

Galvez, T. et al. Mapping the agonist-bcing site of GABA(B) type 1 subunit sheds light on the activation process of GABA(B) receptors. *J. Biol. Chem.* **275**, 41166–41174 (2000).

Galvez, T. et al. Ca(2+)-induced activation of functional GABA(B) receptors: involvement of serine 269 of the GABAB(B1) subunit. *Mol. Pharmacol.* **57**, 419–426 (2000).

Kniazeit, J. et al. Closed state of both binding domains of homodimeric mGlu receptors is required for full activity. *Nature Struct. Mol. Biol.* **11**, 706–713 (2004).

Furukawa, H., Singh, S. K., Mannusco, R. & Gouaux, E. Subunit arrangement and function in NMDA receptors. *Nature* **438**, 185–192 (2005).

Kammerer, R. A. et al. Heterodimerization of a functional GABA(B) receptor is mediated by parallel coiled-coil alpha-helices. *Biochemistry* **38**, 13263–13269 (1999).

Supplementary Information is available in the online version of the paper.

Acknowledgements We thank W. A. Hendrickson and R. Kass for advice and support; I. Berger for the gift of pBDm vector; K. Rajashankar, K. Perry, S. Banerjee, F. Murphy, I. Kouznetzov and D. J. Neau for help with data collection; Y. Chen for technical assistance; and M. Evelyn for writing the manuscript. This work was supported by the American Heart Association grant SDG083183N and the National Institute of Health grant R01GM088454 (both to Q.R.F.). Q.R.F. is an Irma Hirsch Career Scientist, Pew Scholar, McKnight Scholar and Schaefer Scholar.

Author Contributions Q.R.F. initiated the study and designed the experiments; Y.Q., Q.R.F., M.B., L.M. and F.W. performed experiments and analysed data; Q.R.F. and Y.Q. wrote the paper.

Author Information Atomic coordinates and diffraction data for the structures reported here are deposited in the RCSB Protein Data Bank under accession codes 4M0Q, 4M0F, 4MRF, 4MR9, 4MRM, 4MS1, 4MS3 and 4MS4. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to Q.R.F. (qrf3@columbia.edu).
METHODS
Protein expression and purification. The extracellular domains of human GBR1 and GBR2 were separately cloned into the pFBDM vector29 for expression in baculovirus-infected insect cells. The GBR1 isoform GBR1b was used in this study. The GBR1bVFT construct contained residues 48–459, with the signal peptide of baculovirus envelope surface glycoprotein gp67 attached at the N terminus and a Flag tag at the C terminus. The GBR2VFT construct contained residues 1–466 and a C-terminal Flag tag, as previously described26. S9 insect cells were co-infected with recombinant GBR1bVFT and GBR2VFT baculoviruses at 23 °C for 96 h. The GBR1bVFT:GBR2VFT complex was purified from cell supernatant by anti-Flag antibody (M2) affinity chromatography followed by gel filtration chromatography (Superdex 200, GE Healthcare). The GCP54626-ANT-GBR1bVFT:GBR2VFT complex was produced in the presence of 10 mM GCP54626-ANT throughout expression and 20 mM GCP54626-ANT during purification. The (R)-baclofen-Ago-GBR1bVFT:GBR2VFT complex was expressed and purified in the presence of 100 μM (R)-baclofen and the GABA-GBR1bVFT:GBR2VFT complex was produced in the presence of 100 μM GABA.

Crystalization and data collection. Crystals of the apo-GBR1bVFT:GBR2VFT complex were grown at 4 °C in 10% PEG 3350, 20% glycerol and 0.12 M Na acetate, pH 7.0. Crystals of various antagonist-bound GBR1bVFT:GBR2VFT complexes were obtained under the same condition as the apo complex. Specifically, the GCP54626-ANT-bound hexamer was crystallized using protein that was purified in the presence of GCP54626-ANT. The apo-GBR1bVFT:GBR2VFT complex was also co-crystallized with 10 mM of each of the following antagonists: GCP43813-ANT, GCP35348-ANT, SCH50911-ANT, (R)-2-OH-saclofen-ANT and (R)-phaclofen-ANT. All of the crystals were directly frozen from drops.

The agonist-bound (R)-baclofen-Ago-GBR1bVFT:GBR2VFT complex was crystallized at 20 °C from 20% PEG 2000, 15% glycerol, 0.2 M NH₄Cl and 0.1 M Na cacodylate, pH 5.2, in the presence of 10 mM (R)-baclofen. Crystals of the GABA-GBR1bVFT:GBR2VFT complex were grown at 20 °C from 18% PEG 2000, 5% glycerol, 0.15 M NaCl and 0.1 M Na cacodylate, pH 5.0, in the presence of 10 mM GABA. The crystals were frozen in a cryoprotecting solution containing 20% glycerol and all other components of the crystallization solution.

Native data for the different complexes were collected at the 24ID-C and 24ID-E beamlines of the Advanced Photon Source. Diffraction data for the apo complex and the GCP46381-ANT, GCP53548-ANT, SCH50911-ANT and GABA-bound complexes were integrated using XDS30 and scaled with SCALA31. Data for the GCP54626-ANT, (S)-2-OH-saclofen-ANT and (R)-phaclofen-ANT and (R)-baclofen-Ago-bound complexes were integrated and scaled using HKL200032.

Structure determination. The structure of the apo-GBR1bVFT:GBR2VFT complex was solved by molecular replacement. The position of GBR2VFT was identified using the structure of free GBR2VFT (PDB code 4F11; ref. 28) as the search model. The location of GBR1bVFT was found by using the individual L1 and L2 domains of GBR2VFT as the search probes. A complete atomic model of the apo-GBR1bVFT:GBR2VFT complex was developed through a succession of manual building and iterative refinement. The final model contained the GBR1bVFT residues 48–368 and 377–459, the GBR2VFT residues 53–292, 300–379 and 385–466, and part of the Flag tag at the C termini of both subunits. Carbohydrate residues were also attached to Asn 323 and Asn 365 of GBR1bVFT, and to Asn 404 of GBR2VFT.

All of the antagonist-bound GBR1bVFT:GBR2VFT structures were solved by molecular replacement using the apo-GBR1bVFT:GBR2VFT structure as the search model. For each complex, the bound antagonist was modelled into the residual electron density map obtained in the final rounds of refinement. All of the antagonist-bound structures contained the GBR1bVFT residues 48–368 and 377–459, the GABA-GBR1bVFT:GBR2VFT complex contained the GBR1bVFT residues 50–368 and 377–459; the GABA-GBR1bVFT:GBR2VFT complex contained the GBR1bVFT residues 50–84, 92–337, 344–368 and 377–459. Both agonist-bound structures contained the GBR2VFT residues 50–291 and 302–466, and part of the Flag tag at the C termini of both GBR1bVFT and GBR2VFT. Carbohydrate residues were attached to Asn 404 of GBR2VFT.

Molecular replacement searches were carried out using Phaser33. Model building was performed with COOT34. Structural refinement was executed using Buster35. Ramachandran statistics were calculated for each structure using Molprobity36. Positional and structural comparisons were performed using Lsqman37. Software installation support was provided by SGGrid38.

Cell surface expression. Full-length human GBR1b and GBR2 were individually cloned into a pcDNA3.1+ (+) vector (Invitrogen) for expression in human embryonic kidney (HEK293) cells. A Flag tag was inserted after the signal peptide of GBR1b, and an HA tag was placed after the signal peptide of GBR2. Mutants of GBR1b and GBR2 were constructed using the QuikChange mutagenesis system (Stratagene).

HEK293 T/17 cells (ATCC) were co-transfected with Liptecogene 2000 (Invitrogen) with the GBR1b and GBR2 plasmids. Cells permeabilized with 0.5% Triton X100 were used to determine the total expression levels of GBR1b and GBR2 in transfected cells. Untreated cells were used to determine the cell surface expression level of each subunit. The amount of surface protein detected for each construct was normalized to that found in the total cell lysate.

The cells were blocked with 5% milk, and then incubated with mouse anti-Flag M1 antibody (Sigma) as the primary antibody to measure GBR1b expression. Similarly, mouse anti-HA antibody HA.11 clone 16B12 (Covance) was used to detect GBR2. Donkey anti-mouse IRDye 800-labelled antibody (LiCor) was used as the secondary antibody in both cases. Fluorescent signals were measured with an Odyssey Infrared Imagier (LiCor). The results of three independent experiments were used for statistical analysis. All of the mutants reported here were expressed on the cell surface at levels comparable to that of wild-type GABAA receptor.

Agonist-stimulated ([35S]GTPγS binding. HEK293 T/17 cells were transiently transfected with full-length GBR1b and GBR2 plasmids. The cells were collected in 50 mM HEPES, pH 7.4, to obtain the membrane fraction. Membranes were suspended in an assay buffer containing 50 mM Tris, pH 7.7, 100 mM NaCl, 12 mM MgCl₂, 1.8 mM CaCl₂ and 0.2 mM EGTA to approximately 400 μg protein per millilitre. The membrane homogenates were incubated with increasing concentrations of GABA in the presence of 10 μM GDP ([35S]GTPγS ([1.250 Ci mmol⁻¹]) was then added to a final concentration of 0.5 nM. After incubation at room temperature for 45 min, unbound [35S]GTPγS was removed by centrifugation. The amount of bound [35S]GTPγS was measured using a Beckman LS6500 liquid scintillation counter. Nonspecific binding was measured in the presence of 20 μM unlabelled GTPγS. Basal activity was determined in the absence of GABA. The basal activity of the wild-type receptor was used to calculate the percentage stimulation of the double cysteine mutant receptor GBR1b-Thr198Cys GBR2-Gln206Cys under non-reducing conditions. The reduced ([35S]GTPγS binding activity of the double cysteine mutant (~60% of the wild-type value) could be attributed to the effect of the mutations themselves, because introduction of a single cysteine mutation into either subunit also caused a decrease in agonist response.

To measure [35S]GTPγS binding under reducing conditions, the membrane homogenates were pre-incubated with 1 mM DTT before the addition of various concentrations of GABA and 10 μM GDP. The presence of DTT reduced the basal activity of all different combinations of wild-type and cysteine mutant receptors. The percentage stimulation of each receptor mutant was calculated on the basis of the wild-type response obtained under the same condition. Data analysis was performed using the nonlinear regression algorithms in PRISM (GraphPad Software). Data points represent average ± s.e.m. of triplicate measurements.

Radioligand binding assay. HEK293 T/17 cells were transiently transfected with full-length GBR1b and GBR2 plasmids. Cell membranes were suspended in an assay buffer containing 20 mM Tris, pH 7.4, 110 mM NaCl, 5.6 mM glucose, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 4.7 mM KC1 and 1.8 mM CaCl₂ to approximately 400 μg protein per millilitre. [35S]GTPγS ([25 Ci mmol⁻¹]) was added to the reaction mixture to final concentrations ranging from 0.5 to 20 nM. After incubation at room temperature for 30 min, unbound [35S]GTPγS was removed by centrifugation. The amount of bound [35S]GTPγS was measured by liquid scintillation counting. Nonspecific binding was measured in the presence of 10 μM unlabelled GABA. Data analysis was performed using the nonlinear regression algorithms in PRISM. Data points represent average ± s.e.m. of triplicate measurements.

Disulphide design and western blot analysis. The structure of (R)-baclofen-Ago-GBR1bVFT:GBR2VFT was used for the rational design of disulphide bonds at the LR2–LR2 heterodimer interface. The residue pair, GBR1b-Thr198 and GBR2-Gln206, was identified by the software DISULPHIDE BY DESIGN26 to have the proximity and geometry required for disulphide formation when mutated to cysteines.

©2013 Macmillan Publishers Limited. All rights reserved
The Thr198Cys and Gln206Cys mutations were engineered into full-length GBR1b and GBR2 in pcDNA3.1(+) respectively.

HEK293 T/17 cells were transiently transfected with equal amounts of the full-length GBR1b and GBR2 plasmids. Cells were harvested in a buffer containing 20 mM Tris, pH 7.5, 150 mM NaCl and 1% dodecyl-maltoside. After the insoluble materials were removed by centrifugation, the supernatant was analysed by 4–15% SDS polyacrylamide gel electrophoresis in the absence and presence of 100 mM DTT. In addition, formation of a disulphide-linked heterodimer between the cysteine mutant pair GBR1b-Thr198Cys and GBR2-Gln206Cys was analysed under two different conditions in the absence of any ligand, and in the presence of 10 mM GABA. Heterodimer formation of all other samples was analysed in the presence of 10 mM GABA. The samples were transferred to polyvinylidene fluoride membranes. After blocking with 5% milk, the membranes were incubated with a primary antibody. Mouse anti-Flag M1 antibody (Sigma) was used to detect the GBR1b protein. Mouse anti-HA antibody HA.11 clone 16B12 (Covance) was used to probe GBR2. Both were followed by an alkaline phosphatase (AP)-conjugated anti-mouse secondary antibody. Proteins were visualized by the colorimetric method.

50. Berger, I., Fitzgerald, D. J. & Richmond, T. J. Baculovirus expression system for heterologous multiprotein complexes. Nature Biotechnol. 22, 1583–1587 (2004).

51. Kabsch, W. Xds. Acta Crystallogr. D 66, 125–132 (2010).
52. Evans, P. Scaling and assessment of data quality. Acta Crystallogr. D 62, 72–82 (2006).
53. Otwinowski, Z. & Minor, W. Processing of X-ray diffraction data collected in oscillation mode. Methods Enzymol. 276, 307–326 (1997).
54. Kerr, D. I., Ong, J., Doolette, D. J., Schafer, K. & Prager, R. H. The (S)-enantiomer of 2-hydroxysclofen is the active GABAB receptor antagonist in central and peripheral preparations. Eur. J. Pharmacol. 287, 185–189 (1995).
55. Frydenvang, K. et al. GABAB antagonists: resolution, absolute stereochemistry, and pharmacology of (R)- and (S)-phaclofen. Chirality 6, 583–589 (1994).
56. McCoy, A. J. et al. Phaser crystallographic software. J. Appl. Crystallogr. 40, 658–674 (2007).
57. Emsley, P. & Cowtan, K. Coot: model-building tools for molecular graphics. Acta Crystallogr. D 60, 2126–2132 (2004).
58. Roveri, P., Blanc, E., Vonrhein, C., Evans, G. & Bricogne, G. Modelling prior distributions of atoms for macromolecular refinement and completion. Acta Crystallogr. D 56, 1316–1323 (2000).
59. Chen, V. B. et al. MolProbity: all-atom structure validation for macromolecular crystallography. Acta Crystallogr. D 66, 12–21 (2010).
60. Novotny, M., Madsen, D. & Kleywegt, G. J. Evaluation of protein fold comparison servers. Proteins 54, 260–270 (2004).
61. Morin, A. et al. Collaboration gets the most out of software. eLife 2, e01456 (2013).
62. Dombkowski, A. A. Disulfide by Design: a computational method for the rational design of disulfide bonds in proteins. Bioinformatics 19, 1852–1853 (2003).