Structures of metabotropic GABA\textsubscript{B} receptor

Stimulation of the metabotropic GABA\textsubscript{B} receptor by \(\gamma\)-aminobutyric acid (GABA) results in prolonged inhibition of neurotransmission, which is central to brain physiology\(^1\). GABA\textsubscript{B} belongs to family C of the G-protein-coupled receptors, which operate as dimers to transform synaptic neurotransmitter signals into a cellular response through the binding and activation of heterotrimERIC G proteins\(^2,3\). However, GABA\textsubscript{B} is unique in its function as an obligate heterodimer in which agonist binding and G-protein activation take place on distinct subunits\(^4,5\). Here we present cryo-electron microscopy structures of heterodimeric and homodimeric full-length GABA\textsubscript{B} receptors. Complemented by cellular signalling assays and atomistic simulations, these structures reveal that extracellular loop 2 (ECL2) of GABA\textsubscript{B} has an essential role in relaying structural transitions by ordering the linker that connects the extracellular ligand-binding domain to the transmembrane region. Furthermore, the ECL2 of each of the subunits of GABA\textsubscript{B} caps and interacts with the hydrophilic head of a phospholipid that occupies the extracellular half of the transmembrane domain, thereby providing a potentially crucial link between ligand binding and the receptor core that engages G proteins. These results provide a starting framework through which to decipher the mechanistic modes of signal transduction mediated by GABA\textsubscript{B} dimers, and have important implications for rational drug design that targets these receptors.

The neurotransmitter GABA is primarily responsible for synaptic inhibition throughout the nervous system, via activation of GABA\textsubscript{A} ion channels and pre- and postsynaptic GABA\textsubscript{B} receptors. GABA\textsubscript{B} stimulation of the G\(_{i/o}\) class of heterotrimeric G proteins results in a prolonged decrease in neuronal excitability via the inhibition of adenyl cyclase and voltage-gated Ca\textsuperscript{2+} channels, as well as the opening of G protein-coupled inward-rectifying potassium channels\(^6,3,6\). Abnormal execution of GABA\textsubscript{B} signalling is responsible for several neuropsychiatric diseases, and the receptor is an attractive drug target for a range of disorders, including drug addiction, pain, epilepsy, spasticity, anxiety and gastro-oesophageal reflux disease\(^7,8\).

There are about 20 G-protein-coupled receptors (GPCRs) in family C, including GABA\textsubscript{B}, the metabotropic glutamate receptors (mGlus) to mGlu8), the calcium-sensing receptor, two taste receptors and several orphan receptors\(^9,10\). These receptors operate as obligate dimers, in which each subunit is composed of a bilobed extracellular ligand-binding domain (termed the Venus flytrap (VFT)) and a 7-transmembrane domain (7TM) that are connected via a linker\(^11\). Previous crystallographic studies of the VFT from various members of family C (including GABA\textsubscript{B}) have shown that agonist binding rearranges the VFT in a way that would bring the linker regions into close proximity\(^12,13\). All GPCRs in family C contain a cysteine-rich domain within the linker region—with the exception of GABA\textsubscript{A}, which has a relatively short linker. Recent structures of mGlu5 have shown that the cysteine-rich domain interacts with ECL2 of the transmembrane region, thereby transducing conformational changes 120 Å away from the ligand-binding site on the VFT to the 7TM (where G-protein activation occurs)\(^14\). Given that GABA\textsubscript{B} lacks a cysteine-rich domain, the structural communication between an agonist-bound VFT and the 7TM remains unclear.

Furthermore, unlike other GPCRs in family C, GABA\textsubscript{B} is an obligate heterodimer of dissimilar subunits—GABA\textsubscript{B1} and GABA\textsubscript{B2}\(^15\). Agonist binding occurs only on the VFT of GABA\textsubscript{B1}, and G-protein coupling and activation occurs exclusively through GABA\textsubscript{B2}\(^16\). Therefore, besides its pharmacological interest, GABA\textsubscript{B} presents an ideal model system to study trans-activation mechanisms of dimeric family C GPCRs. However, such studies have been limited by the lack of structural information on full-length GABA\textsubscript{B}, restricting our ability to understand how agonist binding to GABA\textsubscript{B1} results in G-protein activation on the intracellular side of GABA\textsubscript{B2}. Here we use single-particle cryo-electron microscopy (cryo-EM), atomistic simulations and cellular signalling assays to obtain structural and mechanistic insights into full-length GABA\textsubscript{B} receptor complexes.

For structural studies, we co-expressed recombinant constructs of human GABA\textsubscript{B1} and GABA\textsubscript{B2} in insect cells and purified the GABA\textsubscript{B} heterodimer by tandem affinity chromatography in the presence of the inverse agonist CGP53845\(^17,18\). We determined the structure of the inactive detergent-solubilized GABA\textsubscript{B} heterodimer at a global indicated resolution of 3.6 Å (Fig. 1, Extended Data Figs. 1, 2a, Extended Data Table 1). Our cryo-EM map resolved the entire GABA\textsubscript{B} apart from the C-terminal coil–coil domain, which appears to be flexible in relation to the transmembrane regions. Additionally, our focused map

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of the extracellular region at a resolution of 3.5 Å provided improved density for the linker regions, and assisted with modelling (Extended Data Fig. 1). The asymmetric protomers (GABAB₁ and GABAB₂) share a similar secondary structure and arrangement, but are distinguished by differential glycosylation and a well-resolved ligand density within the GABAB₁ VFT that is absent in GABAB₂ (Fig. 1, Extended Data Figs. 3, 4a). The upper VFT lobes of GABAB form a junction, whereas the lower VFT lobes are separated by about 20 Å (Fig. 1, Extended Data Fig. 3). The CGP55845 ligand adopts a horseshoe-like conformation (which was confirmed by GemSpot™) that closely resembles the crystal structure of the GABAB₁ VFT in complex with the inhibitor CGP5462612,16 (Extended Data Fig. 3). We also observe a small spherical density in GABAB₁ that the GABAB₁ VFT in complex with the inhibitor CGP55845, gold; phospholipids, orange; glycosylation, blue and pink). Elongated densities at the 7TM interface are probably lipid or detergent molecules (yellow). elongated densities for the linker regions, and assisted with modelling (Extended Data Fig. 1). The asymmetric protomers (GABAB₁ and GABAB₂) share a similar secondary structure and arrangement, but are distinguished by differential glycosylation and a well-resolved ligand density within the GABAB₁ VFT that is absent in GABAB₂ (Fig. 1, Extended Data Figs. 3, 4a). The upper VFT lobes of GABAB form a junction, whereas the lower VFT lobes are separated by about 20 Å (Fig. 1, Extended Data Fig. 3). The CGP55845 ligand adopts a horseshoe-like conformation (which was confirmed by GemSpot™) that closely resembles the crystal structure of the GABAB₁ VFT in complex with the inhibitor CGP5462612,16 (Extended Data Fig. 3). We also observe a small spherical density in GABAB₁ that appears to be interacting with the backbone carbonyl of G277, in addition to several anionic groups and a tyrosine, which raises the possibility that a divalent cation resides in that location (Extended Data Fig. 3e).

Inactive GABAB assumes an overall morphology similar to that of the apo-state structure of mGlu514, the most substantial differences between these two arise within the linker region (Fig. 1b). Bridging the VFT and 7TM, an approximately 20-residue linker forms a β-sheet in conjunction with the ECL2. Notably, the length of the GABAB₁ receptor ECL2 is nearly twice that of mGlu5 (Fig. 1, Extended Data Fig. 4b). In the absence of cysteine-rich domains, the β-sheet structure of the GABAB₁ linker in complex with ECL2 orders this region, with the additional ECL2 length thus allowing coupling between the 7TM and VFT for signal transduction. Our molecular dynamics simulations support the structural stabilization of the linker through the β-sheet formation. After 200 ns, in all simulations the linker and ECL2 continued to adopt a stable structure even in the absence of the VFTs, although we did observe a slight downward rotation in β-sheet orientation (Extended Data Fig. 5). To further examine the involvement of ECL2 in receptor activation, we used a functional assay with a chimeric Gαo/q, in which the Gαo-coupled GABAB receptor can couple to the PLC pathway3,18. We measured the accumulation of the downstream metabolite inositol monophosphate (IP₁) by LiCl using an established assay19, and thereby monitored the GABA-stimulated and basal receptor activity (Fig. 1c). ECL2 shortening was generally inhibitory to the proper membrane trafficking of the subunits of GABAB. Accordingly, we normalized the transfected DNA to obtain similar expression levels between constructs (Extended Data Fig. 6a). The partial deletion of the ECL2 of GABAB₁ (residues 627–634)—comprising the unstructured tip of the loop nearest the VFT—produced an increase in basal activity, but did not affect the GABA maximal response (Eₘ₉₉) when expressed with wild-type GABAB₂ (Fig. 1d). These findings indicate that abrogating the ECL2-linker allows flexibility in the GABAB₁ VFT relative to the rest of the receptor dimer, resulting in activation through the GABAB₂ VFT–7TM route in the absence of agonist.20. By contrast, the partial deletion of the ECL2 of GABAB₂ (residues 631–638) did not affect basal activity, but produced an increase in GABA E₉₉ and a decrease in GABA potency when compared to wild-type receptor expressed at similar levels at the cell surface—indicating that the ECL2 of GABAB₂ may be partially inhibitory (Fig. 1d, Extended Data Fig. 6a). When both receptors contain a truncated ECL2, we observed a decrease in GABA E₉₉ which suggests that at least one VFT must be structurally coupled through the extended ECL2-linker for full activation (Fig. 1d). Collectively, these data support a bimodal transactivation mechanism of GABAB, in which agonist binding on the GABAB₁ receptor can proceed from the GABAB₁ agonist-binding site, directly promote G-protein activation, and also activate the GABAB₂ 7TM directly through the GABAB₂ VFT.

Besides of the VFT and the C-terminal coiled–coil, we observe that inactive GABAB forms an additional dimer interface through trans-membrane (TM3 and TM5 from each monomer of the heterodimer (Figs. 1, 2). The interface is formed by ionic interactions between residues on the intracellular side of each receptor—H572 in TM3 and E673 in TM5 of GABAB₁, and H579 in TM3 and E677 in TM5 of GABAB₂—and is...
Further stabilized through aromatic residues along the same helices (Fig. 2b, c). We also observed elongated densities, probably corresponding to glyco-diosgenin (CDN) and/or cholesteryl hemisuccinate (which were used in purification), packed between the extracellular sides of the two 7TM regions, which may also stabilize the interface (Fig. 1a, Extended Data Fig. 2i). The dimer interface contrasts with that of the inactive mGlur receptor, in which a distance of about 16 Å separates the two 7TM regions14 (Fig. 2a). To probe the importance of these interactions, we mutated the TM3–TM5 interface and performed IP, accumulation assays (Fig. 2d, e, Extended Data Fig. 6). Notably, mutation of either H579 or E677 on GABA_B2 to alanine, or mutation of both E673 and H579 on GABA_B1 to alanine, increased the basal activity of the receptor, which suggests that the TM3–TM5 interface is inhibitory to signalling in the absence of agonist, which further supports the transactivation mechanism described above (Fig. 2, Extended Data Fig. 6b–e). When expressed at the cell surface alone, GABA_B1(H579A/E677A) exhibited a slight increase in basal activity compared to GABA_B2 alone (Extended Data Fig. 6d). These findings further support a role of an intrarotamer HS79–E677 interaction in GABA_B2 to assist stabilizing an auto-inhibited state, and are consistent with the lack of constitutive activity by the GABA_B1(R549A) (Δ631–638) mutant.

The most unexpected observation in the transmembrane region of both monomers of GABA_B1 is a wishbone-shaped density that occupies the extracellular half of each 7TM core. The bifurcated density, which is better resolved within the GABA_B1 helical bundle, corresponds to a phospholipid (Fig. 3, Extended Data Fig. 2). Our consideration of the size and shape of the density, the surrounding amino acid environment within the 7TM core and the known phospholipid composition in Spodoptera frugiperda (SF9) insect cells led us to infer that the density in GABA_B1 and probably GABA_B2 corresponds to phosphatidylethanolamine (Fig. 3, Extended Data Fig. 7). However, it is possible that the observed density is the result of a heterogeneous mixture of phospholipids. The observation of the phospholipid is particularly intriguing, as—to our knowledge—no other GPCR structure has revealed a two-chained phospholipid within this space. Although a lipid-activated subfamily within family A GPCRs exists, known ligands are single-acyl-chain lipids, eicosanoids and sterols24. Notably, GABA_B1 residues in the extracellular loops (including ECL2) and TM3 of GABA_B1 coordinate the polar headgroup of the phospholipid, resulting in a ‘lid’ over the 7TM cavity that resembles the lipid-binding GPCRs of family A. The remaining hydrophilic atoms in the lipid appear solvent-exposed, whereas the lipid tails are buried deep into the hydrophobic portion of the transmembrane cavity (Fig. 3b, c). The presence of the 7TM lipid in our cryo-EM structures suggests it is bound tightly, as it is also suggested by its retention during detergent solubilization of the receptor. Additionally, a conserved TM6 tryptophan—which acts as an activating ‘toggle-switch’ in other GPCRs of family C22,23—is replaced by cysteine in both GABA_B1 and GABA_B2 (Extended Data Fig. 4c, d). This replacement is essential, as any probable tryptophan rotamer at that position would clash sterically with the bound phospholipid.

The presence of lipid in the 7TM core suggests this lipid may have a physiological role in the structural and functional integrity of the transmembrane bundles, occupying a region that corresponds to the ligand-binding site in other classes of GPCR (Extended Data Fig. 4). Moreover, given the role of the ECL2 in coupling the VFT to the 7TM regions, it is conceivable that interactions of the phospholipid with the ECL2 are essential for increasing the stability of the linker region and also participate in relaying structural transitions from the VFT to the 7TM core. Mutations designed to displace lipid tails in the 7TM core (GABA_B1(R549A) and GABA_B2(R556A)) results in increases in both GABA_B1(R549A) and GABA_B2(R556A), with both GABA_B1(R549A) and GABA_B2(R556A), which further suggests the role of phospholipids in the structure and function of GABA_B2 (Extended Data Fig. 4f, g). Collectively, these results indicate that destabilization of phospholipid in the core affects receptor activation, and it is thus conceivable that the lipid directly modulates receptor activity as a result of ECL2 movements in native GABA_B. To further probe the role of phospholipid, we used a range of atomistic simulations with the GABA_B receptor system. We initiated simulations of the GABA_B7TM and linker with the VFT removed, both with and without lipid.

The simulations revealed that without lipid the transmembrane helices begin to collapse into the core, with mean decreases in cavity volume of about 30% (Fig. 3, Extended Data Fig. 5, Supplementary Data 1, 2); this suggests a strongly coupled interplay between the phospholipid and 7TM core. Alternatively, multiple simulations that started without

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**Fig. 3 Phospholipid binds within the transmembrane cores of GABA_B.**

a. Electron microscopy map clipped to show the location of phospholipid within GABA_B1 of the heterodimer. b. Ribbon representation of phosphatidylethanolamine within GABA_B1, with boxed region (c) to show the structural environment of the headgroup. d. GABA concentration response for wild-type GABA_B1 (red), and mutants designed to displace phospholipid (green) or de-stabilize the phospholipid headgroup (blue). e, f. Molecular dynamics simulations show a collapse of the transmembrane cavity when lipid is absent. e. Violin plot of cavity-volume ensemble data. f. Representative top-down view of the GABA_B1 ribbon and stick model from molecular dynamics simulations at 200 ns.
lipid in the transmembrane helices showed lipid tails from the bilayer entering the receptor hydrophobic cavity below residue Y661, at a site akin to that at which the tail protrudes in our cryo-EM structure. Peripheral lipid-tail insertion in simulations correlated with circumvention of the collapse of the 7TM cavity (Extended Data Fig. 5). In one simulation, after 200 ns we observed a lipid that had inserted both of its hydrocarbon tails into the receptor core. Extending the simulation by an additional 100 ns revealed the headgroup had moved over the top of the receptor, indicating lipid entry may occur as a stepwise process in which the lipid-tail enters by sliding between TM5 and TM6, and is followed by the entire lipid (Extended Data Fig. 5). Although it is probable that lipid insertion into the 7TM core occurs concurrently with helix insertion into the membrane during protein folding, these results suggest a clear tendency for phospholipids to insert at that position in a mature receptor.

Pharmaceutical agents targeting the GABA<sub>B</sub> receptor and other GPCRs of family C are proposed to function either at the orthosteric ligand-binding site or allosterically within the transmembrane core, as with orthosteric ligands of family A GPCRs. However, the bound phospholipid appears to occlude the binding of allosteric modulators analogous to those used to target other family C receptors<sup>44</sup>. Consequently, potential allosteric modulators of GABA<sub>B</sub> would need to either displace the core lipid or bind at an alternative site peripheral to the bundle.

Although we obtained pure GABA<sub>B1</sub> heterodimer by tandem affinity purification of receptor constructs with distinct tags, when both subunits contain an N-terminal Flag tag we also purified a considerable fraction (~40%) of GABA<sub>B2</sub> homodimers. In cells, an endoplasmic-reticulum retention signal within the GABA<sub>B2</sub> coil–coil domain prevents GABA<sub>B2</sub> from reaching the plasma membrane, unless this domain is masked by the GABA<sub>B2</sub> C terminus or other interacting partners<sup>23,26</sup>. Thus, the presence of GABA<sub>B2</sub> homodimer in our preparation is probably due to the persistence of internal membranes during the purification procedure. However, multiple studies have previously suggested a physiological role for GABA<sub>B2</sub> homodimers within some cell types of the nervous system and gastrointestinal tract that express GABA<sub>B2</sub> and that replacement of one of the 7TMs of the GABA<sub>B2</sub> homodimer with that of the GABA<sub>B1</sub> receptor (thus enabling G-protein coupling) produces a constitutively active receptor that is not additionally stimulated by GABA<sub>B1</sub> agonists<sup>33</sup>. Hence, the relative positioning of the VFT–as observed in the structure of the GABA<sub>B2</sub> homodimer—appears to be sufficient to stabilize an active conformation, presumably through the linkage of VFT to TM6 to reorient the helical interface. Because GABA<sub>B2</sub> in the homodimer is bound to an inverse agonist, the adoption of an ‘active-like’ global conformation of the VFT must be irrespective of the ligand, possibly owing to differences in the interaction interface of the VFT N-terminal lobes in comparison to the same interface in the heterodimer.

Collectively, our structures, cellular signalling assays and atomistic simulations provide crucial insights into mechanistic aspects of GABA<sub>B</sub> signalling. The extended ECL2 and its interaction with the linker region appears to compensate for the lack of a cysteine-rich domain in

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Fig. 4 | Inhibitor-bound GABA<sub>B1</sub> homodimers adopt a VFT orientation similar to that of active mGlu5. a, VFT overlay of structures of GABA<sub>B1</sub> heterodimer (tan and teal) in inactive (top) and active (bottom) state (PDB 4MS4<sup>12</sup>), with the model of GABA<sub>B2</sub> homodimer (blue). The upper lobe interaction interface (boxed) of the GABA<sub>B1</sub> homodimer matches that of the active state of the GABA<sub>B2</sub> heterodimer. b, The GABA<sub>B1</sub> subunit of the heterodimer (teal) and homodimer (blue) were aligned to the 7TM, revealing differences in VFT and linker positioning. c, Top-down view of superposed 7TM of GABA<sub>B1</sub> heterodimer (tan and teal) and homodimer (blue), showing the marked difference in the protomer interface. d, Top-down view of superimposed 7TM of agonist-bound mGlu5 (purple) (PDB 6N51<sup>14</sup>) and GABA<sub>B1</sub> homodimer (blue).
GABA_B compared to all other family C receptors, and thus also transduce conformational changes from the VFT to the 7TM regions. The phospholipid that occupies the 7TM core is important for the integrity of the transmembrane domains in both subunits of GABA_B, while also structurally coordinating the critical ECL2 region. Our findings support a model in which agonist binding to GABA_B results in VFT dimerization that reorients the protomers via the linker–ECL2. Such a conformational change would drive the 7TMs to twist away from an auto-inhibited state mediated by the inactive TM3–TM5 interface, thereby forming a new TM6 helical interface. Although currently unclear, the activating transitions within the GABA_B 7TMs are probably mediated both through the newly formed TM6–TM6 interface and propagation through its own ECL2, as supported by our functional assays. An intriguing possibility is that the phospholipid may act as a sensor of changes in VFT and ECL2 conformations that would result in activating transitions within the 7TM of GABA_B to prime it for G-protein engagement. Addressing these questions will require detailed structural studies of the active-state heterodimer alone, and in complex with a G protein engaging the receptor core. The present work—along with recent studies on mGlus5—forms a starting structural framework for deciphering the signal transduction mechanism of GABA_B and family C GPCRs in the context of full-length receptors.

Online content
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1. Bettler, B., Kaupmann, K., Mosbacher, J. & Gassmann, M. Molecular structure and physiological functions of GABA_B receptors. Physiol. Rev. 84, 835–887 (2004).
2. Mannoury la Cour, C., Herbelles, C., Pasteau, V., de Nanteuil, G. & Millan, M. J. Influence of amino acids switches receptor specificity of Gq to that of Gi. Nature 363, 274–276 (1993).
3. Franek, M. et al. The heteromeric GABA-B receptor recognizes G-protein α subunit agonists and antagonists: pharmacological properties and therapeutic possibilities. Expert Opin. Invest. Drugs 6, 1319–1325 (1997).
4. Malcangio, M. GABA_B receptors and pain. Neuropharmacology 136 (Pt A), 102–105 (2018).
5. Pin, J. P., Galvez, T. & Prézeau, L. Evolution, structure, and activation mechanism of family C/G-protein-coupled receptors. Pharmacol. Ther. 98, 325–354 (2003).
6. Bräuner-Osborne, H., Wellendorph, P. & Jensen, A. A. Structure, pharmacology and therapeutic prospects of family C G-protein coupled receptors. Curr. Drug Targets 8, 169-184 (2007).

11. Chun, L., Zhang, W. H. & Liu, J. F. Structure and ligand recognition of class C GPCRs. Acta Pharmacol. Sin. 33, 312–323 (2012).
12. Geng, Y., Bush, M., Mosyak, L., Wang, F. & Fan, Q. R. Structural mechanism of ligand activation in human calcium-sensing receptor. eLife 6, e36662 (2016).
13. Koehl, A. et al. Structural insights into the activation of metabotropic glutamate receptors. Nature 566, 79–84 (2019).
14. Margeta-Mitrovic, M., Jan, Y. N. & Jan, L. Y. A trafficking checkpoint controls GABA_B receptor heterodimerization. Neuron 27, 97-106 (2000).
15. Mukherjee, R. S., McBride, E. W., Beinborn, M., Dunlap, K. & Kopin, A. S. Point mutations in either subunit of the GABA_B receptor confer constitutive activity to the heterodimer. Mol. Pharmacol. 70, 1406-1413 (2006).
16. Robertson, M. J., van Zundert, G. C. P., Borrelli, K. & Skiiniotis, G. GemSpot: a pipeline for robust modeling of ligands into cryo-EM maps. Structure 28, 707-716 (2020).
17. Conklin, B. R., Farfel, Z., Lustig, K. D., Julius, D. & Bourne, H. R. Substitution of three amino acids switches receptor specificity of Gq to that of Gi. Nature 363, 274–276 (1993).
18. Trinquet, E. et al. Iso-Myo-Inositol 1-phosphate as a surrogate of iso-myoinositol 1,4,5-trisphosphate to monitor G protein-coupled receptor activation. Anal. Biochem. 358, 126–135 (2006).
19. Liu, J. et al. Molecular determinants involved in the allosteric control of agonist affinity in the GABA_B receptor by the GABA_B2 subunit. J. Biol. Chem. 279, 15824–15830 (2004).
20. Audet, M. & Stevens, R. C. Emerging structural biology of lipid G protein-coupled receptors. Proc. Natl. Acad. Sci. USA 28, 292–304 (2019).
21. Doré, A. S. et al. Structure of class C GPCR metabolotropic glutamate receptor 5 transmembrane domain. Nature 911, 557–562 (2014).
22. Frangia, A. & Fan, Q. R. Structural biology of GABA_B receptor. Neuropharmacology 136 (Pt A), 68–79 (2018).
23. Gasparrini, F. & Spooren, W. Allosteric modulators for mGlus receptors. Curr. Neuropharmacol. 5, 187–194 (2007).
24. Pagano, A. et al. C-terminal interaction is essential for surface trafficking but not for heteromeric assembly of GABA_B receptors. J. Neurosci. 21, 1189–1202 (2001).
25. Balasubramanian, S., Teissére, J. A., Raju, D. V. & Hall, R. A. Hetero-oligomerization between GABA_A and GABA_B receptors regulates GABA_B receptor trafficking. J. Biol. Chem. 279, 18840–18850 (2004).
26. Hyland, N. P. & Cryan, J. F. A gut feeling about GABA: focus on GABAB receptors. Front. Pharmacol. 1, 124 (2010).
27. Ng, T. K. & Yung, K. K. Differential expression of GABA_AR1 and GABA_AR2 receptor immunoreactivity in neurochemically identified neurons of the rat neostriatum. J. Comp. Neurol. 433, 458–470 (2001).
28. Burman, K. J. et al. GABA_B receptor subunits, R1 and R2, in brainstem catecholamine and serotonin neurons. Brain Res. 970, 35–46 (2003).
29. Calver, A. R. et al. The expression of GABA_AR1 and GABA_AR2 receptor subunits in the CNS differs from that in peripheral tissues. Neuroscience 100, 155–170 (2000).
30. Gassmann, M. et al. Redistribution of GABA_AR1 protein and atypical GABA_AR1 responses in GABA_AR2-deficient mice. J. Neurosci. 24, 6086–6097 (2004).
31. Margeta-Mitrovic, M., Jan, Y. N. & Jan, L. Y. Ligand-induced signal transduction within heterodimeric GABA_B receptor. Proc. Natl. Acad. Sci. USA 98, 14643–14648 (2001).
32. Xue, L. et al. Rearrangement of the transmembrane domain interfaces associated with the activation of a GPCR hetero-oligomer. Nat. Commun. 10, 2765 (2019).
33. Binet, V. et al. The heptahelical domain of GABA_AR3 is activated directly by CGP9930, a positive allosteric modulator of the GABA_AR3 receptor. J. Biol. Chem. 279, 29085–29091 (2004).
34. Galvez, T. et al. Allosteric interactions between GB1 and GB2 subunits are required for optimal GABA_AR2 receptor function. EMBO J. 20, 2152–2159 (2001).
**Methods**

No statistical methods were used to predetermine sample size. The experiments were not randomized and investigators were not blinded to allocation during experiments and outcome assessment.

**Cloning**

The cDNA clone for human GABA<sub>B</sub><sub>1</sub> receptor (accession NM_005458) in pcDNA3.1<sup>®</sup> was obtained from the cDNA Resource Center (www.cdna.org); and the cDNA clone for human GABA<sub>B</sub><sub>2</sub> purchased from Horizon Discovery (accession BC050532, clone ID 5732186). Primers were designed to include a haemagglutinin (HA) signal sequence in the place of authentic signal sequences of each receptor, thus removing the first 29 residues of GABA<sub>B</sub><sub>1</sub> and 41 residues of GABA<sub>B</sub><sub>2</sub>. Both the authentic signal sequence and the HA sequence are cleaved during processing; therefore, the substitution does not result in a change of sequence in the mature receptor. For purification of receptors for cryo-EM studies, GABA<sub>B</sub> constructs were subcloned into the pFastBacDual vector (Invitrogen) with N-terminal Flag epitope (DYKDDDDK) following the HA signal sequence and/or C-terminal hexa-histidine (His<sub>6</sub>) tags, so that the following constructs were produced: HA−Flag−GABA<sub>B</sub><sub>1</sub>(30−844)−His<sub>6</sub>, HA−GABA<sub>B</sub><sub>2</sub>(30−844)−His<sub>6</sub> and HA−Flag−GABA<sub>B</sub><sub>2</sub>(41−941). For signalling assays, the HA−Flag−GABA<sub>B</sub><sub>1</sub>(30−844)−His<sub>6</sub> and a HA−HA−GABA<sub>B</sub><sub>2</sub>(41−941) construct (in which the Flag tag was replaced by the HA tag) were subcloned into pcDNA3.1<sup>®</sup>. The primers (Integrated DNA Technologies) used to subclone GABA<sub>B</sub> included: EcoRI-HA-Flag-GABA<sub>B</sub><sub>1</sub>, 5′-GGCGCGGATCCATAAGAGCATCTAGCCGTGACACCTCTCTGCTTGGTTGCTCCACATGATTAGTGGTTGCTTTAAGAAGATGAC-3′; GABA<sub>B</sub><sub>1</sub>−Sall, 5′-GGCCCGTCGACTTAAATGATGATGATGGTGCTTATAAAGCAAATGCAC-3′; GABA<sub>B</sub><sub>2</sub>(30−844)−His<sub>6</sub>, 5′-GGCCCGTCGACTTAAATGATGATGATGGTGCAGGCCCGAGACCATGAC-3′; and GABA<sub>B</sub><sub>2</sub>-SalI, 5′-GGCGCGCGTCGACTTAATGATGATGATGGTGCAGGCCCGAGACCATGAC-3′; GABA<sub>B</sub><sub>2</sub>-His6-SalI, 5′-GCGCGCGTCGACTTAAATGATGATGATGGTGCAGGCCCGAGACCATGAC-3′; GABA<sub>B</sub><sub>1</sub>-SalI, 5′-GCGCGCGTCGACTTAATGATGATGATGGTGCAGGCCCGAGACCATGAC-3′; GABA<sub>B</sub><sub>1</sub>-His6-SalI, 5′-GCGCGCGTCGACTTAAATGATGATGATGGTGCAGGCCCGAGACCATGAC-3′; and GABA<sub>B</sub><sub>2</sub>-His6-SalI, 5′-GCGCGCGTCGACTTAAATGATGATGATGGTGCAGGCCCGAGACCATGAC-3′. To generate GABA<sub>B</sub> mutants, PCR reactions were performed with either Pfu Turbo or Q5 polymerase using the following pairs of primers and pcDNA3.1<sup>®</sup> neo containing either HA−Flag−GABA<sub>B</sub> or HA−GABA<sub>B</sub>: GABA<sub>B</sub><sub>1</sub>-HindIII, 5′-GGTTGCGTGGCCAGCCAGCTTC-3′ and 5′-GAAGACCCGCGGCCAACACC-3′; GABA<sub>B</sub><sub>1</sub>-EcoRI, 5′-CTCGTTCTTGCTGAGCTGATGATGATGGTGCAGGCCCGAGACCATGAC-3′; and GABA<sub>B</sub><sub>2</sub>-EcoRI, 5′-CTCGGAGAGTCGCTGCCATCTTCAA-3′ and 5′-TTGAAGATGGCCTGGTGTTCGCCGATTACAAGGACGACGATGACAAGTCCACTCCCC-3′.

**Expression and purification**

S<sub>9</sub> insect cells (Expression Systems) were coinfected at a density of about 2.0 × 10<sup>7</sup> cells/ml with HA-Flag-GABA<sub>B</sub><sub>1</sub>, His<sub>6</sub> or HA-GABA<sub>B</sub><sub>2</sub>, His<sub>6</sub> baculovirus and either HA-Flag-GABA<sub>B</sub><sub>2</sub> or His<sub>6</sub> or HA-GABA<sub>B</sub><sub>2</sub>, His<sub>6</sub> baculovirus at a multiplicity of infection between 3.0 and 5.0. During expression, cells were treated with 5 μM CGP55845 (Hello Bio). At 48 h post-infection, cells were collected by centrifugation, washed once with phosphate-buffered saline containing protease inhibitors (leupeptin, soybean trypsin inhibitor, N-p-tosyl-L-phenylalanine chloromethyl ketone, tosyl-t-lysin-chloromethane hydrochloride, phenylmethylsulfonyl fluoride, aprotinin, bestatin and pepstatin) and 5 μM CGP55845. Cell lysis was achieved through nitrogen cavitation in buffer containing 20 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 10 μM CGP55845, 2 mM MgCl<sub>2</sub>, nuclease and protease inhibitors. The whole-cell lysate was centrifuged at 1,000 g to remove nuclei and unbroken cells. The supernatant was centrifuged at 100,000 g to isolate the membrane fraction. Membranes were resuspended by Dounce homogenization in buffer containing 20 mM HEPES, pH 7.5, 150 mM NaCl, 2 mM MgCl<sub>2</sub>, 1 mM EDTA, 2 mg/ml iodoacetamide, 10 μM CGP55845, 1% n-dodecyl β-D-maltoside (DDM), 0.2% sodium cholate, 0.2% cholesterol hemisuccinate (CHS), nuclease and protease inhibitors. Solubilized membranes were clarified by centrifugation at 100,000 g, and the supernatant was loaded onto a pre-equilibrated column of anti-DYKDDDDK G1 affinity resin (Genscript). The resins was washed with buffer A (20 mM HEPES, pH 7.5, 150 mM NaCl, 10 μM CGP55845 and protease inhibitors) with 0.1% DDM, 0.02% CHS, 0.02% His<sub>6</sub> and 0.2 mg/ml DYLKDDEE peptide. The eluate was then loaded onto a pre-equilibrated Nickel-NTA column. Washes with buffer A containing 0.1% DDM and 0.02% CHS; and the buffer was exchanged in six steps to buffer B supplemented with 0.2% DDM, 0.02% CHS, followed by a two-step exchange into buffer A containing 0.004% DDM and 0.0004% CHS. Protein was eluted from the Ni-NTA resin with buffer B containing 0.004% DDM, 0.0004% CHS and 500 mM imidazole. The resulting eluate was concentrated by centrifugal filtration with a 50-kDa molecular-weight cut off, and subsequently run on a Superose 6 size-exclusion column (GE Healthcare). Samples were pre-screened for sample quality by negative stain transmission electron microscopy and then immediately prepared on cryo-EM grids.

**Cryo-EM data collection**

For the GABA<sub>B</sub><sub>1</sub>−GABA<sub>B</sub><sub>2</sub> heterodimer, 3.5 μl of sample was applied at a concentration of 3–5 mg/ml to glow-discharged holey carbon grids (Quantifoil R2/1.3). The grids were blotted using an FEI Vitrobot Mark IV (Thermo Fisher Scientific) at 18 °C and 100% humidity, and plunge-frozen into liquid ethane. Two datasets were used to produce the final structure. For both data collections, cryo-EM imaging was performed on a Titan Krios (Thermo Fisher Scientific) electron microscope equipped with a K3 Summit direct electron detector (Gatan). The microscope was operated at 300 kV accelerating voltage, at a magnification of 57,050 × in counting mode resulting in a magnified pixel size of 0.8521 Å. For the first dataset, movies were obtained at an exposure rate of 14.19 electrons per Å<sup>2</sup> per s with defocus ranging from −1.5 to −2.7 μm. The total exposure time was 2.98 s over 57 frames per movie stack. For the second dataset, movies were obtained at an exposure rate of 21.43 electrons per Å<sup>2</sup> per s with defocus ranging from −1.2 to −2.5 μm. The total exposure time was 2.99 s including 50 frames per movie stack.

Cryo-EM grids for the GABA<sub>B</sub><sub>2</sub> homodimer at a concentration of 5.0 mg/ml were prepared similarly to the heterodimer. Cryo-EM imaging was performed on a Titan Krios electron microscope equipped with a K3 Summit direct electron detector (Gatan). The microscope was operated at 300 kV accelerating voltage, at a magnification of 57,050 × in counting mode resulting in a magnified pixel size of 0.8521 Å. For the first dataset, movies were obtained at an exposure rate of 14.19 electrons per Å<sup>2</sup> per s with defocus ranging from −1.5 to −2.7 μm. The total exposure time was 2.98 s over 57 frames per movie stack. For the second dataset, movies were obtained at an exposure rate of 21.43 electrons per Å<sup>2</sup> per s with defocus ranging from −1.2 to −2.5 μm. The total exposure time was 2.99 s including 50 frames per movie stack.

**Image processing and 3D reconstructions**

Dose-fractionated image stacks were subjected to beam-induced motion correction and dose-weighting using MotionCor2<sup>®</sup>. Contrast transfer function (CTF) parameters for each non-dose weighted micrograph were determined by Gctf<sup>®</sup> for the homodimer and dataset 1 of the heterodimer, and by CTFIND-4.1<sup>®</sup> for dataset 2 of the heterodimer. For all datasets, particle selection, 2D and 3D classification were performed.
on a binned dataset (pixel size 1.72 Å and 4.24 Å for the heterodimer and homodimer, respectively) using RELION (versions 3.0 and 3.1)\(^{41}\). The two datasets for the heterodimer were processed individually before being combined following a Bayesian polishing step. A total of 538,957 particles from 1,324 micrographs and 2,062,083 particles from 8,991 micrographs were extracted using semi-automated particle selection for the heterodimer datasets 1 and 2, respectively. Both particle sets were then separately subjected to three rounds of 2D classification and two rounds of 3D classification. Particles in both sets were subjected to Bayesian polishing individually and then combined for a total of 286,140 particles. The merged dataset was fit for CTF parameters (per particle defocus and astigmatism, per micrograph B-factor) and estimated for anisotropic magnification and beam-tilt. A final 3D refinement was followed by post-processing using a mask that excluded the GDN micelle density. A focused refinement was also carried out using a mask encompassing the VFT and linker regions of GABA\(_b\). For the GABA\(_b\) homodimer structure, a total of 2,278,113 particles were extracted from 5,602 micrographs using semi-automated particle selection. Particles were subjected to multiple rounds of 2D and 3D classification, until a subset of 282,811particles was selected for the final map. The particle set underwent multiple rounds of CTF parameter fitting and was subjected to Bayesian polishing before 3D refinement and post-processing of the final map. UCSF Chimera\(^{42}\) was used for map and model visualization.

**Model building**

The initial model for the VFT was taken from the inactive-state VFT crystal structure (PDB 4MR7\(^{7}^{\text{a}}\)) and the initial structure of the transmembrane domain of GABA\(_{b}\) was generated as a homology model from the inactive cryo-EM structure of mGlu5 (PDB 6NS2\(^{7}^{\text{b}}\)) (38% sequence similarity to GABA\(_b\)) using Schrödinger’s Prime homology modelling\(^{43}\). Both components were placed into the GABA\(_b\) cryo-EM map using ‘fit-in-map’ function of Chimera. The linker, intracellular loops and extracellular loops of GABA\(_b\) were interactively adjusted into the electron microscopy map using Coot (version 0.8.9.1e)\(^{44}\) and the resulting model of the GABA\(_b\) linker–TM7 was then used to generate a homology model of GABA\(_b\) using Schrödinger’s Prime homology modelling, which was also placed into the map in Chimera. Iterative rounds of interactive model adjustment in Coot followed by real-space refinement in Phenix (version 1.17-1-3660)\(^{45}\), using secondary structure restraints in addition to the default restraints, were performed to improve the modelling. The resulting structure of GABA\(_b\) was sufficiently different to the original homology model such that the root mean square deviation (r.m.s.d.) between the homology model and the final model is non-trivial (about 2.0 Å in the transmembrane helices alone). Once confidence in the side-chain placement was reached for the ligand-binding cleft on GABA\(_b\), the GemSpot pipeline\(^{46}\) was used to model the inhibitor, CGP53845, into the map. After further improvement, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE) was modelled in the transmembrane pocket of GABA\(_b\) and GABA\(_b\) with GemSpot. Final model refinement was performed with Phenix\(^{47}\).

**Molecular dynamics simulations and analysis**

To prepare the system for molecular dynamics simulations, the low-resolution features of the map were used to manually build intracellular loop 2 (ICL2) into the model of the GABA\(_b\) inactive heterodimer using Coot. The system was then prepared in Maestro, version 2019-4 (Schrödinger) to build any stubbed side chains and determine protonation states. The VFTs were removed from the heterodimer to produce a truncated construct starting at residues T461 for GABA\(_{b}\) and T468 for GABA\(_b\), thus containing only the linkers and the transmembrane domains. The Orientations of Proteins in Membranes (OPM)\(^{46}\) webserver was used to orient the system with respect to a membrane plane and the CHARMM-GUI\(^{48}\) was used to generate a PDB file of the system in either a 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and cholesterol bilayer or a 3:1 POPC:POPE and cholesterol bilayer. Approximate dimensions for the system were 105 × 105 × 110 Å for a total of 240 lipid and 7 cholesterol molecules. This bilayer was then solvated in TIP3P water with 150 mM sodium chloride ions balanced to achieve charge neutrality. The salt concentration of 150 mM is consistent with the conditions at which the receptor was purified. POPE was used for the lipid in the transmembrane binding sites of GABA\(_b\) and GABA\(_b\).

The PDB file for the full solvated system was prepared in VMD (version 1.9.3)\(^{49}\) for simulation in NAMD (version 2.13)\(^{48}\) to produce a protein structure file (psf). The OPLS-AA\(^{50}\) force field was used for the protein, and OPLS-AA\(^{50}\) was used for the lipids, cholesterol and ions. Disulfide bonds were placed between C546 and C644 in GABA\(_b\) and C533 and C648 in GABA\(_b\), and both the N and C termini were blocked with capping groups, acetylated N termini and N-methylamide C termini. NAMD was used to run molecular dynamics simulations, in which all phases used periodic boundary conditions with nonbonded interactions smoothed starting at 10 Å to 12 Å, with long-range interactions treated with the particle mesh Ewald method. Systems were minimized for 2,000 steps and then slowly heated in the NPT ensemble with a Langevin thermostat and a Nosé–Hoover Langevin piston barostat set at 1 atm with a period of 30 fs and a decay of 25 fs. A 2-fs time-step was used with the SHAKE\(^{51}\) and SETTLE\(^{52}\) algorithms. Heating occurred from 0 to 310 K in increments of 20 K with 0.4 ns of simulation at each increment. Harmonic restraints of 1 kcal per mol per Å\(^2\) were used during heating on all non-hydrogen atoms of the protein and lipids. The system was then equilibrated with 1 kcal per mol per Å\(^2\) harmonic restraints on all protein and lipid non-hydrogen atoms for 10 ns followed by another 10 ns of equilibration with 1 kcal per mol per Å\(^2\) harmonic restraints on non-hydrogen backbone atoms. Finally, 1 kcal per mol per Å\(^2\) harmonic restraints were applied to only Ca atoms for 2 ns before being stepped down to 0.5 kcal per mol per Å\(^2\) for 2 ns, 0.3 kcal per mol per Å\(^2\) for 2 ns, and then removed. The first 30 ns of unrestrained molecular dynamics were also discarded as equilibration.

All trajectories were downsampled by 10× for analysis. Cavity volume was calculated with Epوك (1.0.5)\(^{53}\) in VMD\(^{49}\) on trajectories that had been aligned to either GABA\(_b\) or GABA\(_b\) from the starting structure. The cavity region was defined to include the binding region of the hydrophobic tails of the lipid. Transmembrane–transmembrane distances were calculated in VMD based on the Ca position of residues: 3.33, 4.50, 5.40, 6.54, and 7.28 in the Ballesteros–Weinstein\(^{44}\) numbering scheme.

**Transfection and seeding of cells for signalling assays**

HEK293 cells (ATCC CRL-1573) were transfected with expression vector DNA encoding the two GABA\(_b\) receptor protomers and a chimeric G\(_{q10}\) subunit (five C-terminal amino acids of G\(_{q}\) were exchanged with those of G\(_{o}\)) to allow the G\(_{o}\)-coupled GABA\(_b\) receptor to activate PLC and induce IP\(_3\) and intracellular Ca\(^{2+}\) release\(^{5}\). Before transfection, cells were brought into suspension by trypsinization and resuspension to 0.18 million cells per ml in growth medium (D-MEM, Gibco 10566016; supplemented with 10% fetal bovine serum, Gibco 10270106; 1% sodium pyruvate, Gibco 11360039; 1% MEM non-essential amino acids, Gibco 11410068; and 1% penicillin–streptomycin Solution, Gibco 15140122). For each 1 ml of cell suspension transfected, a total of 1 μg DNA in 25 μl OptiMEM (Gibco 51985) was incubated for 20 min with a mixture of 57 μl OptiMEM and 3 μl FuGene6 (Promega E2692). After FuGene6–DNA complex formation, the mixture was added directly to the cell suspension, mixed thoroughly and cells seeded with 100 μl cell suspension in appropriate 96-well plates. Of the 1 μg DNA per ml cell suspension, the amount of expression vector DNA encoding the chimeric G\(_{q10}\) was 0.5 μg/ml cell suspension in all experiments. The amount of GABA\(_b\) encoding DNA was varied between 7.8 ng and 0.25 μg for each of the appropriate 96-well plates. Of the 1 μg DNA per ml cell suspension, the amount of expression vector DNA encoding the chimeric G\(_{q10}\) was 0.5 μg/ml cell suspension in all experiments.
was performed. DNA corresponding to 62.5 ng DNA per ml cell suspension of each of the protomers (wild type or mutants) were mixed and serially diluted twofold 5 times, typically down to 3.9 ng DNA per ml cell suspension. The transfected cell suspension was seeded at 100 μg/ml both in clear poly-L-lysine coated 96-well plates for IP, accumulation assays and in white poly-L-lysine coated 96-well plates for cell-surface enzyme-linked immunosorbent assay (ELISA) assays.

**IP, accumulation assays**

The IP₂ assays for wild-type and mutant receptors were performed essentially as previously described

Forty-eight hours after transfection, the growth medium was replaced with HBSS buffer (HBSS (Gibco 14025), 20 mM HEPES pH 7.5, 1 mM CaCl₂, 1 mM MgCl₂, and 0.1% BSA) supplemented with BSA to 0.5% and incubated at 37 °C for 3–4 h. For characterization of the TM3–TM5 protomer-interface mutants for basal activity, the HBSS + 0.5% BSA buffer was replaced with 100 μl HBSS buffer, followed by addition of 50 μl HBSS buffer containing LiCl (150 mM) to give a final concentration of 50 mM LiCl. After incubation for 1 h at 37 °C the IP₂ accumulation was stopped by addition of 40 μl CisBio IP-One Tb HTRF Kit (CisBio, 621PAPEC) lysis buffer. The accumulated IP₂ levels were determined according to the manufacturer’s instructions and as previously described.

For the generation of GABA concentration–response curves, the compounds were diluted in three times the final concentration in HBSS buffer containing 60 mM LiCl. The assay was started, first by replacing the HBSS + 0.5% BSA buffer with 100 μl HBSS buffer, followed by addition of 50 μl of the above compound dilutions to give a final LiCl concentration of 20 mM. The IP₂ accumulation assay was stopped and assayed as described above after incubation for 1 h at 37 °C. Data were calculated as the amount of IP₂ formed per well or normalized to the basal IP₂ level, and fitted by nonlinear regression using GraphPad Prism.

**Cell-surface ELISA assay**

Surface expression levels of wild-type and mutant GABA₂ receptors were determined using a direct ELISA against the N-terminal GABA₂a₁b Flag tag and the N-terminal GABA₂a₂b HA tag, as previously described. Transfected cells were seeded in white poly-D-lysine-coated 96-well plates. Forty-eight hours after transfection, cells were washed once with 100 μl per well DPBS + 1 mM CaCl₂ (wash buffer). Following fixation with 50 μl per well 4% paraformaldehyde solution for 5 min at room temperature, cells were washed twice with 100 μl wash buffer and blocked with 100 μl per well blocking solution (3% dry milk, 1 mM CaCl₂, 50 mM Tris-HCl, pH 7.5) for 30 min at room temperature, followed by addition of 75 μl per well HRP-conjugated anti-Flag antibody (Sigma Aldrich, A8592), or HRP-conjugated anti-HA antibody (R&D systems HAM060), both diluted 1:2,000 in blocking solution, and allowed to incubate for 1 h at room temperature. The plates were then washed four times with 100 μl per well blocking solution followed by four washes with wash buffer. The amount of surface-expressed receptors was detected by adding 60 μl wash buffer and 20 μl HRP substrate (Bio-Rad, 170-5060) per well, incubating for 10 min and measuring of luminescence in an EnVision plate reader (Perkin Elmer).

**Statistics and reproducibility**

Detailed results and description of statistical analyses used in this Article can be found in Supplementary Table 1. Data and error bars in Figs. 1c, d are mean ± s.e.m. from at least five independent experiments. In Fig. 1c, data of co-expressed wild-type GABA₂a₁b (6.3 ng) with wild-type GABA₂a₂b (6.3 ng), wild-type GABA₂a₁b (3.1 ng) with wild-type GABA₂a₂b (3.1 ng), and wild-type GABA₂a₁b (1.6 ng) with wild-type GABA₂a₂b (1.6 ng) represent n = 7 independent experiments; data of co-expressed wild-type GABA₂a₁b (0.8 ng) with wild-type GABA₂a₂b (0.8 ng), solely expressed wild-type GABA₂a₁b (6.3 ng), solely expressed wild-type GABA₂a₂b (3.1 ng), and untransfected HEK293 cells represent n = 6 independent experiments. In Fig. 1d, data of co-expressed GABA₂a₁b (Δ627–634) with wild-type GABA₂a₂b, and wild-type GABA₂a₁b with GABA₂a₁b (Δ631–638) represent n = 7 independent experiments; and co-expressed GABA₂a₁b (Δ627–634) with GABA₂a₂b (Δ631–638) represent n = 5 independent experiments. Data in Fig. 2d are representative of one experiment performed in triplicate and repeated independently at least three times with similar results (n = 3), data points and error bars are mean ± s.e.m. Data in Fig. 2e are mean ± s.e.m. from at least three independent experiments; data for co-expressed GABA₂a₁b (H572A/E673A) with wild-type GABA₂a₂b, and wild-type GABA₂a₁b with GABA₂a₂b(H579A/E677A) represent n = 3 independent experiments; and data for wild-type GABA₂a₁b with wild-type GABA₂a₂b represent n = 7 independent experiments. Data in Fig. 3d are mean ± s.e.m. from at least four independent experiments; co-expressed GABA₂a₁b(L553W) with GABA₂a₂b(L560W) represent n = 5 independent experiments, and data for wild-type GABA₂a₁b with wild-type GABA₂a₂b represent n = 7 independent experiments. Ensemble data in Fig. 3e represent distribution over a 200-ns time course, for 5 simulations per condition; each violin represents n = 37,500 time points.

**External computational resource use**

The Extreme Science and Engineering Discovery Environment (XSEDE) resource comet-gpu through sdsc-comet allocation TG-MCB190153 was used in preparation of this Article.

**Reporting summary**

Further information on research design is available in the Nature Research Reporting Summary linked to this paper.

**Data availability**

All data generated or analysed during this study are included in the Article and its Supplementary Information. Cryo-EM maps of GABA₂ heterodimer and GABA₂a₁b homodimer have been deposited in the Electron Microscopy Data Bank under accession codes EMD-21533 and EMD-21534, respectively. The atomic coordinates of GABA₂ heterodimer and GABA₂a₁b homodimer have been deposited in the Protein Data Bank under the accession codes 6W2X and 6W2Y, respectively.
51. Ryckaert, J.-P., Ciccotti, G. & Berendsen, H. J. C. Numerical integration of the cartesian equations of motion of a system with constraints: molecular dynamics of n-alkanes. J. Comput. Phys. 23, 327–341 (1977).

52. Miyamoto, S. & Kollman, P. A. Settle – an analytical version of the shake and rattle algorithm for rigid water models. J. Comput. Chem. 13, 952–962 (1992).

53. Laurent, B. et al. Epoch: rapid analysis of protein pocket dynamics. Bioinformatics 31, 1478–1480 (2015).

54. Ballesteros, J. A. & Weinstein, H. in Methods in Neurosciences vol. 25 (ed. Sealfon, S. C.) 366–428 (Academic, 1995).

55. Hilger, D. et al. Structural insights into ligand efficacy and activation of the glaucon receptor. Preprint at https://www.biorxiv.org/content/10.1101/660837v1 (2019).

56. Toews, J. et al. XSEDE: accelerating scientific discovery. Comput. Sci. Eng. 16, 62–74 (2014).

57. Scheres, S. H. Processing of structurally heterogeneous cryo-EM data in RELION. Methods Enzymol. 579, 125–157 (2016).

58. Afonine, P. V. et al. New tools for the analysis and validation of cryo-EM maps and atomic models. Acta Crystallogr. D 74, 814–840 (2018).

59. Kato, K., Goto, M. & Fukuda, H. Regulation by divalent cations of 3H-baclofen binding to GABAB sites in rat cerebellar membranes. Life Sci. 32, 879–887 (1983).

60. Bowery, N. G., Hill, D. R. & Hudson, A. L. Characteristics of GABAergic receptor binding sites on rat whole brain synaptic membranes. 1983. Br. J. Pharmacol. 120 (Suppl), 452–467, discussion 450–451 (1997).

61. Haga, K. et al. Structure of the human M2 muscarinic acetylcholine receptor bound to an antagonist. Nature. 482, 547–551 (2012).

62. Marheineke, K., Grünewald, S., Christie, W. & Reiländer, H. Lipid composition of Spodoptera frugiperda (Sf9) and Trichoplusia ni (Tn) insect cells used for baculovirus infection. FEBS Lett. 441, 49–52 (1998).

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Author contributions M.M.P.-S. designed and cloned GABAB constructs, expressed and purified all proteins and collected and processed cryo-EM data. M.J.R. built and refined the structure from cryo-EM density maps and set up, performed and analysed molecular simulations. A.B.S. and O.P. assisted with cryo-EM data collection and processing. J.M.M. performed and analysed cellular signalling experiments. M.M.P.-S., M.J.R., J.M.M. and G.S. interpreted results. M.M.P.-S., M.J.R., J.M.M. and G.S. wrote the manuscript with O.P. and A.B.S. providing input. G.S. supervised the project.

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Extended Data Fig. 1 | Sample preparation, cryo-EM processing and reconstruction of GABA<sub>B</sub> heterodimer. 

a, Purification scheme for GABA<sub>B</sub>. 

b, c, Representative cryo-EM micrograph of 10,315 collected (b) and 2D class averages (c) of GABA<sub>B</sub> dimers. 

d, Flow chart outlining the cryo-EM processing workflow using RELION<sup>57</sup>; the global resolutions of the full-length structure and VFT focused structures were 3.6 Å and 3.5 Å, respectively, at 0.143 Fourier shell correlation (FSC) as calculated by RELION. 

e, f, Gold-standard FSC curve of half-maps calculated using RELION and Phenix Mtrae<sup>58</sup> (e), and map-to-model validation curves generated through Phenix Mtrae (f). 

g, Local resolution of cryo-EM maps. 

h, Angular distribution of projections used in final cryo-EM reconstruction. 

i, Ordered cryo-EM densities (light yellow), probably corresponding to GDN and/or cholesteryl hemisuccinate, are found at the TM5 interface of GABA<sub>B1</sub> and GABA<sub>B2</sub>. 
Extended Data Fig. 2 | Agreement between cryo-EM map and model.

a, Electron microscopy density and model for GABA\textsubscript{B1} heterodimer complex; transmembrane helices of GABA\textsubscript{B1}, transmembrane helices of GABA\textsubscript{B2}, linker region, bound phosphatidylethanolamine (PE) and ligand CGP55845. Densities visualized within UCSF Chimera\textsuperscript{42} and zoned at 2.2 with threshold set to 0.0142, with the exception of the following: GABA\textsubscript{B1}-bound lipid, GABA\textsubscript{B1}-bound CGP55845 and GABA\textsubscript{B2} linker, in which thresholds of 0.01, 0.0189 and 0.0127 were used, respectively.

b, Electron microscopy density and model for GABA\textsubscript{B1} homodimer; transmembrane helices and linker region of both protomers, 7TM-bound PE and ligand CGP55845. Densities were zoned at 2.2 and threshold set to 0.016, apart from CGP55845 and the ECL2 in both protomers, in which a threshold of 0.03 or 0.02 was used, respectively.
Extended Data Fig. 3 | Binding of CGP55845 and cation to GABA$_B$ VFT.

**a**, Model of CGP55845 within the VFT of GABA$_{B1b}$. The entire ligand is confined by W65 and W278 of GABA$_{B1b}$, which form hydrophobic interactions with the chlorinated ring of CGP55845. **b**, Schematic of interacting residues on GABA$_{B1b}$ with the inhibitor, CGP55845. GABA$_{B1b}$ residues S153 and S130 form hydrogen bonds with oxygen atoms of the phosphate group, and H170 and E349 form a hydrogen bond and a salt bridge with the amine group of the ligand, respectively. π–π stacking occurs between the chlorinated ring structure of CGP55845 and W278, and W65 provides hydrophobic packing on the opposing side of the ring. S130, H170, E349 and W65 are all substantially different residues in GABA$_{B2}$, precluding ligand binding. Residues are color-coded corresponding to their properties: light blue, hydrophilic; orange, anionic; green, hydrophobic; yellow, glycine; and grey, cysteine. Interaction lines are also color-coded according to their type: light blue, side-chain hydrogen bonding; grey, backbone hydrogen bonding; blue–red gradient, salt bridge; and green, π–π stacking. **c**, Overlay of CGP55845-bound GABA$_B$ cryo-EM structure (tan and teal) with CGP54626-bound GABA$_B$ crystal structure (pink) (PDB 4MR7) or apo-state GABA$_B$ crystal structure (grey) (PDB 4MQE), resulting in r.m.s.d. values of 1.30 Å and 1.28 Å, respectively. **d**, Comparison of ligand pose between CGP55845 (yellow) and CGP54626 (blue), which differs from CGP55845 only in a substitution of an aromatic ring in place of cyclohexane. **e**, Spherical density surrounded by anionic residues within the VFT supports a cation (magenta) at that site. The presence of a metal ion would be consistent with the observation that calcium and other divalent ions affect ligand affinity, and examination of the deposited scattering factors for the high-resolution VFT crystal structure (PDB 4MR7) reveals positive difference density also consistent with a cation at this site.
Extended Data Fig. 4 | Comparison of structures across GPCR classes.

a, GABAB protomers share similar secondary and overall structure.
b, Comparison of mGlu5 and GABAB 7TM and ECL2–linker shown from side view.
c, Top-down view of GABAB1, GABAB2, mGlu5 (PDB 6N52^[4]) and family A M2 acetylcholine receptor (PDB 3UON^[6]) with sidechains corresponding to the toggle-switch motif shown. Phospholipid space-filling model is included in grey within GABAB1 and GABAB2.
d, Sequence alignment of human GABAB receptors with mGlu5 and M2 receptors, comparing canonical GPCR activation motifs: TM3–TM6 ionic lock (blue), toggle-switch motif (green) and FXPKXY motif (yellow). Sequences are aligned to motifs within each transmembrane helix and transmembrane helical secondary structure is underlined. Residues in GABAB sequences differing from canonical motifs are outlined in pink. The cysteine residue that replaces the toggle-switch tryptophan is highlighted in pink.
Extended Data Fig. 5 | Atomistic simulations of phospholipid structural stabilization and entry. a, The results of three out of seven total simulations of GABA$_B$$_7$TM and linker in the absence of core-bound lipid after 200 ns. The results show the extent of lipid (green and purple) entry in GABA$_B$$_2$ (grey) in the simulations versus the experimental structure (tan) with PE (orange). One of the trajectories was extended an additional 100 ns (rightmost panel) and lipid entry was observed to progress towards the core. b, Representative side view of the GABA$_B$ ribbon and stick model from simulations at 200 ns, showing persistence of the ECL2 and β-sheet structure even in absence of the VTs. c, d, Violin plots of ensemble distances between GABA$_B$$_1$ (d) and GABA$_B$$_2$ (e) TM helices in simulations with and without core-bound lipid. Distances were measured from the Ca atoms of the following residues in GABA$_B$$_1$: L550 (TM3), W611 (TM4), L653 (TM5), M707 (TM6) and A716 (TM7); and the following residues in GABA$_B$$_2$: T557 (TM3), W615 (TM4), L657 (TM5), F711 (TM6) and Q720 (TM7). Simulations were run over 200 ns and violin plots represent $n = 37,500$ data points and 5 simulations per condition. e, 7TM cavity volume measured over time for individual simulations. Average cavity volume of phospholipid-bound receptor is shown as dashed line, thick lines indicate rolling averages of 5.33 ns and thin lines represent raw data.
Extended Data Fig. 6 | Functional analysis of GABA_B mutants.

**a**, Comparative normalized surface-expression levels of constructs. Surface-expression levels of wild-type and mutant GABA_B receptors were determined using a direct ELISA against the N-terminal GABA_B1 Flag tag and the N-terminal GABA_B2 HA tag. Surface-expression levels were normalized to the surface expression of a wild-type GABA_B receptor when 6.3 ng Flag-tagged GABAB1 and 6.3 ng HA-tagged GABA_B2 DNA was used for transfection. The line x = y indicates similar surface expression of GABA_B subunits as evident from transfection with lower amounts of Flag-tagged GABAB1 and HA-tagged GABA_B2. Values above the line have greater GABA_B1 surface expression relative to GABAB2, and values below the line have greater GABA_B2 surface expression relative to GABAB1. GABA_B1 did not reach the cell surface (data point hidden behind triangle at the coordinate (0,0) in the left subpanel).

**b**, **c**, Mutations of ionic residues forming the interface of GABA_B1 (**b**) and GABAB2 (**c**) result in increased constitutive activity of the receptor. To achieve a range of expression levels of the mutants in **b** and **c**, the DNA amounts transfected were serially diluted twofold from 3.3 ng (mutants in **b**) or 6.3 ng (mutants in **c**) of each subunit, respectively. **d**, **e**, GABA_B2(H579A/E677A) expressed without GABA_B1 shows a moderate increase in basal activity over wild-type GABA_B2 expressed alone (**d**), although surface expression (**e**) of the wild-type GABA_B2 subunit was higher than of GABA_B2(H579A/E677A).

**f**, **g**, Mutation of lipid coordinating residues (blue triangle) increase the constitutive activity of GABA_B1 (**f**) and GABAB2 (**g**), whereas mutations displacing the lipid tails from the 7TM core (purple triangle) result in decreased basal activity of the receptor when compared to wild-type receptor of similar receptor surface expression. **h**, Key to colours and symbols used in **a**, **f**, and **g** with DNA transfection amounts indicated in parentheses. Data are representative of one experiment performed in triplicate and repeated independently at least three times with similar results (n = 3 independent experiments), error bars represent mean ± s.e.m.
Extended Data Fig. 7 | Modelling of phospholipid into GABA$_B$. a, Schematic of GABA$_B$ residues interacting with the polar headgroup of PE. The terminal amine (-NH$_3^+$) forms a salt bridge with residue D714 in ECL3, and the phosphate group is coordinated by S710 of ECL3, R549 of TM3 and the backbone nitrogen of the C644 of ECL2. b–d, As our receptor purification did not contribute additional lipid, we considered the known lipid composition of Sf9 insect cells. Four primary phospholipids are present in Sf9 insect cells: phosphatidylcholine (43%), phosphatidylethanolamine (32%), phosphatidylinositol (23%) and cardiolipin (4%)\textsuperscript{62}. It was apparent from the map that the lipid had only two carbon chains, immediately excluding cardiolipin as it has four hydrocarbon tails. A comparison of the map and the binding site residues led to the decision to model PE (b) into the pocket using the GemSpot pipeline\textsuperscript{17}, which produced good cross-correlation with favourable interactions. To further confirm our selection, analysis of overlays of phosphatidylcholine (c) and phosphatidylinositol (d) over the docked model revealed phosphatidylcholine is unlikely given that the interactions with the cation appear to be primarily salt bridges, rather than the cation–π interactions that more commonly coordinate choline in proteins\textsuperscript{63}. Although phosphatidylinositol may make favourable interactions, our map does not appear to support such a large moiety in the headgroup position. Thus, PE is the most likely lipid to reside in the structure and was therefore used in the models.
Extended Data Fig. 8 | Cryo-EM processing workflow of GABA<sub>B1b</sub> homodimer.  

**a.** Flow chart outlining the cryo-EM processing of the GABA<sub>B1b</sub> homodimer.  

**b.** Representative micrograph of 5,602 collected (b) and 2D class averages (c).  

**d.** Local resolution of cryo-EM maps.  

**e.** Angular distribution of projections used in the final cryo-EM reconstruction.  

**f, g.** Gold-standard FSC curve of half-maps calculated using RELION and Phenix Mtriage<sup>46</sup> (f), and map-to-model validation curves generated through Phenix Mtriage (g).  

Global indicated resolutions of the full-length structure and VFT structure were 3.2 Å and 3.1 Å, respectively, at 0.143 FSC as calculated by RELION.  

**h.** Electron microscopy maps of active mGlu5 (purple) (EMD-0345<sup>14</sup>), and homodimeric GABAB<sub>1b</sub> (green). The GABA<sub>B1b</sub> homodimer adopts an overall architecture similar to that of active mGlu5.
| Data collection and processing | Data Set #1 | Data Set #2 | Data Set #3 |
|-------------------------------|-------------|-------------|-------------|
| Magnification (x)             | 57,050      | 57,050      | 47,198      |
| Voltage (kV)                  | 300         | 300         | 300         |
| Electron exposure (e-/Å²)     | 56.5        | 64.2        | 49.7        |
| Defocus range (µm)            | -1.5 - -2.7 | -1.2 - -2.5 | -0.9 - -2.5 |
| Pixel size (Å)                | 0.8521      | 0.8521      | 1.06        |
| Symmetry imposed              | C1          | C1          | C1          |
| Initial particle images (no.) | 538,957     | 2,062,083   | 2,278,113   |
| Final particle images (no.)   | 286,140 (Combined Data Sets) | 282,811 |
| Map resolution (Å)            | 3.6 Å       | 3.2 Å       |
| FSC threshold                 | 0.143       | 0.143       |
| Map resolution range (Å)      | 3.3 - 4.4   | 3.0 - 4.4   |
| Map sharpening B factor (Å²)  | -183.273    | -84.088     |

**Refinement**

Initial model used (PDB code) 4MR7, 6N52 Homology GABA_{B1B} of 6W2X (this work)

Model resolution (Å) 3.7 3.4

FSC threshold 0.5 0.5

**Model composition**

- Non-hydrogen atoms 9860 9721
- Protein residues 1345 1327
- Ligands 3 4

**B factors (Å²)**

- Protein 15.17 57.57
- Ligand 28.97 59.82

**R.m.s. deviations**

- Bond lengths (Å) 0.004 0.007
- Bond angles (°) 0.608 0.763

**Validation**

- MolProbity score 1.75 2.02
- Clashscore 5.55 9.98
- Poor rotamers (%) 0.00 0.12

**Ramachandran plot**

- Favored (%) 92.92 91.66
- Allowed (%) 7.08 8.34
- Disallowed (%) 0.00 0.00
Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Research policies, see Authors & Referees and the Editorial Policy Checklist.

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

- n/a Confirmed
- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- The statistical test(s) used AND whether they are one- or two-sided
- Only common tests should be described solely by name; describe more complex techniques in the Methods section.
- A description of all covariates tested
- A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted. Give P values as exact values whenever possible.
- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection

CryoEM Data was collected automatically on a Titan Krios (FEI) using SerialEM.

Data analysis

The following software were used in this study: MotionCor2, Gctf, CTFFIND-4.1, Relion3.0, Relion3.1, USCF Chimera 1.14, Coot (0.8.9.1e1), Phenix (1.17.1-3660), GSN, Maestro (2019-4), OPM, CHARMM-GUI, VMD (1.9.3), NAMD (2.13), Epox [1.0.5], GraphPad Prism 8.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

All data generated or analyzed during this study are included in this article and the Supplementary Information. CryoEM maps of GABAB heterodimer and GABAB1b homodimer have been deposited in the Electron Microscopy Data Bank under accession codes EMD-21533 and EMD-21534, respectively. The atomic coordinates of GABAB heterodimer and GABAB1b homodimer have been deposited in the Protein Data Bank under the accession codes 6W2X and 6W2Y, respectively.
# Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

- **Life sciences**
- **Behavioural & social sciences**
- **Ecological, evolutionary & environmental sciences**

For a reference copy of the document with all sections, see [nature.com/documents/nr-report/cg-summary-flat.pdf](http://nature.com/documents/nr-report/cg-summary-flat.pdf)

## Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | Sample sizes were not predetermined by statistical methods. For cryoEM data, sample sizes were determined/limited by time availability of the microscope. |
|-------------|-----------------------------------------------------------------------------------------------------------------------------|
| Data exclusions | No data was systematically excluded. Generation of maps from cryo-EM particles involves use of Relion (3.0 or 3.1) to sort particles, and remove damaged or poor quality particles to achieve a high-resolution final reconstruction. |
| Replication | Cellular signalling assays were replicated in at least three independent experiments for each condition tested. All replication attempts were successful. |
| Randomization | No randomization was attempted or needed. |
| Blinding | No blinding was attempted or needed. |

## Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

### Materials & experimental systems

| n/a | Involved in the study |
|-----|-----------------------|
| □ | Antibodies |
| □ | Eukaryotic cell lines |
| □ | Palaeontology |
| □ | Animals and other organisms |
| □ | Human research participants |
| □ | Clinical data |

### Methods

| n/a | Involved in the study |
|-----|-----------------------|
| □ | ChIP-seq |
| □ | Flow cytometry |
| □ | MRI-based neuroimaging |

### Antibodies

**Antibodies used**

- HRP-conjugated anti-FLAG antibody (Sigma Aldrich, A8592) and HRP-conjugated anti-HA antibody (R&D Systems HAM001).

**Validation**

Antibodies are validated by supplier. No additional validation was conducted by authors of this study.

### Eukaryotic cell lines

**Policy information about cell lines**

- **Cell line source(s)**: HEK293 cells (ATCC® CRL-1573™)
  Sf9, Expression Systems, Cat 94-0015
- **Authentication**: Cell lines are maintained by the supplier. No additional authentication was performed by the authors of this study.
- **Mycoplasma contamination**: Cell lines are tested by manufacturer for contamination and no additional testing was performed by the authors of this study.
- **Commonly misidentified lines (See ECLAC register)**: None used.