GABA<sub>A</sub> receptor signalling mechanisms revealed by structural pharmacology

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Type-A γ-aminobutyric acid (GABA<sub>A</sub>) receptors are ligand–gated chloride channels with a very rich pharmacology. Some of their modulators, including benzodiazepines and general anaesthetics, are among the most successful drugs in clinical use and are common substances of abuse. Without reliable structural data, the mechanistic basis for the pharmacological modulation of GABA<sub>A</sub> receptors remains largely unknown. Here we report several high-resolution cryo–electron microscopy structures in which the full–length human α1β2γ2L GABA<sub>A</sub> receptor in lipid nanodiscs is bound to the channel–blocker picrotoxin, the competitive antagonist bicuculline, the agonist GABA (γ-aminobutyric acid), and the classical benzodiazepines alprazolam and diazepam. We describe the binding modes and mechanistic effects of these ligands, the closed and desensitized states of the GABA<sub>A</sub> receptor gating cycle, and the basis for allosteric coupling between the extracellular, agonist–binding region and the transmembrane, pore–forming region. This work provides a structural framework in which to integrate previous physiology and pharmacology research and a rational basis for the development of GABA<sub>A</sub> receptor modulators.

In vertebrates, GABA<sub>A</sub> receptors mediate both phasic and tonic neuronal inhibition in the adult central nervous system<sup>1–3</sup>. Their dysfunction leads to channelopathies associated with epilepsy, insomnia, anxiety and chronic pain<sup>4</sup>. GABA<sub>A</sub> receptors are among the most important human drug targets owing to their many allosteric sites, which bind compounds with anticonvulsant, anti-anxiety, analgesic, sedative and anaesthetic properties<sup>5,6</sup>. Some of these—such as benzodiazepines (BZDs), which are typically positive allosteric modulators—entered clinical use decades before the identity of their receptors was known<sup>7</sup>, and were crucial for the isolation<sup>8</sup> and subsequent cloning<sup>9</sup> of GABA<sub>A</sub> receptors. Other GABA<sub>A</sub> receptor ligands are important research tools, including the antagonists picrotoxin (PTX) and bicuculline (BCC)<sup>5,6</sup>. The binding modes and conformational effect of most GABA<sub>A</sub> receptor allosteric modulators are unknown. Docking attempts have had to rely on models based on the structures of homologous proteins, including the Caenorhabditis elegans glutamate-gated chloride channel α (GluCl)<sup>11</sup>, the Torpedo nicotinic acetylcholine receptor<sup>12</sup> or the human homopentamic β3 GABA<sub>A</sub> receptor<sup>13</sup>. Crystallographic and single-particle cryo-electron microscopy (cryo-EM) studies have revealed the interactions of GABA<sub>A</sub>-receptor–derived constructs with neurosteroids<sup>14–16</sup>, the agonist GABA<sub>A</sub><sup>17–19</sup>, and the BDZ-site ligands brexazeni (BRZ)<sup>20</sup> and flumazenil (FLZ)<sup>18,20</sup>. However, owing to the use of engineered receptors, some of which are structurally damaged by the presence of detergents, the interpretability of these models is limited.

Functional studies on small-molecule GABA<sub>A</sub> receptor modulators have also raised numerous questions. We will list here just a few examples. It is not known why the two agonist (GABA) binding sites, which should be structurally identical, are not functionally equivalent<sup>21</sup>. The mechanism of action of BZDs is also unclear, despite their widespread use as sedatives and anxiolytics. Moreover—unlike newer compounds such as brexazeni and flumazenil—classical BZDs including diazepam (DZP, also known by the trade name Valium) and alprazolam (ALP, also known by the trade name Xanax) act specifically through GABA<sub>A</sub> receptors containing α1, α2, α3 or α5, but not α4 or α6 subunit types<sup>22,23</sup>. The basis for this specificity is not known. The plant alkaloid PTX, which is one of the most widely used GABA<sub>A</sub> receptor antagonists<sup>24</sup>, is thought to act as a channel blocker. However, its competitive antagonistic properties suggest that additional binding sites might exist<sup>25,26</sup>. Another broadly used GABA<sub>A</sub> receptor antagonist, BCC, is thought to act competitively, although its preference for binding resting over desensitized receptors suggests that it might also act allosterically<sup>27</sup>. How reagents such as PTX and BCC actually bind, as well as how they work, is not yet known.

To address these questions, here we present five structures of the human synaptic α1β2γ2L GABA<sub>A</sub> receptor in complex with PTX, PTX and GABA (PTX/GABA), BCC, DZP and GABA (DZP/GABA), and ALP and GABA (ALP/GABA). We establish the binding modes and structural effect of these ligands and explain the molecular basis for their function. To obtain structures in which GABA<sub>A</sub> receptors can be observed in physiologically relevant conformations, we used a full-length receptor variant from a thoroughly characterized cell line<sup>28</sup> and reconstituted it in a lipid bilayer<sup>28</sup>. Our results lay the foundation for understanding the fundamental principles of the action of small molecules on heteromeric synaptic GABA<sub>A</sub> receptors.

**Picrotoxin and GABA binding modes**

We first solved the structure of the α1β2γ2L receptor in complex with PTX (800 μM) to a nominal resolution of 3.1 Å (Fig. 1a, Extended Data Fig. 1a–f, Extended Data Table 1, Supplementary Video 1). PTX is an equimolar mix of two highly similar compounds, picrotin and picrotoxin. Our structure illustrates picrotoxin, which is known to be the more active compound, fully sequestered in the channel pore between the M2 2' and 9' rings (Fig. 1b, c, Extended Data Fig. 1g, Supplementary Video 2). This agrees both with mutagenesis and electrophysiology
results that implicate the 2′, 6′ and 9′ M2 residues in PTX binding, and with suggestions that PTX becomes ‘trapped’ in closed/resting channels of GABA_α and Gly receptors upon agonist wash-off. The hydrophobic isoprenyl moiety is surrounded by the 9′ Leu ring, whereas the exocyclic oxygen atoms in the main PTX body form putative hydrogen bonds with the 6′ ring (Fig. 1b, c). In picrotin, the isoprenyl group is replaced by a polar tertiary alcohol; this is not compatible with 9′ Leu ring interactions, which explains why picrotin is less active.

We next determined a GABA_α receptor structure in complex with PTX (800 μM) and the neurotransmitter GABA (5 μM) to a nominal resolution of 3.04 Å (Fig. 2a, Extended Data Fig. 2a–c, Extended Data Table 1, Supplementary Video 3). The orthosteric GABA-binding sites are located at the two β3^{{}\text{1}}/α1^{{}\text{1}} interfaces (the principal (+) and complementary (−) annotation of subunit faces is used here), capped by the extracellular loops-C of the β subunits. Similar to the Torpedo nAChR α subunits, in the agonist-free GABA_α receptor the β loops-C adopt ‘open’ (outwards-projecting) conformations, whereas in the PTX/GABA-bound state they are ‘closed’ (Extended Data Fig. 2d). Strong electron microscopy densities are present in the two orthosteric ligand-binding sites, enabling unambiguous modelling of GABA molecules in the ‘aromatic boxes’ formed by β3 Tyr157, β3 Phe200, β2 Tyr205 and β1 Tyr205. In β2 Tyr205 and β1 Tyr205, the GABA amino group engages in a cation–π interaction with β3 Tyr205 and a network of hydrogen bonds.

**Fig. 1 | Structure of the α1β3γ2L GABA_α receptor in complex with picrotoxin.** a, Cryo-EM map of the PTX-bound α1β3γ2L GABA_α receptor viewed from the extracellular space (left) and parallel to the membrane plane (right). The PTX-binding site is boxed. b, c, Side-on (b) and top-down (c) views of PTX (carbon atoms in pink, oxygen atoms in red) bound to the channel pore, with the amino acid side chains lining the site shown as sticks. Dashed lines indicate hydrogen bonds.

**Fig. 2 | Conformational impact of GABA binding to the α1β3γ2L GABA_α receptor.** a, Cryo-EM map of the PTX/GABA-bound α1β3γ2L receptor viewed from the extracellular space (left) and parallel to the membrane plane (right). b, One GABA-binding pocket viewed from the extracellular space. GABA is shown in ball-and-stick representation with the atoms coloured as follows: carbon, khaki; oxygen, red; nitrogen, blue. c, Plot of the pore radii for the receptor bound to PTX (black dashed line) and PTX/GABA (green line). d, Superposition of ECDs from the PTX-bound (grey) and the PTX/GABA-bound (red/blue/yellow) receptor structures based on the global TMD alignment. Subunits were radially translated away by 10 Å from the pore axis to enable better visualization of conformational changes in the ECD upon GABA binding. GABA-induced ECD rotation is stated as the rotation angle around the ECD rotation axes, and the direction of motion is shown. e, f, Superposition of α1 ECDs from PTX-bound and PTX/GABA-bound structures reveals conformational changes induced by GABA binding in the orthosteric pockets at β3-B^1/α1-A^- (e) and β3-E^-/α1-D^- (f) interfaces. Dashed lines indicate hydrogen bonds, π–π stacking, π–cation interactions and salt bridges.
PTX inhibition is not caused by blocking an open pore, but by inducing pore radius to ~1.5 Å (Fig. 2c, Extended Data Fig. 2i). This suggests that phobic α1′ adopts the same conformation—all five M2 helices present the hydro -phobic PTX/GABA-bound structures, the transmembrane domains (TMDs) (Extended Data Fig. 2f–h). Unexpectedly, in both PTX-bound and the PTX/GABA-bound structures, both of which have closed state TMDs, it is possible to visualize the initial conformational changes at the two agonist-binding sites upon the binding of GABA. Closure of [33 loops–C—likely to be the first step in the process—triggers remodelling of the β3-B′/α1-A′ and the β3-E′/α1-D′ interfaces, which leads to an anticlockwise (looking down the pore axis from the extracellular space) asymmetric rotation of all subunit ECDs (Fig. 2d). This state is stabilized by new hydrogen–bond networks involving the Ω3Gly158, Ω3Tyr205 and α1Arg85 residues. The cation–π interaction between α1Arg85 and Ω3Tyr195 breaks, and the α1Arg85 side chain moves to the periphery of the interface (Fig. 2e, f). At the β3-B′/α1-A′ interface, these changes enable the β3-Subunit to move closer to the α1-A subunit by 1.5 Å (as measured between Ω3Gly33Cα and α1Pro80Cα), and the total buried surface between the two subunit ECDs increases by 184 Å² (Extended Data Table 2, Extended Data Table 2). In the GABA-bound conformation, α1Phe15 and β3Phe31 residues form a hydrogen–π interaction and the side chains of α1Arg85 and β3Asp163 form new salt bridges, further interlocking the β3-E′/α1-D′ ECD interface and increasing its total buried surface area by 234 Å² (Fig. 2f, Extended Data Table 2, Supplementary Video 5).

The ‘incomplete’ closure of the β3-B′/α1-A′ interface in the PTX/GABA-bound structure, relative to the β3-E′/α1-D′ interface, defines an intermediate conformation from which GABA can presumably dissociate more readily. Notably, in the desensitized (high affinity to GABA) receptor states desribed later in this paper—both β3′/ α1′ interfaces close to the same extent. We conclude that the PTX/GABA-bound structure represents a pre-active receptor state, in which agonist-induced conformational changes at the ECD level are not large enough to perturb the closed/resting TMD conformation.

**Mechanism of bicuculline antagonism**

Using a highly efficient competitive antagonist, BCC was expected to induce a true GABA receptor closed state. We first verified whether BCC could close the α1β3γ2L receptor that had been pre-bound to Mb38. In whole-cell voltage-clamp experiments, application of a 42-s pulse of Mb38 produced currents (Fig. 3a) that were inhibited (102 ± 7%; n = 4) by the co-application of bicuculline (100 µM) (Fig. 3b) in a reversible fashion (Fig. 3c). We solved the cryo-EM structure of the α133γ2L heteromer in complex with Mb38 and BCC to a nominal resolution of 3.69 Å (Fig. 3d, Extended Data Fig. 3a–c, Extended Data Table 1). Electron microscopy densities corresponding to BCC were observed at both of the orthosteric agonist sites (Extended Data Fig. 3d), where the hydrophobic nature of the phthalide and isoxinoline rings of BCC enable its interaction with ‘aromatic box’ residues (Ω3Tyr157, β3Phe200, β3Tyr205 and α1Phe65 (Fig. 3e, Supplementary Video 6). Relative to the PTX-bound structure, the β3-B and β3-E subunit loops–C flex inward (by around 2.2 Å at the tip; Extended Data Fig. 3e) in order to accommodate BCC, but they retain overall ‘open’ conformations (Extended Data Fig. 3f). The channel pore of the BCC-bound α133γ2L receptor is fully closed by the M2 residues at three levels—9′, 2′ and 2″ (Fig. 3f)—and its subunit conformations are almost identical to those of the PTX-bound structure.
The cryo-EM map of the α(1)β2γ2L GABA_A receptor in desensitized states induced by GABA and alprazolam or GABA and diazepam.

The binding of BCC at the orthosteric sites prevents closure of the β3/α1 subunit interfaces and rotation of the ECDs, and also stabilizes TMDs in the closed state, thus inactivating the channel.

**Benzodiazepine binding modes and mechanisms**

We next solved cryo-EM structures of the α1β2γ2L receptor in complex with GABA and ALP, as well as with GABA and DZP, to nominal resolutions of 3.26 Å and 3.58 Å, respectively (Fig. 4a–c, Extended Data Fig. 4a–f, Extended Data Table 1, Supplementary Video 7). In both structures, GABA molecules are bound to the orthosteric agonist pockets and ALP or DZP occupy the canonical BZD-binding site at the α1/β2 interface, where they form extensive interactions (Fig. 4d, e). The densities of ALP and DZP were well defined, enabling us to distinguish the fused benzene-diazepine from the pendant phenyl rings (Extended Data Fig. 4g, h, Supplementary Video 8).

The binding modes of ALP and DZP shown here are in agreement with the results of cysteine crosslinking experiments, in which isothiocyanate substitutions at the DZP C7 position reacted with cysteines introduced at the α1His102, α1Asn103 and γ2Asn60 positions, and isothiocyanate introduced at DZP C3 reacted with the α1Ser206Cys and α1Thr207Cys mutants44. The chlorine atoms at the C8 and C7 positions in ALP and DZP, respectively, interact with the α1His102 side chain. In the α4 and α6 subunits the equivalent positions are occupied by arginine residues, the larger side chains of which would sterically clash with ALP and DZP. This could explain why classical BZDs do not act on GABA_A receptor subtypes containing α4 or α6 subunits.22,45,46 The ALP-bound and DZP-bound structures also demonstrate that BZDs with a pendant phenyl ring do not share the binding mode reported for the benzodiazepine antagonist FLZ and the partial agonist BRZ.18,20 (Extended Data Fig. 5a–e). Compared with ALP and DZP, both FLZ and BRZ bind deeper into the BZD pocket and higher from the pocket floor, which is delineated by the side chain of γ2Asn60. This could be due to additional hydrogen bonds between the hydroxyl group of γ2Thr142 and either the imidazole nitrogen or the ester carbonyl of FLZ and BRZ.20 (Extended Data Fig. 5c, e).

In the DZP/GABA-bound map, we also observed two strong densities at sites in the β3/α1 TMD interfaces that are thought to be the binding sites of general anaesthetics39 (Extended Data Fig. 4i, Supplementary Video 9). These densities are not present in the ALP/GABA-bound map and have a distinct shape that is attributable to DZP. In these pockets, the DZP A ring forms hydrophobic interactions with β3Met286 and β3Phe289 and the phenyl ring points towards α1Pro233 (Fig. 4f, Extended Data Fig. 4i). Our structure corroborates previous electrophysiology and mutagenesis data, which suggested the existence of secondary DZP-binding sites that are responsible for the anaesthetic activity and bhipasic GABA_A receptor potentiation at higher DZP concentrations40,48.

We next compared the α1/β2 interfaces in PTX-, PTX/GABA-, BCC-, ALP/GABA- and DZP/GABA-bound structures. Notably, the binding of ALP or DZP induced only minor rearrangements in the BZD pocket: γ2Asn60 adopted a different rotamer (Extended Data Fig. 5e) and the tip of the α1 loop-C flexed outwards by around 0.6 Å (Extended Data Fig. 5f, g). We therefore propose that BZDs such as ALP and DZP act as 'connectors' to stabilize the weakest ECD interface, α1-D+/γ2-C− (Extended Data Table 2), and facilitate the concerted ECD rotation upon GABA binding. Nanobody 3B (Nb3B) and Nb3B might act similarly by 'crosslinking' the α1-A−/β3-E− and α1-D+/γ2-C− interfaces20,29. FLZ antagonizes classical BZDs by competing for the same pocket; however, because it interacts largely with the α1-D+/β3-E− face18,20, its binding would not confer the same inter-subunit connectivity benefits (Extended Data Fig. 5b, c).

The channel-pore radii in the ALP/GABA-bound and the DZP/GABA-bound structures are 2.6 Å and 2.3 Å, respectively, at the 9’ level, and are further constricted to 1.6 Å and 1.8 Å by the −2’ residues (Fig. 4g). Whole-cell voltage-clamp experiments indicate that a prolonged exposure to a combination of GABA (10 mM), Mb38 (2 μM) and diazepam (100 μM) leads to the complete desensitization of the α1β2γ2L receptor (Fig. 4h). Subunit comparison between ALP/GABA-bound and DZP/GABA-bound structures reveals that they adopt similar conformations (Extended Data Fig. 6); this illustrates a desensitized state of the receptor, in which it is bound to the agonist but the channel is closed only at the −2’ gate (Supplementary Video 7).
Allosteric interactions between ECDs and TMDs

To understand how the GABA-induced ECD rotation induces conformational changes in the TMD region, we compared ALP/GABA-bound and PTX-bound structures (Fig. 5a, b). In the presence of ALP, GABA binding leads to equal closure of both β3/α1′ interfaces and a larger ECD rotation compared to the pre-open PTX/GABA-bound structure (Fig. 5a, Extended Data Figs. 2j, k, 7a, b, Supplementary Video 10). This conformational change of the ECD triggers a clockwise rotation of the TMD. The resulting tilt of M2 helices moves the 9 Leu side chains away from the channel pore and towards the inter-subunit interfaces (Fig. 5b, Extended Data Fig. 7c). These motions are transmitted through interactions between the β1–β2 and the M2–M3 loops, whereas the β6–β7 (Cys) loops act as pivot points and the TMD bundles rotate as rigid bodies (Extended Data Figs. 7d, 8a–f, Supplementary Video 10).

The relative flexibility in the M2–M3 loops appears to regulate the efficiency of the ECD–TMD signal transduction. In α1 and γ2 subunits, the highly conserved arginine residues at the M2 19′ positions interact with and rigidify the M2–M3 loops. However, in β3 subunits, the strictly conserved Lys279 residues displace the 19′ Arg269 side chains, causing them to rotate and wedge in between the M1 and M2 helices of the neighbouring α1 subunits. This restricts the β3 TMD rotations (Extended Data Fig. 8g–l), possibly dampening signal transduction. In α1 and γ2 subunits, positions equivalent to β3Lys279 are occupied by conserved threonine residues, which do not clash with 19′ Arg (Extended Data Fig. 8g–l). The M2–M3 loops of β3 therefore seem to be more flexible than those of α1 and γ2 (Extended Data Fig. 7d, Supplementary Video 10). In agreement with these observations, α1/β3/γ2 receptors containing β3Lys279Thr mutations are 20-fold more sensitive to GABA relative to the wild-type receptors.

We propose that GABA-induced signalling can be described by a ‘lock-and-pull’ mechanism. GABA binding triggers loops-C closure in β subunits, initiating rotation of their ECDs, which become ‘locked’ to the neighbouring α′ interfaces. These conformational changes ‘pull’ the other ECDs, and transfer to the TMDs, leading to a concerted anticlockwise rotation. The inclusion of a BZD strengthens the α1/γ2–ECD interface to facilitate these motions (Fig. 5c). In β3 subunits, signal transduction to TMDs is modulated by the flexible M2–M3 loops, whereas the ECD rotations of α and γ subunits couple to the TMDs more efficiently because their M2–M3 loops are more rigid (Fig. 5d).

**Conclusion**

The structures presented here illustrate how important pharmacological compounds, used broadly in research and in the clinic, interact with a full-length human heteromeric GABA<sub>A</sub> receptor to modulate its conformation and function. Picrotoxin must initially bind to an open channel pore and subsequently stabilizes a closed/resting receptor state, which explains its simultaneous channel blocker and allosteric antagonist activities. Bicuculline occupies the agonist-binding sites. However, unlike GABA, it cannot drive the rotation of β subunits and therefore stabilizes the closed channel pore. Comparison of agonist-free and GABA-bound structures delineates the molecular mechanism by which neurotransmitter binding to the β3/α1′ interfaces prompts a global rotation of ECD regions, initially in an asymmetrical fashion, and explains how different subunit types transduce this conformational change to their TMDs. We also characterize the binding sites of two major classical benzodiazepines—alprazolam and diazepam—and define their primary role as stabilizers of the α′/γ′ interface, facilitating the concerted motion of GABA<sub>A</sub> receptor subunits. This work underlines the potential of cryo-EM to study the interactions of drugs with challenging yet highly valuable human membrane-protein targets. Specifically, these structures might lead to the rational design of safer and more specific anxiolytic, sedative, hypnotic and anticonvulsant drugs.

**Online content**

Any methods, additional references, Nature Research reporting summaries, source data, statements of data availability and associated accession codes are available at https://doi.org/10.1038/s41586-018-0832-5.

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METHODS

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

GABA<sub>3</sub> receptor production, purification and nanodisc reconstitution. Human tri-heteromeric α1β3γ2L was expressed in E. coli WK6 cells. Cell cultures were grown at 37 °C (120 r.p.m., 8% CO<sub>2</sub>) and the majority of N-linked glycans were restricted to the immature, endoplasmic-reticulum-type Man<sub>4</sub>GlcnAc<sub>2</sub>. Cells were collected by centrifugation around 24 h after doxycycline addition. All steps of purification were performed at 4 °C or on ice.

The experiments were not randomized and the investigators were not blinded to allocation during experiments and outcome assessment.

Cryo-Electron microscopy (Cryo-EM). A circular permutant of the extracellular adhesin domain of comprising nanobodies (Nbs) fused to larger scaffold proteins (J.S., manuscript in preparation) was fused to the α1 subunit N terminus and a rhodopsin-1D4 tag (TETSQV APA) was inserted into the first α helix of the mature domain. The periplasmic protein in HEK293S cells producing MbNb38 was produced as previously described<sup>53</sup>. After nanodisc reconstitution, the MbNb38 receptor expression was induced by the addition of doxycycline (2 mg ml<sup>−1</sup>) for 30 h and then blotted for 5.5 s before plunge-freezing the grids into liquid ethane cooled by liquid nitrogen. Plunge-freezing was performed using a Vitrobot Mark IV (Thermo Fisher Scientific) at approximately 100% humidity and 14.5 °C.

Cryo-EM image collection and processing. Cryo-EM data were collected on a 300 kV Titan Krios microscopes (Thermo Fisher Scientific) fitted with a GIF Quantum energy filter (Gatan) and Volta Phase Plate (Thermo Fisher Scientific). Micrographs were recorded in counting mode using K2 Summit (Gatan) or Falcon 3EC (Thermo Fisher Scientific) direct electron detectors. For sample-specific collection parameters, see Extended Data Table 2.

Datasets were analysed using the same basic processing pipeline in RELION 3.0<sup>60</sup> as described below. First, MotionCor2<sup>57</sup> and Gctf<sup>58</sup> wrappers in RELION 3.0 were used to motion-correct movies, and to estimate the contrast transfer function and phase shift parameters, respectively. Poor-quality images were discarded after manual inspection. For particle picking, a Gaussian blob was used as a template to auto-pick particles from a small set of micrographs and 2D classification was performed. Selected 2D classes were used as templates for auto-picking particles from all micrographs. Two rounds of reference-free 2D classifications were performed and well-aligned 2D classes showing clear GABA<sub>3</sub> receptor projections were selected for 3D reconstruction. An initial reference-free 3D model was generated in RELION 3.0 using stochastic gradient descent (SGD) methodology<sup>57</sup>. Selected particles were 3D-refined (‘gold standard’ refinement) and Bayesian polishing of particles was performed<sup>60</sup>. Next, the polished particles were classified into eight 3D volumes without particle alignment. Particles from volumes with the highest resolution were combined and 3D-refined using a soft mask and solvent-flattened Fourier Shell Correlation (FSC<sub>0.143</sub>) as a criterion for convergence. Beam tilt correction and particle contrast transfer function refinement implementations in RELION 3.0 were applied, resulting in a final 0.1–0.2 Å increase in resolution. Notably, for the DZP/GABA-bound GABA<sub>3</sub> receptor dataset, beam tilt correction helped to increase the resolution from 3.82 Å to 3.58 Å. The resolution was estimated using relion_postprocess with the FSC criteria of 0.143. Local map resolution was estimated with Monomol<sup>45</sup>.

The initial atomic model used in this work was obtained from the truncated α1β3γ2 heteromer structure<sup>20</sup>. First, ECDs and TMDs from this model were fit to the PTX/GABA-bound α1β3γ2 heteromer map (<sup>3.04 Å nominal resolution</sup>) as rigid bodies using UCSF Chimera<sup>62</sup>. COOT<sup>63</sup> was used to adjust the model and build the regions absent in the truncated receptor form. The model was then subjected to several rounds of global refinement and minimization in real space using phenix_real_space_refine<sup>44</sup>. The resulting model served as a starting point for the other structures, applying the same refinement strategy. The geometry constraint files for small-molecule ligands used in the refinement were generated using the Grade Web Server (Global Phasing). The quality assessment of geometry in all models was performed using the MolProbity<sup>30</sup> Web server. For the refinement protocol validation, each final model coordinates were displaced by 0.5 Å and 1.0 Å, respectively against the starting model and the new models were re-refined using RELION 3.0. The refinement with the highest model and map FSC<sub>0.143</sub> was chosen for further analysis.

Micrographs were recorded in counting mode using K2 Summit (Gatan) or Falcon 3EC (Thermo Fisher Scientific) direct electron detectors. For sample-specific collection parameters, see Extended Data Table 2.

Subunit interface surface and associated free energy parameter analysis was performed using the PDBEPIA server<sup>27</sup>. Pocket volumes were calculated using the HOLE<sup>28</sup> plug-in in Coot. Structural figures were prepared using UCSF Chimera<sup>62</sup>. Global TMD alignments were performed using the align command in PyMol (Schrodinger). TMD boundaries for global alignments are as follows: α1 residues 233–309 and 392–418, α3 residues 218–302 and 419–447, α2 residues 233–319 and 411–436. ECD alignments were performed using the match command in UCSF Chimera. Molecule boundaries for the α1β3γ2 heteromer structure<sup>20</sup> were applied via a quad-channel super-perfusion pipette coupled to a piezoelectric 4-way valve (Camlab). Subunits were aligned against each other using the super-perfusion solution in <1 ms as described previously<sup>40</sup>. Data were acquired using C2SMs (Molecular Devices). For short drug applications, data were acquired at 10 kHz and filtered at 5 kHz, whereas for longer applications, data were collected at 2 kHz and filtered at 1 kHz. Cells or
patches were continually perfused with a bath solution consisting of (in mM): 145 NaCl, 5 KCl, 10 HEPES, 2 CaCl₂, 1 MgCl₂ and 10 glucose, pH 7.4 (adjusted with NaOH). The pipette solution consisted of (in mM): 140 KCl, 10 HEPES, 1 EGTA, 2 MgCl₂ and 2 Mg-ATP, pH 7.3 (adjusted with KOH). For whole-cell recordings, the open pipette resistances ranged from 1.3–2.5 MΩ, and for outside-out patches they ranged from 6 to 10 MΩ. For whole-cell recordings, cell capacitance ranged from 5 to 13 pF and series resistance ranged from 0.2 to 2.5 MΩ. Series resistances were electronically compensated by >85% with a lag of 10 μs. Cells and patches were voltage-clamped at −52 mV unless otherwise stated. The liquid junction potential of ~2 mV between the bath and pipette solution was corrected post-recording.

**Electrophysiology data analysis.** Clampfit 9 (Molecular Devices) was used to measure the peak current amplitudes. Current traces were normalized and prepared for presentation in Origin 6 (OriginLab Corporation). All data are presented as mean ± s.d. Statistical analysis was performed using Prism 6 (GraphPad Software).

**Materials.** All reagents for electrophysiology solutions were purchased from either Millipore Sigma or Thermo Fisher Scientific. Diazepam, doxycycline and DMSO were purchased from Millipore Sigma. Bicuculline and kifunensine were purchased from Tocris. A stock solution of bicuculline (100 mM) was prepared fresh each day in DMSO. The stock solution of diazepam (500 mM) in DMSO was stored at −80 °C. A working solution of diazepam (100 μM) in the bath solution was prepared fresh daily and was dissolved by sonication. In control experiments, patches exposed to GABA (10 mM) in the presence of DMSO (0.02% used in application) did not differ from those exposed to GABA (10 mM) alone. The small Mb38-elicited currents were enhanced by a similar small amount by 0.1% DMSO (84 ± 41% (n = 3)).

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

**Data availability**
All data are available from the corresponding authors and/or included in the manuscript or Supplementary Information. Atomic coordinates for PTX-, PTX/GABA-, BCC-, ALP/GABA- and DZP/GABA-bound structures have been deposited in the Protein Data Bank with accession codes 6HUG, 6HUJ, 6HUK, 6HUO and 6HUP, respectively, and the cryo-EM density maps have been deposited in the Electron Microscopy Data Bank with accession codes EMD-0275, EMD-0279, EMD-0280, EMD-0282 and EMD-0283, respectively.
Extended Data Fig. 1 | Single-particle cryo-EM analysis of human α1/β3/γ2L GABA<sub>A</sub> receptor bound to the channel-blocker picrotoxin. 

a, Representative micrograph of the PTX-bound GABA<sub>A</sub> receptor particles embedded in vitreous ice. b, Representative 2D class averages. c, FSC curves for the 3D reconstruction using gold-standard refinement in RELION<sup>56</sup>. Curves are shown for the phase randomization, unmasked, masked and phase-randomization-corrected masked maps. d, Validation of model refinement protocol. Curves are shown for model versus summed map (FSC<sub>full</sub>), model refined in half-map 1 versus half-map 1 (FSC<sub>work</sub>) and model refined in half-map 1 versus half-map 2 (FSC<sub>free</sub>). e, The final, unsharpened cryo-EM map coloured by local resolution (estimated using MonoRes<sup>61</sup>) shown at a higher contour level (left) and at a lower level (middle and right) to highlight the nanodisc belt and flexible intracellular domains (ICDs). f, Angular distribution of particle projections. The map of the GABA<sub>A</sub> receptor–PTX complex is shown in teal. g, Cryo-EM density segments for the PTX-binding site between residues 2' and 9' of the M2 transmembrane helices.
Extended Data 2 | See next page for caption.
Extended Data 2 | Structural and electrophysiological analyses of human \(\alpha133\gamma2L\) GABA\(_A\) receptor in complex with PTX and GABA.

a, FSC curves for the 3D reconstruction of the GABA\(_A\) receptor bound to PTX and GABA. Curves are shown for the phase randomization, unmasked, masked and phase-randomization-corrected masked maps. b, Validation of the model refinement protocol. Curves are shown for model versus summed map (FSC\(_{\text{full}}\)), model refined in half-map 1 versus half-map 1 (FSC\(_{\text{mask}}\)) and model refined in half-map 1 versus half-map 2 (FSC\(_{\text{mask}}\)). c, The final, unsharpened cryo-EM map coloured by local resolution (estimated using MonoRes\(^{41}\)). d, Superposition of the PTX-bound and PTX/GABA-bound \(\alpha133\gamma2\) receptor based on the global TMD alignment. The GABA-induced movements of loop-C in each of the \(\beta3\) subunits are highlighted by green lines between C\(_\alpha\) atoms of Thr202 residues. GABA is shown as spheres (carbon, khaki; nitrogen, blue; oxygen, red). e, Cryo-EM density segments showing GABA-binding sites in the PTX/GABA-bound structure. f–h, Representative whole-cell current traces elicited from the same HEK293 cell by three 8.8-s pulses of GABA (5 \(\mu\)M) plus Mb38 (2 \(\mu\)M), each separated by a 1-min wash: control (f); one second after the start of the second 8.8-s pulse, PTX (800 \(\mu\)M) was co-applied for 4 s (g); wash control showing full recovery (h). PTX inhibited currents by 106 \(\pm\) 2.6\% (mean \(\pm\) s.d.; \(n=6\) cells). In addition, the protocol was repeated with outside-out patches (117 \(\pm\) 9\% (mean \(\pm\) s.d.; \(n=5\) patches). i, Globally superposed PTX-bound and PTX/GABA-bound \(\alpha133\gamma2\) receptor transmembrane domains viewed from the extracellular space. Side chains of 9' Leu residues are shown as sticks, whereas PTX is represented as balls and sticks. j, k, Superposition of \(\alpha1\) subunit ECDs from PTX-bound and PTX/GABA-bound \(\alpha133\gamma2\) receptors reveal the relative \(\beta3\) ECD motions towards \(\alpha1\) ECDs, as viewed from outside of the receptor (j) and from the vestibule (k). Differences in distances (Å) between the selected C\(_\alpha\) atoms in the complexes without and with GABA are indicated by lines. The PTX-bound structure is shown in grey and the PTX/GABA-bound structure is coloured by subunit (\(\alpha1\), red; \(\beta3\), blue; \(\gamma2\), yellow). © 2019 Springer Nature Limited. All rights reserved.
Extended Data Fig. 3 | Structural analysis of human α1β3γ2L GABA<sub>A</sub> receptor bound to the competitive antagonist bicuculline. a, FSC curves for the 3D reconstruction of the GABA<sub>A</sub> receptor bound to BCC. Curves are shown for the phase randomization, unmasked, masked and phase-randomization-corrected masked maps. b, Validation of the model refinement protocol. Curves are shown for model versus summed map (FSC<sub>full</sub>), model refined in half-map 1 versus half-map 1 (FSC<sub>work</sub>) and model refined in half-map 1 versus half-map 2 (FSC<sub>free</sub>). c, The final, unsharpened cryo-EM map coloured by local resolution (estimated using MonoRes<sup>61</sup>). d, Cryo-EM density maps of the BCC-binding pockets. e, Superposition of the PTX-bound and BCC-bound α1β3γ2 receptor based on the global TMD alignment. The BCC-induced movements of loop-C in each of the β3 subunits are highlighted by green lines between C<sub>α</sub> atoms of Thr202 residues. BCC is shown as spheres (carbon, khaki; nitrogen, blue; oxygen, red). f, Superposition of individual subunits from the PTX-bound and BCC-bound GABA<sub>A</sub> receptor structures. r.m.s.d. values (Å) for equivalent C<sub>α</sub> in the entire subunits are shown. Loops-C are marked by arrows. The PTX-bound structure is shown in grey and the BCC-bound structure is coloured by subunit (α1, red; β3, blue; γ2, yellow).
Extended Data Fig. 4 | Structural analysis of human α1/β3/γ2L GABA<sub>A</sub> receptor in complexes with alprazolam and diazepam. a, FSC curves for the 3D reconstruction of the GABA<sub>A</sub> receptor bound to ALP. Curves are shown for the phase randomization, unmasked, masked and phase-randomization-corrected masked maps. b, Validation of the model refinement protocol. Curves are shown for model versus summed map (FSC<sub>full</sub>), model refined in half-map 1 versus half-map 1 (FSC<sub>work</sub>) and model refined in half-map 1 versus half-map 2 (FSC<sub>free</sub>). c, The final, unsharpened cryo-EM map coloured by local resolution (estimated using MonoRes<sup>61</sup>). d–f, Same as a–c but for the GABA<sub>A</sub> receptor bound to DZP. g–i, Cryo-EM density maps of the ligand binding sites: ALP binding in the BZD pocket (g), DZP binding in the BZD pocket (h), DZP binding in the general-anaesthetic pocket at the β3/α1− interface (i). Ligands are shown as sticks, with atom colouring as follows: ALP carbons, cyan; DZP carbons, teal; nitrogen, blue; chlorine, green. Side chains of residues lining the binding pockets are shown as sticks and are numbered. Dotted circles highlight the difference between the structures of alprazolam and diazepam.
Extended Data Fig. 5 | The classical benzodiazepines flumazenil and bretazenil bind to the same benzodiazepine pocket in GABA_A receptors, but use different modes. ALP/GABA-bound and DZP/GABA-bound structures are coloured by subunit (α1, red; β3, blue; γ2, yellow), whereas the other superposed structures are shown in grey. Loop-C is shown in a coil representation, to enable better visualization of the BZD pocket. a, Structural formulae of flumazenil (FZL) and (S)-bretazenil (BRZ). b, c, Superposition of γ2 subunit ECDs from the DZP-bound α1/β3/γ2 and FZL-bound α1/β2/γ2 receptor structures reveals the FZL 16 (white) position in the BZD pocket relative to DZP (teal). Side-on (b) and top-down (c) views of the pocket are presented. d, e, Same as b, c but the structural alignment shows the relative position of the BRZ 15 (white). Grey dashed lines indicate hydrogen bonds that FZL and BRZ form with γ2Thr142 in the BZD pocket. f, Superposition of γ2 subunit ECDs from the PTX-, PTX/GABA-, BCC-, DZP/GABA- to ALP/GABA-bound γ2 ECD illustrates conformational changes of the BZD pocket associated with ALP/DZP binding: an outward movement of loop-C; rearrangement of γ2Tyr58 and γ2Phe77 side chains; and a change in the γ2Asn60 rotamer. g, Superposition of the α1-D subunit ECDs from the PTX-bound and ALP/GABA-bound α1/β3/γ2 structures shows that ALP binding causes only a minimal outwards motion of the α1 loop-C, by 0.8 Å as measured between Ser206_Cα atom positions.
Extended Data Fig. 6 | Superposition of individual subunits from ALP-bound and DZP-bound α1β3γ2L GABA\textsubscript{A} receptor structures. Superposition of the individual subunits from the DZP-bound (grey) and ALP-bound (α1, red; β3, blue; γ2, yellow) GABA\textsubscript{A} receptor structures. r.m.s.d. values are in the range of 0.38–0.41 Å for α1 (343–344 equivalent C\textalpha positions), 0.37–0.40 Å for β3 (334–336 equivalent C\textalpha positions) and 0.47 Å for γ2 subunits (330 equivalent C\textalpha positions). Loops-C are marked by arrows.
Extended Data Fig. 7 | Structural analysis of PTX-bound and ALP/GABA-bound α1/β3γ2L GABA_A receptor structures. The PTX-bound structure is shown in grey and the ALP/GABA-bound structure is coloured by subunit (α1, red; β3, blue; γ2, yellow). a, b, Superposition of α1 subunit ECDs from PTX-bound and ALP/GABA-bound GABA_A receptors reveals the relative β3 ECD motions towards α1 ECDs, as viewed from outside of the receptor (a) and from the vestibule (b). Differences in distances (Å) between the selected Cα atoms in the complexes without and with GABA are indicated with lines. c, Individual subunits from the PTX-bound and the ALP/GABA-bound GABA_A receptor structures superposed on the basis of the global TMD alignment (ALP/GABA TMD over PTX TMD). Angles between vectors representing M2 helices and the pore axis of the PTX-bound structure are shown. Side chains of residues at the −2 and the 9ʹ positions are shown. d, Superposition of TMDs from PTX-bound and ALP/GABA-bound structures. r.m.s.d. values (Å) are shown for entire TMDs and for the M2–M3 loops (see Methods for boundary definitions).
Extended Data Fig. 8 | Conformational differences at the ECD–TMD interfaces between PTX-bound (closed) and ALP/GABA-bound (desensitized) α1β3γ2L GABA_A receptor structures. The PTX-bound structure is shown in grey and the ALP/GABA-bound structure is coloured by subunit (α1, red; β3, blue; γ2, yellow). The TMDs of the principal subunits of PTX-bound and ALP/GABA-bound structures were superposed enabling visualization of relative movements of neighbouring ECDs and TMDs. Structural rearrangements of the ECD–TMD interface between α1-A and β3-E subunits. Same as a, b, but for γ2-C and β3-B subunits. Same as a, b, but for β3-B and α1-A subunits. Amino acid residues present in the β1–β2 loop tip in each subunit are shown, the Cαs for these residues are represented as spheres and the distances of displacement indicated. The strictly conserved M2–M3 loop proline residue interacting with the β1–β2 loop is shown for each subunit. β1–β2 loop motions are indicated by curved arrows. Conformational differences in the kM2–M3 loop and 19’ Arg side chain positions between the PTX-bound and the ALP/GABA-bound structures shown for α1-A (g, h), γ2-C (i, j) and β3-B (k, l) subunits. Neighbouring subunit M1 and M2 helices are shown as cylinders. Dashed lines indicate putative hydrogen bond interactions between amino acid side chains and main-chain carboxyls.
## Extended Data Table 1 | Cryo-EM data collection, refinement and validation statistics

|                        | PTX       | PTX/GABA  | BCC       | ALP/GABA  | DZP/GABA  |
|------------------------|-----------|-----------|-----------|-----------|-----------|
|                        | EMDB-0275 | EMDB-0279 | EMDB-0280 | EMDB-0282 | EMDB-0283 |
|                        | PDB: 6HUG | PDB 6HJU | PDB 6HUK | PDB 6HJO  | PDB 6HUP  |

### Data collection and processing

| Parameter                                | PTX       | PTX/GABA  | BCC       | ALP/GABA  | DZP/GABA  |
|------------------------------------------|-----------|-----------|-----------|-----------|-----------|
| Microscope, location                     | Krios-S,  | Krios-II, | Krios-II, | Krios-II, | Krios-I,  |
|                                          | STRUBI    | MRC-LMB   | MRC-LMB   | MRC-LMB   | MRC-LMB   |
| Magnification                            | 75,000    | 75,000    | 75,000    | 75,000    | 130,000   |
| Voltage (kV)                             | 300       | 300       | 300       | 300       | 300       |
| Detector                                 | Falcon 3EC with VPP | Falcon 3EC with VPP | Falcon 3EC with VPP | Falcon 3EC with VPP | K2 Summit with GIF |
| Pixel size (Å)                           | 1.055     | 1.07      | 1.07      | 1.07      | 0.89      |
| Electron exposure (e⁻/Å³)                | 30        | 30        | 30        | 30        | 62        |
| Exposure length (s)                      | 60        | 60        | 60        | 60        | 14        |
| Dose rate (e⁻/pixel/s)                   | 0.5       | 0.55      | 0.55      | 0.55      | 4.5       |
| Frame number                             | 75        | 75        | 75        | 75        | 40        |
| Defocus range (μm)                       | -0.7 to -0.5 | -0.7 to -0.5 | -0.7 to -0.5 | -0.7 to -0.5 | -3.6 to -2.4 |
| Micrographs collected (no.)              | 803       | 794       | 988       | 815       | 768       |
| Micrographs selected (no.)               | 664       | 659       | 964       | 617       | 593       |
| Initial particle images (no.)            | 205,673   | 292,669   | 489,434   | 210,073   | 233,543   |
| Final particle images (no.)              | 56,269    | 67,604    | 30,536    | 39,050    | 55077     |
| Symmetry imposed                         | C1        | C1        | C1        | C1        | C1        |
| Map resolution (Å)                       | 3.10      | 3.04      | 3.69      | 3.26      | 3.58      |
| FSC threshold                            | 0.143     | 0.143     | 0.143     | 0.143     | 0.143     |
| Map resolution range (Å)*                | 2.6-6.0   | 2.6-6.0   | 2.4-6.0   | 2.7-6.0   | 2.4-6.0   |

### Refinement

| Parameter                                | PTX       | PTX/GABA  | BCC       | ALP/GABA  | DZP/GABA  |
|------------------------------------------|-----------|-----------|-----------|-----------|-----------|
| Initial model used (PDB code)            | N/A       | N/A       | N/A       | N/A       | N/A       |
| Model resolution (Å)*                    | 3.15      | 3.13      | 3.75      | 3.34      | 3.66      |
| FSC threshold                            | 0.5       | 0.5       | 0.5       | 0.5       | 0.5       |
| Model resolution range (Å)               | 3.15      | 3.13      | 3.75      | 3.34      | 3.66      |
| Map sharpening B factor (Å²)              | -73       | -82       | -128      | -79       | -119      |

### Protein composition

| Component                          | PTX       | PTX/GABA  | BCC       | ALP/GABA  | DZP/GABA  |
|------------------------------------|-----------|-----------|-----------|-----------|-----------|
| Protein residues                   | 1,817     | 1,814     | 1,817     | 1,810     | 1,810     |
| Non-hydrogen atoms                 | 15,297    | 15,263    | 15,308    | 15,235    | 15,273    |
| Protein atoms                      | 14,788    | 14,763    | 14,788    | 14,733    | 14,733    |
| N-linked glycan atoms              | 394       | 372       | 372       | 372       | 372       |
| PTX atoms                          | 21        | 21        | -         | -         | -         |
| GABA atoms                         | -         | 14        | -         | 14        | 14        |
| BCC atoms                          | -         | -         | 54        | -         | -         |
| ALP atoms                          | -         | -         | -         | 22        | -         |
| DZP atoms                          | -         | -         | -         | -         | 60        |
| PIP2 atoms                         | 94        | 94        | 94        | 94        | 94        |

### R.m.s. deviations

| Parameter             | PTX       | PTX/GABA  | BCC       | ALP/GABA  | DZP/GABA  |
|-----------------------|-----------|-----------|-----------|-----------|-----------|
| Bond lengths (Å)      | 0.005     | 0.005     | 0.009     | 0.005     | 0.013     |
| Bond angles (°)       | 0.81      | 0.79      | 0.78      | 0.81      | 1.11      |

### Validation

| Parameter             | PTX       | PTX/GABA  | BCC       | ALP/GABA  | DZP/GABA  |
|-----------------------|-----------|-----------|-----------|-----------|-----------|
| MolProbity score      | 1.45      | 1.50      | 1.53      | 1.50      | 1.66      |
| Clashscore            | 4.64      | 4.55      | 4.97      | 4.66      | 5.86      |
| Poor rotamers (%)     | 0         | 0         | 0         | 0         | 0         |

### Ramachandran plot

| Parameter             | PTX       | PTX/GABA  | BCC       | ALP/GABA  | DZP/GABA  |
|-----------------------|-----------|-----------|-----------|-----------|-----------|
| Favored (%)           | 96.65     | 96.03     | 96.04     | 96.19     | 95.07     |
| Allowed (%)           | 3.35      | 3.97      | 3.96      | 3.81      | 4.93      |
| Disallowed (%)        | 0         | 0         | 0         | 0         | 0         |

*Local resolution range.

*Resolution at which the FSC between map and model is 0.5.
Extended Data Table 2 | Analysis of interfaces between α1β3γ2L GABA<sub>A</sub> receptor subunits

| Subunit interface | Interface area* (Å<sup>2</sup>) | ΔiG<sup>†</sup> (kcal/mol) |
|-------------------|---------------------------------|---------------------------|
|                   | ECD | TMD | Full subunit | ECD | TMD | Full subunit |
| PTX               |     |     |             |     |     |             |
| α1-A'/β3-E'       | 1536| 1182| 2797        | -11.5| -23.7| -35.7       |
| β3-E'/α1-D'       | 1246| 1583| 2905        | -12.3| -30.4| -43.8       |
| α1-D'/γ2-C'       | 1400| 1229| 2781        | -5.8 | -25.2| -32.3       |
| γ2-C'/β3-B'       | 1619| 1203| 2895        | -13.7| -20.4| -34.7       |
| β3-B'/α1-A'       | 1251| 1652| 2990        | -13.3| -31.1| -45.7       |
| Mb38/α1-A         | 810 | -   | 829         | -6.0 | -   | -6.1        |
| Mb38/β3-E         | 232 | -   | 232         | -2.1 | -   | -2.1        |
| PTX/GABA          |     |     |             |     |     |             |
| α1-A'/β3-E'       | 1534| 1224| 2856        | -11.9| -24.8| -35.8       |
| β3-E'/α1-D'       | 1480| 1534| 3113        | -13.2| -28.9| -43.6       |
| α1-D'/γ2-C'       | 1423| 1221| 2781        | -6.4 | -25.0| -32.4       |
| γ2-C'/β3-B'       | 1618| 1204| 2911        | -13.8| -21.6| -36.5       |
| β3-B'/α1-A'       | 1435| 1618| 3166        | -14.5| -29.8| -45.9       |
| Mb38/α1-A         | 822 | -   | 834         | -7.0 | -   | -7.1        |
| Mb38/β3-E         | 248 | -   | 248         | -2.3 | -   | -2.3        |
| BCC               |     |     |             |     |     |             |
| α1-A'/β3-E'       | 1531| 1180| 2810        | -10.5| -24.5| -36.1       |
| β3-E'/α1-D'       | 1309| 1555| 2982        | -14.0| -29.4| -44.4       |
| α1-D'/γ2-C'       | 1426| 1155| 2737        | -7.9 | -23.4| -32.6       |
| γ2-C'/β3-B'       | 1589| 1214| 2894        | -12.7| -21.1| -34.9       |
| β3-B'/α1-A'       | 1319| 1602| 3043        | -11.8| -30.0| -43.7       |
| Mb38/α1-A         | 815 | -   | 830         | -6.2 | -   | -6.3        |
| Mb38/β3-E         | 248 | -   | 248         | -2.9 | -   | -2.9        |
| GABA/ALP          |     |     |             |     |     |             |
| α1-A'/β3-E'       | 1563| 1167| 2833        | -11.8| -23.3| -35.8       |
| β3-E'/α1-D'       | 1501| 1353| 2960        | -13.8| -24.0| -39.9       |
| α1-D'/γ2-C'       | 1346| 1051| 2512        | -5.6 | -19.5| -25.1       |
| γ2-C'/β3-B'       | 1620| 1220| 2937        | -11.9| -22.0| -34.4       |
| β3-B'/α1-A'       | 1510| 1415| 3034        | -14.4| -25.8| -40.9       |
| Mb38/α1-A         | 834 | -   | 834         | -5.8 | -   | -5.9        |
| Mb38/β3-E         | 238 | -   | 238         | -2.6 | -   | -2.6        |
| GABA/DZP          |     |     |             |     |     |             |
| α1-A'/β3-E'       | 1527| 1103| 2733        | -11.2| -24.0| -36.4       |
| β3-E'/α1-D'       | 1453| 1273| 2838        | -13.3| -22.1| -37.0       |
| α1-D'/γ2-C'       | 1324| 1063| 2505        | -4.1 | -23.5| -28.4       |
| γ2-C'/β3-B'       | 1564| 1168| 2846        | -13.2| -19.7| -33.3       |
| β3-B'/α1-A'       | 1473| 1377| 2955        | -12.9| -23.3| -37.1       |
| Mb38/α1-A         | 794 | -   | 800         | -5.5 | -   | -5.7        |
| Mb38/β3-E         | 231 | -   | 231         | -2.4 | -   | -2.4        |

*Buried surface area per interface (sum of monomer areas buried at the interface, divided by 2, calculated using PDBePISA<sup>67</sup>).

†ΔiG (solvation energy gain at complex formation) is the change of the solvation energy of a subunit due to interface formation, in kcal mol<sup>-1</sup>, calculated using PDBePISA<sup>67</sup>.)
Corresponding author(s): A. Radu Aricescu

Statistical parameters

When statistical analyses are reported, confirm that the following items are present in the relevant location (e.g. figure legend, table legend, main text, or Methods section).

|   | Confirmed |
|---|-----------|
| n/a | □ | ✗ |
|   | The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement |
| □ | An indication of 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 statistics 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 suitable. |
| □ | 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 |
| □ | Clearly defined error bars |
| □ | State explicitly what error bars represent (e.g. SD, SE, CI) |

Our web collection on statistics for biologists may be useful.

Software and code

Policy information about availability of computer code

Data collection

FEI EPU, Clampex 8.2

Data analysis

MotionCor2, Gctf-v.1.18, RELION 2.1, RELION 3.0, MonoRes, Scipion, UCSF Chimera v1.12, Pymol v2.0.7, Coot 0.8.9, Phenix 1.13, MolProbity, PDBePISA, HOLE, Clampfit 9.0., Graphpad Prism 6, Igor Pro, Origin 6.

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 upon request. 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

Atomic coordinates of all protein models were deposited in the Protein Data Bank. Cryo-EM density maps were deposited in the Electron Microscopy Data Bank.
Field-specific reporting

Please select 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/authors/policies/ReportingSummary-flat.pdf

Life sciences study design

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

| Sample size | Sample sizes were estimated on the basis of previous studies using similar methods and analyses that are widely published. |
|-------------|------------------------------------------------------------------------------------------------------------------|
| Data exclusions | A small number of the acquired cryo-EM movies were discarded owing to poor ice, excessive movement or defocus. |
| Replication | All attempts to replicate data were successful. |
| Randomization | Randomization is not relevant to this study. |
| Blinding | Blinding is not relevant to this study. |

Reporting for specific materials, systems and methods

Materials & experimental systems

- n/a Involved in the study
- X Unique biological materials
- X Antibodies
- X Eukaryotic cell lines
- X Palaeontology
- X Animals and other organisms
- X Human research participants

Methods

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

Unique biological materials

Policy information about availability of materials

Obtaining unique materials: No restrictions.

Antibodies

Antibodies used: Rho 1D4 antibody was purchased from the University of British Columbia. The Mb38 megabody is available upon request.

Validation: The nanobody Nb38, used to design the megabody Mb38 as described in methods, was validated by surface plasmon resonance and published elsewhere (doi: https://doi.org/10.1101/338343).

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s): The cell line (based on ATCC CRL-3022) expressing the human alpha1beta3gamma2L GABAA receptor has been previously described (PMID 24288268).

Authentication: Authentication was not performed for this study.

Mycoplasma contamination: Mycoplasma testing was not performed for this study.
Commonly misidentified lines
(See ICLAC register)

N/A