Molecular mechanisms of metabotropic GABA<sub>B</sub> receptor function

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Metabotropic γ-aminobutyric acid G protein–coupled receptors (GABA<sub>B</sub>) represent one of the two main types of inhibitory neurotransmitter receptors in the brain. These receptors act both pre- and postsynaptically by modulating the transmission of neuronal signals and are involved in a range of neurological diseases, from alcohol addiction to epilepsy. A series of recent cryo-EM studies revealed critical details of the activation mechanism of GABA<sub>B</sub>. Structures are now available for the receptor bound to ligands with different modes of action, including antagonists, agonists, and positive allosteric modulators, and captured in different conformational states from the inactive apo to the fully active state bound to a G protein. These discoveries provide comprehensive insights into the activation of the GABA<sub>B</sub> receptor, which not only broaden our understanding of its structure, pharmacology, and physiological effects but also will ultimately facilitate the discovery of new therapeutic drugs and neuromodulators.

INTRODUCTION

γ-Aminobutyric acid (GABA) is a key neurotransmitter in the central nervous system (CNS) and is responsible for the inhibition of neurons (1). In the synaptic cleft, GABA is sensed by two types of receptors, GABA<sub>A</sub> and GABA<sub>B</sub>. Ionotropic GABA<sub>A</sub> receptors are pentameric ligand-gated ion channels that mediate fast responses (milliseconds) by counteracting potentials through increasing the Cl<sup>-</sup> permeability of the neuronal membrane (2, 3). Metabotropic GABA<sub>B</sub> receptors are G protein–coupled receptors (GPCRs), which, on the other hand, elicit slow (hundreds of milliseconds) and sustained activity (4) by triggering signal transduction pathways with downstream effectors such as ion channels and adenylyl cyclases, mainly via G<sub>i/o</sub> proteins (Fig. 1). GABA<sub>B</sub> receptors act both pre- and postsynaptically, where they either block neurotransmitter release through the inhibition of voltage-gated Ca<sup>2+</sup> channels or induce hyperpolarization of the neuron by opening G protein–gated inwardly rectifying K<sup>+</sup> (GIRK) channels (5). Given the central role of GABA<sub>B</sub> in neurobiology, it is implicated in a broad spectrum of neurological and psychiatric disorders, such as epilepsy (6), spasticity (7), stress (8), sleep disorders (9), neuropathic pain (10), and depression and anxiety (11). GABA<sub>B</sub> has also been strongly linked to drug addiction, where the systemic administration of the selective GABA<sub>B</sub> agonist baclofen inhibits alcohol, cocaine, morphine, and heroin self-administration in rats (12) and is under investigation as a treatment for alcohol addiction in human patients (https://clinicaltrials.gov/ct2/show/NCT02596763).

GABA<sub>B</sub>, together with metabotropic glutamate (mGlu), calcium sensing (CaS), and taste (TAS) receptors, forms class C of GPCRs (13). GABA<sub>B</sub> receptor was the first GPCR for which the functional entity was demonstrated to be an obligate heterodimer, consisting of two subunits GB1 and GB2, and each subunit individually is thought to be not capable of signaling (14–16). Each receptor subunit comprises an extracellular Venus flytrap (VFT) domain, connected by a short linker to the canonical seven-transmembrane domain (TMD). There are two major identified isoforms of GB1: GB1a [961 amino acids, UniProt (17) ID Q9UBS5] and GB1b (844 amino acids, UniProt ID O75899), which differ in either the presence (GB1a) or absence (GB1b) of two “sushi” domains (SD1 and SD2) at the N terminus. The main role of the sushi domains is trafficking and cell surface stabilization; heterodimers containing GB1 interact with amyloid precursor protein (APP) directing them into axons of glutamatergic neurons, while both isoforms traffic into dendrites (18, 19). The C termini of both subunits contain a coiled-coil (CC) motif, which has two known functions: The formation of the CC has a positive effect on the heterodimerization of GABA<sub>B</sub>, while this interaction also masks an endoplasmic reticulum (ER) retention signal on GB1, ensuring that, predominantly, heterodimers are trafficked toward the plasma membrane (20). Receptor activation has been proposed to consist of a unique allosteric mechanism, where binding of an agonist in the VFT of GB1 results in a series of conformational rearrangements, which are translated into the TMD of GB2 to trigger G protein signaling (21).

Five recent studies have described high-resolution reconstructions of the near full-length GABA<sub>B</sub> heterodimer by single-particle cryo–electron microscopy (cryo-EM) (22–26). In particular, it was possible to map the activation pathway by capturing several discrete conformational states: inactive (apo and antagonist bound), two intermediates (agonist bound), active [agonist and positive allosteric modulator (PAM)] bound, and active in complex with G protein (agonist, PAM, and G protein bound). These structures provide detailed insights into the unique activation mechanism of GABA<sub>B</sub>, where upon agonist binding, large conformational rearrangements of the VFTs are translated to the TMDs, ultimately leading to signaling via G proteins.

Despite tremendous efforts to develop novel drugs, only few targeting GABA<sub>B</sub> are Food and Drug Administration–approved, one of which is the muscle relaxant baclofen (27). Several drug candidates showed promising initial results but were ultimately abandoned due to severe side effects (i.e., seizures, sedation, and respiratory depression) as well as the development of tolerance and dependence. We believe that structural insights in the intricate activation mechanism of GABA<sub>B</sub> receptors offer opportunities for pharmaceutical
intervention by compounds with functional properties distinct from classical agonists, particularly by allosteric modulators.

In this review, we will describe our current understanding of the unique activation mechanism of GABA<sub>B</sub> on a molecular level, which provides fundamental insights into the biology of this important neurotransmitter receptor. We will emphasize the structural aspects of receptor activation while also placing these novel findings in the context of physiology, pathology, and drug development.

**HISTORICAL PERSPECTIVE AND MAJOR MILESTONES OF GABA<sub>B</sub> RESEARCH**

While known as a metabolite in plants and bacteria since the beginning of the 20th century, GABA was not actively studied until its discovery as the major amine present in the brain in 1950 (28, 29). The inhibitory function of GABA was first proposed in the late 1950s (30); however, its role remained controversial for another decade. Since the late 1960s and the 1970s, research had accelerated,
firmly establishing GABA as an inhibitory neurotransmitter acting through a receptor (31). Eventually, Bowery and colleagues (32, 33) found distinct GABA binding sites in 1980, coining the term GABA<sub>B</sub> receptor to distinguish it from the previously known GABA<sub>A</sub> receptor. The physiological roles of GABA<sub>B</sub> were uncovered in a series of studies in the late 1980s (34); however, it has taken another decade to clone the two subunits and establish the heterodimeric nature of the receptor, as reported in three seminal back-to-back publications in 1998 (14–16). Cloning GABA<sub>B</sub> opened up a venue for studying molecular mechanisms of its function, where a high-resolution receptor structure would have been critical but remained elusive, resulting in a divide-and-conquer approach for structure determination. During the past several years, crystallography and nuclear magnetic resonance (NMR) revealed the structure of the VFTs and the details of its orthosteric ligand-binding site (35, 36), the structure of the CC domain that regulates receptor trafficking (20), that of the GB2 C-terminal peptide in complex with an auxiliary protein potassium channel tetramerization domain (KCTD) regulating GABA<sub>B</sub> signaling (37, 38), and the structure of the sushi domains SD2 (39) and SD1 in complex with a peptide derived from soluble amyloid-precursor protein (40). Last, 70 years after the discovery of GABA in the brain, five research groups (22–26) independently determined 12 cryo-EM structures of nearly full-length GABA<sub>B</sub> heterodimer in complex with different ligands and in different conformational states (Table 1), shedding light on its molecular mechanisms of signal transduction across the membrane.

**STRUCTURE DETERMINATION OF THE FULL-LENGTH GABA<sub>B</sub> HETERO DIMER**

The breakthrough in GABA<sub>B</sub> structure determination was enabled by technological advancements in cryo-EM, as well as receptor expression and purification. Most important recent improvements include optimized protocols for cryo-EM sample preparation, particularly using accumulated knowledge of suitable detergents for membrane protein structure determination (41), substantially faster data collection strategies by exploiting beam shift (42), better access to high-end instrumentation, and markedly improved data processing pipelines (43–45). Near full-length constructs of GB1 and GB2, lacking the N-terminal sushi domains and flexible C-terminal tails, were transiently coexpressed in insect (Sf9) (22, 24) or mammalian (HEK293GnTI − or HEK293F) (23, 25, 26) suspension cells using baculovirus- or polyethylenimine-mediated transfection. Sodium butyrate was added 10 to 18 hours after transfection to increase protein expression in mammalian cells (23, 25, 26). Cells were harvested 48 hour (S9) or 48 to 90 hours [HEK293 (human embryonic kidney 293)] after transfection, and receptors were extracted from isolated membranes using either n-dodecyl-β-D-maltosylpolyoxoside/cholesteryl hemisuccinate (CHS) (22, 24) or lauryl maltose neopentyl glycol/CHS (23, 25, 26). The detergents used for extraction were replaced by digitonin (22) or glyco-diosgenin/CHS (24, 26) during receptor purification. Solubilized receptors were purified in the presence or absence of the corresponding ligands using dual-affinity chromatography with two different affinity tags (Flag, His, or enhanced green fluorescent protein) situated on GB1 and GB2 subunits, respectively. Park et al. (23) used ion-exchange chromatography instead of the second affinity purification step. Mao et al. (25) further assembled and captured GABA<sub>B</sub> in complex with a heterotrimERIC G<sub>i</sub> protein and a stabilizing antibody scFv16. In all cases, purified and concentrated receptors were run through a size exclusion column packed with Superose 6 (or Superose Increase) resin before applying them to EM grids, blotting, and plunge-freezing in liquid ethane (22, 24–26) or propane/ethane (23). Kim et al. (26) added 0.1% octyl-β-D-glucoside (OG) to the sample before transferring it to the grids to alleviate the problem of preferred receptor orientation, which often occurs in case of elongated molecules, such as GABA<sub>B</sub>, trapped in thin ice. Addition of OG results in lowering the surface tension and can lead to increased ice thickness, allowing to capture a broader range of receptor orientations, however, in expense of a higher noise limiting the overall resolution. Despite the variations in the sample preparation protocols and potential limitations due to the use of detergent rather than membrane environment, the GABA<sub>B</sub> structures obtained by different groups show remarkable consistency, highlighting the robustness of the applied approaches.

**STRUCTURE OF THE GABA<sub>B</sub> HETERODIMER**

The overall assembly of the GABA<sub>B</sub> heterodimer follows the class C GPCR topology of a dimeric receptor with a large extracellular ligand-binding domain (Fig. 2). The N-terminal extracellular domains, each consisting of two lobes, LB1 and LB2, are referred to as “Venus flytrap domains” (VFTs), since the GB1 VFT can “trap” ligands in its orthosteric binding site, located between the two lobes. Each VFT is connected to a canonical TMD via a stalk domain (Fig. 2A). This stalk consists of a relatively rigid, twisted three-stranded β sheet, formed by the linker connecting the VFT and TMD together with the long extracellular loop 2 (ECL2) of the TMD. A patch of charged residues provides additional stabilization between the stalk and the VFT (Fig. 3B). The stalk domain is unique to GABA<sub>B</sub>, while all other class C receptors have a cysteine-rich domain as a connection between their extracellular and TMDs (Fig. 2B).

The GB1a isoform contains two N-terminal sushi domains, SD1 and SD2, with sequences homologous to complement control protein (CCP) domains, also known as short consensus repeats, which are present in the regulator of complement activation protein family (46). Although each of the sushi domains forms two intact disulfide bonds, only SD2 adopts a compact fold, while SD1 appears to be natively disordered (39). SD1 has been found, however, essential for binding to extracellular matrix and APPs. Structures of intact SD2 and of SD1 in complex with a 9-mer peptide derived from APP were solved by NMR (39, 40). Both structures adopt a similar fold [root mean square distance (RMSD) = 2.1 Å] consisting of a small β sheet, connected by ordered loops.

The structure of the C-terminal CC domain shared between GB1 and GB2 was determined by x-ray crystallography (20). The CC resembles a classic “knobs-into-holes” motif with an extensive network of intersubunit hydrogen bonds. This crystal structure gave insights into the heterodimerization of the two subunits and revealed that the di-leucine ER retention signal is buried within the interface, facilitating trafficking of the GB1–GB2 heterodimer to the cell surface (20). The active state GABA<sub>B</sub> structure of Shaye et al. (22) showed an elongated density protruding from the intracellular side of GB2 at a 20° angle relative to the membrane surface, which was tentatively assigned to the CC domain. However, the local resolution was not sufficient for unambiguous building and inclusion of this domain in the final model. This observation could hint at a possibility that, besides trafficking, the CC domain could also play a...
Table 1. Summary of available GABA<sub>B</sub> structures and details of structure determination methods. CHS, cholesteryl hemisuccinate; GDN, glyco-diosgenin; GFP, green fluorescent protein; LMNG, lauryl maltose neopentyl glycol.

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<th>Resolution (Å)</th>
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regulatory role in receptor signaling, which could warrant further investigations.

The orthosteric ligand-binding site is located at the interface between LB1 and LB2 of GB1 (Fig. 4). On the basis of the available x-ray and cryo-EM structures, both agonists and antagonists are anchored by several polar residues—such as S247, S248, S270, H287, E466—and a key aromatic residue W182 of LB1. Binding of an agonist leads to the closure of these two lobes by engaging two bulky aromatic LB2 residues, Y367 and W395, sandwiching the ligand.

Park et al. (23) and Papasergi-Scott et al. (24) both described putative calcium binding to the LB2 lobe of GB1, in close proximity to the orthosteric binding site of GABA_B (Fig. 3A). While recent structures of receptors across different GPCR classes have started to hint at a broader role of ions in ligand binding and receptor modulation (47, 48), notably, calcium was not identified in the previous high-resolution crystal structures of GABA_B VFT (36), where water molecules were modeled at corresponding sites. In the past, several studies reported a positively modulating effect of a broad range of divalent cations on GABA_B (36, 49–51), while Park et al. (23) have revealed the ion identity using mass spectrometry. The positive modulation could be explained by a stabilizing effect of the ion on the conformation of the loop containing W395, which is one of the key residues in LB2 that interacts with both agonists and antagonists (Fig. 4, C to F).

 Unexpectedly, several groups (23–26) also identified endogenous phospholipid binding sites, located within both TMDs of the heterodimer (Fig. 3C and D). Phospholipid binding has been suggested to confer a positive effect on receptor stability and integrity, as well as to modulate signaling. The locations of these sites within GABA_B TMDs correspond to orthosteric ligand-binding sites in class A and B GPCRs and allosteric sites in class C and F receptors; however, no double-chain phospholipid binding in those pockets has been previously observed. The identity of these lipids was probed by mass spectrometry and assigned as PE (phosphatidylethanolamine) 38:5 (GB1) and PC (phosphatidylcholine) 38:2 (GB2) (23). The overall binding mode of the endogenous phospholipids appears to be conserved between the two subunits, with an intricate network of interactions with most TM helices, except for TM1 and TM4, forming a large interface contact area of $\approx 1000 \text{ Å}^2$. The head group of each lipid is anchored through polar interactions with conserved K3.50 in TM3 and histidine in ECL2 (H760 in GB1 and H647 in GB2), as well as with R714 in ECL3 of GB2. The port of lipid entry into the TMD is most likely located between TM5 and TM6, as one of the acyl tails protrudes through a gap between these two helices in both subunits. Mutational studies suggested that PC 38:2 acts as a negative allosteric modulator (NAM), as it appears to stabilize the inactive state of GABA_B (24); however, further studies are required to fully understand the biological function of these interactions.

### INACTIVE STATE OF GABA_B

All five cryo-EM publications (22–26) present the receptor in an inactive state (three apo- and three antagonist-bound structures, Table 1). Their VFTs are very similar within an RMSD_VFT of $\approx 0.8$ to $1.2 \text{ Å}$ and align equally well with the previously published x-ray structures of apo- and antagonist-bound states of the VFTs (36). Antagonists, CGP54626 and CGP55845, are anchored in the orthosteric site of GB1 VFT by polar interactions with LB1 residues S247, S270, H287, E466, similarly to agonists; however, they have bulky substituents at both amino and phosphinic acid ends interacting with Y367 and W395 from LB2 in its open conformation (Fig. 4, E and F). This observation could hint toward the mechanism of these antagonists acting like a “doorstop,” preventing the VFT from closing for activation, which is consistent with their function as inverse agonists in constitutively active receptor mutants (52). Kim et al. (26) used a NAM CLH304a along with the antagonist CGP54626 for obtaining their structure [Protein Data Bank (PDB) ID 7CUM]; however, no density for the NAM was observed. The TMD conformations between the six structures are very similar (RMSD_TMD $\approx 1.1$ to $1.2 \text{ Å}$, all showing a heterodimer interface with the proposed inter-subunit latch at the intracellular tips of TM5 and TM3, consisting of H689$^{3.55}$, E790$^{5.60}$ in GB1 and H579$^{3.55}$, E677$^{5.60}$ in GB2, which locks the receptor in its inactive state (Fig. 3E). Notably, Park et al. (23) and Kim et al. (26) described an extensive network of cholesterol molecules surrounding this exact interface, potentially stabilizing the intersubunit latch. In addition, in both subunits, the intracellular ends of TM3 and TM6 are constrained by an ionic lock between K$^{3.50}$ and D$^{6.35}$, which is conserved in class C GPCRs (53).

Comparing the inactive state structures overall, the most pronounced differences are found in the relative orientation of the VFTs with respect to the TMDs, of which all five are captured in a slightly different relative angle (within $2^\circ$), with no apparent correlation between apo- and antagonist-bound states, in line with Shaye et al.’s (22) molecular dynamics (MD) simulations that proposed some freedom of motion of the VFT relative to the TMD in the inactive state. As a caveat, this relative motion could also be explained by the fact that all above mentioned data processing schemes either involved separate treatment of the VFT and TMDs, and subsequent merge of the individually refined maps, or used local refinement strategies, allowing for a flexible hinge between the two domains, to obtain high-resolution reconstructions.

### GABA_B ACTIVE STATE

Three of the five studies (22, 25, 26) were able to obtain a high-resolution reconstruction of the active state of GABA_B. In all three structures, a PAM was required in addition to an agonist to lock the
Fig. 2. Comparison of GABA B structure and activation with mGluR5. (A) Structures of GABA B in apo inactive [Protein Data Bank (PDB) ID 6VJM, left] and agonist-bound active (6UO8, right) states are shown in side views (top) and looking on TMDs from the extracellular side (bottom). (B) Similar views as in (A) for mGluR5 receptor in apo inactive (6N52, left) and agonist-bound active (6N51, right) states. Membrane boundaries are shown as a gray slab. GABA B is the only member of class C GPCRs lacking the cysteine-rich domain (CRD); instead, its elongated ECL2 forms a three-stranded β sheet together with a linker connecting VFT and TMD, termed stalk. A common mechanism of activation in class C GPCRs involves a twisting motion of each subunit along the (pseudo-)symmetry axis in which TM5s move apart while TM6s approach closer forming the (hetero-)dimeric interface. The distances shown in lower panels are measured between Ca atoms of residues 5.41 of both subunits (Fig. 4) (22, 25, 26). Both PAMs have amphiphilic properties, allowing them to partition into and penetrate through the membrane. Mao et al. (25) and Kim et al. (26) used rac-BHFF as PAM but found that the (+)-BHFF enantiomer fits better in the experimental density, in agreement with pharmacological data (54). (+)-BHFF occupies a predominantly hydrophobic pocket made of residues GB1-Y810 (6UO8) with GB2-N698 (6N51) and GB1-N811 (6N52) with GB2-Y697 (6N51). The ionic lock between TM3 and TM6 in GB2 is broken, and the intracellular ends of TM3, TM4, and TM5 are shifted by 4 to 6 Å with respect to their positions in the inactive state, opening up a cleft on the intracellular side of GB2 for G protein binding. All three structures are similar (RMSD < 2 Å), showing overall rearrangements required for the activation of GABA B, while indicating that two structurally different PAMs are binding to the same allosteric site at the heterodimer TMD interface. However, comparing these three structures, the Shaye et al. (22) structure (PDB ID 6UO8) appears slightly bulkier overall—based both on the longer distance between the VFT lobes (~ 2.5 Å, measured between Ca atoms of GB1-Q577 and GB2-K467) and on a slightly looser packing of the TMDs (~ 2 Å longer TM5-TM5 distance, measured between Ca atoms of GB1-Y774 (5,44) and GB2-Y661 (5,44)). In summary, this suggests that the active state conformation of GABA B may slightly differ depending on the specific combination of an agonist and PAM; however, it does not seem to depend on the presence of a G protein.

PAM BINDING SITES COMPARISON

One of the most notable discoveries in the reported cryo-EM structures was the identification of the PAM binding sites at the interface of the two TMDs in the active state of GABA B. Notably, two chemically distinct PAMs, GS39783 (6UO8) and (+)-BHFF (7C7Q and 7CA3), were found to bind at overlapping sites, buried inside the membrane and composed of residues from TM5, TM6, and TM7 of both subunits (Fig. 4) (22, 25, 26). Both PAMs have amphiphilic properties, allowing them to partition into and penetrate through the membrane. Mao et al. (25) and Kim et al. (26) used rac-BHFF as PAM but found that the (+)-BHFF enantiomer fits better in the experimental density, in agreement with pharmacological data (54). (+)-BHFF occupies a predominantly hydrophobic pocket made of residues GB1-A788 (5,48), GB1-Y789 (5,59), GB1-M807 (6,41), GB1-Y810 (6,44), GB2-K690, GB2-Y691, and GB2-M694 (6,41), and it forms one hydrogen bond with GB1-K792 (5,13). On the other hand, GS39783 is anchored by a hydrogen bond with GB2-N698 (6,45) and a stacking interaction with GB1-Y810 (6,44), as well as by extensive hydrophobic and polar interactions with GB1-Y789 (5,59), GB1-K792 (5,13), GB1-M807 (6,41), GB2-M694 (6,41), and GB2-Y697 (6,44).

G PROTEIN BINDING

Mao et al.’s study (25) is the only one of the five studies that describes a structure of GABA B in complex with a G i protein. Initially, the authors tried capturing the structure of the complex using a
full-length GABA_B, which resulted in sample aggregation. Removing the CC increased the yield of the receptor and facilitated the formation of GABA_B-Gi complex. To assemble the complex, HEK293F cells expressing truncated GABA_B-ΔCC construct were combined with SF9 cells expressing Gi1 protein, followed by addition of an agonist baclofen, a PAM rac-BHFF, an apyrase to hydrolyze guanosine 5′-triphosphate, and a Gi protein–stabilizing antibody scFv16. Purified complex was used to collect single-particle cryo-EM data, revealing three distinct major conformations identified by 3D classification. Further refinement produced three reconstructions that differed only by the binding position of the Gi1 protein: (i) 17% of particles were found in a state termed B1 (8.8-Å resolution) in which Gi1 is bound to GB1, (ii) 74% of particles were found in a B2a state (6.8 Å) in which Gi1 is bound to GB2, and (iii) 9% of particles were found in a B2b state (8.6 Å) in which Gi1 is also bound to GB2 but in a different orientation from the B2a state (25). This distribution agrees with the asymmetric model of GABA_B signaling in which G protein couples predominantly to GB2. Although it was not possible to model the Gi1 protein unambiguously, its Gi1 subunit appears to be in an activated nucleotide-free form based on the separated densities for the α-helical (AHD) and Ras-like domains. Compared to class A GPCRs, the opened upon activation cleft on the intracellular side of GB2 is very shallow, preventing the C-terminal α5 helix of Gi1 from the insertion in the GB2 TMD. This leads to a much smaller Gi1 protein–binding interface in which the α5 helix interacts mostly with intracellular loops and the tip of TM3, resulting in a more flexible binding of Gi1 that allows multiple conformations, where the B2a state is the most stable, while the B2b state in which Gi1 is rotated about 90° may represent an intermediate state.

**GB1 HOMODIMER**

While the primary signaling unit of GABA_B is a heterodimer, it has been shown that each subunit can form homodimers (55) and that the two subunits can assemble into higher-order oligomers (56). Papasergi-Scott et al. (24) observed that, along with the GB1-GB2 heterodimer, they could purify a GB1 homodimer. Subsequently, they collected cryo-EM data for a GB1 homodimer bound to an antagonist CGP55845, which resulted in a 3.2-Å resolution structure (PDB ID 6W2Y) with each subunit occupied by the antagonist and arranged in a twofold symmetry (24). In the antagonist-bound homodimer, the VFTs of each subunit adopt a fully open conformation, very similar to the GB1 subunit of the antagonist-bound x-ray structure (4MR7, RMSD = 0.86 Å), locked in an inactive conformation (36). The overall conformation of the homodimer, however, is similar to the conformation of the active state heterodimer in which the LB2 lobes of both subunits come in contact, rearranging the TMDs to interact along TM6. Despite resembling the active-like subunit arrangement, there are no conformational changes within TMDs, and the homodimer is unable to bind to G protein and other transducers.

**ACTIVATION MECHANISM OF GABA_B**

In addition to two major stable receptor conformations, active and inactive, Shaye et al. (22) observed two distinct intermediate states (int-1 and int-2) along the receptor activation trajectory in the presence of an agonist (Fig. 5). Both intermediate states have comparatively lower resolution (6.3 and 4.8 Å), as they are likely present in a dynamic equilibrium, preventing assignment of most amino acids. These

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*Fig. 3. Structural features of GABA_B.* Left: Overall view of apo GABA_B in the inactive conformation (PDB ID 6WIV). (A) Representative model of Ca2+-binding site in GB1 bound to antagonist CGP55845 (6W2V). (B) In each subunit, VFT is connected to TMD via a stalk domain that is stabilized by a network of electrostatic interactions between positively (blue) and negatively (red) charged residues. (C and D) Binding sites for endogenous phospholipids: PC 38:2 in GB2 TMD (C) and PE 38:5 in GB1 TMD (D). Receptor surface is colored by hydrophobicity (125), where more hydrophobic residues are shown in darker shade of green and charged residues are colored using the same color scheme as in (B). (E) Intersubunit lock between TM3 and TM5 at the heterodimeric interface stabilizes inactive conformation. (F) Activation-related ionic lock between TM3 and TM6 in GB1 TMD. Ligand and lipid molecules are shown as sticks with carbon atoms colored in sand, oxygen in red, nitrogen in blue, chlorine (A) and phosphor (C and D) in light green, sulfur in yellow, and calcium ion in magenta. GB1 and GB2 subunits are colored in blue and gold, respectively.
intermediate state structures, nevertheless, provide important insights into the activation pathway of GABA<sub>B</sub>. They add crucial details to the large conformational rearrangements that delineate the activation pathway, where, first, the LB lobes of the GB1-VFT close upon agonist binding (int-1 state), likely accompanied by breaking the intersubunit latch between the two TM5s. After the closure of the VFT, a rearrangement of the entire heterodimer interface is observed, which closes the gap between the LB2 domains of the two VFTs while forming a new TMD interface along TM6s (int-2 state).

The presence of these intermediate states highlights that GABA<sub>B</sub> activation is decoupled into at least two main transitions, as opposed to a single concerted motion. The second intermediate state—in contrast to the final active state structures—also demonstrates that the GB2 TMD remains in the inactive conformation, with the TM6-TM3 ionic lock still intact. Only the addition of a PAM that stabilized this state for structure determination allowed capturing the final step of the activation-related rearrangements, namely, straightening and displacements of intracellular parts of TM3 to TM5, which open a cleft that accommodates G protein binding. These findings have general implications for our understanding of GABA<sub>B</sub> as they showcase a tightly regulated activation mechanism that is conserved between the two subunits.

Comparing the conserved motifs in class A and C receptors in more detail, notable similarities and differences can be attributed to their different modes of action become apparent. (i) The ionic lock between K<sup>3.50</sup> and E/D<sup>6.35</sup>, conserved in class C receptors, is also observed in the inactive state in both GABA<sub>B</sub> subunits (61). A similar conserved lock is present in class A GPCRs between the residues R<sup>1.50</sup> and D/E<sup>6.30</sup>, where mutational disruptions of the lock resulted in increased basal activity (62, 63). It should be noted in this context that generic residue numbering differs between GPCR classes and depends on the intraclass conservation of a residue rather than its absolute position relative to the lipid bilayer (64). The ionic lock appears to play an important role in GABA<sub>B</sub> signaling, since it is broken in GB2 in the active state, and mutating GB2-K<sup>1.50</sup> to an acidic residue results in a complete loss of function (63). (ii) The FxP<sup>7.50</sup>KxY motif at the intracellular end of TM7 is highly conserved in class C GPCRs and shares similarity with the NP<sup>7.50</sup>xxY motif of class A. In class A, Y<sup>7.53</sup> plays a critical role in stabilizing the TM3-TM7 interface in the active state. However, in class C, Y<sup>7.53</sup> (mGluR) or R/I<sup>7.53</sup> (GB1/2) faces toward the outside of the helical bundle and does not make any contacts with TM6. On the other hand, K<sup>7.51</sup> participates in a network of hydrogen bonds with the ionic lock through N<sup>2.39</sup>, S625<sup>ICL1</sup> in GB1 and S515<sup>ICL1</sup> in GB2, thereby stabilizing the intracellular segment of the TMD. (iii) The residue W<sup>6.48</sup> from the highly conserved FxxCWxP<sup>6.50</sup> motif in class A GPCRs was proposed to act as a “toggle switch,” changing its conformation upon activation (65). In addition, F<sup>6.44</sup> belongs to the P-I-F motif that rearranges upon agonist binding (66), initiating a cascade of conformational changes that result in the large-scale movements of TM5 and TM6, and enables the receptor to engage with G protein. The mGlu receptors, on the other hand, feature a highly conserved W<sup>6.50</sup> residue at the equivalent position; however, in mGluR<sub>C</sub>, it holds a different conformation, forming a bridge between TM5 and TM6 (53). In both GB1 and GB2 of GABA<sub>B</sub>, this residue is replaced with a Cys residue to

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**Fig. 4. Structural details of orthosteric ligands and PAM binding to GABA<sub>B</sub>.** (A and B) Binding of PAMs at the heterodimeric TMD interface: GS39783 (PDB ID 6UO8) (A) and (+)-BHFF (7C7Q) (B). (C and D) Binding of agonists in the orthosteric site of GB1 VFT: SKF97541 (6UO8) (C) and baclofen (7C7Q) (D). (E and F) Binding of antagonists in the orthosteric site of GB1 VFT: CGP55845 (6W2X) (E) and CGP54626 (7C7S) (F). (G) Orthosteric ligand-binding site of GB1 VFT in the apo state (6VJM). Ligands are shown as sticks with carbon atoms colored in sand, oxygen in red, nitrogen in blue, chlorine and phosphor in light green, sulfur in dark green, and fluorine in pale blue. GB1 and GB2 subunits are colored in blue and gold, respectively. In (C) to (G), LB1 and LB2 of GB1 subunit are colored in blue and teal, respectively.
avoid the steric clash with the phospholipid occupying the TMDs and allows the hydrophobic chain of the lipid to enter a cavity created at the interface of TM5 and TM6 of both subunits.

IMPLICATIONS OF GABAB STRUCTURES FOR UNDERSTANDING DISEASE AND DRUG DISCOVERY

Mutational model of GABAB and its correlation to different diseases

GABAB is considered the oldest member of class C GPCRs (67). Given the importance of GABA as a major inhibitory neurotransmitter in the brain, it is perhaps expected that the amino acid sequence of GABAB receptor is evolutionarily constrained. This results in an exceptionally low number of naturally occurring variants: Among the 19,704 human Ensembl transcripts in the gnomAD (v 2.1.1) database of naturally occurring sequence variants in more than 15,000 genomes and more than 125,000 exomes (68, 69), GB1 and GB2 both rank in the 99th percentile (127th/151st, respectively) and first and second among 697 GPCRs mapping to human transcripts, in terms of the lowest observed number of missense mutations (i.e., nucleotide changes leading to an amino acid substitution), normalized for gene length and sequence context (70, 71). In particular, both subunits display fewer than half (ratios of 0.42 and 0.44, respectively) of the number of expected missense mutations, i.e., are strongly intolerant of (such) variations.

We limit the following analysis of variants to missense mutations, since their effect can be most immediately rationalized based on the experimental GABAB structures that have now become available. When mapping the location of the observed variants onto these structures, it becomes apparent that they are underrepresented in areas associated with protein function and ligand binding, as described above. For example, there are no missense mutations within the orthosteric ligand-binding site or ion-coordinating residues of GB1, the ionic lock is unaffected, as well as the intersubunit latch and the FxPKxY motif in both subunits. A single missense mutation has been observed for a residue linking the ionic lock and the FxPKxY motif (GB2-N2520.39T); two missense mutations in the nonpolar interaction interface between TM5s that is associated with the inactive receptor state (GB1-L7795.49V and GB2-L6695.52S); and a small number of missense mutations near the PAM binding site between the intracellular tips of TM5 and TM6 (GB1-A7885.58S, T7915.61N, R8036.37Q/P, and G8066.40V). A number of variants slightly higher than that observed for microswitches map to residues in the proximity of the two endogenous phospholipids, most of which preserving the hydrophobic character of this region.

While mapping these mutations to motifs of known or presumed function in GABAB and other GPCRs earmarks them for more detailed molecular studies, several missense mutations in GABAB have already been reported to have strong association to disease, particularly the Rett syndrome and epileptic encephalopathy (72–74). Mapping the most notable variants—such as GB2-A5673.43T, GB2-G6936.40W, GB2-S6956.42I, GB2-I7056.52N, and GB2-A7076.54T—onto the available GABAB structures revealed their distribution across the GB2 TMD. In particular, GB2-A5673.43T is situated at the bottom of the lipid-binding crevice of GB2 and has been found to impair receptor signaling efficacy (73). Of the other residues, GB2-I7056.52N points toward the lipid bilayer, which could potentially destabilize the TMD and affect TM6–TM6 dimerization, while GB2-A7076.54T is close to the opening through which the lipid enters the pocket. Residues GB2-G6936.40W and GB2-S6956.42I are at the interface of TM6 with TM5 and TM7, respectively, close to the allosteric binding site.

Pharmacological modulation of the GABAB receptor

While benzodiazepines are widely used as stereotypical modulators of GABA receptors with sedative, hypnotic, anticonvulsant, and
muscle relaxant properties, fewer approved drugs target GABA_B (75–77). We will give a brief overview of compounds targeting GABA_B described to date, spanning a variety of pharmacological efficacies including orthosteric agonists and antagonists, as well as positive and NAMs (Fig. 6), and highlight their clinical application.

The inability of the endogenous agonist, GABA (in structures with PDB IDs 4MS3 and 7CA3), and its derivatives to efficiently penetrate the blood-brain barrier (BBB) (78) has motivated the development of baclofen (79, 80) (in 4MS4 and 7C7Q), which has been approved as a muscle relaxant and antispasmodic agent in the United

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**Fig. 6. Chemical structures of select GABA_B ligands.** Compounds used for structural studies described in this review are highlighted in bold, and, in the following, ChEMBL (126) identifiers or PubChem (127) identifiers are given where available. Clinical trial identifiers (clinicaltrials.gov) and indications are listed where available. Agonists: GABA (CHEMBL96), GHB (γ-hydroxybutyric acid; CHEMBL1342, approved for cataplexy, NDA 021196), Lesogaberan (CHEMBL448343, phase 2 clinical trial for gastroesophageal reflux disease, NCT01043185), CGP27492 (CHEMBL112203), CGP35024 (CHEMBL112710), baclofen (CHEMBL701, approved antispasmodic and muscle relaxant, NDA 017851), arbaclofen (CHEMBL301742) (phase 3 clinical trials for autism spectrum disorder and fragile X syndrome, NCT01282268 and NCT01706523), phenibut (CHEMBL315818, approved in Russia), and tolbut (PubChem ID 49344). Antagonists: CGP36216 (CHEMBL329521), GCP36742 (CHEMBL112797, phase 2 clinical trials for Alzheimer’s disease, NCT00093951), SCH50911 (CHEMBL1895916), CGP54626 (CHEMBL1213187), CGP55845 (CHEMBL455185), phaclofen (CHEMBL1255941), saclofen (CHEMBL312403), 2-hydroxysaclofen (CHEMBL1256573), and tramiprosate (CHEMBL149082, phase 3 clinical trials for Alzheimer’s disease, NCT00088673). PAMs: GS39783 (CHEMBL392394), BHF177 (CHEMBL2346820), ADX71441, fendiline, CGP7930 (CHEMBL1256697), and BHFF (CHEMBL2322946). Negative allosteric modulator: compound 14 (CHEMBL3326377).
States in 1977. The subsequent identification of its bioactive enantiomer R-(-)-baclofen (arbaclofen) (81, 82) and its binding selectivity profile has helped to uncover the existence of a distinct subset of GABA binding sites and the GABA_B receptor subtype a few years later (32). Since baclofen requires active transport across the BBB (83), attempts to target GABA_B in the CNS prompted the development of more readily bioavailable formulations and prodrugs (84–89). γ-Hydroxybutyric acid (GHB), which is metabolized into GABA and itself acts as a weak agonist of GABA_B (90, 91), displays anesthetic and intoxicating properties and is thought to elicit its effects through a mechanism involving an additional receptor (79, 90, 92, 93). Approved for treatment of cataplexy and narcolepsy (91, 94, 95), GHB and its salts are controlled substances notorious for their illegal and recreational use. While BBB penetration is required for CNS action, a different strategy is used for targeting peripheral GABA_B receptors. Thus, Lesogabaran, an agonist with poor brain penetration to avoid CNS side effects, has reached phase 2 clinical trials for the treatment of gastroesophageal reflux disease where it failed to show significant efficacy, halting its further development (96).

To date, no GABA_B receptor antagonist has found clinical approval, although they are promising agents for mediating antidepressant (97), behavioral, and neuroprotective effects (98). Functionally, antagonizing the inhibition of GABA-mediated neurotransmitter release such as glutamate, noradrenaline, or serotonin could enhance their function, thereby indirectly modulating their downstream effectors. The first selective antagonists described were phaclofen (in 4MRM), saclofen (in 4MQF), and 2-hydroxysaclofen (99–101). Notably, extensions of the methyl moiety of the agonist CGP35024 (SKF97541, in 6U08, 6U09, and 6UAO) (102) were shown to confer antagonism [CGP36216 and CGP36742 (103, 104)]. Antagonists CGP36742 and tramiprosate (homotaurine) have been investigated as treatments of Alzheimer’s disease (105, 106), with the latter still available as a dietary supplement in the United States, while the orally active SCH50911 (107) has been in preclinical development as a treatment for absence seizures. The low affinity of all of these antagonists limits their usefulness as tool compounds (79), prompting development of compounds with nanomolar affinity such as CGP54626 (16) (in 4MRK, 7C75, and 7CUM) and the related CGP55845 (108) (in 6W2X and 6W2Y).

Improvement of ligand potency and selectivity as well as bioavailability remains an active topic of research that has motivated the exploration of a multitude of molecular decorations of the GABA scaffold. The high-resolution GABA_B structures help to rationalize structure-activity relationship data for many of these compounds and better understand the functional consequence of the ligand pharmacophore on receptor function. GABA consists of a carboxylic acid and an amino group connected by a three-carbon aliphatic chain. Substitution of the amino group in GABA can decrease its in vivo activity, demonstrating nociceptive responses at lower doses compared to baclofen and without any sedation effect (111). However, replacing the methyl group with bulkier ethyl or butyl converts the full agonist SKF97541 into a partial agonist CGP36216 or an antagonist CGP36742, respectively. We attribute this behavior to a two-step transition involved in VFT closure. Initially, an agonist binds to the LB1 of GB1 governed by a network of hydrogen bonds between the amino and carboxyl (or phosphinic acid) groups of the ligand and residues in the binding pocket. The amino group interacts with E466 and H287 while the carboxylic or phosphinic group forms a network of hydrogen bonds with S270 and S247 (Fig. 4). In addition, the central aliphatic chain of the ligand makes van der Waals contacts with W182. Removing the amino or carboxy group of the ligand reduces polar interactions rendering it inactive and therefore unable to bind to GB1 VFT. The second step involves the closure of GB1 VFT that adds interactions with residues in the second lobe. These interactions are mainly hydrophobic or aromatic and are mediated by several residues such as W395, V318, and F319 in the LB2 that patches the ligand between the two lobes. In addition, Y367 stabilizes agonist binding by forming a hydrogen bond to ligand’s carboxy/phosphinic acid end via its carbonyl group and making van der Waals interactions with the hydrophobic core of the ligand. Adding bulky groups to either end of the GABA scaffold or increasing the length of its aliphatic chain renders the ligand an antagonist by introducing steric clashes with Y367 and/or W395 and preventing the closure of LB2.

Allosteric modulators as novel opportunity for therapeutic intervention

Allosteric modulators promise safer alternatives to orthosteric synthetic ligands as they can modulate receptor signaling without replacing the action of endogenous ligands. PAMs have the ability to potentiate response to endogenous agonists while binding at distal sites (108). They have little to no intrinsic activity and open up chemical space and the possibility for specific receptor modulation without being constrained to the endogenous ligand chemotype. Their ability to fine-tune receptor response to endogenous agonists where physiologically relevant, and to potentiate effects of co-administered exogenous agonists, minimizes their abuse potential and makes them a sought-after class of compounds for clinical applications (112–114). In case of GABA_B receptor, a number of PAMs have been identified (Fig. 6), with CGP7930 (115), followed by pyrimidine-based GS39783 (116) (in 6U08), prototypical examples of PAMs that have found initial promise in animal models related to substance abuse, and showed anxiolytic effects, motivating their continued clinical development. The fluorinated benzofuranone derivative rac-BHFF (in 7C7Q and 7CA3) was obtained in the process of optimization of CGP7930. More recently, the CGP7930 scaffold has been modified to obtain the first NAM, compound 14 (117), also known as CLH304a. Its molecular similarity to the PAM CGP7930, juxtaposed with the disappearance of the well-defined PAM binding site formed by heteromeric contacts around the intersubunit latch region, warrants the future mechanistic exploration of NAMs.

CONCLUSIONS

Recent comprehensive cryo-EM studies described in this review have markedly furthered our understanding of the structure-function relationship of the metabotropic GABA_B receptor and shed new light on its complex biology and pharmacology. High-resolution structures of the near full-length receptor obtained in several conformational states provide detailed insights into the unique asymmetric activation mechanism of this heterodimeric receptor. Binding of an agonist in the GB1 VFT triggers a series of concerted motions leading...
to conformational changes in GB2 TMD, priming it for interactions with and activation of G proteins. This activation mechanism is fundamentally different from that of class A, B, and F GPCRs in which the hallmark of activation involves large-scale shifts of TM5, TM6, and TM7 on the intracellular side, opening a large cleft for engagement of G proteins and β-arrestins. On the contrary, in GABA\(_B\) and, likely, other class C receptors, the motion of TM6 in the active state is constrained by the hetero(or homo)-dimeric interface, while, instead, TM3, TM4, and TM5 of GB2 shift upon activation, creating a relatively shallow groove, mostly within intracellular loops, which could explain the high plasticity of GABA\(_B\) interactions with G proteins and difficulties in capturing the structure of such complexes. The activation transition of GABA\(_B\) occurs in at least three discrete steps involving two intermediate states, and these states could help to analyze effects of specific mutations and potentially be targeted by NAMs. In addition to these mechanistic insights into receptor activation, new structures revealed previously allotropic sites for calcium in GB1 VFT, endogenous phospholipids inside of activation, new structures revealed previously unknown allosteric sites for calcium in GB1 VFT, endogenous phospholipids inside of both TMDs, and PAMs at the interface between two TMDs.

Besides the advances of our understanding of GABA\(_B\), a list of important open questions regarding its structure and function remains. Although difficult to achieve, a high-resolution structure of a GABA\(_B\)-Gi complex would unveil a critical milestone, structurally constraining the recognition of the receptor activation trajectory and helping to decipher G protein recognition, selectivity, and activation mechanisms. Along the same lines, structural identification of NAM binding sites would provide essential templates for structure-based design of novel therapeutic modulators.

In addition to G proteins and KCTDs, high-resolution proteomics identified at least 20 soluble and membrane proteins that interact with GABA\(_B\) (118). It has been proposed, therefore, that GABA\(_B\) functions within macromolecular signaling complexes with defined architecture but diverse composition, which could explain their complex biology. Isolating such complexes and determining their structures would further refine signaling mechanisms of this receptor. GABA\(_B\) is also directly modulated by various toxins such as α-conotoxin, derived from the venom of marine snails of the Conus genus, which has been identified as a potential analgesic (119). It is completely unknown how these toxins are binding to GABA\(_B\), which will be a matter of future biochemical and structural investigations.

All structures described in this review represent hetero- or homodimers. However, GABA\(_B\) is also known to form higher-order oligomers such as tetramers (56). Future studies will be required to find out how these oligomers can be stabilized, as upon detergent solubilization, most of the receptors regress to their dimeric state. Another interesting avenue are heterodimers between different types of receptors, such as GB1 and CaSR, which have been described recently (120).

Last, while structural snapshots provide important high-resolution static views of stable conformational states and complexes, our understanding of the receptor function is incomplete without information about receptor dynamics, obtained by complementary spectroscopic techniques (121). Recent advances in sample preparation and labeling methods as well as in single-molecule fluorescence, NMR, electron paramagnetic resonance, and computer modeling approaches should translate into accumulation of data on each state’s free energy, dwell time, and exchange rates, providing a comprehensive view of the receptor’s free energy landscape (122). Ultimately, time-resolved serial femtosecond crystallography at x-ray–free electron sources shows promise to record molecular movies of proteins undergoing conformational changes with high spatial and temporal resolution (123, 124). Taking it all together, we expect that our understanding of GABA\(_B\) function will advance to a new level within the next few years.

Note added in proof: A high-resolution cryo-EM structure of GABA\(_B\)-Gi complex is now available (https://doi.org/10.1038/s41586-021-03507-1).

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Molecular mechanisms of metabotropic GABA<sub>B</sub> receptor function
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