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Activation of the γ-Aminobutyric Acid Type B ($GABA_B$) Receptor by Agonists and Positive Allosteric Modulators

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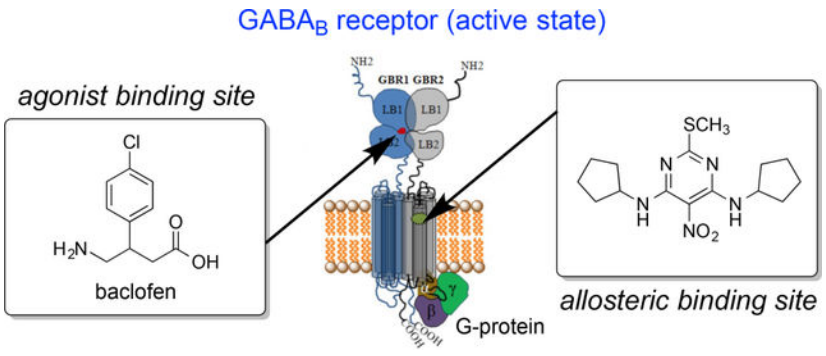
Abstract

Since the discovery of the $GABA_B$ agonist and muscle relaxant, baclofen, there have been substantial advancements in the development of compounds that activate the $GABA_B$ receptor as agonists or positive allosteric modulators. For the agonists, most of the existing structure–activity data applies to understanding the role of substituents on the backbone of GABA as well as replacing the carboxylic acid and amine groups. In the cases of the positive allosteric modulators, the allosteric binding site(s) and structure–activity relationships are less well defined; however, multiple classes of molecules have been discovered. The recent report of the X-ray structure of the $GABA_B$ receptor with bound agonists and antagonists provides new insights for the development of compounds that bind the orthosteric site of this receptor. From a therapeutic perspective, these data have enabled efforts in drug discovery in areas of addiction-related behavior, the treatment of anxiety, and the control of muscle contractility.

Graphical Abstract



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Author Contributions

The manuscript was written through contributions of all authors.

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Introduction

In the central nervous system, the primary inhibitory neurotransmitter is γ-aminobutyric acid (GABA). Its major roles are to regulate the release of other neurotransmitters and the excitation of neurons. These actions are modulated by three types of receptors: $GABA_A$, $GABA_B$, and $GABA_A\rho$ (also known as $GABA_C$). These three receptors have served as a rich source of targets for the pharmaceutical industry and many drugs have been developed as ligands for these sites.[1] The ionotropic $GABA_A$ receptor, a ligand-gated chloride-ion channel that suppresses neuronal excitability, is the most extensively targeted receptor. $GABA_A$ receptors are pentamers assembled around a central pore, and sixteen distinct subunits have been identified (six α, three β, three γ, δ, ε, θ, and π).[2] Typically, $GABA_A$ receptors consist of two identical α subunits, two identical β subunits, and one "other" subunit. The differential subunit assembly may give rise to considerable diversity in channel composition. Three major classes of pharmaceuticals, the barbiturates (e.g., phenobarbital), the benzodiazepines (e.g., diazepam), and the nonbenzodiazepine sedative/hypnotics (e.g., zaleplon and zolpidem), are ligands that bind to the $GABA_A$ receptor.[1, 3] The $GABA_A\rho$ receptors are also chloride-ion channels that are homo-pentameric assemblies of ρ1–3, and perhaps, other subunits.[2] Though they are ionotropic receptors, they have a distinct pharmacology from $GABA_A$ receptors, being resistant to both the $GABA_A$ antagonist bicuculline and the sedative/hypnotic drugs that target the $GABA_A$ receptors.[4, 5] Currently, no pharmaceuticals target the $GABA_A\rho$ receptor as a primary mechanism of action.[1, 6]

The $GABA_B$ receptor is an unusual G-protein coupled (i.e., metabotropic) receptor in that it exists as a heterodimer of $GABA_{B1}$ and $GABA_{B2}$ subunits.[7] These receptors can inhibit the release of many neurotransmitters, such as dopamine, serotonin, and acetylcholine, via a G-protein-dependent inhibition of neuronal voltage-gated Ca^{2+} channels.[8] However, activation of $GABA_B$ receptors also reduces neuronal excitability via activation of G-protein regulated inwardly rectifying K^+ (GIRK or Kir3) channels.[8] Finally, $GABA_B$ receptors regulate intracellular signaling by inhibiting adenylyl cyclase activity.[9] $GABA_B$ receptors have an important function in the neuronal pathophysiology of many CNS diseases and disorders including anxiety, depression, epilepsy, autism spectrum disorder, stroke, drug addiction, and the neurodegenerative disorders: Huntington's disease, Parkinson's disease, and Alzheimer's disease.[10, 11] These receptors also have an established role in muscle spasticity disorders,[12] pain,[13] gastro-esophageal reflux disease (GERD),[14] and schizophrenia.[15] In contrast to the highly targeted $GABA_A$ receptor, currently the only FDA-approved drug to target the $GABA_B$ receptor is baclofen, a muscle relaxant. Specifically, baclofen is indicated for the relief of muscle spasticity in patients with multiple sclerosis and spinal cord injuries; however, baclofen is used off-label to treat many other conditions. One such off-label use is in the treatment of GERD[14] and other peripherally acting $GABA_B$ agonists are under evaluation for the treatment of GERD.[16, 17] $GABA_B$ agonists, including baclofen,[10] and $GABA_B$ positive allosteric modulators[18] have shown promise in numerous studies investigating their ability to prevent relapse in the treatment of drug and alcohol addiction,[19, 20] as well as in the treatment of other neurological disorders.[21, 22] In addition, the combination of baclofen and acamprosate was recently shown to be neuroprotective in a rodent model of Alzheimer's disease.[23]

Despite its FDA approval for the treatment of muscle spasticity and a growing number of off-label and potential uses, baclofen is far from an ideal drug molecule. Baclofen has low penetration into the brain,[24] a property which may be ascribed to transport out of the brain via the organic acid transporter.[25] Additionally, baclofen has a short duration of action, a narrow therapeutic window, and a rapid tolerance.[26] One notable advance is the investigational compound, arbaclofen placarbil, which serves as a prodrug of (*R*)-baclofen and was developed to enhance oral absorption compared to the parent compound.[26] Although baclofen can be administered intrathecally via an implanted pump to reduce the doses required to achieve relief of muscle spasms and reduce peripheral side effects, additional innovations are needed. Thus, development of $GABA_B$ agonists or positive allosteric modulators with excellent brain/CNS penetration would provide a substantial improvement over baclofen in many therapeutic applications.

The extensive drug development efforts on the neurotransmitter, GABA, and the GABA receptors have provided many analogues of GABA. Correspondingly, the structure of GABA is embedded in the pharmaceuticals, baclofen, gabapentin, pregabalin, and vigabatrin (Figure 1). Although they display highly similar structures, the targets of these drugs are quite varied and some do not act on the GABA pathways.[1] Gabapentin is an anticonvulsant, and despite its structural resemblance to GABA, gabapentin does not bind GABA receptors or influence the production or uptake of GABA.[27] Pregabalin, which is used as an analgesic for neuropathic pain and as an anticonvulsant, also does not bind to GABA receptors or affect concentrations of GABA in the brain. Vigabatrin is an anticonvulsant and its mechanism of action is the inhibition of GABA transaminase, an enzyme responsible for the degradation of GABA.[28] These agents further demonstrate the tremendous value in understanding the structure and the function of GABA and its receptors. The recent report in 2013 of the X-ray structure of the $GABA_B$ receptor, along with co-crystallized agonists and antagonists, provides a major breakthrough in this area, and a great foundation for the protein structure-based drug design of new modulators of this type of receptor.[29] This perspective will serve as a guide of the existing structure-activity data for the activation of the $GABA_B$ receptor. The most significant and current potential for activation of the $GABA_B$ receptor is in the areas of alcoholism and drug abuse, and for example, three reviews focused on the preclinical and clinical studies in these therapeutic areas have appeared in the literature in 2014.[18, 30, 31]

The $GABA_B$ receptor mediates slow and prolonged synaptic inhibition through G_i or G_o proteins, and malfunctions of the $GABA_B$ receptor can be associated with various kinds of neurological disorders, including spasticity and epilepsy.[29, 32–34] The functional unit of the $GABA_B$ receptor is a heterodimeric assembly of the two subunits GBR1b ($GABA_{B1}$) and GBR2 ($GABA_{B2}$)[9, 35–37] containing 844 and 941 amino acids, respectively.[38] Figure 2 depicts the complete $GABA_B$ receptor system, including its active and inactive (or resting) states. The extracellular Venus flytrap (VFT) domain (also called ectodomain) of the GBR1b subunit contains the recognition site for orthosteric ligands (agonists and competitive antagonists),[29, 39–41] and no ligand binding occurs in the GBR2 subunit[29, 40, 41] (Figure 2). The ectodomain of GBR2 directly interacts with the GBR1b ectodomain to enhance agonist affinity and is required for the normal trafficking of the GBR1b to the cell surface as well as for G-protein activation, *via* coupling with its heptahelical transmembrane domain.[37, 42, 43]

There is no X-ray crystal structure available for the complete $GABA_B$ receptor system. However, crystal structures for the stable heterodimeric assembly of the extracellular VFT module of the human GBR1b ($GBR1b_{VFT}$) and GBR2 ($GBR2_{VFT}$) in ligand-free state (apo state) and in the presence of various agonists and antagonists have recently been determined.[29] These structures have provided important insights into the $GABA_B$ architecture, molecular mechanisms of receptor heterodimerization, ligand recognition and receptor activation. The structural information also establishes a good basis for efficient design and discovery of new $GABA_B$ receptor modulators.

Agonists and Antagonists

The first major agonist of the $GABA_B$ receptor, baclofen, was synthesized in 1962, and it was originally designed as a lipophilic analogue of GABA that would display enhanced permeability through the blood-brain barrier.[12] Although baclofen does accumulate in the brain, this action is known to be aided by the large amino acid transporter.[25, 44] On the other hand, its distribution is restricted because baclofen is effluxed by the organic anion transporters.[24, 25] The pharmaceutical is a racemic mixture, but it was resolved and the (*R*)-(–)-enantiomer is substantially more active than the other enantiomer.[45] For example, (*R*)-(–)-baclofen inhibits binding of [^{3}H]baclofen to $GABA_B$ receptors in cat cerebellum with IC_{50} = 0.015 μM but (*S*)-(+)-baclofen inhibits with IC_{50} = 1.77 μM in this assay (100-fold difference in potency).[46] Analogues of baclofen have been produced to understand the role of the carboxylic acid, amine, and *para*-chlorophenyl group.[12] Replacement of the *para*-chlorophenyl group of baclofen with a 2-chlorothienyl group, compound **1**, provides an active agonist with IC_{50} = 0.61 μM in the (*R*)-(–)-[^{3}H]baclofen displacement assay, but substitution with a benzofuran produces a weak antagonist of the $GABA_B$ receptor (Figure 3).[47, 48] Removal of the chlorine atom of baclofen results in another potent $GABA_B$ agonist, phenibut, and in a similar fashion, the racemic mixture is used, however (*R*)-phenibut displays the majority of the agonist activity at the $GABA_B$ receptor.[49] For example, in radioligand-binding experiments, the affinity for racemic phenibut is K_i = 177 μM compared to 92 μM for (*R*)-phenibut and 6 μM for racemic baclofen. Adding ether substituents to the 3-position of baclofen provided the analogue **2**, a potent $GABA_B$ agonist that stimulates currents at 60-fold lower concentration than (*R*)-baclofen in patch clamp experiments measuring the induction of $GABA_B$ receptor-mediated GIRK currents in rat hippocampal slices.[50] Also, the compound **2** appears to cross the blood brain barrier as it demonstrates activity in rodent models of hypothermia. Replacing the aromatic substituent at the 3-position of baclofen with a hydroxyl group produces amino-3-hydroxybutanoic acid (GABOB).[51] (*R*)-(–)-GABOB is an agonist of the $GABA_B$ (10-fold less potent than racemic baclofen in binding experiments from rat brain isolates) and $GABA_A\rho$ receptors and (*S*)-(+)-GABOB is an agonist of the $GABA_A$ receptor and a partial agonist of the $GABA_B$ receptor.[52] In these two cases, the configuration of the 3-position is critical to distinguish between agonist and antagonist behavior at the $GABA_B$ receptor, yet the agonist, (*R*)-(–)-GABOB, does not display the hydroxyl group on the same face as the *para*-chlorophenyl group of (*R*)-baclofen. The derivative (*S*)-(+)-5,5-dimethyl-2-morpholineacetic acid (SCH50911)[53] can be envisioned to be a cyclized version of (*S*)-(+)-GABOB, and this analogue acts as an antagonist of the $GABA_B$ receptor.[53] Specifically, SCH50911 inhibits the binding of GABA

to $GABA_B$ receptors from rat brain with IC_{50} = 1.1 μM, but shows no binding affinity for the $GABA_A$ receptor. Exchanging the hydroxyl group of (*R*)-(–)-GABOB with fluorine provides (*R*)-3-fluoro-GABA **3** that is an agonist of the $GABA_A$[54] and $GABA_A\rho$[55] receptors. Compounds **1**–**3**, phenibut, GABOB, and SCH50911 bear a strong structural resemblance to baclofen by displaying the γ-aminobutyric acid core, yet they demonstrate that subtle changes can produce antagonists, eliminate activity at the $GABA_B$ receptor, or impart activity at other GABA receptors.

During the search for $GABA_B$ receptor ligands, a major divergence occurred when the carboxylic acid of γ-aminobutyric acid was replaced with potential bioisosteres such as sulfonic acids, phosphonic acids, and phosphinic acids.[56] Although this change predominately results in the creation of antagonists of the $GABA_B$ receptor, the structure–activity relationships are of significance in the context of ligand design. For example, the phosphinic acid derivatives incorporate an additional alkyl substituent over the carboxylic acid and this component can aid in designing specific action across the receptors. (*R*)-Phaclofen, the phosphonic acid analogue of baclofen, was one of the first discovered antagonists of the $GABA_B$ receptor,[57] followed closely by (*S*)-2-hydroxy-saclofen (Figure 4).[58] (*S*)-2-Hydroxy-saclofen is 10-fold more potent an antagonist at the $GABA_B$ receptor than (*R*)-phaclofen. Additionally, (*S*)-2-hydroxy-saclofen can be compared to GABOB due to similar placement of the hydroxyl substituent. The phosphinic acid analogue of GABA, **4** (CGP27492),[46] is a potent agonist (IC_{50} = 0.0024 μM) at the $GABA_B$ receptor in the [^{3}H]baclofen binding assay in cat cerebellum and is nearly 15-fold more potent than baclofen.[46] The methyl phosphinic acid analogue, **5** (CGP35024),[46] is also an agonist of the $GABA_B$ receptor and is five times more potent than baclofen. Fluorine and hydroxyl substituents at the 3-position are well tolerated similar to these modifications of GABA. For example, the phosphinic acid, **6** (CGP44532),[59] bears a 3-hydroxy group and retains activity as a $GABA_B$ receptor agonist.[59] Furthermore, this compound demonstrates a 2-fold enhancement in potency compared to baclofen and an improved pharmacokinetic and CNS profile compared to baclofen in studies on *Rhesus* monkeys and rodents (rotarod test).[46] The phosphinic acid, lesogaberan, displays a 3-fluoro substituent and functions as a $GABA_B$ receptor agonist.[16, 17] It demonstrates binding affinity over 40-fold more potent (K_i = 5.1 nM for inhibition of [^{3}H]GABA binding) than baclofen in rat brain GABA receptors yet lesogaberan primarily exhibits peripheral effects.[60] Moreover, lesogaberan displays 87-fold more potent $GABA_B$ agonist activity (EC_{50} = 8.6 nM) in human recombinant $GABA_B$ receptors compared to racemic baclofen.[60] On the other hand, lesogaberan is a contrast to its carboxylic acid equivalent **3**, because the phosphonic acid derivative (lesogaberan) shows agonist activity at $GABA_B$, whereas the subtle divergence in structure causes its absence in the carboxylic acid derivative **3**.

The size of the alkyl substituent on the phosphinic acid plays a key role in distinguishing between agonist activity and antagonist activity at the $GABA_B$ receptor. Specifically, the methyl substituent is present on **5** and **6** and these compounds are agonists.[46] As the size of the substituent increases in the case of the fluoromethyl group of **7** (CGP47656),[46] the action of the molecule at the $GABA_B$ receptor is as a partial agonist. Further increases in size to the ethyl and butyl groups in **8** (CGP36216) and (3-aminopropyl)butylphosphinic

acid (CGP36742),[61] respectively, result in derivatives that display activity as antagonists of the $GABA_B$ receptor.[61] The phosphinic acid bearing a methylcyclohexyl group, **9** (CGP46381),[61] is also a potent antagonist. Moreover, (3-aminopropyl)diethoxymethylphosphinic acid (CGP35348)[61] is a potent $GABA_B$ antagonist that penetrates the blood brain barrier upon i.p. administration.[61, 62]

Another important structural modification is the addition of benzyl substituents to the nitrogen atom, because this manipulation usually imparts antagonist activity at the $GABA_B$ receptor in the nanomolar range. A representative group is the *meta*, *para*-dichlorobenzyl group as compounds (2*S*)-3-[[(1*S*)-1-(3,4-diclorophenyl)ethyl]amino-2-hydroxypropyl](cyclohexylmethyl)phosphinic acid (CGP54626) and (2*S*)-3-[[(1*S*)-1-(3,4-dichlorophenyl)ethyl]amino-2-hydroxypropyl](phenylmethyl)phosphinic acid (CGP55845) are both highly potent antagonists[63]: compounds CGP54626 and CGP55845 display nanomolar potency as antagonists against mammalian $GABA_B$ receptors bound to bullfrog brain membranes (Figure 5).[63] Antagonists with other substituted benzyl groups are well known in the literature.[12] Studies on the conformational restriction of γ-aminobutyric acid into a five-membered ring provided 4-aminocyclopent-1-enecarboxylic acid **10** (4-ACPCA) that displays antagonist activity at the $GABA_A\rho$ receptor and some agonist activity at the $GABA_A$ receptor.[6] The design and synthesis of additional analogues of the cyclic scaffold produced compounds, such as **11**, with enhanced activity at the $GABA_A\rho$ receptor but replaced the agonist activity at the $GABA_A$ receptor with some activity at the $GABA_B$ receptor, in *Xenopus laevis* oocytes expressing recombinant GABA receptors. A class of structurally distinct agonists of the $GABA_B$ receptor was discovered in which each active compound displays a difluoromethyl ketone with a β-hydroxyl substituent (e.g., compounds **12** and **13**).[64] Compound **12** displays agonist activity in HEK293 cells expressing human $GABA_B$ receptors at a level that is 10-fold less potent than racemic baclofen; however, no activity was observed at the $GABA_A$ receptor. The fluorine atoms were critical, as demonstrated by the loss of activity for the non-fluorinated analogue of compound **12**. Methylation of the β-hydroxyl substituent also eliminated activity at the $GABA_B$ receptor. Radiolabeled ligands of the $GABA_B$ receptor have been developed for understanding the presence of the receptor and these agents have been primarily produced from the potent antagonists displaying a *N*-benzyl group.[12] For example, [H^3]CGP54626, **14** ([I^{125}]CGP64213),[65] and **15** ([I^{125}]CGP71872)[66] are potent radiolabeled ligands for the $GABA_B$ receptor.[66] The latter two compounds are powerful antagonists and present some of the largest substituent groups on the phosphinic acid. The azidophenol in **15** is the site of incorporation of the I^{125}, and it also displays potency in the nanomolar range with a K_D value of 1 nM.[66]

Structure and Activation of the $GABA_B$ Receptor

The $GBR1b_{VFT}$ and $GBR2_{VFT}$ subunits of $GABA_B$ receptor have similar bi-lobed architecture (33% sequence identity), like other class-C GPCRs, and each subunit contains two distinct domains, LB1 and LB2 (Figure 6). However, these two subunits have different interdomain arrangements. The ligand binding occurs within a large extracellular VFT module located at the crevice between the LB1 and LB2 domains of the $GBR1b_{VFT}$ subunit. The ligand-binding subunit $GBR1b_{VFT}$ can transit between open and closed conformations

that correspond to resting (ligand-free or antagonist-bound) and active (agonist-bound) $GABA_B$ states, respectively. In contrast, the $GBR2_{VFT}$ subunit has identical conformations in both resting and active states of the $GABA_B$ receptor.[29] The crystal structures of the $GBR1b_{VFT}$:$GBR2_{VFT}$ complex bound to the antagonists CGP54626, **9**, CGP35348, SCH50911, (*S*)-2-OH-saclofen, and (*R*)-phaclofen and to the agonists GABA and (*R*)-baclofen were published in 2013.[29] All of the six antagonist-bound $GABA_B$ crystal structures resemble those of the apo or ligand-free structure in terms of both the heterodimeric arrangement as well as in the topology of individual subunits (Figures 6A and 6C). The ligand-binding crevice of $GBR1b_{VFT}$ remains open when bound to an antagonist. Agonist binding induces large conformational changes within the heterodimeric complex, leading to the closed conformation of the $GABA_B$ receptor (Figures 6B and 6D).[29]

In both the resting (open) and active (closed) states of the $GABA_B$ receptor, $GBR1b_{VFT}$ and $GBR2_{VFT}$ subunits interact through their LB1 domains (Figures 2 and 6). The LB1–LB1 heterodimeric interface involves formation of several hydrogen bonds (H-bond) or ion-dipole interactions, a few salt bridges, and a strong array of van der Waals (vdW) and hydrophobic contacts. Although the LB2 domains of the $GBR1b_{VFT}$ and $GBR2_{VFT}$ subunits remain separated in the resting state, agonist binding induces another heterodimeric interaction between the LB2 domains of the two subunits of the $GABA_B$ receptor.[29, 68] The LB1–LB1 heterodimeric interface involves multiple interfacial H-bond interactions between several polar residues including the $GBR1b_{VFT}$ residues Thr198, Glu201 and Ser225, and the $GBR2_{VFT}$ residues Asp204, Gln206, Asn213 and Ser233. The LB1–LB1 interaction is mediated by the helices, but the LB2–LB2 interaction prevalent in the open or active state is mediated by loop-strand-helix motifs from each domain of the $GBR1b_{VFT}$ and $GBR2_{VFT}$ subunits. The agonist-induced formation of the LB2–LB2 heterodimeric interface is important for $GABA_B$ receptor activation and has been confirmed by alanine-scanning mutagenesis.[29] In particular, the three deeply buried tyrosine residues (Tyr113 and Tyr 117 of $GBR1b_{VFT}$ and Tyr118 of $GBR2_{VFT}$) at the heterodimer interface are critical for heterodimer interaction and receptor activation.[43]

All of the agonists and antagonists bind within a large extracellular VFT module situated at the crevice between the LB1 and LB2 domains of the $GBR1b_{VFT}$ subunit. Depending on the nature of bound ligand (antagonist or agonist), the ligand-binding subunit $GBR1b_{VFT}$ attains open or closed conformations that correspond to the resting (inactive) and active $GABA_B$ states, respectively[29] (Figures 2 and 6). Structurally, all of the co-crystallized agonists and antagonists are derivatives of GABA. Figure 7 depicts the $GABA_B$ amino acids and waters that interact directly or indirectly with the bound agonists and antagonists. The implication from the X-ray co-crystal structures is that agonist binding favors the closed conformation, and antagonist binding stabilizes the open conformation of the $GBR1b_{VFT}$ subunit. Furthermore, LB1 residues are necessary for recognition of both the agonist and antagonist, but the LB2 residues are primarily required for agonist recognition only (Figures 6 and 7).

The receptor–antagonist interactions are mediated mostly by multiple H-bonds with the LB1 domain of the $GBR1b_{VFT}$ subunit, and the LB2 domain interacts sparsely, as apparent in the available multiple X-ray crystal structures of the antagonist-bound $GABA_B$ receptor. The α-acid groups like carboxylic, phosphoric, sulfonic, *etc.*, at one end form H-bonds with the

LB1 residues Ser130 and Ser153, and the primary or secondary γ-amino group at the other end forms H-bonds to His170 and Glu349 (Figure 7C). The three-carbon linker present in all of the antagonists makes vdW interaction with Trp65 but its main role is to provide appropriate separation between the acid and amino end groups. Depending on structural features present in the antagonists, they exhibit other distinct interactions with the LB1 residues that are specific to them. For example, the β-hydroxyl group of CGP54626 and (*S*)-2-OH-saclofen exhibits H-bonding interactions with the LB1 residue that are specific to them. In addition, there are some common water-mediated H-bonds with the LB1 residue Ser131 that have been observed with all of the studied antagonists except SCH50911 and (*R*)-phaclofen.[29]

As found for antagonists, the endogenous agonist GABA and its synthetic derivative (*R*)-baclofen, which includes a β-(4-chlorophenyl) substituent, exhibit a common set of H-bonding interactions (Ser130 and Ser153 at one end, and His170 and Glu349 at the other end) and vdW contacts (Trp65) with the LB1 residues. However, unlike what is found for antagonists, these two agonists exhibit direct H-bonds with the LB2 residue, Tyr250, and strong vdW contacts with various LB2 residues (Figure 7). These additional interactions are due to a notable transition of the LB2 domain of the GBR1b subunit during receptor activation characterized by the closed state. This distinction is evidenced from X-ray crystal structures of $GABA_B$-complexed with agonists and antagonists. The backbone Cα–Cα movements of Tyr250 and Trp278 from the active state to the inactive (resting) state are 7.21 and 6.08 Å, respectively (Figure 7), suggesting a phenomenal transition of the LB2 domain (GBR1b subunit) between the closed and inactive $GABA_B$ states. Most notably, the LB2 residue Tyr250 which forms an ion-dipole interaction with the negatively ionizable carboxylate group of the agonists GABA and (*R*)-baclofen remains far away and no H-bonding or ion-dipole interactions are apparent with Tyr250 in any of the antagonist-bound X-ray structures. Instead, a few water molecules are found to reside close to the antagonists and interact with the α-acid groups of antagonists (Figure 7C). The LB2 residue, Trp278, is highly flexible, exhibiting a wide rotameric adaptation of different conformations that can help the GABA receptor to recognize ligands (agonists or antagonists) of variable sizes in order to maintain ligand-binding affinity and specificity (Figures 7 and 8).[29] The importance of the LB1 and LB2 residues has been implicated through mutagenesis studies (Table 1).

Each antagonist contains a bulky substituent at either one or both of the α- and γ-positions, and these apparently inhibit the domain closure of the $GBR1b_{VFT}$ subunit (Figures 2 and 6). The bulky substituent of the antagonist interacts with the LB2 domain residues Tyr250 and Trp278 (Figure 7C), which possibly prevents the LB1 and LB2 domains of the $GBR1b_{VFT}$ subunit from approaching each other (Figures 6, 7C, and 8B). In particular, this would be expected to prevent the LB2 domain from undergoing the substantial transition that happens during the receptor activation characterized by a closed $GABA_B$ state. The two antagonists (*S*)-2-OH-saclofen and (*R*)-phaclofen contain sulfonic and phosphinic acid motifs, respectively, that are structurally analogous to the carboxylic acid of agonist (*R*)-baclofen. Their acid motifs assume tetrahedral coordination geometries that are observed to be incompatible with the active or closed state conformation of the LB2 residue Tyr250.[29] It is also suggested that these acid motifs at the α-position, being comparatively bulky relative to carboxylate, push the 4-chlorophenyl group located at the β-position towards the γ-amino

end of each antagonist, and hence could possibly create steric hindrance with the LB2 residues Ile276 and Trp278 that undergo significant transition during domain closure of the $GBR1b_{VFT}$ subunit.

Positive Allosteric Modulators and Negative Allosteric Modulators

Three major structural classes of positive allosteric modulators (PAMs) have been described for the $GABA_B$ receptor in the literature: pyrimidines, di-*tert*-butylbenzenes, and thiophenes. These PAMs display no or some partial agonistic activity when applied alone in most assays, but enhance both the potency and efficacy of $GABA_B$ agonists (competitive or orthosteric ligands).[38, 41] PAMs have been aggressively pursued in the context of drug design, because they are less likely to result in receptor desensitization than traditional $GABA_B$ agonists.[71] Indeed, one of the typical drawbacks of baclofen use is that tolerance develops rapidly; therefore, this class of potential therapeutic agents was developed to display less tolerance and have an increased propensity for long-term use. The pyrimidine-based PAM, *N,N'*-dicyclopentyl-2-(methylthio)-5-nitro-pyrimidine-4,6-diamine (GS39783),[72] was one of the first, potent molecules for allosteric activation of the $GABA_B$ receptor (Figure 9). GS39783 enhances the affinity of GABA at recombinant $GABA_B$ receptors in the [^{35}S]GTPγS assay 8-fold and increases the maximal intrinsic efficacy for GABA 2.2-fold.[72] Derivatives with alternative *N*-alkyl substituents were prepared along with analogues in which the thiomethyl group is exchanged for alkyl groups or a methoxy; however, GS39783 displays the highest potency. Other pyrimidine-derived compounds, such as **16** and **17**, were designed with a trifluoromethyl group to eliminate the potential metabolic liability associated with the nitro group[73] of GS39783.[74] Compounds **16** and **17** displayed 106% and 139% potentiation compared to 192% for GS39783 in GABA-induced simulation of GTP(γ)^{35}S binding. Pyrimidine **18** (BHF177)[74] was also produced during optimization efforts upon GS39783, and two similar analogues are compounds **19** and **20**. These compounds display the trifluoromethyl group but lack one of the alkyl aniline groups. Compound **18** is as potent as GS39783, but **19** and **20** display less than half of the potency of GS39783 in potentiating the effects of GABA. The binding site of the positive allosteric modulator, GS39783, was identified by point mutations in the heptahelical TM domain of the GBR2 subunit ($GBR2_{TM}$). Specifically, the mutations G760T and A780P in TM6 were critical for allosteric activation, because mutations of these TM6 residues led to conversion of the PAM into an agonist.[75] These sites are assumed to be involved in stabilizing the $GBR2_{TM}$ domain into an inactive conformation. It is proposed that the closure of the $GBR1_{VFT}$ domain signifying the active $GABA_B$ state conformation results in a new conformation of the heterodimer through an inter-subunit major rearrangement of the extracellular VFT domain. This new conformation, in turn, stabilizes the active state conformation of the $GBR2_{TM}$ domain for coupling with the G-protein. Recently, Matsushita and colleagues revealed through fluorescence resonance energy transfer (FRET) studies that the extracellular VFT domain undergoes a major rearrangement, but the heptahelical TM domain of the GBR2 subunit undergoes a minor intra-TM rearrangement, during the receptor activation by an agonist.[76] They also proposed that such a mode of receptor activation, involving a major inter-subunit rearrangement without any apparent intra-TM (intra-helical) structural changes, appears to

be a common feature which applies to both the $GABA_B$ receptor and the metabotropic glutamate receptor 1α (mGluR1α), another class-C GPCR family member.[76]

The calcium ion Ca^{2+} (a known PAM for the $GABA_B$ receptor) is proposed to stabilize the closed state of the extracellular $GBR1_{VFT}$ domain, but other PAMs bind to and stabilize the active conformation of the $GBR2_{TM}$ domain. These PAMs, other than Ca^{2+}, may bind to their recognition sites ($GBR2_{TM}$ domain) when applied alone, but may not initiate the relative movement of the extracellular VFT domains of the two heterodimeric subunits the way that agonists do.[41] The first discovered PAM was 2,6-di-*tert*-butyl-4-(3-hydroxy-2,2-dimethyl-propyl)-phenol (CGP7930)[77] and it is not based on a pyrimidine scaffold (Figure 10).[77] CGP7930 displays EC_{50} = 5.37 μM in GABA-induced stimulation of GTP(γ)^{35}S binding at 1 μM GABA. During the characterization of the binding site of CGP7930 through various studies on the wild-type and chimeric $GABA_B$ subunits, Binet and colleagues revealed that only the heptahelical TM domain of the GBR2 subunit ($GBR2_{TM}$) is required and sufficient for allosteric action.[78] CGP7930 was then optimized into the fluorinated derivative, (*R,S*)-5,7-di-*tert*-butyl-3-hydroxy-3-trifluoromethyl-3*H*-benzofuran-2-one (*rac*-BHFF),[79] and separation of the enantiomers and subsequent biological evaluation demonstrated that (+)-BHFF was the potent PAM.[79] The major structural advance was tethering the primary alcohol of CGP7930 with the phenolic hydroxyl group to form a cyclic lactone. At a concentration of 0.3 μM, *rac*-BHFF increased the potency of GABA by 15.3-fold with efficacy of 149% in GTP(γ)^{35}S-binding assays compared to 87.3-fold increase and 181% efficacy with (+)-BHFF. The 2-(acylamino)thiophenes are the most recent class of PAMs to be discovered, and key members are methyl 2-(1-adamantanecarboxamido)-4-ethyl-5-methylthiophene-3-carboxylate (COR627) and methyl 2-(cyclohexanecarboxamido)-4-ethyl-5-methylthiophene-3-carboxylate (COR628),[80] which were originally identified by a virtual screening protocol.[80] Extensive optimizations led to the discovery of thiophene **21** that displays substantial activity in potentiating baclofen-induced sedation/hypnosis in mice, is active after intragastric administration, and fails to affect phenobarbital-induced sedative/hypnosis.[81] The identification of new PAMs of the $GABA_B$ receptor continues to be an area of substantial interest due to the therapeutic potential, and new molecules are appearing in the literature, such as the PAM ADX71441 (structure not disclosed).[82] Additionally, the arylalkylamines were reported to display activity as PAMs of the $GABA_B$ receptor,[83] but subsequent evaluation in GTP(γ)^{35}S-binding assays in the presence of GABA did not support that these molecules act in this fashion on the $GABA_B$ receptor.[84]

Negative allosteric modulators (NAMs) for the $GABA_B$ receptor were not known until 2014 and the first report described the discovery of a compound designed from CGP7930.[85] The group of molecules acts as non-competitive antagonists of the $GABA_B$ receptor and they display the di-*tert*-butyl phenol scaffold that is present in other PAMs, such as CGP7930. The analogue **22** is the most active NAM and it does not bind into the orthosteric binding site of the $GABA_B$ receptor (Figure 11). The compound **22** decreased GABA-induced production of IP3 in HEK cells overexpressing $GABA_B$ receptors with IC_{50} = 37.9 μM. Even though it bears a common electrophilic group, an enone, specificity for the target was

demonstrated for $GABA_B$ versus other GPCR class C members such as mGluR1, mGluR2, and mGluR5.

Conclusions

Since the discovery of the $GABA_B$ agonist and muscle relaxant, baclofen, there have been substantial advancements in the development of compounds that activate this receptor as agonists or PAMs. For the agonists, most of the existing structure–activity data applies to understanding the role of substituents on the backbone of GABA as well as replacing the carboxylic acid and amine groups. In the cases of the PAMs, the allosteric binding site(s) and structure–activity relationships are less well defined; however, multiple classes of molecules that act as PAMs have been discovered. Despite the preclinical and clinical investigations with PAMs, the fundamental question that remains to be answered is whether they will be therapeutically effective. Further studies are needed to characterize the therapeutic potential of these compounds. In terms of $GABA_B$ receptor structural information, there is now sufficient knowledge of the orthosteric site and recognition modes of various representative $GABA_B$ agonists and antagonists, but there is still a lack of knowledge of the allosteric site(s). A key topic of future exploration is whether multiple and distinct allosteric sites exist within $GABA_B$ receptors, especially in view of observations that some of the $GABA_B$ receptor PAMs (e.g. CGP7930) behave as allosteric agonists. The available X-ray crystal structures of agonist- and antagonist-bound $GABA_B$ receptors have established a platform for the use of state-of-the-art protein structure-based drug design tools to discover new ligands. From a therapeutic perspective, these data will enable additional efforts in drug discovery in areas of addiction-related behavior, the treatment of anxiety, and the control of muscle contractility. A major clinical question is whether modulation of the $GABA_B$ receptor can be used to effectively benefit patients afflicted with drug or alcohol addiction, as there is a substantial need to develop new agents for these conditions. Because the $GABA_B$ pathway is crucial in so many CNS related disorders, the stage is set for future investigations on this receptor to determine if it can be as pharmacologically lucrative as the extensively exploited $GABA_A$ receptor.

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ABBREVIATIONS

CNS	central nervous system
FRET	fluorescence resonance energy transfer
GABA	γ-aminobutyric acid
$GABA_A$	γ-aminobutyric acid type A
$GABA_B$	γ-aminobutyric acid type B
$GABA_C$	γ-aminobutyric acid type C
GABOB	amino-3-hydroxybutanoic acid
GBR1b	γ-aminobutyric acid receptor subunit 1b

GBR2	γ-aminobutyric acid receptor subunit 2
GERD	gastro-esophageal reflux disease
GIRK	G-protein-coupled inwardly-rectifying potassium channel
GPCR	G-protein coupled receptor
HEK	human embryonic kidney
i.p.	intraperitoneal
IP3	inositol triphosphate
Kir3	potassium channel, inwardly-rectifying, member 3
LB	ligand binding
mGluR1α	metabotropic glutamate receptor 1α
NAM	negative allosteric modulator
PAM	positive allosteric modulator
TM	transmembrane
vdW	van der Waals
VFT	Venus flytrap

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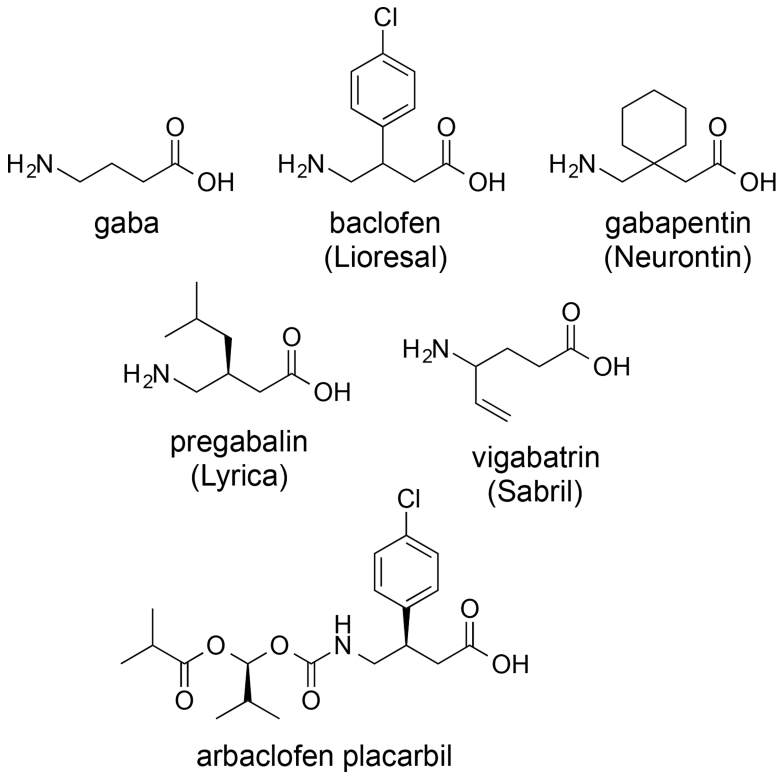


Figure 1.
Structure of GABA and pharmaceuticals based on the structure of GABA.

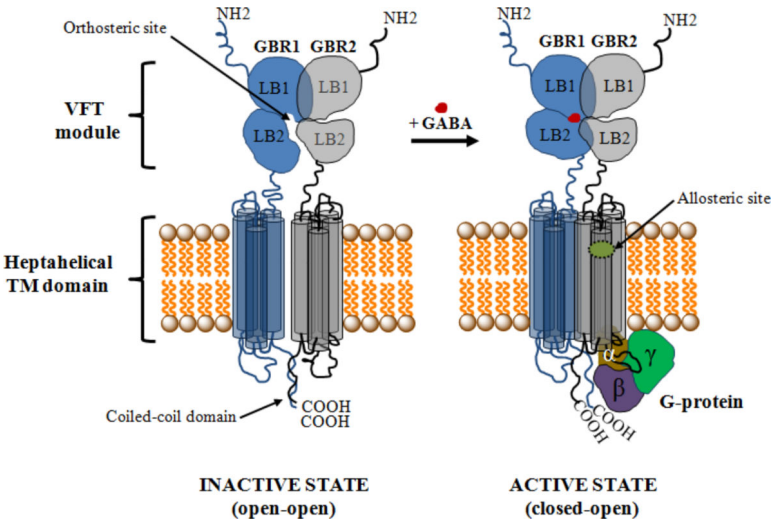


Figure 2.

Schematic representation of the structure of the $GABA_B$ receptor in resting (inactive) and active states. In the resting state, the GBR1b and GBR2 subunits attain an open-open state, but during receptor activation only the GBR1b subunit transits closed (resulting in a closed-open state). The orthosteric site resides in the GBR1b subunit of the VFT module, and the allosteric site is proposed to lie in the heptahelical TM domain of the GBR2 subunit, based on mutagenesis experiments (see main text). The GBR2 subunit couples with a G-protein for further signaling.

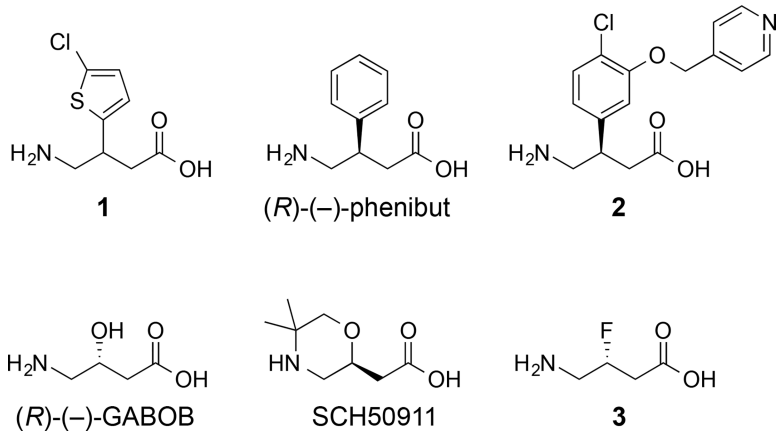


Figure 3.

Ligands of the $GABA_B$ receptor that display the structure of GABA. Compounds **1**, **2**, (*R*)-(–)-phenibut and (*R*)-(–)-GABOB are agonists of the $GABA_B$ receptor. SCH50911 is an antagonists of the $GABA_B$ receptor. Compound **3** is an agonist of the $GABA_A$ and $GABA_A\rho$ receptors.

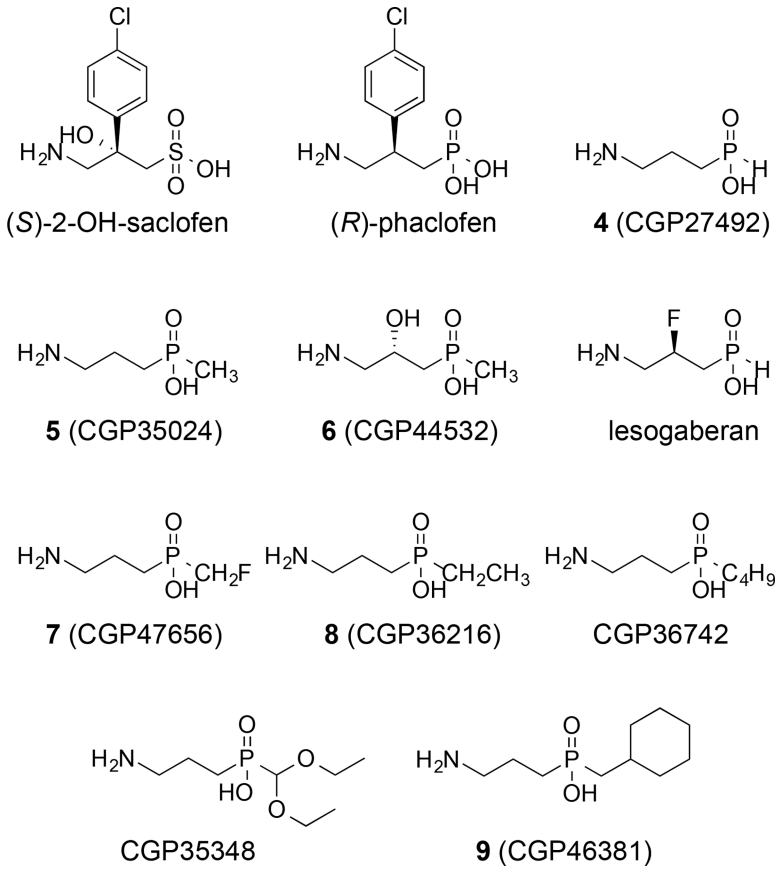


Figure 4.

Analogues of GABA and baclofen in which the carboxylic acid is replaced. Compounds **4**–**6** and lesogaberan are agonists of the $GABA_B$ receptor, and **7** is a partial agonist of the $GABA_B$ receptor. (*S*)-2-hydroxy-saclofen, (*R*)-phaclofen, **8**, CGP36742, CGP35348, and **9** are antagonists of the $GABA_B$ receptor.

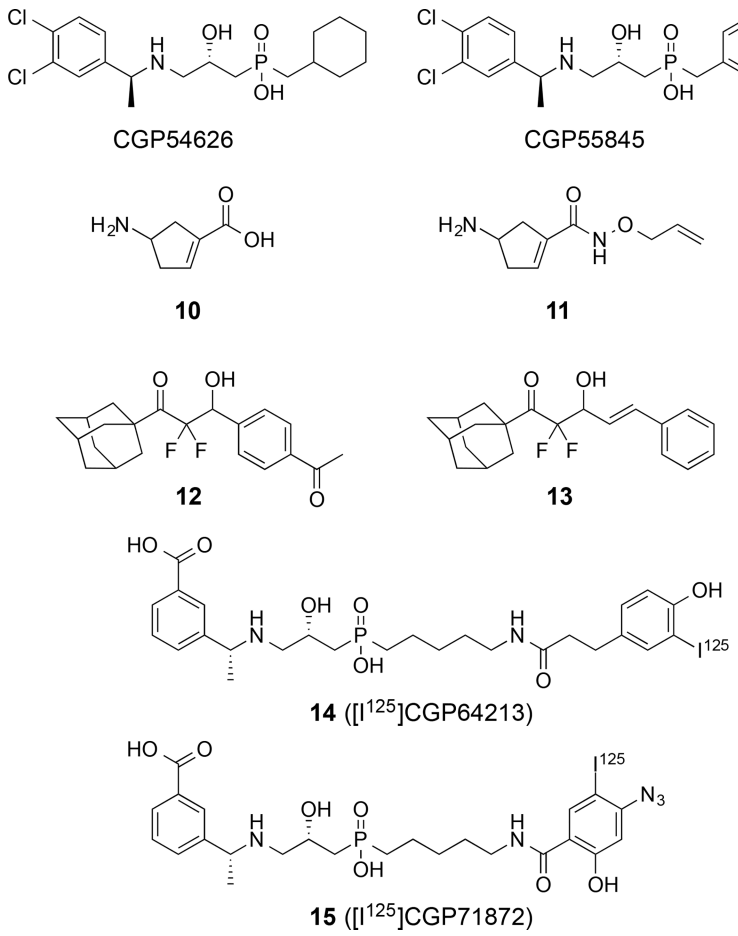


Figure 5.

Additional ligands of the $GABA_B$ receptor. CGP54626, CGP55845, **14**, and **15** are antagonists of the $GABA_B$ receptor. Compounds **10** and **11** are antagonists of the $GABA_A\rho$ receptor. Compounds **12** and **13** are agonists of the $GABA_B$ receptor.

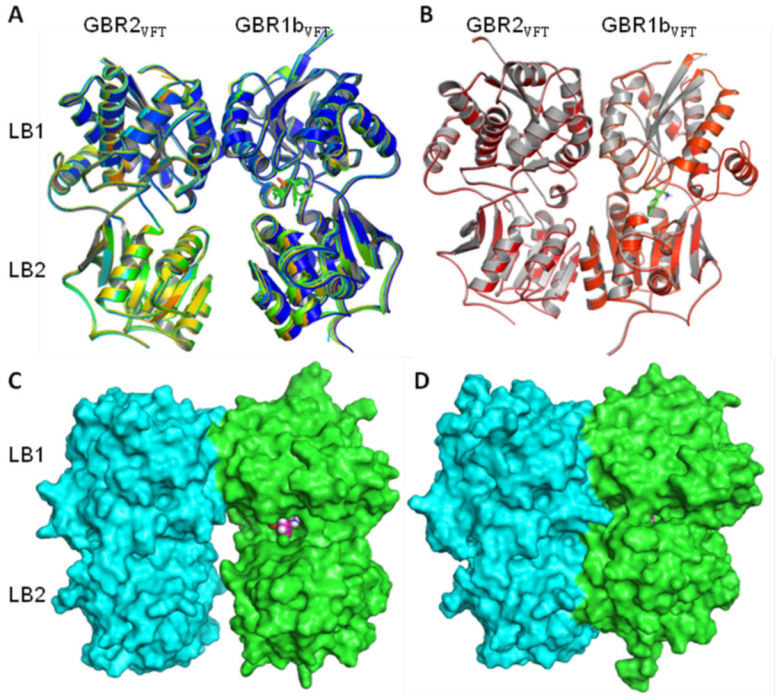


Figure 6.

Cartoon depiction of superposed apo (blue) and antagonist-bound crystal structures (**A**) and of agonist-bound crystal structures (**B**) of the $GBR1b_{VFT}$:$GBR2_{VFT}$ complex. Molecular surface of $GBR1b_{VFT}$:$GBR2_{VFT}$ assembly bound to antagonist (*R*)-phaclofen (**C**), and that bound to agonist (*R*)-baclofen (**D**). The resting and antagonist-bound (inactive) $GABA_B$ receptors are found in an open state but the agonist-bound (active) receptor adjusts to a closed state. This image was generated using the PyMol molecular graphics software.[67]

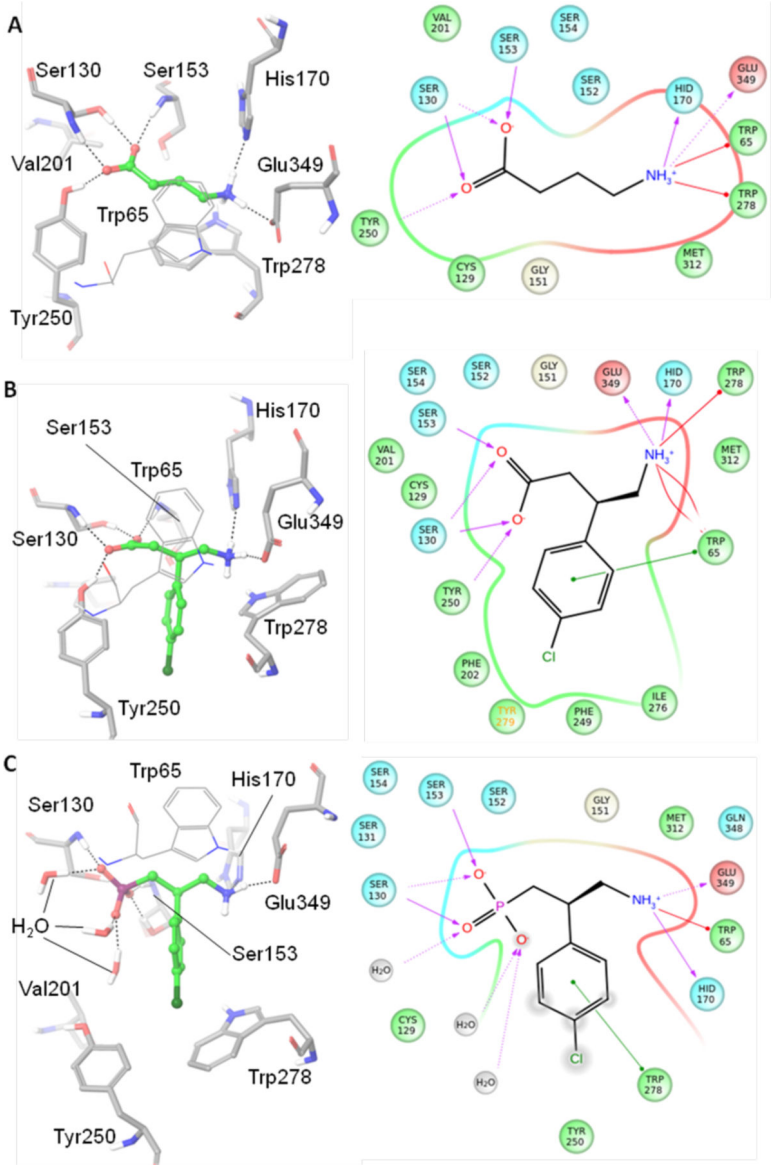


Figure 7.

Depiction of the $GABA_B$ amino acids and waters that interact directly or indirectly with bound agonists, GABA (**A**) and (*R*)-baclofen (**B**), or antagonist, (*R*)-phaclofen (**C**). 3D- (those that include the exact 3-dimensional geometry) and 2D-depictions of the protein-ligand interactions are on the left and right sides, respectively. These images were generated using the Schrödinger suite.[69]

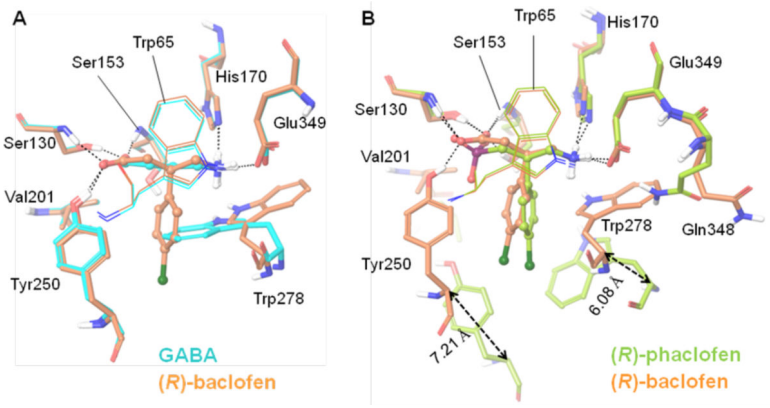


Figure 8.

Binding mode of ligands as shown in X-ray crystal structures.[29] (**A**) Depiction of the differences in binding site residues of $GABA_B$ bound to the agonists, GABA (cyan) and (*R*)-baclofen (orange). The LB2 residue Trp278 is highly flexible, and undergoes rotameric transition to accommodate the bulky substituent *p*-chlorophenyl of (*R*)-baclofen. Other residues are conserved. (**B**) Depiction of the differences in binding site residues of the $GABA_B$ bound to the agonist (*R*)-baclofen (orange) and the antagonist (*S*)-phaclofen (green). As shown, the LB2 lobe of GBR1b undergoes major changes to govern the closed state of $GABA_B$. The LB2 residues undergo major perturbation, especially Val201, Tyr250, and Trp278, which line the binding site during agonist binding (activation of the $GABA_B$ receptor). On the contrary, most of the LB1 residues, except for Gln348, are conserved in the resting (inactive) and active states of the $GABA_B$ receptor. Gln348 undergoes rotameric transition to allow a high degree of freedom to Trp278. The LB2 domain of the GBR1b subunit undergoes a major shift during the activation process. For example, the backbone Cαs of Tyr250 and Trp278 move 7.21 and 6.08 Å, respectively, between active and resting (inactive) states. These images were generated using the Schrödinger suite.

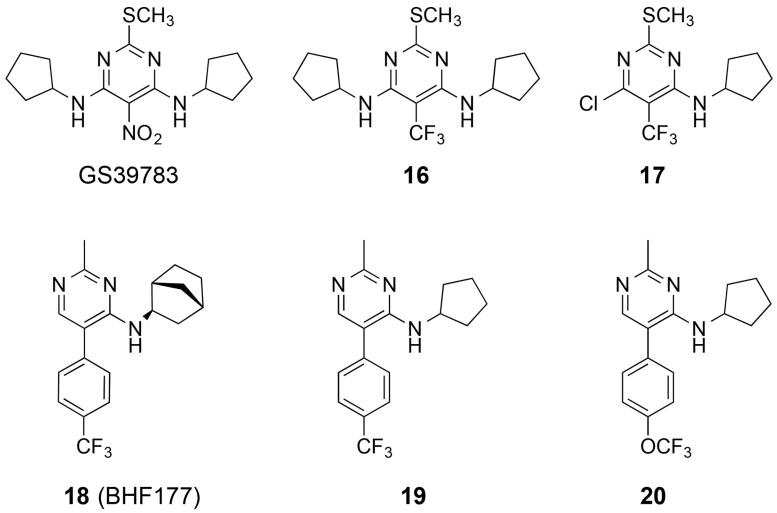


Figure 9.
Pyrimidine-based allosteric modulators of the $GABA_B$ receptor.

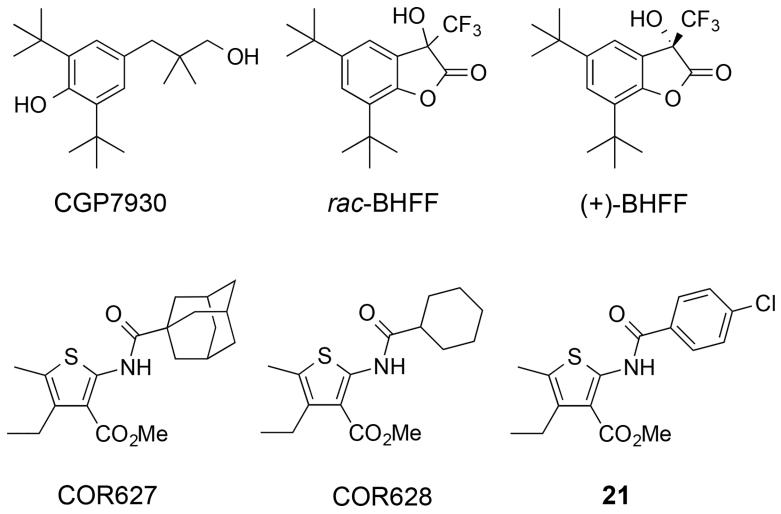
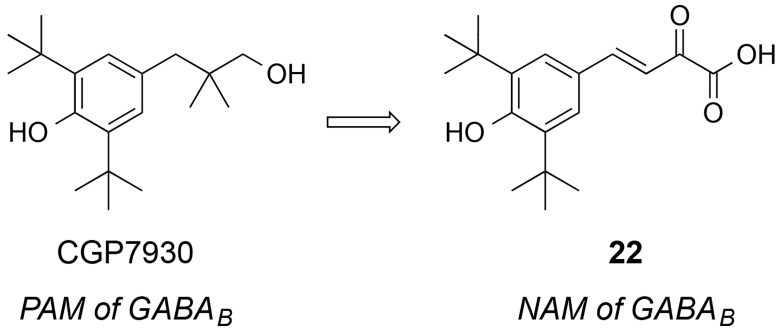


Figure 10.
Additional positive allosteric modulators of the $GABA_B$ receptor.



CGP7930 **22**

PAM of GABA$_B$ *NAM of GABA$_B$*

Figure 11.
First negative allosteric modulator of the $GABA_B$ receptor.

Table 1.

The importance of LB1 and LB2 residues, as implicated through mutagenesis studies.

Mutation	Resulting effects
Tyr118Ala	Disruption of heterodimeric interaction between $GBR1b_{VFT}$ and $GBR2_{VFT}$[43]
Tyr113Ala, Tyr117Ala	Weakening of heterodimeric interaction between $GBR1b_{VFT}$ and $GBR2_{VFT}$[43]
Ser130Ala	Complete loss of binding of antagonist **14**[65, 70]
Ser153Ala	Moderate effect on ligand binding[65, 70]
Gly151Val	Loss of binding of antagonist [^{3}H]-CGP54626; drastic loss of receptor function[43]
Glu349Ala	Substantial loss of binding of antagonist **14**; loss of potency of agonists GABA and baclofen[65, 70]
Trp65Ala	Substantial loss of ligand binding and receptor function[29]
His170Ala	Complete loss of antagonist binding; lowering of the maximum agonist-induced [^{35}S]GTPγS binding to half that of wild-type level[29]
Trp278Ala	No effect on the binding of antagonist [^{3}H]-CGP54626, but a decrease in its potency; detrimental effect on receptor activation; decrease in agonist potency[29]
Tyr250Ala	No effect on the binding of antagonist [^{3}H]-CGP54626, but a decrease in agonist response and potency[29]