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Differentiating Enantioselective Actions of GABOB: A Possible Role for Threonine 244 in the Binding Site of $GABA_C$ ρ_1 Receptors

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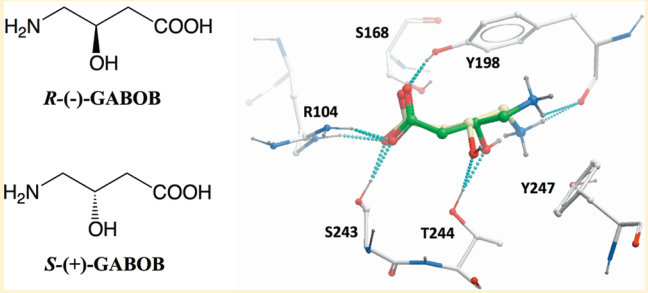
Supporting Information

ABSTRACT: Designing potent and subtype-selective ligands with therapeutic value requires knowledge about how endogenous ligands interact with their binding site. 4-Amino-3-hydroxybutanoic acid (GABOB) is an endogenous ligand found in the central nervous system in mammals. It is a metabolic product of GABA, the major inhibitory neurotransmitter. Homology modeling of the $GABA_C$ ρ_1 receptor revealed a potential H-bond interaction between the hydroxyl group of GABOB and threonine 244 (T244) located on loop C of the ligand binding site of the ρ_1 subunit. Using site-directed mutagenesis, we examined the effect of mutating T244 on the efficacy and pharmacology of GABOB and various ligands. It was found that mutating T244 to amino acids that lacked a hydroxyl group in their side chains produced GABA insensitive receptors. Only by mutating ρ_1T244 to serine (ρ_1T244S) produced a GABA responsive receptor, albeit 39-fold less sensitive to GABA than $\rho_1$wild-type. We also observed changes in the activities of the $GABA_C$ receptor partial agonists, muscimol and imidazole-4-acetic acid (I4AA). At the concentrations we tested, the partial agonists antagonized GABA-induced currents at ρ_1T244S mutant receptors (Muscimol: $\rho_1$wild-type, EC_{50} = 1.4 μM; ρ_1T244S, IC_{50} = 32.8 μM. I4AA: $\rho_1$wild-type, EC_{50} = 8.6 μM; ρ_1T244S, IC_{50} = 21.4 μM). This indicates that T244 is predominantly involved in channel gating. *R*-(−)-GABOB and *S*-(+)-GABOB are full agonists at $\rho_1$wild-type receptors. In contrast, *R*-(−)-GABOB was a weak partial agonist at ρ_1T244S (1 mM activates 26% of the current produced by GABA EC_{50} versus $\rho_1$wild-type, EC_{50} = 19 μM; I_{max} 100%), and *S*-(+)-GABOB was a competitive antagonist at ρ_1T244S receptors ($\rho_1$wild-type, EC_{50} = 45 μM versus ρ_1T244S, IC_{50} = 417.4 μM, K_B = 204 μM). This highlights that the interaction of GABOB with T244 is enantioselective. In contrast, the potencies of a range of antagonists tested, 3-aminopropyl(methyl)phosphinic acid (3-APMPA), 3-aminopropylphosphonic acid (3-APA), *S*- and *R*-(3-amino-2-hydroxypropyl)methylphosphinic acid (*S*-(−)-CGP44532 and *R*-(+)-CGP44533), were not altered. This suggests that T244 is not critical for antagonist binding. Receptor gating is dynamic, and this study highlights the role of loop C in agonist-evoked receptor activation, coupling agonist binding to channel gating.



KEYWORDS: *T244, channel gating, enantioselective actions of GABOB, loop C, coupling agonist binding, channel gating*

4-Amino-3-hydroxybutanoic acid (GABOB) is an endogenous molecule found within the CNS, possessing anticonvulsant properties.[1] It is formed by two metabolic pathways: either by the metabolism of putrescine to γ-aminobutyric acid (GABA) and then hydroxylation of GABA at the third carbon (C3),[2,3] or by the hydroxylation of putrescine to 2-hydroxyputrescine and then oxidative N-dealkylation to GABOB.[3] There are conflicting results concerning the concentration of GABOB in the CNS, with concentration ranging between <0.01 and 4.8 μmol/g for rat and bovine brain, respectively. However, the precursor to GABOB, 2-hydroxyputrescine, is found throughout the brain, and Noto and his colleagues have quantified the amount of 2-hydroxyputrescine in rat cerebellum (2.44 ± 0.49 pg/μg protein) and hippocampus (1.52 ± 0.20 pg/μg protein).[4]

Introducing a hydroxyl group at the C3 position of GABA generates a stereogenic center, thus producing the enantiomers *R*-(−)-GABOB and *S*-(+)-GABOB (Figure 1). Of the two enantiomers, *R*-(−)-GABOB is the more potent anticonvulsant[2] and has been shown to have a greater inhibitory effect on induced seizures in cat brain and rabbit motor cortex.[5,6]

Like GABA, GABOB mediates its inhibitory effects by activating ionotropic ($GABA_A$ and $GABA_C$) and metabotropic ($GABA_B$) receptors. Both isomers of GABOB act as agonists at all three classes of GABA receptors. However, there are differences in their potencies: The affinity of *S*-(+)-GABOB is

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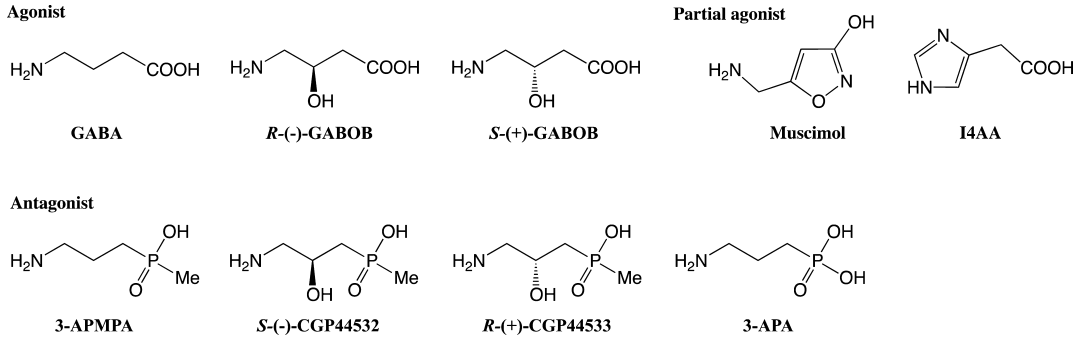

Figure 1. Structures of agonists: GABA, *R*-(−)-GABOB, *S*-(+)-GABOB, partial agonists; muscimol, I4AA and antagonists; 3-APMPA, *S*-(−)-CGP44532, *R*-(+)-CGP44533, 3-APA.

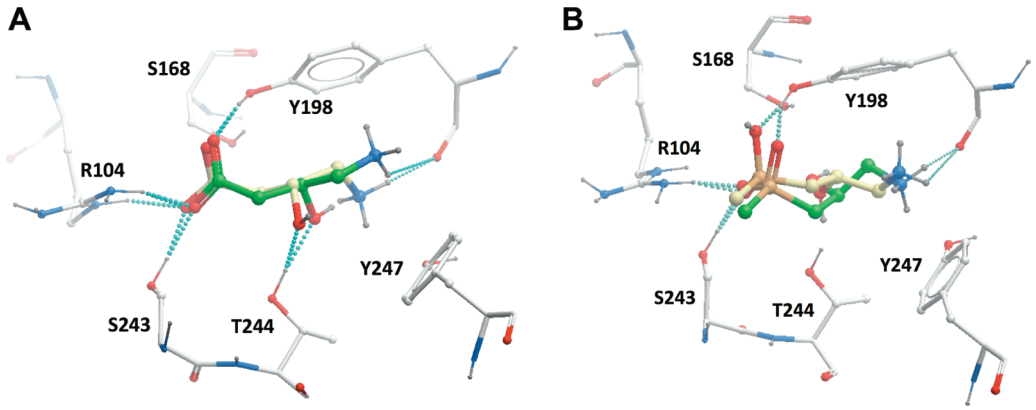

Figure 2. Ligands docked into the ρ_1 receptor GABA binding site: (A) Hydroxyl group of *R*-(−)-GABOB (yellow) and *S*-(+)-GABOB (green) forming H-bond with the hydroxyl group of T244. Selected H-bonds are depicted with dashed cyan lines. (B) Hydroxyl group of *R*-(+)-CGP44533 (yellow) and *S*-(−)-CGP44532 (green) not interacting with T244. Selected H-bonds are depicted with dashed cyan lines.

higher than that of *R*-(−)-GABOB at $GABA_A$ receptors.[7,8] In contrast, *R*-(−)-GABOB is the more potent agonist at $GABA_B$ and $GABA_C$ receptors.[9,10] While *S*-(+)-GABOB shows similar efficacy for $GABA_C$ receptors, it acts as a partial agonist on $GABA_B$ receptors.[11]

Of particular interest to this study is the ionotropic $GABA_C$ receptor. $GABA_C$ receptors are members of the Cys-loop superfamily of ligand-gated receptors which include nicotinic acetylcholine (nACh), serotonin type-3A ($5\text{-}HT_{3A}$), strychnine-sensitive glycine, $GABA_A$ receptors, and invertebrate glutamate-gated chloride channels (GluCl)[12−15] and share a similar tertiary and identical quaternary pentameric structure.[16] $GABA_C$ receptors are formed by ρ subunits (ρ_1−ρ_3) and exist as homopentameric receptors with distinct pharmacology compared that of $GABA_A$ and $GABA_B$ receptors.[17,18] They are highly expressed in retina and in distinct areas of the brain: cerebellum,[19] hippocampus,[20] superior colliculus,[21] and lateral amygdala.[22] Experimental evidence suggests that $GABA_C$ receptors are potential therapeutic targets for the treatment of myopia,[23] anxiety,[22] memory,[24] and sleep-related disorders.[25]

The orthosteric binding sites of $GABA_C$ receptors are located at the interface of two ρ subunits within the N-terminal domain. Residues located on five discontinuous loops (referred to as loop A-E) within each subunit contribute to GABA binding.

In this study, we docked the enantiomers of GABOB in our previously reported homology model of the ρ_1 receptor binding site[26] (Figure 2) in order to elucidate the key interactions between the receptor and ligands. Threonine at position 244 (T244) in the ρ_1 subunit is located in loop C of the ligand binding site. This residue is highly conserved in chloride selective Cys-loop receptors, such as the $GABA_{A/C}$ and glycine receptors.[27,28] Our homology model predicts a possible hydrogen-bond (H-bond) formation between the acidic group of GABA and the hydroxyl group of T244[29] and a possible H-bond between the C3-hydroxyl group of GABOB and T244 (Figure 2). In addition, the model predicts there are no H-bonds between the C3-hydroxyl group of the phosphinic analogue of GABOB, *S*- and *R*-(3-amino-2-hydroxypropyl)-methylphosphinic acid (*S*-(−)-CGP44532 and *R*-(+)-CGP44533) and T244 (Figure 2). However, a possible H-bond was predicted with methyl phosphonate analogues of GABA, possibly as a result of the extra oxygen in the compound.[30] To validate these predictions, we mutated T244 to explore the effect on affinity and efficacy of these ligands using molecular biology and electrophysiology techniques. Partial agonists were also examined to explore the effect of the mutation on channel gating.

RESULTS AND DISCUSSION

Mutation at Threonine 244. The role of the hydroxyl group on the side-chain of the threonine residue at position 244 (T244) was explored by mutating T244 to alanine (T244A), glycine (T244G), serine (T244S), valine (T244 V), isoleucine (T244I), leucine (T244L), and phenylalanine (T244F). As previously reported, only the serine mutant (T244S) produced receptors that elicited a chloride current in response to GABA.[27] The observed GABA EC_{50} value at ρ_1T244S mutant receptors was increased by 39-fold compared to $\rho_1$wild-type

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receptors (Table 1), indicating that GABA has reduced potency for the mutant receptor. This is consistent with previous findings[27] (Figure 3). Altered GABA sensitivity indicates either a change in GABA binding for the orthosteric binding site or a change in receptor gating. Homology modeling predicts that the acidic group of GABA may form a H-bond with the hydroxyl group of T244,[29] indicating that the change in GABA potency observed at the ρ_1T244S mutant is the result of altered binding. However, there are many limitations to inferences derived from homology modeling, particularly with respect to conformational changes that occur throughout the structure initiated by ligand binding leading to channel opening. We performed further pharmacological experiments to determine if the model accurately predicts the role of T244 during the dynamic receptor gating process.

Table 1. Pharmacological Data for Agonists and Antagonists at ρ_1Wild-type and ρ_1T244S Mutant Receptors Expressed in *Xenopus* Oocytes

	$\rho_1$wild-type receptors	ρ_1T244S mutant receptors
GABA	EC_{50} = 0.8 ± 0.1 μM	EC_{50} = 31.5 ± 4.5 μM
Muscimol	EC_{50} = 1.4 ± 0.2 μM[a]	IC_{50} = 32.8 ± 2.2 μM
I4AA	EC_{50} = 8.6 ± 1.0 μM[a]	IC_{50} = 21.4 ± 1.7 μM
R-(−)-GABOB	EC_{50} = 19 μM[b]	1 mM activates 26.0 ± 0.8%[d]
S-(+)-GABOB	EC_{50} = 45 μM[b]	IC_{50} = 417.4 ± 7.0 μM K_B = 204.0 ± 14.3 μM[e]
3-APMPA	IC_{50} = 0.75 μM[c]	IC_{50} = 0.64 ± 0.03 μM
S-(−)-CGP44532	IC_{50} = 17 μM[b]	IC_{50} = 16.6 ± 1.0 μM
R-(+)-CGP44533	IC_{50} = 5 μM[b]	IC_{50} = 28.8 ± 2.4 μM
3-APA	IC_{50} = 20.8 ± 3.3 μM	IC_{50} = 33.1 ± 2.0 μM

[a]Data from ref 31. [b]Data from ref 10. [c]Data from ref 39. All Data are the mean ± SEM (n = 4−13 oocytes). [d]Percentage activation by *R*-(−)-GABOB (1 mM) compared to the current produced by a submaximal concentration of GABA (30 μM, EC_{50}). Data are the mean ± SEM (n = 3 oocytes). Figure 6A showing the weak agonist effect of *R*-(−)-GABOB. [e]K_B value is the mean ± SEM (n = 4).

Mutation of T244 to amino acids that lack a hydroxyl group in their side chain (T244A, T244G, T244V, T244I, T244L, and T244F) resulted in a lack of GABA-mediated responses, even when GABA was tested at a concentration of 30 mM (n = 9−15). This suggests that a hydroxyl group at position 244 in $GABA_C$ ρ_1 receptors is important for GABA induced receptor activation. To assess the effect of the T244 mutation on receptor gating, we examined the activity of the partial agonists, muscimol and imidazole-4-acidic acid (I4AA), at ρ_1T244S mutant receptors. The intrinsic efficacy of muscimol at 100 μM produced 79% of the maximum response of GABA at ρ_1 wild-type receptors[31] and produced 2.4 ± 0.8% (n = 5) of the maximum response of GABA (1 mM) at ρ_1T244S mutant receptors (Figure 4A and E). We did not observe I4AA (1 mM, n = 4) acting as an agonist at ρ_1T244S mutant receptors (Figure 4B). However, at the concentrations we tested, the predominant effects of muscimol and I4AA were as antagonists (Muscimol; IC_{50} = 32.8 ± 2.2 μM, n = 6, I4AA; IC_{50} = 21.4 ± 1.7 μM, n = 4) (Figure 4C, D, and F, Table 1). The conversion of a partial agonist to antagonist at mutant receptors infers that while the compound still binds to the receptor, they are unable to activate or gate the receptor. Thus, our results demonstrate that mutation of T244 alters receptor gating.

Interestingly, we and others[27] show that mutating T244 has a much more dramatic effect on GABA potency compared to mutating the adjacent serine residue (S243) of the ρ_1 subunit.[32] Mutation of serine 243 to alanine (S243A) afforded mutant receptors that were functional, with GABA potency reduced only by approximately 2-fold compared to ρ_1 wild-type.[32] The lack of a hydroxyl group in the residue at 243 did not strongly change the potency of GABA. However, our results confirm the fact that removal of the hydroxyl group at T244 was not tolerated,[27] indicating that the T244 has a more important role in ρ_1 receptor GABA mediated channel gating than S243.

T244 is located in loop C within the orthosteric binding site. Studies using crystal structures of the related acetylcholine binding protein (AChBP) have shown that, upon agonist binding, loop C undergoes a distinctive binding conformation, resulting in constriction of the binding site.[33,34] Loop C directly interacts with ligands, suggesting that a conformational change in loop C is one of the first steps which contributes to the channel gating. Other studies using rate-equilibrium free energy relationships (REFER)[35] examined the sequence of conformational changes during nACh receptor channel gating and found the conformational changes at the extracellular domain of the receptor before the channel gating. In another study using REFER,[36] it was found that residues in loop C of the mouse ACh receptor α-subunit and the surrounding area undergo initial stages of gating motion upon changes in receptor conformation. Taken together, these studies provide strong evidence that loop C is involved in agonist-mediated channel gating. Our homology modeling of the ρ_1 subunit predicts that T244 may be involved in GABA binding.[29] In agreement with this prediction, introduction of the T244S mutation into ρ_1 resulted in an approximately 39-fold change in GABA potency, which is consistent with that previously reported.[27] However, changes in the efficacy of the partial agonists muscimol and I4AA were observed. Both muscimol and I4AA acted as antagonists at the mutant receptor. These findings, along with data from other groups,[33,34,36] strongly suggest that mutation of T244 predominantly alters receptor gating. Considering the location of the residue in the binding site, it is likely that T244 is also involved in the early stages of the conformational changes that are initiated by ligand binding. It cannot be altogether ruled out that the T244 residue can directly interact with ligands at the binding site.

Effect of GABOB at ρ_1T244S Receptors. The enantiomers of GABOB are full agonists at ρ_1 receptors,[10] but at ρ_1T244S receptors it was found that *R*-(−)-GABOB and *S*-(+)-GABOB exert opposite pharmacological effects: *R*-(−)-GABOB is a weak partial agonist (1 mM activates 26% of the current produced by GABA EC_{50}; n = 3), while *S*-(+)-GABOB is a competitive antagonist (K_B = 204.0 ± 14.3 μM, n = 4) (Figures 5 and 6, Table 1). The change in GABOB pharmacology observed with the introduction of the T244S mutation infers that the interaction between the enantiomers and the hydroxyl group of T244 differs. Threonine possesses two chiral centers and it is L-threonine that is naturally found in proteins. In contrast, L-serine has only one chiral center. The major differences between L-threonine and L-serine lie in their side chain volume and the rotational freedom of the hydroxyl group. Specifically, the position of the hydroxyl group of threonine is restricted due to the additional methyl group in the side chain. This restriction and added volume appears to stabilize threonine 244 in a conformation that is optimal for agonist interaction. Indeed, our experimental data suggests that

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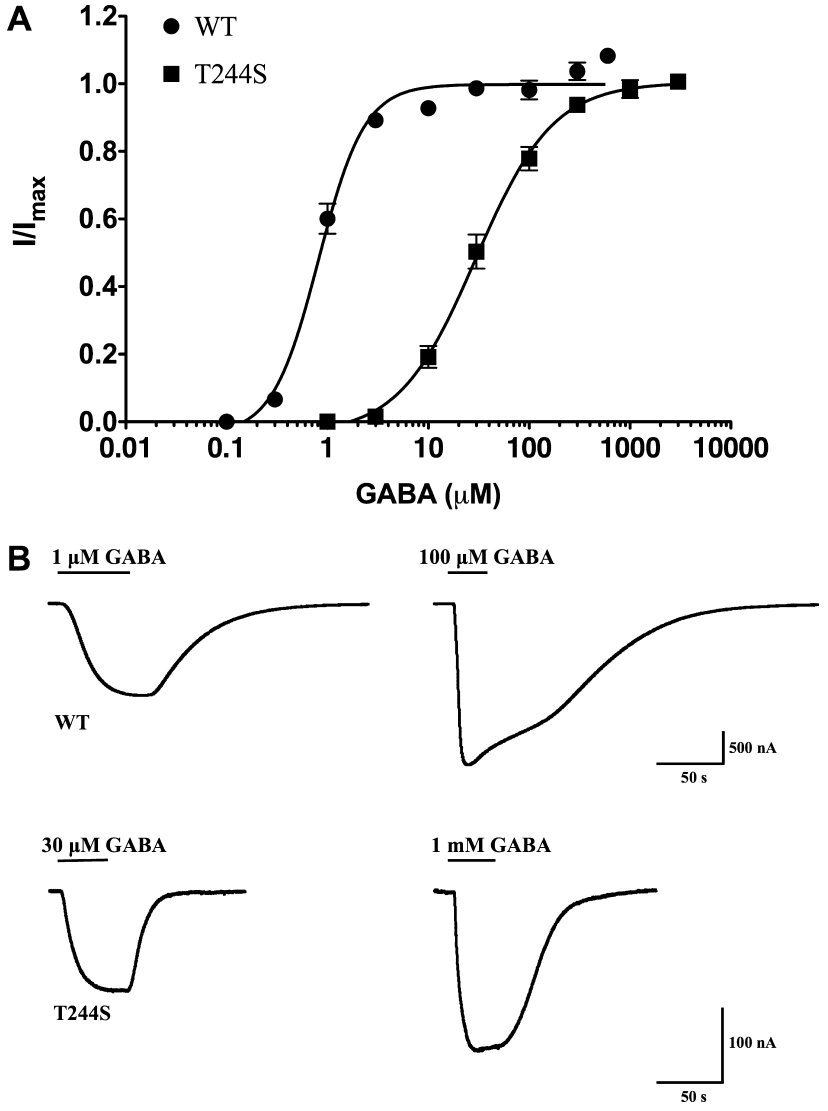

Figure 3. (A) Normalized concentration−response curves from responses to GABA for $\rho_1$wild-type and ρ_1T244S mutant receptors expressed in *Xenopus* oocytes. Each data point represents the mean ± SEM (n = 4−7). All data are normalized with I_{max}, which refers to their maximum current. (B) Example of responses for GABA at $\rho_1$wild-type and ρ_1T244S receptors at approximately EC_{50} and I_{max} GABA concentrations.

L-threonine at position 244 in the ρ_1 ligand binding site is preferred in agonist-induced activation compared to serine (Table 1).

The weak partial agonist activity of *R*-(−)-GABOB at ρ_1T244S receptors suggests that *R*-(−)-GABOB can interact with the serine hydroxyl group, but not as strongly as with the hydroxyl group of the original threonine residue. Thus, the increased rotational freedom of serine may allow the amino acid residue side chain to adopt conformations that are unable to interact with *R*-(−)-GABOB. In contrast, *S*-(+)-GABOB, like muscimol and I4AA, acts as a competitive antagonist at ρ_1T244S receptors. This implies that *S*-(+)-GABOB can still bind, but it is unable to facilitate activation, suggesting that the mutation has removed the ability of the molecule to gate the receptor, possibly due to a lack of interaction between the serine hydroxyl and *S*-(+)-GABOB.

An alternative explanation may be postulated from our homology modeling studies and the role of loop C in the active conformation of the receptor. Our previous homology modeling studies[26,29] have shown the possible formation of H-bonds between S243 or T244 with the carboxylate group of GABA. Modeling studies with GABOB show the possible formation of a H-bond with the hydroxyl group of GABOB. Upon ligand binding, loop C is thought to close over or engulf the agonist, and with this movement coupling agonist binding to channel gating.[33,34] It is possible that T244 initially forms a H-bond with the hydroxyl group of GABOB and that gating of the receptor may require the H-bond to break and make an alternative H-bond with the carboxylate group in order to stabilize the subsequent conformational change of loop C. The hydroxyl group of the serine side chain has more rotational freedom than threonine and may therefore adopt conformations that do not interact with the hydroxyl of *S*-(+)-GABOB and interact only weakly with *R*-(−)-GABOB. Thus, *S*-(+)-GABOB is a competitive antagonist at the mutant. On the other hand, while serine may preferentially interact with the hydroxyl group of *R*-(−)-GABOB, it may also be capable of forming a H-bond with the carboxylate group. Therefore, *R*-(−)-GABOB is a very weak partial agonist at the ρ_1T244S receptors.

Insertion of a methyl at the C2 position of *trans*-4-aminocrotonic acid (TACA) to give *trans*-4-amino-2-methyl-

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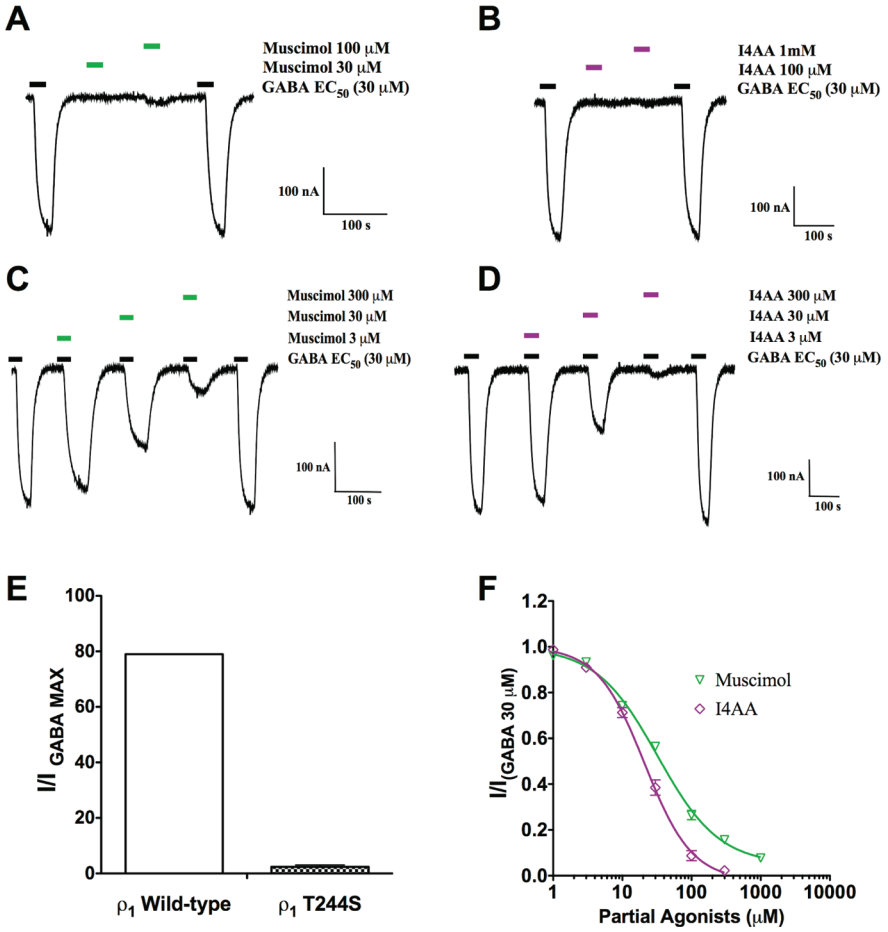

Figure 4. Effect of partial agonists at ρ_1T244S mutant receptor expressed in *Xenopus* oocytes: (A) Sample current trace showing the effect of muscimol (green) (30 and 100 μM) at ρ_1T244S mutant receptors. (B) Sample current trace showing the effect of I4AA (purple) (100 μM and 1 mM) at ρ_1T244S mutant receptors. (C) Sample current trace showing inhibition of GABA EC_{50} 30 μM (black) by muscimol concentrations, 3, 30, and 300 μM (green). (D) Sample current trace showing inhibition of GABA EC_{50} 30 μM (black) by I4AA concentrations, 3, 30, and 300 μM (purple). (E) Bar graphs showing the difference in muscimol efficacy at $\rho_1$wild-type and ρ_1T244S mutant receptors. Muscimol (100 μM) produced 79%[31] and 2.4 ± 0.8% (n = 5) of the maximum response of GABA at $\rho_1$wild-type and ρ_1T244S mutant receptors respectively. (F) Inhibitory concentration response curves for muscimol (green) and I4AA (purple) at ρ_1T244S mutant receptors. Data are the mean ± SEM (n = 4–6 oocytes). All data are normalized with $I_{(GABA\_30\mu M)}$ concentrations.

but-2-enoic acid (2-MeTACA) results in a similar change in activity.[37] TACA is a full agonist at ρ_1 receptors; however, 2-MeTACA is an antagonist. Initial studies suggested that this change in activity was due to steric factors. However, we now speculate that the methyl group at the C2 position may interact unfavorably with T244 and interrupt any possible H-bond with carboxylate group of 2-MeTACA, preventing gating of the receptor and therefore 2-MeTACA acts as an antagonist at ρ_1 wild-type receptors.

Antagonist Activity at ρ_1T244S Receptors. We further investigated the role of T244 on ρ_1 receptor antagonists (Figure 7). The methylphosphinic and phosphonic acid analogues of GABA, 3-aminopropyl(methyl)phosphinic acid (3-APMPA) and 3-aminopropylphosphonic acid (3-APA), respectively, and the methylphosphinic acid analogues of GABOB, *S*-3-amino-2-hydroxypropyl)methylphosphinic acid (*S*-(−)-CGP44532), *R*-3-amino-2-hydroxypropyl)-methylphosphinic acid (*R*-(+)-CGP44533), showed no agonist activity (100 μM, n = 5) at ρ_1T244S receptors. This is consistent with the compounds acting as antagonists at ρ_1 wild-type receptors.[10,38,39] Our homology model does not predict any H-bonds between T244 and the hydroxyl group of the GABOB analogues, *R*-(+)-CGP44533 and *S*-(−)-CGP44533 (Figure 2). In addition, no H-bond was predicted for 3-APMPA, while a H-bond was observed between the phosphonic acid group of 3-APA and T244 in the model (Figure 1 Supporting Information), similar to what was observed with the methylphosphonate.[30] Electrophysiological data demonstrated that the IC_{50} values observed at ρ_1T244S mutant receptors for 3-APMPA and *S*-(−)-CGP44533 were unchanged compared to ρ_1 wild-type receptors (Table 1). In contrast, the IC_{50} values for *R*-(+)-CGP44533 and 3-APA were increased by approximately 6-fold and 2-fold, respectively, at ρ_1T244S receptors (Table 1). These subtle changes in IC_{50} values suggest T244 is not important for antagonist affinity, as predicted by the homology model, and therefore, the effect of the T244S mutation on antagonist activity was not as drastic as that observed for the agonists.

While our homology model is based on the AChBP in order to develop our hypothesis, our pharmacological results demonstrate the model's predictive value. However, there are several limitations with using a homology model based on the AChBP crystal structure: Crystal structures are representative of a fixed structure, but by definition the receptor changes

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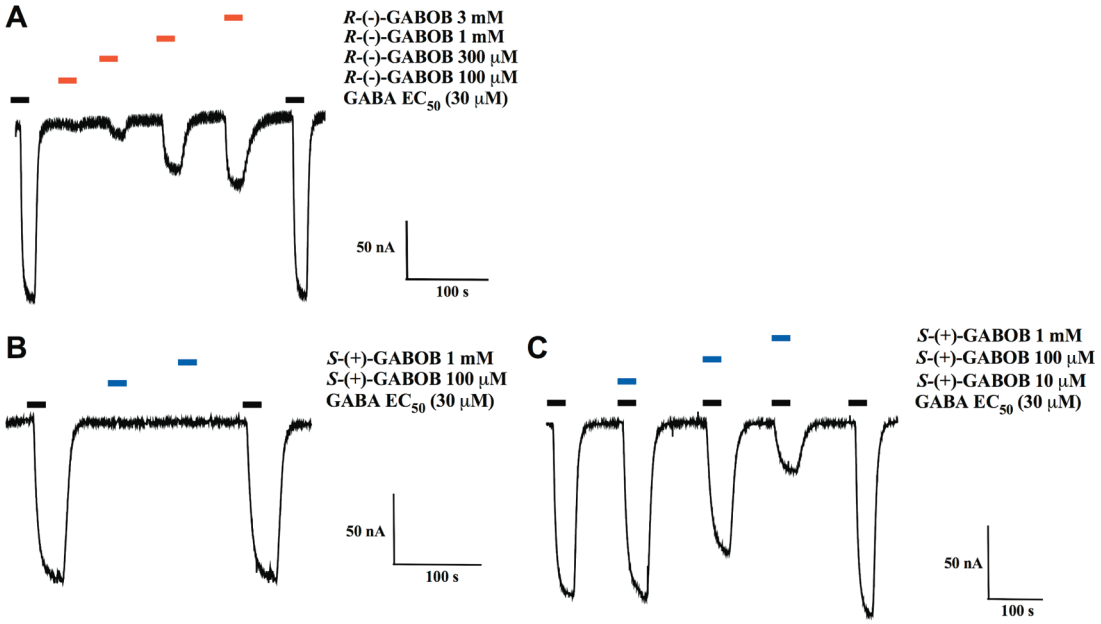

Figure 5. Effect of the enantiomers of GABOB on ρ_1T244S mutant receptor expressed in *Xenopus* oocytes: (A) Sample current trace showing weak agonist effect of *R*-(−)-GABOB on ρ_1 T244S mutant receptor. GABA EC_{50} 30 μM (black) activates the receptor, allowing Cl^- ions to pass through the pore. Application of *R*-(−)-GABOB (red), 100 μM, 300 μM, 1 mM, and 3 mM concentrations, activates the receptor in concentration dependent manner. (B) Sample current trace showing the effect of *S*-(+)-GABOB (blue) on ρ_1T244S mutant receptor. *S*-(+)-GABOB did not exhibit agonist effects at concentration 100 μM and 1 mM. (C) Sample current trace showing inhibition of GABA EC_{50} 30 μM (black) by *S*-(+)-GABOB (blue) concentrations,10 μM, 100 μM, and 1 mM.

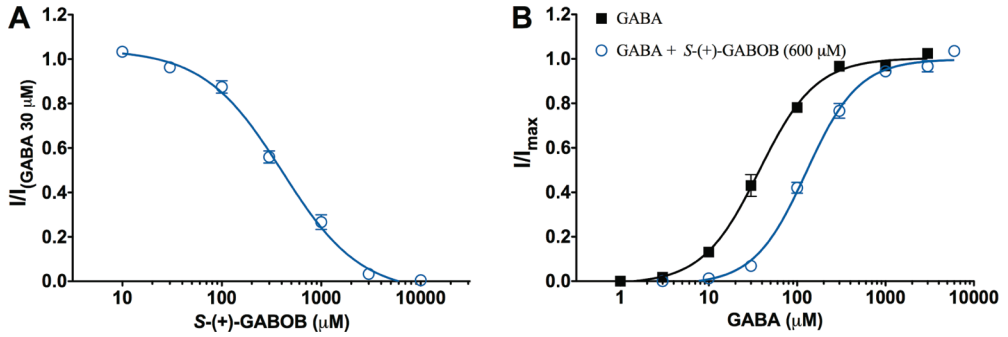

Figure 6. Pharmacology of *S*-(+)-GABOB on ρ_1T244S mutant receptor expressed in *Xenopus* oocytes: (A) Inhibitory concentration−response curve for *S*-(+)-GABOB on ρ_1T244S mutant receptors. Each data point represents the mean ± SEM (n = 6). All data are normalized with $I_{(GABA\_30\mu M)}$. (B) Concentration−response curves of GABA alone (black, n = 4) and GABA in presence of *S*-(+)-GABOB 600 μM (blue, n = 4) on ρ_1T244S mutant receptor. Each data point represents the mean ± SEM (n = 4). All data are normalized with I_{max}.

structure during the channel gating process, and also will include a variety of agonist-bound, antagonist-bound, and closed and open states. Our model also did not include transmembrane domains, and it is reasonable to assume that the overall structure of the receptor is dependent on the presence of this domain. Third, there are other related crystal structures that have recently been published, including the GluCl channel[40] that has higher sequence homology to the $GABA_C$ receptor and includes the transmembrane regions. Ultimately, however, the pharmacology is different between receptors and homology models will not necessarily be predictive of these differences. To develop the best understanding of structural differences between related receptors, hypotheses built on homology models tested by pharmacological experiments and further refining these models as we have pursued here will be the most useful method to understand ligand-induced channel gating.

In conclusion, threonine at position 244 is critical for ρ_1 receptor pharmacology. A hydroxyl group on the side chain of the amino acid at position 244 in ρ_1 receptors is crucial for agonist-mediated activation. This is consistent with previous findings.[27] In addition, T244 plays an important role for the enantioselective actions of GABOB, with *S*-(+)-GABOB converted to a competitive antagonist, while *R*-(−)-GABOB acts as a weak partial agonist at ρ_1T244S mutant receptors.

While mutating T244 had a significant effect on agonist activity, antagonist activity remained similar to $\rho_1$wild-type. Studies using the crystal structure of AChBP from *Aplysia californica* reported that loop C did not exhibit significant changes in conformational change upon antagonist binding.[33] However, the interaction between T244 and antagonists may have minor effects on the stability of antagonist binding, resulting in the small changes in affinity of *R*-(+)-CGP44533 and 3-APA at ρ_1T244S receptors. In contrast, T244 in loop C is involved in receptor gating upon agonist binding. Thus, this

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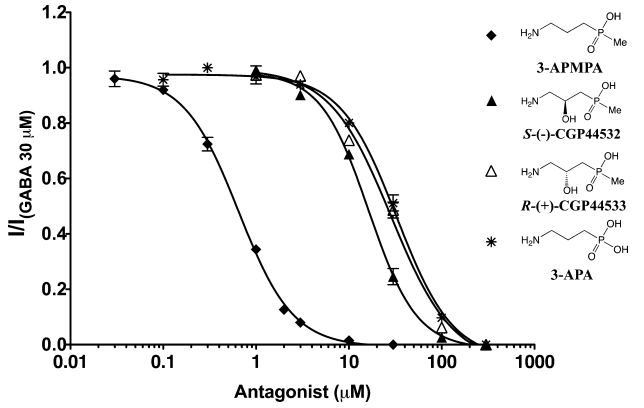

Figure 7. Inhibitory concentration−response curves for 3-APMPA, *S*-(−)-CGP44532, *R*-(+)-CGP44533 and 3-APA on ρ_1T244S receptors expressed in *Xenopus* oocytes. Each data point represents the mean ± SEM (n = 5−13 oocytes). All antagonists were tested with the presence of GABA EC_{50} (30 μM). All data are normalized with $I_{(GABA\_30\mu M)}$.

study confirms that mutation of T244 has a significant influence on agonist potency and efficacy and highlights the dynamic role of loop C in channel gating with T244 playing a critical role in agonist-evoked receptor activation.

METHODS

Chemicals. 3-APA ((3-aminopropylphosphonic) acid) and 3-APMPA (3-aminopropyl(methyl)phosphinic acid) were synthesized according to the previously published methods.[41,42] GABA (γ-aminobutyric acid), muscimol, and I4AA (imidazole-4-acidic acid) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). The enantiomers of GABOB (4-amino-3-hydroxybutanoic acid) and (3-amino-2-hydroxypropyl)methylphosphinic acid were gifts from Dr. Wolfgang Froestl (formerly Novartis, Switzerland) and Prof. Povl Krogsgaard-Larsen (University of Copenhagen, Denmark).

Site-Directed Mutagenesis. Alanine, glycine, serine, valine, isoleucine, leucine, and phenylalanine mutations were generated at the position 244 of human $GABA_C$ ρ_1 subunit by using sense and antisense oligonucleotide primers (Table 1, Supporting Information) and the QuickChange II Site-directed Mutagenesis kit protocol (Stratagene, La Jolla, CA). All mutations were verified by DNA sequencing to confirm fidelity (Australian Genome Research Facility, Australia). The plasmids containing ρ_1 wild-type and mutations inserts were linearized with Xba-I, and T7 mMESSAGE mMACHINE kit (Ambion, Austin, TX) was used for mRNA synthesis.

Expression of $GABA_C$ ρ_1 Wild-type and Mutant Receptors in *Xenopus* Oocytes. Oocytes from *Xenopus laevis* (South Africa clawed frogs) were harvested as described previously[43] in accordance with the National Health and Medical Research Council of Australia's ethical guidelines and approved by the University of Sydney Animal Ethics Committee. Stage V−VI oocytes were injected with 10−15 ng cRNA and then stored at 18 °C in ND 96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.5) supplemented with 2.5 mM sodium pyruvate, 0.5 mM theophylline, 50 μg mL^{-1} gentamycin, and 2.5 mg mL^{-1} tetracycline.

Electrophysiological Recordings. Two to six days after injections, the activity was measured via two-electrode voltage clamp recording using Geneclamp 500 amplifier (Axon Instruments, Foster City, CA), a MacLab 2e recorder (AD Instruments, Sydney, NSW, Australia), and Chart version 5.5.6 program as previously described.[44] Briefly, oocytes expression receptors were clamped at −60 mV with continuous flow of ND96 buffer. Antagonist effects were tested in the presence of GABA EC_{50} concentration (30 μM) on ρ_1T244S mutant receptors and the effects were evaluated for their inhibitory concentration−response actions.

Data Analysis. Current responses were normalized to the maximum GABA-activated current recorded in the same cell and expressed as a percentage of this maximum and fitted by least-squares to the Hill equation (eq 1). GABA concentration response curves were generated using GraphPad PRISM 5.02 (GraphPad software San Diego, CA).

$$I = I_{max}[A]^{n_H}/(EC_{50}{}^{n_H} + [A]^{n_H}) \quad (1)$$

where I is the current response to a known concentration of agonist, I_{max} is the maximum current obtained, [A] is the agonist concentration, EC_{50} is the concentration of agonist at which current response is half maximal, and n_H is the Hill coefficient.

Dissociation equilibrium constants (K_B) were estimated via the Schild equation (eq 2).

$$K_B = [B]/([A]/[A^*] - 1) \quad (2)$$

where [B] is the antagonist concentration, [A] is the EC_{50} of GABA in the presence of antagonist, and [A*] is the EC_{50} of GABA in the absence of antagonist. Data are expressed as mean ± standard error of the mean (SEM).

IC_{50} values were calculated using eq 3. The inhibitory concentration curves were generated using GraphPad PRISM 5.02.

$$I = I_{max}[A]^{n_H}/(IC_{50}{}^{n_H} + [A]^{n_H}) \quad (3)$$

where I is the peak current at a given concentration of agonist, I_{max} is the maximal current generated by the concentration of agonist, [A] is the concentration of GABA, IC_{50} is the antagonist concentration, which inhibits 50% of the maximum GABA response, and n_H is the Hill coefficient.

Homology Modeling and Docking Studies. A homology model of $GABA_C$ ρ_1 receptor ligand binding site was generated using the crystal structure of *L. stagnalis* AChBP[28] as template (PDB ID: 1I9B).[45] The amino acid sequence of $GABA_C$ ρ_1 receptor (accession code: P24046) as obtained from NCBI[46] was aligned on the template. Sequence alignments were based on the results of Adamian et al.,[47] and performed on each of the five subunits of the AChBP complex using Prime v2.1 (Prime, version 2.1, Schrödinger, LLC, New York). The "bldstruct" command in Prime was used to merge and build the five alignments, resulting in the $GABA_C$ ρ_1 pentameric model. The OPLS_2005 all-atom force field was used for energy scoring of the protein and surface generalized Born (SGB) continuum solvation model for treating solvation energies and effects. The predicted model was then prepared for docking by using protein preparation wizard, wherein hydrogens were added, bond orders assigned, and disulfide bonds created. Finally, the corrected structure was optimized by restrained minimization using "impref minimization" by selecting hydrogens only so that heavy atoms were left untouched. Docking studies were conducted by using "Glide" software as provided in Maestro (Glide, version 5.6, Schrödinger, LLC, New York). A docking model was generated by forming a receptor grid around the active site formed by two adjacent $GABA_C$ ρ_1 monomers. All the possible ionization states of the ligands at pH 7.4 were generated and then docked flexibly in to the active site using extra-precision (XP) mode.[48]

ASSOCIATED CONTENT

Supporting Information

Information on the oligonucleotide primers, 3-APMPA and 3-APA docked into the ρ_1 receptor GABA binding site. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

Participated in research design: I.Y., N.A., and M.C. Conducted experiments: I.Y., M.R.D., and N.G. Performed data analysis: I.Y., N.A., J.E.C., J.R.H., and M.C. Wrote or contributed to the writing of the manuscript: I.Y., N.A., J.E.C., G.A.R.J., J.R.H., M.C.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

3-APMPA, 3-aminopropyl(methyl)phosphinic acid; 3-APA, 3-aminopropylphosphonic acid; *S*-(−)-CGP44532 and *R*-(+)-CGP44533, (3-amino-2-hydroxypropyl)methylphosphinic acid; AChBP, acetylcholine binding protein; CNS, central nervous system; K_B, dissociation equilibrium constant; EC_{50}, effective concentration that activates 50% of the maximum response; GABA, γ-aminobutyric acid; GABOB, 4-amino-3-hydroxybutanoic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; H-bond, hydrogen bond; I4AA, imidazole-4-acidic acid; IC_{50}, effective concentration that inhibits 50% of GABA EC_{50}; LGIC, ligand-gated ion channel

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