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Point mutations in the transmembrane region of $GABA_{B2}$ facilitate activation by the positive modulator GS39783 in the absence of the $GABA_{B1}$ subunit

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Running Title:

Positive modulator GS39783 activates $GABA_{B2}$ subunits

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Abbreviations:

CRC, concentration response curve
ECD, N-terminal extracellular domain
DMEM, Dulbecco's Modified Eagle Medium
dGB1, dGB2, drosophila $GABA_B$ receptor subunits $GABA_{B1}$, $GABA_{B2}$, respectively
GABA, γ-aminobutyric acid
rGB1, rGB2, rat $GABA_B$ receptor subunits $GABA_{B1}$, $GABA_{B2,}$, respectively
rGB1a, rat $GABA_{B1}$ receptor subunit, isoform 1a
GPCR, G-protein coupled receptor
GS39783, N,N'-Dicyclopentyl-2-methylsulfanyl-5-nitro-pyrimidine-4,6-diamine
GTP(γ)S, guanosine 5'-O-(3-thiotriphosphate)
$G\alpha o_A$, G protein αo, isoform A
HA, hemagglutinin
HEK293 cells, human embryonic kidney cells
IPSP, inhibitory postsynaptic potential
mGluR, metabotropic glutamate receptor
TM, transmembrane domain

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ABSTRACT

$GABA_B$ receptors are heterodimers of two subunits, $GABA_{B1}$ (GB1) and $GABA_{B2}$ (GB2). Agonists such as GABA and baclofen bind to the GB1 subunit only whereas GB2 is essential for G protein activation. Positive allosteric modulators enhance the potency and efficacy of agonists at $GABA_B$ receptors and are of particular interest as they lack the sedative and muscle relaxant properties of agonists. In this study we aimed to characterize the interaction of the positive modulator GS39783 (N,N'-Dicyclopentyl-2-methylsulfanyl-5-nitro-pyrimidine-4,6-diamine) with the $GABA_B$ receptor heterodimer. Using functional GTPγS binding assays we observed positive modulation by GS39783 in different vertebrate species but not in drosophila. However, co-expression of drosophila GB1 with rat GB2 yielded functional receptors positively modulated by GS39783. Together with data from rat/drosophila GB2 subunit chimeras, this pointed to a critical role of the GB2 transmembrane region for positive modulation. We further characterized GS39783 function using point mutations. GS39783 positively modulated GABA responses but also showed considerable agonistic activity at heterodimers containing a mutant rat GB2 subunit with three amino acids substitutions in transmembrane domain VI. Surprisingly, in contrast to wild-type rat GB2, this mutant subunit was also activated by GS39783 when expressed without GB1. The mutations of both G706T and A708P are necessary and sufficient for activation and identify a key region for the effect of GS39783 in the GB2 transmembrane region. Our data show that mutations of specific amino acids in GB2 can induce agonism in addition to positive modulation and facilitate GB2 activation in the absence of GB1.

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INTRODUCTION

$GABA_B$ receptors are the metabotropic receptors for γ-aminobutyric acid (GABA) and modulate inhibitory and excitatory neurotransmission (reviewed in Bettler et al., 2004). Presynaptic $GABA_B$ receptors, via inhibition of Ca^{2+}-channels, inhibit the release of several neurotransmitters and neuropeptides whereas postsynaptically located receptors activate potassium channels and induce slow inhibitory postsynaptic potentials (IPSPs). $GABA_B$ receptors belong to the family C of G protein coupled receptors (GPCRs) with the agonist binding pocket being constituted by the large N-terminal extracellular domain (ECD). Native $GABA_B$ receptors are heterodimers composed of two subunits, $GABA_{B1}$ (GB1) and $GABA_{B2}$ (GB2). The receptors are exceptional among GPCR heterodimers in that only one subunit, GB1, constitutes the GABA binding domain whereas activation of G proteins is mediated only through the second subunit, GB2. Mutagenesis studies and the lack of evolutionary conservation suggest that the ECD of GB2 does not form a binding pocket for a natural ligand (Kniazeff et al., 2002). Genetic inactivation of either the GB1 or the GB2 subunit abolishes physiological $GABA_B$ receptor responses demonstrating that GB1 and GB2 are essential subunits of all brain $GABA_B$ receptors (reviewed in Bettler et al., 2004)

Baclofen is a selective $GABA_B$ receptor agonist and used clinically as muscle relaxant (Bowery, 2006). Although $GABA_B$ receptors represent a potentially interesting target for several neurological and psychiatric diseases the exploration and use of baclofen for such indications is hampered by its sedative and muscle relaxant effects. Positive allosteric modulators of $GABA_B$ receptors such as CGP7930 and GS39783 have recently been identified (Urwyler et al., 2001, 2003). These molecules enhance both the potency and the maximal efficacy of GABA but have little or no intrinsic agonistic efficacy on their own. *In vivo*, the effect of positive allosteric modulators are dependent on the endogenously released agonists, thus the compounds potentiate ongoing synaptic activity only (reviewed in Christopoulos, 2002; Christopoulos and Kenakin, 2002; Jensen and Spalding, 2002). Therefore, the principle of positive modulation provides an interesting avenue for the development of new pharmacotherapies targeting $GABA_B$ receptors as differential *in vivo* pharmacological profiles of positive modulators compared to agonists are expected. Indeed, it has been shown that GS39783 lacks the sedative and muscle relaxant properties of baclofen, while activity in animal models of drug abuse

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and anxiety suggest that $GABA_B$ receptor positive modulation induces desired pharmacological effects (Cryan et al., 2004; Smith et al., 2004; Slattery et al., 2005).

The binding sites of allosteric inhibitors and positive modulators of several other family C GPCRs have been localized to the transmembrane domain (Litschig et al., 1998; Pagano et al., 2000; Knoflach et al., 2001; Schaffhauser et al., 2003; Lavreysen et al., 2003; Jiang et al., 2005). Binet et al (2004) recently provided evidence that the $GABA_B$ receptor positive modulator CGP7930 interacts with the transmembrane domain of the GB2 subunit, however specific amino acids important for positive modulator function have not been identified yet.

In the present study, we aimed to characterize the binding site for the $GABA_B$ receptor positive modulator GS39783. We used interspecies combinations of receptor subunits from drosophila and rat to map the interaction of GS39783 with the $GABA_B$ receptor heterodimer. We identified specific amino acids in the transmembrane domain of GB2 which are important for GS39783 function and show that mutation of selective residues can switch positive modulation to agonistic effects. Our results also support the notion that GB2 subunits can function independently of GB1.

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MATERIALS AND METHODS

Cloning of drosophila $GABA_B$ receptor subunits. *Drosophila melanogaster* GB1 and GB2 cDNAs (dGB1, dGB2) were re-cloned by PCR. Adult and embryo poly A(+) RNAs were purchased from Clontech (6947-1 and 6945-1) and reverse transcribed (Gibco cDNA synthesis module) using random and oligo-dT primers. Primers were designed from published sequences (Mezler et al., 2001): dGB1: 5'CAC CAT GAC AAG TGA TGG TGC TGT TAC G and 5'-TAG TTC CAT GCA CCA GGT ACT CTA CTC; dGB2: 5'-CAC CTC TGG GAC TAA GCA AGC TGC CCA and 5'-CTT GTA GGC GGC GCG AGT CAT ATG. PCRs were carried out using Pfu polymerase (Stratagene; 58ºC, 35 cycles, 5 min extension) and the products cloned into pcDNA3-topo (Invitrogen) and sequenced.

Rat/drosophila GB2 subunit chimeras and point mutations. Mutant receptor subunits were constructed by PCR (Phusion polymerase, Finnzymes) using the 'splicing by overlap extension' method as described (Horton et al., 1989). The boundary sequences in rat/ drosophila GB2 subunit chimeras were: PPKD_RTLI (N-terminus rat, TM and C-terminus drosophila) and PPKD_RTII (N-terminus drosophila, TM and C-terminus rat). For chimeras within the GB2 transmembrane region the splice sites were chosen within conserved sequences in the connecting loops: KLIK_MSSP (after TM I); ETLC_TARA (after TM II); KKII_KDYQ (after TM III); YSME_HHEN (after TM IV); TRNV_SIPA (after TM V); LTRD_RKDL (after TM VI); LRTN_PQGV (after TM VII). C-terminal hemagglutinin (HA)-tags (YPYDVPDYA) were added to mutant subunits containing C-terminal drosophila sequences to facilitate expression analysis by western blots. For the introduction of point mutations (Fig. 3) two complementary mutant primers sequences were designed (35-45 nucleotides) and used in PCR reactions (25 cycles, 50 ng template, 62ºC annealing) together with forward primer 5'-ATC TCA GGG AAG ACT CCA CAG (rGB2-f) or reverse primer 5-TCC CTC CAG GCG TGA CGT GCT C (rGB2-r). PCR products were joined in a second amplification with primers Apa1-f and Ale1-r (10 cycles). The DNA fragments were gel-purified (Qiaex, Qiagen), digested with Apa1/AleI and used to replace a corresponding wild-type Apa1/AleI fragment of rat GB2 cloned into pC1-neo (Promega). Similarly, rGB1 mutant subunits were constructed using primers 5'-CTG CTC ACTG GCA CTG GCT GC and 5'-GCG GCC GCG CGG CCG CTC AGG GAG ATC CTT CTC CAT G

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together with mutant primers. Final PCR products were digested with BstEII/NotI and used to replace a corresponding wild-type fragment of rat $GABA_{B1a}$ (rGB1a) in pC1-neo. For the construction of drosophila GB2 (dGB2) point mutations PCRs were done with primers 5'-CTT GTG GAG TAC GAC AGA CTG C (dGB2-f) and 5'-TAC CGA CGT TGG AGC CAC CTG (dGB2-r) together with mutant primers. Joined PCR products were used to replace a BaeI/ BstEII fragment of dGB2-HA cloned into pcDNA3.1-topo (Invitrogen). To introduce mutations into chimeric subunits (N-term rat, TM drosophila) PCRs were done with primers rGB2-f and dGB2-r, and the ApaI/ BstEII digested products used to replace corresponding wild-type fragments. All constructs were sequenced. For the mutations the first character and the number indicate amino acid (single letter code) and position of the targeted amino acid, respectively, according to the translations of accessions Y10369; AJ011318, and AF318273 including the signal peptide (rGB1a, rGB2, and dGB2, respectively). The second character indicates the amino acid substitution introduced (from drosophila GB2 or rat GB1). For key amino acids also the numbers according to the generic system proposed by Ballesteros and Weinstein (1995) are indicated in brackets.

Transient expression of plasmid constructs and membrane preparation. HEK293FT cells (Invitrogen) were cultured in DMEM, 10% fetal calf serum, 1mg/ml G418 (Invitrogen) supplemented with non-essential amino acids. Cells were co-transfected with $GABA_B$ receptor plasmids and the G protein $G\alpha o_A$ (rat Gnao; NM_017327) cloned in pCD-PS (8 μg plasmid DNA, Fugene). For expression of heteromeric $GABA_B$ receptors 2-3 μg GB1, 1-2 μg GB2, and 4 μg $G\alpha o_A$ was used; to express GB2 subunits individually 4 μg GB2 and 4 μg $G\alpha o_A$ was used. Cells were harvested two days after transfection. The cells were scraped of the dishes in phosphate buffered saline, homogenized using glass-glass homogenizers and centrifuged 30 min, 4ºC at 20000g. After resuspension in buffer the pellet was re-homogenized and centrifuged again. Membranes were resuspended in GTPγ^{35}S binding assay buffer, the protein concentrations determined using a BCA protein assay kit (Novagen), and the membranes used immediately in GTPγ^{35}S binding assays.

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GTPγ^{35}S binding assay. The assay mixtures contained 10-40 µg of membranes in 50 mM Tris-HCl buffer pH 7.7, 10 mM $MgCl_2$, 1.8 mM $CaCl_2$, 100 mM NaCl, 10 µM guanosine 5'-diphosphate (Sigma), 0.2 nM ^{35}S-GTPγS, and test compounds (Urwyler et al, 2001). 96-well Packard Pico-plates (300 µl volume) were used. The reagents were incubated for 60 min at room temperature and subsequently filtered (Packard unifilter-GF/C). After two washes with assay buffer as above the plates were dried for one hour at 50°C, 50 µl of scintillation solution (Microscint) was added and the radioactivity counted. Counts were normalized to 20 µg of membrane protein. Prism 3.0 or 4.0 software (Graph Pad software, San Diego, CA) was used for all data calculations. Basal levels were determined in the absence of test compounds. In all figures except Fig. 1 signals are expressed as cpm above basal. The datapoints in figures are means (± sem) calculated from triplicate determinations. Statistical comparisons were done using a t-test (two-tailed, unpaired; $p < 0.05$ was considered significant).

Western blot. Cell membrane preparations were resuspended in sample buffer (62.5 mM Tris pH 6.8, 2% (w/v) sodium dodecyl sulfate, 0.01% (w/v) bromophenol blue, 5% (v/v) ß-mercaptoethanol, 25% glycerol), shaked for 45 min at room temperature and loaded on 7.5% SDS acrylamide gels (Bio-Rad). After electrophoretic transfer the membranes were incubated for 1 hour at room temperature in PBS containing 0.1% Tween 20; 5% fat-free powdered milk (PBST/milk). Antibody AbC22 (directed against C-terminal sequences of rGB2; Kaupmann et al., 1998) was applied overnight at 4ºC in PBST/milk. Incubation with horseradish-peroxydase-conjugated anti-rabbit antibody (Cell Signaling was for 1 hour at room temperature. For detection of HA-tagged dGB2 a peroxydase-conjugated anti-HA antibody (Roche) was applied overnight at 4ºC. Peroxydase activity was detected using Supersignal West Pico substrate (Pierce) and Kodak MR-1 X-ray films (Amersham Biosciences).

Compounds. GS39783 and CGP7930 (2,6-Di-tert-butyl-4-(3-hydroxy-2,2-dimethyl-propyl)-phenol) were synthetized in house. 10 mM stock solutions were prepared in dimethyl sulfoxyde and

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subsequently diluted in assay buffer. GABA was obtained from Fluka; 100 mM stock solution were prepared in H_2O.

RESULTS

Vertebrate but not drosophila $GABA_B$ receptors are positively modulated by GS39783. We aimed to characterize the molecular interaction of the $GABA_B$ receptor modulator GS39783 with the $GABA_B$ receptor heterodimer. The identification of amino acid residues important for the GS39783 function is hampered as mapping approaches using chimeras between different mammalian receptor subtypes, strategies which have successfully been applied for example to identify binding sites for metabotropic glutamate receptor modulators, were not possible as additional subunits to GB1 and GB2 are not known. In order to identify a possible means to identify critical residues for GS39783 function we explored $GABA_B$ receptor positive modulation in different species, using functional GTPγ^{35}S binding assays (Fig. 1). GABA at 1 μM or 20 μM significantly stimulated GTPγ^{35}S binding using native $GABA_B$ receptor preparations from different vertebrate species as well as with membranes from cells expressing cloned drosophila $GABA_B$ receptors. In the presence of GS39783 the GABA signal was enhanced at rat, fish and chicken but not at drosophila $GABA_B$ receptors (Fig. 1a). Concentration response curves (CRCs) support the conclusion that rat but not drosophila $GABA_B$ receptors are positively modulated by GS39783 (Fig. 1b, c).

The transmembrane domain of GB2 is important for positive modulation by GS39783. We then explored interspecies combinations of drosophila and rat $GABA_B$ receptor subunits. When drosophila GB1 (dGB1) was co-expressed with rat GB2 (rGB2) concentration-dependent positive modulation of the GABA signal by GS39783 was observed (Fig. 1d). The overall stimulation levels by GABA at dGB1/rGB2 heterodimers appear reduced compared to rat receptors (rGB1/rGB2), possibly because of less efficient heterodimerization or because of a somewhat reduced GABA potency. The potency of GS39783 however in modulating the GABA response at the interspecies heterodimer was similar compared to wild-type rat $GABA_B$ receptors (1.0 ± 0.5 versus 0.3 ± 0.03 μM, respectively; n = 4). The *vice versa* combination, i.e. rat GB1 (rGB1) co-expressed with drosophila GB2 (dGB2), however

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was not activated by GABA and therefore was not informative with regard to positive modulation (not shown). These data suggested a critical role of GB2 subunit for the activity of GS39783.

To investigate further which receptor domains are important for positive modulation drosophila/rat GB2 subunit chimeras were constructed and investigated in functional GTPγ^{35}S binding assays (Fig 2). With a first set of GB2 subunit chimeras we aimed to show if the binding site of GS39783 resides within the seven transmembrane (TM) region or the ECD of the GB2 subunit. A GB2 subunit chimera in which the ECD of dGB2 was fused to the TM region of rGB2 was positively modulated by GS39783, upon co-expression with either rat or drosophila GB1 (Fig. 2a). The stimulation levels for the application of GABA in the presence of GS39783 were significantly higher than the levels obtained with GABA applied alone ($p < 0.05$ versus 1 mM GABA, $n = 3$). The converse chimera, i.e. the ECD of rGB2 fused to the TM domain of dGB2, yielded functional receptors responsive to activation by GABA, but which were not positively modulated by GS39783. These data indicated that the TM spanning region or C-terminal intracellular sequences of rGB2 are critical for positive modulation. No significant stimulation with GABA or GS39783 was observed when rGB2 subunit chimeras were expressed without GB1.

To further delineate which part of the TM region is important for positive modulation, mutant GB2 subunits were generated in which individual TM helices (TM1-7) of rGB2 where introduced into the drosophila TM region. As template a chimera was used in which the ECD from rGB2 was fused to the TM region of dGB2 (Fig. 2b). Receptors containing this chimeric GB2 subunit were not responsive to GS39783 (Fig. 2a); hence we hypothesized that by successive addition of TM helices from rGB2 positive modulation by GS39783 would be gained at one point. However, none of the mutants which contained TM helices from both drosophila and rat yielded functional receptors, as shown by lack of stimulation with GABA in the GTPγ^{35}S binding assay (Fig. 2b). Therefore, these mutants were not informative for the GS39783 mapping purpose. Only after all TMs from rGB2 had been introduced (Fig. 2b) stimulation by GABA was obtained and receptors containing this mutant subunit were positively modulated by GS39783 as expected. The C-terminal intracellular sequence of this mutant subunit was derived from drosophila, thus excluding the C-terminal rGB2 sequence for being important for GS39783 function.

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Identification of individual amino acids in the GB2 TM domain affecting GS39783 function. The abovementioned data suggested that subtle changes (i.e. point mutations) were required to further elucidate which transmembrane helices are critical for positive modulation. To identify candidate amino acids a sequence alignment of TMs from rat and drosophila GB2 together with different family C GPCRs was used (Fig. 3a). Candidate residues for mutagenesis were identified based on the following criteria: 1) conservation in rat but not drosophila GB2; or 2) conservation in rGB2 but not in rGB1. rGB1 and rGB2 subunits share only 42% sequence identical amino acids in the TM region and the similarity between rat and drosophila GB2 is also very limited (52% identical residues in the TM). Therefore many amino acids fulfill the 'candidate' criteria as above. We investigated amino acids in TMs II to VII with a focus on residues which have previously been shown to be involved in the binding of allosteric modulators to other family 3 GPCRs (reviewed in Jensen & Spalding, 2004). Respective candidate amino acids in rGB2 were mutated to the corresponding residues present in dGB2, or to the corresponding residue present in rGB1. By this approach we expected to obtain functional rGB2 subunits which, upon co-expression with rGB1, are not positively modulated if the mutated amino acid is crucial for GS39783 activity. A summary of all amino acids investigated (> 50) is shown in Fig. 3b. Whenever possible several adjacent point mutations were combined in one construct. Each rGB2 mutant was transiently co-expressed in HEK293FT cells together with rGB1 and cell membranes analyzed in GTPγ^{35}S binding assays. To assess positive modulation by GS39783 the stimulatory effect of 10 μM GABA in the presence of 10 μM GS39783 was compared to the response obtained with 10 μM GABA applied alone. The signal obtained with GABA applied alone serves as readout for functionality of the mutant protein. In addition the effect of 10 μM GS39783 applied alone was measured (Fig. 3b).

GABA stimulated GTPγ^{35}S binding at the majority of rGB1/mutant rGB2 heterodimers confirming the functionality of the rGB2 proteins (Fig. 3b). Receptors containing a rGB2 mutant subunit (G706T, A708P, S710T in TM VI) were considerably activated by GS39783 when the modulator was applied without GABA ($p < 0.01$ versus basal; two-tailed t-test, unpaired). In this mutant rGB2 subunit residues corresponding residues from rGB1 had been introduced. Heterodimeric

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receptors containing another rGB2 mutant subunit with three amino acid substitutions at the extracellular face of TMVII (N718D, V719L, Q720T) were functionally activated by GABA, but the response was not significantly enhanced in the presence of GS39783 ($p = 0.21$). In this rGB2 subunit three amino acids were mutated to corresponding residues in dGB2. A few mutant subunits did not yield to considerable functional activation by GABA in the GTPγ^{35}S binding assay, although expression of GB2 subunit was confirmed by western blots (not shown). At all other functional mutants GS39783 did not stimulate significantly when applied alone but enhanced the GABA signal. Although absolute stimulation levels varied, probably dependent on expression levels, we concluded that most likely GS39783 function is not affected in these mutants.

The two rGB2 subunits with mutations in TMVI and VII described above (underlined in Fig. 3b) were investigated further as they identify candidate amino acids potentially being involved in GS39783 function. CRCs in the GTPγ^{35}S binding assay for the mutant G706T, A708P, S710T in TMVI after co-expression with wild-type rGB1 are shown in Fig. 4a, b. GABA application in the absence and presence of modulator revealed that GS39783 significantly increased the potency and maximal efficacy of GABA (Fig. 4a, Table 1) but also stimulated GTPγ^{35}S binding when applied alone. By contrast, GS39783 did not stimulate GTPγ^{35}S binding at wild-type receptors when applied alone but significantly increased GABA potency and efficacy as expected (Fig. 4e). CRCs for GS39783 confirmed stimulation of GTPγ^{35}S binding in the absence or presence of 10 μM GABA (Fig. 4b), stimulation which was not observed at wild-type receptors (Fig. 4f). The basal counts in GTPγ^{35}S binding assays were not significantly different from wild-type receptors (not shown) suggesting that the mutations introduced did not markedly affect constitutive receptor activity. Noteworthy, the TM VI mutations introduced led to a somewhat increased GABA potency compared to wild-type controls whereas as the potency of GS39783 in modulating the GABA response was similar (Table 1). In summary these data suggested a switch to agonistic activity of GS39783 at $GABA_B$ heterodimers containing this mutated rGB2 subunit.

CRCs for the rGB2(N718D, V719L, Q720T) mutation in TMVII are shown in Fig. 4c, d. GS39783 positively modulates the GABA signal with similar potency as at wild-type receptors,

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however the maximal effect appeared reduced (Fig. 4e, f; Table 1). We concluded that the mutations introduced did not completely abolish positive modulation but impaired the efficacy of GS39783.

We have also generated a number of point mutations in dGB2 aimed to generate 'gain of function' mutants. Mutations were introduced into wild-type dGB2 and the mutants co-expressed with dGB1. Selected mutations (e.g the mutations in TMVII described above) were also constructed into a GB2 subunit chimera (ECD from rat, TM from drosophila; Fig. 2), and co-expressed with rGB1. Susceptibility to GS39783 however was not obtained (not shown).

The mutant subunit rGB2(G706T, A708P, S710T) is activated by GS39783 in the absence of GB1. We further investigated the rGB2(G706T, A708P, S710T) mutant in TMVI in GTPγ^{35}S binding assays without co-expression of rGB1 (Fig. 5). To our surprise GS39783 concentration-dependently activated this mutant subunit when expressed alone whereas wild-type rGB2 subunits were not activated (Fig. 5a). The EC_{50} values for the agonistic effect of GS39783 was 1.0 ± 0.2 μM (n = 3), thus in a similar range as its EC_{50} for positive modulatory activity at rGB1/rGB2 heterodimers (0.3 μM, Table 1). To ensure that no endogenous GB1 subunits were present in the membrane preparation used we also measured GABA responses (Fig. 5b). GABA-induced GTPγ^{35}S binding was not observed using membranes from rGB2(G706T, A708P, S710T) and wild-type rGB2 transfected cells (Fig. 5b). This rules out the presence of GB1 in the membrane preparation used and supports previous observations that GABA does not bind to the GB2 subunit (Kniazeff et al., 2002). We concluded that the mutant rGB2 subunit G706T, A708P, S710T can be activated by GS39783 independently of the GB1 subunit.

Mutations of G706T (6.51) and A708P (6.53) in rGB2 are necessary to confer agonistic activity to GS39783. To identify which of the point mutations introduced into rGB2(G706T, A708P, S710T) are important to confer agonistic activity to GS39783 mutant subunits containing all possible permutations of the three amino acid exchanges were constructed and analyzed (Fig. 6). After co-expression with rGB1 GABA-stimulated GTPγ^{35}S binding as well as positive modulation by GS39783 was observed with all constructs confirming functionality of the mutant rGB2 proteins (Fig. 6a). When

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expressed without GB1 the rGB2(G706T, A708P, S710T) mutant was activated by GS39783 as expected. A combination of G706T (6.51) and A708P (6.53) mutations in rGB2 led to similar activation levels whereas GS38783 was inactive at all other combinations including single point mutations (Fig. 6b; Table 1). Of note, the activation of mutant rGB2 subunits by GS39783 in this assay is substantial. The stimulation levels of 10 μM GS39783 at mutant subunits were similar to the effect of 10 μM GABA in the presence of GS39783 at rGB1/ rGB2 heterodimers (Fig. 6b). In summary, these data showed that the mutations in rGB2 of G706T and A708P are necessary and sufficient to confer agonistic activity to GS39783 and render the rGB2 subunit active independently of GB1.

We also measured the activity of a structurally different positive modulator compound, CGP7930 (Urwyler et al., 2001) on the rGB2 mutants described above (Fig. 6c). CGP7930 significantly activated both rGB2(G706T, A708P, S710T) and rGB2(G706T, A708P) ($p < 0.01$; $n = 4$), however the agonistic efficacy was reduced to approximately 25 percent of the response obtained with GS39783.

The mutations introduced (Gly > Thr and Ala > Pro) are conservative exchanges. Non-conservative mutations at these positions (G706D and A708D) did not result in functional receptors upon co-expression with rGB1 (not shown). In rGB2(G706T, A708P, S710T) corresponding amino acids present in rGB1 had been introduced. Thus the observation that the agonistic activity is gained by these mutations is surprising and raises the question if there may be a binding site for GS39783 also on the GB1 subunit. We constructed the reverse mutations in rGB1 (T>G (6.51); P>A (6.53)) however, when co-expressed with rGB2, we did not observe significant differences compared to wild-type receptors (not shown).

DISCUSSION

Using interspecies combinations and chimeras of drosophila and rat $GABA_B$ receptor subunits we have shown that GS38783 interacts with the GB2 subunit and localized its binding site within the transmembrane region. Point mutations identified critical residues for GS39783 agonistic effects in

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TM VI. The mutations of selective amino acids switched positive modulation to agonism and led to GB2 subunits which where activated independently of the GB1 subunit.

The combination of drosophila GB1 (dGB1) and rat GB2 (rGB2) subunits yielded functional $GABA_B$ receptor heterodimers activated by GABA whereas the *vice versa* combination, rGB1 co-expressed with dGB2, was not functional. However, functionality was obtained when rGB1 was co-expressed with drosophila/rat GB2 subunit chimeras containing either the N-terminal or the transmembrane/C-terminal part from rat GB2 (Fig. 2). $GABA_B$ receptor heterodimer formation is mediated via interactions between C-terminal coiled-coil motifs of GB1 and GB2 subunits but also involves allosteric contact sites between the TM and extracellular sequences. In fact, the deletion of coiled-coil motifs in GB1 and GB2 does not prevent heterodimer formation emphasizing the importance of additional contacts between subunits (Pagano et al., 2001). The observation that functional receptors can be generated by the interspecies subunit combinations as above was unexpected as the sequence conservation between rat and drosophila subunits is very limited (51 and 44 percent identical residues for GB1 and GB2, respectively; bestfit alignment). It is likely that the contact sites between subunits are evolutionary highly conserved between species and further investigation of sequence conservation in drosophila and rat subunits could provide a strategy to identify residues critical for heterodimer formation.

We attempted to further delineate the binding site of GS39783 using drosophila/ rat GB2 chimeras with junctions within the TM region (Fig. 2b), however none of these chimeras yielded functional receptors after co-expression with GB1. The limited sequence conservation (52 percent identical residues in TM) may impair functional interactions between TM helices from the different species. Certainly it is not possible to generalize from our observations that functional GB2 subunits combining TMs from drosophila and rat can not be generated in principle. The precise junctions and composition may be critical and only a few chimeras have been generated so far.

A set of rGB2 point mutations were constructed, some of which affected GS389783 function. When co-expressed with rGB1, a mutant rGB2 subunit with three amino acids substitutions in transmembrane domain VI was considerably activated by GS39783 in the absence of GABA. Surprisingly, in contrast to wild-type rGB2, this mutant was also activated by GS39783 when

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expressed without GB1. The mutations G706T (6.51) and A708P (6.53) are necessary and sufficient for activation and identify a key region for the effect of GS39783 in transmembrane VI of the rGB2 subunit. Notably, homologous residues in metabotropic glutamate receptor mGluR1, in the calcium sensing receptor and in serotonin receptors (5HT-4) have previously been demonstrated to be involved in the effects of negative allosteric modulators and inverse agonists (Hu et al., 2006; Malherbe et al., 2003; Joubert et al., 2002). Furthermore, Surgand et al. (2006) predicted based on chemogenomic analysis of TM binding cavities of GPCRs that small-sized $GABA_B$ allosteric modulators might interact with TM VI residues 6.48 or 6.51. Our data support the validity of these predictions and emphasize the importance of TM VI for the effects of the positive modulator GS39783.

The molecular effects of the amino acid substitutions introduced on positive modulator binding and receptor activation however are not understood to date. An important question is whether the aforementioned residues are directly involved in GS39783 binding or whether the mutations have indirect effects, such as facilitation or alteration of GS39783 induced conformational changes of the TM helices. Since the key mutations introduced as above did not abolish GS39783 function a definite answer to this question is not yet possible. A caveat is that the low (micromolar) potency of currently available positive modulator compounds such as GS39783 does not allow the use of respective radioligand derivatives to investigate if binding affinities are affected. In the functional GTPγS binding assay used in this study non-conservative mutations at positions 6.48 and 6.51 in rGB2 (G706D; A708D) disrupted not only GS39783 but also GABA responses, therefore conclusions as to whether GS39783 binding was impaired are not possible. Of note, the rGB2(G706T, A708P) mutations induced agonistic activity of GS39783 but did not alter its potency in positively modulating GABA responses at heterodimeric receptors (Table 1). Therefore, the mutations identify critical residues important for agonism rather than positive modulation. In support of a key role of TM VI for allosteric modulation, modeling studies by Malherbe et al. (2003) suggest that conserved amino acids in TM VI of metabotropic glutamate receptors are key for the transition between allosteric states. In addition to G706T (6.51) and A708P (6.53) identified in this study it is likely that additional amino acids in other TMs are important for GS39783 function. These residues could be conserved between subunits and species and therefore could have escaped identification by the strategy used in this study.

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Our observation that the rGB2(N718D, V719L, Q720T) mutations affected efficacy but not potency of positive modulation by GS39783 may suggest importance of ligand interactions at the extracellular face of TMVII.

In the rGB2(G706T, A708P) mutant subunit amino acids were exchanged to the corresponding homologues present in rGB1 (Fig. 3). The observation that these mutations did not impair but rather induced responsiveness to GS39783 was therefore very surprising. It remains possible that GS39783 also binds to the rGB1 subunit. On the other hand, in the present study we did not obtain additional evidence for GS39783 interaction with GB1. The potency of GS39783 on rGB1/ rGB2 heterodimers was similar compared to its potency on mutant rGB2 subunits (Table 1). Because the GB1 subunit does not activate effector systems (Margeta-Mitrovic et al., 2001) binding assays with more potent positive modulator radioligands are required to further elucidate if there is a molecular interaction also with GB1.

In a recent study Binet et al. (2004) reported activation of wild-type GB2 subunits by a different positive modulator compound, CGP7930, which led to the conclusion that the modulator in fact is a partial GB2 agonist. In the present study GS39783 did not activate at all individually expressed GB2 subunits, and agonistic activity was strictly dependent on the mutations introduced as described above. Furthermore, even at co-expressed wild-type rGB1 and rGB2 subunits significant agonistic activity of GS39783 (ago-allosterism, see Schwartz and Holst; 2006) was not observed. It is likely that apparent partial agonistic activity of positive modulator compounds depends on the expression systems used and requires assays with considerable receptor reserve, such as the inositol phosphate production assay as used by Binet et al. (2004). Similar observations have been made for both CGP7930 and GS39783 in a cAMP assay, using a cell line stably expressing $GABA_B$ receptors (Urwyler et al., 2005). However, the lack of significant agonistic activity of GS39783 observed in the present study is in agreement with *in vitro* assays (Urwyler et al., 2003) and with *in vivo* experiments where orally applied GS39783 lacked effects on its own but, together with a threshold concentration of the agonist baclofen, significantly decreased cyclic AMP formation in the rat striatum in a dose-dependent fashion (Gjoni et al., 2006).

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The observation that two conservative mutations in GB2 conferred agonistic activity to a synthetic compound has several implications. In support of previous notion (Binet et al., 2004) our data suggest that GB2 subunits may function independently of GB1. The developmental regulation and localization of GB1 and GB2 subunits in the brain do not always match precisely (reviewed in Bettler et al., 2004). It remains to be investigated if GB2 fulfills receptor functions independently of GB1 *in vivo*. Critical residues for agonistic activation of GB2 subunits by GS39783 were identified in TM VI. This may indicate conservation of binding site cavities for allosteric enhancers between $GABA_B$ receptors and other family C GPCRs. Homology modeling and docking studies are therefore warranted. Further, it seems evident that significant activation of $GABA_B$ receptors may be achieved via agonism at the GB2 subunit. Therefore, the GB2 subunit may represent a useful site to develop novel $GABA_B$ receptor agonists.

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FOOTNOTES

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LEGENDS FOR FIGURES

Fig. 1. GS39783 positive modulates different vertebrate but not drosophila $GABA_B$ receptors. **a**, GTPγ^{35}S binding assays to investigate positive modulation by GS39783 in different species. Rat, chicken and fish (salmon) brain membranes and membranes from cloned drosophila $GABA_B$ receptors expressed in HEK293FT cells were used. The responses are normalized to the stimulation obtained with a saturating concentration of GABA (1 mM; dotted line). GS39783 enhances GABA induced GTPγ^{35}S binding at rat, chicken ($p < 0.001$) and fish membranes ($p < 0.05$) but not at drosophila $GABA_B$ receptors ($n = 3$). **b-d**, Concentration response curves (CRCs) for GS39783 using membranes from cells expressing rat (**b**) or drosophila (**c**) GB1 and GB2 receptor subunits, and from cells co-expressing drosophila GB1 (dGB1) and rat GB2 (rGB2) (**d**). GTPγ^{35}S binding assays were done using membrane preparations from receptor subunits expressed in HEK293FT cells. The dotted lines in **b-d** indicate basal levels and stimulation levels with 1 mM GABA and 10 μM GABA (corresponding to a maximal and submaximal effect of GABA when applied alone, respectively). Note that the origins of y axes in **b-d** were set close to the basal levels. Data from one experiment are shown which was repeated at least three times.

Fig. 2. The transmembrane (TM) region of rat GB2 is critical for positive modulation by GS39783. **a**, GB2 subunit chimeras were generated by PCR and transiently co-expressed with either rat or drosophila GB1 in HEK293FT cells. Membranes were harvested two days after transfection and investigated in GTPγ^{35}S binding assays. The sequence composition in each chimera is indicated (black: rat, grey: drosophila origin); GB1 (1) and GB2 (2) subunits are indicated. **b**, GB2 subunit chimeras with junctions within the TM region did not lead to functional receptors when co-expressed with rat GB1. Activation by GABA was observed only after addition of all TM helices from rat. Data from one experiment are shown which was repeated at least three times. The expression of mutant proteins was confirmed by western blots (not shown).

Fig. 3. Point mutations in the TM region of rGB2. **a**, Sequence alignment of rat and drosophila GB1 and GB2 subunits with metabotropic glutamate receptors mGluR1 and mGluR5 and the Ca-sensing

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receptor (CaSR). Amino acids in rGB2 investigated in this study are marked by asterisks. The arrows mark the mutations G706T, A708P, S710T (simultaneous mutations of these residues in rGB2 leads to activation by GS39783 when applied alone) and N718D, V719L, Q720T (blunted effect of GS39783). **b**, Characterization of different rGB2 point mutations in the GTPγ^{35}S binding assay. Mutants were constructed by PCR and transiently co-expressed with rGB1 in HEK293FT cells together with the G protein Gαo_A. GTPγ^{35}S binding was measured after stimulation with 10 μM GABA, with 10 μM GABA in the presence of 10 μM GS39783, and with 10 μM GS39783 applied alone. The mutants G706T, A708P, S710T (activated by GS39783 when applied alone) and N718D, V719L, Q720T (blunted effect of GS39783) are underlined. For the mutants GB2(S530T, A532) and GB2(F535I, L536F, F537L) no significant GABA signal was obtained although expression of the mutant protein was confirmed in western blots (not shown). Alanine mutations of these residues were generated. GB2(S530A) yields to functional receptors positively modulated by GS39783 whereas GB2(F535A, L536A, F537A) appears non-functional. The data are from single experiments (triplicate determinations ± sem) which were repeated three times; WT (wild-type: co-expressed rGB1/rGB2).

Fig. 4. Characterization in the GTPγ^{35}S binding assay of heterodimeric receptors containing the rGB2(G706T, A708P, S710T) and GB2(N718D, V719L, Q720T) mutant subunits in comparison to wild-type receptors. Wild-type or mutant rGB2 subunits were co-expressed with rGB1. **a, c, e,** Concentration response curves (CRCs) for GABA without and in the presence of 10 μM GS39783. The dotted line in (**a**) denotes the level of stimulation with 10 μM GS39783 applied alone. **b**, **d, f,** CRCs for GS39783 without or in the presence of 10 μM GABA. The dotted lines denote the level of stimulation with 10 μM GABA applied alone. Agonistic activity of GS39783 in addition to positive modulation is observed for heterodimeric receptors containing the mutant rGB2(G706T, A708P, S710T) subunit. The data shown are from single experiments (triplicate determinations ± sem) which were repeated at least three times. The EC_{50} values calculated from at least three independent experiments are given in Table 1.

Fig. 5. GS39783 activates the rGB2(G706T, A708P, S710T) subunit when expressed without rGB1. CRCs in the GTPγ^{35}S binding for the rGB2(G706T, A708P, S710T) mutant subunits in comparison to wild-type receptors. **a**, GS39783 concentration-dependently induces GTPγ^{35}S binding at mutant but not at wild-type rGB2. **b,** GABA is inactive at both mutant and wild-type rGB2. rGB2 subunits were expressed in HEK293FT cells without GB1. The data shown are from one experiment which was repeated at least three times. The EC_{50} values are listed in Table 1.

Fig. 6. Mutations of G706T and A708P are necessary and sufficient to confer agonistic efficacy of GS39783 on rGB2 subunits expressed in the absence of GB1. All permutations of the three amino exchanges in the rGB2(G706T, A708P, S710T) mutant were generated and analyzed in GTPγ^{35}S binding assays. **a**, Co-expression with rGB1 demonstrates functionality of mutant subunits. **b**, Simultaneous mutations of G706T and A708P in rGB2 are necessary and sufficient to confer agonistic efficacy to GS39783. Western blots with the rGB2 specific antibody AbC22 (Kaupmann et al., 1998) confirm expression of mutant subunits. The rGB2 immunoreactive band (110 kda) is indicated. **c.** Activity of the $GABA_B$ receptor positive modulator CGP7930 (Urwyler et al., 2001) on the rGB2 mutants G706T, A708P, S710T and G706T, A708P. Cells were transfected with GB2 plasmids as indicated and the membranes assayed in parallel for the effect of CG7930 and GS39783. CGP7930 activates the GB2 mutant subunits ($p < 0.01$ versus basal, $n = 4$), however the efficacy is reduced compared to GS39783. The same plasmid preparations were used in a, b, c.

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TABLES

Table 1. Potencies of GABA and GS39783 at key mutant GB2 subunits in the GTPγ^{35}S binding assay.

	GABA EC_{50} (μM)	GABA, 10 μM GS39783 EC_{50} (μM)	GS39783 EC_{50} (μM)	GS39783, 10 μM GABA EC_{50} (μM)	Maximal effect (%)
rGB2(G706T,A708P,S710T) + rGB1	2.07 ± 0.39	0.11 ± 0.03	0.89 ± 0.22	0.27 ± 0.13	165 ± 18
rGB2(N718D,V719L,Q720T) + rGB1	5.03 ± 0.64	0.37 ± 0.10	n.d.	0.40 ± 0.04	120 ± 9
rGB2 + rGB1	12.06 ± 3.16	0.87 ± 0.27	no effect	0.33 ± 0.03	253 ± 29
rGB2(G706T,A708P,S710T)	no effect	n.d	0.88 ± 0.28	0.96 ± 0.24	n.a.
rGB2(G706T,A708P)	no effect	n.d	0.51 ± 0.16	0.83 ± 0.10	n.a.
rGB2	no effect	n.d	no effect	no effect	n.a.

Concentration response curves for GABA and GS39783, in the absence or presence of 10 μM GS39783 and 10 μM GABA, respectively, were measured using membranes form transiently transfected HEK293FT cells. Data are shown for wild-type and key mutant rat GB2 (rGB2) subunits after co-expression with rat GB1 (rGB1), and for individually expressed rGB2 mutants where agonistic activity of GS39793 was observed. The maximal effects were determined in GABA concentration response curves in the presence 10 μM GS39783, and are expressed as percentage of the maximal response obtained with GABA alone. The data are means ± sem from 3 to 7 independent experiments (triplicate determinations); n.a.; not applicable; n.d., not determined.

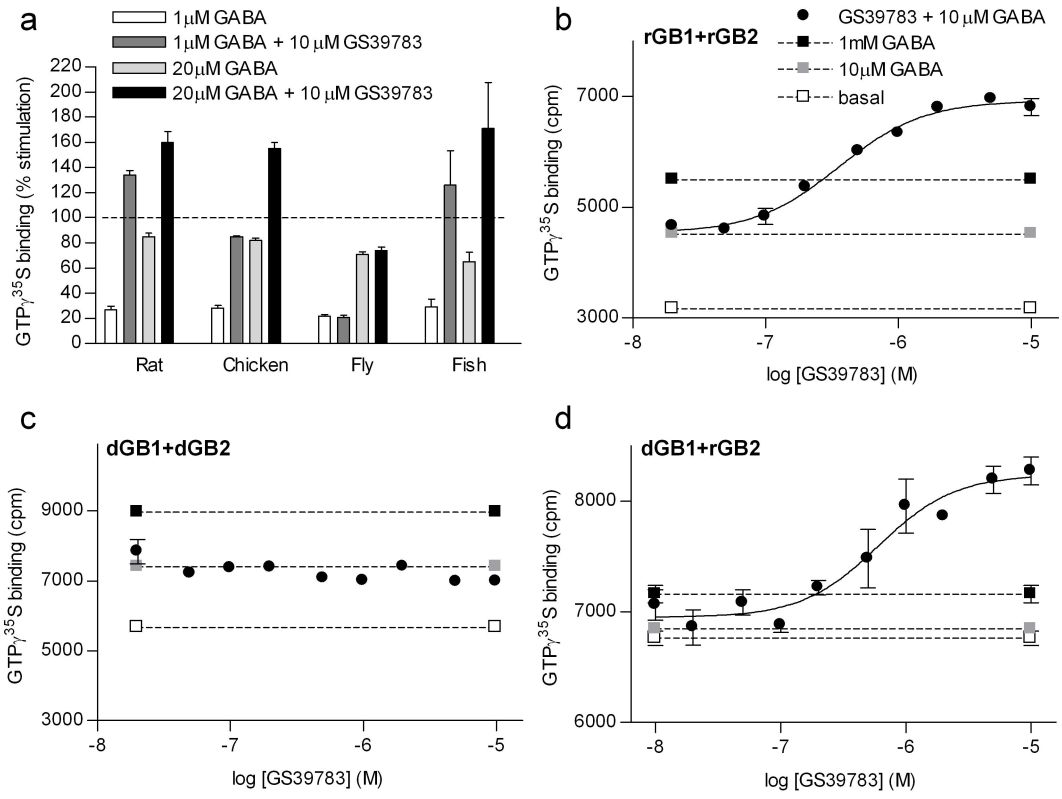
a
1μM GABA
1μM GABA + 10μM GS39783
20μM GABA
20μM GABA + 10μM GS39783
GTPγ35S binding (% stimulation)
Rat
Chicken
Fly
Fish
b
rGB1+rGB2
GS39783 + 10μM GABA
1mM GABA
10μM GABA
basal
GTPγ35S binding (cpm)
log [GS39783] (M)
c
dGB1+dGB2
GTPγ35S binding (cpm)
log [GS39783] (M)
d
dGB1+rGB2
GTPγ35S binding (cpm)
log [GS39783] (M)

Fig. 1

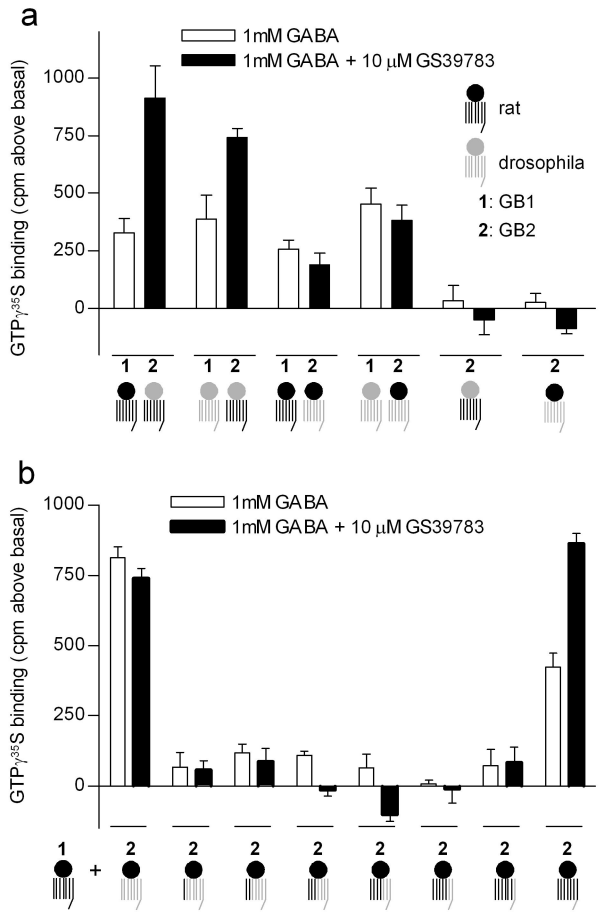
a
1mM GABA
1mM GABA + 10 μM GS39783
rat
drosophila
1: GB1
2: GB2
GTPγ35S binding (cpm above basal)
1000
750
500
250
0
1 2
1 2
1 2
1 2
2
2
b
1mM GABA
1mM GABA + 10 μM GS39783
GTPγ35S binding (cpm above basal)
1000
750
500
250
0
1
+
2
2
2
2
2
2
2
2

Fig. 2

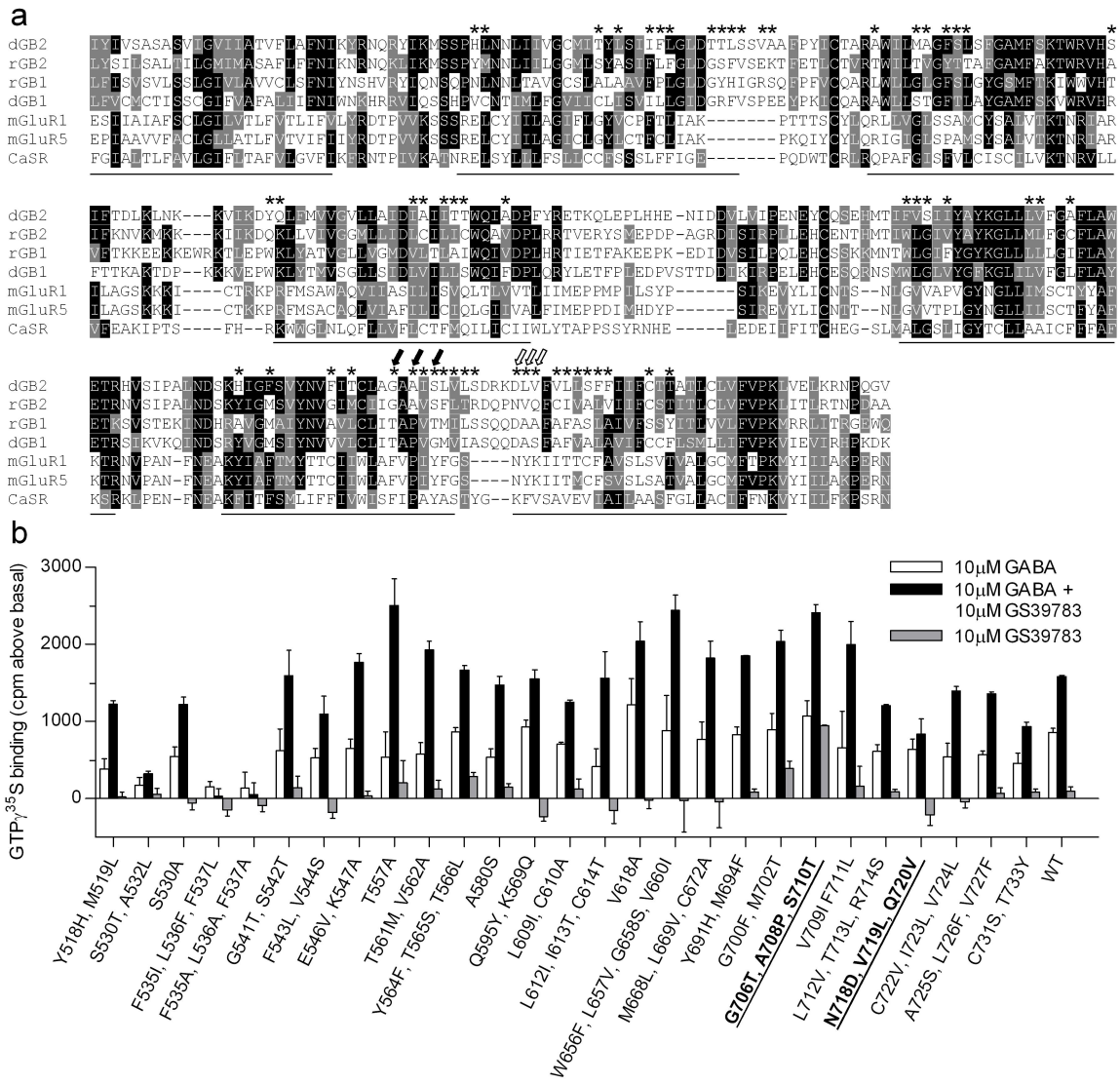
a
dGB2
rGB2
rGB1
dGB1
mGluR1
mGluR5
CaSR
b
GTPγ35S binding (cpm above basal)
3000
2000
1000
0
10μM GABA
10μM GABA + 10μM GS39783
10μM GS39783
Y518H, M519L
S530T, A532L
S530A
F535I, L536F, F537L
F535A, L536A, F537A
G541T, S542T
F543L, V544S
E546V, K547A
T557A
T561M, V562A
Y564F, T565S, T566L
A580S
Q595Y, K569Q
L609I, C610A
L612I, I613T, C614T
V618A
W656F, L657V, G658S, V660I
M668L, L669V, C672A
Y691H, M694F
G700F, M702T
G706T, A708P, S710T
V709I F711L
L712V, T713L, R714S
N718D, V719L, Q720V
C722V, I723L, V724L
A725S, L726F, V727F
C731S, T733Y
WT

Fig. 3

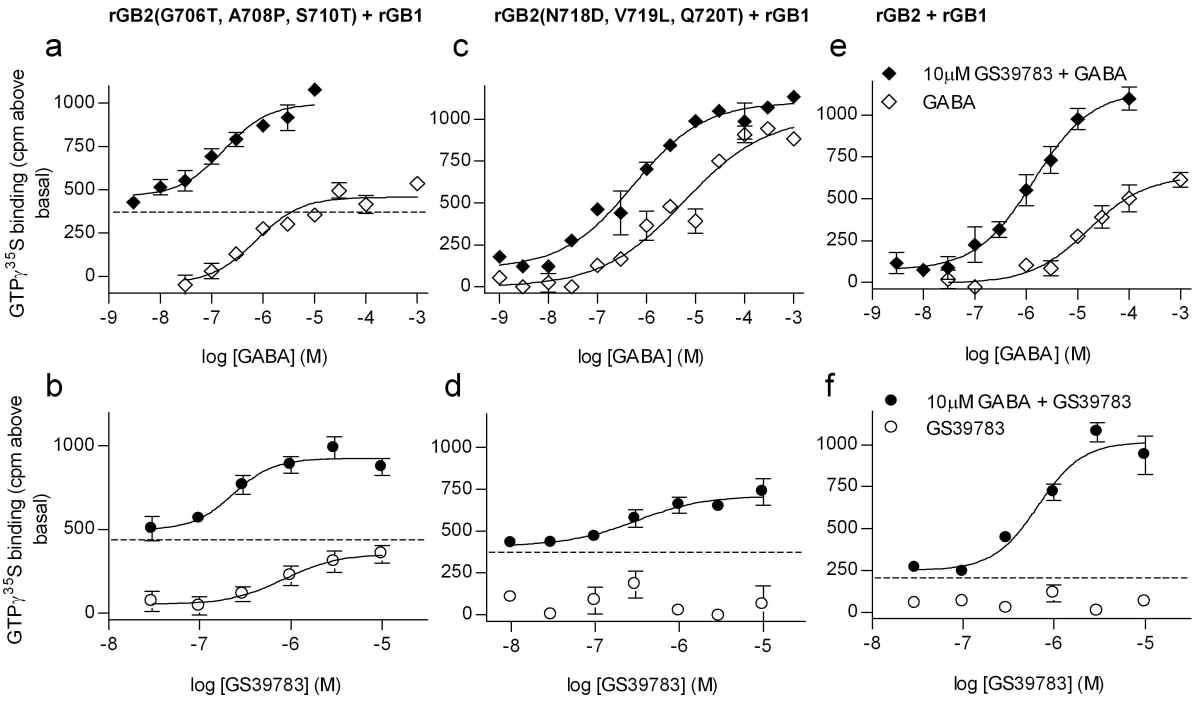
rGB2(G706T, A708P, S710T) + rGB1
rGB2(N718D, V719L, Q720T) + rGB1
rGB2 + rGB1
a
c
e
b
d
f
GTP-γ-35S binding (cpm above basal)
log [GABA] (M)
log [GS39783] (M)
10μM GS39783 + GABA
GABA
10μM GABA + GS39783
GS39783

Fig. 4

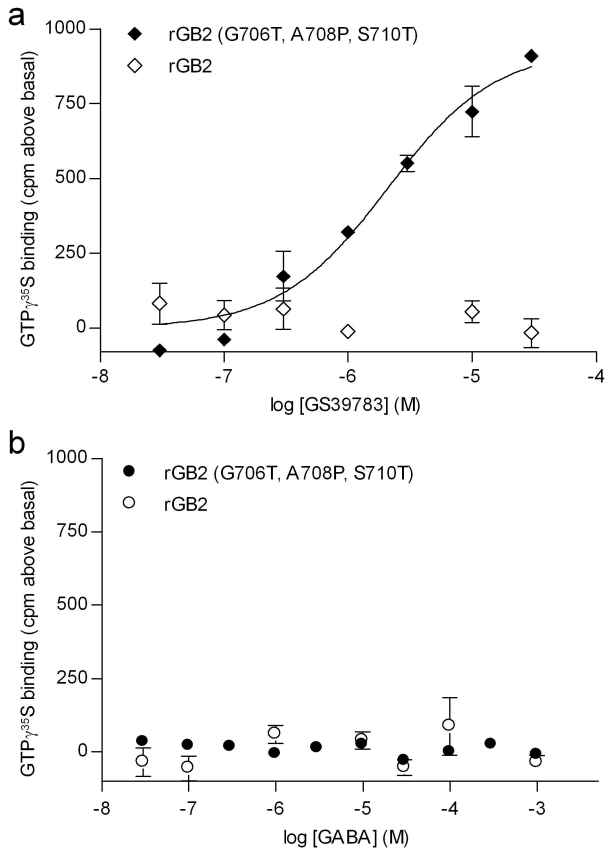
a
rGB2 (G706T, A708P, S710T)
rGB2
GTPγ35S binding (cpm above basal)
1000
750
500
250
0
-8
-7
-6
-5
-4
log [GS39783] (M)
b
rGB2 (G706T, A708P, S710T)
rGB2
GTPγ35S binding (cpm above basal)
1000
750
500
250
0
-8
-7
-6
-5
-4
-3
log [GABA] (M)

Fig. 5

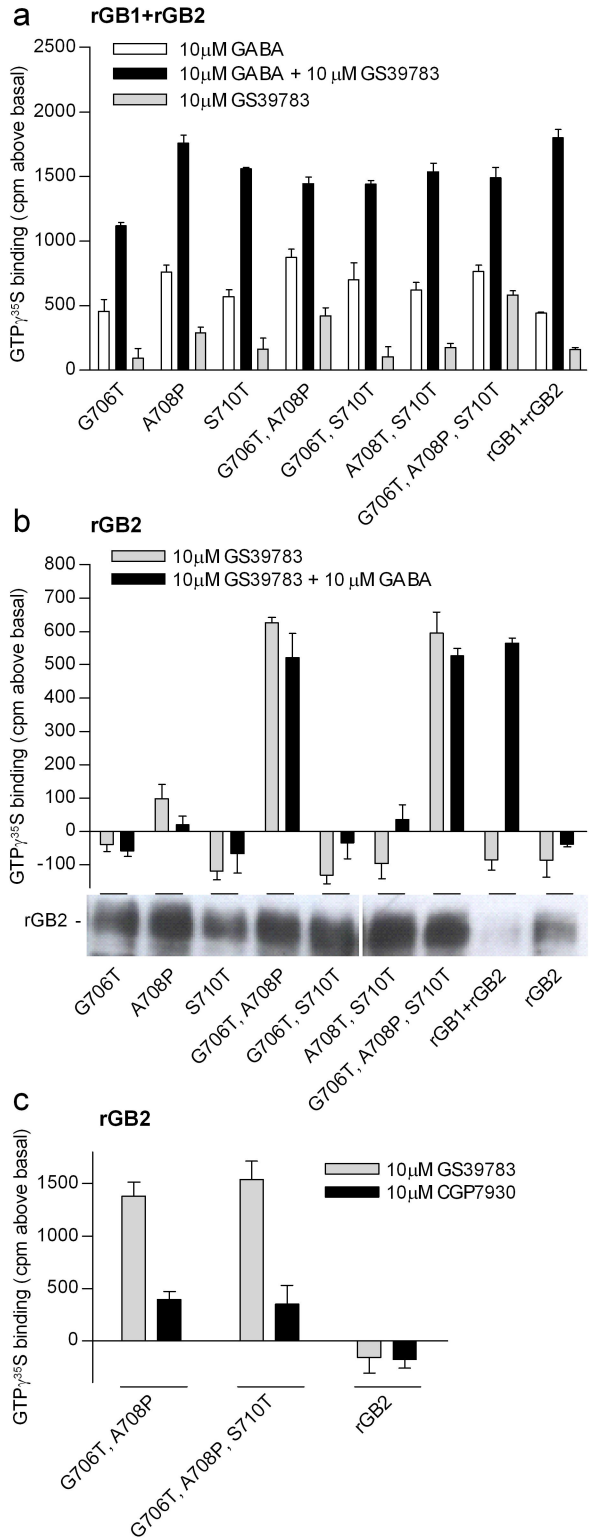
a
rGB1+rGB2
10μM GABA
10μM GABA + 10 μM GS39783
10μM GS39783
GTPγ35S binding (cpm above basal)
2500
2000
1500
1000
500
0
G706T
A708P
S710T
G706T, A708P
G706T, S710T
A708T, S710T
G706T, A708P, S710T
rGB1+rGB2
b
rGB2
10μM GS39783
10μM GS39783 + 10 μM GABA
GTPγ35S binding (cpm above basal)
800
700
600
500
400
300
200
100
0
-100
rGB2 -
G706T
A708P
S710T
G706T, A708P
G706T, S710T
A708T, S710T
G706T, A708P, S710T
rGB1+rGB2
rGB2
c
rGB2
10μM GS39783
10μM CGP7930
GTPγ35S binding (cpm above basal)
1500
1000
500
0
G706T, A708P
G706T, A708P, S710T
rGB2

Fig. 6