Activation of constitutive 5-hydroxytryptamine<sub>1B</sub> receptor by a series of mutations in the BBXXB motif: positioning of the third intracellular loop distal junction and its G<sub>o,α</sub> protein interactions

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Constitutive activity of the recombinant human 5-hydroxytryptamine<sub>1B</sub> (5-HT<sub>1B</sub>) receptor (RC code 2.1.5HT.01.B) was investigated by mutagenesis of the BBXXB motif (in which B represents a basic residue and X a non-basic residue) located in the C-terminal portion of the third intracellular loop. In contrast with wild-type 5-HT<sub>1B</sub> receptors, three receptor mutants (Thr<sup>313</sup> → Lys, Thr<sup>313</sup> → Arg and Thr<sup>313</sup> → Gln) increased their agonist-independent guanosine 5′-[γ-<sup>35</sup>]<sup>S</sup>CTPGTP binding response by 26–41%. This activity represented approx. 30% of the maximal response induced by 5-HT and could be reversed by the inverse agonists methiothepin and 3-(3-dimethylaminopropyl)-4-hydroxy-N-(4-pyrindin-4-yl phenyl)-benzenamide (GR 55562). Enhanced agonist-independent and agonist-dependent 5-HT<sub>1B</sub> receptor activation was provided by co-expression of a pertussis toxin-resistant rat G<sub>αi</sub> Cys<sup>303</sup> → Ile protein. The wild-type 5-HT<sub>1B</sub> receptor displayed a doubling in basal activity, whereas a spectrum of enhanced basal activities (313–571%) was observed with a series of diverse amino acid substitutions (isoleucine, glycine, asparagine, alanine, lysine, phenylalanine, glutamine and arginine) at the 5-HT<sub>1B</sub> receptor position 313 in the presence of pertussis toxin (100 ng/ml). Consequently, the constitutive 5-HT<sub>1B</sub> receptor activity can be modulated by the mutation of Thr<sup>313</sup>, and displays a graded range between 11% and 59% of maximal 5-HT<sub>1B</sub> receptor activation by 5-HT. No clear pattern is apparent in the framework of traditionally cited amino acid characteristics (i.e. residue size, charge or hydrophobicity) to explain the observed constitutive activities. The various amino acid substitutions that yielded enhanced activity are unlikely to make similar intramolecular interactions within the 5-HT<sub>1B</sub> receptor. It is hypothesized that the positioning of the junction between the third intracellular loop and transmembrane domain VI is altered by mutation of Thr<sup>313</sup> in the BBXXB motif and thereby unmask G<sub>αi</sub>-protein interaction points.

Key words: guanine nucleotide-binding protein-coupled receptors, inverse agonist, mutagenesis.

INTRODUCTION

Several lines of research have revealed that a number of guanine nucleotide-binding protein-coupled receptors (GPCR) demonstrate constitutive activity in the absence of agonist in their native state, by reconstitution in phospholipid vesicles, in recombinant heterologous receptor-expression systems and in transgenic mice [1–6]. This led to the concept that GPCR change their conformation spontaneously or by mutation and oscillate between active and inactive receptor conformations [7]. Receptors exist in an equilibrium between both conformations: an inactive conformation that is structurally constrained and unable to bind a G<sub>α</sub>-protein subunit, and an active conformation that can interact productively with a GTP-bound G<sub>α</sub> subunit. Certain ligands, termed inverse agonists or negative antagonists, seem capable of driving activated GPCR to the inactive conformation [8]. Negative antagonism is demonstrated when a ligand binds to a receptor that exhibits some basal activity, which is then decreased. If ligand efficacy represents the differential affinity of a ligand for the active and/or the inactive conformation of the receptor, then a zero efficacy, which would be required for a pure neutral antagonist, might be uncommon, because this would require the same ligand to exhibit a similar affinity for both receptor states [9]. Numerous drugs previously classified as neutral antagonists might actually act as inverse agonists [9]; such a reclassification might have pharmacological and therapeutical implications [6].

A widely accepted model used to describe the activation of GPCR by agonists is the ternary complex model, which accounts for the co-operative interactions between receptor, G-protein and agonist [10]. This model has recently been extended to accommodate the observation that many receptors can activate G-proteins in the absence of agonist and that mutations in different structural domains of the GPCR can enhance the agonist-independent activity [8,11]. The extended ternary complex model also accounts for the effects of different classes of drug (full agonists, partial agonists, neutral antagonists and inverse agonists) on receptor signalling [12].

The C-terminal portion of the third intracellular loop has been suggested to be involved in constraining the GPCR in the inactive (G-protein-uncoupled) conformation [13]. Mutagenesis studies of the BBXXB motif (in which B represents a basic residue and X a non-basic residue) in the third intracellular loop of κ-, α-, δ- and μ-opioid receptors demonstrated constitutively active mutants [13–16]. Several anti-psychotic drugs displayed inverse

Abbreviations used: CHO, Chinese hamster ovary; GPCR, guanine-nucleotide-binding protein-coupled receptors; GR 55562, 3-(3-dimethylaminopropyl)-4-hydroxy-N-(4-pyrindin-4-yl phenyl)-benzenamide; GR 125743, N-[4-methoxy-3-(4-methyl-piperazin-1-yl)-3-methyl-4-pyridin-4-yl-benzamid; GR 127935, 2′-methyl-4′-(5-methyl-1,2,4-oxadiazol-3-yl)biphenyl-4-carboxylic acid [4-methoxy-3-(4-methylpiperrazin-1-yl)phenyl]amide; [γ<sup>35</sup>S]GTP[S], guanosine 5′-[γ-<sup>35</sup>S]triphosphate; 5-HT, 5-hydroxytryptamine (serotonin); PTX, Bordetella pertussis toxin; SB 224289, 1′-methyl-5′-[2′-methyl-4′-(5-methyl-1,2,4-oxadiazol-3-yl)biphenyl-4-carbonyl]-2,3,6,7-tetrahydrosphingosine[furo[2,3-f]indole-3,4-piperidine]; TM, transmembrane domain.

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agonist activity by inhibiting the constitutive activation of phosphatidylinositol hydrolysis by mutant Cys<sup>222</sup>→Lys 5-HT<sub>1A</sub> receptor [15]. Similarly, mianserin and mesulergine displayed inverse agonist activity by decreasing basal levels of phosphatidylinositol hydrolysis stimulated by mutant Ser<sup>312</sup>→Lys 5-HT<sub>1B</sub> receptors [16] in accordance with the observations for native 5-HT<sub>2</sub> receptors expressed in NIH 3T3 cells [17] or f<sub>5</sub> cells [18]. Otherwise, constitutively active mutant (Thr<sup>273</sup>→Lys) 5-HT<sub>2</sub>-adrenergic receptors provide apparently opposite results (i.e. positive efficacy) for ligands that have so far been characterized as antagonists and receptor interactions, such that some antagonists are capable of stabilizing an active conformation at the new binding interface generated by the mutation.

The present paper reports on constitutively active mutant 5-HT<sub>1B</sub> receptors. One particular amino acid (Thr<sup>392</sup>) was exchanged in the BBX<sub>B</sub> motif (Arg<sup>504</sup>Lys-Ala-Thr-Lys) by each of the natural amino acids. Because the agonist-independent guanosine 5'-[γ<sup>32</sup>P]triphosphate ([<sup>32</sup>P]GTP[S]) binding responses were clearly measurable only with a few receptor mutants, analysis was also performed by co-expression with a G<sub>A</sub> Cys<sup>501</sup>→Ile protein resistant to Bordetella pertussis toxin (PTX) [23]. The local cellular G-protein concentration has been shown to regulate the constitutive receptor activity of 5-HT<sub>1A</sub> and muscarinic receptors [24,25]. The 5-HT<sub>1B</sub> receptor mutants were analysed in terms of their structure–activity relationship by measuring 5-HT<sub>1B</sub> receptor-mediated agonist-independent and antagonist experiments, 1 μM methiothepin, unless indicated otherwise. pEC<sub>50</sub> values were defined as the ligand concentration that showed 50% of its own maximal stimulation of [GTP]<sup>S</sup> binding. Maximal stimulation of [GTP]<sup>S</sup> binding was obtained. pIC<sub>50</sub> values represent the ligand concentration at which 50% of its own maximal stimulation of [GTP]<sup>S</sup> binding was obtained. pEC<sub>50</sub> values represent the concentration at which 50% of the agonist activity was inhibited. pEC<sub>50</sub> values were calculated as K<sub>i</sub> = B/(A′/A − 1), where B is the concentration of the antagonist and A and A′ are the EC<sub>50</sub> values of 5-HT.
measured in the absence or the presence of antagonist respectively. Statistical analysis was performed with Student’s t test.

**Immunological G$_{\alpha}$ protein detection**

Membrane preparations of CHO-K1 cells containing either the wild-type 5-HT$_{1B}$ or 5-HT$_{1B}$ receptor mutants in the presence of the G$_{\alpha}$ Cys$^{331}$ → Ile protein were prepared as described above. Total proteins were separated by SDS/PAGE [12.5 % (w/v) gel], as described [30]. Thereafter the proteins were blotted to a nylon membrane by semi-dry electrotransfer (23 V, 45 min) in 192 mM glycine/20 % (v/v) methanol/25 mM Tris/HCl buffer (pH 8.3). Proteins were probed with a polyclonal antibody raised against a peptide corresponding to residues 18–33 of the rat G$_{\alpha}$ protein. The incubation was performed in PBS containing 0.1 % (w/v) Tween 20, 5 % (w/v) dried non-fat milk and the antibody at a dilution of 1:1000. Proteins were detected with an anti-rabbit IgG antibody coupled to alkaline phosphatase with the use of a colorimetric reaction [0.12 mM Nitro Blue Tetrazolium chloride monohydrate/0.12 mM 5-bromo-4-chloroindol-3-yl phosphate p-toluidine salt/5 mM MgCl$_2$ in 100 mM diethanolamine (pH 9.6)].

**Protein content**

Membrane protein levels were estimated by using a Bio-Rad kit; BSA was used as a standard [31].

**Materials**

The ABI Prism 310 Genetic Analyser and the Big Dye terminator cycle sequencing kit were from Perkin–Elmer (Foster City, CA, U.S.A.). The pCR3.1 expression vector was from Invitrogen (San Diego, CA, U.S.A.). CHO-K1 cells were obtained from A.T.C.C. (Manassas, VA, U.S.A.). The G$_{\alpha}$ and the anti-rabbit IgG antibodies were from Calbiochem (La Jolla, CA, U.S.A.). 5-HT and the substrates for the colorimetric assay by using a Bio-Rad kit; BSA was used as a standard [31].

**RESULTS**

**[$^{35}$S]GTP[S] binding responses by 5-HT$_{1B}$ receptors**

In contrast with plasmid-transfected CHO-K1 cells, 5-HT$_{1B}$ receptors displayed, on transient expression, a 5-HT-dependent [$^{35}$S]GTP[S] binding response (Figure 1). The basal [$^{35}$S]GTP[S] binding response was enhanced 2.5-fold with 5-HT (10 $\mu$M), whereas it was virtually unaffected by the inverse agonist methiothepin (1 $\mu$M). Mutation of Thr$^{313}$ in the 5-HT$_{1B}$ receptor to the basic residue arginine displayed a significantly ($P < 0.05$) enhanced basal response (44±9 %) and a similar (P > 0.05) maximal 5-HT response (Figure 1). The enhanced basal [$^{35}$S]GTP[S] binding response was fully blocked by methiothepin (1 $\mu$M). To analyse further whether the 5-HT$_{1B}$ receptor mutant Thr$^{313}$ → Arg was the most effective mutation to enhance constitutive 5-HT$_{1B}$ receptor activity, a series of 18 other amino acid mutations at the Thr$^{313}$ position in the 5-HT$_{1B}$ receptor was constructed and assayed for 5-HT$_{1B}$ receptor expression and for agonist-independent and agonist-dependent [$^{35}$S]GTP[S] binding responses. Apart from the 5-HT$_{1B}$ receptor mutant Thr$^{313}$ → Arg, none of the investigated mutants displayed a significantly enhanced basal [$^{35}$S]GTP[S] binding response. The amount of inhibition of basal [$^{35}$S]GTP[S] binding by methiothepin (1 $\mu$M) was 20–28 %, for the mutants with lysine, arginine and glutamine. Similar results were obtained with GR 55562 (1 $\mu$M, results not shown). The enhanced constitutive receptor activity was not related to an increased 5-HT$_{1B}$ receptor expression. Specific [$^{35}$S]GTP[S] binding was, apart from the 5-HT$_{1B}$ receptor mutant Thr$^{313}$ → His, either similar to or at least half that of the wild-type 5-HT$_{1B}$ receptor (Figure 2B). The 5-HT$_{1B}$ receptor mutant Thr$^{313}$ → His expressed a small amount of specific [$^{35}$S]GTP[S] binding response (Figure 2C). The 5-HT-mediated [$^{35}$S]GTP[S] binding response was for most of the 5-HT$_{1B}$ receptor mutants either unchanged from that of the wild-type 5-HT$_{1B}$ receptor or attenuated by 48–59 % ($P < 0.05$) for the mutants with either a proline, glutamate or aspartate residue (Figure 2C). Sumatriptan also behaved as an efficacious agonist, with $E_{max}$ values close to those of 5-HT (results not shown). In contrast, GR 125743 (1 $\mu$M) clearly demonstrated properties of a partial agonist (Figure 2D); its maximal effect for the 5-HT$_{1B}$ receptor mutants with an arginine or glutamine residue attained the response mediated by 5-HT at the wild-type 5-HT$_{1B}$ receptor.

**[$^{35}$S]GTP[S] binding responses by 5-HT$_{1B}$ receptors on co-expression of the rat G$_{\alpha}$ protein**

Co-expression of the 5-HT$_{1B}$ receptor with a rat G$_{\alpha}$ protein was investigated to enhance the resolution of constitutive 5-HT$_{1B}$

**Figure 1** [$^{35}$S]GTP[S] binding responses of wild-type (wt) 5-HT$_{1B}$ receptor and 5-HT$_{1B}$ receptor mutant Thr$^{313}$ → Arg in CHO-K1 cells

[$^{35}$S]GTP[S] ([$^{35}$S]GTPyS) binding responses were performed with 0.5 nM [$^{35}$S]GTP[S] either in the absence (Basal), or the presence of 1 $\mu$M methiothepin (Methio.) or 10 $\mu$M 5-HT as described in the Materials and methods section. Results are means ± S.E.M. for six or seven independent transfection experiments, each performed in duplicate. The basal condition compared with methiothepin treatment was significantly (P < 0.05) different for the Thr$^{313}$ → Arg mutant.
Figure 2. [35S]GTP[S] binding responses and expression levels of wild-type (wt) 5-HT1B receptor and 5-HT1B receptor mutants Thr313→Xaa in CHO-K1 cells.

(A) [35S]GTP[S] ([35S]GTPγS) binding responses were obtained as the difference between the binding of [35S]GTP[S] (0.5 nM) performed in either the absence or the presence of 1 μM methiothepin as described in the Materials and methods section. Results are expressed as the net increase of basal [35S]GTP[S] binding compared with the respective methiothepin-mediated [35S]GTP[S] binding response for each mutant receptor. (B) The 5-HT1B receptor density was estimated by measuring the specific binding of [3H]GR 125743. This was obtained as the difference between total and non-specific (in the presence of 10 μM 5-HT) radioligand binding. (C) Maximal [35S]GTP[S] binding responses were obtained as the difference between the responses mediated by 5-HT (10 μM) and by methiothepin (1 μM), and are expressed as percentages of that obtained with the 5-HT1B receptor mutant Thr313→Arg (234 ± 35 fmol/mg of protein). (D) GR 125743-mediated stimulation of [35S]GTP[S] binding responses was obtained as the difference between the response mediated by GR 125743 (1 μM) and methiothepin (1 μM), and are expressed as percentages of the 5-HT-mediated response as mediated by the 5-HT1B receptor mutant Thr313→Arg. Results are means ± S.E.M. for five independent transfection experiments, each performed in duplicate.

Figure 3. Effect of co-expression of rat Gα Cys351→Ile protein and treatment with PTX on [35S]GTP[S] binding responses of wild-type 5-HT1B receptor and 5-HT1B receptor mutant Thr313→Arg in CHO-K1 cells.

[35S]GTP[S] ([35S]GTPγS) binding responses were determined with 0.5 nM [35S]GTP[S] either in the absence (Basal) or the presence of 1 μM methiothepin (Methio.) or 10 μM 5-HT as described in the Materials and methods section. (A) wild-type 5-HT1B; (B) wild-type 5-HT1B plus PTX (100 ng/ml); (C) wild-type 5-HT1B plus Gα Cys351→Ile; (D) wild-type 5-HT1B plus Gα Cys351→Ile plus PTX (100 ng/ml); (E) 5-HT1B receptor mutant Thr313→Arg plus Gα Cys351→Ile plus PTX (100 ng/ml). Results are means ± S.E.M. for three (C), five (D), or seven (A) independent transfection experiments, each performed in duplicate. The basal condition compared with methiothepin treatment was significantly (P < 0.05) different in (D) and (E).

Figure 4(A) shows dose-dependent Gα Cys351→Ile protein expression in CHO-K1 membranes at different plasmid concentrations for the 5-HT1B receptor mutant Thr313→Arg. Constitutive and 5-HT-dependent 5-HT1B receptor activity were similar between co-expression with 0.1 μg and with 3 μg of the Gα Cys351→Ile protein plasmid (results not shown). Subsequent experiments were therefore performed with 3 μg of either the wild-type 5-HT1B or the mutant 5-HT1B receptor plasmid and 3 μg of the Gα Cys351→Ile plasmid to ensure sufficient Gα protein ex-

receptor activation because the 5-HT1B receptor-to-G-protein stoichiometry might be unfavourable in the overexpression of 5-HT1B receptor protein in CHO-K1 cells. To ascertain the specific coupling of the 5-HT1B receptor to a co-expressed Gα protein rather than to endogenous Gα subunits proteins of CHO-K1 cells, a putative PTX-resistant Gα Cys351→Ile protein was assayed

[23]. The basal [35S]GTP[S] binding response was increased to 220 ± 45 fmol/mg of protein on co-expression of the wild-type 5-HT1B receptor with a Gα Cys351→Ile protein in the presence of PTX (100 ng/ml), and attenuated by methiothepin (−54 ± 6 %) to the basal [35S]GTP[S] binding level of the wild-type 5-HT1B receptor protein in the absence of Gα, Cys351→Ile protein (Figure 3). The wild-type 5-HT1B receptor displayed a PTX-sensitive 5-HT-mediated [35S]GTP[S] binding response in the absence of Gα, Cys351→Ile protein (Figure 3B), whereas the 2.4-fold 5-HT-mediated increase and methiothepin-mediated decrease in [35S]GTP[S] binding were fully resistant to PTX on co-expression of a Gα Cys351→Ile protein (Figure 3D). A comparison with the 5-HT1B receptor mutant Thr313→Arg plus the Gα Cys351→Ile protein (Figure 3E) displayed a similar PTX-resistant 5-HT-mediated stimulus, a 2-fold increase in basal [35S]GTP[S] binding response and its blockade by methiothepin. Figure 4(A) shows dose-dependent Gα Cys351→Ile protein expression in CHO-K1 membranes at different plasmid concentrations for the 5-HT1B receptor mutant Thr313→Arg. Constitutive and 5-HT-dependent 5-HT1B receptor activity were similar between co-expression with 0.1 μg and with 3 μg of the Gα Cys351→Ile protein plasmid (results not shown). Subsequent experiments were therefore performed with 3 μg of either the wild-type 5-HT1B or the mutant 5-HT1B receptor plasmid and 3 μg of the Gα Cys351→Ile plasmid to ensure sufficient Gα protein ex-
response mediated by 5-HT at the 5-HT receptor displayed a spectrum of activities between 26% and 57% of the wild-type 5-HT receptor. An attenuation of 26–57% concentration–response range of the 5-HT receptor mutants in comparison with the wild-type 5-HT receptor. This variation was not related to the observed rank order of 5-HT receptor-mediated constitutive activity, as shown in Figure 5(A). In contrast to the histidine receptor mutant, each of the amino acid substitutions at position 313 in the 5-HT receptor yielded, by co-expression of the G protein, an elevated basal [35S]GTP[S] binding response ranging from 69% to 571%. The 5-HT receptor mutants Thr → Arg displayed agonist-independent responses that were greater than that for the wild-type 5-HT receptor. Methiothepin (1 μM) reversed for each of the mutants the enhanced basal [35S]GTP[S] binding responses to the level of the wild-type 5-HT receptor in the absence of the G protein (T). The enhanced constitutive receptor activity was not a consequence of an increased 5-HT receptor expression as estimated by measuring the specific binding of [3H]GR 125743 (Figure 5B). Maximal [35S]GTP[S] binding responses as determined by 5-HT (10 μM) were similar for most of the 5-HT receptor mutants in comparison with the wild-type 5-HT receptor. An attenuation of 26–57% in the 5-HT-mediated response (P < 0.05 compared with wild-type 5-HT receptor) was observed with the receptor mutants containing a glutamate, a tryptophan or a cysteine residue. The partial agonist GR 125743 displayed a spectrum of activities between 26% and 95% of the response mediated by 5-HT at the 5-HT receptor mutant Thr → Arg (Figure 5D). Figure 6 compares a series of concentration–response curves of ligands that have been previously reported to display antagonist properties at 5-HT receptors. They yielded either stimulation or inhibition of [35S]GTP[S] binding by wild-type 5-HT receptors with the rat G protein. The corresponding E_max and pEC values are summarized in Table 1 and are compared with their pK_i values for the wild-type 5-HT receptor. Each

![Diagram](image)
of the ligands under investigation displayed half-maximal \(^{35}\)S[GTP]S binding responses at concentrations similar to their 5-HT agonist.[\(^{35}\)S]GTP[S] binding affinity for the wild-type 5-HT\(_{1B}\) receptor. 1-Naphthylpiperazine, GR 125743, and ritanserin inhibited at submicromolar and micromolar potencies slightly less than basal [\(^{35}\)S]GTP[S] binding. Ketanserin (10 \(\mu M\)) was virtually inactive towards the [\(^{35}\)S]GTP[S] binding response. SB 224289 (30 and 100 nM) antagonized the 5-HT-mediated concentration–response curve with a pK\(_{i}\) of 8.22, close to its affinity for the wild-type 5-HT\(_{1B}\) receptor (Figure 6C).

### Table 1

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### Discussion

The present paper reports on the modulation of agonist-independent and agonist-dependent activation of the recombinant human 5-HT\(_{1B}\) receptor by mutation of Thr\(^{313}\) in the BBX\(_B\) motif located in the C-terminal portion of the third intracellular loop. Wild-type 5-HT\(_{1B}\) receptors yielded a low (less than 10 \(\%\)) and variable level of constitutive activity in CHO-K1 cells. Mutation of Thr\(^{313}\) in the 5-HT\(_{1B}\) receptor to either a lysine, an arginine or a glutamine residue increased constitutive activity by 26–41 \(\%\), which was reversed by inverse agonists. Increased constitutive receptor activation was provided by co-expression of the 5-HT\(_{1B}\) receptor with a PTX-resistant rat Go protein. Thus, this response was amplified with a series of diverse amino acid substitutions (isoleucine, glycine, asparagine, alanine, lysine, phenylalanine, glutamine and arginine) at 5-HT\(_{1B}\) receptor position 313. Enhanced basal activity was maximal (approx. 500 \(\%\)) with the exception of the mutant Thr\(^{313}\)Ile protein, with the exception of the mutant

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pattern is apparent in the framework of traditionally cited amino acid characteristics such as residue size, charge or hydrophobicity. The study also illustrates that each of the ligands previously characterized as 5-HT<sub>1A</sub> antagonists behave, in the presence of the G<sub>α</sub> Cys<sup>335</sup> → Ile protein, either as partial agonists or inverse agonists.

Similar constitutively active mutations in the BBXXB motif have been reported for α<sub>1A</sub>-adrenergic (Ala<sup>295</sup> [13]), α<sub>2A</sub>-adrenergic (Thr<sup>272</sup> [14]), 5-HT<sub>3A</sub> (Cys<sup>292</sup> [15]) and 5-HT<sub>3C</sub> (Ser<sup>312</sup> [16]) receptors. Replacement by the basic residue lysine in each of these receptor subtypes induces, as in the 5-HT<sub>1B</sub> receptor at position 313, an enhanced constitutive activity. An arginine residue is also well tolerated in the α<sub>1A</sub>-adrenergic and 5-HT<sub>1B</sub> receptors, whereas a histidine residue is not readily admitted at this position. Otherwise, a glutamate residue has also been shown to increase basal activity of α<sub>1A</sub>-adrenergic, α<sub>2A</sub>-adrenergic and 5-HT<sub>3A</sub> receptors in contrast with 5-HT<sub>1B</sub> receptors. The other acidic residue, aspartate, does not improve constitutive activity for both α<sub>1A</sub>-adrenergic and 5-HT<sub>1B</sub> receptors.

The mutation of Thr<sup>313</sup> might disrupt interactions that hold the 5-HT<sub>1B</sub> receptor in an inactive conformation and/or facilitate its interactions with G-proteins. Wild-type 5-HT<sub>1B</sub> receptors exhibited a low level of constitutive activity, whereas the mutation of Thr<sup>313</sup> might selectively destabilize the inactive receptor conformation so that in the absence of ligands up to 28% (i.e. mutant Thr<sup>313</sup> → Arg) of the receptors are in an active conformation and 72% in an inactive conformation. Co-expression with the G<sub>α</sub> Cys<sup>335</sup> → Ile protein resulted in an enhanced proportion of active 5-HT<sub>1B</sub> receptors, ranging from 19% to maximally 59% for the wild-type 5-HT<sub>1B</sub> (Thr<sup>313</sup>) receptor and its Thr<sup>313</sup> → Arg mutant respectively. It is possible that the hydroxy group of Thr<sup>313</sup> is directly involved in a constraining interaction or in critical post-translational modifications (e.g. phosphorylation), hydrogen-bonding or other intramolecular protein interactions, because these would be destroyed by mutation of Thr<sup>313</sup> to a glycine or an alanine residue. These substitutions produce a 5-HT<sub>1B</sub> receptor phenotype that was less constrained. The mutants with a tyrosine or serine residue, which also contain a hydroxy group, showed similar activities to that of threonine. Otherwise, the hydroxy group of Thr<sup>313</sup> is unlikely to be involved in the agonist-activation mechanism of the 5-HT<sub>1B</sub> receptor. With the exception of proline and the acidic residues aspartate and glutamate at position 313, which caused lower levels (48–58%) of 5-HT<sub>1B</sub> receptor activation, the other mutants either did not affect or affected only slightly the 5-HT<sub>1B</sub>-mediated receptor activation. Another argument against a direct involvement of the hydroxy group of the threonine residue in constraining the 5-HT<sub>1B</sub> receptor interactions might be the presence of an alanine, a cysteine or a serine residue at the same position in α<sub>1A</sub>-adrenergic, 5-HT<sub>3A</sub> and 5-HT<sub>3C</sub> receptor subtypes shown to yield a low degree of constitutive activity in their native states [13,15,16].

The junction between the third intracellular loop and transmembrane domain (TM) VI of GPCR has been proposed to fold into a α-helical amphiphilic domain that extends the α-helix of TM VI into the cytoplasm [32,33]. This region might either be embedded into the plasma membrane or protrude into the cytoplasm, depending on the structure of the GPCR. The basic residues of the BBXXB motif face towards one side of the putative α-helix; their hydrophilic character probably excludes them from TM VI. Mutation of Thr<sup>313</sup> in the 5-HT<sub>1B</sub> receptor, facing the same side as the basic residues Arg<sup>318</sup>, Lys<sup>314</sup> and Lys<sup>311</sup>, to a basic residue increases the positively charged character of this putative α-helix. This feature might release this helix from the plasma membrane and thereby facilitate interactions with G-proteins. A similar model can be envisaged for the amino acids glutamine and asparagine. The constitutive activity of the 5-HT<sub>1B</sub> receptor is also increased by exchanging Thr<sup>313</sup> for the non-polar residues phenylalanine and isoleucine. This might increase the hydrophobic character of the BBXXB motif and probably modify its positioning with regard to TM VI and consequently modify G-protein interactions. Alternatively, mutagenesis of the Thr<sup>313</sup> residue might modify intramolecular bonds (i.e. with the other intracellular domains) and thereby modify the receptor activation state.

Although the physiological implications of constitutively active 5-HT<sub>1B</sub> receptor mutations remain unclear, the identification of mutations in thyrotropin receptors in patients with thyroid adenomas [3], rhodopsin receptors in retinitis pigmentosa [34], parathyroid hormone receptors in dwarfishism [35] and luteinizing hormone receptors in precocious puberty [36] suggests a role for natural GPCR mutations in disease pathophysiology. In each case, the expression of these mutant receptors in vitro in mammalian cell lines resulted in the activation of appropriate second messenger systems in the absence of agonist, thereby confirming the constitutively active nature of the mutated receptor identified in vivo. Proliferation of human small-cell lung carcinoma cell lines has been reported to be mediated by 5-HT<sub>1B</sub> receptors [37]. It would be interesting to test the hypothesis that mutations in the 5-HT<sub>1B</sub> receptor might be involved in the pathophysiology of various disease states.

Ligands previously characterized as antagonists at the wild-type 5-HT<sub>1B</sub> receptor demonstrated in this study either a positive (GR 125743, GR 127935 and 1-naphthylpiperazine) or a negative efficacy (ritanserin, GR 55562, SB 224298 and methiothepin) at concentrations relevant to their affinities for the wild-type 5-HT<sub>1B</sub> receptor. The 5-HT<sub>1B</sub> receptor model systems discussed here permit the unambiguous definition of ligands as partial agonists, neutral antagonists or inverse agonists. The lack of ligands that do not modify the basal G<sub>α</sub>[S]GTP[S] binding response at 5-HT<sub>1B</sub> receptors suggests 5-HT<sub>1B</sub>-neutral antagonists might be relatively uncommon. This is not surprising because this would require the same ligand to exhibit a similar affinity for both the active and the inactive receptor conformation.

In summary, the present study demonstrates that the 5-HT<sub>1B</sub> receptor can be transformed to a constitutively activated form either by a single amino acid (Thr<sup>313</sup>) mutation, by G<sub>α</sub> protein co-expression or by both. The constraint of 5-HT<sub>1B</sub> receptor activation can be relieved by mutation with a series of various amino acids. It is likely that the positioning of the junction between the third intracellular loop and TM VI is altered by mutation of Thr<sup>313</sup> in the BBXXB motif, thereby unmasking G<sub>α</sub> protein interaction points.

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REFERENCES


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