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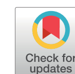
Selectivity in agonist and antagonist binding to Serotonin$_{1A}$ receptors *via* G-protein coupling



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A R T I C L E I N F O

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A B S T R A C T

G protein-coupled receptors (GPCRs) constitute the largest superfamily of membrane proteins in higher eukaryotes, and facilitate information transfer from the extracellular environment to the cellular interior upon activation by ligands. Their role in diverse signaling processes makes them an attractive choice as drug targets. GPCRs are coupled to heterotrimeric G-proteins which represent an important interface through which signal transduction occurs across the plasma membrane upon activation by ligands. To obtain further insight into the molecular details of interaction of G-proteins with GPCRs, in this work, we explored the selectivity of binding of specific agonists and antagonists to the serotonin$_{1A}$ receptor under conditions of progressive G-protein inactivation. The serotonin$_{1A}$ receptor is an important neurotransmitter receptor belonging to the GPCR family and is a popular drug target. By use of a number of agents to inactivate G-proteins, we show here that the serotonin$_{1A}$ receptor displays differential discrimination between agonist and antagonist binding. Our results show a reduction in binding sites of the receptor upon treatment with G-protein inactivating agents. In addition, G-protein coupling efficiency was enhanced when G-proteins were inactivated using urea and alkaline pH. We envision that our results could be useful in achieving multiple signaling states of the receptor by fine tuning the conditions of G-protein inactivation and in structural biology of GPCRs bound to specific ligands.

1. Introduction

G protein-coupled receptors (GPCRs) are extremely diverse and constitute the largest superfamily of membrane proteins in higher eukaryotes and characterized by seven transmembrane domain architecture [1–3]. GPCRs respond to various kinds of stimuli (including endogenous and exogenous ligands) and facilitate signal transduction from the extracellular environment to the cellular interior. They are involved in the regulation of numerous signaling pathways and mediate important physiological processes such as cellular differentiation, immune responses and entry of pathogens [3]. As a consequence, GPCRs have emerged as major drug targets and represent attractive candidates for the development of new drugs in all clinical areas [3–7]. More importantly, the fact that ligands for many GPCRs are yet to be discovered, gives rise to the exciting possibility that orphan receptors could be drug targets for diseases for which currently available drugs are not effective [8].

GPCRs are integral membrane proteins coupled to heterotrimeric G-proteins (consisting of α, β and γ subunits) that act as the interface through which signal transduction occurs across cell membranes. Upon ligand binding, the receptor undergoes conformational changes and activates G-proteins, leading to dissociation of the α and βγ dimer subunits and initiation of several downstream signaling pathways [2,9]. In spite of the existing diversity in ligands and ligand binding regions exhibited by the GPCR family, there is evidence to suggest that a common mechanism is involved in the process of GPCR activation [10,11]. Several hypotheses have been proposed to explain GPCR activation, among which the two-state model was one of the early ones to elucidate key aspects of GPCR function [12]. Subsequently, experimental studies indicated that GPCRs exist in multiple conformational

Abbreviations: 5-HT$_{1A}$R, 5-hydroxytryptamine$_{1A}$ receptor; BCA, bicinchoninic acid; CAPS, 3-[cyclohexylamino]-1-propanesulfonic acid; CHO, Chinese Hamster Ovary; GTP-γ-S, guanosine-5′-*O*-(3-thiotriphosphate); 8-OH-DPAT, 8-hydroxy-2(di-*N*-propylamino)tetralin; *p*-MPPF, 4-(2′-methoxy)-phenyl-1-[2′-(*N*-2″-pyridinyl)-*p*-fluorobenzamido]ethyl-piperazine; PMSF, phenylmethylsulfonyl fluoride

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states rather than an active and inactive state proposed in the two-state model [13]. However, the details involved in the process of GPCR activation are not fully understood due to the complexity associated with the process.

Among the GPCR family, serotonin receptors constitute a major class of GPCRs that act as neurotransmitter receptors in the brain [14,15], and are responsible for the initiation and modulation of cognitive and behavioral functions. The serotonin$_{1A}$ receptor is one of the extensively studied members in the serotonin receptor family [16–21]. A major factor contributing to this is the early development and availability of the selective agonist 8-OH-DPAT [22,23]. Importantly, the serotonin$_{1A}$ receptor is a key drug target for diseases ranging from neuropsychiatric disorders to cancer [19,20]. Previous work from our group has comprehensively demonstrated the crucial role of membrane lipids such as cholesterol [24–29] and sphingolipids [26,30] in the organization, dynamics, endocytosis and function of the serotonin$_{1A}$ receptor.

Upon activation by ligands, the serotonin$_{1A}$ receptor couples to the adenylate cyclase system *via* inhibitory (G_i) class of G-proteins [31,32]. From a pharmacological perspective, ligands are classified based on their interactions with GPCRs and are categorized according to their potency to elicit a given biological response [33,34]. Agonists are defined as ligands that activate GPCRs upon binding and generate a physiological response over and above the basal activity (activity in the absence of any ligand). On the other hand, antagonists (sometimes termed as neutral antagonists) neither stimulate GPCR activation, nor inhibit its basal activity [33,34]. In this context, previous work by us [35,36] and others [37] has demonstrated differential discrimination of G-protein coupling to the serotonin$_{1A}$ receptor by specific agonists and antagonists using conditions that uncouple the receptor/G-protein complex. This discrimination has its origin in the fact that agonists predominantly bind to receptors that are coupled to G-proteins with high affinity, whereas antagonists bind to all receptors, regardless of their G-protein coupling state. The differential biological response of GPCRs upon stimulation by agonist or antagonist therefore could be utilized as a pharmacological tool to gain a better understanding of receptor function.

Determining the structure of GPCRs constitutes a major step in understanding their function and mechanism of action. In recent years, high-resolution crystal structures of several GPCRs have been solved [38]. In addition, structures of GPCRs bound to G-proteins, and a GPCR/G-protein/β-arrestin complex have been resolved by x-ray crystallography [39–41]. A caveat associated with x-ray crystallography of GPCRs is that flexible loops (which are critical for GPCR function) [42] are usually truncated and stabilized by a monoclonal antibody or substituted with lysozyme [43–45], since the intrinsic conformational flexibility of the loops poses a problem for x-ray crystallography. Another major issue related to GPCR crystal structures is the fact that receptors are crystallized in the lipidic cubic phase whose physiological relevance could be somewhat tenuous [46]. In a recent development, cryo electron microscopy has emerged as a superior technique which is being utilized to obtain insights related to structure and function of GPCRs [47–49].

The interaction of receptor with G-proteins is an essential step in GPCR activation and detailed information on various aspects of this interaction would enable us to improve our understanding of receptor function. Upon GPCR activation, concerted structural rearrangements occur in the extramembranous and transmembrane regions [50,51], which are translated to signaling events inside cells. Such dynamic changes in receptor conformation make the study of interaction of GPCRs with G-proteins a challenging task. To obtain molecular details on the interaction of the serotonin$_{1A}$ receptor with G-proteins, in this work, we explored the selectivity of receptor binding to specific agonists and antagonists upon treatment with agents which are known to inactivate G-proteins. Our results show that the serotonin$_{1A}$ receptor exhibits differential discrimination between agonist and antagonist binding in the presence of G-protein inactivating agents. We observed a reduction in binding sites of the receptor upon treatment with G-protein inactivating agents. Interestingly, G-protein coupling efficiency of serotonin$_{1A}$ receptors is enhanced when G-proteins are inactivated using urea and alkaline pH. We believe that these results provide a new perspective of receptor/G-protein interaction. In addition, our results could have implications in biased signaling by GPCRs.

2. Materials and methods

2.1. Materials

3-[Cyclohexylamino]-1-propanesulfonic acid (CAPS), EDTA, EGTA, gentamycin sulfate, $MgCl_2$, $MnCl_2$, iodoacetamide, penicillin, pertussis toxin, 4-(2′-methoxy)-phenyl-1-[2′-(*N*-2″-pyridinyl)-*p*-fluorobenzamido] ethyl-piperazine (*p*-MPPF), phenylmethylsulfonyl fluoride (PMSF), serotonin hydrochloride, sodium azide, streptomycin, sucrose, Tris, trypsin and urea were purchased from Sigma Chemical Co. (St. Louis, MO). [^{3}H]8-hydroxy-2(di-*N*-propylamino)tetralin ([^{3}H]8-OH-DPAT, specific activity 141 Ci/mmol) and [^{3}H]*p*-MPPF (specific activity 74.2 Ci/mmol) were purchased from MP Biomedicals (Santa Ana, CA). Bicinchoninic acid (BCA) assay reagent for protein estimation was from Pierce (Rockford, IL). GF/B glass microfiber filters were from Whatman International (Kent, UK). Guanosine-5′-*O*-(3-thiotriphosphate) (GTP-γ-S) and trypsin inhibitor (from chicken egg white) were purchased from Roche Applied Science (Mannheim, Germany). D-MEM/F-12 (Dulbecco's Modified Eagle Medium: nutrient mixture F-12 (Ham) (1:1)), fetal calf serum, and geneticin (G418) were from Invitrogen/Life Technologies (Carlsbad, CA). Water was purified through a Millipore (Bedford, MA) Milli-Q system and used throughout. Fresh bovine brains were acquired from a local slaughterhouse within 10 min of death, and the hippocampal region was cautiously dissected out. The hippocampi were immediately flash frozen in liquid nitrogen and stored at −80 °C till further use.

2.2. Methods

2.2.1. Preparation of native hippocampal membranes

Native hippocampal membranes were prepared as described previously [52]. The final pellet was suspended in a minimum volume of buffer A (50 mM Tris, pH 7.4), homogenized using a hand-held Dounce homogenizer, flash frozen in liquid nitrogen and stored at −80 °C. See Supplementary Material, Section 1.1 for more details.

2.2.2. Urea treatment of native hippocampal membranes

The treatment of native hippocampal membranes with urea was carried out as described previously [53] with minor modifications. Briefly, hippocampal membranes were resuspended at a protein concentration of ~2 mg/ml and treated with varying concentrations of urea (2–8 M) in buffer A. After 30 min incubation on ice, membranes were centrifuged at 50,000 ×*g* for 10 min. The pellet was resuspended in buffer A and centrifuged again at 50,000 ×*g* for 10 min to remove residual urea. The final membrane pellet was resuspended in buffer A, homogenized using a hand-held Dounce homogenizer and used immediately for radioligand binding assays.

2.2.3. Treatment of native hippocampal membranes with trypsin

Hippocampal membranes were treated with trypsin as described previously [54] with some modifications. Membranes were resuspended at a protein concentration of ~2 mg/ml in buffer A. Trypsin was added at concentrations ranging from 1- to 100 μg/ml to membranes at room temperature (~23 °C). After 30 min incubation at room temperature, the reaction was terminated by adding trypsin inhibitor (200 μg/ml). After further incubation for 5 min at ~23 °C, the tubes were placed on ice and radioligand binding activity was measured immediately. In addition, we carried out a protein fragmentation assay to show that trypsin is active on membrane proteins in the

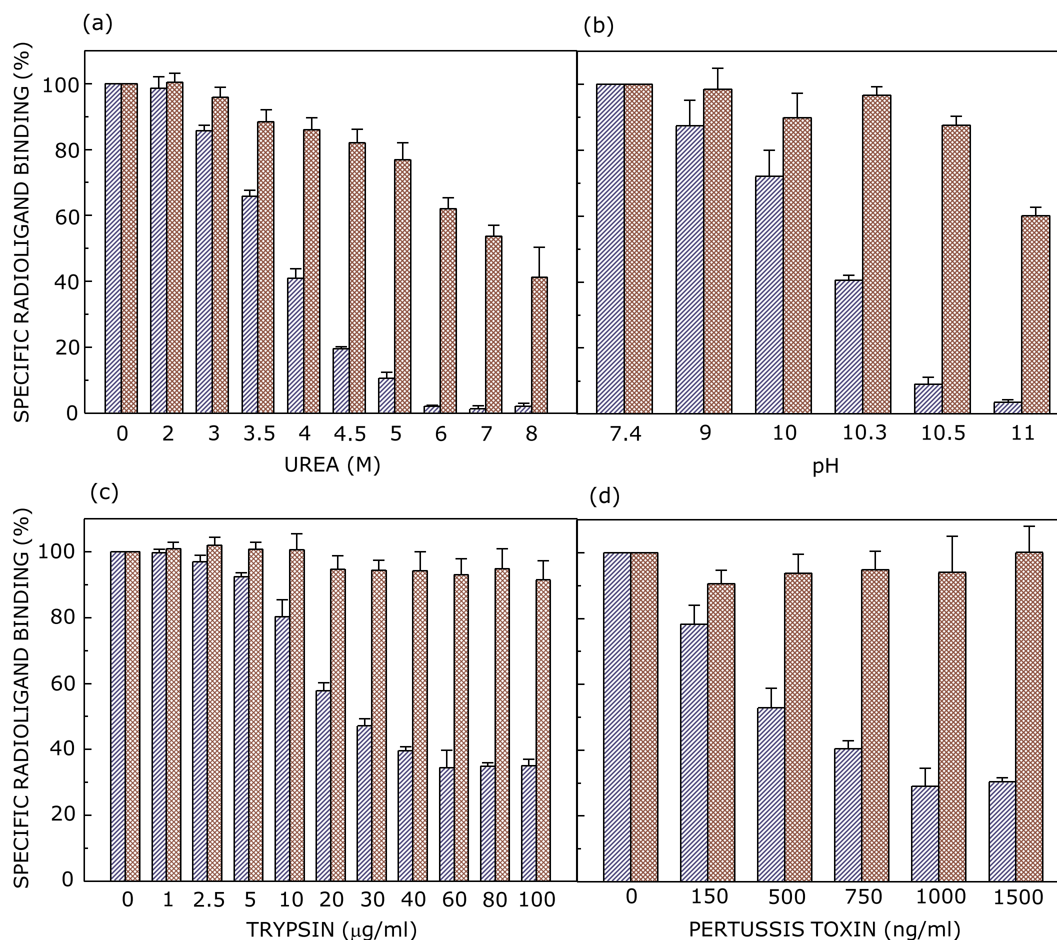

Fig. 1. Specific radioligand binding to serotonin$_{1A}$ receptors upon treatment with G-protein inactivating agents. Binding of specific agonist [^{3}H]8-OH-DPAT and antagonist [^{3}H]*p*-MPPF to serotonin$_{1A}$ receptors in hippocampal membranes upon treatment with urea (panel a), alkaline pH (b) and trypsin (c). (d) Binding of specific agonist and antagonist to serotonin$_{1A}$ receptors in membranes from CHO-5-HT$_{1A}$R cells upon treatment with pertussis toxin. Agonist binding is represented by blue hatched bars whereas antagonist binding is represented by maroon crisscrossed bars in all four panels. Values are expressed as percentages of specific radioligand binding obtained in either native hippocampal membranes or CHO-5-HT$_{1A}$R cell membranes without any treatment. Data represent means ± S.E. of duplicate points from at least three independent experiments. See Materials and methods for other details.

concentrations used in our experiments (see Fig. S1).

2.2.4. Incubation of hippocampal membranes at alkaline pH

Native hippocampal membranes were incubated with CAPS buffer of desired alkaline pH as described previously [55] with some modifications. Briefly, membranes were resuspended at a protein concentration of ~2 mg/ml in CAPS buffer of various alkaline pH. After incubation for 30 min at room temperature (~23 °C), membranes were centrifuged at 50,000 ×*g* for 10 min. The pellet was resuspended in buffer A, homogenized as described before and used immediately for radioligand binding assays.

2.2.5. Cells and cell culture

Chinese Hamster Ovary (CHO) cells stably expressing the serotonin$_{1A}$ receptor (termed as CHO-5-HT$_{1A}$R) were maintained in D-MEM/F-12 (1:1) medium supplemented with 2.4 g/l of sodium bicarbonate, 10% fetal calf serum, 60 μg/ml penicillin, 50 μg/ml streptomycin, 50 μg/ml gentamycin sulfate (complete medium), and 0.2 mg/ml G418 in a humidified atmosphere with 5% CO_2 at ~37 °C.

2.2.6. Pertussis toxin treatment and cell membrane preparation

CHO-5-HT$_{1A}$R cells were grown in complete medium in presence of varying concentrations of pertussis toxin (150–1500 ng/ml) for 24 h before experiments. Following treatment, CHO-5-HT$_{1A}$R cell membranes were prepared as described previously [56]. See Supplementary Material, Section 1.2 for more details.

2.2.7. Radioligand binding assays

Receptor radioligand binding assays in native and treated hippocampal membranes were performed as described previously [52]. Radioligand binding assays with membranes isolated from control and pertussis toxin treated CHO-5-HT$_{1A}$R cells were carried out as described previously [56]. See Supplementary Material, Section 1.3 for more details.

2.2.8. Saturation binding assays

Saturation binding assays were carried out to estimate binding parameters in control and membranes treated with G-protein inactivating agents, as described previously [52,56]. The concentration of bound radioligand (RL_{bound}) was determined using the equation:

$$RL_{bound} = 10^{-9} \times B/(V \times SA \times 2220)\ M \tag{1}$$

where B is the bound radioactivity in disintegrations per minute (dpm), V is the assay volume in ml, and SA is the specific activity of the radioligand. Saturation binding data could be fitted best to a one-site ligand binding equation given below:

$$RL_{bound} = B_{max}x/(K_d + x) \tag{2}$$

where x is the total ligand concentration, K_d is the dissociation constant and B_{max} is the number of maximum binding sites. Values of K_d and

B_{max} were determined by nonlinear regression analysis of binding data using GraphPad Prism software, version 4.0 (San Diego, CA). See Supplementary Material, Section 1.4 for more details.

2.2.9. Determining G-protein coupling efficiency by GTP-γ-S sensitivity assays

G-protein coupling efficiency to serotonin$_{1A}$ receptors in control and membranes treated with G-protein inactivating agents was measured utilizing GTP-γ-S sensitivity assays as described previously [52,56]. The concentrations of GTP-γ-S leading to half-maximal inhibition (IC_{50}) of specific agonist binding were calculated by nonlinear regression fitting of the data to a four parameter logistic function [57]:

$$B = a[1 + (x/I)^s]^{-1} + b \tag{3}$$

where B is the binding of specific agonist in presence of GTP-γ-S normalized to binding observed at the lowest concentration of GTP-γ-S used (10^{-12} M in cell membranes and 10^{-11} M in hippocampal membranes), x denotes concentration of GTP-γ-S, a is the range (y_{max}-y_{min}) of the fitted curve on the ordinate (y-axis), I is the IC_{50} concentration, b is the background of the fitted curve (y_{min}) and s is the slope factor.

3. Results and discussion

3.1. Specific radioligand binding to serotonin$_{1A}$ receptors upon treatment with G-protein inactivating agents

In order to assess the selectivity between agonist and antagonist binding to serotonin$_{1A}$ receptors in the presence of agents that inactivate G-proteins, we measured specific radioligand binding to serotonin$_{1A}$ receptors in hippocampal membranes. We utilized various strategies for inactivating G-proteins such as a chaotropic agent (urea), a protease (trypsin) and alkaline pH. Fig. 1 shows the specific agonist ([^{3}H]8-OH-DPAT) and antagonist ([^{3}H]*p*-MPPF) binding to serotonin$_{1A}$ receptors in G-protein inactivated conditions. As shown in the figure, a progressive reduction in specific agonist binding was observed in all cases, thereby implying the dependence of agonist binding to G-protein coupling. In contrast, the corresponding change in specific antagonist binding was much less pronounced.

Urea is known to denature soluble and peripheral membrane proteins without affecting integral membrane proteins [58–60]. Chaotropic agents such as urea act by disrupting hydrophobic interactions that stabilize native protein conformation [53]. Urea has been previously utilized for uncoupling G-proteins from various GPCRs expressed in insect or mammalian cell lines [53,61–63]. In case of treatment with urea (see Fig. 1a), we observed a sharp reduction in specific agonist binding in the concentration range of 3–5 M. Agonist binding was almost abolished beyond 5 M urea, whereas the antagonist binding was retained to ~40% of native membranes at the highest concentration of urea used (see Fig. 1a).

Side chains of charged amino acid residues in proteins experience the pH of the environment. The charge on these pH-sensitive residues could help in maintaining protein conformation and modulate protein function. Pre-treatment of membranes in alkaline pH has been shown to affect the activity of G-proteins and subsequent activation of adenylate cyclase [55,64,65]. Incubation of membranes in high alkaline condition (pH 11) resulted in complete loss of agonist binding, while ~60% reduction in activity was observed at pH 10.3 (Fig. 1b). Such pH-dependent agonist binding activity has previously been reported for GPCRs such as the serotonin$_{1A}$ receptor and the β$_2$-adrenergic receptor [66,67]. On the contrary, antagonist binding was retained up to ~88% at pH 10.5, and with further increase in pH to 11, ~60% antagonist binding was retained with respect to control membranes (see Fig. 1b).

Trypsin is a serine protease which cleaves proteins at the carboxyl side of the amino acids lysine or arginine [68]. The $G\alpha_i$ class of G-proteins contains ~48 sites susceptible to cleavage by trypsin and could be one of the major targets of trypsin as proposed previously [54,69]. In addition, the serotonin$_{1A}$ receptor contains 44 sites (including two putative sites in transmembrane helices) prone to cleavage by trypsin [67]. Importantly, one of the sites in the serotonin$_{1A}$ receptor is localized in the conserved DRY motif which is crucial for G-protein coupling of GPCRs [70]. Fig. 1c shows that agonist binding was markedly reduced upon treatment of membranes with trypsin with the half-maximal effect at ~30 μg/ml of trypsin. The reduction in agonist binding could be due to proteolytic degradation by trypsin at susceptible sites in the G-protein and/or the receptor. On the other hand, antagonist binding was found to be predominantly invariant upon trypsin treatment. Interestingly, a significant population of the serotonin$_{1A}$ receptor (~35%) retained agonist binding activity after trypsin treatment, even at the highest concentration of trypsin (100 μg/ml). This could possibly be due to lack of accessibility of trypsin to certain sites.

Treatment of cells with pertussis toxin is known to specifically abolish receptor and G-protein coupling by ADP-ribosylation of $G_{i/o}$ subtype of G-proteins [71,72]. As mentioned earlier, the serotonin$_{1A}$ receptor is negatively coupled to the adenylate cyclase system through pertussis toxin sensitive G-proteins (G_i/G_o) [31,32]. As a control, we monitored specific radioligand binding to serotonin$_{1A}$ receptors from native and pertussis toxin treated CHO-5-HT$_{1A}$R cells (CHO cells heterologously expressing the serotonin$_{1A}$ receptor). The human serotonin$_{1A}$ receptor heterologously expressed in CHO cells displays characteristic pharmacological features similar to the native hippocampal receptor [56]. Cells treated with pertussis toxin showed a progressive reduction in agonist binding with increasing concentrations of the toxin, while antagonist binding exhibited no appreciable change across the concentration range used (see Fig. 1d).

Taken together, the reduction in agonist binding to serotonin$_{1A}$ receptors in hippocampal and CHO-5-HT$_{1A}$R cell membranes upon treatment with G-protein inactivating agents could be majorly attributed to the disruption in G-protein coupling since agonist binding to serotonin$_{1A}$ receptors was affected to a much greater extent than antagonist binding. These results are in agreement with our earlier work where we showed that agonist binds selectively to G-protein coupled receptors, while antagonist binds to all receptors regardless of their state of G-protein coupling [35].

It is interesting to note that there is a difference in the pattern of reduction exhibited by agonists and antagonists in case of urea denaturation. For example, while agonist binding was almost abolished at high concentration of urea (6–8 M), even antagonist binding (which should not depend on G-protein coupling) displayed significant reduction (~60%) at the highest urea concentration used. This is in contrast with other inactivating agents (see Fig. 1(b–d)), where the antagonist binding appears to be more or less invariant even at high concentrations of inactivating agents (with a minor exception at very high pH). The antagonist binding site of the serotonin$_{1A}$ receptor is predicted to be localized in the transmembrane region [73,74]. The appreciable reduction even in antagonist binding at very high urea concentrations could possibly be due to denaturation of membrane spanning regions of the serotonin$_{1A}$ receptor, since urea is known to diffuse through the membrane bilayer [75] and has been proposed to perturb polar and hydrophobic interactions in the bilayer [53,76].

3.2. Alteration in binding sites in serotonin$_{1A}$ receptors upon treatment with G-protein inactivating agents

The reduction in specific agonist binding to serotonin$_{1A}$ receptors (Fig. 1) could be either due to reduction in affinity of the receptor to the agonist or loss in ligand binding sites, or a combination of both. In order to obtain further insight on the mechanism of reduction in agonist binding in G-protein inactivated conditions, we carried out saturation binding assays under these conditions. Saturation binding analysis for binding of the specific agonist ([^{3}H]8-OH-DPAT) in control and hippocampal membranes treated with the inactivating agents was carried

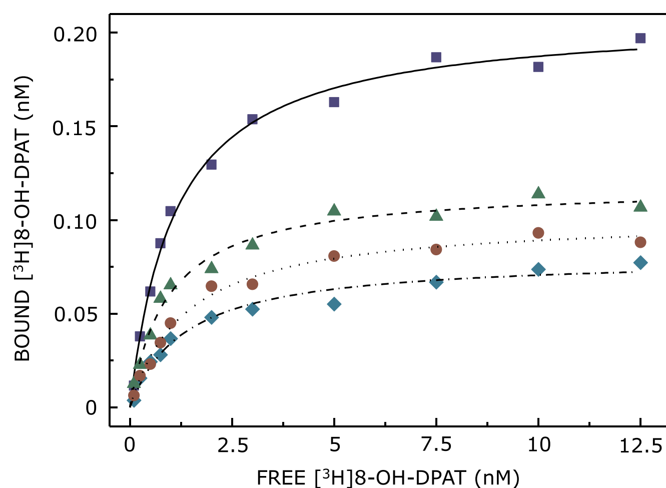

Fig. 2. Saturation binding analysis in the presence of G-protein inactivating agents. Saturation binding analysis was performed for binding of the specific agonist [^{3}H]8-OH-DPAT to serotonin$_{1A}$ receptors in hippocampal membranes upon treatment with various G-protein inactivating agents. Representative plots for bound [^{3}H]8-OH-DPAT with increasing concentrations of [^{3}H]8-OH-DPAT are shown for native membranes (■), and membranes treated with trypsin (▲), urea (●) and alkaline pH (◆). The concentration of [^{3}H]8-OH-DPAT was 0.1–12.5 nM. Values of dissociation constant (K_d) and maximum binding sites ($B_{\max}$) obtained upon analysis of saturation binding data are shown in Table 1. The curves shown are non-linear regression fits to the experimental data obtained using Eq. (2). Data represent means ± S.E. of duplicate points from three independent experiments. See Materials and methods, and Table 1 for more details.

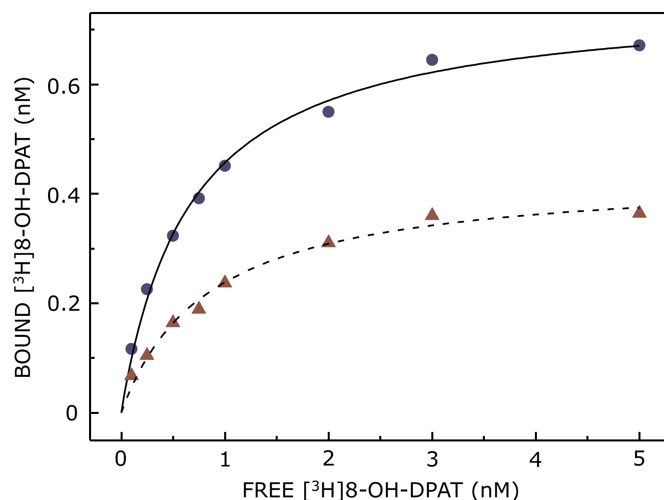

Fig. 3. Saturation binding analysis for specific agonist binding to serotonin$_{1A}$ receptors upon treatment with pertussis toxin. Representative plots for bound specific agonist with increasing concentrations of [^{3}H]8-OH-DPAT are shown for membranes from control (●) and pertussis toxin (▲) treated CHO-5-HT$_{1A}$R cells. The concentration of [^{3}H]8-OH-DPAT was 0.1–5 nM, whereas the concentration of pertussis toxin was 500 ng/ml. Values of dissociation constant (K_d) and maximum binding sites ($B_{\max}$) obtained upon analysis of saturation binding data are shown in Table 1. The curves shown are non-linear regression fits to the experimental data obtained using Eq. (2). Data represent means ± S.E. of duplicate points from three independent experiments. See Materials and methods, and Table 1 for other details.

Table 1
Effect of G-protein inactivating agents on specific [^{3}H]8-OH-DPAT binding to serotonin$_{1A}$ receptors[a].

Condition	$B_{\max}$ (fmol/mg of protein)	K_d (nM)
Native membranes	175.7 ± 14.3	1.00 ± 0.11
Urea-treated membranes	83.0 ± 11.1[b]	1.71 ± 0.20
Trypsin-treated membranes	118.2 ± 5.5[b]	0.87 ± 0.03
Membranes treated at pH 10.3	104.6 ± 10.1[b]	1.31 ± 0.21
CHO-5-HT$_{1A}$R cells	852.2 ± 38.3	0.77 ± 0.05
Pertussis toxin treated cells	583.8 ± 60.3[b]	1.27 ± 0.18

[a] The binding parameters shown represent means ± S.E. from three independent experiments, while saturation binding data shown in Figs. 2 and 3 are from a representative experiment. Urea, trypsin and pertussis toxin concentrations were 4 M, 30 μg/ml and 500 ng/ml, respectively. See Materials and methods for more details.

[b] A significant decrease in $B_{\max}$ ($p < 0.05$) was observed relative to corresponding control in all cases, whereas the change in K_d was found to be not significant.

out and representative binding plots obtained are shown in Fig. 2. Data from saturation binding assays were analyzed and values of binding parameters, *i.e.*, maximum binding sites (B_{max}) and dissociation constant (K_d), are shown in Table 1. In native hippocampal membranes, the B_{max} value was 175.7 fmol/mg, whereas in presence of urea, trypsin and alkaline pH, considerably lower B_{max} values of 83 (~53% reduction), 118.2 (~33% reduction) and 104.6 fmol/mg (~40% reduction), respectively, were obtained. This shows a significant reduction in the number of ligand binding sites upon inactivation of G-proteins relative to native hippocampal membranes. On the other hand, the agonist binding affinity to serotonin$_{1A}$ receptors under these conditions does not change significantly, as indicated by no significant variation in K_d values (see Table 1). The reduction in specific agonist binding to the serotonin$_{1A}$ receptor (Fig. 1) could therefore be attributed to a decrease in binding sites in serotonin$_{1A}$ receptors upon inactivation of G-proteins.

In addition, we performed saturation binding analysis for specific agonist ([^{3}H]8-OH-DPAT) binding in control and pertussis toxin treated CHO-5-HT$_{1A}$R cells. The representative saturation binding plots are shown in Fig. 3 and analysis of the binding curves yielded a B_{max} value of 852.2 fmol/mg in control CHO-5-HT$_{1A}$R membranes. Treatment with pertussis toxin resulted in a significantly lower B_{max} value of 583.8 fmol/mg (~32% reduction; see Table 1). This was accompanied by no significant change in ligand binding affinity (see Table 1). Taken together, our results suggest that there is a reduction in the number of binding sites without any change in receptor binding affinity under G-protein inactivating conditions.

3.3. G-protein coupling efficiency to serotonin$_{1A}$ receptors in the presence of G-protein inactivating agents

The canonical signaling of GPCRs takes place *via* G-proteins upon activation by specific agonists and this process has been shown to be regulated by guanine nucleotides [2,9]. GTP-γ-S is a non-hydrolyzable analog of GTP which uncouples the typical cycle of guanine nucleotide exchange and has been widely used to explore G-protein coupling efficiency to GPCRs [77]. It has been shown that the serotonin$_{1A}$ receptor specifically activates the G_i/G_o class of G-proteins, when stimulated by its specific agonist in CHO cells and in hippocampal membranes [56,78,79]. We previously showed that the serotonin$_{1A}$ receptor displays a transition from a high affinity to a low affinity state upon uncoupling of G-proteins using GTP-γ-S [35]. In order to assess the efficiency of G-protein coupling to serotonin$_{1A}$ receptors in the presence of G-protein inactivating agents, we carried out GTP-γ-S sensitivity assays under these conditions (see Figs. 4 and 5). Fig. 4 shows a characteristic reduction in binding of the specific agonist ([^{3}H]8-OH-DPAT) to serotonin$_{1A}$ receptors with increasing concentrations of GTP-γ-S in hippocampal membranes. The half-maximal inhibition concentrations (IC_{50}) derived from non-linear regression analysis of the inhibition curves reflect the efficiency of G-protein coupling. The IC_{50} values of

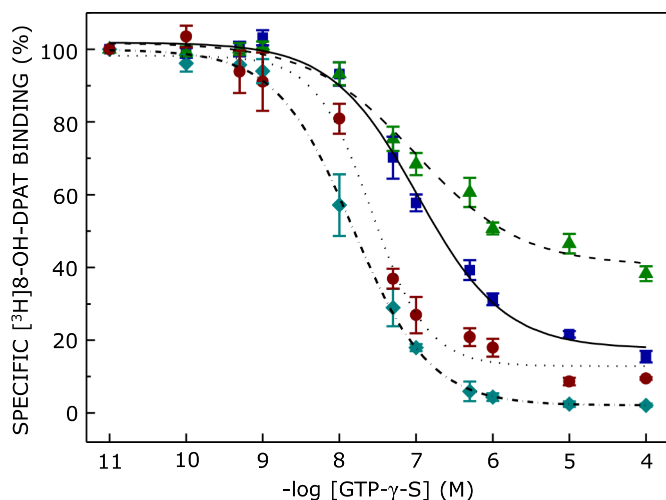

Fig. 4. G-protein coupling efficiency to serotonin$_{1A}$ receptors upon G-protein inactivation. The efficiency of G-protein coupling to hippocampal serotonin$_{1A}$ receptors in the presence of inactivating agents was determined by monitoring the sensitivity of binding of specific agonist [^{3}H]8-OH-DPAT to the receptor in the presence of GTP-γ-S. Upon increasing the concentration of GTP-γ-S, binding of [^{3}H]8-OH-DPAT in control membranes (■), and membranes treated with trypsin (▲), urea (●) and alkaline pH (◆) display characteristic inhibition. The corresponding half-maximal inhibition concentrations (IC$_{50}$) of GTP-γ-S, which are indicative of G-protein coupling efficiency to the receptor are shown in Table 2. Values are expressed as percentages of specific agonist binding obtained at the lowest concentration of GTP-γ-S. The curves in the figure are non-linear regression fits to the experimental data obtained using Eq. (3). Data represent means ± S.E. of duplicate points from at least three independent experiments. See Materials and methods, and Table 2 for other details.

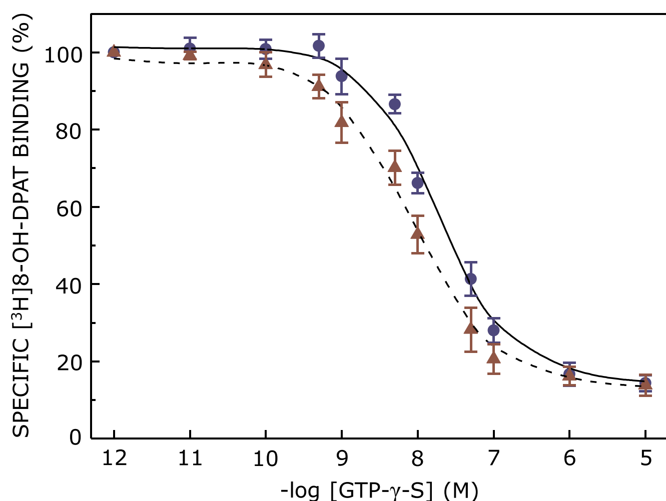

Fig. 5. Efficiency of G-protein coupling to serotonin$_{1A}$ receptors upon treatment with pertussis toxin. G-protein coupling efficiency in membranes from control and pertussis toxin treated CHO-5-HT$_{1A}$R cells, obtained by monitoring the sensitivity of [^{3}H]8-OH-DPAT binding to the receptor in presence of GTP-γ-S. With increasing concentrations of GTP-γ-S, specific binding of [^{3}H]8-OH-DPAT in control membranes (●) and membranes treated with pertussis toxin (▲) exhibit characteristic reduction. The corresponding half-maximal inhibition concentrations (IC$_{50}$) of GTP-γ-S, which reflect G-protein coupling efficiency to the receptor are shown in Table 2. The concentration of pertussis toxin was 500 ng/ml. Values are expressed as percentages of specific agonist binding obtained at the lowest concentration of GTP-γ-S. The curves in the figure are non-linear regression fits to the experimental data obtained using Eq. (3). Data represent means ± S.E. of duplicate points from at least three independent experiments. See Materials and methods, and Table 2 for other details.

Table 2
Effect of G-protein inactivating agents on the efficiency of G-protein coupling to serotonin$_{1A}$ receptors[a].

Condition	IC$_{50}$ (nM)
Native membranes	104.9 ± 16.5
Urea-treated membranes	14.8 ± 1.5[b]
Trypsin-treated membranes	99.6 ± 26.4
Membranes treated at pH 10.3	26.1 ± 4.9[b]
CHO-5-HT$_{1A}$R cells	3.7 ± 0.52
Pertussis toxin treated cells	1.7 ± 0.24[b]

[a] The binding parameters shown represent means ± S.E. from at least three independent experiments. Urea, trypsin and pertussis toxin concentrations were 4 M, 30 μg/ml and 500 ng/ml, respectively. See Materials and methods for more details.
[b] The decrease in IC$_{50}$ was found to be significant ($p < 0.01$) relative to corresponding control in all cases.

GTP-γ-S that induce inhibition of agonist binding in case of control membranes was found to be ~105 nM, in agreement with our previous results [52]. The inhibition curves for agonist binding to the receptor in hippocampal membranes treated with urea and alkaline pH are shown in Fig. 4. As evident from the figure, these curves exhibit significant shifts toward lower concentrations of GTP-γ-S relative to native hippocampal membranes. As a result, the IC$_{50}$ values corresponding to these curves are considerably lower (~15 and 26 nM), for treatment with urea and alkaline pH, respectively (see Table 2). It is therefore apparent that agonist binding to the serotonin$_{1A}$ receptor under these conditions is more sensitive to GTP-γ-S, thereby indicating increased G-protein coupling efficiency of the receptor. In contrast, in trypsin-treated membranes, the inhibition curve does not display any appreciable shift relative to native hippocampal membranes, and the IC$_{50}$ value obtained (~100 nM) is not significantly different from control value (~105 nM). These results could possibly indicate heterogeneity in G-protein coupling efficiency, since treatment with urea and alkaline pH could possibly target the pool of G-proteins which are weakly coupled to the receptor. Our results could therefore imply heterogeneity in the pool of G-proteins, possibly coupled to GPCRs with varying efficiencies (see Fig. 6). The refractory nature of the results with trypsin treatment could be due to the fact that sites in G-protein/receptor that are crucial for G-protein coupling could be inaccessible for proteolytic cleavage by trypsin (see Fig. 1c) due to topological constraints.

In addition, we measured the G-protein coupling efficiency utilizing GTP-γ-S sensitivity assay in membranes from control and pertussis toxin treated CHO-5-HT$_{1A}$R cells. The inhibition curves obtained are shown in Fig. 5. Analysis of these curves yielded IC$_{50}$ value of 3.7 nM for control, whereas a significantly lower value of 1.7 nM was obtained upon treatment of cells with pertussis toxin. This is indicative of enhanced G-protein coupling efficiency in cells treated with pertussis toxin.

Several approaches have been employed to inactivate G-proteins such as treatment with alkaline pH, urea, pertussis toxin, high temperature and detergents [36,53–55,71,80–83]. We have previously shown that agonists and antagonists can differentially discriminate G-protein coupling to hippocampal serotonin$_{1A}$ receptors [35]. In addition, we monitored ligand binding and G-protein coupling efficiency of serotonin$_{1A}$ receptors at high temperature and demonstrated irreversible inactivation of G-protein coupling in a temperature-dependent manner [36]. In the present work, we explored the selectivity in agonist and antagonist binding to serotonin$_{1A}$ receptors utilizing ligand binding and G-protein coupling upon treatment with agents which inactivate G-proteins. In general, agonist binding was found to be more sensitive to the presence of G-protein inactivating agents relative to antagonist binding. Analysis of saturation binding data revealed a decrease in maximum binding sites in the serotonin$_{1A}$ receptor rather than change in agonist binding affinity. Importantly, G-protein coupling efficiency

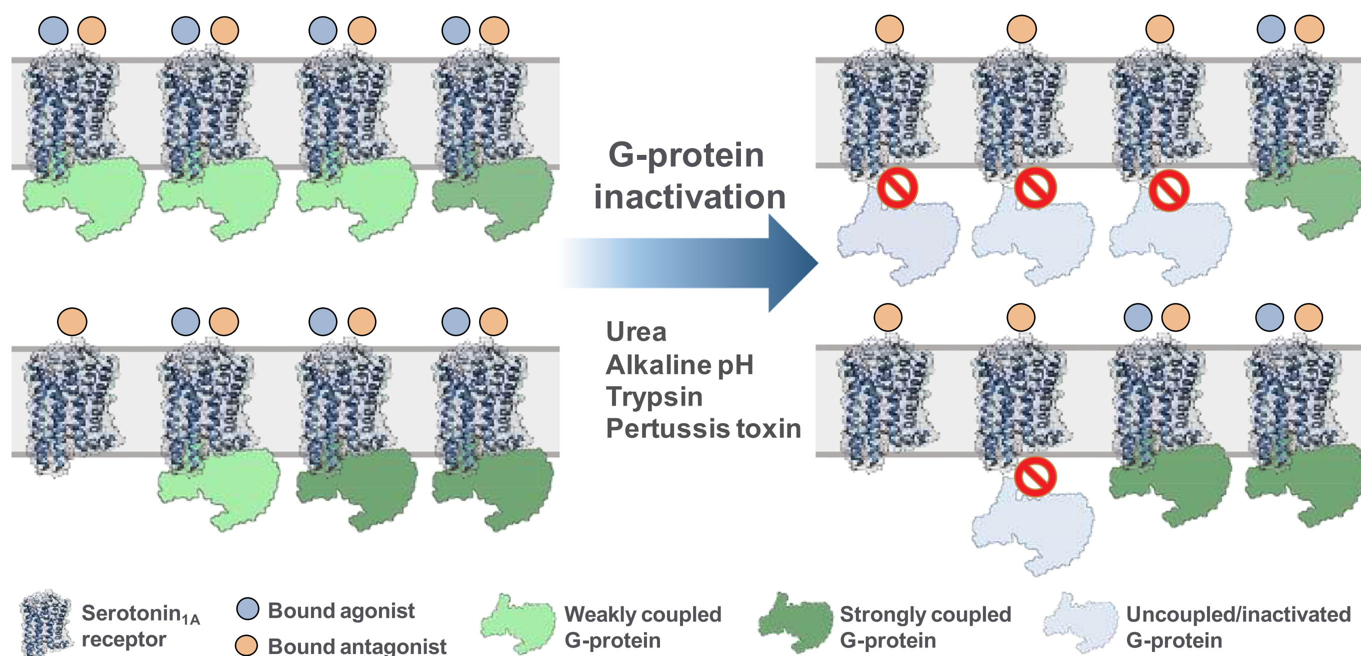

Fig. 6. A schematic model showing the heterogeneity in G-protein pools upon inactivation of G-proteins. Specific agonists (light blue circle) exclusively bind to serotonin$_{1A}$ receptors (dark blue) that are coupled to G-proteins, whereas specific antagonists (peach circle) bind to all receptors, regardless of their G-protein coupling state. The gray horizontal lines represent the boundary of cell membranes. The serotonin$_{1A}$ receptor is associated with a weakly coupled pool (light green) and a strongly coupled pool (dark green) of G-proteins. Inactivation of G-proteins using a variety of agents resulted in selective inactivation of weakly coupled G-proteins, which contribute to reduction in specific agonist binding (Fig. 1) and maximum binding sites (Figs. 2 and 3). Such selective uncoupling of the weakly coupled pool of G-proteins leaves the strongly coupled pool unaffected and this is reflected in the enhanced G-protein coupling efficiency (Figs. 4 and 5). Our results could imply heterogeneity in the pool of G-proteins, possibly coupled to GPCRs with varying efficiencies.

was enhanced upon treatment with G-protein inactivating agents such as urea and alkaline pH, with the exception of trypsin. This is indicative of different levels of stringency associated with various pharmacological readouts such as ligand binding and G-protein coupling. Our results show that treatment with G-protein inactivating agents led to enhanced G-protein coupling efficiency, implying that they possibly target/inactivate the pool of G-proteins which are weakly coupled to the receptor with low affinity (see Fig. 6). Such selective uncoupling of the weakly coupled pool of G-proteins leaves the strongly coupled pool unaffected. We believe that coupling efficiencies of different pools of G-proteins to the receptor could be one of the mechanisms implicated in the action of the inactivating agents, thereby elucidating the observed heterogeneity in G-protein coupling efficiency to serotonin$_{1A}$ receptors.

G-proteins go through a variety of co/post translational modifications in the α and γ subunits, which helps not only in proper localization of G-proteins in membranes but also distribution of the complex and its subunits to appropriate membrane microdomains upon activation [84–87]. The different types of lipid modifications observed in case of heterotrimeric G-proteins include myristoylation, palmitoylation and isoprenylation [88]. A working model of GPCR activation envisages GPCRs to be localized in nonrandom microdomains in biological membranes. Upon activation by agonists, presence of specific lipids could be essential for driving the necessary conformational change of GPCRs. Interestingly, specific lipids have been reported to be important for the function and localization of G-proteins [89–91]. The observed changes in G-protein coupling (Fig. 4) by inactivating agents could have its origin in the modification of lipid/G-protein interaction which would eventually affect receptor/G-protein interaction. Moreover, the contribution of any possible change in membrane physical properties (such as fluidity or curvature) induced by the inactivating agents in altering receptor conformation cannot be ruled out. In addition, treatment with inactivating agents could result in changes in receptor oligomerization due to modulation of lipid-protein interaction in cell membranes.

Our results show that G-protein inactivating agents provide a handle to modulate signaling by GPCRs by selectively inhibiting agonist binding. This could be useful in achieving varying signaling states of the receptor by fine tuning the conditions of G-protein inactivation, and in crystallization of GPCRs bound to either agonists or antagonists, allowing structure determination of GPCRs in specific conformational states. Another interesting application could be in cases where inactivation of endogenous G-proteins is necessary.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbamem.2020.183265.

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