

Temperature-dependent interaction of the bovine hippocampal serotonin_{1A} receptor with G-proteins

Vaishali Javadekar-Subhedar and
Amitabha Chattopadhyay*

Centre for Cellular and Molecular Biology, Uppal Road,
Hyderabad 500 007, India

Summary

The ligand binding and G-protein coupling of the bovine hippocampal 5-HT_{1A} receptor as a function of temperature was monitored. There is an almost complete and irreversible loss in agonist binding at 50°C. However, the antagonist binding is reduced only by 50%, and this could be reversed if the temperature is lowered to 25°C. Interestingly, the agonist binding of the 5-HT_{1A} receptor in membranes exposed to 50°C is inhibited to a much lesser extent by GTP- γ -S, a non-hydrolysable analogue of GTP, indicating uncoupling of the 5-HT_{1A} receptor to G-proteins at 50°C. We propose that high temperature selectively and irreversibly inactivates G-proteins thereby affecting G-protein-receptor interaction and agonist binding of the 5-HT_{1A} receptor.

Keywords: Temperature, 5-HT_{1A} receptor, GTP- γ -S, bovine hippocampus, G-protein.

Abbreviations: BCA, bicinchoninic acid; BSA, bovine serum albumin; GTP- γ -S, guanosine-5'-O-(3-thiotriphosphate); 5-HT, 5-hydroxytryptamine; 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; *p*-MPPI, 4-(2'-methoxy)-phenyl-1-[2'-(*N*-2''-pyridinyl)-*p*-iodobenzamido]ethyl-piperazine; PMSF, phenylmethylsulfonyl fluoride; Tris, *tris*-(hydroxymethyl)amino-methane.

Introduction

Serotonin (5-hydroxytryptamine or 5-HT) is an intrinsically fluorescent [1], biogenic amine that acts as a neurotransmitter and is found in a wide variety of sites in the central and peripheral nervous system [2]. Serotonergic signalling appears to play a key role in the generation and modulation of various cognitive and behavioural functions such as sleep, mood, pain, addiction, locomotion, sexual activity, depression, anxiety, alcohol abuse, aggression and learning [3–5]. Disruptions in serotonergic systems have been implicated in the aetiology of mental disorders such as schizophrenia, migraine, depression, suicidal behaviour, infantile autism, eating disorders and obsessive compulsive disorder [4,6,7].

Serotonin exerts its diverse actions by binding to distinct cell surface receptors, which have been classified into many groups [8]. Serotonin receptors are members of a super-family of seven transmembrane domain receptors [9] that

couple to GTP-binding regulatory proteins (G-proteins) [10]. Among the 14 sub-types of serotonin receptors, the G-protein coupled 5-HT_{1A} receptor is the best characterized one for a variety of reasons [11]. The hippocampal 5-HT_{1A} receptor is negatively coupled to the adenylate cyclase system through G_i-proteins [12]. We earlier partially purified and solubilized the 5-HT_{1A} receptor from bovine hippocampus in a functionally active form [13,14] and have shown modulation of ligand binding by metal ions, guanine nucleotides, alcohols and covalent modifications of the disulfide and sulfhydryl groups [11,15–19].

In this study, we monitored ligand binding and G-protein coupling of the 5-HT_{1A} receptor from bovine hippocampal membranes as a function of temperature, and observed striking differences between the agonist and the antagonist binding to the 5-HT_{1A} receptor at 50°C. Agonist binding at 50°C shows a drastic reduction, which is found to be irreversible since lowering the temperature does not result in recovery of binding. Interestingly, these features are not observed in the case of antagonist binding. Based on the previously reported differential discrimination of G-protein coupling to the 5-HT_{1A} receptor by the agonist and the antagonist [17], we demonstrate that irreversible inactivation of G-protein coupling to the 5-HT_{1A} receptor takes place in a temperature-dependent manner.

Results

Figure 1 shows the effect of temperature on the specific binding of the agonist [³H]8-OH-DPAT and the antagonist [³H]*p*-MPPF to bovine hippocampal 5-HT_{1A} receptors. There is a drastic reduction in specific agonist binding with increase in temperature. The specific binding obtained when the assays were carried out at 25°C served as a control. Agonist binding is reduced to 58% of control at 37°C and there is a progressive reduction in binding with further increase in temperature. At 50°C, agonist binding is almost completely abolished (i.e. 98% reduction). In sharp contrast to this, the reduction in specific antagonist binding with increase in temperature is considerably less and 83% of control is retained at 37°C. Even at 50°C, more than 50% of the original antagonist binding activity is retained. The sensitivity of the agonist and antagonist binding to increase in temperature therefore appears to be markedly different.

The binding parameters of the agonist [³H]8-OH-DPAT determined by Scatchard analysis under these conditions are shown in Table 1. As can be seen from the table, both binding affinity and number of binding sites show an overall decrease with increase in temperature, and, at 45°C, both these parameters show almost 50% reduction. Binding parameters at 50°C could not be determined owing to high background.

*To whom correspondence should be addressed.
e-mail: amit@ccmb.res.in

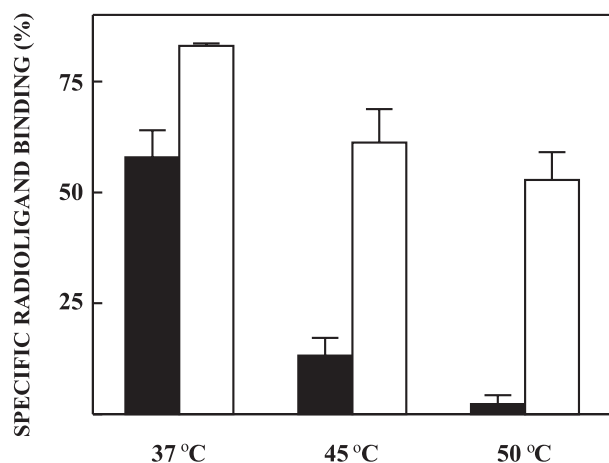


Figure 1. Effect of temperature on the specific binding of the agonist [^3H]8-OH-DPAT (filled bars) and the antagonist [^3H]p-MPPF (empty bars) to the 5-HT $_{1A}$ receptor from bovine hippocampal membranes. Values are expressed as a percentage of the specific binding obtained when the assays were carried out at 25°C. The data shown are the means \pm SEM of triplicate points from three independent experiments. See Experimental procedures for other details.

Although selective 5-HT $_{1A}$ agonists such as 8-OH-DPAT were discovered long back [20], the development of selective 5-HT $_{1A}$ antagonists has been relatively slow and less successful. A few years back, two specific antagonists for the 5-HT $_{1A}$ receptor, p-MPPI and p-MPPF, were introduced [21,22]. These compounds bind specifically to the 5-HT $_{1A}$ receptor with high affinity that is equivalent to the affinity displayed by the specific agonist 8-OH-DPAT. Thus, the inhibition constants (K_i) of p-MPPI and p-MPPF to the binding of 8-OH-DPAT in rat hippocampal homogenates have been found to be 2.6 and 3.3 nM, respectively [21].

The basis of the differential sensitivity of the agonist and antagonist binding to increase in temperature could be linked to coupling of the receptor to G-proteins. We have shown earlier that the agonist 8-OH-DPAT and the antagonist p-MPPF differentially discriminate G-protein coupling of 5-HT $_{1A}$ receptors from bovine hippocampus [17]. In other words, while the agonist binds only to those receptors that are coupled to G-proteins, the antagonist binds to all receptors irrespective of their state of G-protein coupling. Interestingly, our previous results with bovine 5-HT $_{1A}$ receptors showed that the binding affinity of the agonist [^3H]8-OH-DPAT to the receptor is reduced when the G-protein coupling of the receptor is compromised [17]. The reduction in binding affinity of the 5-HT $_{1A}$ receptor (see Table 1) could be due

Table 1. Binding parameters of [^3H]8-OH-DPAT binding to 5-HT $_{1A}$ receptors from bovine hippocampal membranes at high temperatures^a.

Temperature (°C)	K_d (nM)	B_{max} (fmol/mg of protein)
25	0.74 \pm 0.05	92 \pm 9.30
37	0.72 \pm 0.06	60 \pm 1.49
45	1.66 \pm 0.20	48 \pm 2.88

^aBinding parameters shown in the table represent the means \pm SEM of duplicate points from three independent experiments. See Experimental procedures for other details.

to the loss of G-protein coupling of the receptor due to perturbation of the G-protein conformation caused by thermal denaturation. An alternative possibility could be that the receptor conformation itself is altered owing to high temperature resulting in loss of agonist binding. However, the retention of significant specific antagonist binding (> 50%) even at the highest temperature at which binding is checked (50°C) rules this out as a major cause.

In order to check the reversibility of the reduction in specific ligand binding to the 5-HT $_{1A}$ receptor at high temperature, we pre-treated (incubated) membranes at specific high temperatures, followed by rapid cooling to 25°C, and, subsequently, ligand binding assays were carried out. The specific ligand binding activity of membranes at 25°C in the absence of any pre-treatment at high temperatures served as control. Figure 2 shows that the agonist binding could be recovered to a large extent when membranes pre-treated at 37°C are brought back to 25°C. This indicates that any change in G-protein organization is reversible to a considerable extent when the increase in temperature is moderate (37°C). However, as the temperature for the pre-treatment is increased, there is a progressive loss in the recovery of agonist binding with only 15% being recovered when membranes are pre-treated at 50°C. This shows that the change in G-protein organization induced by such high temperatures is irreversible to a large extent and cannot be reversed by lowering the temperature.

Interestingly, the antagonist binding of pre-treated membranes displays predominant reversibility in all cases (see Figure 2). This is true even when the pre-treatment is carried out at 50°C since 88% of the antagonist activity could be recovered at 25°C. This reinforces our earlier contention that the basis of the differential sensitivity of the agonist and

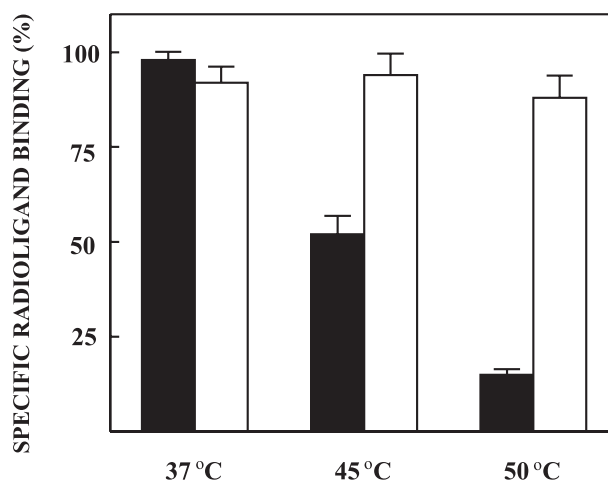


Figure 2. Effect of pre-treatment at high temperature on the specific binding of the agonist [^3H]8-OH-DPAT (filled bars) and the antagonist [^3H]p-MPPF (empty bars) to the 5-HT $_{1A}$ receptor from bovine hippocampal membranes. Membranes were incubated at high temperatures and then rapidly cooled to 25°C followed by ligand binding assay at 25°C. Values are expressed as a percentage of the specific binding obtained when the assays were carried out at 25°C in the absence of any pre-treatment at high temperature. The data shown are the means \pm SEM of triplicate points from three independent experiments. See Experimental procedures for other details.

antagonist binding to increase in temperature could have its origin in G-protein coupling of the receptor. We have shown earlier that the antagonist binding is insensitive to the extent of G-protein coupling [17]. This implies that the antagonist binding would be insensitive to any perturbation to the G-protein structure induced by temperature or other means. This is also found to be true for alterations of G-protein structure due to chemical denaturation by urea (V. Javadekar-Subhedar and A. Chattopadhyay, unpublished data).

The binding parameters of the agonist [³H]8-OH-DPAT determined by Scatchard analysis under conditions of pre-treatment at high temperatures followed by cooling to 25°C are shown in Table 2. The most striking change is observed for membranes pre-treated at 50°C compared with control membranes (see Table 1). As shown in Table 2, there is a considerable reduction in binding affinity as well as number of binding sites even after lowering the temperature to 25°C, again demonstrating the irreversible nature of the change. This explains the rather poor recovery of agonist binding for membranes pre-treated at 50°C, as shown in Figure 2. These results indicate that the conformational changes in G-proteins induced at high temperatures are irreversible to a great extent.

Our results suggest that the differential sensitivity of the agonist and antagonist binding to increasing temperature is based on the extent of coupling of the receptor to G-proteins. This can be further tested by monitoring the agonist binding to the receptor in the presence of GTP-γ-S, a non-hydrolysable analogue of GTP. Guanine nucleotides are known to regulate agonist binding of G-protein coupled receptors [23]. We have previously shown that the specific agonist [³H]8-OH-DPAT binding to hippocampal 5-HT_{1A} receptors is sensitive to guanine nucleotides and the specific binding is inhibited with increasing concentrations of GTP-γ-S [15,17]. Figure 3 shows the inhibition of specific agonist binding to the 5-HT_{1A} receptor in membranes pre-treated at 50°C by GTP-γ-S in a characteristic concentration-dependent manner. Membranes in the absence of any pre-treatment at high temperature served as control. It is apparent from the figure that inhibition of agonist binding by GTP-γ-S is less pronounced in the case of membranes pre-treated at 50°C resulting in a shift in the inhibition curve toward higher concentrations of GTP-γ-S. This indicates that the concentration of GTP-γ-S necessary to bring about the same change in agonist binding is more in the case of pre-treated membranes. In other words, pre-treatment at high temperature effectively makes the system less sensitive to the effect of GTP-γ-S, which demonstrates that the extent of G-protein

Table 2. Binding parameters of [³H]8-OH-DPAT binding to 5-HT_{1A} receptors from bovine hippocampal membranes after pre-treatment at high temperatures^b.

Temperature (°C)	K _d (nM)	B _{max} (fmol/mg of protein)
37	0.81 ± 0.09	104 ± 10.80
45	0.76 ± 0.02	86.66 ± 11.60
50	1.97 ± 0.02	41.66 ± 14

^bBinding parameters shown in the table represent the means ± SEM of duplicate points from three independent experiments. See Experimental procedures for other details.

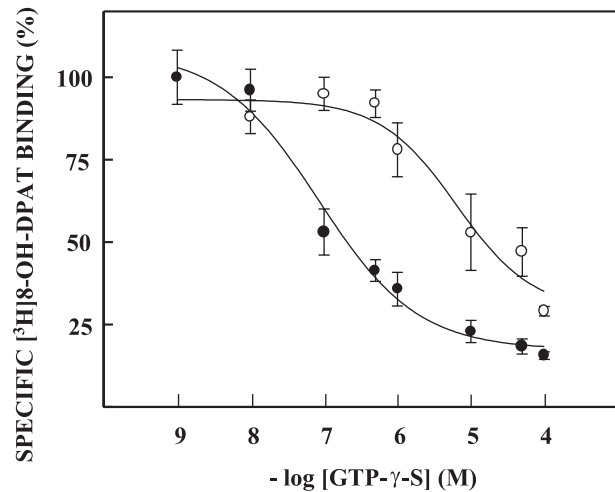


Figure 3. Effect of increasing concentrations of GTP-γ-S on the specific binding of the agonist [³H]8-OH-DPAT to the 5-HT_{1A} receptor from bovine hippocampal membranes pre-treated at 50°C (O) as described in Figure 2. The specific agonist binding to membranes at 25°C in the absence of any pre-treatment at high temperature served as control (●). Values are expressed as a percentage of the specific binding obtained in the absence of GTP-γ-S. The data points are the means ± SEM of triplicate points from three independent experiments. See Experimental procedures for other details.

coupling is reduced, probably due to alterations in the organization of G-proteins at high temperature. This conclusion is supported by a comparison of the half maximal inhibition concentrations (IC₅₀) for inhibition by GTP-γ-S under these conditions (see Table 3). The IC₅₀ value in case of membranes treated at 50°C (6.30 μM) shows an increase by ~80-fold compared with control membranes (0.08 μM). This shows that G-protein coupling is drastically altered for these membranes.

Discussion

Inactivation of G-proteins by factors such as alkaline pH [24], detergents [25,26] bacterial toxins [27], and denaturants [28] has been reported earlier. In case of muscarinic and dopamine receptors, heat pre-treatment has been reported to affect G-protein coupling to the receptors [29–31]. In this study, we monitored ligand binding and G-protein coupling of the bovine hippocampal 5-HT_{1A} receptor at high temperatures. Our results show striking differences in sensitivity of the agonist and antagonist binding to an increase in temperature and pre-treatment at high temperatures. The basis of this differential sensitivity is shown to be differential

Table 3. IC₅₀ values for inhibition of specific [³H]8-OH-DPAT binding to 5-HT_{1A} receptors from native and high temperature pre-treated bovine hippocampal membranes by GTP-γ-S.

Membrane condition	IC ₅₀ (μM)
Native membranes at 25°C without pre-treatment	0.08
Membranes pre-treated at 45°C	0.18
Membranes pre-treated at 50°C	6.30

G-protein coupling of the receptor under various conditions as demonstrated by monitoring agonist binding in presence of GTP- γ -S. These results imply that G-proteins are irreversibly inactivated at high temperature.

The possibility of thermal denaturation of the receptor itself upon exposure to high temperature should be considered. Near complete recovery of the antagonist binding upon cooling membranes pre-treated at high temperatures (see Figure 2) suggests that the receptor was not damaged to a great extent, and any conformational changes of the receptor were predominantly reversible in nature. It is difficult quantitatively to ascertain from our data whether there is any change in the receptor conformation due to high temperature. We cannot totally rule out, therefore, the possibility that the receptor undergoes some change induced by temperature. Even if this is true, this cannot be a major factor since the thermal stability of transmembrane proteins is known to be very high [32]. For example, the thermostability of the analogous 5-HT₄ receptor, which belongs to the family of G-protein-coupled serotonin receptors, has recently been demonstrated [33]. This receptor does not undergo any thermal denaturation even when it is incubated at 45°C for 3 h. On the other hand, G-proteins are soluble in nature (although attached to membranes by fatty acylation) and thus are more likely to be thermally denatured and inactivated at high temperatures. This is supported by the recent observation that for different types of G-protein coupled receptors (including the 5-HT_{1A} receptor) expressed in different cell lines, it is the G-proteins coupled to the receptors rather than the receptors themselves, that are inactivated when exposed to the denaturant urea [28]. It is worthwhile mentioning here that the conformation of the sub-type of G-proteins that is coupled to the hippocampal 5-HT_{1A} receptor, namely G_i [12], has previously been reported to be sensitive to temperature [34].

In summary, we report here the irreversible inactivation of G-proteins coupled to the bovine 5-HT_{1A} receptor at high temperatures possibly due to thermal denaturation. These results imply that it is possible to use high temperature as one of the agents for selective inactivation of G-proteins without affecting the receptor. This may be useful in cases where inactivation of endogenous G-proteins is necessary.

Experimental procedures

EDTA, EGTA, MgCl₂, MnCl₂, PMSF, Tris, iodoacetamide, polyethylenimine, serotonin, sodium azide, and sucrose were obtained from Sigma Chemical Co. (St Louis, MO, USA). [³H]8-OH-DPAT (specific activity 124.0 Ci/mmol) and [³H]*p*-MPPF (specific activity 66.2 Ci/mmol) were purchased from DuPont New England Nuclear (Boston, MA, USA). *p*-MPPF was from Research Biochemicals International (Natick, MA, USA). GTP- γ -S was purchased from Boehringer Mannheim (Germany). BCA reagent kit for protein estimation was obtained from Pierce (Rockford, IL, USA). All other chemicals used were of the highest available quality. GF/B glass micro-fibre filters were from Whatman International (Kent, UK). Fresh bovine brains were obtained from a local slaughterhouse within 10 min of death and the hippocampal region carefully dissected out. The hippocampi were immediately flash frozen in liquid nitrogen and stored at -70°C until needed.

Native hippocampal membranes were prepared as described earlier [15]. Bovine hippocampal tissue (~100 g) was homogenized

as 10% (w/v) in a polytron homogenizer in buffer A (2.5 mM Tris, 0.32 M sucrose, 5 mM EDTA, 5 mM EGTA, 0.02% sodium azide, 0.24 mM PMSF, 10 mM iodoacetamide, pH 7.4). The homogenate was centrifuged at 900 × g for 10 min at 4°C. The supernatant was filtered through four layers of cheesecloth and the pellet was discarded. The supernatant was further centrifuged at 50 000 × g for 20 min at 4°C. The resulting pellet was suspended in 10 volumes of buffer B (50 mM Tris, 1 mM EDTA, 0.24 mM PMSF, 10 mM iodoacetamide, pH 7.4) using a hand-held Dounce homogenizer and centrifuged at 50 000 × g for 20 min at 4°C. This procedure was repeated until the supernatant was clear. The final pellet (native membranes) was re-suspended in a minimum volume of 50 mM Tris buffer (pH 7.4), homogenized using a hand-held Dounce homogenizer, flash frozen in liquid nitrogen and stored at -70°C till further use. Protein concentration was determined using BCA reagent with BSA as a standard [35].

Ligand binding assays at various temperatures were carried out as described by Harikumar and Chattopadhyay [15] with some modifications. Tubes in duplicate containing 1 mg of total protein in a total volume of 1 ml of buffer C (50 mM Tris, 1 mM EDTA, 10 mM MgCl₂, 5 mM MnCl₂, pH 7.4) for agonist binding or in buffer D (50 mM Tris, 1 mM EDTA, pH 7.4) for antagonist binding, were incubated at different temperatures in the presence of 0.29 nM [³H]8-OH-DPAT or 0.5 nM [³H]*p*-MPPF. The necessary incubation time was found to be less than 2 min at the temperatures used in these experiments to attain equilibrium for ligand binding except for assays performed at 25°C when the incubation time was 1 h. Non-specific binding was determined by performing the assay in the presence of 10 μM of unlabelled 5-HT (for agonist binding) and unlabelled *p*-MPPF (for antagonist binding). The incubation was terminated by rapid filtration under vacuum in a millipore multiport filtration apparatus through Whatman GF/B (1.0 μm pore size) 2.5 cm diameter glass micro-fibre filters which were pre-soaked in 0.15% (w/v) polyethylenimine for 3 h [36]. The filters were then washed twice with 5 ml of ice-cold water, dried and the retained radioactivity was measured in a Packard Tri-Carb 1500 scintillation counter using 5 ml of scintillation fluid. In experiments involving pre-treatment at high temperatures, membranes were heated to various temperatures in a Julabo F-25 water bath. The time of pre-treatment was same as that of incubation at high temperatures and was less than 2 min in all cases. The samples were then rapidly cooled to 25°C and ligand binding assays were performed at 25°C as described above.

Saturation binding assays were carried out using varying concentrations (0.1–7.5 nM) of radio-labelled agonist ([³H]8-OH-DPAT) with native membranes containing 1 mg of total protein. Non-specific binding was measured in the presence of 10 μM unlabelled 5-HT. Binding assays were carried out at various temperatures as mentioned above. Binding data were analysed as described earlier [11]. The concentration of the bound ligand (RL*) was calculated from the equation:

$$RL^* = 10^{-9} \times B / (V \times SA \times 2220) \text{ M}$$

where B = bound radioactivity in disintegrations per min (dpm) (i.e. total dpm – non-specific dpm), V is the assay volume in millilitres, and SA is the specific activity of the radioligand. Scatchard plots (i.e. plots of [RL*]/[L*] vs. [RL*]) were analysed using Sigma-Plot (version 3.1) in an IBM PC. The dissociation constants (K_d) were obtained from the negative inverse of the slope, determined by linear regression analysis of the plots (r = 0.85–0.92). The B_{max} values were obtained from the intercept on the abscissa. The B_{max} values reported in Tables 1 and 2 have been normalized with respect to the amount of native membrane used.

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