Multiple conformations of 5-HT_{2A} and 5-HT_{2C} receptors in rat brain. An autoradiographic study with [¹²⁵I](±)DOI

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Summary

Earlier autoradiographic studies with the 5-HT₂ receptor agonist [¹²⁵I](±)DOI in human brain showed unexpected biphasic competition curves for various 5-HT_{2A} antagonists. We have performed similar studies in rat brain regions with selective 5-HT_{2A} (M100907) and 5-HT_{2C} (SB242084) antagonists together with ketanserin and mesulergine. The effect of GTP analogs on antagonist competition was also studied. Increasing concentrations of Gpp(NH)p or GTPγS resulted in a maximal inhibition of [¹²⁵I](±)DOI specific binding of approximately 50%. M100907 competed biphasically in all regions. In presence of 100μM Gpp(NH)p, M100907 still displaced biphasically the remaining [¹²⁵I](±)DOI binding. Ketanserin showed biphasic curves in some regions, and monophasic curves in others. In the latter, Gpp(NH)p evidenced an additional high affinity site. SB242084 competed biphasically in brainstem nuclei and monophasically in the other regions. In most areas, SB242084 affinities were not notably altered by Gpp(NH)p. Mesulergine competed monophasically in all regions without alteration by Gpp(NH)p.

These results conform with the extended ternary complex model of receptor action: receptor exist as an equilibrium of multiple conformations, i.e., ground (R), partly activated (R*) and activated G-protein coupled (R*G) conformation/s. Thus, [¹²⁵I](±)DOI would label multiple conformations of both 5-HT_{2A} and 5-HT_{2C} receptors in rat brain and M100907 and ketanserin would recognize these conformations with different affinities.

Keywords: Receptor autoradiography, G-protein coupling, inverse agonist

Introduction

The group of serotonin type 2 receptors (5-HT₂ receptors) is comprised by three subtypes: 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C} receptors. They share several characteristics in common such as some degree of sequence homology as well as the stimulation of the same primary effector system (phospholipase C, PLC) via G-protein coupling (Hoyer et al. 1994; Barnes and Sharp 1999). In addition, the 5-HT₂ receptor family is peculiar when compared to other G-protein coupled receptors because they are downregulated upon antagonist treatments in some cases (Roth et al. 1998; Yadav et al. 2011). As a consequence of this structural homology, the pharmacological characterisation of each of these receptors has been difficult due to the lack of selective drugs that really differentiate among them (Baxter et al. 1995). Since the first description of 5-HT₂ receptors by means of radioligand techniques (Peroutka and Snyder 1979), several radiolabeled drugs have been used to study these receptors. Some of them are phenylalkylamines such as DOB (4-bromo-2,5-dimethoxyphenyl-isopropilamine) and DOI (4-iodo-2,5-dimethoxyphenyl-isopropilamine) in their radiochemical forms [³H]DOB and [¹²⁵I]DOI respectively. These compounds behave as agonists at 5-HT₂ receptors and present similar affinities for the three different subtypes (Nelson et al. 1999; Knight et al. 2004). Certain studies performed with both radioligands led to the hypothesis of the existence of different subtypes of 5-HT_{2A} receptors in different mammalian species (McKenna and Peroutka 1989)(Pierce and Peroutka 1989). In contrast, other studies carried out in cell lines heterologously expressing either rat 5-HT_{2A} or human 5-HT_{2A} receptors demonstrated that the different binding sites labelled by [¹²⁵I]DOI and [³H]DOB respectively corresponded to multiple states of a same receptor and not multiple receptor subtypes (Branchek et al. 1990) (Teitler et al. 1990) , being these multiple states a consequence of the receptor coupling to G-proteins. Later studies performed with [¹²⁵I]DOI in native tissues by means of receptor autoradiography described that several antagonists (ketanserin, spiperone and cinanserin) displayed biphasic curves when displacing [¹²⁵I]DOI binding from some human brain regions (Waeber and Palacios 1994). These two binding site populations recognised by the agonist radioligand and some antagonist competitors did not correspond clearly to different receptor subtypes, so it was hypothesised that allosteric phenomena were taking place. Subsequently, more selective antagonists for 5-HT_{2A} and 5-HT_{2C} receptors were synthesized, among them M100907 or volinanserin (formerly MDL100,907) and SB242084 respectively (Kehne et al. 1996; Kennett et al. 1997). We extended previous studies performed in human brain by including these more selective compounds as competitors and by using a permanent cell

line heterologously expressing human 5-HT_{2A} receptors (Lopez-Gimenez et al. 2001); competition assays under these new experimental conditions with selective antagonists for 5-HT_{2A} receptors, i.e. ketanserin and M100907, resulted again in curves displaying two binding sites regardless the sample origin (native tissue or cultured cells). More recently, the functional characterisation of 5-HT_{2A} receptors expressed in CHO cells in parallel with radioligand binding competition assays led to the proposal of a new model of multiple active interconvertible conformations of dimeric receptors to explain this biphasic component observed for some antagonists (Brea et al. 2009).

The aims of the present work were to further analyze the atypical biphasic profile of ketanserin when displacing [¹²⁵I]DOI binding in different rat brain areas, and at the same time to examine the pharmacological behaviour of mesulergine, M100907 and SB242084 in equivalent competition experiments. In an attempt to elucidate the nature of the heterogeneous binding sites labelled by [¹²⁵I]DOI in rat brain, the effect of non-hydrolysable GTP analogues on [¹²⁵I]DOI binding and antagonist displacements was also considered.

Methods

Animal brains

Male Wistar rats (200-300 g) were purchased from Iffa Credo (Lyon, France). The animals were killed by decapitation and the brains were removed quickly and frozen on dry ice. Animal care followed the spanish legislation on "Protection of Animals Used in Experimental and Other Scientific Purposes", in agreement with the E.E.C. regulations on the care of laboratory animals (O.J. of E.C. L358/1 18/12/1986). Tissue sections, 14 μm thick, were cut using a microtome-cryostat (Microm HM500-OM, Walldorf, Germany), thaw-mounted onto APTS (3-aminopropyltriethoxysilane, Sigma, St. Louis, Mo, USA) coated slides, and kept at -20°C until use.

Receptor autoradiography

[^3H]mesulergine (82.0 Ci/mmol) and [^{125}I]($\pm$)DOI (2200 Ci/mmol) were purchased from Amersham (UK) and DuPont NEN (Boston, MA) respectively. Ritanserin, mesulergine, ketanserin and mianserin were purchased from Research Biochemical International (Natick, MA). Gpp(NH)p (5'-guanylylimidodiphosphate), GTP γ S (guanosine-5'-O-(3-thio)triphosphate), 5-HT (5-Hydroxytryptamine) and ($\pm$)DOI (($\pm$)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane) were purchased from Sigma (UK). MDL100,907 ((R)-(+)-4-[1-Hydroxy-1(2,3-dimethoxyphenyl)methyl]N-2-4-fluorophenylethyl)piperidine) was a generous gift from Dr. M. Galvan (Marion Merrell Dow, Strasbourg, France). SB242084 (6-Chloro-5-methyl-1-[6-(2-methylpyridin-3-yloxy)pyridin-3-yl-carbamoyl]indoline) was kindly provided by Smithkline Beecham Pharmaceuticals (UK).

[^{125}I]($\pm$)DOI saturation experiments were carried out within the 0-1.5 nM concentration range to determine total binding. Non-specific binding was determined at three different radioligand concentrations, and the values obtained were used to generate a linear regression curve. To obtain specific binding for each radioligand concentration, the calculated value of non-specific binding was subtracted from the corresponding total binding value. In competition experiments, [^{125}I]($\pm$)DOI was used at a concentration between 100 and 150 pM. Non-specific binding in saturation and competition experiments was defined as that remaining in the presence of 10^{-5}M ritanserin. Experimental procedures were as described (McKenna and Peroutka 1989) with some modifications: cryostat tissue

sections were air dried, pre-incubated for 30 min at room temperature in 50 mM Tris-HCl pH 7.4, 0.1% ascorbate, 4mM CaCl₂, and incubated in the presence of radioligand in the same buffer during 60 min at room temperature. After incubation, tissue sections were washed during 10 min twice in ice cold buffer, dipped in distilled ice cold water and dried rapidly under a cold air stream. Tissues were exposed to tritium-sensitive film (Hyperfilm-³H, Amersham, UK) together with plastic ¹²⁵I-standards (¹²⁵I-microscales, Amersham, UK) for a period of time between 2 and 5 days.

In saturation studies, [³H]mesulergine was used at increasing concentrations between 0-5 nM in rat choroid plexus to obtain values of total binding. Non-specific binding and specific binding was determined as for [¹²⁵I](±)DOI saturation experiments. In competition studies [³H]mesulergine was used at a concentration of 0.5 nM. Non-specific binding in saturation and competition experiments was defined as that remaining in the presence of in the presence of 10⁻⁵M mianserin. The incubation protocol for [³H]mesulergine was as described previously (Pazos and Palacios 1985). Briefly, cryostat tissue sections were air dried, pre-incubated for 15 min at room temperature in 50 mM Tris-HCl pH 7.6, 10 mM, and incubated in the presence of radioligand in the same buffer during 120 min at room temperature. After incubation, tissue sections were washed for 10 min twice in ice cold buffer, dipped in distilled ice cold water and dried rapidly under a cold air stream. Tissues were exposed to tritium-sensitive film (Hyperfilm-³H, Amersham, UK) together with plastic ³H-standards (³H-microscales, Amersham, UK) for 1 week. Quantitative analysis of the autoradiograms obtained with both radioligands was done with a computerized image analysis system (MCID M4, St. Catharines, Ontario, Canada).

Data analysis

Estimates of binding parameters (K_d , B_{max} , IC_{50} and Hill slope) were derived with the GraphPad Prism software (GraphPad Software Inc, San Diego, CA) from the data obtained after quantification of the autoradiograms generated in saturation and competition experiments. The program calculated K_i values according to the Cheng-Prusoff equation (Cheng and Prusoff 1973) using the IC_{50} previously determined in competition experiments, the mean of the K_d for the radioligand in the different brain regions obtained in saturation experiments and the concentration of free radioligand used in competition experiments.

Results

Pharmacological characterisation of [¹²⁵I](±)DOI binding sites

Saturation experiments performed with [¹²⁵I](±)DOI in different rat brain areas resulted in affinity constants (K_d) in the subnanomolar or low nanomolar range (Table 1; Fig. 1), being frontal cortex, claustrum and choroid plexus the brain areas that presented the highest densities of binding sites. Inhibition experiments with Gpp(NH)p, a non-hydrolysable GTP analogue, were carried out to examine the effect of receptor/G-protein uncoupling on [¹²⁵I](±)DOI binding. Figure 2A displays inhibition curves obtained in two representative regions, frontal cortex and choroid plexus, with pIC_{50} values of 6.69 ± 0.19 and 6.72 ± 0.35 respectively (mean $\pm$ s.e.m., $n=4$ animals). In all the regions examined, a maximum of approximately 50% of specific binding was inhibited. The effect of GTP γ S, another GTP analogue, on [¹²⁵I](±)DOI binding was also studied resulting in inhibition parameters similar to those of Gpp(NH)p: pIC_{50} 7.55 ± 0.18 in frontal cortex and 6.85 ± 0.24 in choroid plexus (mean $\pm$ s.e.m., $n=4$ animals) with approximately 50% of maximal inhibition of specific binding in both brain areas (Fig. 2B). For subsequent experiments, Gpp(NH)p at a concentration of 100 μ M was used to uncouple receptors from G-proteins.

Competition studies were carried out at a [¹²⁵I](±)DOI concentration between 100-150 pM. Non-specific binding was determined with 10^{-5} M ritanserin, resulting in a remaining autoradiographic signal hardly distinguishable from film background (Fig. 3B). Four antagonists, M100907, ketanserin, SB242084 and mesulergine, were competed against [¹²⁵I](±)DOI in absence or presence of 100 μ M Gpp(NH)p. Addition of Gpp(NH)p resulted in a reduction of approximately 50% of the specific binding observed in absence of competing drug. Inhibition parameters obtained in competition experiments are summarised in Table 2, and Figure 4 displays inhibition curves for three representative rat brain regions. When [¹²⁵I](±)DOI binding was inhibited by M100907, the data points were best fitted by a two site model in the different rat brain regions considered in this study. The high affinity site was in the low nanomolar range, except in the choroid plexus where this high affinity site displayed 100-fold lower affinity than in the rest of the regions. In presence of Gpp(NH)p, M100907 still displaced the remaining [¹²⁵I](±)DOI binding sites in a biphasic manner in the majority of brain regions, with affinities about two orders of magnitude lower than those observed without the GTP analogue. In some regions (choroid plexus, olfactory tubercle and claustrum) a single binding site was observed in presence of

Gpp(NH)p, with an affinity similar to the low affinity site observed in absence of Gpp(NH)p (Table 2).

The competition of [125 I]($\pm$)DOI with ketanserin resulted in monophasic inhibition curves in forebrain regions (frontal cortex, caudate-putamen, nucleus accumbens, olfactory tubercle and claustrum) and in biphasic curves in the analysed brainstem nuclei (dorsal motor nucleus of vagus, hypoglossal nucleus and facial nucleus). The choroid plexus also presented two binding site populations, with affinity constants similar to those observed in the brainstem nuclei. However, the percentage of high affinity sites was much lower in choroid plexus (Table 2). The presence of Gpp(NH)p in the incubation medium revealed an additional high affinity site in the forebrain areas (Table 2; Fig. 5), whereas no significant changes were observed in the affinity constants of the two sites detected in brainstem nuclei and choroid plexus. In this latter case, however, Gpp(NH)p induced a marked increase (from 20% to 60% approximately) in the percentage of high affinity sites (Table 2).

SB242084 presented monophasic displacement curves against [125 I]($\pm$)DOI in the different rat forebrain areas studied as well as in choroid plexus, although the affinity in this structure was about two orders of magnitude higher. On the other hand, data points corresponding to the brainstem nuclei were best fitted by a two site model (Table 2, Fig. 4), with the high affinity site similar to the one observed in choroid plexus, and the low affinity site similar to the one observed in forebrain regions. In the majority of the rat brain regions, presence of Gpp(NH)p did not induce notable changes in the affinities and relative proportions of high and low affinity sites. However, there were two regions, nucleus accumbens and claustrum, where significant changes were observed: in both regions an additional high affinity site was observed that accounted for 30% approximately of the total [125 I]($\pm$)DOI binding sites in presence of Gpp(NH)p (Table 2; Fig. 5).

Mesulergine was the only antagonist compound that presented a single high affinity site in all the rat brain regions analysed which was not modified by the presence of Gpp(NH)p (Table 2, Fig.5).

[3 H]mesulergine autoradiography studies.

Saturation experiments in absence or presence of 100 μ M Gpp(NH)p were performed with rat brain sections containing choroid plexus resulting in no significant differences either in K_d values (0.61 ± 0.11 nM *versus* 1.05 ± 0.26 nM) as in maximum specific binding sites (836.20 ± 40.9 fmol/mg *versus* 924.30 ± 79.9 fmol/mg). Competition

experiments against [³H]mesulergine in choroid plexus were carried out with the antagonists M100907, ketanserin, SB242084 and mesulergine, and with the agonists 5-HT and (±)DOI. Inhibition constants for each compound are summarised in Table 3. All the competitors displayed monophasic displacement curves, being SB242084 and M100907 the drugs that presented the highest and lowest affinity respectively for [³H]mesulergine binding sites in rat choroid plexus. The presence of Gpp(NH)p did not change the inhibition parameters for any competitor drug. As expected from the results from saturation experiments, addition of 100µM Gpp(NH)p did not alter total [³H]mesulergine binding in absence of competitor drugs.

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Discussion

The aim of the present work was to further explore into the nature of the previously observed biphasic profile of ketanserin and other 5-HT₂ compounds when displacing [¹²⁵I]DOI binding in autoradiographic studies (Waeber and Palacios 1994; Lopez-Gimenez et al. 2001). For this purpose, we have expanded the study in rat brain by including two more selective antagonists (M100907 and SB242084) together with two more classical compounds (ketanserin and mesulergine) already used in the aforementioned report. In addition, the role of receptor/G-protein coupling in this atypical pharmacological profile was also investigated by examining the effect of non-hydrolysable GTP analogues on [¹²⁵I]DOI binding and antagonist displacements. The results obtained from the present series of experiments indicate that the biphasic profile previously shown for ketanserin is also shared by the more selective 5-HT_{2A} antagonist M100907 and this happens both at 5-HT_{2A} and 5-HT_{2C} receptors. Furthermore, the presence of a non-hydrolyzable GTP analogue (Gpp(NH)p) altered the affinities for [¹²⁵I]DOI binding sites displayed by ketanserin, M100907, and SB242084, suggesting the lack of true silent antagonism of these compounds.

After the first description of [¹²⁵I](±)DOI as a radioligand for autoradiography (McKenna et al. 1987), it was later more extensively characterised for the autoradiographic visualisation of 5-HT_{2A} and 5-HT_{2C} receptors in rat brain (Appel et al. 1990). Our present results concerning the affinity constant of [¹²⁵I](±)DOI for its binding sites in rat brain (K_d in the low nanomolar range) agree well with those described previously in autoradiographic studies (McKenna et al. 1989) or in binding studies using rat brain homogenates (Johnson et al. 1987) (McKenna et al. 1987; Glennon et al. 1988). Given the ability of [¹²⁵I](±)DOI to label both 5-HT_{2A} and 5-HT_{2C} receptors, it is first of all necessary to establish the nature of the sites labelled by this radioligand in the different rat brain regions considered in the present study. Very likely, the binding sites labelled by [¹²⁵I](±)DOI correspond to 5-HT_{2A} receptors predominantly in rat forebrain regions, to 5-HT_{2C} receptors in rat choroid plexus and to both receptor subtypes in rat brainstem nuclei. This is supported by several experimental evidences: 1) [¹²⁵I](±)DOI at the concentration used in competition experiments (0.10-0.15 nM) does not label other neurotransmitter receptors different from the 5-HT₂ receptor family in rat brain (Appel et al. 1990). 2) The possibility that [¹²⁵I](±)DOI at the concentration used labelled 5-HT_{2B} receptors in rat brain is very unlikely since it seems that this receptor subtype is not present to a significant extent in the brain of another rodent species (Loric et al. 1992) and no 5-HT_{2B} receptor

mRNA could be detected in rat brain by means of *in situ* hybridisation techniques (unpublished observations). 3) Visualisation of choroid plexus by receptor autoradiography in mouse brain is possible by using [125 I]($\pm$)DOI, [3 H]5-HT or [3 H]mesulergine as radioligands. The complete absence of autoradiographic signal in choroid plexus from genetically engineered mice devoid of expression of 5-HT_{2C} receptors indicates that this is the only receptor labelled by these radiochemicals in this particular structure of rodent brain (Lopez-Gimenez et al. 2002). 4) The pharmacological data obtained in the present study with SB242084, a 5-HT_{2C} receptor selective compound (Kennett et al. 1997), support the 5-HT_{2A} and/or 5-HT_{2C} receptor nature of these [125 I]($\pm$)DOI binding sites. Thus, SB242084 showed monophasic displacement curves in the analyzed forebrain structures with a pK_i close to that described for human 5-HT_{2A} receptors expressed in permanent cell lines (pK_i $\approx$ 6.8), whereas in rat choroid plexus it also presented a single site with a pK_i very similar to that described for human 5-HT_{2C} receptors expressed in permanent cell lines (pK_i $\approx$ 9.0) (Kennett et al. 1997). In addition, SB242084 displayed biphasic competition curves in rat brainstem motor nuclei differentiating two binding site populations: the high affinity site presented an affinity constant very similar to that found in choroid plexus, whereas the low affinity site presented an affinity constant similar to that found in forebrain structures. These results agree with the presence of both 5-HT_{2A} and 5-HT_{2C} receptors in the rat facial, hypoglossal and motor nucleus of the vagus, where the presence of encoding mRNA for both receptors has previously been demonstrated (Pompeiano et al. 1994; Wright et al. 1995). Although the presence of mRNA for both 5-HT_{2A} and 5-HT_{2C} receptors has been described also in some of the forebrain nuclei considered in the present study as models of 5-HT_{2A}-enriched regions (e.g. claustrum, accumbens, olfactory tubercle (Pompeiano et al. 1994) (Wright et al. 1995), the presence of a relevant population of [125 I]($\pm$)DOI-labeled 5-HT_{2C} receptors in these brain regions is unlikely, since had it existed, it would have been detected most probably by SB242084. These findings with SB242084 corroborate the selectivity of this compound for 5-HT_{2C} versus 5-HT_{2A} receptors in rat brain, and indicate its suitability for the pharmacological characterisation of these receptors.

At variance with SB242084, mesulergine displaced [125 I]($\pm$)DOI binding sites in a monophasic manner presenting the same inhibition constant in all the rat brain regions analysed. This was an expected observation since mesulergine presents very similar affinities for both receptor subtypes in rat brain and therefore does not differentiate

between 5-HT_{2A} and 5-HT_{2C} receptors in this species, in contrast to what is observed in pig and human brain (Hoyer et al. 1986).

The pharmacological profile obtained with ketanserin in rat forebrain structures, where a single high affinity site was observed, agrees with the affinity of this compound for 5-HT_{2A} receptors. These results differ from those described in human brain using the same radioligand and competitor: in this case ketanserin displayed biphasic curves when inhibiting [¹²⁵I](±)DOI binding sites in cortex, claustrum and striatum (Waeber and Palacios 1994; Lopez-Gimenez et al. 2001). On the other hand, the biphasic competition curves observed with ketanserin in rat brainstem nuclei most likely correspond to the inhibition of [¹²⁵I](±)DOI labelling of 5-HT_{2A} and 5-HT_{2C} receptors by ketanserin (high and low affinity sites respectively). In contrast, ketanserin presented an unexpected high affinity site in choroid plexus in addition to the lower affinity site which is in accordance with 5-HT_{2C} receptors. Unexpectedly also, competition binding assays performed with the more selective 5-HT_{2A} receptor antagonist M100907, resulted in biphasic displacement curves in all the regions analysed, and independently, therefore, whether [¹²⁵I](±)DOI labelling corresponded to 5-HT_{2A} receptors predominantly (forebrain), to 5-HT_{2C} receptors (choroid plexus) or to both simultaneously (brainstem nuclei).

In an attempt to further analyse the nature of the different [¹²⁵I](±)DOI binding sites detected by the antagonists, competition experiments in the presence of GppNHp, a non-hydrolyzable analogue of GTP were conducted. Previous studies (Appel et al. 1990) have analyzed the effect of GTP analogues (GTPγS and Gpp(NH)p) on [¹²⁵I](±)DOI binding. The results obtained in that study concerning inhibition constants (IC₅₀) as well as the percentage of inhibition of [¹²⁵I](±)DOI specific binding at the maximal concentration of GTP analogue (about 55%), were very similar to the present ones for both analogues. There is, however, a point of disagreement between both studies regarding the effect of GTP analogues on [¹²⁵I](±)DOI binding in rat brain. This discrepancy, the reason for which is unclear at present, consists in the image that Appel and co-workers showed in their report where [¹²⁵I](±)DOI autoradiographic signal is completely inhibited by 100 nM GTPγS, in contrast with their own quantitative data and with our present results (see Fig. 3D). Other studies performed with homogenates of pig choroid plexus and rat and human cortex (Leonhardt et al. 1992) also described a maximal inhibition by Gpp(NH)p of 55-60% of specific [¹²⁵I](±)DOI binding. It seems thus evident that GTP analogues, even at very high concentrations, are unable to completely inhibit the binding of [¹²⁵I](±)DOI in different species. Given that such high concentrations of GTP analogues are thought to

completely uncouple receptors from G-proteins, it can be assumed that the remaining [¹²⁵I](±)DOI specific binding in presence of 100µM Gpp(NH)p corresponds predominantly to uncoupled form/s of the receptors.

The fact that Gpp(NH)p produces alterations in the pharmacological profile obtained for some antagonists suggests that, at the concentration used in competition experiments (100-150 pM), [¹²⁵I](±)DOI labels, for both 5-HT_{2A} and 5-HT_{2C} receptors, different populations of binding sites whose characteristics depend on the degree of receptor/G-protein coupling. When Gpp(NH)p was present during the competition experiments, the binding parameters observed for M100907 and ketanserin were altered in the majority of the rat brain regions analysed as well as those of SB242084 in claustrum and nucleus accumbens. In other words, these antagonists recognized differently the total versus the uncoupled population of [¹²⁵I](±)DOI binding sites. Interestingly, this characteristic seems to be intrinsic to the primary structure of the receptor and therefore not applicable to equivalent receptors in other species. Thus, equivalent studies carried out with the human orthologous of 5-HT_{2A} receptors, either from brain native tissue or heterologously expressed in cell lines, resulted in a different pharmacological profile, i.e., biphasic competition curves were obtained with M100907 and ketanserin whereas SB242084 and mesulergine both displayed a unique low affinity binding site; additionally, no alteration of binding parameters as a consequence of uncoupling receptor to G-proteins by adding GTP analogues was observed (Lopez-Gimenez et al. 2001).

This atypical pharmacology observed in rat brain for some antagonists as well as the effects of Gpp(NH)p on [¹²⁵I](±)DOI-antagonist competition curves may be interpreted based on the extended ternary complex model of receptor-G protein interactions (Lefkowitz et al. 1993). This model proposes that receptors exist as an equilibrium of different states or conformations. Thus, an intermediate or partly activated state/s of the receptor (called R*) exists between the resting or ground state (R) and the activated one coupled with the G-protein (R*G). Agonists (A) may affect the transition from R to AR*G at two different stages. On one hand, presence of the agonist will move the R/R* equilibrium towards a stabilization of R*, because it is for this receptor form that the agonist has higher affinity. On the other hand, it will also stabilize R*G by binding to this complex with higher affinity than to R*. According to this model, the stabilisation of an intermediate state (R*) by agonist means that agonist binding in the absence of G-protein coupling will not reflect the true ground-state (R) of the receptor (Strange 1998) (for an extended review see (Strange 1999)). This model has in fact already been considered to

explain several experimental findings involving 5-HT_{2A} and 5-HT_{2C} receptors in artificial systems. Thus, the relationship between agonist affinity and efficacy observed for agonists at 5-HT_{2A} and 5-HT_{2C} receptors expressed in recombinant systems can be more accurately explained in the light of this extended ternary complex model (Egan et al. 2000; Roth et al. 1997). In a similar manner, the different efficiency to couple to G proteins described for RNA editing isoforms of human 5-HT_{2C} receptors is also compatible with this model (Niswender et al., 1999). RNA editing isoforms, differing also in their efficiency to couple to G proteins, have also been described for rat 5-HT_{2C} receptors (Burns et al. 1997).

In our present experimental procedure, where we used an agonist radioligand ([¹²⁵I](±)DOI) to label 5-HT_{2A} and 5-HT_{2C} receptors in rat brain, we have at least two factors that may change the proportions among these different receptor states: first, the presence of the radioligand itself, that due to its agonist nature could induce receptor conformations and/or, at the same time, select among the different receptor conformations present in the system (Kenakin 1995; Strange 1998) and second, the presence of Gpp(NH)p that uncouples the receptor from G-protein and can therefore modify the equilibrium between R*G, R* and R receptor conformations. With these factors in mind, we could conjecture that M100907 is able to distinguish the different isomerisation forms of both 5-HT_{2A} and 5-HT_{2C} receptors and ketanserin of 5-HT_{2C} receptors. Furthermore, ketanserin (in rat forebrain) and SB242084 (in claustrum and nucleus accumbens) only discriminate different forms of 5-HT_{2A} receptors in presence of Gpp(NH)p, i.e. when the equilibrium among the various forms was altered by uncoupling receptors from G-proteins.

In contrast, mesulergine does not distinguish these different [¹²⁵I](±)DOI binding site populations when this radioligand labels 5-HT_{2A} or 5-HT_{2C} receptors. The results obtained in [³H]mesulergine binding assays in rat choroid plexus corroborate this finding for 5-HT_{2C} receptors: the lack of effect of Gpp(NH)p on [³H]mesulergine binding as well as on agonist (5-HT and (±)DOI) displacement suggests that this radioligand labels with high affinity all the different receptor states/conformations and that the ground state (R) predominates over other conformations of 5-HT_{2C} receptors in choroid plexus. In addition, the 100-fold higher B_{max} obtained for [³H]mesulergine than for [¹²⁵I](±)DOI in this region also indicates substantially different proportions of the various receptor conformational states in favour of the ground state for which agonists present low affinity. This predominance of the R form in the total population of sites labeled by [³H]mesulergine could be the reason why both agonists (5-HT and (±)DOI) do not discriminate the low proportion of high affinity agonist sites also labelled by [³H]mesulergine. The same

argument can be considered to explain the monophasic displacement curves obtained for M100907 and ketanserin when competing for [³H]mesulergine-binding sites, at variance with the biphasic profile observed when these antagonists displaced [¹²⁵I](±)DOI binding sites in rat choroid plexus.

The alterations produced by Gpp(NH)p in the affinity profiles of M100907 (loss of affinity) and ketanserin and SB242084 (gain of affinity) when displacing [¹²⁵I](±)DOI binding sites conform, according to the extended ternary complex model, to the behaviour expected for agonists and inverse agonists respectively. That is, since agonists have higher affinity for the coupled state (R*G) of the receptors, the net effect of uncoupling the receptor/G-protein complex will be a loss of affinity. On the other hand, inverse agonists have higher affinity for the uncoupled, inactive form (R) of the receptor. So, the net effect of adding GppNHp will be an increased affinity of the inverse agonist. In fact, inverse agonist activity has already been described for ketanserin in artificial systems that expressed constitutively active mutant 5-HT_{2A} receptors (Egan et al. 1998) or constitutively active native 5-HT_{2C} receptors (Barker et al. 1994; Hartman and Northup 1996). These two latter studies and others (Westphal and Sanders-Bush 1994) have also demonstrated inverse agonism for mesulergine in 5-HT_{2C} receptors, in contrast with our present data where no indications of such a behaviour were observed for this compound. We have to keep in mind, however, that these results come from different experimental systems: artificial (transfected cells) versus native system (rat brain). In the same way, we want to stress the point that our results were obtained from receptor binding assays, where no direct information is obtained about the possible effect of a given compound on receptor activity. Therefore, no absolutely firm conclusions can be drawn on the agonist/antagonist/inverse agonist nature of a compound solely on the basis of the effects of GppNHp on its affinity for a given receptor. Indeed, no intrinsic activity has been described to date for M100907 and SB242084 (Kehne et al. 1996), although for M100907 an atypical pharmacology analogous to an inverse agonism effect has been observed by means of autoradiographic techniques in rabbit brain depending on the presence of Mg⁺⁺ (Aloyo et al. 1998). Interestingly, studies performed with 5-HT_{2A} receptors with a point mutation (F340L) demonstrated that certain agonists were unable to induce second messenger stimulation although they still displayed high affinity for the receptor (Roth et al. 1997). Concerning the present results, a similar situation could be contemplated for M100907, that is, this compound could interact with the receptors in an agonist-like manner from the receptor binding viewpoint and at the same time behave as an antagonist

concerning the second messenger generation (null intrinsic activity). Nevertheless, it should be kept in mind that the aforementioned second messenger studies performed to date with M100907 and SB242084 have only measured phosphoinositide hydrolysis (phospholipase C activity) and that other second messenger pathways could as well be activated by these drugs when they interact with 5-HT_{2A} or 5-HT_{2C} receptors. Such pleiotropic behaviour, i.e. activation of two effector systems (phosphoinositide hydrolysis and arachidonic acid release) by the same receptor has been reported for both 5-HT_{2A} and 5-HT_{2C} receptors (Berg et al. 1998). This dual measure approach could help elucidate whether these compounds have any efficacy at all at different pathways triggered by 5-HT_{2A} or 5-HT_{2C} receptors. Other measures of receptor functionality such as [³⁵S]GTPγS binding (Adlersberg et al. 2000) may also prove useful in determining the efficacy of these compounds.

In conclusion, the present results lend further support to the existence also in native tissues of multiple conformations of 5-HT_{2A} and 5-HT_{2C} receptors in accordance with the extended ternary complex model of receptor action, and show that some antagonists of the 5-HT₂ receptor family are able to discriminate these multiple receptor states/conformations. Furthermore, the effects of the GTP analogue GppNHp on the displacement of [¹²⁵I](±)DOI binding sites by M100907 on one hand and by ketanserin or SB242084 on the other hand, suggest that these compounds may not be true silent antagonists, but may have some degree of, respectively, positive or negative intrinsic activity at native 5-HT_{2A} and 5-HT_{2C} receptors. The present work warrants future functional studies to elucidate the true agonist/antagonist/inverse agonist nature of these seemingly atypical compounds.

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Table 1. Binding parameters of [¹²⁵I](±)DOI obtained from saturation experiments at different levels of rat brain. Values represent mean±s.e.m of four different animals.

	K_d	B_{max} (fmol/mg tissue)
FCx	1.61±0.48	14.82±2.76
CPu	1.71±0.50	5.95±1.20
Cl	0.81±0.18	12.12±1.31
7	0.67±0.28	1.64±0.31
ChP	0.52±0.13	10.48±1.06

Table 2. Affinity constants of several compounds for [¹²⁵I](±)DOI-labelled receptors in different rat brain regions in absence or presence of 100 µM Gpp(NH)p. Values represent mean±s.e.m of four different animals.

MDL100,907	-logIC_{50h}	-logIC_{50l}	pK_{ih}	pK_{il}	% h	nH
FCx	10.07±0.12	8.24±0.16	10.01	8.26	60±5.1	0.52±0.01
FCx(Gpp(NH)p)	7.98±0.06	5.21±0.41	8.00	5.23	86±2.7	0.79±0.02
CPu	9.30±0.13	7.05±0.24	9.33	7.07	68±5.0	0.54±0.01
CPu(Gpp(NH)p)	7.80±0.15	4.85±0.23	7.82	4.87	55±4.3	0.40±0.02
Acb	9.78±0.27	8.02±0.27	9.81	8.04	47±11.3	0.54±0.01
Acb(Gpp(NH)p)	7.75±0.11	4.99±0.39	7.77	5.02	75±4.8	0.45±0.01
Tu	9.05±0.12	7.18±0.46	9.07	7.21	79±7.9	0.64±0.01
Tu(Gpp(NH)p)		7.58±0.09		7.61		1.00
Cl	9.64±0.19	7.85±0.27	9.67	7.87	65±8.2	0.50±0.01
Cl(Gpp(NH)p)		7.71±0.07		7.73		1.00
10+12	9.13±0.12	6.48±0.28	9.15	6.52	68±4.2	0.47±0.01
10+12(Gpp(NH)p)	7.52±0.15	5.44±0.65	7.52	5.46	78±8.1	0.69±0.01
7	8.87±0.12	5.99±0.22	8.90	6.02	64±4.0	0.42±0.01
7(Gpp(NH)p)	7.78±0.39	5.93±0.29	7.81	5.96	42±12.7	0.49±0.01
ChP	6.98±0.12	5.35±0.65	7.04	5.41	79±9.4	0.77±0.01
ChP(Gpp(NH)p)		6.26±0.09		6.32		1.00
SB242084						
FCx		6.79±0.05		6.81		1.00
FCx(Gpp(NH)p)		6.83±0.07		6.86		1.00
CPu		6.58±0.06		6.61		1.00
CPu(Gpp(NH)p)		7.04±0.07		7.06		1.00
Acb		6.61±0.05		6.63		1.00
Acb(Gpp(NH)p)	9.78±0.49	6.62±0.14	9.80	6.64	26±5.5	0.50±0.01
Tu		6.72±0.07		6.74		1.00
Tu(Gpp(NH)p)		7.00±0.07		7.02		1.00
Cl		6.87±0.05		6.89		1.00
Cl(Gpp(NH)p)	8.56±0.44	6.56±0.23	8.58	6.58	35±9.9	0.59±0.01
10+12	7.70±0.42	6.16±0.23	7.70	6.18	36±15.3	0.67±0.01
10+12(Gpp(NH)p)	8.98±0.18	6.81±0.13	9.00	6.48	40±4.6	0.52±0.01
7	8.23±0.50	6.22±0.21	8.29	6.24	33±11.1	0.58±0.01
7(Gpp(NH)p)	9.24±0.42	6.75±0.10	9.26	6.77	21±4.5	0.67±0.02
ChP	8.61±0.06		8.67			1.00
ChP(Gpp(NH)p)	8.95±0.16		9.01			1.00

Table 2. Continued

ketanserin	$-\log IC_{50h}$	$-\log IC_{50l}$	pK_{ih}	pK_{il}	% h	nH
FCx		7.85±0.06		7.87		1.00
<i>FCx(Gpp(NH)p)</i>	9.28±0.12	7.17±0.12	9.30	7.19	55±3.8	0.54±0.01
CPu		7.96±0.06		7.99		1.00
<i>CPu(Gpp(NH)p)</i>	9.21±0.21	7.03±0.22	9.23	7.05	56±6.5	0.56
Acb		7.95±0.07		7.97		1.00
<i>Acb(Gpp(NH)p)</i>	9.34±0.17	7.18±0.16	9.36	7.21	53±4.4	0.50±0.01
Tu		7.96±0.06		7.98		1.00
<i>Tu(Gpp(NH)p)</i>	9.01±0.15	6.82±0.15	9.03	6.85	52±4.4	0.50±0.01
Cl		7.81±0.08		7.83		1.00
<i>Cl(Gpp(NH)p)</i>	9.29±0.15	7.03±0.17	9.31	7.05	59±4.6	0.49±0.01
10+12	8.16±0.21	6.34±0.34	8.19	6.35	60±9.8	0.53±0.01
<i>10+12(Gpp(NH)p)</i>	8.50±0.11	5.51±0.38	8.53	5.53	75±3.9	0.52±0.02
7	8.16±0.18	6.31±0.52	8.19	6.33	72±10.2	0.60±0.01
<i>7(Gpp(NH)p)</i>	9.03±0.10	5.89±0.16	9.05	5.91	61±2.9	0.36±0.01
ChP	8.37±0.55	6.52±0.17	8.43	6.59	23±9.9	0.69±0.01
<i>ChP(Gpp(NH)p)</i>	8.49±0.22	6.51±0.35	8.55	6.56	61±9.1	0.55±0.01
mesulergine						
FCx	8.52±0.05		8.54			1.00
<i>FCx(Gpp(NH)p)</i>	8.45±0.06		8.47			1.00
CPu	8.43±0.05		8.45			1.00
<i>CPu(Gpp(NH)p)</i>	8.52±0.08		8.54			1.00
Acb	8.65±0.05		8.68			1.20±0.01
<i>Acb(Gpp(NH)p)</i>	8.52±0.07		8.54			1.00
Tu	8.60±0.07		8.62			1.00
<i>Tu(Gpp(NH)p)</i>	8.52±0.08		8.54			1.00
Cl	8.67±0.06		8.70			1.00
<i>Cl(Gpp(NH)p)</i>	8.53±0.06		8.55			1.00
10+12	8.55±0.07		8.58			1.00
<i>10+12(Gpp(NH)p)</i>	8.07±0.04		8.09			1.00
7	8.49±0.06		8.51			1.00
<i>7(Gpp(NH)p)</i>	8.29±0.06		8.31			1.00
ChP	8.76±0.07		8.83			1.00
<i>ChP(Gpp(NH)p)</i>	8.77±0.08		8.83			1.00

h : high affinity site. l : low affinity site. nH : Hill coefficient.

Table 3. Affinity constants of several compounds for [³H]mesulergine-labelled receptors in rat choroid plexus in absence or presence of 100 μM Gpp(NH)p. Values represent mean±s.e.m of four different animals.

	-logIC₅₀	pK_i	nH
MDL100,907	6.44±0.08	6.71	1.00
<i>MDL100,907(Gpp(NH)p)</i>	<i>6.70±0.06</i>	<i>6.98</i>	<i>1.00</i>
ketanserin	7.07±0.05	7.34	1.00
<i>ketanserin (Gpp(NH)p)</i>	<i>7.09±0.05</i>	<i>7.37</i>	<i>1.00</i>
SB242084	8.62±0.06	8.88	1.00
<i>SB242084(Gpp(NH)p)</i>	<i>8.65±0.04</i>	<i>8.91</i>	<i>1.00</i>
5-HT	7.87±0.08	8.13	1.00
<i>5-HT(Gpp(NH)p)</i>	<i>7.74±0.05</i>	<i>7.99</i>	<i>1.00</i>
(±)DOI	7.44±0.05	7.70	1.00
<i>(±)DOI(Gpp(NH)p)</i>	<i>7.32±0.05</i>	<i>7.58</i>	<i>1.00</i>

nH: Hill coefficient.

Figure 1. Saturation analysis of [¹²⁵I](±)DOI binding to rat frontal cortex (A) and rat choroid plexus (B). Each point represents the mean specific binding of four animals.

Figure 2. Inhibition curves of $[^{125}\text{I}](\pm)\text{DOI}$ binding by non-hydrolysable GTP analogues (A: Gpp(NH)p; B: GTP γ S) in rat frontal cortex (■) and rat choroid plexus (□). Each point represents the mean of four animals.

Figure 3. Autoradiographic visualisation of $[^{125}\text{I}](\pm)\text{DOI}$ binding sites in a coronal level of rat brain. (A) Total binding obtained with 100 pM $[^{125}\text{I}](\pm)\text{DOI}$. (B) Remaining non-specific binding in presence of 10 μM ritanserin. (C) and (D) Remaining total $[^{125}\text{I}](\pm)\text{DOI}$ binding in presence of 100 μM Gpp(NH)p and GTP γ S respectively. Bar: 3 mm.

Figure 4. Competition curves of [¹²⁵I](±)DOI in rat frontal cortex (upper panels), facial nucleus (medial panels) and choroid plexus (lower pannels) by MDL100,907 (■), ketanserin (□), SB242084 (●), and mesulergine (○) in absence (left panels) or presence (right panels) of 100μM Gpp(NH)p. On left panels, * indicates the remaining specific binding when adding 100μM Gpp(NH) in absence of competitor drugs.

Abbreviations

Acb	accumbens
ChP	choroid plexus
Cl	claustrum
CPu	caudate-putamen
FCx	frontal cortex
Tu	olfactory tubercle
7	facial nucleus
10	dorsal motor nucleus of vagus
12	hypoglossal nucleus

Fig. 1 Saturation analysis of [¹²⁵I](±)DOI binding to rat frontal cortex (A) and rat choroid plexus (B). Each point represents the mean specific binding of four animals.

214x72mm (72 x 72 DPI)

Fig. 2 Inhibition curves of $[^{125}\text{I}](\pm)\text{DOI}$ binding by non-hydrolysable GTP analogues (A: Gpp(NH)p; B: GTP γ S) in rat frontal cortex (■) and rat choroid plexus (□). Each point represents the mean of four animals.

215x81mm (72 x 72 DPI)

Fig. 3 Autoradiographic visualisation of $[^{125}\text{I}](\pm)\text{DOI}$ binding sites in a coronal level of rat brain. (A) Total binding obtained with 100pM $[^{125}\text{I}](\pm)\text{DOI}$. (B) Remaining non-specific binding in presence of 10 μM ritanserin. (C) and (D) Remaining total $[^{125}\text{I}](\pm)\text{DOI}$ binding in presence of 100 μM Gpp(NH)p and GTP γ S respectively. Bar: 3 mm.
214x127mm (72 x 72 DPI)

Review

Fig. 4 Competition curves of [125 I]($\pm$)DOI in rat frontal cortex (upper panels), facial nucleus (medial panels) and choroid plexus (lower pannels) by MDL100,907 ($\blacksquare$), ketanserin ($\square$), SB242084 ($\bullet$), and mesulergine ($\circ$) in absence (left panels) or presence (right panels) of 100 μ M Gpp(NH)p. On left panels, * indicates the remaining specific binding when adding 100 μ M Gpp(NH) in absence of competitor drugs.
203x245mm (72 x 72 DPI)