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Title Page:

Native rat hippocampal 5-HT_{1A} receptors show constitutive activity.

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Running Title Page:

a) Running title:

Constitutive activity of native rat 5-HT_{1A} receptors.

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Introduction:	566 words
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d) Abbreviations

(+)8-OH-DPAT: (+)8-hydroxy-2-(di-n-propylamino)tetralin; 5-HT: 5-hydroxytryptamine; CHO: Chinese hamster ovary; EDTA: ethylene dinitril-O-tetraacetate; DPM: disintegration per minute; DTT: dithiothreitol; $G\alpha_x$: x subunit of heterotrimeric G-alpha protein; GPCR: G-protein-coupled receptor; GDP: guanosine diphosphate; GTP: guanosine triphosphate; HEK: Human embryonic kidney; SPA: scintillation proximity assay; [^{35}S]GTPγS: guanosine-5'-O-(3-[^{35}S]thio)-triphosphate; Sf9: *Spodoptera frugiperda*.

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Abstract:

Previous studies have shown that human 5-HT_{1A} receptors stably expressed in transfected cell lines show constitutive G-protein activity, as revealed by the inhibitory effect of inverse agonists, such as spiperone, on basal [^{35}S]GTPγS binding. In the present study, we evaluated the constitutive activity of native rat 5-HT_{1A} receptors in hippocampal membranes. Using anti-Gα_o-antibody capture coupled to scintillation proximity assay under low sodium (30 mM) conditions, we observed high basal [^{35}S]GTPγS binding to Gα_o subunits (defined as 100%). In these conditions, 5-HT and the prototypic selective 5-HT_{1A} agonist (+)-8-OH-DPAT, both stimulated [^{35}S]GTPγS binding to Gα_o to a similar extent, raising binding to approximately 130% of basal with pEC_{50} values of 7.91 and 7.87, respectively. The 5-HT_{1A}-selective neutral antagonist WAY100,635 could block these effects in a competitive manner with pKb values (5-HT: 9.57; (+)-8-OH-DPAT: 9.52) that are consistent with its pKi value at r5-HT_{1A} receptors (9.33). In this native receptor system, spiperone and methiothepin reduced basal [^{35}S]GTPγS binding to Gα_o in a concentration-dependent manner to 90% of basal with pIC_{50} of 7.37 and 7.98, respectively. The inhibition of basal [^{35}S]GTPγS binding induced by maximally effective concentrations of spiperone (10 μM) or methiothepin (1 μM) was antagonised by WAY100,635 in a concentration-dependent manner (pKb: 9.52 and 8.87, respectively), thus indicating that this inverse agonism was mediated by 5-HT_{1A} receptors. These data provide the first demonstration that native rat serotonin 5-HT_{1A} receptors can exhibit constitutive activity in vitro.

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Introduction:

Constitutive activity of G-protein-coupled receptors (GPCR) provides a mechanistic basis for inverse agonism, a phenomenon observed in heterologous expression systems where some pharmacological agents are able to inhibit basal activity, as measured in second messenger system assays (de Ligt *et al.*, 2000; Kenakin, 2004). This phenomenon has been described for several GPCR, and a number of clinically relevant drugs have been shown to act as inverse agonists on some GPCR (Milligan, 2003a; Kenakin, 2004). Constitutive activity has been described in a number of recombinant systems and in "physiological" peripheral tissue preparations (de Ligt *et al.*, 2000), but only rarely in native brain tissue, the few exceptions being serotonin 5-HT_{2C} receptors (De Deurwaerdere *et al.*, 2004) and H_3 histaminergic receptors (Morisset *et al.*, 2000). Moreover, there are evidences that mutated GPCR with elevated constitutive activity are associated with some human diseases (Kenakin, 2004; de Ligt *et al.*, 2000; Seifert and Wenzel-Seifert, 2002). A better characterization of this phenomenon may thus improve our understanding of the mechanisms of action of clinically important drugs, and may also help improve future drug development.

Serotonin 5-HT_{1A} receptors, which are members of the GPCR family, are key targets for the treatment of mood disorders including anxiety and depression (Barnes and Sharp, 1999), and may also improve the outcome of psychotic disorders (Meltzer *et al.*, 2003; Millan, 2000; Newman-Tancredi *et al.*, 2005). Several reports have shown that human (h)5-HT_{1A} receptors expressed in stably transfected cell lines showed constitutive activity as revealed by the inverse agonist properties of spiperone and methiothepin in that heterologous expression system (Newman-Tancredi *et al.*, 1997a; Newman-Tancredi *et al.*, 1997b; Stanton and Beer, 1997; McLoughlin and Strange, 2000). The effects of spiperone were concentration-dependent and could be blocked by the selective neutral 5-HT_{1A} receptor

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antagonist WAY100,635. Similarly, h5-HT_{1A} receptors constitutive activity could be demonstrated on a specific coupling to GTP-binding protein G_z when this particular G-protein was co-expressed with h5-HT_{1A} receptor in *Spodoptera frugiperda* (Sf9) cell line (Barr and Manning, 1997), and on $G\alpha_{i3}$ activation in *Chinese hamster ovary* (CHO) cells expressing h5-HT_{1A} receptors (Newman-Tancredi *et al.*, 2002). Constitutive activity of 5-HT_{1A} receptors could also be demonstrated when the receptor was coupled to $G\alpha_{i1}$ in HEK cells using a fusion protein paradigm (Milligan *et al.*, 2001). In light of these observations, several groups have evaluated constitutive activity of 5-HT_{1A} receptors in brain tissue environment (Alper and Nelson, 1998; Odagaki and Fuxe, 1995; Newman-Tancredi *et al.*, 2003b; Odagaki and Toyoshima, 2005a; Odagaki and Toyoshima, 2005b). However, constitutive activity of native 5-HT_{1A} receptors has not been described to date.

Recent advances in [^{35}S]GTPγS binding assays have improved the selectivity of this technique for studying GPCR (Milligan, 2003b). Scintillation proximity assay (SPA) can be coupled with G-alpha selective antibodies in [^{35}S]GTPγS binding assays, allowing detection of the response of a single Gα-protein subtype to pharmacological manipulation within an homogenate (Newman-Tancredi, 2003; Wu and Liu, 2005). The rat hippocampus contains high densities of 5-HT_{1A} receptors (Barnes and Sharp, 1999) that may be essentially coupled to $G\alpha_o$ (Mannoury la Cour *et al.*, 2006). In the present study, we characterized the effect of selected 5-HT_{1A} receptor drugs on a SPA-based $G\alpha_o$-selective immunocapture assay using two agonists (serotonin and (+)-8-OH-DPAT) and two inverse agonists (spiperone and methiothepin). These results indicate that 5-HT_{1A} receptors constitutively activate $G\alpha_o$ subunits in rat brain tissue homogenate. Part of this work has been presented in an abstract form (Martel *et al.*, 2005).

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Materials and methods:

Animals were handled and cared for in accordance with the Guide for the Care and Use of Laboratory Animals (ILAR, NRC, USA, 1996) and the European Directive EEC/86/609, and the protocol (N° 001) was carried out in compliance with French regulations and with local Ethics Committee Guidelines for animal research .

Receptor affinities in rat hippocampal membranes:

Frozen hippocampi of male rats [Ico: OFA SD (I.O.P.S. Caw); Iffa Credo, France] weighing 240-260 g were used for binding studies and were stored at -80° C prior to use. The membrane preparations and binding assays were performed in duplicate as described previously (Assie *et al.*, 1999). Briefly, membranes were homogenized in 20 volumes of cold Tris-HCl buffer (50 mM, pH 7.4 at 25° C), washed with 2 cycles of centrifugation/resuspension, followed by a 10 minute incubation at 37°C which ended with a final cycle of centrifugation/resuspension. Membranes (750 µg tissue/well) were incubated at 23°C in 96 deep well microplates containing 1 nM [^{3}H]8-OH-DPAT, and increasing concentrations of drugs ranging from 10^{-11}M to 10^{-5}M. Following a 2 hour incubation period, the microplate contents were rapidly filtered under vacuum through GF/B unifilter microplates with two rapid 2 ml washes with ice-cold Tris-HCl buffer. Non specific binding was defined with 10 µM 5-HT while total binding was defined in the absence of any displacer. The radioactivity retained on the filters was measured by scintillation spectroscopy (TopCount, PerkinElmer) in 40 µl of scintillation fluid (Microscint 20, PerkinElmer). The results of displacement experiments were analysed using the non-linear regression program PRISM v 4.03 (GraphPad software) which allowed calculation of an IC_{50}. Ki were calculated using the Cheng-Prusoff equation (Cheng and Prusoff, 1973): $Ki=IC_{50}/(1- ([Ligand] / K_DLigand))$ where [Ligand] is the measured concentration of [^{3}H](+)-8-OH-DPAT in the assay and $K_DLigand$ is the affinity of [^{3}H](+)-8-OH-DPAT in that assay (0.75 ± 0.08 nM). Density of 5-HT_{1A} receptors in that assay was 14.3 ± 0.8 fmol/mg fresh tissue (corresponding to approximately 300 fmol/mg proteins).

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Scintillation Proximity Assay (SPA):

Hippocampi from male rats [Ico: OFA SD (I.O.P.S. Caw); Iffa Credo, France] weighing 240-260 g were dissected on a cold plate and frozen at -80˚C until used. Tissues were homogenized in cold HEPES-DTT buffer (20 mM HEPES pH 7.0, 0.2 mM EDTA, 0.2 mM DTT) with 1 mM GTP using a polytron, GTP favouring endogeneous ligand dissociation. The homogenate was incubated at 35˚C for 15 minutes to favour endogenous ligand dissociation. Membranes were washed by 2 cycles of centrifugation at 20 000 X g for 15 minutes at 4˚C and resuspension in cold HEPES-DTT with 1 mM GDP. Final pellet was resuspended in 300 volumes (based on tissue weight) HEPES-DTT buffer containing 5 mM $MgCl_2$, 100 mM (Galpha-selective antibodies assay) or 30 mM (pharmacological assay) NaCl and 50 µM GDP (HEPES-DTT assay buffer).

All reactions were performed at room temperature on 96 well plates in a final volume of 200 µl HEPES-DTT assay buffer. Membranes (8 µg protein/well) were pre-incubated for 30 minutes at room temperature with drugs (agonist +/- antagonist), buffer (to define basal) or 10 µM GTPγS (to define non-specific). At the end of this pre-incubation, 0.4 nM [^{35}S]GTPγS was added and the membranes were incubated for 60 minutes. Incubation was stopped by adding Nonidet NP-40 and the plate was agitated for another 30 minutes before addition of 0.2 µg anti-Gα-selective antibodies to each well. The antibodies used were rabbit polyclonal anti-$G\alpha_{q/11}$, anti-$G\alpha_{i3}$ and anti-$G\alpha_{s/olf}$ from Santa Cruz Biotechnology (Santa Cruz, CA), or mouse monoclonal anti-$G\alpha_o$ and anti-$G\alpha_{i1}$ from Biomol (Plymouth Meeting, PA). Anti-$G\alpha_{q/11}$, anti-$G\alpha_{i1}$ and anti-$G\alpha_{s/olf}$ have been characterized previously by western blot using purified recombinant $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_o$, $G\alpha_{q/11}$ and $G\alpha_{s/olf}$ protein subtypes (Cussac *et al.*, 2002; Cussac *et al.*, 2004), and are highly selective for their respective G-protein subtypes. As described by the manufacturer, we observed crossreactivity of anti-$G\alpha_{i3}$ with $G\alpha_{i1}$ and $G\alpha_{i2}$, with a stronger reaction on $G\alpha_{i3}$ (data not shown). Specificity of anti-Gao was

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assessed by western blot using 50 ng of purified recombinant $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_{o}$, $G\alpha_{q/11}$ and $G\alpha_{s/olf}$ protein subtypes as described previously (Cussac *et al.*, 2002; Cussac *et al.*, 2004). Anti-$G\alpha_{o}$ are highly selective for $G\alpha_{o}$ (Figure 1A). Note that the basal signal for each antibodies will depend both on the affinity and selectivity of these antibodies for the G-protein subtype(s), and the revelation system (affinity of secondary antibodies) and cannot therefore be compared. The primary antibodies were left to react for 60 minutes under agitation before adding 50 µl of the secondary antibodies (anti-rabbit or anti-mouse coupled to scintillation proximity assay beads, Amersham), diluted according to manufacturer's recommendations. The secondary antibodies were left to react for another 60 minutes and the plate was centrifuged at 1 000 X g for 15 minutes at room temperature to take the complex down at the bottom of the well and radioactivity was immediately measured on a top-count radioactivity counter (Perkin-Elmer). Raw DPM data were transformed by subtracting non-specific and normalizing to % of basal [^{35}S]GTPγS binding. All pharmacological parameters were derived from sigmoid non-linear regression using Graphpad Prism version 4.03. For antagonism by WAY100,635, pKb were calculated using the Cheng-Prusoff equation ($Kb=IC_{50}/(1-([Ago]/EC_{50Ago}))$) where [Ago] is the fixed concentration of agonist used for the antagonism assay, and EC_{50Ago} is the EC_{50} of that agonist when tested alone.

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Results:

The Gα protein subtypes activated by 5-HT_{1A} in rat hippocampus were evaluated using SPA-based antibody capture assay with a set of antibodies selective for different Gα-proteins (see materials and methods for details). Table 1 lists the basal [^{35}S]GTPγS binding (in DPM) observed with the various antibodies, and the absolute (DPM) and relative (% BASAL) changes elicited by 10 μM (+)8-OH-DPAT. The maximal basal [^{35}S]GTPγS binding was observed with the anti-$G\alpha_o$-selective antibodies (Figure 1A), and these antibodies also showed the maximal absolute (+4607 DPM) and relative (+71%) increases following (+)8-OH-DPAT treatment (Table 1). A moderate (+46%) increase in relative [^{35}S]GTPγS binding was also observed with anti-$G\alpha_{i3}$ antibodies (Table 1) but absolute [^{35}S]GTPγS binding represented only a minor fraction (+371 DPM, or 8% of the response observed with $G\alpha_o$ on a DPM basis; Table 1). Due to the cross reactivity of anti-$G\alpha_{i3}$ antibodies with $G\alpha_{i1}$ and $G\alpha_{i2}$, this increase may be predominantly due to $G\alpha_{i2}$ and/or $G\alpha_{i3}$ proteins as no change of [^{35}S]GTPγS binding was seen with $G\alpha_{i1}$-selective antibodies (Table 1), thus demonstrating that 5-HT1A receptors do not couple to $G\alpha_{i1}$ in rat hippocampus. We therefore used the $G\alpha_o$-selective antibodies for investigating constitutive activity of 5-HT_{1A} receptors in rat hippocampal membrane preparations.

Low sodium concentrations (30 mM) and relatively high GDP concentrations (50 μM) were used to detect 5-HT_{1A} receptors constitutive activity in this system. Under those conditions, basal [^{35}S]GTPγS labelling of $G\alpha_o$ was about 25 000 DPM, and this basal value was defined as 100% (figures 1 and 2). In these assay conditions, serotonin, the endogenous ligand for 5-HT_{1A} receptors, and the prototypical selective 5-HT_{1A} receptor agonist (+)8-OH-DPAT both increased [^{35}S]GTPγS binding to $G\alpha_o$ in a concentration-dependent manner (Figure 1 and Table 2). The selective neutral 5-HT_{1A} antagonist WAY100,635

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competitively reversed this effect: increasing concentration of the antagonist reversed the effects of maximally effective concentrations (1 μM) of serotonin or (+)8-OH-DPAT (Figure 2 and Table 2).

In this hippocampal tissue homogenate preparation, spiperone and methiothepin showed inverse agonist properties, reducing basal [^{35}S]GTPγS binding to $G\alpha_o$ in a concentration-dependent manner (Figure 1 and Table 2). WAY100,635 partly reversed the maximal inhibition of basal binding induced by a fixed concentration of spiperone (10 μM) or methiothepin (1 μM) (Figure 2 and Table 2). In comparison, when a higher concentration of NaCl (100 mM) was used, spiperone and methiothepin only inhibited [^{35}S]GTPγS binding at very high concentrations (> 1μM; data not shown).

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Discussion:

Human 5-HT_{1A} receptors expressed in recombinant cell lines have been shown to possess constitutive activity as revealed by spiperone and methiothepin inverse agonist properties (see Introduction). Similarly, constitutive activity of h5-HT_{1A} could be demonstrated when the receptor was co-expressed with GTP-binding protein G_z in Sf9 cells (Barr and Manning, 1997) and in HEK cells expressing a fusion protein combining 5-HT_{1A} to $G\alpha_{i1}$ (Milligan *et al.*, 2001), thus suggesting that constitutive activity is an inherent property of 5-HT_{1A} receptors. Nevertheless, multiple factors can influence detection of constitutive activity. For example, the amplitude of response to inverse agonist depends on GPCR/G-protein stoïchiometry: an increase in 5-HT_{1A}/G-protein ratio increasing the amplitude of inverse agonist response (Newman-Tancredi *et al.*, 1997b). It is therefore important to assess constitutive activity of GPCR in an environment where the "natural" GPCR/G-protein ratio is expressed.

The present study is, to our knowledge, the first demonstration of constitutive activity of native rat 5-HT_{1A} receptors. Previous studies using classical [^{35}S]GTPγS binding assay failed to detect inverse agonist properties of spiperone and methiothepin in rat brain tissue (Alper and Nelson, 1998; Newman-Tancredi *et al.*, 2003b; Odagaki and Toyoshima, 2005a; Odagaki and Toyoshima, 2005b). The study by Alper and Nelson did report that methiothepin, but not spiperone, diminished G-protein activation at high concentrations (10 μM), but this effect was not reversed by the selective neutral antagonist WAY100,635 (Alper and Nelson, 1998), indicating that it was not related to 5-HT_{1A} receptors. In contrast, using SPA-based $G\alpha_o$-selective immunocapture on [^{35}S]GTPγS binding assay, the present study demonstrates inverse agonism at native r5-HT_{1A} receptors with both spiperone and methiothepin that was antagonized by WAY100,635. Rat hippocampal 5-HT_{1A} receptors show a strong coupling to $G\alpha_o$ (Mannoury la Cour *et al.*, 2006). Indeed, in the present study,

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comparison of antibodies selective for various Galpha-proteins using SPA-based immunocapture assay showed that the most prominent response of rat hippocampal 5-HT_{1A} receptors to (+)8-OH-DPAT was activation of $G\alpha_o$. The SPA-based $G\alpha_o$-selective immunocapture approach used here thus permitted characterization of the inverse agonist properties of spiperone and methiothepin on 5-HT_{1A} receptors by selectively detecting the G protein showing the best coupling to 5-HT_{1A} receptors in this tissue. Under the present assay conditions, WAY100,635 had no activity of its own at concentrations up to 10 µM, but it antagonised the effect of the agonists and inverse agonists in a competitive manner. Calculated pKb for this antagonism are consistent with pKi values derived from competition binding of WAY100,635 to rat 5-HT_{1A} receptors (Newman-Tancredi *et al.*, 2005) and from pKb previously published on similar assays (Odagaki and Toyoshima, 2005a; Odagaki and Toyoshima, 2005b). These data therefore confirm that the decreases in $G\alpha_o$ labelling induced by spiperone and methiothepin are indeed mediated by interaction at r5-HT_{1A} receptors.

The amplitude of the inverse agonism response observed with spiperone and methiothepin is relatively modest (10% reduction in basal labelling of $G\alpha_o$, or 33% of the amplitude of agonists responses under those conditions). Two factors may contribute to the moderate inverse agonism response of these drugs on 5-HT_{1A} receptors. First of all, both drugs may be partial inverse agonists on 5-HT_{1A} receptors. Although no efficacy data are available for these 2 drugs as inverse agonists on 5-HT_{1A} receptors, this efficacy may be assessed in recombinant systems expressing these receptors alone using a [^{35}S]GTPγS homologous displacement protocol with unlabelled GTPγS (Audinot *et al.*, 2000; Rouleau *et al.*, 2002). Secondly, this assay being performed on tissue, several $G\alpha_o$-coupled receptors may be constitutively active, thus limiting the contribution of 5-HT_{1A} to a fraction of the overall constitutive activity present in that tissue. Indeed, reversal of the response of spiperone and

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methiothepin by WAY100,635 is only partial (two third of effect), suggesting that other constitutively active receptors may be involved in the effect of these 2 drugs (see below).

The demonstration that native r5-HT_{1A} receptors show constitutive activity in vitro may suggest that this phenomenon also occurs *in vivo*. Constitutive activity has also been demonstrated in rat brain tissues for serotonin 5-HT_{2C} receptors (De Deurwaerdere *et al.*, 2004) and H_3 histaminergic receptors (Morisset *et al.*, 2000), and certain human pathologies including metabolic diseases and some cancers are believed to be associated with abnormal levels of GPCR constitutive activity (Kenakin, 2004). It is also possible that certain neuropsychiatric diseases may be associated with GPCR showing abnormal constitutive activity and several clinically relevant drugs have been shown to have inverse agonist properties on GPCR in recombinant systems (Milligan, 2003a; Kenakin, 2004). The present study assessed the level of constitutive activity of 5-HT_{1A} receptors in hippocampal membranes prepared from brains of untreated normal animals. In view of the importance of 5-HT_{1A} receptors in neuropsychiatric disorders (Millan, 2000; Meltzer *et al.*, 2003; Newman-Tancredi *et al.*, 2005), it will be of interest to evaluate whether constitutive activity of central 5-HT_{1A} receptors may be affected by pharmacological or pathophysiological factors. A better understanding of the factors affecting 5-HT_{1A} receptors constitutive activity *in vivo* may thus help improve therapeutic approaches toward neuropsychiatric disorders.

The pharmacological behaviour of inverse agonists *in vivo* may have other physiological consequences. For example, inverse agonists may produce more prominent receptor up regulation than neutral antagonists (Adan and Kas, 2003) and inverse agonists may lead to increases in G-proteins expression levels (Kenakin, 2004). A recent electrophysiological study assessed the effect of two selective 5-HT_{1A} inverse agonists (Rec 27/0224 and Rec 27/0074) on hippocampal and dorsal raphe neurons (Corradetti *et al.*, 2005). In this physiological assay, the 2 inverse agonists fully antagonized the effect of 5-CT

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applications on dorsal raphe neurons, but with a much slower time course to reach steady state than would have been expected from their binding affinities at 5-HT_{1A} receptors, or when compared to the time course to reach steady state with the neutral antagonist WAY100,635. Corradetti and colleagues suggested that this phenomenon may be explained by a slow allosteric shift of the receptor toward an inactive state (Corradetti *et al.*, 2005). Moreover, in contrast to WAY100,635, which could fully antagonize 5-CT-induced hyperpolarisation of hippocampal CA1 neurons, these 2 compounds showed only partial antagonism, suggesting that these drugs may behave differentially on neuronal populations expressing either pre- or post-synaptic 5-HT_{1A} receptors. These observations are consistent with the differential coupling of the pre- (dorsal raphe) and post-synaptic (hippocampus) 5-HT_{1A} receptors suggested by various observations (Hensler, 2002; Mannoury la Cour *et al.*, 2006) as differential response to allosteric effects of inverse agonists may be expected on differentially coupled receptor.

Three technical points should be noted concerning the data in the present study. First, buffer containing low sodium (30 mM) was used as this condition increases GPCR constitutive activity, and may therefore improve identification of inverse agonists with [^{35}S]GTPγS binding assays (de Ligt *et al.*, 2000). The role of sodium may be to allosterically stabilize the uncoupled (inactive) conformation of the receptors (de Ligt *et al.*, 2000), thus low sodium concentrations favouring the constitutively active conformation of the receptor. Allosteric modulation of GPCR ligand binding by sodium is associated with a highly conserved aspartate residue located in the second transmembrane domain, near the intracytoplasmic side of GPCR (Horstman *et al.*, 1990), a residue that may also be critical for GPCR coupling to G-proteins (Odagaki and Toyoshima, 2005a). An important issue is clearly the “physiological” concentration of NaCl affecting the levels of GPCR constitutive activity and this question remains a subject of debate (Newman-Tancredi *et al.*, 2003a).

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Second, it is unlikely that the inverse agonism of methiothepin and spiperone observed here would be due to antagonism of residual endogenous serotonin in the tissue as the homogenate was preincubated for 15 minutes at 35°C in a large excess of buffer containing GTP to favour endogenous ligand dissociation, followed by several membrane washes. Moreover, WAY100,635 had no activity at concentrations up to 10 µM in these assay conditions, providing further indication that no remaining endogenous 5-HT was affecting basal [^{35}S]GTPγS binding.

Third, the inverse agonism elicited by spiperone and methiothepin was not completely reversed by WAY100,635, a residual lowering of $G\alpha_o$ activation persisting even at high antagonist concentrations. This suggests that other receptor systems that couple to $G\alpha_o$ may also be involved. Indeed, both spiperone and methiothepin are known to interact with other receptors at which they have been shown to be inverse agonists. For example, spiperone is acting as an inverse agonist on dopamine D_2 receptors (Roberts and Strange, 2005), and on alpha1 adrenoceptors (Rossier *et al.*, 1999), while methiothepin has inverse agonist properties on 5-HT_{1B} (Newman-Tancredi *et al.*, 2003a) and 5-HT_{1D} receptors (Audinot *et al.*, 2000). Thus, the inhibition of [^{35}S]GTPγS binding to $G\alpha_o$ remaining with maximal WAY100,635 concentrations may be due to an action at other receptors. The present methodology targeting $G\alpha_o$ activation provides a strategy to investigate the action of inverse agonists at other receptor subtypes.

In conclusion, these data generated by antibody capture methodology associated to SPA detection provide the first demonstration that native rat 5-HT_{1A} receptors show constitutive activation of $G\alpha_o$ proteins in an hippocampal tissue environment, suggesting that rat 5-HT_{1A} receptors may be constitutively active *in vivo* at $G\alpha_o$ and/or other G-protein subtypes.

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Legends for Figures:

Figure 1 : A) Validation of the specificity of anti-$G\alpha_o$ antibodies by western blot (see materials and methods for details).

B) Concentration-response curves of [^{35}S]GTPγS binding to $G\alpha_o$ in rat hippocampal membranes. Data are the average of 3-5 independent determinations, and are expressed as percent changes from basal (defined as 100%). Serotonin and (+)-8-OH-DPAT increased [^{35}S]GTPγS binding to $G\alpha_o$ whereas both spiperone and methiothepin reduced basal binding in a concentration dependent manner (see Table 2 for derived constants).

Figure 2 : Reversal by increasing concentrations of WAY100,635 of the effect of a fixed concentration of drugs on [^{35}S]GTPγS binding to $G\alpha_o$ in rat hippocampal membranes. Data are the average of 3-5 independent determinations, and are expressed as percent changes from basal (defined as 100%). (see Table 2 for derived constants).

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Tables:

Table 1 : Characterisation of Gα protein subtypes coupled to 5-HT_{1A} receptors in rat hippocampal membrane preparations. The levels of [^{35}S]GTPγS binding elicited by 10 μM (+)8-OH-DPAT was compared to basal binding (defined as 100%) using an SPA-based immuno-capture assay with a series of characterized Gα-subtype specific antibodies (see materials and methods for details). Results are expressed as raw DPM, and as percent changes from corresponding basal [^{35}S]GTPγS binding and are the mean ± SEM of 4 independent determinations.

	Basal	**10 μM (+)8-OH-DPAT**	
Antibodies	**DPM (N)**		**% chg. from BASAL**
Anti-$G\alpha_{q/11}$	517 ± 20 (4)	614 ± 23 (4)	19 ± 1
Anti-$G\alpha_{s/olf}$	526 ± 33 (4)	586 ± 18 (4)	13 ± 10
Anti-$G\alpha_{i1}$	4302 ± 457 (4)	4027 ± 374 (4)	-6 ± 1
Anti-$G\alpha_{i3}$	822 ± 41 (4)	1193 ± 6 (4)	46 ± 7
Anti-$G\alpha_o$	**6541 ± 615** (4)	**11148 ± 829** (4)	**71 ± 6**

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Table 2: Constants derived from [^{3}H]8-OH-DPAT binding and [^{35}S]GTPγS binding to Gα_o in rat hippocampal membranes. Results are mean ± SEM of 3-5 independent determinations.

Drug	Binding [^{3}H]8-OH-DPAT	[^{35}S]GTPγS binding to Gα_o			+ WAY100,635
	pKi (N)	pEC_{50}	pIC_{50}	Emax (as % Basal) (N)	pK_B (N)
5-HT	8.73 ± 0.04 (3)	7.91 ± 0.06	---	129.3 ± 5.8 (5)	9.57 ± 0.16 (5)
(+)8-OHDPAT	9.15 ± 0.11 (3)	7.87 ± 0.12	---	126.7 ± 2.3 (4)	9.52 ± 0.07 (3)
Spiperone	7.03 ± 0.11 (3)	---	7.37 ± 0.21	91.6 ± 2.1 (4)	9.52 ± 0.20 (4)
Methiothepin	7.60 ± 0.01 (3)	---	7.98 ± 0.25	91.0 ± 0.5 (4)	8.87 ± 0.44 (3)
WAY100,635	9.33 ± 0.03 (3)	---	---	---	---

A

B