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ACCELERATED COMMUNICATION

Native Rat Hippocampal 5-HT_{1A} Receptors Show Constitutive Activity

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ABSTRACT

Previous studies have shown that human 5-hydroxytryptamine (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 guanosine-5′-*O*-(3-[^{35}S]thio)-triphosphate ([^{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-$G\alpha_o$-antibody capture coupled to scintillation proximity assay under low sodium (30 mM) conditions, we observed high basal [^{35}S]GTPγS binding to $G\alpha_o$ subunits (defined as 100%). Under these conditions, 5-HT and the prototypic selective 5-HT_{1A} agonist (+)8-hydroxy-2-(di-*n*-propylamino)tetralin [(+)-8-OH-DPAT] both stimulated [^{35}S]GTPγS binding to $G\alpha_o$ 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 [*O*-methyl-3*H*]-*N*-(2-(4-(2-methoxyphenyl)-1-piperazinyl)ethyl)-*N*-(2-pyridinyl)cyclohexanecarboxamide trihydrochloride (WAY100,635) could block these effects in a competitive manner with pK_b values (5-HT, 9.57; (+)-8-OH-DPAT, 9.52) that are consistent with its pK_i value at r5-HT_{1A} receptors (9.33). In this native receptor system, spiperone and methiothepin reduced basal [^{35}S]GTPγS binding to $G\alpha_o$ in a concentration-dependent manner to 90% of basal with pIC_{50} values 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 antagonized by WAY100,635 in a concentration-dependent manner (pK_b, 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.

Constitutive activity of G-protein-coupled receptors (GPCR) provides a mechanistic basis for inverse agonism, a phenomenon observed in heterologous expression systems in which 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 GPCRs, 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; among the few exceptions are serotonin 5-HT_{2C} receptors (De Deurwaerdere et al., 2004) and H_3 histaminergic receptors (Morisset et al., 2000). Moreover, there is evidence that mutated GPCRs with elevated constitutive activity are associated with some human diseases (de Ligt et al., 2000; Seifert and Wenzel-Seifert, 2002; Kenakin, 2004). 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.

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ABBREVIATIONS: GPCR, G-protein-coupled receptor; WAY100,635, [*O*-methyl-3*H*]-*N*-(2-(4-(2-methoxyphenyl)-1-piperazinyl)ethyl)-*N*-(2-pyridinyl)cyclohexanecarboxamide trihydrochloride; [^{35}S]GTPγS, guanosine-5′-O-(3-[^{35}S]thio)-triphosphate; (+)8-OH-DPAT, (+)8-hydroxy-2-(di-*n*-propylamino)tetralin; SPA, scintillation proximity assay; 5-HT, 5-hydroxytryptamine; DTT, dithiothreitol.

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 (Millan, 2000; Meltzer et al., 2003; 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,b; 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 antagonist WAY100,635. Likewise, h5-HT$_{1A}$ receptor constitutive activity could be demonstrated on a specific coupling to GTP-binding protein G_z when this particular G-protein was coexpressed with h5-HT$_{1A}$ receptor in *Spodoptera frugiperda* (Sf9) cell line (Barr and Manning, 1997) and on Gα_{i3} activation in Chinese hamster ovary 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 Gα_{i1} in human embryonic kidney 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 (Odagaki and Fuxe, 1995; Alper and Nelson, 1998; Newman-Tancredi et al., 2003b; Odagaki and Toyoshima, 2005a,b). 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 Gα-selective antibodies in [^{35}S]GTPγS binding assays, allowing detection of the response of a single Gα-protein subtype to pharmacological manipulation within a 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 Gα_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 Gα_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 Gα_o subunits in rat brain tissue homogenate.

Materials and Methods

Animal Handling. Animals were handled and cared for in accordance with the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, 1996) and the European Directive EEC/86/609, and the protocol 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, L'Arbresele, France] weighing 240 to 260 g were used for binding studies and were stored at −80°C before use. The membrane preparations and binding assays were performed in duplicate as described previously (Assié et al., 1999). In brief, membranes were homogenized in 20 volumes of ice-cold Tris-HCl buffer (50 mM, pH 7.4 at 25°C), washed with two cycles of centrifugation/resuspension, followed by a 10-min incubation at 37°C, and ending with a final cycle of centrifugation/resuspension. Membranes (750 μg of tissue/well) were incubated at 23°C in microplates with 96 deep wells, each containing 1 nM [^{3}H]8-OH-DPAT, and increasing concentrations of drugs ranging from 10^{-11}M to 10^{-5}M. After a 2-h 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. Nonspecific binding was defined with 10 μM 5-HT, whereas total binding was defined in the absence of any displacer. The radioactivity retained on the filters was measured by scintillation spectroscopy (TopCount; PerkinElmer Life and Analytical Sciences, Boston, MA) in 40 μl of scintillation fluid (Microscint 20; PerkinElmer Life and Analytical Sciences). The results of displacement experiments were analyzed using the nonlinear regression program Prism ver. 4.03 (GraphPad Software, San Diego, CA), which allowed calculation of an IC_{50}. K_i were calculated using the Cheng-Prusoff equation (Cheng and Prusoff, 1973): $K_i = IC_{50}/(1 - ([\text{ligand}]/K_D\text{ligand}))$ where [ligand] is the measured concentration of [^{3}H](+)-8-OH-DPAT in the assay and $K_D$ligand 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 of fresh tissue (corresponding to approximately 300 fmol/mg of protein).

Scintillation Proximity Assay. Hippocampi from male rats [Ico: OFA SD (I.O.P.S. Caw); Iffa Credo] weighing 240 to 260 g were dissected on a cold plate and frozen at −80°C until used. Tissues were homogenized in ice-cold HEPES-DTT buffer (20 mM HEPES, pH 7.0, 0.2 mM EDTA, and 0.2 mM DTT) with 1 mM GTP using a Polytron homogenizer (Kinematica, Basel, Switzerland), GTP favoring endogenous ligand dissociation. The homogenate was incubated at 35°C for 15 min. Membranes were washed by two cycles of centrifugation at 20,000*g* for 15 min at 4°C and resuspension in ice-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 NaCl (Gα-selective antibodies assay) or 30 mM NaCl (pharmacological assay), 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 of HEPES-DTT assay buffer. Membranes (8 μg of protein/well) were preincubated for 30 min at room temperature with drugs (agonist ± antagonist), buffer (to define basal) or 10 μM GTPγS (to define nonspecific). At the end of this preincubation, 0.4 nM [^{35}S]GTPγS was added, and the membranes were incubated for 60 min. Incubation was stopped by adding Nonidet P-40, and the plate was agitated for another 30 min before addition of 0.2 μg of anti-Gα-selective antibodies to each well. The antibodies used were rabbit polyclonal anti-G$\alpha_{q/11}$, anti-Gα_{i3}, and anti-G$\alpha_{s/olf}$ from Santa Cruz Biotechnology (Santa Cruz, CA) or mouse monoclonal anti-Gα_o and anti-Gα_{i1} from BIOMOL Research Laboratories (Plymouth Meeting, PA). Anti-G$\alpha_{q/11}$, anti-Gα_{i1}, and anti-G$\alpha_{s/olf}$ have been characterized previously by Western blot using purified recombinant Gα_{i1}, Gα_{i2}, Gα_{i3}, Gα_o, G$\alpha_{q/11}$, and G$\alpha_{s/olf}$ protein subtypes (Cussac et al., 2002, 2004) and are highly selective for their respective G-protein subtypes. As described by the manufacturer, we observed cross-reactivity of anti-Gα_{i3} with Gα_{i1} and Gα_{i2}, with a stronger reaction on Gα_{i3} (data not shown). Specificity of anti-Gα_o was assessed by western blot using 50 ng of purified recombinant Gα_{i1}, Gα_{i2}, Gα_{i3}, Gα_o, G$\alpha_{q/11}$, and G$\alpha_{s/olf}$ protein subtypes as described previously (Cussac et al., 2002, 2004). Anti-Gα_o are highly selective for Gα_o (Fig. 1A). Note that the basal signal for each antibody will depend on the affinity and selectivity of these antibodies for the G-protein subtype(s) and the revelation system (affinity of secondary antibodies) and basal signal therefore cannot be compared between antibodies.

The primary antibodies were left to react for 60 min under agitation before adding 50 μl of the secondary antibodies (anti-rabbit or anti-mouse coupled to scintillation proximity assay beads; GE Healthcare, Little Chalfont, Buckinghamshire, UK), diluted according to manufacturer's recommendations. The secondary antibodies were left to react for another 60 min, and the plate was centrifuged

at 1000*g* for 15 min at room temperature to take the complex down at the bottom of the well, and radioactivity was immediately measured on a TopCount radioactivity counter (PerkinElmer Life and Analytical Sciences). Raw disintegrations-per-minute data were transformed by subtracting nonspecific and normalizing to percentage of basal [^{35}S]GTPγS binding. All pharmacological parameters were derived from sigmoid nonlinear regression using Prism version 4.03. For antagonism by WAY100,635, pK_b were calculated using the Cheng-Prusoff equation ($K_b = IC_{50}/(1 - ([Ago]/EC_{50Ago}))$), 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.

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 disintegrations per minute) observed with the various antibodies, and the absolute (disintegrations per minute) and relative (percentage of basal) changes elicited by 10 μM (+)8-OH-DPAT. The maximal basal [^{35}S]GTPγS binding was observed with the anti-Gα_o-selective antibodies (Fig. 1A), and these antibodies also showed the maximal absolute (+4607 DPM) and relative (+71%) increases after (+)8-OH-DPAT treatment (Table 1). A moderate (+46%) increase in relative [^{35}S]GTPγS binding was also observed with anti-Gα_{i3} antibodies (Table 1), but absolute [^{35}S]GTPγS binding represented only a minor fraction (+371 DPM, or 8% of the response observed with Gα_o on a disintegrations-per-minute basis; Table 1). Because of the cross-reactivity of anti-Gα_{i3} antibodies with Gα_{i1} and Gα_{i2}, this increase may be due predominantly to Gα_{i2} and/or Gα_{i3} proteins, because no change of [^{35}S]GTPγS binding was seen with Gα_{i1}-selective antibodies (Table 1), thus demonstrating that 5-HT1A receptors do not couple to Gα_{i1} in rat hippocampus. We therefore used the Gα_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$_{1A}$ receptor constitutive activity in this system. Under those conditions, basal [^{35}S]GTPγS labeling of Gα_o was approximately 25,000 dpm, and this basal value was defined as 100% (Figs. 1 and 2). Under 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 Gα_o in a concentration-dependent manner (Fig. 1 and Table 2). The selective neutral 5-HT$_{1A}$ antagonist WAY100,635 competitively reversed this effect; increasing concentration of the antagonist reversed the effects of maximally effective concentrations (1 μM) of serotonin or (+)8-OH-DPAT (Fig. 2 and Table 2).

In this hippocampal tissue homogenate preparation, spiperone and methiothepin showed inverse agonist properties, reducing basal [^{35}S]GTPγS binding to Gα_o in a concentration-dependent manner (Fig. 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) (Fig. 2 and Table 2). In comparison, when a higher concentration of NaCl (100 mM) was used, spiperone and methiothepin inhibited [^{35}S]GTPγS binding only at very high concentrations (> 1 μM; data not shown).

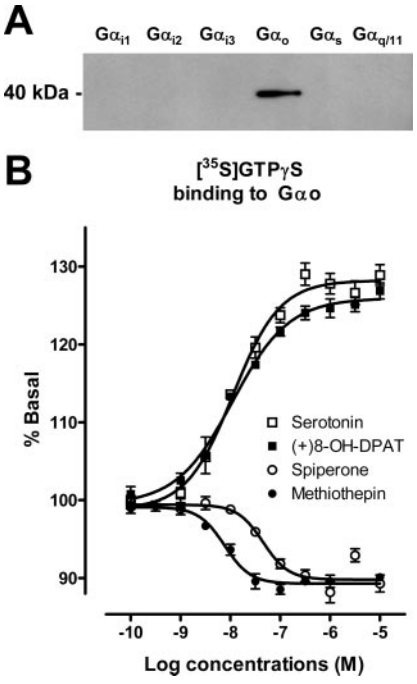

Fig. 1. A, validation of the specificity of anti-Gα_o antibodies by Western blot (see *Materials and Methods* for details). B, concentration-response curves of [^{35}S]GTPγS binding to Gα_o in rat hippocampal membranes. Data are the average of three to five independent determinations, and are expressed as percentage changes from basal (defined as 100%). Serotonin and (+)-8-OH-DPAT increased [^{35}S]GTPγS binding to Gα_o whereas both spiperone and methiothepin reduced basal binding in a concentration dependent manner. See Table 2 for derived constants.

Discussion

Human 5-HT$_{1A}$ receptors expressed in recombinant cell lines have been shown to possess constitutive activity, as revealed by spiperone and methiothepin's inverse agonist properties (see Introduction). Likewise, constitutive activity of h5-HT$_{1A}$ could be demonstrated when the receptor was coexpressed with GTP-binding protein G_z in Sf9 cells (Barr and Manning, 1997) and in human embryonic kidney cells expressing a fusion protein combining 5-HT$_{1A}$ to Gα_{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

TABLE 1

Characterization 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 disintegrations per minute and percentage changes from corresponding basal [^{35}S]GTPγS binding and are the mean ± S.E.M. of four independent determinations.

	Basal	10 μM (+)8-OH-DPAT	
	dpm (N)		*% change from basal*
Antibodies			
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-Gα_{i1}	4302 ± 457 (4)	4027 ± 374 (4)	−6 ± 1
Anti-Gα_{i3}	822 ± 41 (4)	1193 ± 6 (4)	46 ± 7
Anti-Gα_o	6541 ± 615 (4)	11148 ± 829 (4)	71 ± 6

depends on GPCR/G-protein stoichiometry (i.e., an increase in 5-HT$_{1A}$/G-protein ratio increasing the amplitude of inverse agonist response) (Newman-Tancredi et al., 1997b). It is important therefore to assess constitutive activity of GPCR in an environment in which the "natural" GPCR/G-protein ratio is expressed.

The present study is, to our knowledge, the first to demonstrate constitutive activity of native rat 5-HT$_{1A}$ receptors. Previous studies using classic [^{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,b). Alper and Nelson (1998) 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, indicating that it was not related to 5-HT$_{1A}$ receptors. In contrast, using SPA-based Gα_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 Gα_o (Mannoury la Cour et al., 2006). Indeed, in the present study, comparison of antibodies selective for various Gα 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 Gα_o. The SPA-based Gα_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 antagonized 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 pK_b previously published on similar assays (Odagaki and Toyoshima, 2005a,b). These data therefore confirm that the decreases in Gα_o labeling induced by spiperone and methiothepin are indeed mediated by interaction at r5-HT$_{1A}$ receptors.

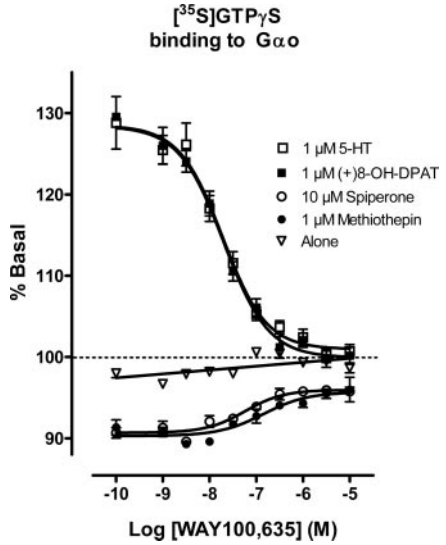

Fig. 2. Reversal by increasing concentrations of WAY100,635 of the effect of a fixed concentration of drugs on [^{35}S]GTPγS binding to Gα_o in rat hippocampal membranes. Data are the average of three to five independent determinations, and are expressed as percentage changes from basal (defined as 100%). See Table 2 for derived constants.

The amplitude of the inverse agonism response observed with spiperone and methiothepin is relatively modest (10% reduction in basal labeling of Gα_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 two drugs as inverse agonists on rat 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 unlabeled GTPγS (Audinot et al., 2000; Rouleau et al., 2002). Second, because this assay is performed on tissue, several Gα_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 methiothepin by WAY100,635 is only partial (two thirds of effect), suggesting that other constitutively active receptors may be involved in the effect of these two drugs (see below).

The demonstration that native r5-HT$_{1A}$ receptors show constitutive activity in vitro suggests 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 pathological conditions, 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 abnormal constitutive activity of some GPCRs, and several clinically relevant drugs have been shown to have inverse agonist properties on GPCRs in recombinant systems (Milli-

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 ± S.E.M. of three to five independent determinations.

Drug	Binding [^{3}H]8-OH-DPAT pK_i (*N*)	[^{35}S]GTPγS Binding to Gα_o			+WAY100,635 pK_B (*N*)
		pEC$_{50}$	pIC$_{50}$	E_{max}	
				% basal (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)				

gan, 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 behavior of inverse agonists in vivo may have other physiological consequences. For example, inverse agonists may produce more prominent receptor upregulation than neutral antagonists (Adan and Kas, 2003), and inverse agonists may lead to increases in G-protein 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 two inverse agonists fully antagonized the effect of 5-CT 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 compared with the time course to reach steady state with the neutral antagonist WAY100,635. Corradetti et al. (2005) suggested that this phenomenon may be explained by a slow allosteric shift of the receptor toward an inactive state. Moreover, in contrast to WAY100,635, which could fully antagonize 5-CT-induced hyperpolarization of hippocampal CA1 neurons, these two compounds showed only partial antagonism, suggesting that these drugs may behave differentially on neuronal populations expressing either pre- or postsynaptic 5-HT_{1A} receptors. These observations are consistent with the differential coupling of the pre- (dorsal raphe) and postsynaptic (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 because 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 favoring 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).

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 because the homogenate was preincubated for 15 min at 35°C in a large excess of buffer containing GTP to favor 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 Gα_o activation persisted even at high antagonist concentrations. This suggests that other receptor systems that couple to Gα_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 acts as an inverse agonist on dopamine D_2 receptors (Roberts and Strange, 2005) and on α_1 adrenoceptors (Rossier et al., 1999), whereas 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 Gα_o remaining with maximal WAY100,635 concentrations may be due to an action at other receptors. The present method targeting Gα_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 with SPA detection provide the first demonstration that native rat 5-HT_{1A} receptors show constitutive activation of Gα_o proteins in an hippocampal tissue environment, suggesting that rat 5-HT_{1A} receptors may be constitutively active in vivo at Gα_o and/or other G-protein subtypes.

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