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Characterization of 5-HT_{1A} receptor-mediated [^{35}S]GTPγS binding in rat hippocampal membranes

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Abstract

Stimulation of [^{35}S]GTPγS binding by serotonin (5-hydroxytryptamine, 5-HT) receptor ligands was characterized in rat hippocampal membranes. The optimized assay contained 30–50 μg protein, 300 μM GDP and 0.1 nM [^{35}S]GTPγS, incubated at 37°C for 20 min. At 10 μM, the 5-HT_{1A} receptor agonist *R*(+)-8-hydroxy-2-(di-*n*-propylamino)tetralin [*R*(+)-8-OH-DPAT] stimulated GTPγS binding from 27.1 ± 2.5 to 45.7 ± 4.2 fmol/mg protein. Increasing the protein concentration did not affect the absolute difference between basal and maximal GTPγS binding nor the EC_{50}, but decreased the percent stimulation. The non-selective agonists serotonin and 5-carboxamidotryptamine were 30–35% more efficacious, whereas the partial agonists buspirone and *S*(−)-8-hydroxy-2-(di-*n*-propylamino)tetralin stimulated GTPγS binding by 19 ± 1 and 43 ± 3%, respectively, compared to *R*(+)-8-OH-DPAT. Neither the 5-HT_2 receptor agonist [(±)1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane HCl] (DOI) nor the 5-HT_{1A} receptor antagonists WAY 100,635 (*n*-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-*n*-(2-pyridinyl) cyclohexanecarboxamide trihydrochloride) and spiperone altered basal GTPγS binding. WAY 100,635 abolished the effect of *R*(+)-8-OH-DPAT, but only reduced the effect of serotonin by 88 ± 3%. Finally, methiothepin antagonized *R*(+)-8-OH-DPAT-stimulated GTPγS binding and reduced basal GTPγS binding by itself. The reduction was not affected by WAY 100,635. We have characterized a method to assess functional activity at 5-HT_{1A} receptors in rat hippocampal membranes by measuring agonist-induced [^{35}S]GTPγS binding. © 1998 Elsevier Science B.V.

Keywords: 5-HT_{1A} receptor; G protein; Guanosine 5′-*O*-(3-[^{35}S]thiotriphosphate) binding; Hippocampus rat; (Functional assay); 8-OH-DPAT (8-hydroxy-2-(di-*n*-propylamino)tetralin)

1. Introduction

Activation of central 5-HT_{1A} receptors elicits a variety of well-characterized biological and cellular responses including stimulation of hormone secretions from the anterior pituitary gland, hypotension and bradycardia, neuronal hyperpolarization, activation of an inwardly rectifying K^+ current and inhibition of forskolin-stimulated adenylyl cyclase as examples (Zifa and Fillion, 1992). These responses occur after agonist binds to the seven transmembrane G protein-coupled 5-HT_{1A} receptor (Hoyer et al., 1994) to activate the G_i and/or G_o protein subtypes (Harrington et al., 1988; Zgombick et al., 1989; Okuhara and Beck, 1994; Oleskevich, 1995). Currently the primary methods available to provide functional assessment of central 5-HT_{1A} receptor activity at the cellular level require intact cells for electrophysiological studies or adenylyl cyclase assays. Recently there have been numerous reports of measuring agonist-stimulated [^{35}S]GTPγS binding, the initial step in signal transduction following G protein activation, as an index of receptor stimulation for a wide variety of neurotransmitters. To date, the only applications of GTPγS binding to study 5-HT_{1A} receptor function have been in heterologous expression systems using cloned human 5-HT_{1A} receptors (Newman-Tancredi et al., 1996a,b, 1997a; Stanton and Beer, 1997). It was the purpose of these studies to develop and characterize 5-HT_{1A} receptor-mediated GTPγS binding in rat hippocampal membranes as a measure of 5-HT_{1A} receptor function using frozen tissue homogenates. We have demonstrated that GTPγS binding can be used to assess 5-HT_{1A} receptor function in membrane preparations of both hippocampus and frontal cortex and thus provides a novel ex vivo

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method to investigate the initial cellular event in transmission of an extracellular signal to an intracellular response.

2. Materials and methods

2.1. Membrane preparation

Hippocampi were obtained from two sources. For some experiments frozen tissue was purchased from Pel-Freez Biologicals (Rogers, AR). Other tissue was obtained from male Sprague–Dawley rats purchased from Harlan Industries (Indianapolis, IN). Rats were killed by decapitation, the brains placed in ice-cold 0.9% NaCl for 1–3 min, the hippocampi dissected free-hand and placed in a vial on ice. The tissue was weighed and frozen at −70°C. Upon defrosting the tissue was homogenized using a Tekmar tissue homogenizer in cold Tris buffer (50 mM Tris base, pH 7.4). The homogenate was centrifuged (39 800 × g, 4°C for 10 min), the supernatant decanted and the remaining pellet resuspended in the same Tris buffer. After homogenization the suspension was incubated at 37°C for 10 min in a shaking water bath. The suspension was then centrifuged, the supernatant decanted and the pellet again washed with cold Tris buffer. After one final centrifugation, the remaining pellet was homogenized in the same Tris buffer at a final concentration of approximately 100 mg tissue/ml. This suspension was frozen in aliquots at −70°C for later use. On the day of the assay, the frozen tissue suspension was defrosted, resuspended in approximately 35 ml Tris buffer and centrifuged as above. This final membrane pellet was suspended in assay buffer (4 mM $MgCl_2$, 160 mM NaCl, 0.267 mM EGTA, 67 mM Tris base, pH 7.4) to produce a protein concentration approximating 200 μg/ml.

2.2. GTPγS binding assay

To each assay tube was added 200 μl deionized water or drug, 200 μl [^{35}S]GTPγS (0.1 nM; the actual determined concentration ranged from 0.07 to 0.20 nM) in ligand buffer (assay buffer containing 1200 μM GDP to produce a final concentration of 300 μM, except as noted), 200 μl assay buffer and 200 μl tissue homogenate (approximately 30–50 μg protein except as noted). In the first two experiments, the assay tubes were incubated for 30 min in a shaking water bath. The third experiment was an incubation time course; the time was varied from 5–120 min. In all subsequent assays the incubation was 20 min. Except in an experiment designed to study the effect of incubation temperature on GTPγS binding, all assays were conducted at 37°C. The reaction was terminated by rapid vacuum filtration through Whatman GF/B filters pre-wet with 20 mM $Na_4P_2O_7 \cdot 10H_2O$ and pre-cooled with 3 washes of 2 ml cold 50 mM Tris buffer. After filtering the reaction mixture, the filters were washed 4 times with 1 ml cold 50 mM Tris buffer, placed into 7 ml scintillation vials and 5 ml Ready Protein scintillation cocktail added. The samples were shaken, allowed to sit for a minimum of 2 h, shaken again and counted (2 min/sample) in a Beckman scintillation counter. Non-specific binding was determined by the amount of [^{35}S]GTPγS bound in the presence of 10 μM unlabeled GTPγS and was subtracted from all samples. Basal GTPγS binding is defined as the specific binding when 200 μl water containing no ligand were added to the assay tube. This is also referred to as agonist-independent binding.

2.3. Data analysis

The protein concentration of each tissue homogenate was determined using the standard Bradford protein assay (Bradford, 1976). The data are presented three ways: (1) fmol GTPγS bound/mg protein calculated directly from the dpm, the specific activity of the [^{35}S]GTPγS and the protein concentration of the tissue homogenate, (2) absolute change from basal (fmol GTPγS bound/mg protein in the agonist-stimulated samples minus basal fmol GTPγS bound/mg protein) and (3) percent stimulation over basal (((fmol GTPγS bound/mg protein in the agonist-stimulated samples ÷ basal fmol GTPγS bound/mg protein) · 100%) − 100). Values represent the mean ± S.E.M. from triplicate determinations in 3–6 separate assays. The EC_{50}, IC_{50} and E_{max} values were calculated by non-linear regression analysis using a four parameter model. The inhibition constant (K_i) was calculated according to the Cheng–Prusoff equation (Cheng and Prusoff, 1973): $K_i = IC_{50}/(1 + A/EC_{50})$, where the IC_{50} was calculated for the antagonist in the presence of a single concentration of agonist (A) and the EC_{50} was calculated for the agonist in the absence of antagonist.

2.4. Materials

Drugs purchased from RBI (Natick, MA) used in these experiments include $R(+)$-8-hydroxy-2-(di-*n*-propylamino)tetralin HBr [$R(+)$-8-OH-DPAT], $S(-)$-8-hydroxy-2-(di-*n*-propylamino)tetralin HBr [$S(-)$-8-OH-DPAT], buspirone HCl, 5-carboxamidotryptamine maleate (5-CT), methiothepin mesylate, spiperone HCl and (±)1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane HCl (DOI). 5-HT creatinine sulfate complex (5-HT) and GDP were purchased from Sigma Chemical Co. (St. Louis, MO). GDP and unlabeled GTPγS were purchased from Boehringer Mannheim Corporation (Indianapolis, IN). WAY 100,635 (*n*-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-*n*-(2-pyridinyl) cyclohexanecarboxamide trihydrochloride) was synthesized at Eli Lilly and Company (Indianapolis, IN). All drugs were dissolved in deionized water and prepared fresh daily. [^{35}S]GTPγS (1250 Ci/mmol) was purchased from NEN Life Science Products (Boston, MA).

3. Results

3.1. Effects of GDP on agonist-stimulated GTPγS binding in hippocampus and frontal cortex

The effects of graded concentrations of GDP on GTPγS binding were determined in two experiments. The data presented in the inset of Fig. 1 demonstrate that GDP causes a marked reduction in basal (i.e. agonist-independent) GTPγS binding in hippocampus and frontal cortex. Furthermore, there is a direct relationship between the GDP concentration in the incubation mix and the stimulation of GTPγS binding produced by 1 μM of the full 5-HT_{1A} receptor agonist *R*(+)-8-OH-DPAT in both brain regions (Fig. 1). The effect of GDP on *R*(+)-8-OH-DPAT-stimulated GTPγS binding is more pronounced in the hippocampus where the 5-HT_{1A} receptor agonist produced a stimulation approximately 100% above basal whereas the best responses in the frontal cortex were only 30–35% over basal. The absolute values (fmol/mg protein) of both basal and agonist-stimulated GTPγS binding decreased at each concentration of GDP (data not presented), but GDP reduced agonist-independent GTPγS binding proportionally more than agonist-stimulated binding, hence, the percent stimulation above basal increased as the GDP concentration increased (Fig. 1). Since the signal was much more robust in the hippocampus, all subsequent studies were conducted only on this tissue using a final concentration of 300 μM GDP.

3.2. Effect of incubation temperature on GTPγS binding in hippocampus

Presented in the inset of Fig. 2 are the results of a representative experiment demonstrating that as incubation temperature increases both agonist-independent and agonist-stimulated GTPγS binding increases. Incubation temperature does not alter the maximal effect (expressed as percent stimulation over basal; main part of Fig. 2) or the EC_{50} (Table 1) produced by *R*(+)-8-OH-DPAT on GTPγS binding in the hippocampus. It was decided from these data that 37°C was the most appropriate incubation temperature since it produced the greatest range in dpm from basal to maximal stimulation without affecting the E_{max} or EC_{50}.

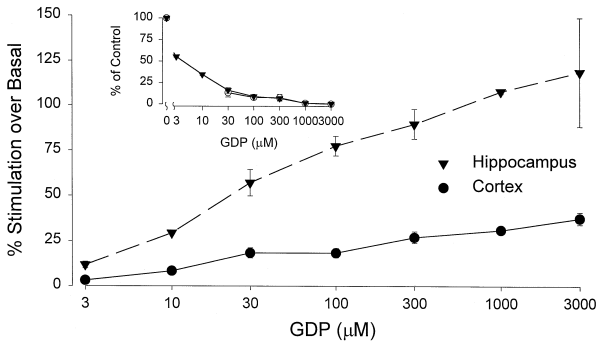

Fig. 1. Effects of GDP on agonist-independent and agonist-stimulated GTPγS binding in hippocampus and frontal cortex. Homogenates of rat hippocampus (triangles) and frontal cortex (circles) were incubated with water (inset) or 1 μM *R*(+)-8-OH-DPAT (main figure) in the presence of GDP (0 to 3000 μM). Values represent the mean ± S.E.M. from 3–6 separate experiments. Although not designated in the figure, there was a significant main effect (hippocampus ≠ frontal cortex) and at all concentrations of GDP greater than 10 μM the percent stimulation in hippocampus was not equal to frontal cortex ($P < 0.05$ by two-way ANOVA followed by Student–Newman–Keuls' post-hoc test).

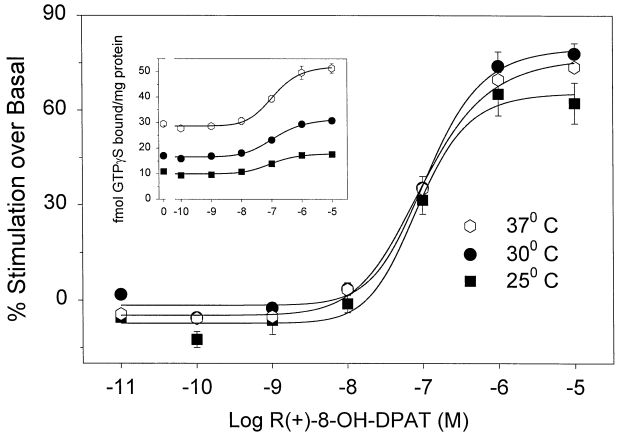

Fig. 2. Effect of incubation temperature on *R*(+)-8-OH-DPAT-stimulated GTPγS binding in hippocampus. Homogenates of rat hippocampus (30–50 μg protein) were incubated for 30 min at 25° (filled squares), 30° (filled circles) or 37°C (open hexagons) in a shaking water bath before the reaction was terminated by rapid filtration. The data are expressed as percent stimulation over basal in the main figure (mean ± S.E.M. from 3–4 separate experiments) and as absolute levels of GTPγS binding in the inset (mean ± S.E.M. of triplicate determinations from a representative experiment).

3.3. Time-course of GTPγS binding in hippocampus

GTPγS binding, when expressed as either total or specific binding, increases linearly over time for at least 120 min under both basal and agonist-stimulated conditions; the results of one typical experiment are presented in Fig. 3. These results are different from what is generally true of radioligand binding assays which typically attain equilibrium within that period of time. Fig. 4 summarizes the data from three separate experiments which show that the greatest relative stimulation produced by either 30 nM or 10

Table 1
Incubation temperature does not alter the potency of *R*(+)-8-OH-DPAT to stimulate GTPγS binding in rat hippocampus

	Incubation temperature		
	25°C	30°C	37°C
EC_{50} (nM)	95 ± 16.9	118 ± 8.4	109 ± 3.6

The values represent the mean ± S.E.M. as determined from 4 separate 6-point concentration–response curves at each temperature (presented in Fig. 2). There were no differences between the values ($P > 0.05$).

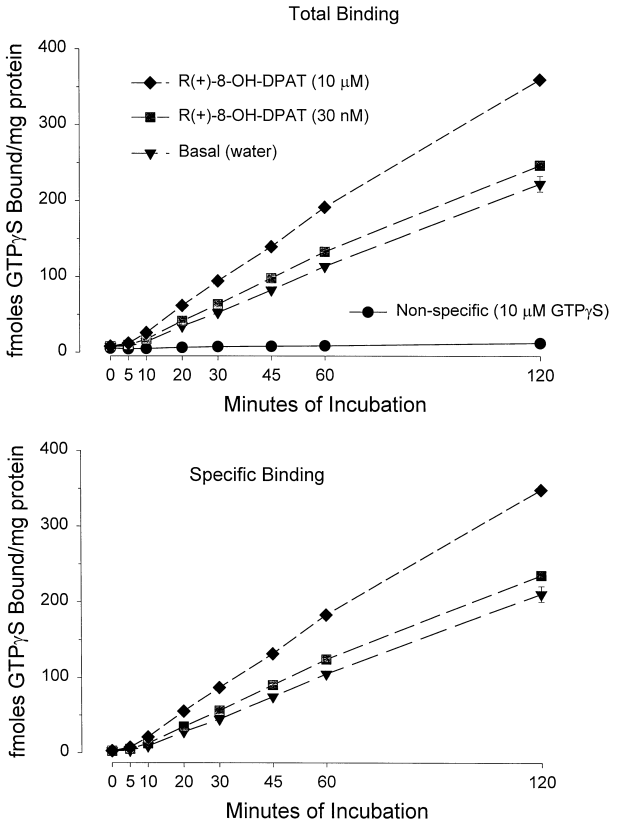

Fig. 3. Effect of incubation time on non-specific, agonist-independent and agonist-stimulated GTPγS binding in hippocampus. Homogenates of rat hippocampus (30–50 μg protein) were incubated for 5–120 min at 37°C in a shaking water bath, or very briefly on ice ('0 min of incubation'), before the reaction was terminated by rapid filtration. Additions to the homogenates included 10 μM unlabeled GTPγS to define non-specific binding (circles), water to define agonist-independent binding (triangles) and either 30 nM (squares) or 10 μM (diamonds) *R*(+)-8-OH-DPAT to define agonist-stimulated binding. The values represent the mean ± S.E.M. of triplicate determinations from a representative experiment. The upper panel presents the data as total binding whereas the lower panel presents the data as specific binding (total minus non-specific binding at the corresponding time point).

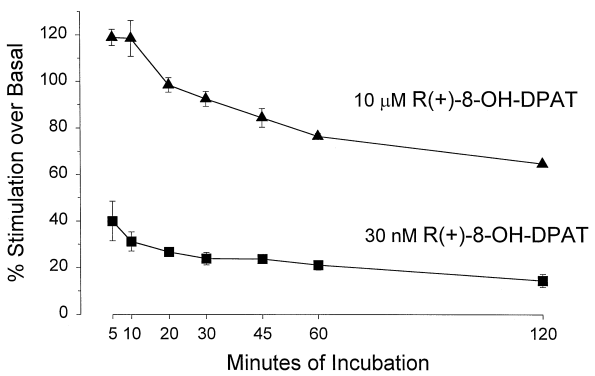

Fig. 4. Effect of incubation time on agonist-stimulated GTPγS specific binding in hippocampus. Values represent the mean ± S.E.M. from 3 separate experiments. See legend for Fig. 3 for further details.

μM of *R*(+)-8-OH-DPAT is observed at shorter incubation times. 20 min was chosen for all experiments because it provided the optimal condition when both dpm in the samples and the percent stimulation above basal were considered.

3.4. Effect of protein concentration on GTPγS binding in hippocampus

All previous experiments were conducted with approximately 30–50 μg of crude membrane protein in each assay tube. We next wanted to determine the effect of altering the amount of protein in each tube without changing any of the other incubation conditions. In this experiment, concentrated membrane homogenates ('undiluted' in Fig. 5) were serially diluted as 1 volume homogenate + 1 volume assay buffer three times; the protein concentration was determined for each preparation and is presented as μg protein/incubation tube in the middle panel. The effects of varying the amount of membrane protein included in the assay on *R*(+)-8-OH-DPAT concentration–response curves are presented in Fig. 5 as the absolute values in the upper panel, the absolute change from basal

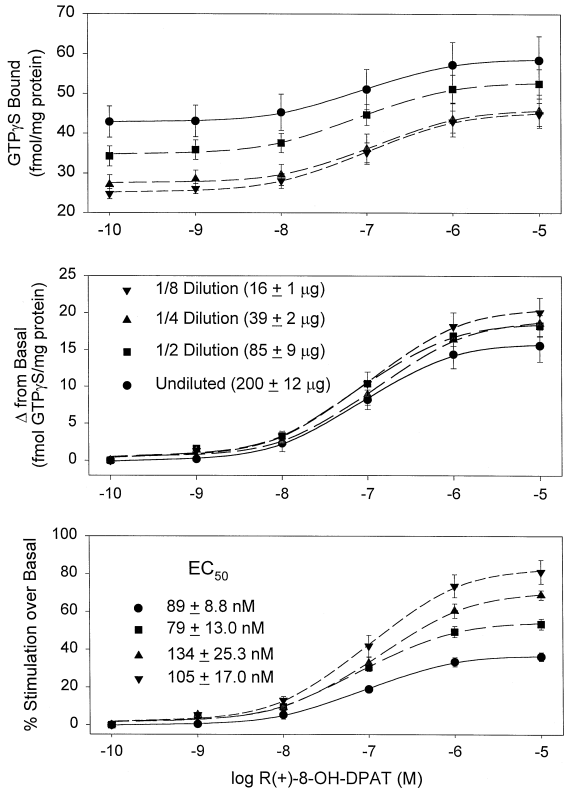

Fig. 5. Effect of protein concentration on GTPγS binding in hippocampus. Hippocampal homogenates were diluted serially in assay buffer as noted in the middle panel. Six-point *R*(+)-8-OH-DPAT concentration–response curves were constructed and analyzed; the EC_{50} values are presented in the bottom panel and are not significantly different from each other ($P > 0.05$ by one-way ANOVA). Values represent the mean ± S.E.M. from 3 separate experiments.

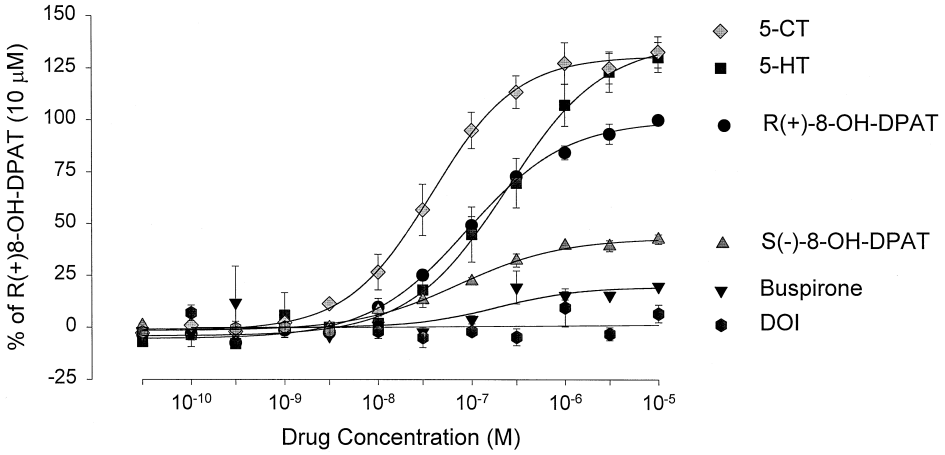

Fig. 6. Effect of 5-HT receptor agonists on GTPγS binding in hippocampus. Hippocampal homogenates were incubated using optimized assay conditions in the presence of 12 concentrations of 5-CT (diamonds), 5-HT (squares), *R*(+)-8-OH-DPAT (circles), *S*(−)-8-OH-DPAT (triangles), buspirone (upside down triangles) or DOI (hexagons). The data are presented as percent of 10 μM *R*(+)-8-OH-DPAT and represent the mean ± S.E.M. from 3 separate experiments. All produced significant stimulation of GTPγS binding above basal except for the 5-HT_2 agonist DOI ($P < 0.05$ by one-way ANOVA).

binding in the middle panel and as the percent stimulation over basal in the bottom panel. As shown previously, *R*(+)-8-OH-DPAT produces consistent concentration-dependent increases in GTPγS binding. Even though the absolute amount of GTPγS bound has been normalized to account for the protein concentration, more GTPγS binding per mg membrane protein is observed when the incubation mixture contains more protein, demonstrated in the upper panel. This is particularly evident at 10^{-10} M *R*(+)-8-OH-DPAT which produced the same amount of GTPγS binding as that produced in the absence of agonist (basal). This once again is very different from what is typically observed in radioligand binding assays.

When the data are presented as absolute change from basal (middle panel of Fig. 5), the results appear somewhat different. Normalization by subtracting agonist-independent binding shifts the agonist-stimulated concentration–response curves to be essentially superimposable, with the most concentrated homogenate producing a slightly lower curve than the others. By contrast, when normalized as percent stimulation over basal (bottom panel, Fig. 5), there is a marked effect of homogenate dilution: the maximal response (but not the EC_{50}) is inversely related to the amount of protein. In all subsequent experiments the tissue homogenates were diluted in an appropriate volume of assay buffer to deliver 30–50 μg membrane protein in a volume of 200 μl into each assay tube.

3.5. Effect of 5-HT receptor agonists on GTPγS binding in hippocampus

The effects of five 5-HT receptor agonists with different affinities, efficacies and specificities at the 5-HT_{1A} receptor were compared to the full, selective 5-HT_{1A} receptor agonist *R*(+)-8-OH-DPAT (Fig. 6). The non-selective agonists 5-HT ($EC_{50} = 257 \pm 73$ nM) and 5-CT ($EC_{50} = 41 \pm 10$ nM) were 30–35% more efficacious than *R*(+)-8-OH-DPAT ($EC_{50} = 92 \pm 13$ nM), whereas the partial agonists buspirone ($EC_{50} = 171 \pm 35$ nM) and *S*(−)-8-OH-DPAT ($EC_{50} = 79 \pm 16$ nM) stimulated GTPγS binding to $19 \pm 1\%$ and $43 \pm 3\%$, respectively, of *R*(+)-8-OH-DPAT. The 5-HT_2 receptor agonist DOI did not alter basal GTPγS binding ($EC_{50} > 10\,000$).

3.6. Effect of WAY 100,635 on agonist-stimulated GTPγS binding in hippocampus

The selective 5-HT_{1A} receptor antagonist WAY 100,635, did not alter GTPγS binding by itself (data not presented), abolished the effect of *R*(+)-8-OH-DPAT, but only reduced the effect of 5-HT by $88 \pm 3\%$ (Fig. 7). The K_i

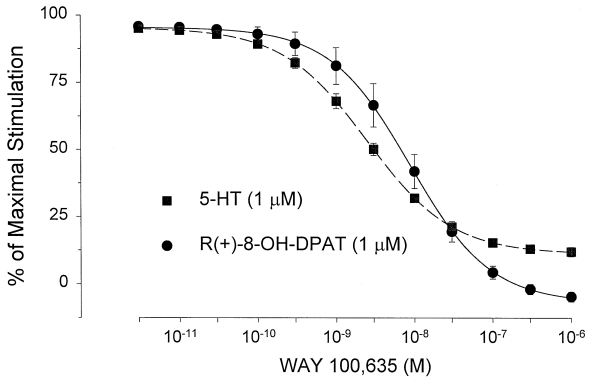

Fig. 7. Effect of WAY 100,635 on agonist-stimulated GTPγS binding in hippocampus. Hippocampal homogenates were incubated in the presence of graded concentrations of WAY 100,635 and 1 μM of either 5-HT (squares) or *R*(+)-8-OH-DPAT (circles). Values represent the mean ± S.E.M. from 3 separated experiments. * significantly different from *R*(+)-8-OH-DPAT at the same concentration of WAY 100,635 ($P < 0.05$).

Table 2
Effect of methiothepin and spiperone on GTPγS binding in hippocampus

	EC_{50}/IC_{50} (nM) (nM)	K_i	Basal fmol GTPγS bound/mg protein	Maximal fmol GTPγS bound/mg protein
$R(+)$-8-OH-DPAT (10^{-10}–10^{-5} M)	146 ± 24	N.C.	19.0 ± 1.9	35.0 ± 4.2[a]
Methiothepin (10^{-10}–10^{-5} M)	N.C.	N.C.	19.8 ± 1.7	17.7 ± 1.6[a]
Spiperone (10^{-10}–10^{-5} M)	N.C.	N.C.	18.5 ± 2.4	18.8 ± 1.5
Methiothepin (10^{-10}–10^{-5} M) + $R(+)$-8-OH-DPAT	72 ± 18	9 ± 2	19.9 ± 1.8	17.7 ± 1.8[a]
Spiperone (10^{-10}–10^{-5} M) + $R(+)$-8-OH-DPAT	240 ± 34	30 ± 6	19.8 ± 2.0	19.0 ± 1.7
WAY 100,635 (10^{-11}–10^{-6} M) + Methiothepin	N.C.	N.C.	18.9 ± 1.7	17.3 ± 1.4[a]

Six-point concentration–response curves were constructed for $R(+)$-8-OH-DPAT, methiothepin and spiperone to assess the effect of these drugs on basal GTPγS binding. In addition, the abilities of methiothepin and spiperone to inhibit 1 μM $R(+)$-8-OH-DPAT-stimulated GTPγS binding was assessed in 6-point concentration–response curves; IC_{50} and K_i values were calculated as measures of the potencies and efficacies of these two compounds as receptor antagonists. Finally, the ability of WAY 100,635 (10^{-11}–10^{-6} M) to inhibit the effect of 10 μM methiothepin on GTPγS binding was also determined. Values represent the mean ± S.E.M. from 3 separate experiments. The maximal response is the GTPγS binding observed at the highest concentration of the ligand that was varied. The K_i was derived according to the Cheng–Prussof equation as described in Section 2.3. N.C. means not able to be calculated from the data.
[a]Significantly different from basal ($P < 0.05$).

values for WAY 100,635 to inhibit the responses to $R(+)$-8-OH-DPAT and 5-HT were calculated using the agonist EC_{50}s from the preceding study and were found to be 0.8 ± 0.19 and 0.5 ± 0.06 nM, respectively. The residual 'non-WAY sensitive' 5-HT-stimulated GTPγS binding (12 ± 2% of maximal) was observed in all three experiments.

3.7. Effect of methiothepin and spiperone on GTPγS binding in hippocampus

Both methiothepin and spiperone were examined in 6-point concentration–response curves as 5-HT_{1A} receptor agonists and antagonists. When tested as agonists, spiperone did not alter GTPγS binding whereas methiothepin produced a small, but statistically significant decrease from basal (Table 2). However, the decrease produced by methiothepin (10 μM) is not due to inverse agonist activity at 5-HT_{1A} receptors since it was not affected by the potent, selective 5-HT_{1A} receptor antagonist WAY 100,635 at concentrations from 10^{-11} to 10^{-6} M. Both methiothepin and spiperone competitively antagonized the stimulation of GTPγS binding produced by 1 μM $R(+)$-8-OH-DPAT, consistent with their known properties as potent non-selective 5-HT_{1A} receptor antagonists.

3.8. Effect of GDP on potency and efficacy of a full, a partial and a putative inverse 5-HT_{1A} receptor agonist

In an earlier experiment we demonstrated that basal GTPγS binding was inversely related to the concentration of GDP in the incubation. To determine if the concentration of GDP and thus the level of basal GTPγS binding could affect the potencies and/or efficacies of 5-HT_{1A} receptor agonists, 6-point concentration–response curves to $R(+)$-8-OH-DPAT, $S(-)$-8-OH-DPAT and spiperone (representing a full, a partial and a putative inverse agonist, respectively) were constructed in the presence of 10, 30 and 300 μM GDP. As shown earlier, basal GTPγS binding is lower in the presence of higher GDP concentrations (Table 3 and upper panel of Fig. 8). Furthermore, spiperone did not decrease GTPγS binding below basal at any concentration of GDP examined (Table 3) suggesting that in our preparation of rat hippocampal membranes spiperone does not behave as an inverse agonist.

Table 3
Spiperone does not alter GTPγS binding in hippocampus

GDP (μM)	GTPγS bound (fmol/mg protein)						
	Spiperone (M)						
	Basal	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
300	28.7 ± 3.4	31.3 ± 6.0	28.0 ± 3.3	26.8 ± 3.4	27.6 ± 4.2	28.0 ± 3.6	27.1 ± 3.0
30	163.3 ± 24.7	161.9 ± 24.8	161.3 ± 24.4	159.0 ± 24.0	159.7 ± 22.1	160.4 ± 23.7	161.0 ± 23.6
10	301.3 ± 42.5	293.0 ± 40.2	285.9 ± 40.0	290.3 ± 44.8	291.5 ± 39.5	301.8 ± 42.3	375.9 ± 123.2

Homogenates of hippocampus were incubated with three concentrations of GDP in the presence of water ('basal') and 6 concentrations of spiperone. Values represent the mean ± S.E.M. from 4 separate experiments. There were no significant differences from basal within any concentration of GDP ($P > 0.05$).

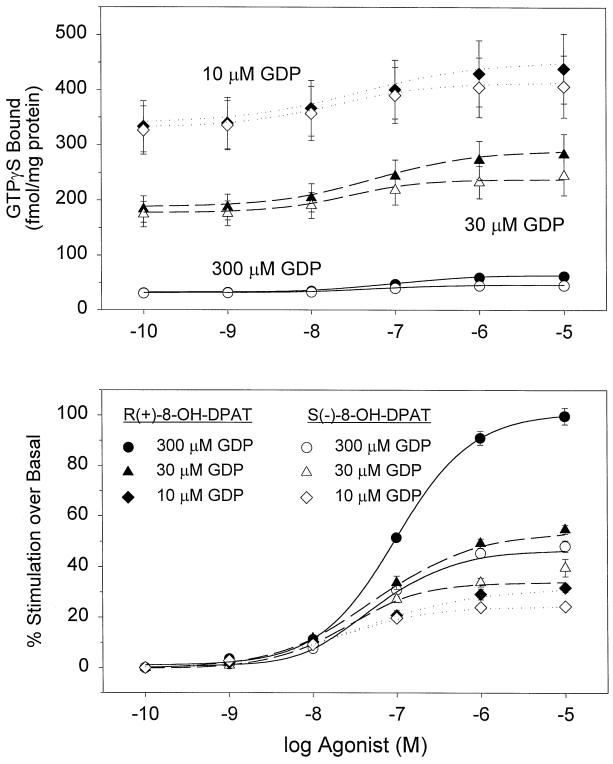

Fig. 8. Effect of GDP on agonist-stimulated GTPγS binding in hippocampus. Hippocampal homogenates were incubated with GDP at concentrations of 10 (diamonds), 30 (triangles) or 300 (circles) μM in the presence of either the full 5-HT_{1A} receptor agonist $R(+)$-8-OH-DPAT (filled symbols) or the partial 5-HT_{1A} receptor agonist $S(-)$-8-OH-DPAT (open symbols). Values represent the mean ± S.E.M. from 4 separate experiments.

The effects of the $R(+)$ and $S(-)$ isomers of 8-OH-DPAT are depicted in Figs. 8 and 9. In Fig. 8 are the concentration–response curves graphically represented as the absolute data in the top panel and as percent stimulation over basal in the bottom panel. Again note in the top panel the dramatic effect that GDP has on GTPγS binding. When the data are normalized it becomes evident that the greatest stimulation for both the full and partial agonist is observed with the GDP concentration at the level for which the assay was optimized; 300 μM. This is presented in Fig. 9 as the E_{max}. An important aspect of this experiment is that the apparent partial agonist activity of $S(-)$-8-OH-DPAT relative to the full agonist $R(+)$-8-OH-DPAT is dependent on the GDP concentration. That is, at 10 μM GDP the E_{max} for the S isomer is 79% of that for $R(+)$-8-OH-DPAT, at 30 μM the relative efficacy is 64% whereas at 300 μM it is only 49%. This is because GDP decreases the maximal effect produced by the full 5-HT_{1A} receptor agonist $R(+)$-8-OH-DPAT more than it decreases the response produced by the partial agonist. In addition, lowering the concentration of GDP in the incubation mix increases the potency (demonstrated as a decrease in the EC_{50} in Fig. 9) of both the full and partial agonists, consistent with the reported effects of guanine nucleotides on [^{3}H]8-OH-DPAT binding in radioligand binding studies (Schlegel and Peroutka, 1986).

4. Discussion

Experiments were designed to characterize 5-HT_{1A} receptor mediated [^{35}S]GTPγS binding in rat hippocampal membranes. The data suggest that the non-selective agonists 5-HT and 5-CT stimulate GTPγS binding through activation of more than just the 5-HT_{1A} receptor: (1) they produce responses greater than those produced by the full, selective 5-HT_{1A} receptor agonist $R(+)$-8-OH-DPAT and (2) the effects of 5-HT were not completely abolished by the selective 5-HT_{1A} receptor antagonist WAY 100,635. In addition, under the conditions of our assay system we were able to detect partial agonists with efficacies as low as

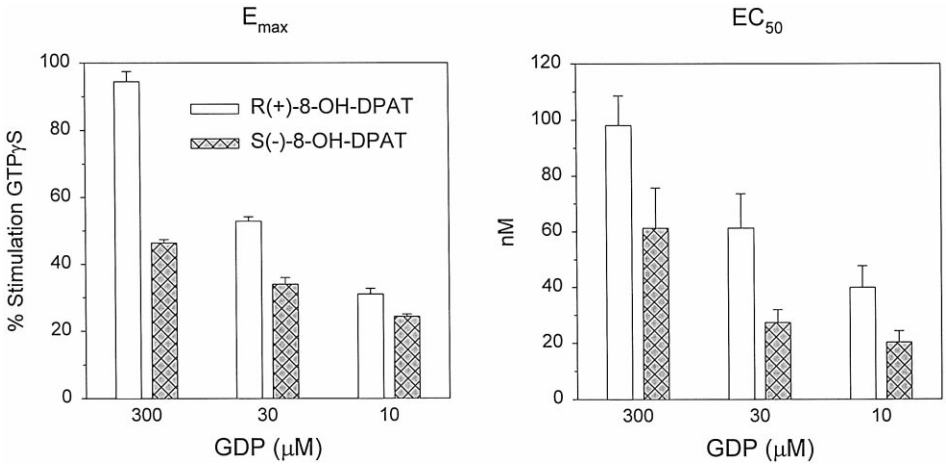

Fig. 9. Effect of GDP on agonist-stimulated GTPγS binding in hippocampus. Data were calculated from the 6-point concentration–response curves shown in Fig. 8 (see legend for details).

19% of *R*(+)-8-OH-DPAT (as in the case of buspirone), did not find any measurable efficacy for the 5-HT_2 receptor agonist DOI and did not observe 5-HT_{1A} receptor-mediated inverse agonist activity for the antagonists WAY 100,635, spiperone or methiothepin.

Neither time nor temperature of incubation produced marked effects on agonist-stimulated GTPγS binding. That is, neither parameter altered the potency or efficacy of *R*(+)-8-OH-DPAT to stimulate GTPγS binding in rat hippocampus, thus these parameters were important simply to optimize the signal to noise ratio.

By contrast, factors such as the amount of membrane protein, GDP and radiolabeled GTPγS did have a major impact on experimental results. Although not presented nor evaluated systematically, small variations in the concentration of [^{35}S]GTPγS added to the incubation mixture appeared to produce marked variations in the absolute amount of GTPγS bound, even with the subtraction of non-specific binding. Therefore, to control for interassay variability the data are presented most frequently as percent stimulation over basal (agonist-independent) binding; this was found to provide the most consistent results from assay to assay.

In our initial GDP study it was found that maximal *R*(+)-8-OH-DPAT-stimulated GTPγS binding was observed in both hippocampus and frontal cortex when the GDP concentration was 100 μM or greater. These concentrations of GDP reduced basal GTPγS binding to less than 10% of control (defined as specific GTPγS binding in the absence of GDP and agonist). In both hippocampus and frontal cortex the greatest difference between agonist-independent and agonist-stimulated GTPγS binding was observed in the presence of GDP concentrations greater than 100 μM. This is greater than what most others have used (Hilf et al., 1989; Chabert et al., 1994; Newman-Tancredi et al., 1996a; Burkey et al., 1997; Selley et al., 1997) and demonstrates the importance of determining optimal assay conditions which may depend on factors such as the G protein (for example, the requirements for G_i- or G_o-coupled receptors are likely to be very different from G_s-coupled receptors (Wieland and Jakobs, 1994)), the specific neurotransmitter receptor of interest and the cell type in which the receptor is expressed, whether native or transfected.

The difference in the response elicited by 1 μM *R*(+)-8-OH-DPAT in the hippocampus as compared to the frontal cortex was greater than anticipated. The binding of [^{3}H](±)-8-OH-DPAT in the hippocampus is about twice that in the frontal cortex (Alper and Nelson, unpublished observations), GDP reduces agonist-independent GTPγS binding similarly in the two brain regions and we have found that the EC_{50} for *R*(+)-8-OH-DPAT to stimulate GTPγS binding is the same in both areas of the brain (data not presented). One might predict that the response produced by 1 μM *R*(+)-8-OH-DPAT in the hippocampus would be approximately double that in the frontal cortex yet under the best circumstance we were able to see about a 3-fold difference. It has been shown previously that (±)-8-OH-DPAT produces a greater inhibition of forskolin-stimulated cAMP accumulation in primary cultures of hippocampus as compared to cortex (Dumuis et al., 1988). Our data suggest that this difference may reside upstream from the cyclase at the level of GTP binding to the G protein or at the receptor/G protein linkage. The precise mechanism for the differential activation of the G protein by *R*(+)-8-OH-DPAT in the hippocampus and frontal cortex is not yet evident.

Our second GDP study revealed even more important information. It was found that the ability to observe partial agonist activity is dependent, in part, on the assay conditions. When GDP was added in less than optimal concentrations, the partial 5-HT_{1A} receptor agonist *S*(−)-8-OH-DPAT behaved nearly as a full agonist due primarily to a decrease in the efficacy of *R*(+)-8-OH-DPAT. Similar results regarding the relationship between efficacies of partial agonists and GDP concentration on GTPγS binding have been reported for opioid agonists at both native and cloned mouse μ-opioid receptors (Selley et al., 1997). One interpretation is that with less than optimal GDP concentrations there is insufficient G protein in the GDP-bound state, the form most favoring agonist activation. This then creates the appearance of 'spare receptors' (i.e. receptors without appropriate coupling to the signal transducing G protein) and thus the inability to differentiate full from partial agonists. The physiological implications are rather profound and suggest that intracellular levels of GDP may, in part, regulate cellular responses to endogenous and exogenous agonists of G protein-coupled receptors.

The results of the protein experiment were a bit unexpected. In standard radioligand binding assays the B_{max} (expressed as fmol ligand bound/mg protein) is not greatly influenced by the amount of protein. That is, the specific binding of the radioligand increases linearly with receptor protein over a wide range. In the GTPγS binding assay we describe, agonist-independent binding behaves similar to standard radioligand binding, but the E_{max} of agonist-stimulated GTPγS binding clearly does not (see Fig. 5). Once the assay conditions were optimized using 30–50 μg protein *per* assay tube, simply doubling the protein addition produced a rather modest decrease in the maximal response (analogous to the B_{max}) without affecting the EC_{50}. Therefore, if a comparison of maximal efficacy is to be made between tissue preparations or between assays on different days, it is critical to ensure that the homogenates used in the assay contain similar amounts of protein. The influence of varying protein concentrations will not necessarily be eliminated through normalization by dividing the amount GTPγS bound by the amount of protein per assay tube.

In previous reports, both methiothepin (Stanton and Beer, 1997) and spiperone (Newman-Tancredi et al., 1997a) were suggested to act as inverse agonists at the 5-HT_{1A} receptor. Our data are somewhat at odds with these obser-

vations, but they are consistent with earlier studies which demonstrated pure antagonist activity for both drugs when assessed in electrophysiological or adenylyl cyclase studies (Schoeffter and Hoyer, 1988; Zgombick et al., 1989; Pauwels et al., 1993). Although methiothepin did decrease basal GTPγS binding in homogenates of hippocampus, we do not feel that it is due to an effect at the 5-HT_{1A} receptor: it was not altered by the potent and selective 5-HT_{1A} receptor antagonist WAY 100,635. However, the effect of methiothepin does not appear to be 'non-specific' (i.e. not receptor mediated) in that methiothepin did not decrease basal GTPγS binding in striatal homogenates ($n = 1$, unpublished data). One explanation is that the decrease in basal GTPγS binding produced by methiothepin in hippocampus is due to inverse agonist activity at another G_i/G_o coupled receptor, such as the 5-HT_{1D} receptor where it also has been suggested to be an inverse agonist (Thomas et al., 1995). Furthermore, our data do not actually conflict with those of Stanton and Beer (1997). Although it was demonstrated that methiothepin decreased basal GTPγS binding in HeLa cells permanently transfected with the cloned human 5-HT_{1A} receptor, there were no data demonstrating that the response could or could not be antagonized by a 5-HT_{1A} receptor antagonist. The greater decrease in basal GTPγS binding by methiothepin reported previously (approximately 45% as compared to our 10%) could be due to differences in expression levels of the receptor, the assay conditions, the coupling between the receptor and the G protein in a native as compared to a transfected cell, or even the cells in which the receptors are expressed (neurons versus HeLa).

It is somewhat more problematic to explain the lack of inverse agonist activity of spiperone in rat hippocampus. The study of Newman-Tancredi et al. (1997a) clearly demonstrated that spiperone produced a modest concentration-dependent inhibition of basal GTPγS binding in Chinese hamster ovary (CHO) cells stably expressing the cloned human 5-HT_{1A} receptor. Furthermore, the effect of spiperone was antagonized by WAY 100,635. Since the basal GTPγS binding in the assay of Newman-Tancredi et al., is much greater than ours when run using the standard 300 μM GDP, we tested spiperone for inverse agonist activity with lower concentrations of GDP in the incubation. Although basal GTPγS binding was increased by decreasing the GDP, we were still unable to observe inverse agonist activity for spiperone. One obvious major difference between the hippocampal homogenates and the CHO cells is the expression density of the 5-HT_{1A} receptors. The CHO cell line reportedly expresses approximately 3 pmol 5-HT_{1A} receptor/mg protein (Newman-Tancredi et al., 1992) whereas the hippocampus expresses approximately 300 fmol/mg protein (unpublished observations). It is assumed that with the 10-fold difference in receptor density, there is likely to be a substantial difference in the stoichiometric relationship between the 5-HT_{1A} receptor and its associated G protein which could account for the lack of inverse agonist activity for spiperone in membranes prepared from rat hippocampus (Newman-Tancredi et al., 1997b).

A simple and plausible explanation for both discrepancies noted above is that we are studying the receptor in its native tissue with its normal complement of G proteins whereas the previous studies suggesting inverse agonism for both spiperone and methiothepin employed artificial expression systems. In such systems the stoichiometry of the receptor/G protein coupling and/or the specific G protein to which the 5-HT_{1A} receptor is coupled has the distinct possibility to be unrelated to the native state (Kenakin, 1996). What is very clear, however, is that neither spiperone nor methiothepin display properties of inverse agonists at the 5-HT_{1A} receptor in our rat hippocampal membrane preparations.

The use of agonist-stimulated GTPγS binding provides an extremely powerful method to study functional responses at G protein-coupled seven transmembrane receptors. It provides data regarding not only affinity which can be obtained using radioligand binding assays, but also efficacy as an agonist, an inverse agonist or an antagonist. This second point is of particular importance because efficacy is determined in the current experiments using frozen membrane preparations which offers major advantages to previously used methods which generally assess function in whole, living cells. One limitation that should be considered is the application of Scatchard analysis to GTPγS binding data. As shown by Northup et al. (1982), the binding of [^{35}S]GTPγS to the guanine nucleotide-binding regulatory protein under activating conditions (i.e. in the presence of Mg^{2+}) is irreversible. Due to these questionable kinetics one must apply Scatchard analysis with caution. A second limitation was originally noted by Lazareno et al. (1993) and was confirmed in the present studies. Since the requirement for GDP depends on the receptor and the expression system (Wieland and Jakobs, 1994) and GDP concentrations clearly alter agonist potency and efficacy in stimulating GTPγS binding (see Figs. 8 and 9), comparing agonist responses between receptors and/or experimental models without considering the assay conditions themselves might lead to inappropriate conclusions. In addition, the requirement for high concentrations of GDP in the GTPγS assay and its effect on agonist binding to the 5-HT_{1A} receptor (Schlegel and Peroutka, 1986) might explain, in part, the apparent greater EC_{50} of the agonists when compared to literature values determined in radioligand binding assays.

Even with this in mind, agonist-stimulated GTPγS binding can be used to determine if a ligand is a receptor agonist, antagonist or inverse agonist (with the caveat that this may be somewhat 'system dependent') or if a treatment (be it in vivo or in vitro) alters receptor function as defined by the first step in signal transduction. It provides a particularly powerful tool for it has the sensitivity to allow both functional (GTPγS binding) and radioligand

binding assays on the same tissue sample from a single animal. That is true, at least, for 5-HT_{1A} receptors in the rat hippocampus and frontal cortex.

In summary, we have characterized a simple, sensitive and reliable ex vivo assay system to assess function of 5-HT_{1A} receptors in membrane preparations derived from rat hippocampus and frontal cortex.

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