ELSEVIER

European Journal of Pharmacology
Molecular Pharmacology Section 288 (1995) 173–186

ejp
molecular pharmacology

The selective 5-HT_{1A} antagonist radioligand [^{3}H]WAY 100635 labels both G-protein-coupled and free 5-HT_{1A} receptors in rat brain membranes

Henri Gozlan [1], Stéphanie Thibault, Anne-Marie Laporte, Lucimey Lima, Michel Hamon *

INSERM U288, Neurobiologie Cellulaire et Fonctionnelle, Faculté de Médecine Pitié-Salpêtrière 91, Boulevard de l'Hôpital, 75634 Paris Cedex 13, France

Received 20 July 1994; revised 26 September 1994; accepted 4 October 1994

Abstract

The tritiated derivative of the novel silent 5-HT_{1A} receptor antagonist WAY 100635 [*N*-(2-(4-(2-methoxyphenyl)-1-piperazinyl)ethyl)-*N*-(2-pyridinyl) cyclohexane carboxamide] was tested as a potential radioligand of 5-HT_{1A} receptors in the rat brain. Binding assays with membranes from various brain regions showed that [^{3}H]WAY 100635 specifically bound to a homogeneous population of sites, with a K_d of 0.10 nM. The regional distribution of [^{3}H]WAY 100635 specific binding sites, as assessed in membrane binding assays and by autoradiography of labelled brain sections, superimposed exactly over that of 5-HT_{1A} receptors specifically labelled by [^{3}H]8-hydroxy-2-(di-*n*-propylamino)tetralin ([^{3}H]8-OH-DPAT). Furthermore, the positive correlation ($r = 0.96$) between the respective pK_i values of a large series of ligands as inhibitors of the specific binding of [^{3}H]WAY 100635 and [^{3}H]8-OH-DPAT in hippocampal membranes indicated that their pharmacological properties were similar. Nevertheless, marked differences also existed between [^{3}H]8-OH-DPAT and [^{3}H]WAY 100635 specific binding, as the former was inhibited by 1-100 μM GTP and GppNHp, whereas the latter was enhanced by these guanine nucleotides. In contrast, Mn^{2+} (1–10 mM) increased the specific binding of [^{3}H]8-OH-DPAT, but inhibited that of [^{3}H]WAY 100635. Treatment of membranes with *N*-ethylmaleimide (1–5 mM) markedly reduced their capacity to specifically bind [^{3}H]8-OH-DPAT, but slightly increased (at 1 mM) or did not affect (at 5 mM) their [^{3}H]WAY 100635 specific binding capacity. Finally, the B_{max} of [^{3}H]WAY 100635 specific binding sites was regularly 50–60% higher than that of [^{3}H]8-OH-DPAT in the same membrane preparations from various brain regions (hippocampus, septum, cerebral cortex). These data are compatible with the idea that whereas [^{3}H]8-OH-DPAT only binds to G-protein-coupled 5-HT_{1A} receptors, [^{3}H]WAY 100635 is a high affinity ligand of both G-protein-coupled and free 5-HT_{1A} receptor binding subunits in brain membranes.

Keywords: 5-HT_{1A} receptor; G-protein; 5-HT_{1A} receptor antagonist; Autoradiography, quantitative; [^{3}H]8-OH-DPAT ([^{3}H]8-hydroxy-2-(di-*n*-propylamino)tetralin); [^{3}H]WAY 100635

1. Introduction

Among the various types of 5-HT receptors which have been identified in the central nervous system (CNS) to date (Boess and Martin, 1994), the G-protein-coupled 5-HT_{1A} receptor is the best known for several reasons. In particular, it was the first of all the 5-HT receptors to be cloned and sequenced (Kobilka et al., 1987; Fargin et al., 1988; Albert et al., 1990). In addition, it was also the first 5-HT receptor for which specific polyclonal antibodies were obtained (Fargin et al., 1988; El Mestikawy et al., 1990), allowing their visualization at the subcellular level in various brain areas, notably the anterior raphe nuclei where the 5-HT_{1A} receptor acts as a somato-dendritic autoreceptor (Sotelo et al., 1990).

The development of a large series of selective ligands also greatly contributed to the present extensive knowledge of 5-HT_{1A} receptors. In particular, the tritiated derivative of 8-hydroxy-2-(n-dipropylamino)tetralin ([^{3}H]8-OH-DPAT) was developed in 1983 as the

* Corresponding author. Tel.: 33 1 4077 9708; Fax: 33 1 4077 9790.
[1] Present address: INSERM U29, Hôpital Port-Royal, 75674 Paris cedex 14, France.

SSDI 0922-4106(94)00159-6

first radioligand for the specific labelling of 5-HT_{1A} receptors in the brain (Gozlan et al., 1983). Later on, several other radioligands were synthesized which generally proved to be as efficient as [^{3}H]8-OH-DPAT for the labelling of these receptors in brain membranes and sections. Thus, [^{3}H]ipsapirone (Dompert et al., 1985), [^{3}H]5-methoxy-3-(di-n-propylamino)chroman (Cossery et al., 1987), and [^{3}H]5-methyl-urapidil (Laporte et al., 1991) were successfully used for studies of 5-HT_{1A} receptor binding sites in the CNS. One of the most significant improvements achieved within this field of research was the synthesis of radioiodinated ligands such as [^{125}I]8-methoxy-2-(*N*-propyl-*N*-(3'-iodo-4'-hydroxyphenyl)-propionamido-*N'*-propylamino) tetralin ([^{125}I]BH-8-Meo-*N*-PAT) (Gozlan et al., 1988) and [^{125}I]trans-8-OH-PIPAT (Zhuang et al., 1993) for the autoradiographic labelling of 5-HT_{1A} receptors in brain sections.

However, all the 5-HT_{1A} receptor radioligands developed so far were derivatives of 5-HT_{1A} receptor agonists (Miquel and Hamon, 1992), which limits their use for the high affinity labelling of only the G-protein-coupled receptors. Indeed, dissociation of the G-protein-5-HT_{1A} receptor complexes results in free 5-HT_{1A} receptor subunits with only low affinity for the agonists, which can no longer be labelled by agonist radioligands (Emerit et al., 1990). Thus, GTP-induced dissociation of G-protein-5-HT_{1A} receptor subunit complexes is well known to result in a decreased B_{max} of specific high affinity [^{3}H]8-OH-DPAT binding sites in rat brain membranes (Hall et al., 1985, 1986). In contrast, the binding of antagonists to G-protein-coupled receptors is generally known to be independent of this coupling (Kobilka, 1992), and therefore antagonist radioligands can be expected to label with high affinity both the receptors which are physically coupled to G-proteins and the free receptor binding subunits. In the case of 5-HT_{1A} receptors, successful labelling by a radioactive antagonist, [^{3}H]spiperone, has been reported by Sundaram et al. (1993). Interestingly, these authors found that instead of a decrease, as observed with agonist radioligands, an increase in the specific binding of [^{3}H]spiperone to 5-HT_{1A} receptors occurred upon dissociation of G-protein-5-HT_{1A} receptor complexes in transfected CHO cells. However, [^{3}H]spiperone is not a specific radioligand of 5-HT_{1A} receptors, and is mainly known as a dopamine D_2 receptor radioligand and a 5-HT_{2A} receptor radioligand (Leysen et al., 1978). In an attempt to characterize an antagonist radioligand which would be selective of 5-HT_{1A} receptors, we studied the binding characteristics of the tritiated derivative of WAY 100635 (*N*-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-*N*-(2-pyridinyl) cyclohexane carboxamide, Fig. 1) which proved to be able to selectively prevent the responses normally evoked by 5-HT_{1A} receptor stimulation

[^{3}H] WAY 100635

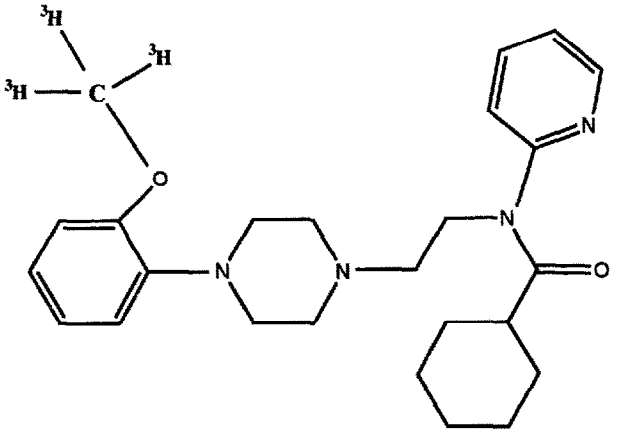

Fig. 1. Chemical structure of [^{3}H]WAY 100635.

(Fletcher et al., 1994; Gurling et al., 1994; Mundey et al., 1994). In vitro binding assays with membranes from various areas of the rat brain and autoradiography with brain sections were used in these studies.

2. Materials and methods

2.1. Animals and treatments

Experiments were performed on adult male Sprague-Dawley rats (250-300 g body weight, Centre d'Elevage R. Janvier, 53940 Le Genest-St Isle, France). They were housed in groups of six and maintained under controlled environmental conditions (21 ± 1°C, 60% relative humidity, 12h/12h light/dark cycle, food and water ad libitum) for at least 7 days before being used.

Some of these animals were treated with clorgyline (5 mg/kg i.p.) 3 hours before death, whereas others received reserpine (5 mg/kg i.p.) 36 h before death. In both cases, control rats were treated with the respective solvents (0.9% NaCl for clorgyline, glacial acetic acid extensively diluted in 0.9% NaCl for reserpine).

2.2. Preparation of membranes for binding assays

Animals were killed by decapitation, and their brains rapidly removed at 4°C and dissected according to the method of Glowinski and Iversen (1966).

Tissues were homogenized in 40 volumes (v/w) of ice-cold 50 mM Tris-HCl (pH 7.4 at 23°C) using a

Polytron disrupter (type PT 10 OD), and homogenates were centrifuged at 40000 × *g* for 20 min at 4°C. The supernatant was discarded and the pellet was washed twice by resuspension in 40 vol Tris-HCl buffer followed by centrifugation. In most cases, the sedimented material was then gently homogenized in 40 vol Tris-HCl and incubated at 37°C for 10 min to remove endogenous 5-HT (Nelson et al., 1978). However, for the membranes from pargyline- or reserpine-treated rats and their respective controls, this step was omitted (see Results). Membranes were then collected by centrifugation and washed three times by 'resuspension-centrifugation' as before. The final pellet was suspended in 10 volumes of 50 mM Tris-HCl, pH 7.4, and aliquots of the resulting suspensions were kept at −80°C before being used for binding assays.

2.3. Measurement of [^{3}H]WAY 100635 binding

Various parameters were tested for the determination of optimal conditions for the specific binding of [^{3}H]WAY 100635 to rat hippocampal membranes (see Results). These conditions were as follows: aliquots (50 μl, corresponding to ~ 0.25 mg membrane proteins) of membrane suspensions were mixed with 50 mM Tris-HCl, pH 7.4, containing [^{3}H]WAY 100635 (0.03–1.4 nM), and where indicated, 10 different concentrations of a given displacing agent, in a total volume of 0.5 ml. Other samples were supplemented with 1 μM 8-OH-DPAT or 1 μM 5-HT for the determination of non-specific binding. All samples were then incubated for 60 min at 25°C, and incubation was stopped by the addition of 3.5 ml ice-cold Tris-HCl buffer and rapid vacuum-filtration through Whatman GF/B filters which had been presoaked in a 0.5% aqueous solution of polyethylenimine for 30 min. Filters were then washed twice with 3.5 ml of ice-cold Tris-HCl buffer, dried and put into vials containing 4 ml of Aquasol® (New England Nuclear, Boston, MA, USA) for radioactivity counting.

2.4. Measurement of [^{3}H]8-OH-DPAT binding

Binding assays were carried out as described for [^{3}H]WAY 100635, except that [^{3}H]8-OH-DPAT was used at 0.25-5.0 nM, and incubation proceeded for only 30 min at 25°C. Furthermore, GF/B filters were not treated with polyethylenimine, but presoaked only with Tris-HCl buffer (Hall et al., 1985).

2.5. Measurement of [^{3}H]5-HT binding

Assays were carried out on 50 μl aliquots of hippocampal membrane suspensions as described by Nelson et al. (1978). Non-specific binding was defined with 0.3 μM 8-OH-DPAT.

All assays were performed in triplicate. Saturation and inhibition curves were analyzed by computer-assisted nonlinear regression analysis using GraphPad and InPlot4 programs. The equation of Cheng and Prusoff (1973) was used for the calculation of K_i values from IC_{50} values.

Proteins were determined according to the method of Lowry et al. (1951) with bovine serum albumin (Sigma) as the standard.

2.6. Autoradiography

Rats were decapitated, and the brain was rapidly removed, frozen in isopentane cooled with dry ice at −30°C, and stored at −80°C for one week. Coronal and horizontal sections (20 μm thick) were cut at −20°C, mounted on glass slides (2.5 × 7.5 cm) coated with a solution of 1% gelatin containing 0.05% chromic potassium sulfate, and stored at −20°C for less than 2 weeks. For the labelling procedure, sections were first equilibrated at room temperature and then preincubated at 25°C for 30 min in 0.17 M Tris-HCl, pH 7.6 (Vergé et al., 1986). Incubation was then performed at 25°C for 60 min in the same (fresh) buffer supplemented with either 1.2 nM [^{3}H]8-OH-DPAT or 0.5 nM [^{3}H]WAY 100635. Labelled sections were then rinsed (2 × 5 min) at 4°C with 0.17 M Tris-HCl, pH 7.6, quickly dipped in ice-cold water (3–5 s) and finally dried under a stream of cold air. Non-specific binding was determined on adjacent sections incubated under the same conditions as above, except that 1 μM 5-HT was added to the incubation medium. Sections were finally apposed to ^{3}H-Hyperfilm (Amersham) in X-ray cassettes for one month at 4°C. Autoradiograms were developed in Kodak Microdol (10 min at 20°C).

2.7. Measurement of 5-HT in tissues

Some of the clorgyline- or reserpine-treated rats and their respective controls were killed by decapitation at the appropriate times (see above), and their brains were removed for the dissection of the hippocampus at 4°C (Glowinski and Iversen, 1966). Tissues were weighed and homogenized individually in 0.5 ml of 0.1 N $HClO_4$ containing 0.5% $Na_2S_2O_5$ and 0.5% disodium EDTA. Homogenates were centrifuged at 30,000 × g for 15 min at 4°C, and the supernatants were neutralized with 2 M KH_2PO_4/K_2HPO_4, pH 7.0, before the addition of ascorbate oxidase (0.1 mg/ml, Sigma). After a second centrifugation as above, the clear supernatants were saved and 20 μl aliquots were injected directly into a high performance liquid chromatography (HPLC) column (Ultrasphere IP, 25 cm,

0.46 cm outside diameter, 5 μm) protected with a Brownlee precolumn (3 cm, 5 μm) (Adrien et al., 1989). The mobile phase consisted of 70 mM KH_2PO_4, 2 mM triethylamine, 0.1 mM disodium EDTA, 1.25 mM octane sulfonic acid and 15% methanol, adjusted to pH 2.78 with solid citric acid. The elution rate was set at 1 ml/min, and electrochemical detection was performed at a potential of 0.72 V. Quantitative determination of 5-HT was made with a Beckman Gold integrator using appropriate standard (see Adrien et al., 1989).

2.8. Statistical calcultations

Data were analyzed statistically by one-way analysis of variance and, in case of significance ($P < 0.05$), the Fisher's test for significant treatment effects was followed by the Student's t test to compare the experimental groups with their respective controls (Snedecor and Cochran, 1967)

2.9. Chemicals

[*O*-methyl-^{3}H]WAY 100635 (69 Ci/mmol) was synthesized by Amersham International (Buckinghamshire, UK) for Wyeth Labs (Taplow, UK) and generously given by Dr. Colin T. Dourish (Wyeth Research UK). [^{3}H]8-OH-DPAT (110 Ci/mmol) was from the Service des Molécules Marquées of CEA (91191 Gif-sur-Yvette, France), and [^{3}H]5-HT (11 Ci/mmol) was from Amersham International.

Other compounds were: clorgyline (May and Baker, Dagenham, UK), reserpine (Ciba-Geigy, Basel, Switzerland), ipsapirone (Troponwerke, Cologne, Germany), (−)tertatolol, 1-[2-(4-fluorobenzoylamino)ethyl]-4-(7-methoxynaphtyl) piperazine (S 14506) and (+)-4-[N-(5-methoxy-chroman-3-yl)-N-propylamino]butyl-8-azaspiro-(4,5)-decane-7,9-dione (S 20499) (Servier, Courbevoie, France), GTP and GppNHp (Boehringer-Mannheim, Germany), methyl-4-(4-(4(1,1,3-trioxo-2H-1,2-benziosothiazol-2-yl)butyl)-1-piperazinyl)-1H-indole-2-carboxylate (SDZ 216-525), mesulergine and clozapine (Sandoz, Basel, Switzerland), 8-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-8-azaspirol[4,5]-decane-7,9-dione (BMY 7378) and buspirone (Bristol-Myers Squibb, Wallingford, CT, USA), lesopitron (Esteve, Barcelona, Spain), 3-(1,2,5,6-tetrahydropyrid-4-yl) pyrrolo[3,2-b]pyrid-5-one (Pfizer, Groton, CT, USA), 5-methoxy-3-(1,2,3,6-tetrahydropyridin-4-yl)-1H-indole (RU 24969) (Roussel-UCLAF, Romainville, France), sumatriptan and ondansetron (Glaxo, Hertfordshire, UK), ketanserin (Janssen, Beerse, Belgium), zacopride (Delalande-Synthélabo, Rueil-Malmaison, France). All other compounds were from Research Biochem. (Natick, MA, USA) or Sigma (St Louis, MO, USA).

3. Results

3.1. Effects of various parameters on the specific binding of [^{3}H]WAY 100635 to rat hippocampal membranes

Binding assays with 0.4 nM [^{3}H]WAY 100635 under conditions normally used for [^{3}H]8-OH-DPAT but without membranes revealed that 8% of total radioactivity (~ 2300 dpm) bound to GF/B filters which had been presoaked with Tris-HCl buffer only. Presoaking the filters in an aqueous solution of 0.5% polyethylenimine reduced this binding by more than 90% (to ~ 220 dpm). This treatment was systematically used in all subsequent assays.

Assays with hippocampal membranes under the conditions described in Materials and methods showed that 1 μM 5-HT could reduce [^{3}H]WAY 100635 binding by at least 90% when the radioligand concentration was ≤ 1.0 nM. Replacement of 50 mM Tris-HCl buffer by 50 mM tricine, 50 mM Hepes or 50 mM sodium phosphate buffer influenced neither the efficacy of 5-HT to inhibit [^{3}H]WAY 100635 binding nor the total amount of [^{3}H]WAY 100635 bound per mg membrane protein (not shown). However, changing the molarity of the Tris-HCl buffer to 10 mM or 100 mM resulted in some decrease (by 25–30%) in the specific binding of [^{3}H]WAY 100635 to rat hippocampal membranes.

As shown in Fig. 2, the specific binding of [^{3}H]WAY 100635 to rat hippocampal membranes was dependent on pH with the highest value at pH 6-6.5, and a

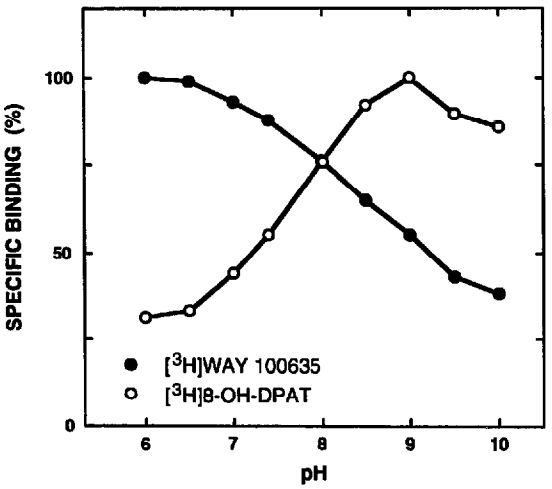

Fig. 2. pH dependency of the specific binding of [^{3}H]WAY 100635 and [^{3}H]8-OH-DPAT to rat hippocampal membranes. Binding assays were performed with 0.4 nM [^{3}H]WAY 100635 or 1.0 nM [^{3}H]8-OH-DPAT as described in Materials and methods except that the incubating mixture, 50 mM Tris-HCl, was adjusted at various pHs (abscissa). The specific binding of each radioligand is expressed as a percentage of its maximal value (at pH 6.0 for [^{3}H]WAY 100635; at pH 9.0 with [^{3}H]8-OH-DPAT). Each point is the mean of triplicate determinations in a typical experiment. Similar data have been obtained in three separate experiments.

progressive decrease in binding capacity with higher pH values. However, at the physiological pH (7.4) that we selected for subsequent assays, the specific binding of [^{3}H]WAY 100635 was still equal to 88% of its maximal value at pH 6.0 (Fig. 2). This pH dependency contrasted with that of [^{3}H]8-OH-DPAT specific binding to the same membrane preparations, as a progressive enhancement in that binding occurred when the pH value increased from 6.5 to 9.0 (Fig. 2).

Binding assays in 50 mM Tris-HCl, pH 7.4, at various temperatures (4°–60°C) for various times (from 6 h at 4°C to 3 min at 60°C) indicated that maximal values of [^{3}H]WAY 100635 specific binding to rat hippocampal membranes were observed at 20–35°C. In contrast, membrane incubation for 3 min at 60°C resulted in an irreversible loss of [^{3}H]WAY 100635 specific binding, which was then ~5% of that measured with membranes kept at 0°C for the same period.

These first series of experiments allowed the selection of the assay conditions described in Materials and methods for all subsequent measurements of [^{3}H]WAY 100635 specific binding to brain membranes.

At 25°C, the specific binding of [^{3}H]WAY 100635 to hippocampal membranes reached equilibrium within 50–60 min (Fig. 3A), and conformed to a second-order plot which was first order with respect to each reaction component. Analysis of the data by the method of Frost and Pearson (1961) gave an association rate constant $k+1 = 0.089\ \text{nM}^{-1} \times \text{min}^{-1}$. Following equilibration after 60 min at 25°C, the [^{3}H]WAY 100635-receptor complex was dissociated by addition of 1 μM of 8-OH-DPAT. First-order reaction plot was rectilinear (Fig. 3B), allowing the calculation of the dissociation rate constant $k-1 = 0.0067\ \text{min}^{-1}$. Accordingly, the dissociation constant K_d calculated from kinetic studies ($k-1/k+1$) was equal to ~0.080 nM.

3.2. *Equilibrium characteristics of [^{3}H]WAY 100635 specific binding to hippocampal membranes: comparison with those of [^{3}H]8-OH-DPAT specific binding*

Binding assays for 60 min at 25°C with increasing concentrations of [^{3}H]WAY 100635 indicated that the specific binding of this radioligand to hippocampal membranes levelled off at concentrations ≥ 1.0 nM. In contrast, the non-specific binding increased linearly with [^{3}H]WAY 100635 concentration. However, this component was always less than 10% of total binding when assays were performed with [^{3}H]WAY 100635 concentrations up to 1.4 nM (Fig. 4A). Scatchard transformation of the saturation curve gave a single slope, allowing the calculation of K_d and B_{max} values (Fig. 4B). Interestingly, the K_d value of [^{3}H]WAY 100635 specific binding, 0.10 nM, was close to that derived from kinetic studies, and about one order of magnitude

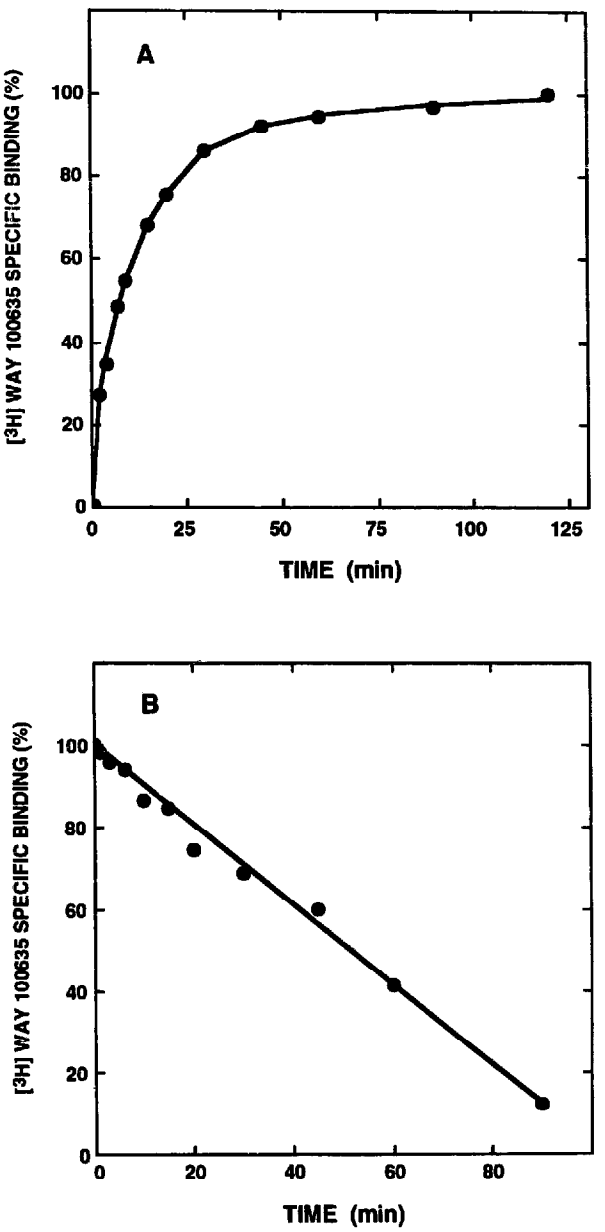

Fig. 3. Association (A) and dissociation (B) time course of specific [^{3}H]WAY 100635 binding to rat hippocampal membranes. (A) Membranes were preincubated for 5 min at 25°C before the addition of [^{3}H]WAY 100635 (0.4 nM), and the incubation proceeded until filtration at the times indicated on abscissa. The specific binding of [^{3}H]WAY 100635 is expressed as a percentage of its value at 120 min. Each point is the mean of triplicate determinations in two independent experiments. Calculation of the association rate constant $k+1$ according to Frost and Pearson (1961) gave a value of $0.089\ \text{nM}^{-1} \times \text{min}^{-1}$. B Membranes were incubated with 0.4 nM [^{3}H]WAY 100635 at 25°C for 60 min (until equilibrium was reached). 8-OH-DPAT (1 μM) was then added (time 0 on the abscissa) and the incubation was stopped at the times indicated, by rapid vacuum filtration. The specific binding of [^{3}H]WAY 100635 is expressed as a percentage of that found at time 0. Each point is the mean of triplicate determinations in two separate experiments. Calculation of the dissociation rate constant $k-1$ gave a value of $0.0067\ \text{min}^{-1}$.

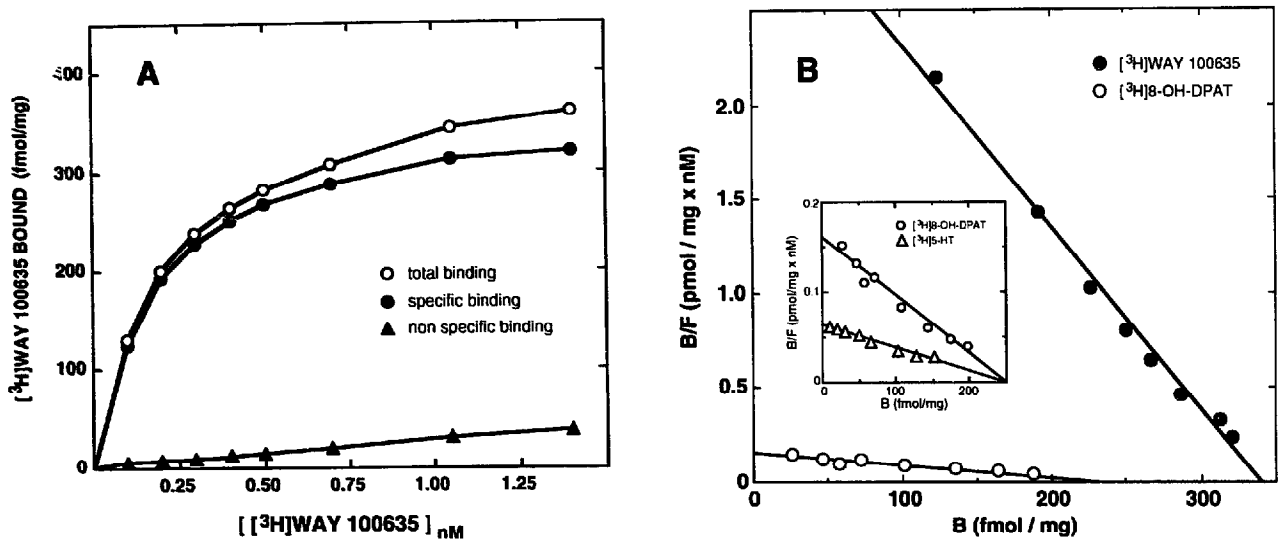

Fig. 4. Concentration curves of [³H]WAY 100635, [³H]8-OH-DPAT and [³H]5-HT binding to rat hippocampal membranes. (A) Binding assays were performed as described in Materials and methods with 0.10–1.40 nM [³H]WAY 100635, in the presence (▲, non-specific binding) or the absence (O, total binding) of 1 μM 5-HT. Each value (in fmol [³H]WAY 100635 bound per mg membrane protein) is the mean of triplicate determinations in two separate experiments. (B) Scatchard plots of the specific binding of [³H]WAY 100635 (● from the data in A), [³H]8-OH-DPAT (O) and [³H]5-HT (Δ) to rat hippocampal membranes. The non-specific binding of [³H]WAY 100135 and [³H]8-OH-DPAT was determined with 1 μM 5-HT, whereas that of [³H]5-HT was determined with 0.3 μM 8-OH-DPAT. B: [³H] ligand specifically bound (in fmol/mg membrane protein); F: free [³H] ligand in the assay mixture, in nM. Each point is the mean of triplicate determinations in two separate experiments.

lower than that of [³H]8-OH-DPAT specific binding (Fig. 4B). Further comparison between the two radioligands indicated that the B_{max} of [³H]WAY 100635 specific binding sites was markedly higher (+50%) than that of [³H]8-OH-DPAT specific binding sites in the same membrane preparation (Fig. 4B). In contrast, the same B_{max} value was found whether 5-HT_{1A} sites were labelled by [³H]8-OH-DPAT or by [³H]5-HT (with 0.3 μM 8-OH-DPAT to determine non-specific binding) (Fig. 4B, inset).

3.3. Regional distribution of [³H]WAY 100635 specific binding - Comparison with that of [³H]8-OH-DPAT specific binding

The data in Fig. 5 show that the specific binding of [³H]WAY 100635 to membranes was highest in the

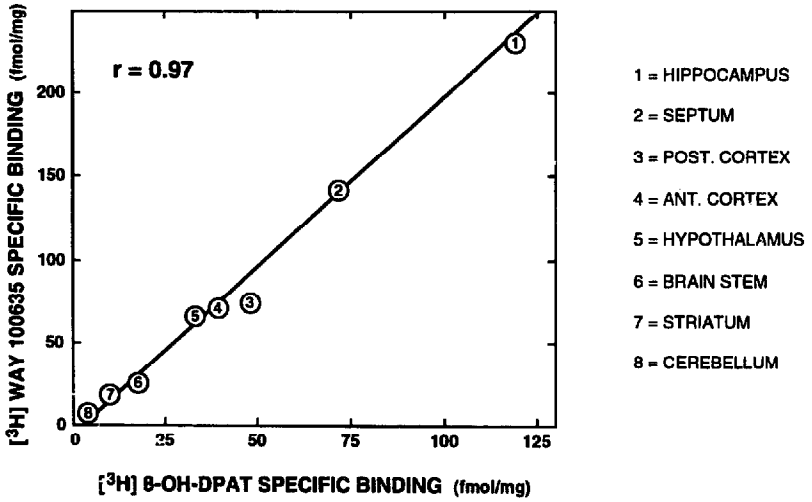

Fig. 5. Comparison of the regional distributions of [³H]WAY 100635 and [³H]8-OH-DPAT specific binding sites. Membranes were prepared from various brain regions, and binding assays were performed with 0.5 nM [³H]WAY 100635 and 0.8 nM [³H]8-OH-DPAT on aliquots of the same membrane preparation for a given brain structure. The specific binding of each radioligand is expressed in fmol per mg membrane protein. Each point is the mean of triplicate determinations in three separate experiments (with less than 15% variations between them). $r = 0.97$ is the coefficient of the positive correlation between [³H]8-OH-DPAT and [³H]WAY 100635 specific binding in the eight areas examined.

Table 1
Characteristics of [^{3}H]WAY 100635 and [^{3}H]8-OH-DPAT specific binding sites in membranes from various brain areas

Brain structure	[^{3}H]WAY 100635		[^{3}H]8-OH-DPAT		R
	K_d (nM)	B_{max} (fmol/mg prot)	K_d (nM)	B_{max} (fmol/mg prot)	
hippocampus	0.12 ± 0.03	350.7 ± 23.7	0.69 ± 0.07	217.0 ± 14.9	1.62
septum	0.12 ± 0.01	221.4 ± 17.0	0.66 ± 0.10	139.6 ± 9.8	1.59
anterior cortex	0.11 ± 0.01	165.0 ± 8.4	0.86 ± 0.11	108.5 ± 11.9	1.52
posterior cortex	0.10 ± 0.01	125.8 ± 7.9	0.74 ± 0.15	78.6 ± 9.4	1.60

Saturation experiments were performed with the same membrane preparations for the two radioligands (0.03–1.4 nM [^{3}H]WAY 100635; 0.25–5.0 nM [^{3}H]8-OH-DPAT). Scatchard transformation of the saturation curves allowed the calculation of respective K_d (in nM) and B_{max} (in fmol/mg membrane protein) values. Each value is the mean ± S.E.M. of 3–5 independent determinations. R is the ratio of the B_{max} of [^{3}H]WAY 100635 specific binding sites to that of [^{3}H]8-OH-DPAT specific binding sites.

hippocampus, followed by the septum > posterior cortex = anterior cortex = hypothalamus > brain stem > striatum > cerebellum. Similar regional differences were found when assays were performed with the same membrane preparations but with [^{3}H]8-OH-DPAT as radioligand. Indeed, a highly significant positive correlation (r = 0.97) existed between the specific binding of these two radioligands in a given brain area (Fig. 5). Saturation studies with membranes from the hippocampus, septum, anterior and posterior cortices showed that the regional differences in [^{3}H]WAY 100635 and [^{3}H]8-OH-DPAT specific binding were only due to variations in the respective B_{max} values, as each K_d value did not vary significantly from one brain area to another (Table 1). As found in the hippocampus, the B_{max} of [^{3}H]WAY 100635 specific binding sites was 50–60% higher than that of [^{3}H]8-OH-DPAT specific binding sites also in the septum and the cerebral cortex (Table 1).

Further studies on the regional distribution of [^{3}H] WAY 100635 specific binding were made using autoradiography. As shown in figs 6 and 7, the autoradiographic labelling by [^{3}H]WAY 100635 superimposed exactly over that by [^{3}H]8-OH-DPAT, with high levels in the septum, hippocampus (notably the gyrus dentatus and CA1 area of Ammon's horn), entorhinal cortex

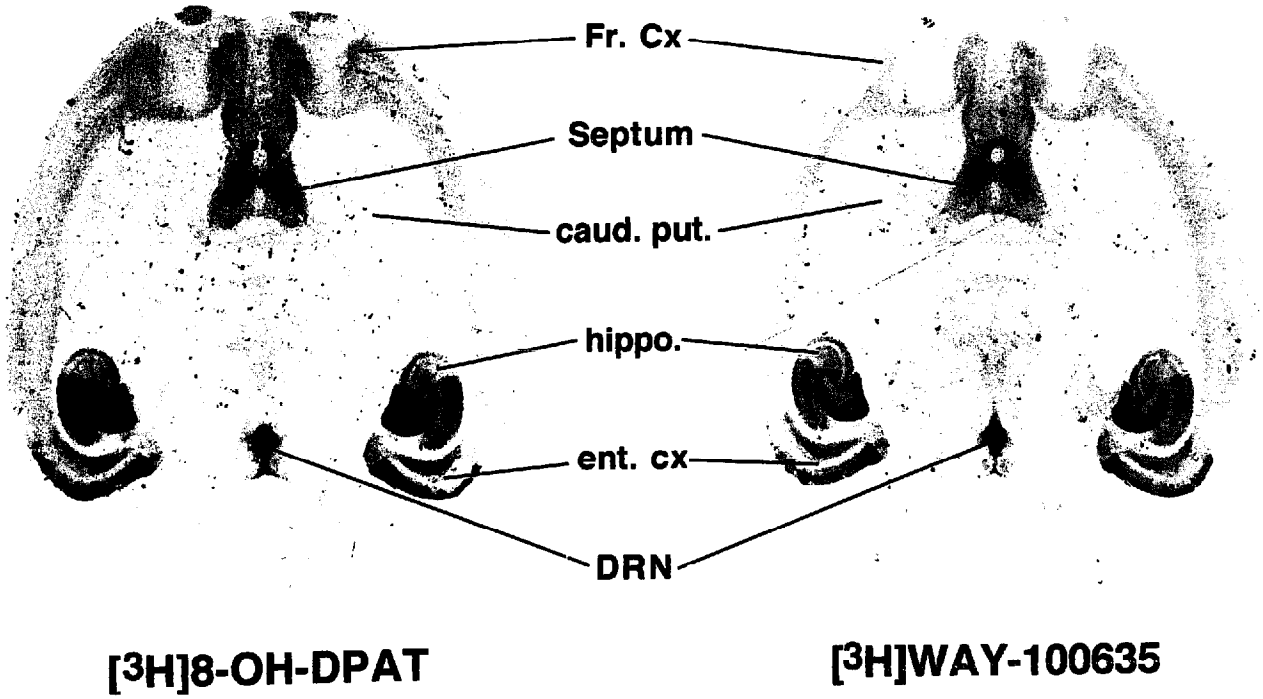

Fig. 6. Autoradiograms of adjacent horizontal brain sections labelled by [^{3}H]8-OH-DPAT or [^{3}H]WAY 100635. Adjacent sections (20 μm) were incubated with 1.2 nM [^{3}H]8-OH-DPAT or 0.5 nM [^{3}H]WAY 100135, and autoradiograms were obtained after exposure of the labelled sections to ^{3}H-Hyperfilm for one month at 4°C. Fr. Cx: frontal cortex; caud. put.: caudate-putamen; hippo.: hippocampus; ent. cx: entorhinal cortex; DRN: dorsal raphe nucleus.

and dorsal raphe nucleus. In contrast, the labelling with either radioligand in the striatum and the substantia nigra (Figs. 6,7) was very low, at the same level as that found in all areas on autoradiograms from brain sections incubated with 1 μM 5-HT (non-specific binding - not shown).

3.4. Pharmacological profile of [^{3}H]WAY 100635 specific binding sites in rat hippocampal membranes

At 1 μM, neither dopamine, noradrenaline, histamine, carbachol, glutamate nor GABA affected the specific binding of [^{3}H]WAY 100635 (at 0.35–0.40 nM

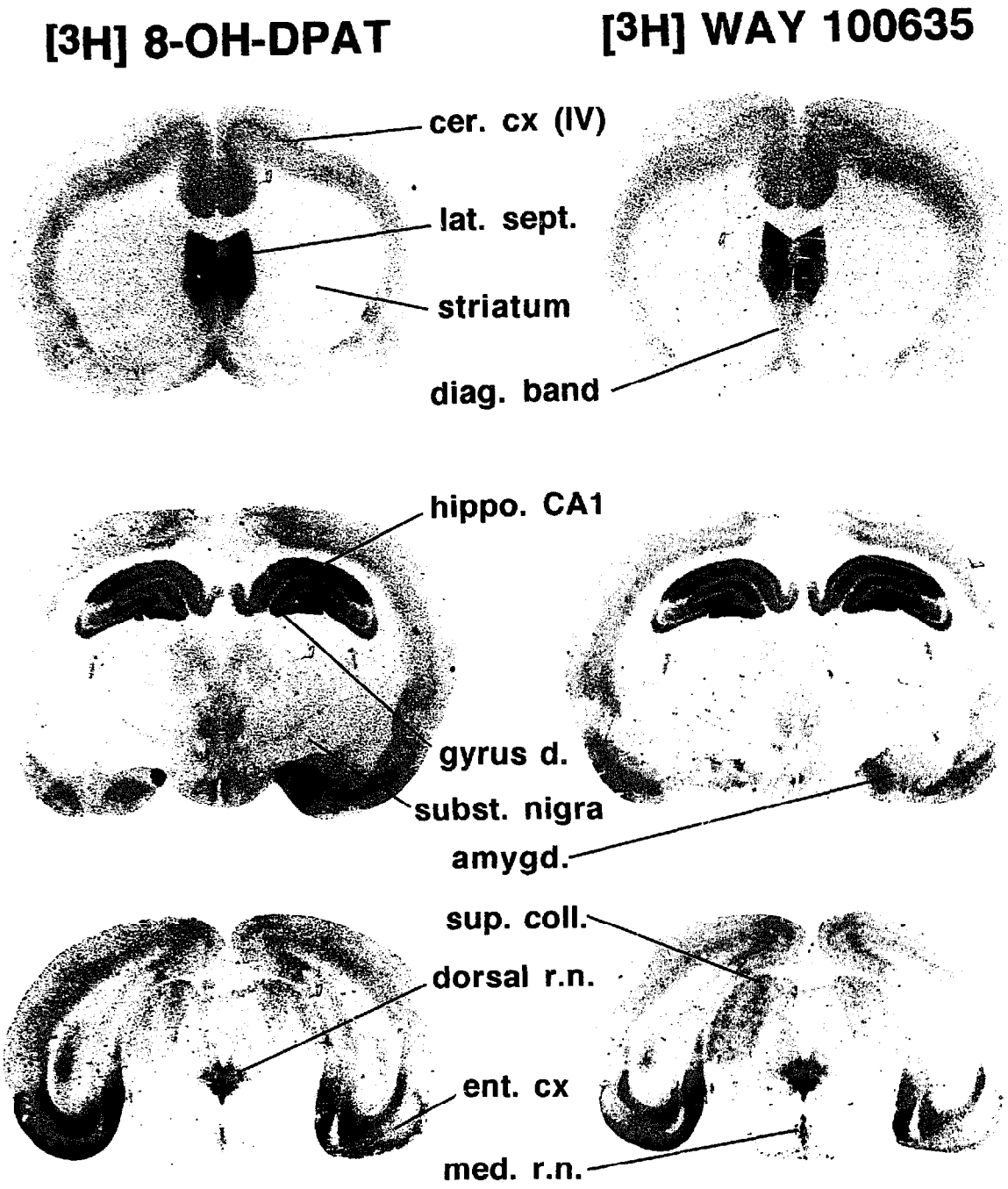

Fig. 7. Autoradiograms of adjacent coronal brain sections labelled by [^{3}H]8-OH-DPAT or [^{3}H]WAY 100635. The same protocol as that described in the legend to Fig. 6 was applied to coronal sections (20 μm) at the level of the striatum (top), dorsal hippocampus (middle) and dorsal raphe nucleus (bottom). cer. cx (IV): layer IV of the cerebral cortex; lat. sept.: lateral septum; diag. band: diagonal band of Broca; hippo. CA1: CA1 area of Ammon's horn in the hippocampus; gyrus d.: gyrus dentatus; subst. nigra: substantia nigra; amygd.: amygdala; sup. coll.: superior colliculi; dorsal r.n.: dorsal raphe nucleus; ent. cx: entorhinal cortex; med. r.n.: median raphe nucleus.

in the assay mixture) to rat hippocampal membranes. In contrast, at this concentration, 5-HT inhibited [^{3}H] WAY 100635 binding to the same non-specific level as that found with 1 μM 8-OH-DPAT. Displacement experiments with a large series of ligands indicated that those with the highest potencies are 5-HT_{1A} receptor ligands such as SDZ 216-525 (Lanfumey et al., 1993), 8-OH-DPAT (Hall et al., 1985), S 14506 (Colpaert et al., 1992), S 20499 (Kidd et al., 1993), ipsapirone (Dompert et al., 1985), with IC_{50} values ≤ 50 nM. In contrast, the 5-HT_{1B} receptor agonist CP 93129 and the 5-HT_{1B}/5-HT_{1D} agonist sumatriptan (see Zifa and Fillion, 1992) displaced [^{3}H]WAY 100635 with IC_{50} values of 10 μM and 15.7 μM, respectively. Similarly, μM concentrations of the 5-HT_2 antagonists mesulergine (IC_{50} = 6.3 μM) and ketanserin (IC_{50} = 3.7 μM) were required to inhibit the specific binding of [^{3}H]WAY 100635 to hippocampal membranes. In addition, the 5-HT_3 receptor antagonists zacopride and ondansetron (Zifa and Fillion, 1992) were inactive when tested at 1.0 μM. In all cases, except with 5-HT (Fig. 8) and a few other agonists: 5-CT, 5-MeO-*N*,*N*-DMT and RU 24969, displacement curves were monophasic with an apparent Hill coefficient close to 1.0. In contrast, the apparent Hill coefficient values were less than one with 5-HT and these agonists (Table 2). Non-linear regression analysis of the corresponding inhibition curves, using the program InPlot 4, allowed the distinction of two classes of [^{3}H]WAY 100635 specific binding sites having high (H) or low (L) affinity for 5-HT, 5-CT, 5-MeO-*N*,*N*-DMT and RU 24969 (Table 2).

Overall, the pharmacological profile of the specific binding sites for [^{3}H]WAY 100635 corresponded to that of [^{3}H]8-OH-DPAT specific binding sites, as a positive correlation (r = 0.96) was found between the respective pK_i values of 16 different compounds as displacing agents at these sites (Fig. 9).

3.5. Effects of Mn^{2+} and guanine nucleotides on the specific binding of [^{3}H]WAY 100635 to hippocampal membranes: comparison with their effects on [^{3}H]8-OH-DPAT specific binding

Increasing concentrations of Mn^{2+} exerted an inhibitory effect on [^{3}H]WAY 100635 specific binding, that reached −30% with 10 mM of the cation (Fig. 10). In contrast, the specific binding of [^{3}H]8-OH-DPAT increased by 70% in the presence of 1–10 mM Mn^{2+} (Fig. 10), in agreement with previous observations (Hall et al., 1985, 1986).

In contrast to Mn^{2+}, GTP and GppNHp were found to inhibit the specific binding of [^{3}H]8-OH-DPAT to rat hippocampal membranes, with IC_{50} values of 1–5 μM (Fig. 10), as already reported elsewhere (Gozlan et al., 1983; Hall et al., 1985, 1986). Conversely, GTP and GppNHp moderately enhanced the specific binding of [^{3}H]WAY 100635 to the same membrane preparations (up to +20% in the presence of 10–100 μM of either guanine nucleotide, Fig. 10). Further experiments with hippocampal membranes prepared from rats treated with either clorgyline or reserpine (see Materials and methods) showed that the effect of GTP might depend on the 5-HT content in tissues. Thus, the GTP-induced increase in [^{3}H]WAY 100635 specific binding was larger with membranes from rats whose hippocampal 5-HT content was enhanced due to MAO blockade by clorgyline (Table 3). In contrast, this effect was lower in membranes from rats treated with reserpine to deplete 5-HT tissue levels (Table 3).

Table 2
Characteristics of the inhibition by 5-HT_1 receptor agonists of [^{3}H] WAY 100635 specific binding to rat hippocampal membranes

Agonist	IC_{50}	nH	$Ki_{(H)}$	$Ki_{(L)}$
5-CT	1.72	0.64	0.12	4.17
5-HT	23.7	0.67	1.41	44.7
RU 24969	30.9	0.66	1.72	56.7
5-MeO-*N*,*N*-DMT	37.5	0.68	1.36	75.9

Binding assays were performed with 0.30 nM [^{3}H]WAY 100635 and 10 different concentrations of each agonist. Non linear regression analysis of the inhibition curves allowed the calculation of the K_i values (in nM) of each agonist at high (H) and low (L) affinity [^{3}H]WAY 100635 specific binding sites. Each value (IC_{50} in nM, nH) is the mean of at least 3 independent determinations with less than 25 percent variations between them. 5-CT: 5-carboxamidotryptamine; 5-MeO-*N*,*N*-DMT: 5-methoxy-*N*,*N*-dimethyltryptamine.

3.6. Effects of N-ethyl-maleimide (NEM) on [^{3}H]WAY 100635 specific binding to hippocampal membranes. Comparison with its effects on [^{3}H]8-OH-DPAT specific binding

In agreement with previous observations (Hall et al., 1986; Stratford et al., 1988), pretreatment of rat hippocampal membranes by NEM resulted in a concentration-dependent reduction in [^{3}H]8-OH-DPAT specific binding, with an almost complete loss of specific binding sites at 5 mM NEM (Table 4). In contrast, binding assays with [^{3}H]WAY 100635 using the same membrane preparations indicated a stimulatory effect of 1 mM NEM on the specific binding of this radioligand, and no significant difference between [^{3}H]WAY 100635 specific binding to control membranes and to those pretreated with 5 mM NEM (Table 4). Displacement experiments with 5-HT showed that NEM pretreatment of hippocampal membranes resulted in a shift to the right of the corresponding inhibitory curve and an increase in the apparent Hill coefficient from 0.67 to 1.0 (Fig. 8). These changes were similar to those induced by the addition of 0.1 mM GTP to the assay mixture of control membranes (Fig. 8). Indeed, the effects of GTP and NEM were not additive, and GTP no longer affected the inhibition of [^{3}H]WAY 100635

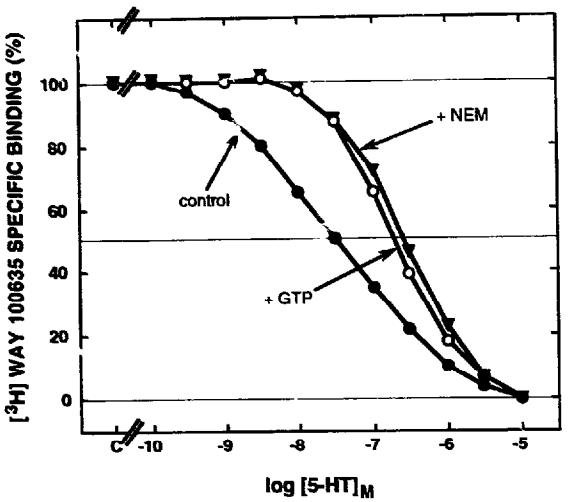

Fig. 8. Inhibition by 5-HT of [^{3}H]WAY 100635 specific binding to rat hippocampal membranes. Effects of GTP and NEM. Binding assays were performed with 0.30 nM [^{3}H]WAY 100635 in the absence (control) or the presence of 0.1 mM GTP. NEM treatment consisted of preincubating the membranes with 5 mM NEM for 30 min at 37°C, followed by extensive washing as described in the legend to Table 4. [^{3}H]WAY 100635 specific binding is expressed as a percentage of that found in the absence of 5-HT (C on abscissa). Each point is the mean of triplicate determinations in three separate experiments, with less than 5% variations between them.

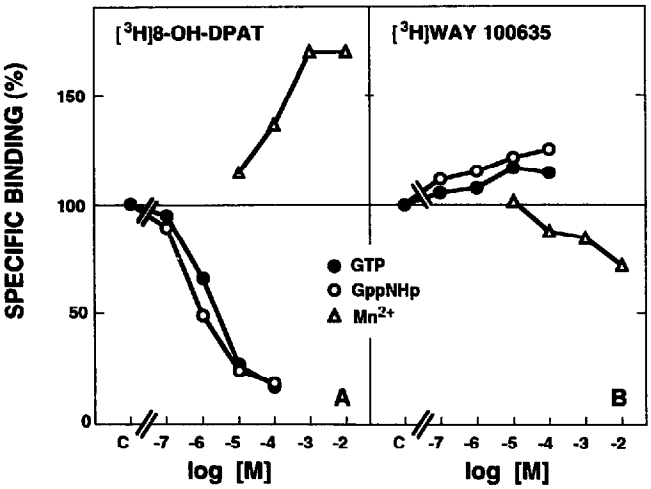

Fig. 10. Differential effects of guanine nucleotides and Mn^{2+} on the specific binding of [^{3}H]8-OH-DPAT (A) and [^{3}H]WAY 100635 (B) to rat hippocampal membranes. Binding assays were performed on the same membrane preparations with 1.0 nM [^{3}H]8-OH-DPAT or 0.3 nM [^{3}H]WAY 100635 in the absence or the presence of various concentrations (abscissa) of GTP, GppNHp or $MnCl_2$. The specific binding of each radioligand is expressed as a percentage of that measured under control conditions, with no additive (C on abscissa). Each point is the mean of triplicate determinations in three separate experiments, with less than 5% variations between them.

specific binding by 5-HT to NEM-pretreated membranes (not shown). In contrast, the inhibition by the antagonists SDZ 216-525 (Lanfumey et al., 1993) and (−)tertatolol (Jolas et al., 1993) of [^{3}H]WAY 100635 specific binding to membranes pretreated by 5 mM NEM exhibited the same characteristics (IC_{50} and nH

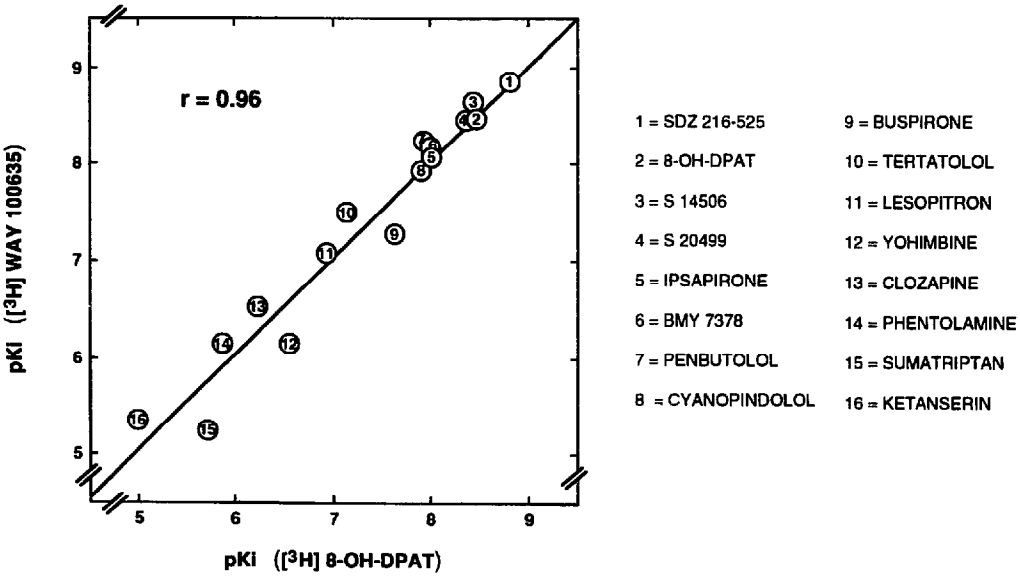

Fig. 9. pK_i values of 16 different compounds as inhibitors of the specific binding of [^{3}H]8-OH-DPAT (abscissa) or [^{3}H]WAY 100635 (ordinate) to rat hippocampal membranes. Each point is the mean of 2-3 separate determinations using 10 different concentrations of the corresponding compound. r is the coefficient of the positive correlation between the two series of pK_i values. 5-HT_{1A} receptor agonists: 8-OH-DPAT, S 14506, S 20499, ipsapirone, buspirone, lesopitron (see Colpaert et al., 1992; Dompert et al., 1985; Haj-Dahmane et al., 1994; Hall et al., 1985; Kidd et al., 1993; Miquel and Hamon, 1992; Zifa and Fillion, 1992). 5-HT_{1A} receptor antagonists: SDZ 216-525, BMY 7378, penbutolol, cyanopindolol, tertatolol (see Jolas et al., 1993; Lanfumey et al., 1993; Miquel and Hamon, 1992; Zifa and Fillion, 1992). Other receptor ligands: yohimbine, clozapine, phentolamine, sumatriptan, ketanserin (see Miquel and Hamon, 1992; Zifa and Fillion, 1992).

Table 3
Effects of clorgyline or reserpine treatment on the effect of GTP on [^{3}H]WAY 100635 specific binding to rat hippocampal membranes

	5-HT (μg/g)	[^{3}H]WAY 100635 specific binding (fmol/mg prot)	
		No addition	0.1 mM GTP
control	0.237 ± 0.011	172 ± 10	202 ± 12 (+17%)
clorgyline	0.734 ± 0.047 *	168 ± 11	219 ± 9 (+30%)
reserpine	0.045 ± 0.009 *	176 ± 6	190 ± 7 (+8%)

Clorgyline (5 mg/kg i.p.) or reserpine (5 mg/kg i.p.) was injected 3h or 36h before sacrifice, respectively. Control rats received the vehicle only. Hippocampal 5-HT levels (in μg/g of fresh tissue) are the means ± S.E.M. of 5 (treated rats) or 10 (pooled controls) independent determinations (* $P < 0.05$ when compared to 5-HT levels in control rats). Binding assays were performed with 0.20 nM [^{3}H]WAY 100635 with or without 0.1 mM GTP. Each value (in fmol [^{3}H]WAY 100635 specifically bound per mg membrane protein) is the mean ± S.E.M. of triplicate determinations in 3 separate experiments. The percent increase due to 0.1 mM GTP is indicated in parentheses.

values) as those found with control membranes (not shown).

4. Discussion

In contrast to most putative 5-HT$_{1A}$ receptor antagonists which have been described so far (Miquel and Hamon, 1992), *N*-tert-butyl-3,4-(2-methoxyphenyl) piperazin-1-yl-2-phenyl-propanamide [(+)WAY 100135] is a really silent competitive antagonist at both pre- and postsynaptic 5-HT$_{1A}$ receptors (Fletcher et al., 1993a), since it prevents the inhibitory effects of agonists on the firing of serotoninergic neurones in the dorsal raphe nucleus and on 5-HT$_{1A}$-dependent adenylate cyclase activity in the hippocampus, as well as various behavioral responses triggered by the stimulation of these receptors (Fletcher et al., 1993b; Lanfumey et al., 1993). However, the potency of (+)-WAY 100135 at 5-HT$_{1A}$ receptors is relatively low ($IC_{50} = 15$ nM against the specific binding of 1 nM [^{3}H]8-OH-DPAT to rat hippocampal membranes, Fletcher et al., 1993b), and not really appropriate for the development of a radioactive derivative to selectively label these receptors in brain membranes and sections. From this compound, however, Fletcher et al. (1994) synthesized a new methoxyphenyl-piperazine derivative, WAY 100635, which also acts as a silent antagonist at both pre- and postsynaptic 5-HT$_{1A}$ receptors (Gurling et al., 1994; Hamon et al., 1994; Mundey et al., 1994), but with a markedly higher potency than that of (+)-WAY 100135. Binding studies with various radioligands (Hamon et al., 1994) indicated that WAY 100635 interacted in the nanomolar range only with 5-HT$_{1A}$ receptor binding sites ($K_i = 0.8$ nM). Among the other receptors tested (5-HT$_1$ subtypes, 5-HT$_{2A}$, 5-HT$_3$, α1- and α2-adrenergic, β1- and β2-adrenergic, D1 and D2 dopamine, GABA$_A$), only the α1 adrenergic receptors labelled by [^{3}H]prazosin (in rat cortical membranes) were recognized by WAY 100635, but with a much lower affinity ($K_i = 360$ nM) than the 5-HT$_{1A}$ receptors (Hamon et al., 1994). Accordingly, it was inferred that the tritiated derivative of WAY 100635 could be a selective high affinity radioligand of 5-HT$_{1A}$ receptors. The present investigations were performed to directly assess this possibility.

Table 4
Effects of membrane treatment with *N*-ethyl-maleimide on the specific binding of [^{3}H]WAY 100635 and [^{3}H]8-OH-DPAT

	[^{3}H]WAY 100635	[^{3}H]8-OH-DPAT
Control	236 ± 19	124 ± 8
NEM 1 mM	306 ± 16 * (+30%)	32 ± 3 * (−74%)
NEM 5 mM	239 ± 20 (+1%)	11 ± 3 * (−91%)

Hippocampal membranes were incubated for 30 min at 37°C in 10 vol (v/w) of 50 mM Tris–HCl, pH 7.4, supplemented with 1 or 5 mM NEM or none. Dithiothreitol (10 mM) was then added to all samples, and membranes were collected by centrifugation at 40,000 ×g for 10 min at 4°C. Membrane pellets were resuspended in 40 vol Tris-HCl buffer, and suspensions were centrifuged as before. This 'washing-centrifugation' cycle was repeated 3 times, and the final pellets were resuspended in 10 vol of 50 mM Tris-HCl, pH 7.4. Binding assays were performed on the same membrane preparations with 0.4 nM [^{3}H]WAY 100635 or 1.0 nM [^{3}H]8-OH-DPAT. Each value (in fmol [^{3}H] ligand specifically bound per mg membrane protein) is the mean ± S.E.M. of triplicate determinations in 3 separate experiments. The percent changes due to NEM treatment are indicated in parentheses.
* $P < 0.05$ when compared to respective control values.

Binding studies with rat hippocampal membranes showed that [^{3}H]WAY 100635 bound mainly to high affinity 5-HT$_1$ receptors, since the occupancy of these receptors by 5-HT (assays with 1 μM 5-HT) resulted in a > 90% decrease in the amount of radioactivity bound to membranes. That these receptors corresponded to the 5-HT$_{1A}$ subtype was confirmed by several observations. The regional distribution of [^{3}H]WAY 100635 specific binding sites superimposed exactly over that of 5-HT$_{1A}$ sites specifically labelled by [^{3}H]8-OH-DPAT (Hall et al., 1985; Vergé et al., 1986; Radja et al., 1991). This conclusion was derived not only from studies with membranes prepared from a few dissected brain regions, but also applied to all the discrete areas examined on autoradiograms of labelled brain sections. In particular, [^{3}H]WAY 100635 binding sites were especially abundant in the gyrus dentatus and CA1 area of Ammon's horn in the hippocampus, lateral septum, entorhinal cortex and dorsal raphe nucleus, but hardly detectable in the striatum, substantia nigra and cere-

bellum, as previously described for 5-HT_{1A} receptors (Radja et al., 1991). The pharmacological profile of [^{3}H]WAY 100635, established with a large series of ligands, was positively correlated (r = 0.96) with that of 5-HT_{1A} receptor binding sites specifically labelled by [^{3}H]8-OH-DPAT in rat hippocampal membranes.

Nevertheless, further comparison of [^{3}H]WAY 100635 and [^{3}H]8-OH-DPAT specific binding to the same membrane preparations revealed striking differences. Thus, [^{3}H]WAY 100635 binding decreased whereas that of [^{3}H]8-OH-DPAT increased when the pH of the assay mixture shifted from 6.0 to 9.0. In addition, the modulatory agents Mn^{2+} and GTP exerted opposite effects on the specific binding of these radioligands. Alkylation by NEM dramatically reduced the capacity of hippocampal membranes to specifically bind [^{3}H]8-OH-DPAT, without any loss of [^{3}H]WAY 100635 specific binding capacity. Finally, in all the brain regions examined, the B_{max} of [^{3}H]WAY 100635 specific binding sites was found to be 50–60% higher than that of [^{3}H]8-OH-DPAT specific binding sites.

Previous studies on 5-HT_{1A} receptor binding sites specifically labelled by [^{3}H]8-OH-DPAT have shown that GTP dissociates the receptor binding subunit from the coupled G-protein, thus resulting in a decreased affinity for the agonist radioligand (Emerit et al., 1990). In addition, Stratford et al. (1988) demonstrated that the loss of high affinity 5-HT_{1A} receptor binding sites in brain membranes exposed to the alkylating agent NEM could be reversed by supplementation of these membranes with exogenous G-proteins, indicating that NEM in fact inactivates G-proteins normally associated with 5-HT_{1A} receptor binding subunits. Interestingly, neither GTP nor NEM decreased [^{3}H]WAY 100635 specific binding, showing that this radioligand probably binds with high affinity to the 5-HT_{1A} receptor binding subunit independently of its possible coupling with G-protein. According to this hypothesis, the lower B_{max} of [^{3}H]8-OH-DPAT specific binding sites compared to that of [^{3}H]WAY 100635 specific binding sites could be explained by the fact that the latter radioligand bound with the same high affinity to both 5-HT_{1A} receptor subunits coupled with G-proteins and free receptor binding subunits, whereas [^{3}H]8-OH-DPAT bound with high affinity only to the G-protein-coupled 5-HT_{1A} receptor subunits. Evidence for the existence of two different binding sites for [^{3}H]WAY 100635 has notably been obtained in displacement studies with 5-HT and other 5-HT receptor agonists, 5-CT, RU 24969 and 5-MeO-*N*,*N*-DMT (see Table 2), as inhibition curves by these ligands yielded an apparent Hill coefficient lower than 1.0, compatible with their interaction at both high (H) affinity and low (L) affinity sites equally labelled by [^{3}H]WAY 100635 in brain membranes. Exposure of membranes to GTP or NEM, which eliminated high affinity binding sites for [^{3}H]8-OH-DPAT, shifted the 5-HT inhibition curve to the right and increased the apparent Hill coefficient to 1.0, as expected of the conversion of all binding sites into a homogeneous population, corresponding to the 5-HT_{1A} receptor binding subunits functionally dissociated from G-proteins. In contrast, antagonists yielded an apparent Hill coefficient close to 1.0, in agreement with the idea that they exhibited the same potency as inhibitors of [^{3}H]WAY 100635 binding to G protein-coupled (H sites) and uncoupled (L sites) 5-HT_{1A} receptors. Interestingly, some agonists or partial agonists such as 8-OH-DPAT, S 14506, S 20499, ipsapirone, buspirone and lesopitron (see Fig. 9) also inhibited [^{3}H]WAY 100635 specific binding with an apparent Hill coefficient not significantly different from 1.0 ($\geq$ 0.85), suggesting that they exhibited similar affinities for the G protein-coupled and uncoupled forms of the 5-HT_{1A} receptor. Accordingly, these agonists might interact with the 5-HT_{1A} receptor at the same site as antagonists, whereas those listed in Table 2 (5-HT, 5-CT, RU 24969 and 5-MeO-*N*,*N*-DMT) might bind at a different site, with a conformation highly dependent on the coupling of the receptor with G protein. In agreement with this interpretation, Lanfumey et al. (1994) reported that the specific binding to rat hippocampal membranes of the tritiated derivative of the potent 5-HT_{1A} receptor agonist S 14506 (Colpaert et al., 1992) exhibited the same characteristics as those of [^{3}H]WAY 100635 binding (same B_{max} value, and insensitivity to GTP and NEM, in particular).

Comparison of the respective B_{max} of [^{3}H]WAY 100635 and [^{3}H]8-OH-DPAT specific binding indicated that the former radioligand bound to more sites than the latter in all the regions examined, further supporting the idea that 5-HT_{1A} sites exist in two forms throughout the brain, one physically coupled to G-proteins and one uncoupled. As the ratio of the respective B_{max} values determined with these two radioligands was relatively constant in the hippocampus, septum, anterior and posterior cortices, it can be inferred that the relative proportion of 5-HT_{1A} receptor binding subunits which coupled to G-proteins did not exhibit marked variations, at least in these areas.

Whereas GTP and GppNHp decrease the specific binding of [^{3}H]8-OH-DPAT and agonists to 5-HT_{1A} receptor binding sites (Hall et al., 1985, 1986), these nucleotides were found to enhance the specific binding of [^{3}H]WAY 100635 to these sites. Interestingly, the latter effect was more pronounced with membranes from rats whose endogenous 5-HT content had been increased by MAO blockade with clorgyline, and, conversely, this effect was reduced after 5-HT depletion due to reserpine treatment. As previously discussed about adenosine A_1 receptors (Prater et al., 1992), it can therefore be proposed that 5-HT still present in membranes could account, at least partly, for the effect

of GTP on [^{3}H]WAY 100635 specific binding. By dissociating the 5-HT_{1A} receptor binding subunit/G-protein complexes, GTP decreases the affinity of the binding sites for endogenous 5-HT, and thus accelerates its removal from the membranes, making more sites available to the antagonist radioligand [^{3}H]WAY 100635. Indeed, Scatchard analyses confirmed that GTP increased the B_{max} of [^{3}H]WAY 100635 specific binding sites in hippocampal membranes from clorgyline-treated rats (unpublished observation). However, a higher intrinsic affinity of the binding site for [^{3}H]WAY 100635 and other antagonists on the 5-HT_{1A} receptor binding subunit functionally dissociated from G-protein might also account for the enhancing effect of GTP on [^{3}H]WAY 100635 specific binding. In fact, using [^{3}H]spiperone as an antagonist radioligand, Sundaram et al. (1993) also noted that GTP increases its specific binding to 5-HT_{1A} receptor sites expressed in transfected CHO cells. Furthermore, as found with GTP, NEM also increased the specific binding of [^{3}H]WAY 100635 to 5-HT_{1A} sites in rat hippocampal membranes, possibly through the functional dissociation of these sites from G-proteins (Stratford et al., 1988).

In conclusion, [^{3}H]WAY 100635 appears to be a selective antagonist radioligand of 5-HT_{1A} receptor binding sites in brain membranes. As it seems to bind with the same high affinity to both G-protein-coupled 5-HT_{1A} receptors and free 5-HT_{1A} receptor binding subunits, whereas [^{3}H]8-OH-DPAT binds with high affinity solely to the G-protein-coupled receptors (Emerit et al., 1990), comparison of the binding characteristics of [^{3}H]WAY 100635 and [^{3}H]8-OH-DPAT in the same membrane preparation should be a useful approach for assessing possible changes in the proportion of 5-HT_{1A} receptors physically associated with G-proteins under various pharmacological, physiological or pathophysiological conditions. In addition, in contrast to a radioactive agonist which rapidly leaves its binding site due to the fall in affinity consecutive to the activation-induced dissociation of the receptor-G-protein complex (Kobilka, 1992), a radioactive antagonist such as [^{3}H]WAY 100635 should persist in binding to its site after this dissociation. Accordingly, [^{3}H]WAY 100635 might be a useful tool for the in vivo labelling of 5-HT_{1A} receptor binding sites in the brain. Indeed, Laporte et al. (1994) recently showed that [^{3}H]WAY 100635 could be successfully used for the selective in vivo labelling of these receptor binding sites in the mouse brain.

Acknowledgements

This research was supported by grants from INSERM, DRET (Contract No. 93/047/BC) and the University of Paris VI (DRED). We are grateful to the Service des Molécules Marquées at CEA (91191 Gif-sur-Yvette, France) and Dr. Colin T. Dourish (Wyeth Research, Taplow, UK) for their generous gifts of [^{3}H]8-OH-DPAT and [^{3}H]WAY 100635, respectively. Pharmaceutical companies (Bristol-Myers-Squibb, Ciba-Geigy, Delalande-Synthélabo, Esteve, Glaxo, Janssen, May and Baker, Pfizer, Roussel-UCLAF, Sandoz, Servier, Tropon-Bayer) are also gratefully acknowledged for their generous gifts of drugs used in the present study. Dr. L. Lima was supported by IVIC, CONICIT and the Fondation pour la Recherche Médicale.

References

Adrien, J., L. Lanfumey, H. Gozlan, C.M. Fattaccini and M. Hamon, 1989, Biochemical and electrophysiological evidence for an agonist action of CM 57493 at pre- and postsynaptic 5-HT_{1A} receptors in brain, J. Pharmacol. Exp. Ther. 248, 1222.

Albert, P.R., Q.Y. Zhou, H.H. Van Tol, J.R. Bunzow and O. Civelli, 1990, Cloning, functional expression and mRNA tissue distribution of the rat 5-HT_{1A} receptor gene, J. Biol. Chem. 265, 5825.

Boess, F.G. and J.L. Martin, 1994, Molecular biology of 5-HT receptors, Neuropharmacology 33, 275.

Cheng, Y.C. and W.H. Prusoff, 1973, Relationship between the inhibition constant (Ki) and the concentration of inhibitor which causes 50 percent inhibition (IC_{50}) of an enzymatic reaction, Biochem. Pharmacol. 22, 3099.

Colpaert, F.C., W. Koek, J. Lehmann, J.M. Rivet, F. Lejeune, H. Canton, K. Bervoets, M.J. Millan, M. Laubie and G. Lavielle, 1992, S 14506: a novel, potent, high-efficacy 5-HT_{1A} agonist and potential anxiolytic agent, Drug Dev. Res. 26, 21.

Cossery, J.M., H. Gozlan, U. Spampinato, C. Perdicakis, G. Guillaumet, L. Pichat and M. Hamon, 1987, The selective labelling of central 5-HT_{1A} receptor binding sites by [^{3}H]5-methoxy-3-(di-n-propylamino)chroman, Eur. J. Pharmacol. 140, 143.

Dompert, W.U., T. Glaser and J Traber, 1985, ^{3}H-TVX Q 7821: identification of 5-HT_1 binding sites as target for a novel putative anxiolytic, Naunyn-Schmiedeberg's Arch. Pharmacol. 328, 467.

El Mestikawy, S., M. Riad, A.M. Laporte, D. Vergé, G. Daval, H. Gozlan and M. Hamon, 1990, Production of specific anti-rat 5-HT_{1A} receptor antibodies in rabbits injected with a synthetic peptide, Neurosci. Lett. 118, 189.

Emerit, M.B., S. El Mestikawy, H. Gozlan, B. Rouot and M. Hamon, 1990, Physical evidence of the coupling of solubilized 5-HT_{1A} binding sites with G regulatory proteins, Biochem. Pharmacol. 39, 7.

Fargin, A., J.R. Raymond, M.J. Lohse, B.K. Kobilka, M.G. Caron and R.J. Lefkowitz, 1988, The genomic clone G-21 which resembles a β-adrenergic receptor sequence encodes the 5-HT_{1A} receptor, Nature 335, 358.

Fletcher, A., I.A. Cliffe and C.T. Dourish, 1993a, Silent 5-HT_{1A} receptor antagonists: utility as research tools and therapeutic agents, Trends Pharmacol. Sci. 14, 441.

Fletcher, A., D.J. Bill, S.J. Bill, I.A. Cliffe, G.M. Dover, E.A. Forster, J.T. Haskins, D. Jones, H.L. Mansell and Y. Reilly, 1993b, WAY 100135: a novel, selective antagonist at presynaptic and postsynaptic 5-HT_{1A} receptors, Eur. J. Pharmacol. 237, 283.

Fletcher, A., D.J. Bill, I.A.Cliffe, E.A. Forster, D. Jones and Y. Reilly, 1994, A pharmacological profile of WAY-100635, a potent and selective 5-HT_{1A} receptor antagonist, Br. J. Pharmacol. 112 (Proceed. Suppl.), 91 P.

Frost, P.A. and R.G. Pearson, 1961, Kinetics and mechanisms. A study of homogeneous chemical reactions, John Wiley and Sons, New York.

Glowinski, J. and L. Iversen, 1966, Regional studies of catecholamines in the rat brain. I. Disposition of [^{3}H]norepinephrine, [^{3}H]dopamine, and [^{3}H]dopa in various regions of the brain, J. Neurochem. 13, 655.

Gozlan, H., S. El Mestikawy, L. Pichat, J. Glowinski and M. Hamon, 1983, Identification of presynaptic serotonin autoreceptors using a new ligand: ^{3}H-PAT, Nature 305, 140.

Gozlan, H., M. Ponchant, G. Daval, D. Vergé, F. Ménard, A. Vanhove, J.P. Beaucourt and M. Hamon, 1988, ^{125}I-Bolton-Hunter-8-methoxy-2-[*N*-propyl-*N*-propylamino] tetralin as a new selective radioligand of 5-HT$_{1A}$ sites in the rat brain. In vitro binding and autoradiographic studies, J. Pharmacol. Exp. Ther. 244, 751.

Gurling, J., M.A. Ashworth-Preece, C.T. Dourish and C. Routledge, 1994, Effects of acute and chronic treatment with the selective 5-HT$_{1A}$ receptor antagonist WAY-100635 on hippocampal 5-HT release in vivo, Br. J. Pharmacol. 112, (Proceed. Suppl.), 299P.

Haj-Dahmane S., T. Jolas, A.M. Laporte, H. Gozlan, A.J. Farré, M. Hamon and L. Lanfumey, 1994, Interactions of lesopitron (E-4424) with central 5-HT$_{1A}$ receptors; in vitro and in vivo studies in the rat, Eur. J. Pharmacol. 255, 185.

Hall, M.D., S. El Mestikawy, M.B. Emerit, L. Pichat, M. Hamon and H. Gozlan, 1985, [^{3}H]8-hydroxy-2-(di-n-propylamino) tetralin binding to pre- and postsynaptic 5-hydroxytryptamine sites in various regions of the rat brain, J. Neurochem. 44, 1685.

Hall, M.D., H. Gozlan, M.B. Emerit, S. El Mestikawy, L. Pichat and M. Hamon, 1986, Differentiation of pre- and postsynaptic high affinity serotonin receptor binding sites using physico-chemical parameters and modifying agents, Neurochem. Res. 11, 891.

Hamon, M., L. Lanfumey, L. Lima, A.M. Laporte, T. Jolas, C.M. Fattaccini and H. Gozlan, Electrophysiological and biochemical investigations with central 5-HT$_{1A}$ receptor antagonists. Third IUPHAR Satellite Meeting on Serotonin, Chicago, August 1994.

Jolas, T., S. Haj-Dahmane, L. Lanfumey, C.M. Fattaccini, E.J. Kidd, J. Adrien, H. Gozlan, B. Guardiola-Lemaitre and M. Hamon, 1993, (-)Tertatolol is a potent antagonist at pre- and postsynaptic serotonin 5-HT$_{1A}$ receptors in the rat brain, Naunyn-Schmiedeberg's Arch. Pharmacol. 347, 453.

Kidd, E.J., S. Haj-Dahmane, T. Jolas, L. Lanfumey, C.M. Fattaccini, B. Guardiola-Lemaitre, H. Gozlan and M. Hamon, 1993, New methoxy-chroman derivatives, 4[*N*-(5-methoxy-chroman-3-yl) *N*-propylamino]butyl-8-azaspiro-(4,5)-decane-7,9-dione [(±)-S 20244] and its enantiomers, (+)-S 20499 and (−)S-20500, with potent agonist properties at central 5-hydroxytryptamine$_{1A}$ receptors, J. Pharmacol. Exp. Ther. 264, 863.

Kobilka, B., 1992, Adrenergic receptors as models for G protein-coupled receptors. Annu. Rev. Neurosci. 15, 87.

Kobilka, B.K., T. Frielle, S. Collins, T. Yang-Feng, T.S. Kobilka, U. Francke, R.J. Lefkowitz and M.G. Caron, 1987, An intronless gene encoding a potential member of the family of the receptors coupled to guanine nucleotide regulatory proteins, Nature 329, 75.

Lanfumey, L., S. Haj-Dahmane and M. Hamon, 1993, Further assessment of the antagonist properties of the novel and selective 5-HT$_{1A}$ receptor ligands (+)-WAY 100135 and SDZ 216-525, Eur. J. Pharmacol. 249, 25.

Lanfumey, L, C. Gaymard, A.M. Laporte, L. Lima, E. Mocaër and M. Hamon, 1994, [^{3}H]S 14506: a novel high affinity radioligand for the specific labelling of 5-HT$_{1A}$ receptors. XIXth C.I.N.P. Congress, Washington, June 1994, Neuropsychopharmacology 10, 93S.

Laporte, A.M., L.E. Schechter, F.J. Bolaños, D. Vergé, M. Hamon and H. Gozlan, 1991, [^{3}H]5-methyl-urapidil labels 5-HT$_{1A}$ receptors and α_1-adrenoceptors in the rat CNS. In vitro binding and autoradiographic studies, Eur. J. Pharmacol. 198, 59.

Laporte, A.M., L. Lima, H. Gozlan and M. Hamon, 1994, Selective in vivo labelling of brain 5-HT$_{1A}$ receptors by [^{3}H]WAY 100635 in the mouse. Eur. J. Pharmacol. 271, 505–514.

Leysen, J.E., C.J.E. Niemegeers, J.P. Tollenaere and P.M. Laduron, 1978, Serotonergic component of neuroleptic receptors, Nature 272, 169.

Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall, 1951, Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193, 265.

Miquel, M.C. and M. Hamon, 1992, 5-HT$_1$ receptor subtypes: Pharmacological heterogeneity, in: Serotonin receptor subtypes: pharmacological significance and clinical implications, eds. S.Z. Langer, N. Brunello and J. Mendlewicz, (Int. Acad. Biomed. Drug Res., Karger A.G., Basel) 1, 13.

Mundey, M.K., A. Fletcher and C.A. Marsden, 1994, Effect of the putative 5-HT$_{1A}$ antagonist WAY 100635 on 5-HT neuronal firing in the guinea pig dorsal raphe nucleus, Br. J. Pharmacol. 112 (Proceed. Suppl.), 93P.

Nelson, D.L., A. Herbet, S. Bourgoin, J. Glowinski and M. Hamon, 1978, Characteristics of central 5-HT receptors and their adaptive changes following intracerebral 5,7-dihydroxytryptamine administration in the rat, Mol. Pharmacol. 14, 983.

Prater, M.R., H. Taylor, R. Munshi and J. Linden, 1992, Indirect effect of guanine nucleotides on antagonist binding to A1 adenosine receptors. Occupation of cryptic binding sites by endogenous vesicular adenosine, Mol. Pharmacol. 42, 765.

Radja, F., A.M. Laporte, G. Daval, D. Vergé, H. Gozlan and M. Hamon, 1991, Autoradiography of serotonin receptor subtypes in the central nervous system. Neurochem. Int. 18, 1.

Snedecor, G.W. and W.G. Cochran, 1967, Statistical methods, Iowa State College Press, Ames, IA.

Sotelo, C., B. Cholley, S. El Mestikawy, H. Gozlan and M. Hamon, 1990, Direct immunohistochemical evidence of the existence of 5-HT$_{1A}$ autoreceptors on serotoninergic neurons in the midbrain raphe nuclei, Eur. J. Neurosci. 2, 1144.

Stratford, C.A., G.L. Tan, M.W. Hamblin and R.D. Ciaranello, 1988, Differential inactivation and G-protein reconstitution of subtypes of [^{3}H]5-hydroxytryptamine binding sites in brain. Mol. Pharmacol. 34, 527.

Sundaram, H., A. Newman-Tancredi and P.G. Strange, 1993, Characterization of recombinant human serotonin 5-HT$_{1A}$ receptors expressed in chinese hamster ovary cells. [^{3}H]spiperone discriminates between the G-protein-coupled and -uncoupled forms, Biochem. Pharmacol. 45, 1003.

Vergé, D., G. Daval, M. Marcinkiewicz, A. Patey, S. El Mestikawy, H. Gozlan and M. Hamon, 1986, Quantitative autoradiography of multiple 5-HT$_1$ receptor subtypes in the brain of control or 5,7-dihydroxytryptamine-treated rats, J. Neurosci. 6, 3474.

Zhuang, Z.P., M.P. Kung and H.F. Kung, 1993, Synthesis of (R,S)-trans-8-hydroxy-2-[*N*-*n*-propyl-*N*-(3'-iodo-2'-propenyl)amino]tetralin (trans-8-OH-PIPAT): a new 5-HT$_{1A}$ receptor ligand, J. Med. Chem. 36, 3161.

Zifa, E. and G. Fillion, 1992, 5-Hydroxytryptamine receptors, Pharmacol. Rev. 44, 401.