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[^{3}H]Alnespirone: A novel specific radioligand of 5-HT$_{1A}$ receptors in the rat brain

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Abstract

Determination of the optimal assay conditions for the specific binding of a tritiated derivative of the novel potential anxiolytic drug alnespirone (S-20499, (+)-4-[*N*-(5-methoxy-chroman-3-yl)-*N*-propylamino]butyl-8-azaspiro-(4,5)-decane-7,9-dione) allowed the demonstration that this radioligand bound with a high affinity (K_d = 0.36 nM) to a homogeneous class of sites in rat hippocampal membranes. The pharmacological properties of [^{3}H]alnespirone specific binding sites matched exactly (r = 0.95) those of 5-HT$_{1A}$ receptors identified with [^{3}H]8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT) as radioligand. Furthermore, membrane binding experiments and autoradiographic labeling of tissue sections showed that the regional distribution of [^{3}H]alnespirone specific binding sites in the rat brain and spinal cord superimposed over that of 5-HT$_{1A}$ receptors specifically labeled by [^{3}H]8-OH-DPAT. However, the differential sensitivity of [^{3}H]alnespirone and [^{3}H]8-OH-DPAT specific binding to various physicochemical effectors (temperature, pH, Mn^{2+}, *N*-ethyl-maleimide) supports the idea that these two agonist radioligands did not recognize 5-HT$_{1A}$ receptors exactly in the same way. These differences probably account for the reported inability of alnespirone, in contrast to 8-OH-DPAT, to induce some 5-HT$_{1A}$ receptor-mediated behavioural effects in rats. © 1997 Elsevier Science B.V.

Keywords: 5-HT (5-hydroxytryptamine, serotonin); 5-HT$_{1A}$ receptor; Binding; Autoradiography; Physicochemical parameter; [^{3}H]Alnespirone

1. Introduction

Among the numerous serotonin (5-hydroxytryptamine, 5-HT) receptors identified to date (Hoyer et al., 1994), the 5-HT$_{1A}$ receptor subtype has been the matter of extensive studies since the discovery that the effects of 8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT) in the central nervous system (CNS) are essentially mediated through 5-HT$_{1A}$ receptor stimulation (Hjörth et al., 1982; Gozlan et al., 1983). The development of several agonist radioligands and, more recently, of the selective antagonist radioligand [^{3}H]*N*-[2-[4-(2-methoxyphenyl)-1-piperazinyl] ethyl]-*N*-(2-pyridinyl) cyclohexanecarboxamide ([^{3}H]WAY 100635, Gozlan et al., 1995; Khawaja, 1995) allowed the autoradiographic mapping of 5-HT$_{1A}$ receptors in the CNS of various species. Although [^{3}H]WAY 100635 was found to bind to approx. 60% more sites then [^{3}H]8-OH-DPAT, both [^{3}H]WAY 100635- and [^{3}H]8-OH-DPAT-specific binding sites exhibit exactly the same distribution pattern, typical of 5-HT$_{1A}$ receptors, with a marked enrichment in limbic areas such as the hippocampus, the septum, the frontal and entorhinal cortices and the amygdala (Marcinkiewicz et al., 1984; Pazos and Palacios, 1985; Gozlan et al., 1995). In addition, 5-HT$_{1A}$ receptor binding sites are also abundant in the raphe nuclei where they are located on the cell bodies and dendrites of serotoninergic neurones (Vergé et al., 1986; Sotelo et al., 1990).

In different areas of the brain, 5-HT$_{1A}$ receptor agonists stimulate the 5-HT$_{1A}$ receptors with various intrinsic activities. Thus, anxiolytic-like effects of 8-OH-DPAT and azapirones, such as ipsapirone, gepirone and buspirone, have been ascribed to the selective stimulation of somato-

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dendritic 5-HT_{1A} autoreceptors within the anterior raphe nuclei (Jolas et al., 1995). On the other hand, the antidepressant-like effects of the same drugs apparently involve the selective stimulation of 5-HT_{1A} receptors located in postsynaptic areas with respect to serotoninergic projections, such as the septum (Martin et al., 1990). Indeed, important differences have been noted between the respective pharmacological, biochemical and regulatory properties of 5-HT_{1A} receptors depending on their location in the anterior raphe nuclei or postsynaptic target areas of serotoninergic neurones, which led some authors to postulate the existence of 5-HT_{1A} receptor subtypes (Blier et al., 1993; Clarke et al., 1996). Different subtypes would be expressed in the raphe nuclei and the hippocampus for instance, thereby explaining why spiperone and (−)-pindolol efficiently block the effects of 5-HT_{1A} receptor agonists in the former but not the latter area (Blier et al., 1993; Romero et al., 1996).

Among drugs which exhibit differential actions at 5-HT_{1A} receptors in the raphe nuclei on one hand and postsynaptic target areas of serotoninergic neurones on the other hand, the potent agonist alnespirone (S-20499, (+)-4-[*N*-(5-methoxy-chroman-3-yl)-*N*-propylamino]butyl-8-azaspiro-(4,5)-decane-7,9-dione) has been the matter of special interest (Porsolt et al., 1992; Kidd et al., 1993). As expected of its agonist action at somatodendritic 5-HT_{1A} autoreceptors in the dorsal raphe nucleus, alnespirone produced a marked inhibition of the firing of serotoninergic neurones, and a decrease in 5-HT release and turnover in their projection areas (Kidd et al., 1993). Furthermore, relevant behavioural paradigms clearly demonstrated that alnespirone exhibits anxiolytic-like properties through its stimulatory effect at 5-HT_{1A} autoreceptors (Griebel et al., 1992; Barrett et al., 1994; Curle et al., 1994). However, in contrast to other 5-HT_{1A} receptor agonists such as 8-OH-DPAT, alnespirone does not cause the '5-HT syndrome' which normally results from the stimulation of postsynaptic 5-HT_{1A} receptors (Scott et al., 1994). Furthermore, alnespirone was shown to neither mimic nor attenuate the forepaw treading due to 5-HT_{1A} receptor stimulation in reserpine-pretreated rats (Scott et al., 1994), therefore suggesting that this drug does not interact with the postsynaptic 5-HT_{1A} receptors responsible for this behavioural effect.

Because of its differential actions at 5-HT_{1A} receptors in the raphe nuclei versus postsynaptic target areas of serotoninergic projections, alnespirone should be especially useful for investigating further the respective properties of pre (i.e., somatodendritic)- and post-synaptic 5-HT_{1A} receptors. On this basis, [^{3}H]alnespirone was synthesized and used as a radioligand for the characterization of the pharmacological properties and regional distribution of corresponding specific binding sites in comparison with 5-HT_{1A} receptors identified with [^{3}H]8-OH-DPAT as the prototypical radioligand (Marcinkiewicz et al., 1984; Hall et al., 1985).

2. Materials and methods

2.1. Animals

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

All the procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national and international laws and policies (Council directive # 87.848, October 19, 1987, Ministère de l'Agriculture et de la Forêt, Service Vétérinaire de la Santé et de la Protection Animale, permission # 0299 to M.H.).

2.2. Chemicals

[^{3}H]Alnespirone (92 Ci/mmol) was generously supplied by the Institut de Recherches Internationales Servier (Courbevoie, France). [^{3}H]8-OH-DPAT (110 Ci/mmol) was a gift of the Service des Molécules Marquées of CEA (Gif-sur-Yvette, France). [^{3}H]5-HT (11 Ci/mmol) was obtained from Amersham International (Les Ulis, France).

The other compounds used were: ipsapirone (Troponwerke, Köln, Germany), alnespirone, 1-[2-(4-fluorobenzoylamino)ethyl]-4-(7-methoxynaphtyl) piperazine (S-14506) and (−)-tertatolol (Servier), GTP and γ-imino-guanosine triphosphate (GppNHp, Boehringer-Mannheim, Mannheim, Germany), buspirone (Bristol-Myers Squibb, Wallingford, CT, USA), ondansetron (Glaxo, Hertfordshire, UK), ketanserin and domperidone (Janssen, Beerse, Belgium), 5-HT-creatinine sulfate (Merck, Darmstadt, Germany), pargyline (Abbott, Chicago, IL, USA), *N*-ethyl-maleimide (Sigma, St Louis, MO), prazosin, butaclamol, idazoxan and 8-OH-DPAT (Research Biochem., Natick, MA), dithiothreitol (Fluka, Buchs, Switzerland), WAY-100635 (Wyeth, Taplow, UK), lesopitron (Dr. Esteve Laboratories, Barcelona, Spain).

2.3. Methods

2.3.1. Preparation of membranes

Rats were killed by decapitation and their brains were rapidly removed and dissected according to the method of Glowinski and Iversen (1966). Brain structures were homogenized in 40 volumes (v/w) of ice-cold 50 mM Tris–HCl, pH 7.4, using a Polytron disrupter (type PT 10 OD). The homogenates were centrifuged at 40 000 × *g* for 20 min and the supernatant was discarded. The pellet was washed twice by resuspension in 40 volumes of Tris–HCl buffer followed by centrifugation (40 000 × *g*, 20 min, 4°C) and resuspension. The sedimented material was then

suspended in 40 volumes of Tris–HCl buffer and incubated at 37°C for 10 min in order to remove endogenous 5-HT (Nelson et al., 1978). The membranes were then collected by centrifugation and washed another 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 until they were used for binding assays.

2.3.2. Binding assays

2.3.2.1. [^{3}H]alnespirone . Various parameters were tested in order to determine the optimal conditions of [^{3}H]alnespirone binding assays (see Section 3). The procedure finally used was as follows: Membrane suspensions were thawed and gently homogenized with a Polytron disrupter, and 50 μl aliquots, corresponding to about 0.25 mg of membrane proteins, were mixed with various concentrations (0.1–4.0 nM) of [^{3}H]alnespirone and 50 mM Tris–HCl, pH 7.4, containing 0.05% bovine serum albumin, in a final volume of 0.5 ml. Non-specific binding was determined from similar samples supplemented with 10 μM 5-HT. All samples were incubated for 1 h at 25°C, and incubation was stopped by adding 3.5 ml of ice-cold 50 mM Tris–HCl, pH 7.4, followed by rapid vacuum filtration (using a Brandel cell harvester) through Whatman GF/B filters which had been presoaked in an aqueous solution of 0.05% bovine serum albumin for 30 min. After three additional washes with 3.5 ml of ice-cold Tris buffer, filters were dried and transferred into plastic vials containing 4.5 ml of Aquasol® scintillation fluid (New England Nuclear, Boston, MA, USA) for radioactivity counting.

2.3.2.2. [^{3}H]8-OH-DPAT. The assay conditions previously described by Hall et al. (1985) were used with minor modifications. Briefly, aliquots (50 μl) of membrane suspensions were mixed with 50 mM Tris–HCl, pH 7.4, containing various concentrations (0.3–6.0 nM) of [^{3}H]8-OH-DPAT (final volume 0.5 ml), and the mixtures were incubated for 1 h at 25°C. Assays were stopped by rapid filtration as described above for the measurement of [^{3}H]alnespirone binding except that Whatman GF/B filters were presoaked with 50 mM Tris–HCl buffer, pH 7.4. Non-specific binding was determined from similar samples supplemented with 10 μM 5-HT.

2.3.2.3. [^{3}H]5-HT. Aliquots (50 μl) of hippocampal membrane suspensions were mixed with 50 mM Tris–HCl, pH 7.4, supplemented with 4 mM $CaCl_2$, 5.7 mM ascorbic acid, 10 μM pargyline, plus various concentrations (0.3–8.0 nM) of [^{3}H]5-HT (final volume: 0.5 ml; see Nelson et al. (1978) for details). Incubations proceeded for 1 h at 25°C, and samples were filtered through Whatman GF/B filters under the same conditions as those used for the measurement of [^{3}H]8-OH-DPAT binding. Non-specific binding of [^{3}H]5-HT was determined in the presence of 0.3 μM 8-OH-DPAT.

All assays were performed in triplicate. Data analysis was done by computer-assisted non-linear regression analysis, using GraphPad and Inplot4 programs. K_i values were calculated from IC_{50} values using the equation of Cheng and Prusoff (1973). Protein concentrations were determined according to the method of Lowry et al. (1951).

2.3.3. Treatment of membranes by N-ethyl-maleimide

Aliquots (1 ml, corresponding to approx. 5 mg proteins) of hippocampal membrane suspensions were diluted 10-fold with 50 mM Tris–HCl, pH 7.4, and then supplemented with various concentrations of *N*-ethyl–maleimide (0.25–5 mM). Samples were incubated for 30 min at 25°C before the addition of dithiothreitol at the same concentration as that of *N*-ethyl-maleimide in order to neutralize the latter alkylating agent. Membranes were then washed with 3 cycles of suspension/centrifugation as described above (Section 2.3.1) before being used for binding assays.

2.3.4. Quantitative autoradiography

Rats were killed by decapitation and their brains were rapidly removed and frozen at −30°C, using isopentane cooled with dry ice. After a storage at −80°C for 1–2 weeks, coronal sections (20 μm) were cut at −20°C and thaw-mounted onto gelatin-coated slides which were then stored at −20°C for less than two weeks.

For the labeling procedure, slides with frozen tissue sections on were first brought to room temperature within 15 min and then preincubated for 30 min in 0.17 M Tris–HCl, pH 7.6, at 20°C. Incubation proceeded for 1 h at 20°C in fresh buffer supplemented with 0.6–1.0 nM [^{3}H]alnespirone or 1–2 nM [^{3}H]8-OH-DPAT. In some experiments, various concentrations (0.1 nM–10 μM) of alnespirone were also added to the [^{3}H]8-OH-DPAT containing incubation medium. Non-specific binding was estimated from adjacent sections incubated in the same solutions supplemented with 10 μM 5-HT. After incubation, the sections were washed by dipping the slides into ice-cold buffer twice for 5 min, then quickly into distilled water. They were dried in a stream of cold air and exposed in the dark to ^{3}H-Hyperfilm (Amersham) for 1 month at 4°C. The autoradiographs were developed in Microdol (Kodak, Rochester, NY, USA) for 10 min at 20°C. Optical density on the autoradiographic films was measured using a Biocom image analyzer and converted to fmol [^{3}H]alnespirone or [^{3}H]8-OH-DPAT specifically bound per mg tissue by comparison with ^{3}H-labeled standards (Amersham). Concentration-curves for the inhibition of [^{3}H]8-OH-DPAT binding by alnespirone were fitted by non-linear regression analysis using GraphPad. The equation of Cheng and Prusoff (1973) was used for the calculation of K_i values from IC_{50} values.

3. Results

3.1. Effects of various physico–chemical parameters and agents on the specific binding of [^{3}H]alnespirone to rat hippocampal membranes – Comparison with [^{3}H]8-OH-DPAT specific binding

3.1.1. Effect of temperature

As shown in Fig. 1, the specific binding of both radioligands increased as a function of temperature between 4°C and 20–25°C. A decrease in the specific binding was then observed at higher temperatures, and this effect was more pronounced with [^{3}H]8-OH-DPAT (−78% at 40°C compared to 20°C) than with [^{3}H]alnespirone (−34% at 40°C compared to 20°C). Subsequent assays were performed at 25°C to ensure optimal binding of [^{3}H]alnespirone. Under this condition, the non-specific binding amounted to less than 20% of total binding in assays with 0.1–2.0 nM [^{3}H]alnespirone.

3.1.2. Effect of pH

In contrast to [^{3}H]8-OH-DPAT, whose specific binding increased markedly as a function of pH increase from 6.5 to 9.0, [^{3}H]alnespirone showed an opposite dependence on pH since a progressive decrease in its specific binding was noted from pH 6.5 to 9.5 (Fig. 2). Subsequent assays were performed at the physiological pH value, 7.4, because it ensured the specific binding of each radioligand to reach a high percentage (65–85%) of its maximum at extreme pH values (pH 6.5 for [^{3}H]alnespirone and pH 9.0 for [^{3}H]8-OH-DPAT).

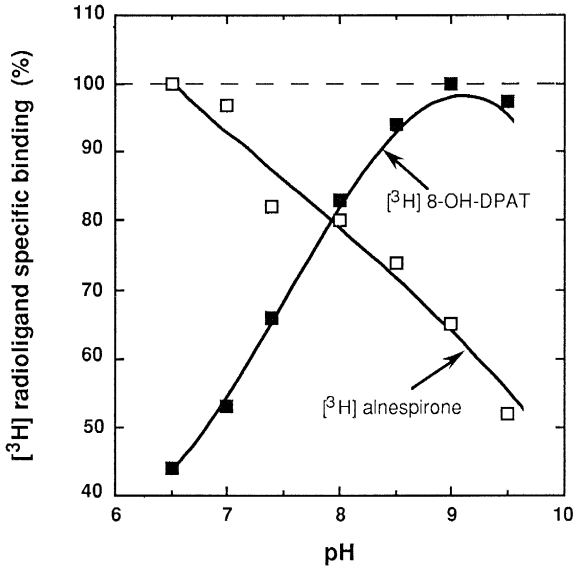

Fig. 2. pH-dependence of the specific binding of [^{3}H]alnespirone and [^{3}H]8-OH-DPAT to rat hippocampal membranes. Assays were performed as described in Section 2 except that the pH of the assay buffer varied from 6.5 to 9.5. The specific binding of each radioligand is expressed as a percentage of the respective maximal value (at pH 6.5 for [^{3}H]alnespirone and pH 9.0 for [^{3}H]8-OH-DPAT). Each point is the mean of triplicate determinations in three separate experiments, with less than 5% variations among them.

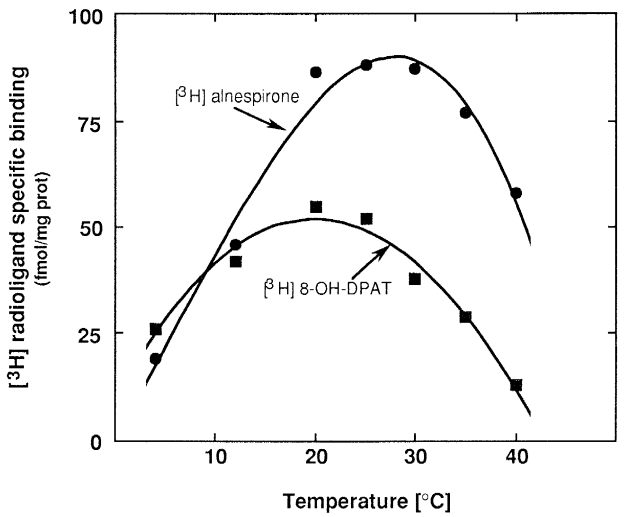

Fig. 1. Temperature dependence of the specific binding of [^{3}H]alnespirone and [^{3}H]8-OH-DPAT to rat hippocampal membranes. Assays were carried out with the same membrane preparations under the conditions described in Section 2 (with 0.30 nM [^{3}H]alnespirone or 0.50 nM [^{3}H]8-OH-DPAT), except that the temperature varied from 4 to 40°C. The time of incubation was adapted (4°C: 240 min; 12°C: 120 min; 20°C: 90 min; 25°C: 60 min; 30°C: 45 min; 35°C: 30 min; 40°C: 22 min) in order to allow maximal binding at each temperature tested. Each point is the mean of triplicate determinations (in fmol [^{3}H]alnespirone or [^{3}H]8-OH-DPAT specifically bound per mg membrane protein) in three separate experiments (with less than 5% variations among them).

3.1.3. Effect of presoaking filters

To reduce the non-specific binding of [^{3}H]alnespirone to glass fiber filters, presoaking of the filters with solutions of bovine serum albumin at different concentrations was tried. A concentration of 0.05% bovine serum albumin was found to maximally reduce (−60%) the non-specific binding of the radioligand. In contrast, presoaking the filter with polyethyleneimine (at 0.5% in water) resulted in an increase (+70%) in [^{3}H]alnespirone non-specific binding. Assays with [^{3}H]8-OH-DPAT showed that neither bovine serum albumin nor polyethyleneimine significantly affected its non-specific binding to glass fiber filters. Subsequently, 0.05% bovine serum albumin was systematically used for presoaking filters in all [^{3}H]alnespirone binding assays. Under this condition, assays with 0.1–2.0 nM [^{3}H]alnespirone gave a non-specific binding that reached only 5–8% of total binding to rat hippocampal membranes.

3.1.4. Effect of pretreatment with N-ethyl-maleimide

Pretreatment of rat hippocampal membranes with *N*-ethyl-maleimide dramatically reduced the specific binding of both radioligands (Fig. 3). At a concentration of 0.5 mM *N*-ethyl-maleimide, [^{3}H]8-OH-DPAT specific binding was

reduced to approx. 20% of that found with control membranes, whereas [^{3}H]alnespirone specific binding was still at approx. 50% of that found with the same control membranes. This difference persisted at higher concentrations of the alkylating agent, showing that the specific binding of [^{3}H]alnespirone was less sensitive than that of [^{3}H]8-OH-DPAT to the inhibitory action of pretreatment with *N*-ethyl-maleimide (Fig. 3).

3.1.5. Effects of GTP, GppNHp and Mn^{2+}

GTP and its less hydrolyzable imino analogue, GppNHp, exerted a negative influence on the specific binding of both radioligands. As shown in Fig. 4, the inhibition by the two nucleotides was concentration-dependent, up to a maximal effect (−65%) observed with 0.1–1.0 mM of GTP or GppNHp. No clearcut difference was seen between the effects of each nucleotide on the specific binding of [^{3}H]8-OH-DPAT or [^{3}H]alnespirone (Fig. 4). Thus, the concentrations for half maximal inhibition of either binding were approx. 0.26 and 2.8 μM for GppNHP and GTP, respectively.

In contrast to guanyl nucleotides, Mn^{2+} (added as $MnCl_2$ in the assay medium) slightly increased the specific binding of [^{3}H]alnespirone to rat hippocampal membranes (Fig. 4). The maximal effect (+15%) was noted with 10 μM of the divalent cation. Similarly, an increase in the specific binding of [^{3}H]8-OH-DPAT occurred upon the addition of Mn^{2+} to the assay medium, with a maximum (+40%) at 10 μM of the cation. At higher concentrations, Mn^{2+} induced a progressive, concentration-dependent, decrease in the specific binding of both [^{3}H]alnespirone and [^{3}H]8-OH-DPAT (Fig. 4).

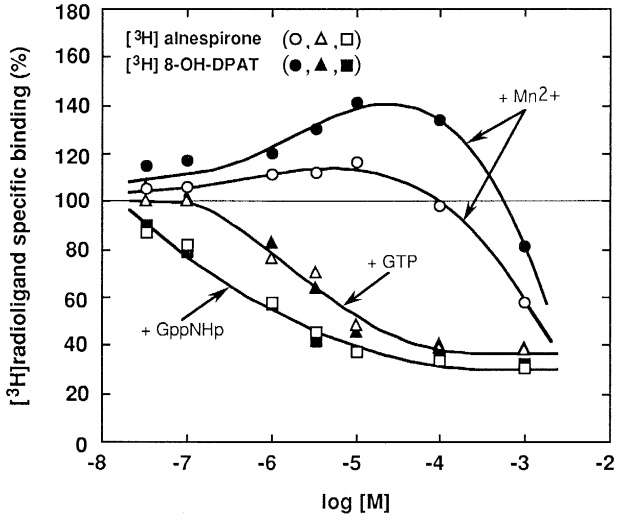

Fig. 4. Effects of GTP, GppNHp and Mn^{2+} on the specific binding of [^{3}H]alnespirone and [^{3}H]8-OH-DPAT to rat hippocampal membranes. Binding assays were performed under standard conditions for each radioligand (see Section 2). Data are expressed as percentages of the specific binding of each radioligand in the absence of additives. Each point is the mean of triplicate determinations in three separate experiments. Inter-experiment variations were less than 4%.

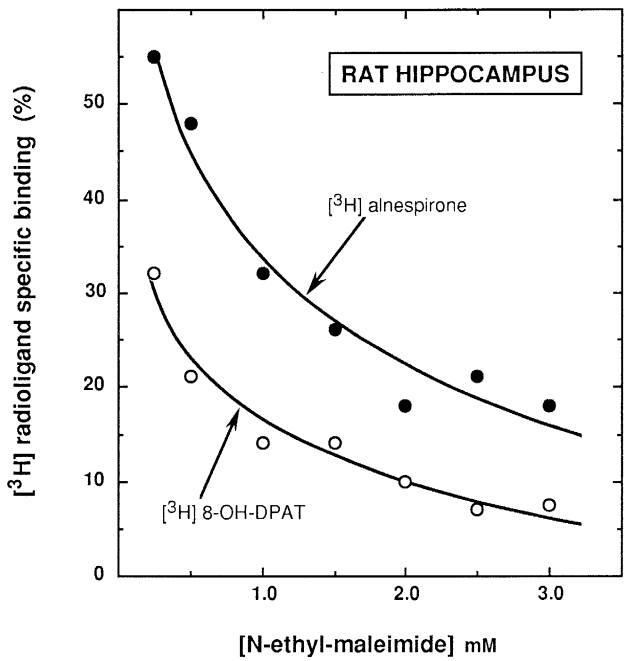

Fig. 3. Effects of *N*-ethyl-maleimide pretreatment on the specific binding of [^{3}H]alnespirone and [^{3}H]8-OH-DPAT to rat hippocampal membranes. Pretreatment with various concentrations of *N*-ethyl-maleimide (abscissa) was performed as described in Section 2. Hippocampal membranes were then washed extensively and used for the measurement of the specific binding of [^{3}H]alnespirone and [^{3}H]8-OH-DPAT under standard assay conditions. Data are expressed as percentages of the specific binding of each radioligand to membranes which were preincubated in the absence of *N*-ethyl-maleimide. Each point is the mean of triplicate determinations in three separate experiments, with less than 4% variations among them.

3.2. Characterization of [^{3}H]alnespirone specific binding sites

3.2.1. Saturation studies

Binding assays with increasing concentrations of [^{3}H]alnespirone revealed a limited capacity of specific binding in rat hippocampal membranes. Conversion of the direct saturation curve according to Scatchard (1949) yielded a single slope, suggesting the existence of a single homogeneous population of specific binding sites (Fig. 5A). Calculations from the Scatchard representation indicated a K_d value of 0.36 nM and a B_{max} value of 195 fmol/mg protein.

Further assays in the presence of varying concentrations of 8-OH-DPAT (0.5, 1.5, 4 nM) showed that this 5-HT_{1A} receptor agonist markedly reduced the slope value but did not significantly change the intersection of the curve with the *x* axis (Fig. 5A). Thus, the B_{max} value remained unchanged whereas the K_d value increased to 0.48, 0.82 and 2.56 nM in the presence of 0.5, 1.5 and 4 nM 8-OH-DPAT, respectively.

As illustrated in Fig. 5B, the specific binding of [^{3}H]8-

OH-DPAT to rat hippocampal membranes also yielded a single slope in the Scatchard's representation, corresponding to a K_d value of 1.0 nM and a B_{max} value of 190 fmol/mg protein. Addition of alnespirone (0.5 and 1.5 nM) to the assay medium resulted in a decrease in the slope value without any significant change in the intersection of the curve with the x axis. Accordingly, as expected of a competitive inhibitor, alnespirone increased the K_d value of [^{3}H]8-OH-DPAT specific binding sites (to 1.20 and 2.94 nM with 0.5 and 1.5 nM alnespirone, respectively) but did not alter their B_{max} value (Fig. 5B).

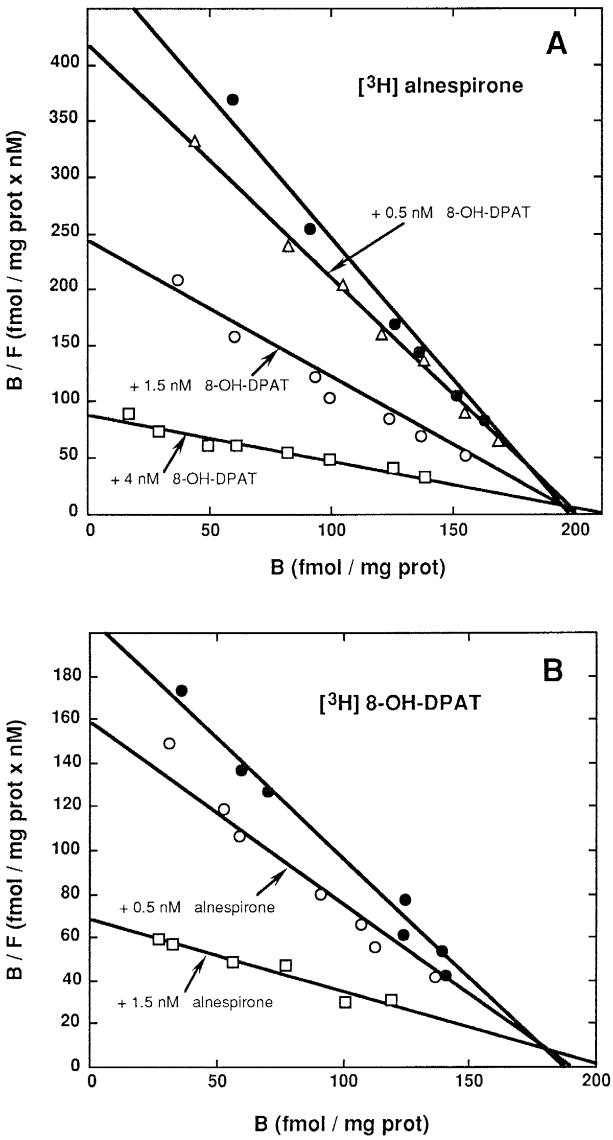

Fig. 5. Competitive interactions between alnespirone and 8-OH-DPAT at specific binding sites in rat hippocampal membranes. Saturation studies were performed with: (A) various concentrations of [^{3}H]alnespirone (0.1–4.0 nM) in the absence or the presence of three different fixed concentrations (0.5, 1.5 and 4 nM) of 8-OH-DPAT or (B) with various concentrations of [^{3}H]8-OH-DPAT (0.3–6.0 nM) in the absence or the presence of two different fixed concentrations (0.5 and 1.5 nM) of alnespirone. Scatchard (1949) representations of the data (B: bound [^{3}H]radioligand; F: free [^{3}H]radioligand) are based on triplicate determinations in three separate experiments.

Table 1
K_i values of various ligands as inhibitors of the specific binding of [^{3}H]alnespirone to rat hippocampal membranes

Compound	K_i (nM)
WAY-100635	0.20 ± 0.03
S-14506	0.53 ± 0.06
8-OH-DPAT	1.33 ± 0.16
Alnespirone	1.48 ± 0.12
5-HT	5.67 ± 1.58
Ipsapirone	7.24 ± 1.45
Buspirone	8.71 ± 0.85
(−)Tertatolol	17.56 ± 2.78
Lesopitron	39.15 ± 7.85
Idazoxan	316.2 ± 41.3
Butaclamol	1047 ± 143
Domperidone	1176 ± 204
Prazosin	1738 ± 219
Ketanserin	2399 ± 307
Ondansetron	23900 ± 2600

Binding assays were carried out with 0.5 nM [^{3}H]alnespirone under standard conditions, using 10 different concentrations of each inhibitory compound. Inhibition curves yielded an apparent Hill coefficient close to unity for all the compounds tested. K_i values were calculated from IC_{50} values according to Cheng and Prusoff (1973). Each value is the mean ± S.E.M. of at least 3 independent determinations.

In another series of experiments with rat hippocampal membranes, [^{3}H]5-HT was used as a radioligand for the specific labeling of 5-HT_{1A} receptors, i.e., with 0.3 μM 8-OH-DPAT for the determination of non-specific binding. Scatchard plots also gave a single slope with a K_d value of 3.2 nM and a B_{max} value of 200 fmol/mg protein (not shown).

3.2.2. Pharmacological properties of [^{3}H]alnespirone specific binding sites

Assays with increasing concentrations of various drugs indicated that 5-HT_{1A} receptor ligands were especially potent to reduce the specific binding of [^{3}H]alnespirone to rat hippocampal membranes. Thus, nanomolar concentrations of the 5-HT_{1A} receptor antagonist WAY 100635, and of the 5-HT_{1A} agonists 8-OH-DPAT, alnespirone and 1-[2-(4-fluorobenzoylamino)ethyl]-4-(7-methoxynaphthyl) piperazine (S-14506), were sufficient to inhibit [^{3}H]alnespirone binding (Table 1). In contrast, ligands of other receptor types such as the 5-HT_{2A} receptor antagonist, ketanserin, the 5-HT_3 receptor antagonist, ondansetron, the dopamine D_2 receptor antagonists, domperidone and butaclamol, and the α-adrenoceptor antagonists prazosin (α_1) and idazoxan (α_2) were active only at much higher concentrations (Table 1). Comparison of the respective K_i values of the 15 different ligands tested as inhibitors of [^{3}H]alnespirone specific binding to rat hippocampal membranes with their K_i values as inhibitors of [^{3}H]8-OH-DPAT specific binding to the same membrane preparations

revealed a highly significant positive correlation ($r = 0.95$, $P < 0.001$) between the two series of values (Fig. 6).

3.3. Regional distribution of [^{3}H]alnespirone specific binding sites

Binding assays with membranes prepared from various brain regions showed that the highest levels of [^{3}H]alnespirone specific binding were found in the hippocampus, followed by the septum, the cerebral cortex, the brain stem and the hypothalamus. Only very low levels of [^{3}H]alnespirone specific binding were detected in the striatum and the cerebellum (Fig. 7). As illustrated in Fig. 7, this distribution matched exactly that of [^{3}H]8-OH-DPAT specific binding sites, and a significant correlation ($r = 0.99$, $P < 0.001$) was found between the respective labeling by [^{3}H]alnespirone and [^{3}H]8-OH-DPAT throughout the rat brain.

Autoradiographic investigations allowed a more detailed examination of the regional distribution of [^{3}H]alnespirone specific binding sites in the rat CNS. It thus appeared that the lateral septum, the hippocampus (especially the dentate gyrus and CA1 area of Ammon's horn), the entorhinal cortex, the dorsal raphe nucleus and the superficial layers of the dorsal horn of the spinal cord were enriched in these sites (Fig. 8). In addition, a moderate density of [^{3}H]alnespirone binding sites was found throughout the cerebral cortex, particularly in layer IV. As shown in Fig. 8, the overall regional distribution of autoradiographic labeling by [^{3}H]alnespirone matched exactly that obtained with [^{3}H]8-OH-DPAT throughout the rat CNS. Quantitative estimates of [^{3}H]alnespirone and [^{3}H]8-OH-DPAT specific binding to 6 selected areas on coronal brain sections allowed the demonstration of a significant correlation ($r = 0.96$, $P < 0.001$) between the respective labeling by the two radioligands (Table 2).

Experiments that consisted of incubating sections with

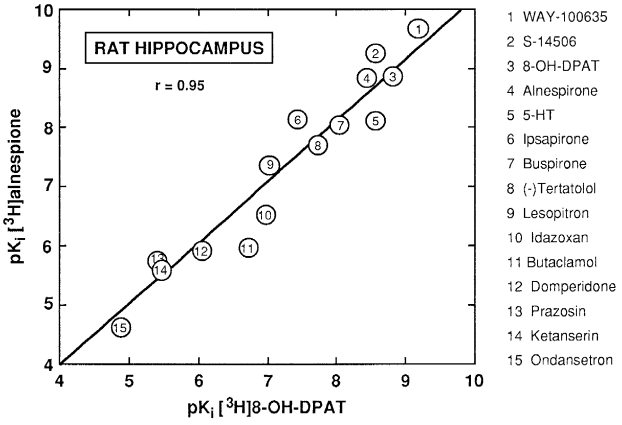

Fig. 6. Comparison of respective pK_i values of 15 different compounds as inhibitors of the specific binding of [^{3}H]alnespirone (ordinate) or [^{3}H]8-OH-DPAT (abscissa) to the same hippocampal membrane preparations. Binding assays were carried out with 0.5 nM [^{3}H]alnespirone or 1.0 nM [^{3}H]8-OH-DPAT and 10 different concentrations of each compound tested. pK_i ($-\log K_i$) values were calculated according to Cheng and Prusoff (1973). Each point is the mean of at least 3 independent determinations (individual pK_i values ± S.E.M. derived from [^{3}H]alnespirone binding assays are listed in Table 1). r = coefficient of the correlation between the two series of respective pK_i values.

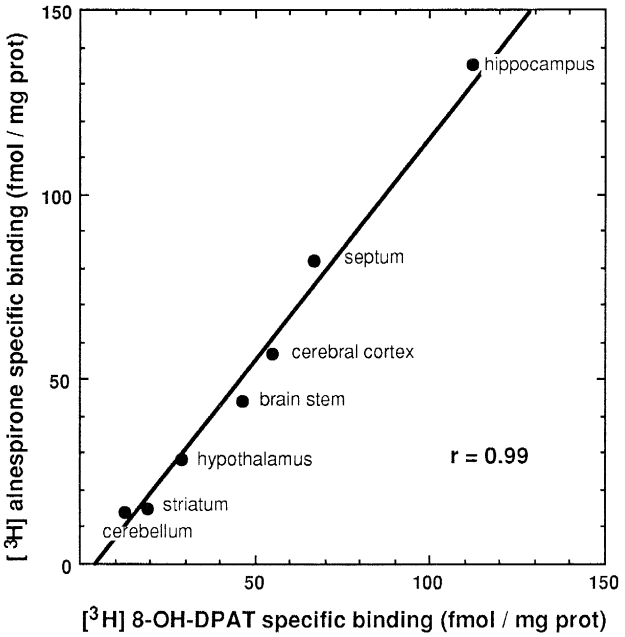

Fig. 7. Comparison of the respective regional distributions of [^{3}H]alnespirone (ordinate) and [^{3}H]8-OH-DPAT (abscissa) specific binding sites in the rat brain. Binding assays were performed with the same membrane preparations for each dissected region, and 0.55 nM [^{3}H]alnespirone or 1.2 nM [^{3}H]8-OH-DPAT under standard conditions. Each point is the mean of triplicate determinations in three separate experiments (with less than 4% variations among them). r = coefficient of the correlation between [^{3}H]alnespirone (ordinate) and [^{3}H]8-OH-DPAT (abscissa) specific binding in the 7 areas examined.

Table 2
Comparison of the respective autoradiographic labelling of selected areas in rat brain sections exposed to [^{3}H]alnespirone or [^{3}H]8-OH-DPAT

Brain area	[^{3}H]radioligand specific binding (fmol/mg tissue)	
	[^{3}H]alnespirone	[^{3}H]8-OH-DPAT
Hippocampus		
– dentate gyrus	173.2 ± 5.5	134.8 ± 2.4
– CA1	139.0 ± 5.0	96.1 ± 2.1
Septum	149.3 ± 5.9	120.5 ± 6.3
Dorsal raphe nucleus	101.1 ± 8.6	89.2 ± 10.8
Entorhinal cortex	106.2 ± 5.2	69.0 ± 2.3
Frontal cortex (layer IV)	61.8 ± 3.8	44.8 ± 1.7

Coronal brain sections (20 μm) were incubated with 1.0 nM [^{3}H]alnespirone or 2.0 nM [^{3}H]8-OH-DPAT, and exposed to autoradiographic films as described in Section 2. Optical density values were converted to fmol [^{3}H]alnespirone or [^{3}H]8-OH-DPAT specifically bound per mg tissue from the calibration curve drawn with tritium labeled standards (Amersham). Each value is the mean ± S.E.M. of 16 independent determinations (on 16 sections prepared from 3 rat brains). The correlation coefficient between both series of values is equal to $r = 0.96$.

both [^{3}H]8-OH-DPAT (1.7 nM) and increasing concentrations of alnespirone (0.1–10 nM) showed a concentration-dependent homogeneous decrease in the specific autoradiographic labeling in all areas. Thus, quantitative determinations indicated that alnespirone was equipotent to inhibit the specific binding of [^{3}H]8-OH-DPAT in the dorsal raphe nucleus (K_i = 0.19 nM) and the dentate gyrus of the hippocampus (K_i = 0.15 nM) (Fig. 9).

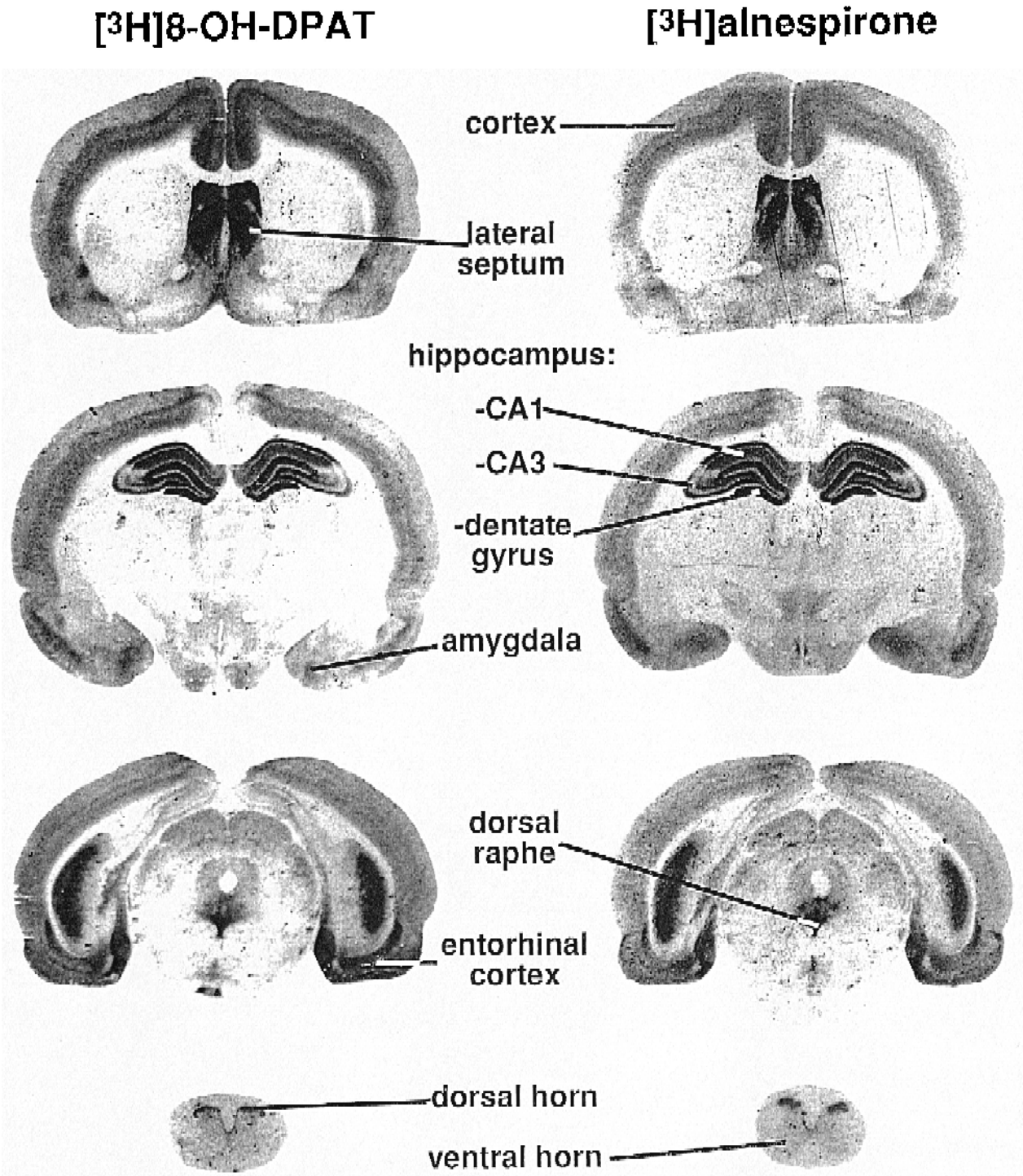

Fig. 8. Autoradiographs of coronal sections of the rat brain and spinal cord labeled by [^{3}H]alnespirone or [^{3}H]8-OH-DPAT. Coronal sections (20 μm) were labeled with 0.60 nM [^{3}H]alnespirone or 1.0 nM [^{3}H]8-OH-DPAT, and apposed to Hyperfilm for 1 month at 4°C. CA1 and CA3: specific fields in Ammon's horn of the hippocampus.

4. Discussion

The present study shows that [^{3}H]alnespirone binds with high affinity to a homogeneous population of specific sites in the rat brain. Extensive characterization of these sites indicates that they correspond to 5-HT$_{1A}$ receptors previously identified using the selective agonist radioligand [^{3}H]8-OH-DPAT (Gozlan et al., 1983; Hall et al., 1985).

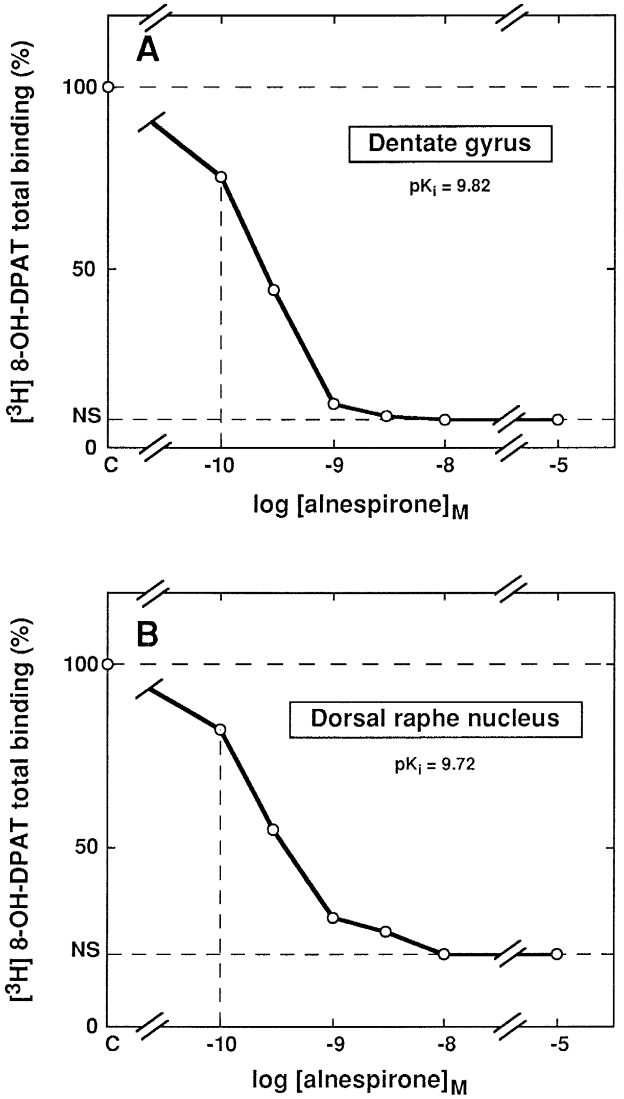

Fig. 9. Quantitative autoradiographic analysis of alnespirone-induced inhibition of [^{3}H]8-OH-DPAT binding in the dentate gyrus (A) and the dorsal raphe nucleus (B) of rat brain sections. Coronal sections (20 μm) at the level of the dorsal hippocampus or the dorsal raphe nucleus were incubated with 1.0 nM [^{3}H]8-OH-DPAT plus various concentrations (abscissa) of unlabeled alnespirone. Corresponding autoradiographs were obtained as described in Section 2. Bound [^{3}H]8-OH-DPAT was quantified at the levels of the dentate gyrus of the hippocampus and the dorsal raphe nucleus, and expressed as percentages of corresponding values for autoradiographs from sections incubated with [^{3}H]8-OH-DPAT alone. Each point is the mean of 8–12 independent determinations (with less than 5% variations among them) in sections from two rat brains. NS: non-specific binding (determined from sections incubated with [^{3}H]8-OH-DPAT plus 10 μM 5-HT). pK_i ($-\log K_i$) values were calculated from IC$_{50}$ values according to Cheng and Prusoff (1973).

As expected from the labeling of the same receptor population, the regional distribution of high affinity [^{3}H]alnespirone specific binding sites matched exactly that of [^{3}H]8-OH-DPAT specific binding sites. The highest levels of labeling by both radioligands were found in the hippocampus, followed by the septum, the cerebral cortex and the hypothalamus, whereas only very low levels of labeling were detected in the striatum and the cerebellum. Further investigations using the autoradiographic technique demonstrated that [^{3}H]alnespirone binding sites were especially concentrated in the dentate gyrus and the CA1 area of Ammon's horn in the hippocampus, the lateral septum, the entorhinal cortex and the dorsal raphe nucleus, like that previously reported for 5-HT$_{1A}$ receptors specifically labeled by [^{3}H]8-OH-DPAT (Marcinkiewicz et al., 1984; Pazos and Palacios, 1985; Radja et al., 1991).

Studies of the inhibition of [^{3}H]alnespirone specific binding to rat hippocampal membranes by various ligands revealed that 5-HT$_{1A}$ receptor agonists and antagonists were the most potent in this respect with K_i values in the nanomolar range. In contrast, micromolar to millimolar concentrations of ligands acting at α_1-, α_2-adrenoceptors, dopamine D$_2$ receptors and 5-HT$_{2A}$ or 5-HT$_3$ receptors were required to prevent the specific binding of [^{3}H]alnespirone to its high affinity sites in rat hippocampal membranes. In all cases, the inhibition curves gave a Hill coefficient not significantly different from 1.0, as expected from interactions between [^{3}H]alnespirone and unlabeled ligands at the level of a single homogeneous population of specific binding sites. That these sites corresponded to 5-HT$_{1A}$ receptors was further confirmed by the existence of a highly significant correlation ($r = 0.95$) between the respective potencies of a series of 15 different compounds to inhibit both [^{3}H]alnespirone and [^{3}H]8-OH-DPAT specific binding in the same hippocampal membrane preparations. Furthermore, Scatchard plots of the inhibition of [^{3}H]alnespirone specific binding by 8-OH-DPAT showed that the latter ligand increased the K_d without changing the B_{max} of [^{3}H]alnespirone high affinity specific binding sites, as expected from a competitive interaction between [^{3}H]alnespirone and 8-OH-DPAT at the same sites. Reciprocally, unlabeled alnespirone was found to competitively inhibit the specific binding of [^{3}H]8-OH-DPAT to hippocampal membranes, with a resulting increase in the corresponding K_d value and no change in the B_{max} value. Finally, [^{3}H]alnespirone and [^{3}H]8-OH-DPAT were found to bind to the same number of high affinity sites in the same hippocampal membrane preparations ($B_{max} = 190$–200 fmol per mg membrane protein), as expected from the selective labeling of 5-HT$_{1A}$ receptors by the two radioligands. Indeed, the same population of specific binding sites could also be labeled by [^{3}H]5-HT provided that

non-specific binding was determined in the presence of 8-OH-DPAT at a concentration high enough (0.3 μM) to saturate selectively 5-HT_{1A} binding sites (Hall et al., 1985).

Previous studies of the pharmacological properties of alnespirone indicated that this compound markedly inhibits both the firing of serotoninergic neurones within the dorsal raphe nucleus and the 5-HT_{1A} receptor-dependent adenylate cyclase activity in rat hippocampal membranes (Kidd et al., 1993). Furthermore, these effects can be prevented by 5-HT_{1A} receptor antagonists, demonstrating that alnespirone is an agonist at both presynaptic (i.e., somato-dendritic) and postsynaptic 5-HT_{1A} receptors in the dorsal raphe nucleus and the hippocampus, respectively (Porsolt et al., 1992; Kidd et al., 1993). In line with this interpretation, we presently found that [^{3}H]alnespirone specific binding could be inhibited by GTP and its less hydrolyzable analogue, GppNHp, in the same way as [^{3}H]8-OH-DPAT specific binding. In contrast, guanine nucleotides have been shown to enhance the specific binding of the selective antagonist radioligand [^{3}H]WAY 100635 to 5-HT_{1A} receptors in rat hippocampal membranes (Gozlan et al., 1995). Extensive studies of the inhibitory effect of GTP and GppNHp on [^{3}H]8-OH-DPAT high affinity binding showed that this effect results from the dissociation of the receptor binding subunit from its coupled G protein, demonstrating that only the G protein-coupled receptors are recognized with high affinity by the agonist radioligand (Emerit et al., 1990; Gozlan et al., 1995). Another method for inactivating the coupling between the receptor binding subunit and its associated G protein consists of treating the membranes by *N*-ethyl-maleimide (Stratford et al., 1988). Under these conditions, the specific binding of the antagonist radioligand [^{3}H]WAY 100635 is unaltered whereas that of the agonist radioligand [^{3}H]8-OH-DPAT is markedly reduced (Gozlan et al., 1995). Similarly, the specific binding of [^{3}H]alnespirone was significantly decreased in hippocampal membranes which had been pretreated with *N*-ethyl-maleimide, further confirming the agonist nature of the interaction of this radioligand with postsynaptic 5-HT_{1A} receptors.

Interestingly, the concentration-dependent inhibition by *N*-ethyl-maleimide of the specific binding of [^{3}H]alnespirone was less pronounced than that of [^{3}H]8-OH-DPAT, suggesting that these two agonists did not interact exactly in the same way with 5-HT_{1A} receptors. Furthermore, the enhancing effect of Mn^{2+} on the specific binding of [^{3}H]alnespirone was also less striking than that observed on [^{3}H]8-OH-DPAT specific binding in the same membrane preparations. Since both the inhibitory effect of *N*-ethyl-maleimide and the enhancing effect of Mn^{2+} very probably result from opposite changes in the degree of coupling between the receptor binding subunit and its associated G protein (Gozlan et al., 1995), these differences would suggest that the specific binding of [^{3}H]alnespirone was, to some extent, less dependent on this coupling than the specific binding of [^{3}H]8-OH-DPAT. Accordingly, the nature of the interaction of alnespirone with 5-HT_{1A} receptors might not be exactly the same as that of the prototypical agonist 8-OH-DPAT. In line with this interpretation, differences were observed in the temperature dependence of the specific binding of [^{3}H]alnespirone and [^{3}H]8-OH-DPAT, which indicated that binding of the former radioligand was less sensitive to heat denaturation than that of the latter in the same membrane preparations. Furthermore, the pH dependence of the specific binding of [^{3}H]alnespirone was also markedly different from that of [^{3}H]8-OH-DPAT specific binding. Thus, a progressive decrease in [^{3}H]alnespirone specific binding to rat hippocampal membranes was found as a function of pH increase from 6.5 to 9.5, whereas, in contrast, [^{3}H]8-OH-DPAT binding progressively increased with this change in pH, in agreement with previous observations (Hall et al., 1986). Interestingly, the specific binding of the selective 5-HT_{1A} receptor antagonist radioligand, [^{3}H]WAY 100635, was also found to decrease as a function of pH increase from 6.5 to 9.0 (Gozlan et al., 1995). Since pH effects on the specific binding of radioligands depend, at least partly, on charged aminoacid residues within the binding site, the differences noted above would suggest that these aminoacid residues may not be identical for [^{3}H]alnespirone and [^{3}H]WAY 100635 on one hand and for [^{3}H]8-OH-DPAT on the other hand. Nevertheless, the competitive interaction between alnespirone and 8-OH-DPAT for the same category of specific sites indicates that their respective binding sites should overlap to some extent.

Previous studies of the specific binding of a tritiated derivative of another potent 5-HT_{1A} receptor agonist, S-14506 (Colpaert et al., 1992), also demonstrated that this molecule does not interact with 5-HT_{1A} receptors in the same way as the prototypical agonist 8-OH-DPAT (Lima et al., 1995). In particular, neither guanine nucleotides nor *N*-ethyl-maleimide pretreatment were found to inhibit the specific binding of [^{3}H]S-14506 to rat hippocampal membranes, in contrast to that found with [^{3}H]8-OH-DPAT (Hall et al., 1986). Furthermore, the specific binding of [^{3}H]S-14506 progressively decreased as a function of pH increase from 6.5 to 9.0 (Lima et al., 1995), like that presently observed with [^{3}H]alnespirone and in contrast with that found with [^{3}H]8-OH-DPAT (Hall et al., 1986). These observations also led to the conclusion that the two agonists S-14506 and 8-OH-DPAT do not bind exactly to the same site within the 5-HT_{1A} receptor (Lima et al., 1995).

Comparison of the behavioural effects of alnespirone and 8-OH-DPAT has shown that the latter but not the former agonist was able to trigger the '5-HT syndrome' in adult rats (see Section 1). In particular, the 'piano playing' and the 'flat body posture', two typical components of this syndrome, were observed in 8-OH-DPAT-treated rats but not in those treated with alnespirone (Scott et al., 1994). Possible differences in the potency of alnespirone at 5-HT_{1A} receptors from one region to another in the CNS (down to

a hypothetical very low potency at the 5-HT_{1A} receptors responsible for the '5-HT syndrome') cannot account for the inability of this agonist to trigger the corresponding behaviours because autoradiographic investigations showed that alnespirone was equipotent to inhibit the specific binding of [^{3}H]8-OH-DPAT in the dorsal raphe nucleus, the hippocampus and all other brain areas. However, the present data also established that alnespirone and 8-OH-DPAT do not interact exactly in the same way with 5-HT_{1A} receptors, and the resulting differences in 5-HT_{1A} receptor activation might well exhibit marked regional variations. Indeed, several lines of evidence support the idea that somatodentritic 5-HT_{1A} autoreceptors in the dorsal raphe nucleus and postsynaptic 5-HT_{1A} receptors, notably in the hippocampus, are functionally different (Blier et al., 1993; Clarke et al., 1996; Romero et al., 1996; Hamon, 1997), and this might also contribute to explain why alnespirone is an active agonist at 5-HT_{1A} autoreceptors (Kidd et al., 1993), but is essentially inactive at postsynaptic 5-HT_{1A} receptors whose activation triggers the '5-HT syndrome' (Scott et al., 1994). Whether agonists such as 8-OH-DPAT activate equally 5-HT_{1A} receptors throughout the CNS whereas other agonists, such as alnespirone, possibly because of their different interaction with these receptors, have a variable efficacy from one cell type to another (notably because of the receptor coupling with different G proteins; see Raymond et al., 1993; Mulheron et al., 1994; Oleskevich, 1995) will have to be further investigated with 5-HT_{1A} agonists that belong to various chemical classes. In line with the concept of regional differences in the efficacy of 5-HT_{1A} receptor ligands in brain, it has already been recognized that compounds that act as antagonists at postsynaptic 5-HT_{1A} receptors in the hippocampus, such as 8-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]8-azaspiro-(4,5)-decane-7,9-dione (BMY 7378), 8-[2-(2,3-dihydro-1,4-benzodioxin-2-yl)methylamino]-8-azaspiro-(4,5)-decane-7,9-dione (MDL-73005EF) and 1-(2-methoxyphenyl)-4-[(phthalimido)butyl]piperazine (NAN-190), can be agonists at somatodendritic 5-HT_{1A} autoreceptors in the dorsal raphe nucleus (see Hamon, 1997). The presence of a large reserve of 5-HT_{1A} receptors in the latter area (Cox et al., 1993) but not in the hippocampus (Yocca et al., 1992) very probably contributes to these regional differences. Accordingly, it can be assumed that regional variations in 5-HT_{1A} receptor reserve may also account, at least in part, for the apparently full 5-HT_{1A} receptor agonist activity of alnespirone in the dorsal raphe nucleus (Kidd et al., 1993), and its partial agonist activity at 5-HT_{1A} receptors in postsynaptic target areas of serotoninergic projections (Scott et al., 1994).

In conclusion, [^{3}H]alnespirone is a novel agonist radioligand for the selective labeling of 5-HT_{1A} receptors in the CNS. Its higher affinity than that of [^{3}H]8-OH-DPAT should make this novel radioligand of particular interest for the labeling of 5-HT_{1A} receptors in brain areas where these sites are poorly abundant.

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