Electrophysiological, biochemical, neurohormonal and behavioural studies with WAY-100635, a potent, selective and silent 5-HT\textsubscript{1A} receptor antagonist

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Abstract

Although considerable progress has been made in characterising the 5-HT\textsubscript{1A} receptor using agonists, partial agonists or non-selective antagonists, further studies of 5-HT\textsubscript{1A} receptor partial agonists have been hindered by the lack of highly selective antagonists. The term 'silent' antagonist has been used for such compounds in order to distinguish them unequivocally from several 5-HT\textsubscript{1A} receptor partial agonists which were initially designated 'antagonists'. In this report we provide a comprehensive review of the biochemical, pharmacological and behavioural properties of the first potent, selective and silent 5-HT\textsubscript{1A} receptor antagonist, WAY-100635 (N-{2-[4-(2-methoxyphenyl)--piperaziny]ethy}-N-(2-pyridiny)cychexanecarbxamide trihydrochloride). WAY-100635 had an IC\textsubscript{50} (displacement of specific [\textsuperscript{3H}]8-OH-DPAT binding to 5-HT\textsubscript{1A} receptors in the rat hippocampus) of 1.35 nM and was >100-fold selective for the 5-HT\textsubscript{1A} site relative to a range of other CNS receptors. [\textsuperscript{3H}]WAY-100635 was also characterised as the first 5-HT\textsubscript{1A} antagonist radioligand, displaying the same regional distribution of binding sites as [\textsuperscript{3H}]8-OH-DPAT in rat brain. As would be expected for the binding of an antagonist to a G-protein-coupled receptor, the B\textsubscript{max} of [\textsuperscript{3H}]WAY-100635 specific binding was consistently 50–60% greater than that of the agonist radioligand, [\textsuperscript{3H}]8-OH-DPAT. Mn\textsuperscript{2+}, but not guanine nucleotides, inhibited [\textsuperscript{3H}]WAY-100635-specific binding. [\textsuperscript{3H}]WAY-100635 was also shown to bind selectively to brain 5-HT\textsubscript{1A} receptors in vivo, following intravenous administration to mice. In vitro electrophysiological studies demonstrated that WAY-100635 had no 5-HT\textsubscript{1A} receptor agonist actions, but dose-dependently blocked the effects of agonists at both the postsynaptic 5-HT\textsubscript{1A} receptor in the CA1 region of the hippocampus, and the somatodendritic 5-HT\textsubscript{1A} receptor located on dorsal raphe 5-HT neurones. In vivo, WAY-100635 also dose-dependently blocked the ability of 8-OH-DPAT to inhibit the firing of dorsal raphe 5-HT neurones, and to induce the '5-HT syndrome', hypothermia, hyperphagia and to elevate plasma ACTH levels. In the mouse light/dark box anxiety model, WAY-100635 also dose-dependently blocked the ability of 8-OH-DPAT to inhibit the firing of dorsal raphe 5-HT neurones, and to induce the '5-HT syndrome', hypnoteremia, hyperphagia and to elevate plasma ACTH levels. In the mouse light/dark box anxiety model, WAY-100635 induced anxiolytic-like effects. WAY-100635 had no intrinsic effect on cognition in the delayed-matching-to-position model of short-term memory in the rat, but reversed the disruptive effects of 8-OH-DPAT on motor/motivational performance. These data clearly demonstrate that WAY-100635 is the first potent, selective and silent 5-HT\textsubscript{1A} receptor antagonist. Furthermore, [\textsuperscript{3H}]WAY-100635 is the first antagonist radioligand to become available for 5-HT\textsubscript{1A} receptor binding studies both in vitro and in vivo. The positive effects of WAY-100635 in an anxiety model also indicate that a postsynaptic 5-HT\textsubscript{1A} receptor antagonist action may contribute to the anxiolytic properties of 5-HT\textsubscript{1A} receptor partial agonists.

Keywords: WAY-100635; 5-HT\textsubscript{1A} receptor; Silent antagonist; Radioligand; Binding; Behaviour; ACTH; Anxiety; Cognition

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1. Introduction

Of the various classes of serotonin (5-hydroxytryptamine, 5-HT) receptor identified to date, the 5-HT1A subtype has been the subject of most attention since the synthesis and characterization of the first selective ligand, 8-hydroxy-2-(di-n-propylamino)tetrailin (8-OH-DPAT), by Hjorth et al. [28]. The subsequent availability of an increasing number of potent and selective agonists has led to the demonstration of an involvement of 5-HT1A receptors in 5-HT-mediated brain function and behaviour [29]. Thus, 5-HT1A receptor agonist agonists appear to possess antidepressant- and/or anxiolytic-like properties, and one, buspirone, is presently used for the therapeutic treatment of anxiety and depression [13,46]. However, buspirone acts as a partial agonist at the 5-HT1A receptor and it is unclear whether its anxiolytic properties are due to a pre-synaptic agonist action [12], a post-synaptic antagonist action, or a combination of both [13]. The development of silent 5-HT1A receptor antagonists provides an opportunity to assess the importance of pre- and post-synaptic 5-HT1A receptors in anxiety disorders ([15], see below).

5-HT1A receptors may also be involved in cognitive processes and it has been proposed by Bowen et al. [5] that 5-HT1A receptor antagonists may be beneficial in the treatment of the cognitive deficits in Alzheimer’s disease. Thus, a loss of corticocortical glutamatergic neurones may contribute to the early cognitive impairments observed in Alzheimer’s disease. The activity of the remaining glutamatergic cells could potentially be enhanced by administering 5-HT1A receptor antagonists which will block the hyperpolarizing action of endogenous 5-HT that is mediated by 5-HT1A receptors. Consistent with this hypothesis, administration of the 5-HT1A receptor agonist, 8-OH-DPAT, has been reported to impair learning and memory performance in the rat (e.g., [7,30]).

The development of antagonists that act selectively at 5-HT1A receptors has been slow and there have been many 'false dawns'. Until recently, most of the drugs described as 5-HT1A antagonist antagonists have lacked potency and/or selectivity. This is notably the case for some β-adrenoceptor antagonists such as (-)-propranolol, (-)-pindolol, (-)-penbutolol and (-)-tertanolol, which have relatively high potency as 5-HT1A receptor blockers [29], but, obviously, are not selective, since they were first characterized as blockers of β-adrenoceptors. In addition, most of the postulated antagonists which have recently been synthesised have proved (like buspirone) to be partial agonists at postsynaptic 5-HT1A receptors and also act as full agonists at the somatodendritic 5-HT1A autoreceptors located in the raphe nuclei (see [15]). Significant progress was achieved during the last two years with the characterisation of (S)WAY-100135 (N-tert-butyl-3,4-(2-methoxy-phenyl)piperazin-1-yl-2-phenylpropanamide) which selectively prevents the responses induced by the stimulation of both postsynaptic and somatodendritic 5-HT1A receptors. However, the potency of this antagonist is relatively low (IC50 = 15 nM against the specific binding of 1 nM [3H]8-OH-DPAT to hippocampal 5-HT1A receptors [16], and some of its effects in vivo have been ascribed to the blockade of x1-adrenoceptors. In this paper we describe the properties of a novel antagonist, WAY-100635 (N-[2-[(2-methoxyphenyl)-1-piperazinyl]ethyl]-N-(2-pyridinyl)cyclohexane-carboxamide), with a much higher potency and selectivity for 5-HT1A receptors than WAY-100135 [17]. WAY-100635 is demonstrated to be a potent antagonist of the behavioural and physiological effects of the 5-HT1A receptor agonist 8-OH-DPAT in rodents and also to possess anxiolytic properties. Studies with the tritiated derivative of WAY-100635 show that this compound is a highly selective radioligand which can be used for in vitro as well as in vivo labelling of 5-HT1A receptors. Additional in vivo binding studies were also recently described using [11C]WAY-100635 [43]. These were the first reported in vivo binding studies in animals using a positron-emitting 5-HT1A antagonist radioligand, as a prelude to the first positron emission tomography (PET) imaging studies of central 5-HT1A receptors in humans [44].

2. Materials and methods

2.1. Radioligand binding experiments

2.1.1. Brain membrane studies in vitro

Adult male Sprague-Dawley rats (250–300 g body weight) were killed by decapitation, and their brains rapidly removed at 4°C and dissected [19]. Tissues (in most cases, hippocampi) were homogenized in 40 vol (v/w) of ice-cold 50 mM Tris-HCl (pH 7.4 at 23°C) using a Polytron disrupter (type PT10 OD), and homogenates were centrifuged at 40,000 x 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. The sedimented material was then gently homogenized in 40 vol Tris-HCl buffer and incubated at 37°C for 10 min to remove endogenous 5-HT [40]. Membranes were then collected by centrifugation and washed three times by 'resuspension-centrifugation' as before. The final pellet was suspended in 10 vol 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.

Binding assays with [3H]WAY-100635, [3H]8-OH-DPAT or [3H]5-HT were as described in detail elsewhere [16,20,25,40]. In brief, aliquots of membrane suspensions (50 μl, corresponding to ~0.25 mg mem-
brane proteins) were mixed with 50 mM Tris-HCl, pH 7.4, containing $[^{3}H]$WAY-100635 (0.03–1.4 nM), $[^{3}H]$8-OH-DPAT (0.25–5.0 nM) or $[^{3}H]$5-HT (0.5–6.0 nM, plus 10 µM pargyline, 5.7 mM ascorbic acid and 4 mM CaCl$_2$), 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, 1 µM 5-HT or 0.3 µM 8-OH-DPAT for the determination of the non-specific binding of $[^{3}H]$WAY-100635, $[^{3}H]$8-OH-DPAT or $[^{3}H]$5-HT, respectively. All samples were incubated for 60 min at 25°C, and incubation was stopped by rapid filtration through Whatman GF/B filters (which had been pre-soaked in 0.5% polyethylenimine for 30 min in the case of $[^{3}H]$WAY-100635 binding assays).

All assays were performed in triplicate. Saturation and inhibition curves were analyzed by computer-assisted non-linear regression analysis using GraphPad and InPlot4 programmes. The equation of Cheng and Prusoff [8] was used for the calculation of $K_i$ values from IC$_{50}$ values.

2.1.2. Autoradiography

Rats were decapitated, and the brain was rapidly removed, frozen in isopentane cooled with solid CO$_2$ at −30°C, and stored at −80°C for 1 week. Coronal and horizontal sections (20 µm thick) were cut at −20°C, mounted on glass slides (2.5 x 7.5 cm) coated with a solution of 1% gelatin containing 0.05% chromic potassium sulphate, and stored at −20°C for less than 2 weeks. For the labelling procedure, sections were first equilibrated at room temperature and then pre-incubated at 25°C for 30 min in 0.17 M Tris-HCl, pH 7.6 [48]. 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 x 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 1 month at 4°C. Autoradiograms were developed in Kodal Microdol (10 min at 20°C).

2.1.3. In vivo labelling with $[^{3}H]$WAY-100635

Experiments were performed on male CD1 mice (25–30 g body weight, Centre d’Elevage R. Janvier). Each mouse was gently introduced and maintained in a Plexiglas cylinder, with the tail protruding. The tail was dipped in water at 45°C for ~5 s in order to induce a vasodilatation, and 250 µl of a $[^{3}H]$WAY-100635 solution in 0.9% NaCl (30.4 µCi/ml) was slowly injected over 5 s into a caudal vein. Animals were returned to individual cages for 1 h and then killed by decapitation. Blood was collected from the trunk vessels, and the brain and peripheral organs (heart, liver, lung, kidney, spleen, intestine) were removed. The brain was then dissected on an ice-cold plate, and the dissected structures, blood and peripheral tissues were homogenized in 10–20 vol (v/w) of distilled water. Aliquots (50–100 µl) of homogenates were mixed with 4 ml of Aquasol scintillation liquid (New England Nuclear, Boston, MA, USA) for radioactivity counting.

2.2. Electrophysiological experiments

2.2.1. In vitro recording of the electrical activity of serotoninergic neurones in the dorsal raphe nucleus

These experiments were performed on young (~100 g body weight) Sprague-Dawley male rats (Centre dʼElevage R. Janvier, 53940 Le Genest-St Isle, France) because brain tissues from these animals are much more resistant than those from adults to the transient anoxic conditions that occur during the preparation of the slices [11].

Animals were decapitated and their brains rapidly removed and placed in an ice-cold Krebs’ solution bubbled continuously with an O$_2$/CO$_2$ mixture (95:5%). A block of tissue containing the dorsal raphe nucleus (DRN) was cut into frontal sections (0.3 mm thick, using a vibratome) while immersed in Krebs’ buffer at 4°C. The entire procedure took 6 to 10 min. After sectioning, the slices containing the DRN were allowed to recover for 1 h at room temperature in an artificial cerebrospinal fluid (ACSF) of the following composition (in mM): NaCl: 126, KCl: 3.5, NaH$_2$PO$_4$: 1.2, MgCl$_2$: 1.3, CaCl$_2$: 2.0, glucose: 11, and NaHCO$_3$: 25, adjusted to pH 7.3 by a continuous bubbling of O$_2$/CO$_2$.

For each experiment, a single slice was transferred to a recording chamber [24] through which flowed ACSF (2 ml/min) at 35°C. Extracellular recordings were then made with a single barrel micropipette (15 MΩ) filled with 2 M NaCl. The micropipette was implanted into the DRN area, which could be easily located in the midline of the slice, between the medial longitudinal fasciculi extending dorsally towards the aqueduct. In all cases, serotoninergic neurones in the brain slices were induced to fire by adding 3 µM phenylephrine (α$_2$-adrenoceptor agonist) to the ACSF throughout the superfusion experiments (see [47]).

When a cell was recorded, it was identified on line as serotoninergic using the following criteria: biphasic action potentials of 2 to 3 ms duration and a slow (0.5–2.0 spikes/s) and regular pattern of discharge [47]. Baseline activity was recorded for 10 min before the infusion of drugs into the chamber via a three-way tap system. Complete exchange of fluids occurred within 2 min of the arrival of a new solution in the chamber, and we observed that a 2.5 min infusion of a drug was
enough to induce maximal effects. The electric signals were fed into a high input impedance amplifier, an oscilloscope and an electronic ratemeter triggered by individual neuronal spikes. The integrated firing rate was computed and recorded graphically as consecutive 10 s samples. The effects of each drug were evaluated by comparing the mean discharge frequency during 2 min before their addition to the superfusing ACSF, and 2–3 min after the end of the drug infusion, when the resulting changes in firing frequency reached their maximal amplitude.

2.2.2. Intracellular recording of hippocampal pyramidal cells

Young male Sprague-Dawley rats (100–200 g body weight) were decapitated and the hippocampi rapidly removed then immersed in ice-cold ACSF (as above) continuously bubbled with O₂/CO₂. Slices (0.4 mm thick) were cut with a Vibratome (1000 Lancer, UK) from the middle of the hippocampus and kept in oxygenated ACSF for at least 1 h at room temperature (20–23°C). A single slice was then placed on a nylon mesh and completely immersed in a small chamber to be superfused at a constant flow rate of 2–3 ml/min with oxygenated ACSF at 30–32°C. Drugs were administered through a three-way tap system, and complete exchange of the chamber volume occurred within 1 min.

CA1 pyramidal neurones were recorded in current clamp mode with electrodes filled with either 2 M potassium methylsulphate (50–100 MΩ) or 3 M KCl (35–50 MΩ) [41]. Electrical signals were amplified with an Axoclamp 2A (Axon Instruments, Foster City, CA, USA), displayed on an oscilloscope and chart recorder (Easy-Graf, Gould Electronics, Cleveland, OH, USA) and stored on a digital tape (DTR 1200, Biologic, France; 48 KHz sampling frequency) and on a computer using a Digidata 1200 interface and a pClamp6 program (Axon Instruments) for off-line measurements (sampling frequency 3–50 KHz). Only neurones with stable resting membrane potential (range: 59–80 mV) and input resistance (range: 27–95 Megohms) throughout the recording were included in the analysis. When cells appeared to have reached a stable membrane potential, pulses of hyperpolarizing current (200/500 pA, 0.4 s duration) were delivered through the recording electrode to monitor changes in input resistance during drug application.

2.2.3. Dorsal raphe 5-HT neuronal firing in the anaesthetised rat

Recording procedures were essentially those of Haigler and Aghajanian [23]. Male Sprague-Dawley rats (200–300 g; Charles River) were initially anaesthetized with chloral hydrate (400 mg/kg i.p.) and given subsequent i.v. maintenance injections as needed. The animals were placed in a stereotaxic frame in the orientation of König and Klippel [34] after jugular and lateral tail vein cannulation (for additional anaesthetic and drug administration, respectively). Throughout the experiment, body temperature was maintained at 36.5–37.5°C by a heating pad placed beneath the animal. Glass micropipettes were filled with 2% pontamine sky blue in 0.5 M sodium acetate, and tips were broken under microscopic control to a diameter of 1–2 μm, yielding an impedance of 4–6 MΩ. The micropipette was then lowered through a burr hole in the skull to a depth of 1.0 mm above the dorsal raphe nucleus (vertical coordinate 4.5 mm, on the midline and −0.6 mm from interaural zero). The recording electrode was further advanced into the recording site by means of a hydraulic microdrive. Neuronal action potentials were passed through a high input-impedance amplifier and monitored on an oscilloscope and audiomonitor. Action potentials were discriminated from background noise and used to trigger an output which was counted and recorded by an online computer system and plotted on a polygraph. Neurons in the dorsal raphe nucleus were identified from their characteristic waveform and slow rhythmic activity [23]. Once these criteria were satisfied, spontaneous activity was monitored for at least 3–5 min to establish baseline firing rate. Drugs were then administered i.v. via the lateral tail vein cannula. Only one cell was studied in each rat to avoid residual drug effects. ID₅₀ values (dose required to reduce firing to 50% of baseline; n = 5) were calculated using non-linear inverse regression analysis. At the termination of the experiment, pontamine sky blue dye was deposited for 20 min by a 10 μA anodal current for histological confirmation of the recording site and reconstruction of the electrode track. Each animal was then perfused intracardially with Mirsky’s fixative, after which the brain was removed, sectioned at 64 μm, and counterstained with neutral red. Data from recording sites not contained within the histological boundaries of the dorsal raphe nucleus were discarded. The effects of WAY-100635 alone on firing rates were statistically analysed using a repeated measures model in which drug effects were compared with (pre-drug) baseline data by a t-test in conjunction with Satterthwaite’s formula to determine degrees of freedom. ID₅₀ values were calculated by parallel logistic regression and statistically compared using a weighted analysis of variance.

2.3. Neurohormonal and behavioural studies

2.3.1. 5-HT syndrome

Male Sprague-Dawley rats (250–400 g) were used in these studies which were carried out as previously described [16]. To assess agonist activity, drugs were administered intravenously up to a dose of 10 mg/kg and the animals placed in circular Perspex observation chambers for behavioural observations. For antagonist
evaluation, groups of at least 10 animals received vehicle or test compound subcutaneously 30 min before the intravenous administration of 8-OH-DPAT. An ED_{50} for 8-OH-DPAT to induce a 5-HT behavioural syndrome (forepaw treading, flat posture, hyperlocomotion) was determined in each treatment group using a sequential, up/down technique. The 5-HT syndrome was assessed by an observer 'blind' to drug pre-treatment, as present (definite syndrome response) or absent (equivocal or no syndrome response) during the 5 min period immediately following the intravenous administration of 8-OH-DPAT. ED_{50} values (with 95% confidence limits) were calculated from these quantal responses by a modified probit analysis. ED_{50} values were considered to be significantly different if the confidence limits did not overlap.

2.3.2. Hypothermia

Female T/O mice (20–27 g; Tuck; n = 8 per treatment) were housed in groups of eight at an ambient temperature of 20±0.5°C for at least 2 h before measurement of body temperature and drug administration. Body temperature was measured with a thermistor probe (connected to an electronic thermometer) inserted 2 cm into the rectum. Temperatures were measured immediately before the administration of test compounds, again immediately before 8-OH-DPAT administration, and 15 and 30 min following 8-OH-DPAT administration. The hypothermic response to 8-OH-DPAT was measured as the maximum decrease in body temperature recorded in this latter period. A standard 8-OH-DPAT challenge dose of 0.5 mg/kg s.c. was used, since this has been established to induce a sub-maximal response under these conditions [4]. Vehicle or drug pre-treatments were administered subcutaneously 20 min before 8-OH-DPAT. Male Sprague-Dawley rats (200–240 g; n = 8 per treatment) were transferred to the experimental laboratory between 11.00–12.00 h where they were housed in groups of four for at least 2 h before body temperature measurement and drug administration. Body temperatures were measured during light manual restraint of each animal, using a thermistor probe (1.5 mm in diameter), inserted to a standard depth of 4 cm into the oesophagus. Test compounds or vehicle were administered s.c. 20 min before the s.c. injection of a standard challenge dose of 0.25 mg/kg of 8-OH-DPAT. Other methodological details were as described for mice. Data were analysed initially using one-way analysis of variance (ANOVA) before comparing data from pairs of treatment groups using Duncan’s new multiple range test. ED_{50} values (doses of the test compounds to attenuate the hypothermic response to 8-OH-DPAT by 50%) and 95% confidence limits were calculated by Litchfield-Wilcoxon probit analysis.

2.3.3. Hyperphagia

These studies were performed using the method described by Hartley and Fletcher [27]. Briefly, male Sprague-Dawley rats (350–420 g) were housed and tested in individual metal grid cages. Food hoppers containing food pellets (CRM, SDS, Cambridge) were weighed and replaced immediately following drug or vehicle administration. The food hoppers were reweighed at 3 h to allow calculation of the weight of food consumed. Drugs were administered subcutaneously; WAY-100635 being administered immediately before 8-OH-DPAT or clonidine.

2.3.4. ACTH studies

Male adult Sprague-Dawley rats (220–620 g) were anaesthetised with halothane (5%-induction, 2%-maintenance in oxygen), prepared with indwelling cannulae in the left jugular vein and left common carotid artery and allowed to recover. On the day following surgery, the rats had extensions appended to their cannulae so that they could move freely in their home cages throughout the experiment. All experiments were initiated between 09:00 and 10:00 h. Animals were allowed food and water ad libitum at all times during the experiment. To determine the possible intrinsic effects of WAY-100635, a pre-injection arterial blood sample was taken (0.7 ml into EDTA) at −15 min before injection of WAY-100635 or vehicle (0.9%, w/v, saline, 1 ml/kg i.v.) at 0 min. Post-injection samples were taken at 10, 20, 30 and 40 min. A dose response curve to 8-OH-DPAT was constructed using the same procedure. To examine the effect of WAY-100635 on the 8-OH-DPAT-induced response, a pre-injection sample was taken at −30 min, and vehicle or WAY-100635 (1, 3, 10, 30 or 100 µg/kg, i.v.) injected at −15 min. 8-OH-DPAT (100 µg/kg, i.v.) or vehicle was injected at 0 min, and a single post-injection blood sample taken 10 min later. Circulating volume was maintained by replacing the blood removed with saline 0.9% (w/v). Blood samples were centrifuged at 3000 rpm for 15 min at 4°C and the plasma stored at −20°C until assayed in triplicate for ACTH using the Incast RIA kit. In experiments that examined intrinsic effects of 8-OH-DPAT and WAY-100635 changes versus pre-injection levels were calculated and resultant data were analysed by 2 factor ANOVA followed by Fisher’s PLSD post hoc test between treatments. In the experiment examining the effects of WAY-100635 on 8-OH-DPAT-induced changes in ACTH, 1 factor ANOVA followed by Fisher’s Protected Least Significant Difference test was used on the change data (n ≥ 5).

2.3.5. Mouse light/dark box studies

These experiments were carried out using a method based on that described in Bill et al. [3]. Female T/O mice (22–30 g; Tuck) were transferred to the laboratory.
at 09:00 h on the day of an experiment, where they were weighed and allocated to groups of eight or nine. The laboratory was dimly illuminated (50 lux); the only illumination being that of the lighting associated with the light/dark box apparatus. Each experiment was performed between 11:00 and 17:00 h.

The design of the two-compartment open field (light/dark box) was based on that of Crawley [10]. The apparatus comprised a rectangular open-topped box (81 x 36 x 27 cm high) divided into a smaller (27 cm long) and larger (54 cm long) compartment by a partition. The smaller compartment was painted black and illuminated by a dim red light, whereas the larger compartment was painted white and brightly illuminated by a 60 W spotlight located approximately 40 cm above the floor of the box (800 lux illumination). Free access between the two compartments was allowed by an opening (7.5 x 7.5 cm) in the centre of the partition at floor level.

The apparatus was semi-automated in that a system of infra-red beams and detectors monitored: (a) the latency to enter the dark compartment following initial placement in the light compartment; (b) the number of transitions between compartments; and (c) the horizontal motor activity of the mouse in the light or dark compartments. Rearing was monitored by direct observation, either live, or from video-tape recordings. A rear was defined as the raising of both fore-paws clear of the floor, either unsupported or supported by a side-wall. Following drug or vehicle administration, animals were placed individually in the centre of the light compartment (facing away from the partition opening) and allowed to explore the apparatus, undisturbed, for a period of 5 min. Data were analysed by one-way ANOVA and post hoc Newman Keuls tests.

2.3.6. Cognition studies

It is often unclear whether the detrimental effects of 8-OH-DPAT in cognitive tests are due to an action on cognitive processes or to a non-specific motor or motivational effect. The present study addressed this issue and examined the effects of WAY-100635 on the 8-OH-DPAT impairment of performance in the delayed-matching-to-position (DMTP) test of short-term memory in the rat [14].

Male Sprague-Dawley rats maintained at 85% of free-feeding body weight were used. Training and testing were carried out in 8 Camden operant chambers with two retractable levers and a centrally located food magazine, controlled by an Acorn A5000 micro-computer with Arachnid software. A dividing partition fitted to either side of the food magazine prevented mediating behaviour. At the start of a 70 min session, one of two levers was inserted into the box. Following a response this 'sample' lever was retracted. The rat was then required to press the magazine flap on a Fixed Interval (FI) schedule of 0, 4, 8 or 16 s, or 0, 8, 16 or 32 s, before both levers were inserted into the box. A correct response was scored, and a food pellet delivered, if the original sample lever was pressed. An incorrect response initiated a 10 s time-out (TO) period when the alternative lever was pressed. A 30 s intertrial interval followed delivery of food reward or completion of the TO period. Cognitive performance was assessed by percentage of correct choices. Latency to complete trials and number of trials completed measured motor/motivational effects.

WAY-100635 (0.03–3.0 mg/kg) was administered 30 min prior to behavioural testing and 8-OH-DPAT (0.003–0.1 mg/kg) was administered 3 min prior to testing. Data were analysed by one- and two-way ANOVA and post hoc Newman Keuls tests.

2.4. Chemicals

WAY-100635 (N-[2-[(4-(2-methoxyphenyl)-1-piperazinyl)]ethyl] N-[2-(2-pyridinyl)cyclohexanecarboxamide trihydrochloride) was synthesised in the Chemistry Department of Wyeth Research UK [O-methyl-3H]WAY-100635 (69 Ci/mmol) was synthesised by Amersham International (Buckinghamshire, UK). [3H]8-OH-DPAT (110 Ci/mmol) was from the Service des Molécules Marquées of CEA (91191 Gil-sur-Yvette, France), and [3H]5-HT (11 Ci/mmol) was from Amersham International.

Other compounds were: Ipsapirone (Tropovenere, Cologne, Germany), (-)tertatolol, S 14506 and S 20499 (Servier, Courbevoie, France), SDZ 216-525, mesulergine and clozapine (Sandoz, Basel, Switzerland), BMY 7378 and buspirone (Bristol-Myers Squibb, Wallingford, CT, USA), lesopitron (Esteve, Barcelona, Spain), sumatriptan (Gliaxo, Hertfordshire, UK). All other compounds were from Research Biochem. (Natick, MA, USA) or Sigma (St Louis, MO, USA).

3. Results

3.1. In vitro binding studies

The IC50 value for WAY-100635 at 5-HT1A sites was 1.35 ± 0.44 nM. The lowest IC50 value for WAY-100635 at other sites tested was 230 ± 30 nM at the x1-adrenoceptor site (values are mean ± s.e.m of three displacement curves). WAY-100635 was >100-fold selective for 5-HT1A sites relative to the following binding sites: 5-HT1B, 5-HT1D, 5-HT2A, 5-HT2C, 5-HT3, 5-HT4, x2 and x3-adrenoceptors, dopamine (D1, D2, D4), GABAa, GABAβ, histamine (H1, H2, H3), muscarinic (M1, M2, M3).
M₃), nicotinic, NMDA, kainate, quisqualate, central benzodiazepine, opiate (mu, delta, kappa), adenosine (A₁, A₂), reuptake sites (dopamine, noradrenaline, 5-HT, GABA) and ion channels (calcium, N, T and L; chloride, sodium (sites 1 and 2) and potassium (ATP; voltage-dependent; apamin-sensitive).

Among a large series of drugs, only the 5-HT₁₄ ligands such as SDZ 216-525 [35], 8-OH-DPAT, S 14506 [9], S 20499 [32], ipsapirone [26] and BMY 7378 [49] were able to inhibit the specific binding of [³H]WAY-100635 to rat hippocampal membranes at nanomolar concentrations (Fig. 1). In contrast, drugs acting at other receptor types such as the α-adrenoceptor antagonist phenotolamine, the 5-HT₁₆ agonist sumatriptan [29] and the 5-HT₂ antagonist ketanserin [29] were active only at μM concentrations in preventing the specific binding of [³H]WAY-100635 to rat hippocampal membranes (Fig. 1). In all cases, the inhibition curves yielded an apparent Hill coefficient not significantly different from unity. In addition, the non-specific binding which remained in the presence of saturating concentrations of these various drugs was similar, and corresponded to less than 10% of total binding when assays were performed with 1.0 nM [³H]WAY-100635. A significant positive correlation (r = 0.96) was found between the respective potencies of 16 different drugs to inhibit the specific binding of [³H]WAY-100635 on the one hand, and that of [³H]8-OH-DPAT on the other hand, to the same hippocampal membrane preparations (Fig. 1).

Further comparison between the specific binding of [³H]WAY-100635 and that of the selective 5-HT₁₄ receptor radioligand, [³H]8-OH-DPAT, revealed both similarities and differences. In particular, the autoradiograms shown in Fig. 2 clearly show that the distribution of [³H]WAY-100635 binding in the rat brain superimposes over that of [³H]8-OH-DPAT binding. The highest levels of labelling were found in the septum, hippocampus (notably the dentate gyrus and CA1 area of Ammon's horn), entorhinal cortex and dorsal raphe nucleus. In contrast, the labelling with either radioligand in the striatum and the substantia nigra 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).

Saturation studies with the two radioligands revealed marked differences, as the affinity of the specific binding sites was approximately 6 times higher with [³H]WAY-100635 (Kᵣ = 0.10–0.12 nM) than with [³H]8-OH-DPAT (Kᵣ = 0.69–0.86 nM) as radioligand with membranes prepared from all regions examined (hippocampus, septum, anterior and posterior cortices). Furthermore, the Bₘₐₓ of [³H]WAY-100635 specific binding sites was also regularly 50–60% higher than that calculated for [³H]8-OH-DPAT specific binding sites in the same membrane preparations (see Fig. 3 for the hippocampus). In contrast, the Bₘₐₓ of [³H]8-OH-DPAT specific binding sites corresponded exactly to that found for the high affinity [³H]5-HT specific binding sites which could be saturated with 0.3 μM 8-OH-DPAT (Fig. 3).

In agreement with previous reports [25,26], the specific binding of [³H]8-OH-DPAT to rat hippocampal membranes could be inhibited in a concentration-dependent manner by GTP and its non-metabolizable analogue, GppNHp (Fig. 4). Conversely, Mn²⁺ enhanced this binding (Fig. 4). The sensitivity of [³H]WAY-100635 specific binding to these modulatory agents was completely different since the guanine nucleotides were slightly stimulatory whereas Mn²⁺ inhibited this binding in a concentration-dependent manner (Fig. 4). Binding assays with [³H]WAY-100635 showed that the inhibition curve by an agonist such as 8-OH-DPAT, 5-HT or 5-methoxy-N,N-dimethyltryptamine was shifted to the right in the presence of 0.1 mM GppNHp, whereas that by an antagonist such as WAY-100635 itself, spiperone or (−)-tertatolol was unaffected by the nucleotide (not shown).

3.2. In vivo labelling

One hour after the i.v. injection of [³H]WAY-100635, marked regional differences were noted in the accumulation of tritium in the brain and in the peripheral tissues of mice. In peripheral organs, radioactivity was concentrated in the intestine, the kidney and the liver. The lung
also exhibited some capacity to accumulate tritium, whereas the levels of radioactivity in the heart and spleen were similar to those remaining in the blood. Interestingly, pre-treatment with the 5-HT1A antagonist \((S)\)WAY-100135 \[16\] at a dose, 10 mg/kg s.c., high enough to prevent the responses normally elicited by agonists acting at both postsynaptic somatodendritic 5-HT1A receptors, did not modify the absolute levels of radioactivity accumulated in the various peripheral organs studied (Fig. 5B).

As shown in Fig. 5A, the brain area with the highest levels of radioactivity recovered 1 h after the i.v. injection of \([3H]\)WAY-100635 in mice was the hippocampus. Then the accumulation of tritium decreased in the following order: posterior cortex = anterior cortex > brain stem = hypothalamus > striatum > cerebellum. In contrast to the results in the peripheral organs, pre-treatment with 10 mg/kg s.c. of \((S)\)WAY-100135 dramatically reduced the radioactivity accumulated in all the brain regions except the cerebellum.
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Fig. 3. Scatchard plots of the specific binding of [3H]WAY-100635, [3H]8-OH-DPAT and [3H]5-HT to rat hippocampal membranes. The three binding assays were performed on the same membrane preparations. The non-specific binding of [3H]WAY-100635, [3H]8-OH-DPAT and [3H]5-HT was determined with 1 μM 8-OH-DPAT, 1 μM 5-HT and 0.3 μM 8-OH-DPAT, respectively. 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.

Fig. 4. Differential effects of guanine nucleotides and Mn2+ on the specific binding of [3H]8-OH-DPAT and [3H]WAY-100635 to rat hippocampal membranes. Binding assays were performed on the same membrane preparations with 1.0 nM [3H]8-OH-DPAT or 0.3 nM [3H]WAY-100635 in the absence or presence of various concentrations (abscissa) of GTP, GppNHp or MnCl2. 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.

Interestingly, the levels of radioactivity accumulated were not significantly different in the 7 regions examined in mice pre-treated with (S)WAY-100135, and were equal to those found in the cerebellum of mice pre-treated with saline (Fig. 5).

3.3. Electrophysiological evidence of the blockade by WAY-100635 of postsynaptic and somatodendritic 5-HT1A receptors

3.3.1. In vitro studies in brain slices

Intracellular recording of CA1 pyramidal cells in the medial zone of the hippocampus showed that superfusion of slices with 30 μM 5-HT produced a hyperpolarization associated with a decrease of membrane resistance. These effects were evident throughout the period of 5-HT application, and were reversible, as shown by the recovery of basal membrane potential (~ - 70 mV) and resistance within 2 min after the removal of 5-HT from the superfusing ACSF (Fig. 6). Tissue superfusion with 10 nM WAY-100635 alone affected neither the potential nor the input resistance of the membrane of CA1 pyramidal cells. However, during the superfusion of slices with WAY-100635, further exposure of cells to 30 μM 5-HT failed to elicit any hyperpolarization. In fact, in the presence of both 10 nM WAY-100635 and 30 μM 5-HT, a slight depolarization was observed, without any concomitant alteration in membrane input resistance (Fig. 6).

Extracellular recording of the electrical activity of serotonergic neurones within the dorsal raphe nucleus showed that the addition of various 5-HT1A receptor agonists, 5-HT (3 μM), lesopitron (100 nM) and 8-OH-DPAT (10–30 nM), to the superfusing ACSF inhibited the firing frequency of the cells (Fig. 7). Whereas the effect of 5-HT was rapidly reversible, that of lesopitron persisted for ~ 5 min after its removal from the superfusing fluid, and that of 8-OH-DPAT was apparent 20 min or more after cessation of the treatment (Fig. 7). As shown in Fig. 7, the inhibitory effects of 3 μM 5-HT, 100 nM lesopitron and 10 nM 8-OH-DPAT were completely prevented upon tissue superfusion with 10 nM WAY-100635. With the highest concentration of 8-OH-DPAT tested, 30 nM, some reduction in the firing frequency was still observed in spite of the presence of 10 nM WAY-100635. However, this effect was considerably less (~20%) than that observed with the agonist alone (100% blockade for ~ 10 min) (Fig. 7C).

Occasionally, the application of WAY-100635 alone produced some increase in the firing frequency of serotonergic DRN neurones (see, for instance, the integrated firing rate histogram in Fig. 7B). However, this effect was observed only in a limited proportion (~25%) of the recorded cells and was not concentration-dependent (not shown).

3.3.2. In vivo studies in the anaesthetised rat

The effects of WAY-100635 on the inhibition of DRN 5-HT neuronal firing induced by 8-OH-DPAT in the anaesthetised rat are shown in Fig. 8. At doses of 10 and 100 μg/kg, WAY-100635 blocked the inhibition of firing induced by 8-OH-DPAT. Importantly, the administra-
Fig. 5. Effects of pretreatment with (S)WAY-100135 on the accumulation of $^3$H in various brain regions (A), peripheral organs and blood (B) in mice injected with $[^3$H]WAY-100635. (S)WAY-100135 (10 mg/kg) or saline (0.2 ml 0.9% NaCl) was administered subcutaneously 20 min before the i.v. injection of $[^3$H]WAY-100635 (7.6 μCi in 0.25 ml of 0.9% NaCl per mouse), and animals were killed 60 min after the second injection. Tritium accumulated in various brain regions, blood and peripheral organs is expressed in dpm x 10^2/mg fresh tissue or ml (blood). Each bar is the mean ± S.E.M. of data obtained in 5 mice. *P<0.05 when compared to respective values in saline-treated mice.

Fig. 6. Prevention by WAY-100635 of 5-HT-induced hyperpolarization in a CA1 pyramidal cell. Upper trace: Continuous chart record of membrane potential showing the hyperpolarization and decrease of input membrane resistance elicited by the addition of 30 μM 5-HT (2 min, horizontal bar) to the superfusing ACSF. Downward deflections are electrotonic potentials produced by injection of negative current steps through the recording electrode to monitor the cell input resistance. Note that before, during, and after 5-HT application, sample electrotonic responses are displayed at faster chart speed to better show the effect of the indoleamine on input resistance. Middle trace: Same cell as for the upper trace. In the presence of WAY-100635 (10 nM, 21 min), the hyperpolarizing effect of 5-HT is antagonized, and the indoleamine elicited only a small depolarization, not accompanied by any measurable decrease in membrane input resistance. Lower trace: Continuous chart record of the constant current square pulses injected through the electrode (−500 pA, 400 ms, 0.11 Hz). Resting membrane potential: −71 mV. Potassium methylsulphate-filled electrode; Calibrations: 10 mV, 100 s or 1 s; 1 nA, 100 s or 1 s. Similar results were obtained in 5 independent experiments.

3.4. Physiological and behavioural studies

3.4.1. 8-OH-DPAT-induced 5-HT syndrome

Following intravenous administration up to a dose of 10 mg/kg WAY-100635 did not evoke any component of the 5-HT syndrome typically elicited by selective and non-selective 5-HT_1A receptor agonists. The ED_{50} values (with 95% confidence limits, in μg/kg i.v.) for 8-OH-DPAT to induce the behavioural syndrome in saline-pretreated animals and in animals pre-treated with 1, 3 or 10 μg/kg s.c. of WAY-100635 were, respectively: 50 (37–68), 58 (35–96), 100 (80–130)* and 220 (190–260)* [*P<0.05 relative to vehicle controls on the basis that 95% confidence limits do not overlap]. Fig. 9 summarises the results of three separate experiments examining the effects of a wide range of doses of WAY-100635 on the 8-OH-DPAT-induced 5-HT syndrome.

3.4.2. Hypothermia

WAY-100635 administered at doses up to 1 mg/kg s.c. had no intrinsic effect on body temperature in the mouse or the rat. However, WAY-100635 potently and dose-dependently antagonised the hypothermic response to 8-OH-DPAT in both species (Fig. 10). The ED_{50} value of WAY-100635 (dose required to reduce the hypothermic response by 50%) was 10 μg/kg s.c. in both the mouse and rat.
Fig. 7. Prevention by WAY-100635 of the inhibitory effects of 5-HT, lesopitron or 8-OH-DPAT on the electrical activity of DRN serotonergic neurones in vitro. Integrated firing rate (in spikes per 10 s) histograms of serotonergic DRN neurones recorded in brain stem slices and exposed for 2.5 min to either 3 μM 5-HT (A), 100 nM lesopitron (B), 10 or 30 nM 8-OH-DPAT (C). The same treatments were applied during superfusion of brain stem slices with 10 nM WAY-100635 for at least 20 min. Each 5-HT1A receptor agonist was added to the superfusing ACSF 10 min after starting tissue superfusion with WAY-100635, in the same cell for 5-HT (A) and lesopitron (B), in which washing of the effect of the agonist alone could be achieved rapidly. Experiments with 8-OH-DPAT (C) showed that the action of this agonist persisted for a longer period after its removal from the superfusing ACSF. In all cases, the inhibitory action of the agonists on the neuronal discharge could be prevented by WAY-100635. Similar data have been obtained in at least 4 cells for each pharmacological condition.

3.4.3. Hyperphagia
WAY-100635 alone had no significant effect on feeding but completely antagonised the hyperphagia induced by 8-OH-DPAT in free-feeding rats (Fig. 11a). This antagonism was shown to be specific to 5-HT1A receptor mediated hyperphagia, since WAY-100635 had no significant effect on the hyperphagic response to the α2-adrenoceptor agonist, clonidine (Fig. 11b).

3.4.4. ACTH studies
The basal plasma ACTH level was 41.0 ± 1.8 pg/ml. 8-OH-DPAT significantly increased ACTH levels at doses of 100 and 300 μg/kg with maximum increases of 551 and 546%, respectively, occurring 10 min post-injection. WAY-100635 had no intrinsic effect on plasma ACTH at doses up to 100 μg/kg but dose-dependently blocked the effect of 8-OH-DPAT. The minimum
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Fig. 8. Antagonism of 8-OH-DPAT-induced inhibition of dorsal raphe neuronal firing in the anaesthetised rat. Cumulative intravenous dose-response curves for the inhibition of neuronal firing by 8-OH-DPAT were determined in rats pretreated intravenously with saline (○) or WAY-100635 at doses of 1 (●), 10 (■) or 100 (●) µg/kg. Values are mean (+ s.e.m, represented by vertical bars; n = 6-8 rats) firing rates expressed as a percentage of the baseline firing rate recorded before administration of vehicle or drug.

Fig. 9. Antagonism of 8-OH-DPAT-induced 5-HT syndrome in the rat by WAY-100635. WAY-100635 (1-10 mg/kg, s.c.) was administered 30 min before the intravenous administration of 8-OH-DPAT. ED50 values (mg/kg, i.v.) for 8-OH-DPAT to induce the 5-HT syndrome in groups of ten animals were determined. The ED50 of 8-OH-DPAT in saline-pretreated controls is represented by the open symbols. Three separate experiments were performed, each with their own vehicle-pretreated control group, in order to encompass a wide dose range for WAY-100635. WAY-100635, at a dose of 0.003 mg/kg, s.c. or greater, significantly (P<0.05) increased the ED50 for 8-OH-DPAT to induce the 5-HT behavioural syndrome.

The effective dose was 10 µg/kg (39% inhibition); 30 and 100 µg/kg WAY-100635 caused 73 and 108% inhibition, respectively (Fig. 12).

3.4.5. Anxiety studies: mouse light/dark box

WAY-100635 mimicked the effects of standard anxiolytics in this anxiety model in that exploratory activity in the more aversive light compartment of the apparatus was significantly increased at doses which had no significant effect on dark compartment activity (Fig. 13).

Therefore, exploratory rearing in the light compartment was significantly increased at doses of 10 µg-3.0 mg/kg s.c., whereas dark compartment activity was only markedly affected (decreased) at the highest dose of 10 mg/kg s.c. The positive control used in this experiment (diazepam, 0.5 mg/kg, s.c.) also significantly increased light compartment rearing. Similar drug effects on locomotor activity in the two compartments were also seen (results not shown). Several selective 5-HT1A receptor partial agonists and antagonists were evaluated in the mouse light/dark box and as antagonists of 8-OH-DPAT-induced hypothermia in the mouse. There was a highly significant correlation (r=0.89) between functional antagonist potency in preventing 8-OH-DPAT-induced hypothermia and anxiolytic activity for these ligands (Fig. 14).

3.4.6. Cognition studies: delayed matching to position in rats

8-OH-DPAT (3-100 µg/kg) did not impair percentage of correct choices in the DMTP task and at a dose 0.1 mg/kg induced a 50-67% reduction in number of trials completed, indicating a disruption of motor/motivational performance. WAY-100635 had no intrinsic effect on cognition but reversed the disruptive effects of 8-OH-DPAT on motor/motivational performance (Fig. 15).

4. Discussion

In contrast to most of the drugs recently proposed as 5-HT1A receptor antagonists but which are in fact, at best, partial agonists (see [15]), WAY-100635 prevented the electrophysiological effects induced by 5-HT via the stimulation of 5-HT1A receptors both at the postsynaptic (i.e., in the hippocampus) and the somatodendritic level (i.e., in the dorsal raphe nucleus) in vitro and in vivo.

It has been clearly demonstrated that the hyperpolarization of CA1 pyramidal cells in the hippocampus by 5-HT is due to the stimulation of 5-HT1A receptors [1,2,41]. In the present studies, this effect was shown to be completely prevented by WAY-100635, as expected of a silent 5-HT1A receptor antagonist. Indeed, in the presence of WAY-100635, a slight depolarization of CA1 cells was observed, which may have resulted from the unmasking of an activation of 5-HT4 receptors by 5-HT [29].

Interestingly, WAY-100635 exerts an excitatory influence on some serotonergic neurones in the dorsal raphe nucleus, in addition to completely preventing the inhibition of their electrical discharge due to the stimulation of somatodendritic 5-HT1A autoreceptors. However, this excitatory effect was not observed with all cells recorded and was not concentration-dependent. We have previously reported that WAY-100635 increases the firing
of dorsal raphe neurones in freely moving cats and this effect is critically dependent upon the arousal state of the animal [18]. Thus, it appears that WAY-100635 can prevent a tonic inhibitory influence of endogenous 5-HT acting at 5-HT<sub>1A</sub> autoreceptors, or exert an intrinsic 'inverse agonist' action on serotonergic DRN neurones. However, binding studies under appropriate conditions for revealing potential inverse agonist properties [45] did not support the idea that WAY-100635 may be an inverse 5-HT<sub>1A</sub> agonist (Hamon et al., unpublished observations). Therefore, both at the postsynaptic level in the hippocampus, and the somatodendritic level in the dorsal raphe nucleus, the present data clearly demonstrate that WAY-100635 is a potent and silent antagonist at 5-HT<sub>1A</sub> receptors.

Binding studies confirmed that WAY-100635 interacts selectively with the 5-HT<sub>1A</sub> receptor. The IC<sub>50</sub> of WAY-100635 for displacement of specific [<sup>3</sup>H]-8-OH-DPAT binding to rat hippocampal membranes was less than 100-fold that for displacement of specific radioligand binding to other receptor, uptake and ion channel sites. It has also been demonstrated (Hamblin, unpublished observations) that WAY-100635, unlike several other 5-HT<sub>1A</sub> receptor ligands [38], does not have significant affinity for the 5-HT<sub>7</sub> site. Further binding studies using [<sup>3</sup>H]WAY-100635 as radioligand confirmed the selective binding of this molecule to 5-HT<sub>1A</sub> sites. Firstly, the pharmacological profile of [<sup>3</sup>H]WAY-100635 specific binding sites corresponded to that of 5-HT<sub>1A</sub> receptors specifically labelled by [<sup>3</sup>H]8-OH-DPAT in rat hippocampal membranes. Secondly, the regional distribution of [<sup>3</sup>H]WAY-100635 specific binding in the rat brain superimposed exactly over that of 5-HT<sub>1A</sub> receptors labelled with [<sup>3</sup>H]8-OH-DPAT. Nevertheless, differences also exist between the specific binding of these two radioligands. In particular, the specific binding of [<sup>3</sup>H]WAY-100635 was not inhibited by GTP or GppNHp, in contrast to what was found for [<sup>3</sup>H]8-OH-DPAT specific binding. Such a difference was not unexpected, as it is well established that for receptors of the G-protein coupled superfamily, only the binding of agonists, but not that of antagonists, is inhibited by guanine nucleotides [33]. Therefore, the lack of inhibitory influence of GTP and GppNHp on the specific binding of [<sup>3</sup>H]WAY-100635 further confirms that this ligand interacts as an antagonist with 5-HT<sub>1A</sub> receptors. In contrast to agonists, which bind with high affinity solely to G-protein coupled receptors, antagonists bind with high affinity to both G-protein coupled and uncoupled receptor binding subunits [33]. Therefore, if some free 5-HT<sub>1A</sub> receptor binding subunits exist in addition to G-protein-coupled 5-HT<sub>1A</sub> receptors, a greater density of high affinity binding sites would be expected when using [<sup>3</sup>H]WAY-100635 as the radioligand than when using [<sup>3</sup>H]8-OH-DPAT as the radioligand. Indeed, this hypothesis was directly verified in the present study, as the density of [<sup>3</sup>H]WAY-100635 specific binding sites was found to be 50–60% higher than that of [<sup>3</sup>H]8-OH-DPAT specific binding sites in all the brain areas examined [20,31]. Accordingly, this implies that the measurement of both [<sup>3</sup>H]8-OH-DPAT and [<sup>3</sup>H]WAY-100635 specific binding in the same membrane preparation can provide relevant information regarding the proportion of 5-HT<sub>1A</sub> receptor binding subunits which physically interact with G proteins in such membranes, and possible changes in this proportion after various pharmacologically active treatments.
 logical treatments. Thus, the unchanged ratio of $^{[3]}$H$^{3}$WAY-100635 over $^{[3]}$H$^{8}$-OH-DPAT specific binding in various brain regions after chronic treatment with selective 5-HT reuptake inhibitors such as fluoxetine and paroxetine allowed Le Poul et al. [36] to conclude that the physical interaction of 5-HT$^{1A}$ receptor binding subunits with G proteins remains unaltered in the rat brain after this treatment.

Because the specific binding of $^{[3]}$H$^{4}$WAY-100635 to 5-HT$^{1A}$ receptor binding subunits appeared to be independent of their coupling with G proteins, we hypothesized that the dissociation of the receptor-G protein complexes which may occur in vivo due to the occupancy of the receptor by endogenous 5-HT or an exogenous agonist, should not affect the labelling of these receptors by the radioligand. Therefore, the in vivo labelling of 5-HT$^{1A}$ receptors should be possible with $^{[3]}$H$^{3}$WAY-100635. This hypothesis was also directly verified in the present study as we found that the accumulation of tritium in the hippocampus (and in other brain areas) 1 h after i.v. injection of this radioligand in mice could be prevented by the prior occupancy of 5-HT$^{1A}$ receptors by the 5-HT$^{1A}$ receptor antagonist (S)WAY-100135. Interestingly, the radioactivity which persisted in various brain regions in mice pretreated with (S)WAY-100135 was equal to that found in the cerebellum of mice injected with $^{[3]}$H$^{3}$WAY-100635 only, indicating that the cerebellar accumulation of tritium corresponded to ‘non-specific’ binding of $^{[3]}$H$^{3}$WAY-100635. Comparison of this value with the radioactivity accumulated in the hippocampus in non-
pretreated mice indicated that 'specific binding' accounts for ~80% of the latter value.

In vivo studies clearly demonstrated that WAY-100635 lacks intrinsic agonist activity in a range of physiological and behavioural models of central 5-HT1A receptor activation. In contrast, in all models examined (both of presynaptic (somatodendritic) and postsynaptic 5-HT1A receptor function), WAY-100635 was a potent antagonist of responses evoked by the reference 5-HT1A receptor agonist, 8-OH-DPAT. Thus, WAY-100635 blocked 8-OH-DPAT-induced 5-HT behavioural syndrome, hypothermia, hyperphagia, elevation of plasma ACTH and disruption of motor/motivational performance in a cognitive task. WAY-100635 has also been reported to block the the 8-OH-DPAT discriminative cue [42], to prevent the inhibition of hippocampal 5-HT release induced by 8-OH-DPAT in the rat [21,22] and to block dorsal raphe somatodendritic 5-HT1A receptors in the guinea-pig [39] and conscious cat [18]. In these studies WAY-100635 significantly increased raphe 5-HT neuronal cell firing and hippocampal 5-HT release during periods of active waking (but not during quiet waking or sleep) indicating that these cells are under a tonic inhibitory control by endogenous 5-HT.

Since 5-HT1A receptors are thought to be involved in several psychiatric and neurological disorders (e.g., [6,37]) it is feasible that potent and selective 5-HT1A receptor antagonists such as WAY-100635 may have therapeutic utility [15]. In this report we have shown that WAY-100635 was active in the mouse light/dark box model of anxiety, suggesting that blockade of central 5-HT1A receptors can exert an anxiolytic action. This suggestion was further supported by the finding that the antagonist potencies (for attenuation of 8-OH-DPAT-induced hypothermia) of a range of 5-HT1A receptor partial agonists and antagonists were highly correlated with their potencies as anxiolytics in the light/dark box. Thus, the anxiolytic properties of 5-HT1A receptor partial agonists such as buspirone [13] may be due, at least in part, to central 5-HT1A receptor blockade. It has also been proposed [5] that 5-HT1A receptor antagonists may improve the cognitive deficits associated with dementia by facilitating glutamate release from surviving cortical glutamatergic neurones. Our data show that neither 8-OH-DPAT nor WAY-100635 had any selective effect on cognitive performance in the DMTP task suggesting that some previous reports of cognitive impairment induced by 8-OH-DPAT may have been secondary to motor impairment (e.g., [30]). However, a definitive test
of the potential cognitive enhancing effects of 5-HT\textsubscript{1A} receptor antagonists requires examination of the effects of WAY-100635 on cognitive deficits in rats induced by lesions of cortico-cortical glutamate neurones which may provide a more valid animal model of the early cognitive deficits in Alzheimer's disease. Such studies are currently underway in our laboratory.

In conclusion, we have shown that WAY-100635, the first potent, selective and 'silent' 5-HT\textsubscript{1A} receptor antagonist, will be a valuable new addition to the range of selective ligands available for research into the physiological and pathophysiological roles of 5-HT receptor subtypes. Radiolabelled WAY-100635 has also been characterised as the first antagonist radioligand for 5-HT\textsubscript{1A} Receptor binding studies. \[^3\text{H}\]WAY-100635 has been shown to be a suitable radioligand for the in vivo labelling of 5-HT\textsubscript{1A} receptors in rodent brain, and a derivative labelled with a short half-life radioisotope is of considerable potential interest for the visualization and quantification of brain 5-HT\textsubscript{1A} receptors in humans using PET. At the IUPHAR Serotonin Satellite Symposium, the first in vivo binding studies in rodents using \[^{11}\text{C}\]WAY-100635, a positron-emitting radioligand, were reported [43], and this radioligand has now been used to carry out the first CNS 5-HT\textsubscript{1A} receptor PET imaging studies in human volunteers [44].

Acknowledgment

Part of this research was supported by grants from INSERM, DRET (Contract No 93/047/BC) and the University of Paris VI (DRED). INSERM U.288 are grateful to Pharmaceutical companies for generous gifts of drugs used in the present study.

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