

RESEARCH PAPER

Signal transduction and functional selectivity of F15599, a preferential post-synaptic 5-HT_{1A} receptor agonist

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Background and purpose: Activation of post-synaptic 5-HT_{1A} receptors may provide enhanced therapy against depression. We describe the signal transduction profile of F15599, a novel 5-HT_{1A} receptor agonist.

Experimental approach: F15599 was compared with a chemical congener, F13714, and with (+)8-OH-DPAT in models of signal transduction *in vitro* and *ex vivo*.

Key results: F15599 was highly selective for 5-HT_{1A} receptors in binding experiments and in [³⁵S]-GTPγS autoradiography of rat brain, where F15599 increased labelling in regions expressing 5-HT_{1A} receptors. In cell lines expressing h5-HT_{1A} receptors, F15599 more potently stimulated extracellular signal-regulated kinase (ERK1/2) phosphorylation, compared with G-protein activation, internalization of h5-HT_{1A} receptors or inhibition of cAMP accumulation. F13714, (+)8-OH-DPAT and 5-HT displayed a different rank order of potency for these responses. F15599 stimulated [³⁵S]-GTPγS binding more potently in frontal cortex than raphe. F15599, unlike 5-HT, more potently and efficaciously stimulated G_o than G_q activation. In rat prefrontal cortex (a region expressing post-synaptic 5-HT_{1A} receptors), F15599 potently activated ERK1/2 phosphorylation and strongly induced c-fos mRNA expression. In contrast, in raphe regions (expressing pre-synaptic 5-HT_{1A} receptors) F15599 only weakly or did not induce c-fos mRNA expression. Finally, despite its more modest affinity *in vitro*, F15599 bound to 5-HT_{1A} receptors *in vivo* almost as potently as F13714.

Conclusions and implications: F15599 showed a distinctive activation profiles for 5-HT_{1A} receptor-mediated signalling pathways, unlike those of reference agonists and consistent with functional selectivity at 5-HT_{1A} receptors. In rat, F15599 potently activated signalling in prefrontal cortex, a feature likely to underlie its beneficial effects in models of depression and cognition. *British Journal of Pharmacology* (2009) **156**, 338–353; doi:10.1111/j.1476-5381.2008.00001.x; published online 12 January 2009

Keywords: antidepressant; serotonin; 5-HT_{1A}; G-protein; ERK1/2 phosphorylation; autoradiography; c-fos; agonist-directed trafficking of receptor signalling

Abbreviations: DTT, dithiothreitol; ERK1/2, extra-cellular signal regulated kinase; SPA, scintillation proximity assay; SNRI, selective noradrenaline reuptake inhibitor; SSRI, selective serotonin reuptake inhibitor

Introduction

5-HT_{1A} receptors play a key role in 5-hydroxytryptaminergic transmission due to their localization both as pre-synaptic receptors located on 5-HT cell bodies in the raphe nuclei and as post-synaptic receptors in brain areas associated with mood, cognition and pain (Lucki, 1998; Colpaert *et al.*, 2002;

Blier and Ward, 2003; Colpaert, 2006; Millan, 2006). In particular, 5-HT_{1A} receptors are profoundly involved in the mechanism of action of antidepressants (Blier and Ward, 2003; Celada *et al.*, 2004). Thus, 5-HT_{1A} receptor agonists are potently active in rodent models of depression such as the forced swim test, completely reversing upon acute administration the immobility that is taken as a measure of 'helplessness' (De Vry and Schreiber, 1993; De Vry, 1995; Koek *et al.*, 1998; 2001). Conversely, the antidepressant-like activities in rats of the selective 5-HT (serotonin) reuptake inhibitor (SSRI), fluoxetine, the selective noradrenaline reuptake inhibitor, desipramine and the non-selective (5-HT and noradrenaline) re-uptake inhibitor, venlafaxine are inhibited by 5-HT_{1A}

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*Dr. Liesbeth Bruins Slot died on 4 April 2008.

Received 4 June 2008; revised 18 July 2008; accepted 1 August 2008

receptor antagonists (Detke *et al.*, 1995; De Vry *et al.*, 2004; Berrocoso and Mico, 2008). Further, the influence of fluoxetine in a strain of mice selected for their 'depressive' behaviour is accompanied by changes in 5-HT_{1A} receptor expression levels (Naudon *et al.*, 2002; El Yacoubi *et al.*, 2003). In electrophysiological studies in rats, a variety of antidepressant treatments, as well as 5-HT_{1A} agonists, share a common influence: desensitization of pre-synaptic 5-HT_{1A} receptors in the raphe nuclei and increased response of post-synaptic 5-HT_{1A} receptors in limbic regions (e.g. hippocampus; Haddjeri *et al.*, 1998; 1999). When 5-HT neurons are lesioned, eliminating pre-synaptic 5-HT_{1A} receptors, antidepressant-like actions of 5-HT_{1A} agonists in mice are maintained, indicating that reduction of depressive-like behaviour is mediated by post-synaptic 5-HT_{1A} receptors (Matsuda *et al.*, 1995). Further, post-synaptic 5-HT_{1A} receptor control of hippocampal pyramidal neuron firing is amplified by preferential antagonism of pre-synaptic 5-HT_{1A} receptors with pindolol (Romero *et al.*, 1996; Bélique *et al.*, 2000). The involvement of 5-HT_{1A} receptors in depressive states is supported by the decrease in both forebrain and dorsal raphe 5-HT_{1A} receptor binding potential in positron emission tomography scans of depressed humans and primates (Bhagwagar *et al.*, 2004; Shively *et al.*, 2006). Further, antidepressant treatment normalizes 5-HT_{1A} receptor binding potential in depressed subjects (Parsey *et al.*, 2005). Several weeks of treatment with SSRIs are necessary for pre-synaptic 5-HT_{1A} receptors to be desensitized (Invernizzi *et al.*, 1994; Rutter *et al.*, 1994) allowing antidepressant efficacy to manifest itself. Accordingly, combined treatment of depressed patients with SSRIs and pindolol accelerates the onset of therapeutic efficacy of antidepressants (see Artigas *et al.*, 2006). 5-HT_{1A} partial agonists (such as the anxiolytics, buspirone and gepirone) are not effective as antidepressants in monotherapy, probably due to insufficient efficacy at post-synaptic 5-HT_{1A} receptors and/or metabolic instability. However, when feedback inhibition of pre-synaptic 5-HT_{1A} receptors was blocked with pindolol, buspirone did exert clinical antidepressant influence, presumably through activation of post-synaptic 5-HT_{1A} receptors (Blier *et al.*, 1997).

All these data strongly suggest that direct targeting of post-synaptic 5-HT_{1A} receptors with a high-efficacy agonist may provide accelerated and more effective antidepressant therapy. Indeed, although only a single 5-HT_{1A} receptor gene sequence has been identified, post-synaptic 5-HT_{1A} receptors exhibit distinct control of downstream signal transduction events. Thus, chronic administration of fluoxetine or imipramine desensitizes 5-HT_{1A} receptor-mediated G-protein activation in the raphe, hypothalamus and lateral septum but not in frontal cortex or hippocampus (Hensler, 2002; Pejchal *et al.*, 2002; Shen *et al.*, 2002; Castro *et al.*, 2003; see Hensler, 2003 for review). G_o and G_{i2} G-proteins are down-regulated, but G_s up-regulated, in the prefrontal cortex of suicide victims (Dwivedi *et al.*, 2002). Indeed, G_s localization in brain of depressed suicides is preferentially shifted to lipid raft domains, with decreased coupling to adenylyl cyclase (Donati *et al.*, 2008). Pre-synaptic 5-HT_{1A} receptors in raphe are preferentially coupled to G_{i3} G-proteins whereas post-synaptic 5-HT_{1A} receptors in hippocampus and cortex preferentially couple to G_o and G_{i3} proteins (Mannoury *et al.*, 2006). Further, post-synaptic 5-HT_{1A} receptors in hypothalamus also couple

to other G-protein subtypes (G_{i1} and G_z; Raap *et al.*, 1999; Serres *et al.*, 2000). Pre-synaptic 5-HT_{1A} receptors in raphe inhibit inositol phosphate but not adenylyl cyclase formation in rat tissue (Johnson *et al.*, 1997; Marazziti *et al.*, 2002). In contrast, post-synaptic 5-HT_{1A} receptors in hippocampus and cortex inhibit adenylyl cyclase but not inositol phosphate formation (Clarke *et al.*, 1996; Johnson *et al.*, 1997). In *ex vivo* experiments, 5-HT_{1A} receptor activation stimulates extracellular signal-regulated kinase (ERK1/2) phosphorylation in the raphe and the paraventricular nucleus (Sullivan *et al.*, 2005; Crane *et al.*, 2007) but inhibits it in the hippocampus (Chen *et al.*, 2002; Crane *et al.*, 2007), indicating brain region specificity in a 'downstream' signalling response. A single-nucleotide polymorphism (C-1019G substitution; Lesch and Gutknecht, 2004) impairs repression of the 5-HT_{1A} promoter in raphe cells, leading to excessive expression of pre-synaptic 5-HT_{1A} receptors (Lemondé *et al.*, 2003; Parsey *et al.*, 2006a). In patients receiving antidepressant treatments, the C-1019G polymorphism is associated with higher levels of remission failure (Lemondé *et al.*, 2004; Parsey *et al.*, 2006b), probably due to overactive pre-synaptic 5-HT_{1A} receptors. In contrast, in cortical neurons, the C-1019G polymorphism leads to 5-HT_{1A} receptor hypofunction (Czesak *et al.*, 2006), suggesting that increased stimulation of receptors in cortex is necessary to alleviate depressive symptoms.

Thus, drugs exhibiting 5-HT_{1A} receptor functional selectivity (i.e. agonist-directed trafficking of receptor signalling; Kenakin, 1995; Newman-Tancredi *et al.*, 2003) would be expected to exert brain region-specific activity on 5-hydroxytryptaminergic neurotransmission. Recently, we identified a novel, highly selective and efficacious 5-HT_{1A} receptor agonist, F15599. Neurochemical, electrophysiological and behavioural data indicate that this compound preferentially activates post-synaptic 5-HT_{1A} receptors. Thus, F15599 influences frontal cortex pyramidal neuron electrical activity at doses that are an order of magnitude lower than those that inhibit raphe neuron electrical activity (Celada *et al.*, 2007). Further, F15599 increases dopamine release in frontal cortex at low doses (reflecting post-synaptic 5-HT_{1A} receptor activation) whereas higher doses are necessary to inhibit 5-HT release in hippocampus (a pre-synaptic 5-HT_{1A} receptor response; Assié *et al.*, 2006a). The preferential activation of post-synaptic 5-HT_{1A} receptors by F15599 is accompanied by a remarkable capacity to reverse memory/cognition deficits (Auclair *et al.*, 2007) and potent activity in the forced swim test in rats (Assié *et al.*, 2006a).

The present study describes the receptor interaction and signal transduction profile of F15599 both *in vitro* and *ex vivo* in comparison with a chemical congener, F13714 and the more active (+) enantiomer of the prototypical 5-HT_{1A} receptor agonist, 8-OH-DPAT. These ligands displayed distinct signalling profiles, suggesting that functional selectivity of F15599 for specific receptor activation responses is likely to underlie its favourable profile in models of cognitive and mood deficits.

Methods

The activity of F15599 and reference agonists at 5-HT_{1A} receptors was characterized using a series of methods designed to investigate different levels of drug action. These

Table 1 *In vitro* competition binding methods for determination of affinity at various monoamine receptors

Receptor	Tissue	[³ H]Radioligand (nmol·L ⁻¹)	K _d (nmol·L ⁻¹)	Non-specific (μmol·L ⁻¹)	Incubation (h) temperature (°C)	Buffer
h 5-HT _{1A}	CHO cells	8-OH-DPAT (1)	0.68	5-HT (10)	2, 23	A
r 5-HT _{1A}	Rat hippocampus	8-OH-DPAT (1)	0.75	5-HT (10)	2, 23	B
r 5-HT _{1B}	Rat cortex	GR125,743 (1)	0.36	5-HT (10)	2, 23	C
r 5-HT _{2A}	Rat cortex	Ketanserin (0.2)	3.1	Methysergide (10)	0.5, 23	C
p 5-HT _{2C}	Pig cortex	Mesulergine (1)	4.8	Mianserin (10)	1, 23	C
r α ₁	Rat cortex	Prazosin (0.2)	0.07	Phentolamine (5)	2, 23	B
r α ₂	Rat cortex	RX 821002 (0.5)	0.49	Phentolamine (5)	2, 23	B
r D ₁	Rat striatum	SCH 23390 (1)	0.45	SKF 38393 (10)	2, 37	D
r D ₂	Rat striatum	Nemonapride (0.2)	0.05	(+)-Butaclamol (1)	2, 23	D

Further methodological details are given in Newman-Tancredi *et al.* (2007).

D₁, D₂, dopamine receptors; h, human; p, porcine; r, rat; α₁, α₂, α-adrenoceptors; buffer A, HEPES 20 mmol·L⁻¹ pH 7.4; buffer B, Tris HCl 50 mmol·L⁻¹ pH 7.4; buffer C, Tris HCl 50 mmol·L⁻¹ pH 7.4, pargyline 10 μmol·L⁻¹, CaCl₂ 4 mmol·L⁻¹, ascorbic acid 0.1%; buffer D, Tris HCl 50 mmol·L⁻¹ pH 7.4, NaCl 120 mmol·L⁻¹, KCl 5 mmol·L⁻¹.

Table 2 Summary of methods for determination of functional responses at recombinant human and native rat 5-HT_{1A} receptors *in vitro*

Functional measure	Tissue/cell line	Incubation buffer	Incubation time (min) and temperature (°C)	Literature reference
'Total' G-protein activation ^a	C6-h5-HT _{1A} glial cell memb	Buffer A	30, 23	Koek <i>et al.</i> (2001)
'Total' G-protein activation	HeLa-h5-HT _{1A} cell memb	Buffer B	60, 30	Newman-Tancredi <i>et al.</i> (2005)
Gα _i activation	HeLa-h5-HT _{1A} cell memb	Buffer C	60, 23	Newman-Tancredi <i>et al.</i> (2002)
cAMP formation	HeLa-h5-HT _{1A} cell memb	Buffer D	10, 23	Newman-Tancredi <i>et al.</i> (2005)
ERK1/2 phosphorylation	CHO-h5-HT _{1A} whole cells	RPMI serum-free	5, 37	Bruins Slot <i>et al.</i> (2006)
5-HT _{1A} internalization	HEK293-h5-HT _{1A} whole cells	DMEM serum-free	120, 37	Heusler <i>et al.</i> (2008)
'Total' G-protein activation	Rat hippocampal memb	Buffer E	60, 37	Newman-Tancredi <i>et al.</i> (2005)
G _{αo} activation	Rat hippocampal memb	Buffer F	60, 23	Martel <i>et al.</i> (2006)

G-protein activation experiments were carried out by [³⁵S]-GTPγS binding to cell membrane (memb) preparations. Other measures (cAMP formation and ERK1/2 phosphorylation and receptor internalization) were carried out on whole cells.

^a'Total' G-protein activation refers to [³⁵S]-GTPγS binding that does not distinguish between the G-protein subtypes involved.

Buffer A, 20 mmol·L⁻¹ HEPES, pH 7.4, 100 mmol·L⁻¹ NaCl, 3 mmol·L⁻¹ MgCl₂, 30 μmol·L⁻¹ GDP, 0.2 mmol·L⁻¹ ascorbic acid; buffer B, 20 mmol·L⁻¹ HEPES, pH 7.4, 100 mmol·L⁻¹ NaCl, 10 mmol·L⁻¹ MgCl₂, 30 μmol·L⁻¹ GDP, 10 μmol·L⁻¹ pargyline; buffer C, 20 mmol·L⁻¹ HEPES, pH 7.4, 100 mmol·L⁻¹ NaCl, 3 mmol·L⁻¹ MgCl₂, 3 μmol·L⁻¹ GDP; buffer D, DMEM + 10 mmol·L⁻¹ HEPES, pH 7.4, 100 μmol·L⁻¹ forskolin, 100 μmol·L⁻¹ isobutylmethylxanthine; buffer E, 50 mmol·L⁻¹ HEPES, pH 7.4, 150 mmol·L⁻¹ NaCl, 5 mmol·L⁻¹ MgCl₂, 100 μmol·L⁻¹ GDP, 0.2 mmol·L⁻¹ EDTA, 0.2 mmol·L⁻¹ DTT; buffer F, 20 mmol·L⁻¹ HEPES, pH 7.4, 100 mmol·L⁻¹ NaCl, 5 mmol·L⁻¹ MgCl₂, 50 μmol·L⁻¹ GDP, 0.2 mmol·L⁻¹ EDTA, 0.2 mmol·L⁻¹ DTT.

included: (i) ligand binding, to determine affinity directly at 5-HT_{1A} receptors *in vitro* and *in vivo* receptor occupancy in mice; (ii) *in vitro* signal transduction measures representing a cascade of responses to 5-HT_{1A} agonism. These included 'total' G-protein activation (i.e. without distinguishing the subtypes of G-protein involved), G_{αi} and G_{αo} G-protein subtype targeting, adenylyl cyclase inhibition, ERK1/2 phosphorylation and receptor internalization. The purpose was to determine whether the pattern of activation of these different signalling responses was agonist-specific; and (iii) *Ex vivo* measures of ERK1/2 phosphorylation and c-fos gene expression in various brain regions in rat. The object was to determine whether F15599 influenced these responses in a brain region-specific manner. Details of the methods employed are described below. The molecular target nomenclature (receptors, ion channels, etc.) is expressed according to the *BJP* Guide to Receptors and Channels (Alexander *et al.*, 2008).

Animals

Animals were housed and tested in an Association for the Assessment and Accreditation of Laboratory Animal Care International-accredited facility in strict compliance with all applicable regulations and the protocols were carried out in

compliance with French regulations and with local ethical committee guidelines for animal research.

Competition binding methods

Competition binding experiments were carried out using the radioligands, buffer and incubation conditions outlined in Table 1 and described by Newman-Tancredi *et al.* (2007). Experiments at native rat receptors employed brains of male Sprague-Dawley rats [Ico: OFA SD (S.P.F. Caw); Iffa Credo, France], weighing 180–200 g. Animals were decapitated and brains were rapidly removed and stored at –80°C before use in binding assays. For native 5-HT_{2C} receptor binding assays (Pazos *et al.*, 1985a,b), pig cortex was obtained from the local slaughter house.

Binding experiments at human 5-HT_{1A} receptors were carried out using membranes from stably transfected CHO cells as described previously (see references in Table 2). Experiments were terminated by rapid filtration through Whatman GF-B filters. Radioactivity retained on the filters was measured by liquid scintillation spectroscopy. Data from native tissue receptors were analysed using the non-linear curve fitting program KELL RADLIG version 6 (Biosoft, Cambridge, UK; McPherson, 1985) and pK_i values are from at least three experiments, each comprising six to seven concentrations

differing by one log unit interval. Data from human cloned receptor binding experiments were analysed using GraphPad Prism (GraphPad Software Inc., San Diego, CA), and pKi values are expressed as mean \pm SEM of at least three experiments each comprising 7–10 concentrations differing by 0.5 or 1 log unit interval.

Functional responses to F15599 at recombinant human 5-HT_{1A} receptors

The agonist properties of F15599 at h5-HT_{1A} receptors expressed in transfected cell lines were determined *in vitro* for measures of signal transduction representing different levels of intracellular responses: activation of G-proteins, inhibition of adenylyl cyclase activity, phosphorylation of ERK1/2 and induction of (HA-tagged) h5-HT_{1A} receptor internalization. The methodologies employed were as described previously in studies of h5-HT_{1A} receptor signalling (see literature references and methodological summary shown in Table 2). In the case of G-protein activation experiments, two test conditions were used: [³⁵S]-GTP γ S binding to C6-h5-HT_{1A} glial cell membranes and to HeLa-h5-HT_{1A} cell membranes. Membranes from these cell lines express similar levels of h5-HT_{1A} receptors (1110 and 1280 fmol receptor per mg protein respectively). C6-h5-HT_{1A} glial cells have been previously shown to be suitable to discriminate agonists with high efficacy (Pauwels *et al.*, 1997). Thus, lower E_{max} values are observed under these conditions, relative to 5-HT. HeLa-h5-HT_{1A} cells are more sensitive and partial agonists are more easily detected therein (Cosi and Koek, 2000). These cells were used in order to provide a comparison with experiments on cAMP inhibition and G_o activation, which were carried out in the same cell line.

Concentration-response isotherms were analysed by non-linear regression, using GraphPad Prism. The value of the minimum and maximum asymptotes was not fixed. The latter (E_{max}) is expressed as a percentage of the effect observed with 5-HT (10 μ mol·L⁻¹). K_B values of antagonists for inhibition of agonist action were calculated according to Lazareno and Birdsall (1993):

$$K_B = IC_{50} / [1 + (\text{agonist} : EC_{50})]$$

where IC₅₀ = inhibitory concentration₅₀ of antagonist, agonist = concentration of agonist in the test and EC₅₀ = effective concentration₅₀ of agonist.

G-protein activation by F15599 at native rat 5-HT_{1A} receptors in rat brain regions

Following death and removal of brain from the skull, brains were rapidly cooled over liquid nitrogen before dissection. Large brain structures (frontal cortex and hippocampus) were dissected macroscopically while other structures (lateral septum, entorhinal cortex and raphe nuclei) were micro-dissected on a cold plate as described by Palkovits and Brownstein (1987). Briefly, micro-dissections were performed with a 1 mm circular punch on 300- μ m-thick coronal sections in the range from 2400 to 600 μ m anterior to bregma for lateral septum, from 4000 to 8400 μ m posterior to bregma for

entorhinal cortex, and from 6900 to 8400 μ m posterior to bregma for dorsal and median raphe. Tissues and punches were collected in microtubes, frozen on dry ice and stored at -80°C until used.

On the day of experiment, tissues were suspended in 20 volumes (v/w) of ice cold 50 mmol·L⁻¹ HEPES containing 150 mmol·L⁻¹ NaCl, 0.2 mmol·L⁻¹ EDTA, 10 μ mol·L⁻¹ pargyline (pH 7.4 at room temperature), 1 mmol·L⁻¹ GTP, homogenized and incubated at 37°C for 10 min in order to dissociate the endogenous neurotransmitters from the receptors. The homogenate was centrifuged at 20 000 \times g for 15 min at 4°C. The pellet was washed twice by two successive runs of homogenization/centrifugation in the same buffer containing successively 1 mmol·L⁻¹ and 100 μ mol·L⁻¹ GDP instead of GTP. The final pellet was resuspended in 600 volumes (v/w from original weight) of the same buffer containing 100 μ mol·L⁻¹ GDP and 5 mmol·L⁻¹ MgCl₂. Membranes (30–40 μ g protein per tube) were incubated in the presence of different concentrations of drugs ranging from 10⁻¹⁰ to 10⁻⁵ mol·L⁻¹, together with 0.1 nmol·L⁻¹ [³⁵S]-GTP γ S for 1 h at 37°C. Non-specific binding was determined with 10 μ mol·L⁻¹ unlabelled GTP γ S. Other experimental controls included basal and 5-HT (10 μ mol·L⁻¹)-stimulated [³⁵S]-GTP γ S binding.

G-protein subtype targeting experiments using scintillation proximity assay

Rat hippocampi were homogenized in cold HEPES-dithiothreitol (DTT) buffer (20 mmol·L⁻¹ HEPES pH 7.0, 0.2 mmol·L⁻¹ EDTA and 0.2 mmol·L⁻¹ DTT) with 1 mmol·L⁻¹ GTP using a Polytron homogenizer: GTP was added to induce dissociation of endogenous ligand. Homogenates were incubated at 35°C for 15 min to favour endogenous ligand dissociation, and washed by two cycles of centrifugation at 20 000 \times g for 15 min at 4°C and resuspension in cold HEPES-DTT with 1 mmol·L⁻¹ GDP. Final pellets were resuspended in 300 volumes (based on original tissue weight) of HEPES-DTT buffer containing 100 mmol·L⁻¹ NaCl, 5 mmol·L⁻¹ MgCl₂ and 50 μ mol·L⁻¹ GDP (rat hippocampus assay buffer) to give a final protein concentration of 8 μ g per well. Membranes from HeLa-h5-HT_{1A} cells were diluted in 20 mmol·L⁻¹ HEPES pH 7.0 containing 100 mmol·L⁻¹ NaCl, 3 mmol·L⁻¹ MgCl₂ and 3 μ mol·L⁻¹ GDP (HeLa-h5-HT_{1A} assay buffer) to give a final protein concentration of 50 μ g per well. All reactions were performed at room temperature using 96-well plates, in a final volume of 250 μ L of the appropriate assay buffer. Membranes were incubated at room temperature with drugs (10⁻¹¹ to 10⁻⁵ mol·L⁻¹), buffer (to define basal) or 10 μ mol·L⁻¹ GTP γ S (to define non-specific) and 0.4 nmol·L⁻¹ (rat hippocampus) or 0.2 nmol·L⁻¹ (HeLa-h5-HT_{1A}) [³⁵S]-GTP γ S for 60 min. Incubations were stopped by adding Nonidet NP-40 and the plate was agitated for another 30 min before addition of 0.2 μ g anti-G α -selective antibodies to each well. The antibodies used were mouse monoclonal anti-G α_{i1} and anti-G α_o from Biomol (Plymouth Meeting, PA). Antibody specificity was assessed by Western blot as described (Cussac *et al.*, 2002; 2004). Anti-G α_o is highly selective (Martel *et al.*, 2007) while some cross-reactivity was observed for anti-G α_{i1} antibody with G α_{i3} (Cussac *et al.*, 2002). As HeLa-h5-HT_{1A} cells express undetectable levels of G α_{i1} (D. Cussac, unpubl. obs.), the response

detected in this cell line with anti-G α_{i1} antibodies is more likely to correspond to a stimulation of G α_{i3} proteins. The primary antibodies were left to react for 60 min under agitation before adding 50 μ L of the secondary antibodies [anti-mouse coupled to scintillation proximity assay (SPA) beads, GE Healthcare, Amersham, UK], diluted according to manufacturer's recommendations. The secondary antibodies were left to react for another 60 min and the plate was centrifuged at 1000 \times g for 15 min at room temperature and radioactivity was immediately measured on a Top-Count scintillation counter (Perkin-Elmer Life Sciences, Courtaboeuf, France). Raw data [disintegrations per minute (DPM)] were analysed by subtracting non-specific binding and expressed as percentage of basal [35 S]-GTP γ S binding. All pharmacological parameters were derived from sigmoid non-linear regression using Graphpad Prism.

Functional autoradiography: specific activation of 5-HT $_{1A}$ receptors by F15599

[35 S]-GTP γ S autoradiography was carried out essentially as described by Newman-Tancredi *et al.* (2003). Frozen rat brains were cut horizontally in 20 μ m serial sections using a cryostat at -20°C , fixed on microscope slides and were kept frozen until assayed. The assay was performed by pre-incubating the slides at room temperature for 15 min in buffer A (50 mmol·L $^{-1}$ HEPES buffer containing 150 mmol·L $^{-1}$ NaCl, 0.2 mmol·L $^{-1}$ EGTA and 0.2 mmol·L $^{-1}$ DTT) plus 2.5 mmol·L $^{-1}$ GTP, 15 min in buffer A plus 2.5 mmol·L $^{-1}$ GDP and 15 min in buffer A with 2.5 mmol·L $^{-1}$ GDP, 10 mmol·L $^{-1}$ MgCl $_2$ and 100 mU·mL $^{-1}$ adenosine deaminase (= buffer B) plus antagonist. Sections were then incubated for 60 min at 37°C in buffer B containing 0.05 nmol·L $^{-1}$ [35 S]-GTP γ S and drugs. Basal [35 S]-GTP γ S binding was defined as that observed in the absence of drugs, and non-specific binding was defined as the binding in the presence of 100 μ mol·L $^{-1}$ unlabelled GTP γ S. At the end of the incubation period, sections were rapidly washed twice for 2 min in cold buffer B (4°C), and rapidly dried under a flow of cold air. Dried sections together with [14 C] radioactivity standards were placed in X-ray cassettes, apposed to Biomax films and exposed for 4.5 days. Films were developed and radioactivity on sections was quantified using an image analysis system (AIS system, InterFocus Ltd, Linton, UK). Grey levels were converted to nCi·g $^{-1}$ equivalents using [14 C] radioactivity standards, and radioactivity was measured on each structures/sections.

In vivo 5-HT $_{1A}$ receptor binding of F15599 in mice

Male NMRI mice [Ico: NMRI (S.P.F.) Han; Iffa Credo, France], weighing 20–22 g upon arrival, were group-housed (12 mice per cage) in the animal facility, under controlled conditions (12/12 h light/dark cycle: lights on at 7.00 a.m.; ambient temperature $21 \pm 1^{\circ}\text{C}$; humidity $55 \pm 5\%$), with rodent food (AO4, UAR, France) and filtered (0.2 μ m pore diameter) tap water available *ad libitum*. At least 5 days were allowed for adaptation before the mice were used in the experiments. The doses of compounds were expressed as the weight of the base. The compounds were dissolved in distilled water and the administration volume was 10 mL·kg $^{-1}$.

On the day of the experiment, mice were housed individually with free access to food and water. Each animal was treated with the drug or saline and returned to its cage. Thirty minutes later, the mouse was gently introduced and maintained in a Plexiglas cylinder, with the tail protruding, and 4 μ Ci in 0.2 mL (48 pmol) of a [^3H]WAY100635 solution was slowly injected into the caudal vein and the mouse was returned to its cage. One hour after administration of the test compound, the animal was decapitated, the frontal cortex and hippocampus dissected out, weighed and kept frozen on dry ice. At the end of the experiment, the tissue was thawed and homogenized (10 s with an Ultra-Turrax) in 3 mL distilled water. The radioactivity in 0.5 mL samples was counted using liquid scintillation spectroscopy (Packard, TriCarb 2500). Data were expressed as mean \pm SEM dpm·(mg wet tissue weight) $^{-1}$ ($n = 5$ to 8 animals per group). Data were analysed using GraphPad Prism software to determine the ID $_{50}$. For each compound and each brain region, the maximum value of the curve was constrained at the value of control animals. Dose-response data were analysed by one-way ANOVA, followed by Dunnett's test.

Modulation of ERK1/2 phosphorylation by F15599 in rat brain

Adult male Sprague–Dawley rats were injected intraperitoneally (i.p.) with F15599 (0.04–2.5 mg·kg $^{-1}$) or saline ($n = 6$ per group). Thirty minutes after injection, rats were decapitated. In 5-HT $_{1A}$ receptor antagonist studies, WAY100635 (0.63 mg·kg $^{-1}$) or saline was injected subcutaneously 30 min prior to the i.p. injection of F15599 (0.63 mg·kg $^{-1}$ i.p.) or saline, and rats were decapitated 30 min after the i.p. injection. The 0.63 mg·kg $^{-1}$ dose of WAY100635 was chosen from previous data showing this dose to antagonize the effects of the other selective high-efficacy 5-HT $_{1A}$ receptor agonists, F13640 (Bardin *et al.*, 2003; Buritova *et al.*, 2003) and F13714 (Assié *et al.*, 2006b) in the rat.

Following decapitation, rat brains were cooled in vaporized liquid nitrogen for 1 min and the brain regions of interest (entire hippocampus, prefrontal cortex and entire hypothalamus) then immediately dissected. Each dissected brain region was put into Lysing Matrix D tube (MP, Illkirch, France) filled with 700 μ L of cell extraction buffer at 4°C (Biosource, Nivelles, Belgium) supplemented with protease inhibitors, phosphatase inhibitors and urea (7 mol·L $^{-1}$). Tissues were homogenized, centrifuged and supernatant was stored frozen until pERK1/2 quantification. The latter was carried out by quantitative ELISA assay (Bruins Slot *et al.*, 2006), using kits from Biosource (ref. KHO0091) according to the manufacturer's instructions. Samples of homogenized rat brain tissues were assayed for content of pERK1/2 and total ERK1/2 ($n = 6$ and 3 rats per group respectively). For each brain region studied, effects of the treatments were compared by ANOVA with the Fisher test). The pair-wise comparisons of treatment groups were carried out by the contrast method (using the Student's t distribution). The significance level was fixed at 5%.

Induction of c-fos mRNA expression by F15599 in rat brain

For quantitation of c-fos mRNA, rats were treated as above for ERK1/2 phosphorylation and tissue specimens from eight

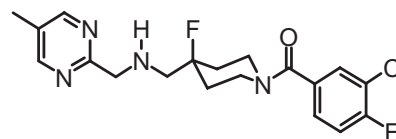
different brain regions (see Figure 7 later) were dissected. These were mechanically homogenized and total RNAs were extracted using a commercially available kit (Rneasy Mini Kit; Qiagen, France) and including a DNase treatment step (Qiagen, France) following the manufacturer's protocol. The quality and quantity of the RNA samples were determined by analysis using an Agilent 2001 Bioanalyser (France). 100 ng RNA (for raphe nuclei) and 1 µg RNA (for others structures) were reverse-transcribed in a 15 µL final volume following the Iscript cDNA synthesis protocol from BioRad (France). Cycle settings were as follows: 5 min at room temperature to allow primer alignment, then 45 min at 42°C and 5 min at 85°C.

Quantitative polymerase chain reaction measures were performed in an IQ thermocycler in 96-well microtitre plates using a 25 µL final volume with a mix composed of 400 nmol·L⁻¹ of specific primers, 7.3 µL of water, 12.5 µL of the iQ SYBR Green Supermix from Biorad (France) and 5 µL of diluted cDNA. Thermal cycling parameters were 2 min at 95°C, followed by 40 cycles at 94°C for 30 s, 30 s at 60°C and 30 s at 72°C. To avoid amplification of contaminating genomic DNA, c-fos primers were placed in two different exons and one of the housekeeping gene Arbp primers (acidic ribosomal phosphoprotein P0) was placed at a junction between two exons. Primers were designed from sequences in the Genbank and Ensembl database using Primer 3 software and I.D.T. (Integrated DNA technologies) software in order to check the absence of dimer or short hairpin sequences. Each primer pair was then validated to verify that their amplification efficacy was ≥95%, and their amplicons were specific (as verified by a melting curve). Primer alignment was then validated by BLAST on NCBI. Primer sequences were as follows: c-fos forward (NM_022197) AAGGGAAAGGAATAAGATGG and c-fos reverse TCTCAAGTTGATCTGTCTCC (94 bp product); Arbp forward (NM_022402) GGTACCATTGAAATCCTGAG and Arbp reverse GTTCAACATGTTTCAGCAGTG (90 bp product). Results, calculated using the comparative 2^{ΔΔCt} method, are shown as a ratio of c-fos expression relative to that of the housekeeping Arbp (i.e. P0) gene. Arbp was previously chosen among six housekeeping genes as being the most stable between different treatments. Histograms (see Figure 7 later) show mean ± SEM from *n* = 6 rats per group. Statistical analysis using SAS software was conducted by ANOVA, followed by Tukey's test, to evaluate differences in c-fos expression between treatment groups. The significance level was fixed at 5%. The mean value of the control group (saline-treated animals) was set at 1 (basal value) and all data were normalized versus the saline-treated group.

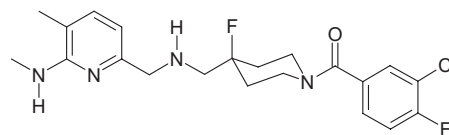
Drugs

F15599 (3-Chloro-4-fluorophenyl-(4-fluoro-4-[(5-methylpyrimidin-2-ylmethyl)-amino]-methyl)-piperidin-1-yl)-methanone) and F13714 (3-chloro-4-fluorophenyl-(4-fluoro-4-[(5-methyl-6-methylaminopyridin-2-ylmethyl)-amino]-methyl)-piperidin-1-yl)-methanone) were synthesized by Jean-Louis Maurel, Medicinal Chemistry Division 1, Centre de Recherche Pierre Fabre (Castres, France), as described earlier (Maurel *et al.*, 2007; Figure 1). 5-HT creatinine sulphate, (+)8-hydroxydipropylaminotryptamine [(+)-8-OH-DPAT] bromohydrate,

F15599



F13714



8-OH-DPAT

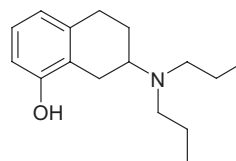


Figure 1 Chemical structures of F15599, F13714 and (+)-8-OH-DPAT.

were purchased from Sigma RBI (St. Quentin Fallavier, France). Drugs were dissolved in distilled water or 10% DMSO at 10⁻³ mol·L⁻¹, and subsequent dilutions were prepared in the appropriate assay buffer.

[³H]8-OH-DPAT (TRK.850: 160–240 Ci·mmol⁻¹), [³H]-GR125,743 (TRK.1046: 50–86 Ci·mmol⁻¹), [³H]mesulergine (TRK.845: 70–85 Ci·mmol⁻¹), [³H]SCH23390 (TRK.876: 60–90 Ci·mmol⁻¹), [³H]RX821002 (TRK.914: 40–70 Ci·mmol⁻¹), [³H]citalopram (TRK.1068: 60–86 Ci·mmol⁻¹), [³H]prazosin (TRK.843: 65–85 Ci·mmol⁻¹), [³H]WAY100635 (TRK.1034: 60–86 Ci·mmol⁻¹) and [³⁵S]-GTPγS (1000–1200 Ci·mmol⁻¹) were purchased from GE Healthcare. [³H]ketanserine (NET-791: 60–90 Ci·mmol⁻¹) and [³H]YM-09151-2 (i.e. [³H]nemonapride; NET-1004: 70–87 Ci·mmol⁻¹) were purchased from Perkin-Elmer Life Sciences.

Results

Receptor binding profile of F15599 at native and recombinant human receptors

In competition binding experiments, F15599 had affinity for both native rat and cloned human 5-HT_{1A} receptors, with pK_i values of about 8.5 (Table 3). The affinity of F15599 is an order of magnitude less than that of 8-OH-DPAT and 35- to 70-fold less than that of F13714. F15599 has no measurable affinity (up to 10 µmol·L⁻¹) for other native receptor binding sites examined (Table 3). In a receptor screen carried out by Cerep (La Celle Levescault, France; data on file), F15599 did not interact significantly with a panoply of other sites including h5-HT_{2A}, h5-HT_{2B}, h5-HT_{2C}, h5-HT₃, 5-HT₄, h5-HT_{5A}, h5-HT₆, h5-HT₇, adrenergic, dopaminergic, muscarinic, histaminergic,

Table 3 Comparison of affinities of F15599, F13714 and (+)8-OH-DPAT for various native receptors determined by competition binding *in vitro*

Receptor	Tissue	F15599	F13714	(+)8-OH-DPAT
h 5-HT _{1A}	CHO cells	8.57 ± 0.05	10.40 ± 0.09 (68)	9.50 ± 0.03 (9)
r 5-HT _{1A}	Rat hippocampus	8.47 ± 0.08	10.01 ± 0.01 (35)	9.15 ± 0.11 (5)
r 5-HT _{1B}	Rat cortex	<5	<5	5.28 ± 0.18
r 5-HT _{2A}	Rat cortex	<5	<5	<5
p 5-HT _{2C}	Pig cortex	<5	<5	5.12 ± 0.05
r α ₁	Rat cortex	<5	7.17 ± 0.05	5.82 ± 0.12
r α ₂	Rat cortex	<5	5.58 ± 0.03	6.52 ± 0.04
r D ₁	Rat striatum	<5	5.59 ± 0.03	<5
r D ₂	Rat striatum	<5	5.89 ± 0.11	6.08 ± 0.03

Affinity is expressed as pK_i ± SEM. Numbers in brackets indicate the affinity ratio versus F15599.

D₁, D₂, dopamine receptors; h, human; p, porcine; r, rat; α₁, α₂, α-adrenoceptors.

adenosine, opiate, cannabinoid, benzodiazepine, GABA, sigma (less than 50% inhibition at 10 µmol·L⁻¹). F15599 also did not significantly interact with calcium (verapamil site), potassium, sodium or chloride channels and did not inhibit MAO-A or MAO-B enzyme activities. F15599 did not interact with 5-HT, dopamine or noradrenaline transporters. Thus, F15599 was 1000-fold selective for 5-HT_{1A} receptors versus all other receptors and binding sites examined.

Activation by F15599 of recombinant human 5-HT_{1A} receptors

In a rigorous *in vitro* system that discriminates high-efficacy agonists ([³⁵S]-GTPγS binding in membranes from C6 glial-h5-HT_{1A} cells), F15599 increased G-protein activation with a pEC₅₀ of 6.41 ± 0.06 and a maximal response of 70 ± 1% relative to that induced by 10 µmol·L⁻¹ 5-HT (mean ± SEM, *n* = 3). The efficacy of F15599 was higher, but its potency lower, than that of F13714 (pEC₅₀ 8.31 ± 0.18, E_{max} 61 ± 5), (+)8-OH-DPAT (pEC₅₀ 7.16 ± 0.09, E_{max} 55 ± 2) and 5-HT (pEC₅₀ 6.64 ± 0.14, E_{max} 94 ± 7). In HeLa-h5-HT_{1A} cell membranes (Figure 2; Table 4), F15599 maximally increased G-protein activation (E_{max} 102%) relative to 5-HT. Maximal efficacy was also observed for inhibition of cyclic AMP formation in the same cell line. However, in all cases, the potency of F15599 was one to two orders of magnitude less than that of F13714 and (+)8-OH-DPAT. In CHO-h5-HT_{1A} cells, F15599 efficaciously stimulated ERK1/2 phosphorylation (pERK) and, in HEK293-h5-HT_{1A} cells, F15599 stimulated HA-tagged h5-HT_{1A} receptor internalization.

F15599 exhibited a distinctive order of potency for these responses: the pEC₅₀ for ERK1/2 phosphorylation (7.81; Table 4) was greater than that for G-protein activation (6.97), which was similar to that for internalization (6.80), which was greater than that for inhibition of cAMP formation (6.46). (+)8-OH-DPAT also showed the highest potency for ERK1/2 phosphorylation but lowest potency for G-protein activation. In contrast, F13714 showed similar potency for ERK1/2 phosphorylation, internalization and G-protein activation. 5-HT showed its highest potency for G-protein activation and inhibition of cAMP formation, with lower potency for ERK1/2 phosphorylation and receptor internalization. Hence, the order of potencies for the different signalling pathways was different for each of the agonists tested (Table 4).

Activation by F15599 of native rat 5-HT_{1A} receptors in membranes from different brain regions

The influence of F15599 on G-protein activation ([³⁵S]-GTPγS binding) in different brain regions was diverse (Table 5). Agonist efficacy, relative to 5-HT (10 µmol·L⁻¹), varied from 54% in prefrontal cortex to 78% in hippocampal membranes. However, the stimulation by 5-HT is partially due to receptors other than 5-HT_{1A} (Newman-Tancredi *et al.*, 2003) such that the relative efficacy of a 5-HT_{1A} receptor 'full agonist' is necessarily less than 100%. As regards agonist potency, compounds differed between brain regions. Thus, in prefrontal cortex, F15599 was 2.2-fold more potent than in hippocampus, and sixfold more potent than in dorsal raphe (based on EC₅₀ ratios). In comparison, in prefrontal cortex, F13714 was 1.5-fold more potent than in hippocampus and 2.9-fold more potent than in dorsal raphe. In contrast, (+)8-OH-DPAT did not differ in potency between prefrontal cortex and hippocampus. In addition, a low slope value was observed for concentration-response curves in raphe membranes and for (+)8-OH-DPAT in prefrontal cortex (see Table 5, legend), suggesting the presence of heterogeneous populations of G-proteins.

In separate experiments, activation of G-proteins induced by F15599 (10 µmol·L⁻¹) (assayed as [³⁵S]-GTPγS binding) in rat hippocampal membranes was completely antagonized by WAY100635, indicating that it is specifically mediated by 5-HT_{1A} receptors (pK_B = 9.27 ± 0.03, *n* = 3).

Preferential activation by F15599 of G_{ai} over G_{ao} G-proteins

In a G-protein subtype targeting procedure (antibody capture coupled to detection by SPA), F15599 activated G_{ai} subunits in HeLa-h5-HT_{1A} cell membranes with modest potency (pEC₅₀ 6.96) but remarkably high efficacy (E_{max} 122%), exceeding that of F13714, (+)8-OH-DPAT and even 5-HT (Figure 3; Table 6).

F15599 also activated G_{ao} subunits in rat hippocampal membranes with high efficacy, similar to that of 5-HT, and exceeding that of F13714 and (+)8-OH-DPAT. However, the potency of F15599 (pEC₅₀ 6.07) was eightfold less than for G_{ai} activation and markedly less than that for activation of 'total' hippocampal G-proteins (i.e. without distinguishing the subtypes; pEC₅₀ 6.85; Table 5). In contrast, F13714 and (+)8-OH-DPAT exhibited potency for G_{ao} that was similar to that for G_{ai}

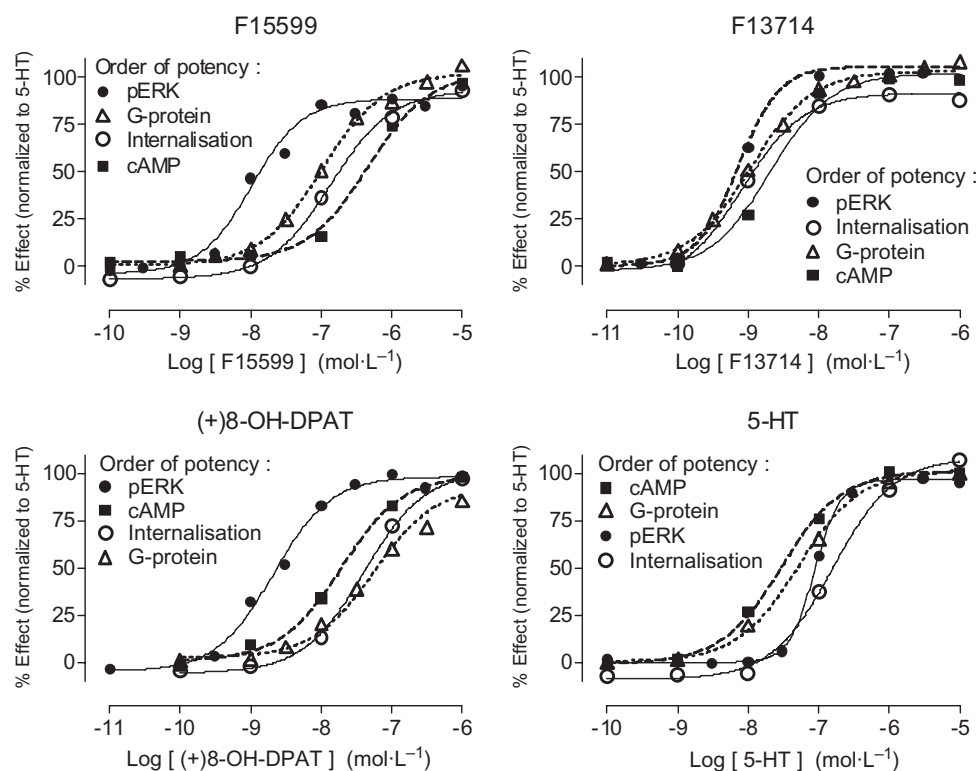


Figure 2 Influence of F15599 and reference agonists on: (i) stimulation of G-protein activation using [35 S]-GTP γ S binding at h5-HT $_{1A}$ receptors in HeLa cell membranes; (ii) inhibition of cAMP accumulation in HeLa-h5-HT $_{1A}$ cells; (iii) stimulation of ERK1/2 phosphorylation at h5-HT $_{1A}$ receptors expressed in CHO cells; and (iv) induction of HA-tagged h5-HT $_{1A}$ receptor internalization in HEK-293 cells. Data are expressed as percentage of the effect induced by a maximally effective concentration of 5-HT (10 μ mol·L $^{-1}$). Values are mean \pm SEM from at least three experiments performed in triplicate or in duplicate. The order of potency of the responses for the different transduction tests is agonist-dependent (see Table 4).

Table 4 Efficacy and potency of F15599 and comparators for stimulation of signal transduction responses in cell lines stably expressing h5-HT $_{1A}$ receptors

Efficacy model	F15599		F13714		(+)8-OH-DPAT		5-HT	
	E_{max} (%)	pEC_{50}	E_{max} (%)	pEC_{50}	E_{max} (%)	pEC_{50}	E_{max} (%)	pEC_{50}
Total G-protein ^a	102 \pm 3	6.97 \pm 0.08	73 \pm 14	9.02 \pm 0.12	107 \pm 3	7.17 \pm 0.05	101 \pm 9	7.32 \pm 0.05
cAMP inhibition	95 \pm 1	6.46 \pm 0.10	100 \pm 1	8.67 \pm 0.02	99 \pm 1	7.77 \pm 0.38	100 \pm 1	7.45 \pm 0.18
ERK1/2 phosphorylation	102 \pm 9	7.81 \pm 0.13	108 \pm 13	9.07 \pm 0.11	100 \pm 3 ^b	8.74 \pm 0.06 ^b	95 \pm 8 ^b	6.86 \pm 0.09 ^b
Receptor internalization	93 \pm 7	6.80 \pm 0.04	92 \pm 7	9.06 \pm 0.16	101 \pm 3	7.38 \pm 0.02	108 \pm 6 ^c	6.80 \pm 0.12 ^c

Efficacy (E_{max}) values are expressed as % of the stimulation induced by saturating concentrations (10 μ mol·L $^{-1}$) of 5-HT.

^aTotal G-protein activation refers to [35 S]-GTP γ S binding that does not distinguish between the G-protein subtypes involved: compare with data in Table 6 on G $_{oi}$ and G $_{ao}$ activation.

^bBruins Slot et al. (2006).

^cHeusler et al. (2008).

activation. Furthermore, they were more potent for G $_{ao}$ activation in hippocampal membranes than for total G-protein activation in the same preparation (compare data in Tables 5 and 6).

Agonism by F15599 at native rat 5-HT $_{1A}$ receptors: functional autoradiography

The influence of a saturating concentration (100 μ mol·L $^{-1}$) of F15599 was examined on [35 S]-GTP γ S binding to horizontal rat brain sections. Incubation with F15599 increased [35 S]-

GTP γ S binding to hippocampus, lateral septum and limbic cortex, areas that express high levels of 5-HT $_{1A}$ receptors (Figure 4). The effect of F15599 was blocked by co-incubation with the selective 5-HT $_{1A}$ receptor antagonist, WAY100635 (10 μ mol·L $^{-1}$). A low concentration (0.1 μ mol·L $^{-1}$) of F13714 exhibited a similar pattern of G-protein activation that was also abolished by WAY100635.

In vivo occupancy of 5-HT $_{1A}$ receptors by F15599

The 5-HT $_{1A}$ receptors in mouse brain were labelled *in vivo* with [3 H]WAY100635. Control (saline-treated) animals

Table 5 Efficacy and potency of F15599 and comparators for stimulation of [³⁵S]-GTPγS binding to membranes prepared from various brain regions

Tissue/cell line	F15599		F13714		(+)-8-OH-DPAT		5-HT	
	<i>E</i> _{max} (%)	<i>pEC</i> ₅₀	<i>E</i> _{max} (%)	<i>pEC</i> ₅₀	<i>E</i> _{max} (%)	<i>pEC</i> ₅₀	<i>E</i> _{max} (%)	<i>pEC</i> ₅₀
Prefrontal cortex	54 ± 3	7.20 ± 0.04	54 ± 6	9.19 ± 0.08	65 ± 2	7.62 ± 0.14	92 ± 5	7.25 ± 0.05
Entorhinal cortex	63 ± 7	7.00 ± 0.14	65 ± 12	9.14 ± 0.01	75 ± 6	7.41 ± 0.05	101 ± 7	7.07 ± 0.08
Hippocampus	78 ± 2	6.85 ± 0.03	77 ± 2	9.02 ± 0.04	77 ± 3	7.59 ± 0.04	101 ± 1	6.99 ± 0.03
Dorsal raphe	68 ± 8	6.42 ± 0.12	64 ± 4	8.73 ± 0.08	n.d.		n.d.	
Median raphe	57 ± 2	6.62 ± 0.06	57 ± 2	8.82 ± 0.07	n.d.		n.d.	

Efficacy (*E*_{max}) values are expressed as % of the stimulation induced by saturating concentrations (10 μmol·L⁻¹) of 5-HT. Slope values of the [³⁵S]-GTPγS binding isotherms did not significantly differ from unity except in: (i) dorsal raphe F15599 0.62 ± 0.07, F13714 0.76 ± 0.03; (ii) median raphe F13714 0.69 ± 0.03; and (iii) prefrontal cortex (+)-8-OH-DPAT 0.59 ± 0.06.

n.d., not determined.

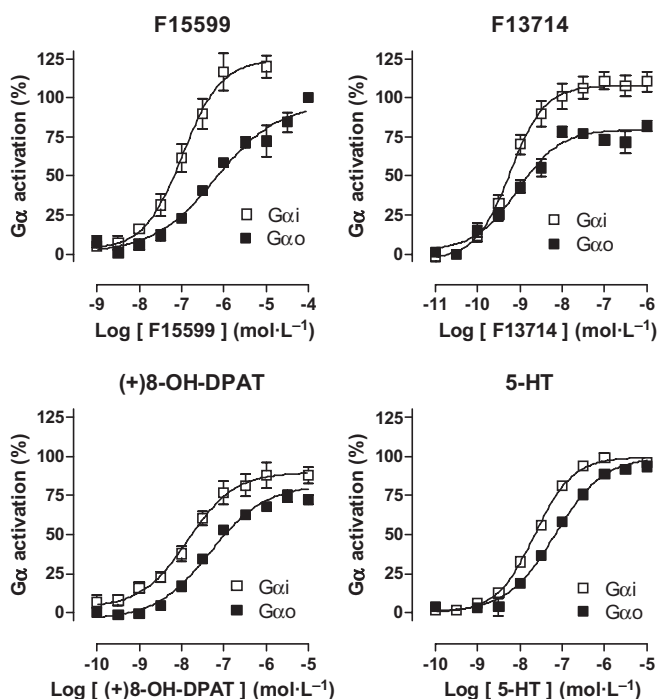


Figure 3 Influence of F15599 and reference agonists on G-protein subtype activation. G_{oi} subunits in HeLa-h5-HT_{1A} cell membranes and G_{oo} subunits in rat hippocampal membranes were targeted by [³⁵S]-GTPγS binding followed by antibody capture coupled to detection by scintillation proximity assay. Data are expressed as percentage of the effect induced by a maximally effective concentration of 5-HT (10 μmol·L⁻¹). Values are mean ± SEM from at least three experiments performed in triplicate or in duplicate.

exhibited radiolabelling of 517 ± 22 dpm·(mg wet weight)⁻¹ in the frontal cortex and 507 ± 22 dpm·(mg wet weight)⁻¹ in the hippocampus. F15599 and F13714 [0.16–640 mg·kg⁻¹ per os (p.o.)] dose-dependently inhibited [³H]WAY100635 binding in these brain structures. The ID₅₀ values were similar in both brain regions (Figure 5). F15599: 2.5 mg·kg⁻¹ [95% confidence interval (CI): 1.0 to 6.4] in frontal cortex and 2.2 mg·kg⁻¹ (95% CI: 0.57 to 8.7) in hippocampus. F13714: 0.95 mg·kg⁻¹ (95% CI: 0.69 to 1.3) in frontal cortex and 0.95 mg·kg⁻¹ (95% CI: 0.63 to 1.23) in hippocampus. The maximal inhibition of labelling by F15599 was similar to that produced by F13714 (26 ± 6% binding remaining,

compared with 22 ± 3%, respectively, in cortex). However, the inhibition curve of F15599 was noticeably shallower than that of F13714, possibly indicating the presence of more than one population of 5-HT_{1A} binding sites.

Modulation of ERK1/2 phosphorylation in rat brain

In preliminary experiments, the induction of ERK1/2 phosphorylation by F15599 was time-dependent: ANOVA indicated a significant effect of time in the hippocampus (*F*_{4,25} = 8.53, *P* < 0.001), prefrontal cortex (*F*_{4,25} = 7.09, *P* < 0.001) and hypothalamus (*F*_{4,25} = 4.14, *P* < 0.05; data not shown). A decrease in pERK1/2 levels is observed in hippocampus, while an increase was observed in prefrontal cortex and hypothalamus. A treatment interval of 30 min (used in all subsequent experiments) was determined, permitting analysis of the effects in the three brain regions.

Dose-response experiments showed a significant effect of treatment in hippocampus (*F*_{4,25} = 8.42, *P* < 0.001), prefrontal cortex (*F*_{4,25} = 20.15, *P* < 0.0001) and hypothalamus (*F*_{4,25} = 9.97, *P* < 0.0001). The 0.63 mg·kg⁻¹ dose of F15599 was the first to reduce hippocampal pERK1/2 levels (–47% relative to basal values, *P* < 0.0001) and to increase pERK1/2 levels in both the prefrontal cortex and hypothalamus (+53% and +74% relative to basal values, *P* < 0.001 for both; Figure 6, left-hand graphs).

Pre-treatment with WAY100635 (0.63 mg·kg⁻¹ subcutaneously) did not modify pERK1/2 levels in saline-injected animals but abolished the F15599 (0.63 mg·kg⁻¹ i.p.)-induced decrease in hippocampal pERK1/2 levels (*P* < 0.001 as compared with saline-saline group) and increase in pERK1/2 levels in prefrontal cortex (*P* < 0.001 as compared with saline-saline group; Figure 6, right-hand graphs). Although in this interaction study with WAY100635, F15599 did not significantly modify the pERK1/2 levels in hypothalamus, the slight increase observed with F15599 was reversed by WAY100635 (Figure 6, right-hand graphs).

In control experiments, quantitative ELISA assays were carried out to determine total ERK1/2 levels, showing that these were not significantly modified by treatment with various doses of F15599 (*n* = 3 rats per group, hippocampus *F*_{4,10} = 0.24, *P* = 0.907; prefrontal cortex *F*_{4,10} = 1.13, *P* = 0.395; hypothalamus *F*_{4,10} = 0.71, *P* = 0.602 respectively; data not shown).

Table 6 Efficacy and potency of F15599 and comparators for stimulation of [³⁵S]-GTPγS binding to G_{ai} and G_{ao} subunits

Tissue/cell line	F15599		F13714		(+)8-OH-DPAT		5-HT	
	E _{max} (%)	pEC ₅₀	E _{max} (%)	pEC ₅₀	E _{max} (%)	pEC ₅₀	E _{max} (%)	pEC ₅₀
G _{ai} (HeLa-h5-HT _{1A} membranes)	122 ± 8	6.96 ± 0.08	111 ± 6	9.15 ± 0.08	90 ± 8	7.79 ± 0.13	100 ± 1	7.63 ± 0.04
G _{ao} (hippocampus)	103 ± 8	6.07 ± 0.06	83 ± 6	9.11 ± 0.16	82 ± 4	7.37 ± 0.06	100 ± 1	7.18 ± 0.07

Efficacy (E_{max}) values are expressed as % of the stimulation induced by saturating concentrations (10 μmol·L⁻¹) of 5-HT.

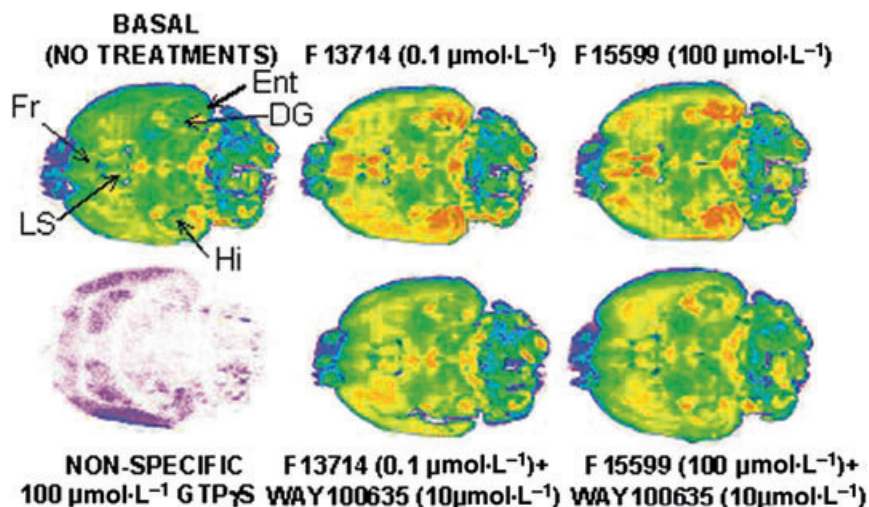


Figure 4 Influence of F15599 and F13714 on G-protein activation in horizontal brain sections. Pseudo-colour images of sections incubated with [³⁵S]-GTPγS and drug treatments: blue/pink indicates low levels of activation while red represents high activation. Images are from a representative experiment. Measurements were performed on sets of serial sections from six animals, each set representing all treatments presented. DG, dentate gyrus; Ent, entorhinal cortex; Fr, frontal cortex; Hi, hippocampus; LS, lateral septum.

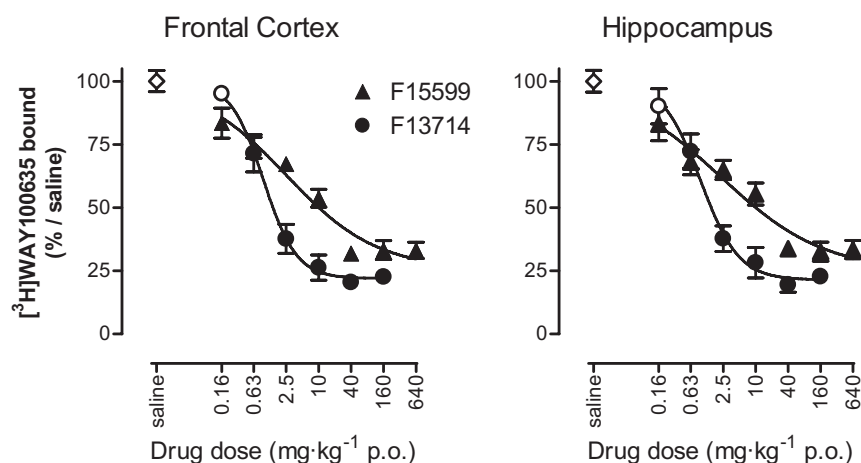


Figure 5 Inhibition of [³H]WAY100635 binding *in vivo* in mouse hippocampus and frontal cortex. Compounds were administered per os (p.o.) 30 min before intravenous injection of the radiotracer. Animals were then killed 30 min later. Each point is the mean ± SEM of five animals. Filled symbols indicate significant difference ($P < 0.05$) from control group (one-way ANOVA followed by Dunnett's test).

Induction of *c-fos* expression in rat brain

The action of F15599 on *c-fos* mRNA was determined using quantitative polymerase chain reaction with Arbp as the housekeeping gene, in different rat brain regions 30 min after i.p. injection of different doses of compound. The most potent action of F15599 was observed in prefrontal cortex where increased *c-fos* expression was observed at the lowest

dose tested (0.16 mg·kg⁻¹). In other brain regions expressing post-synaptic 5-HT_{1A} receptors (hippocampus, septum, entorhinal cortex, hypothalamus), F15599 induced *c-fos* expression at doses of 0.63 mg·kg⁻¹ (Figure 7). In brain regions expressing pre-synaptic 5-HT_{1A} receptors, F15599 induced some *c-fos* mRNA expression in the dorsal raphe nucleus but was totally inactive in median raphe. The mag-

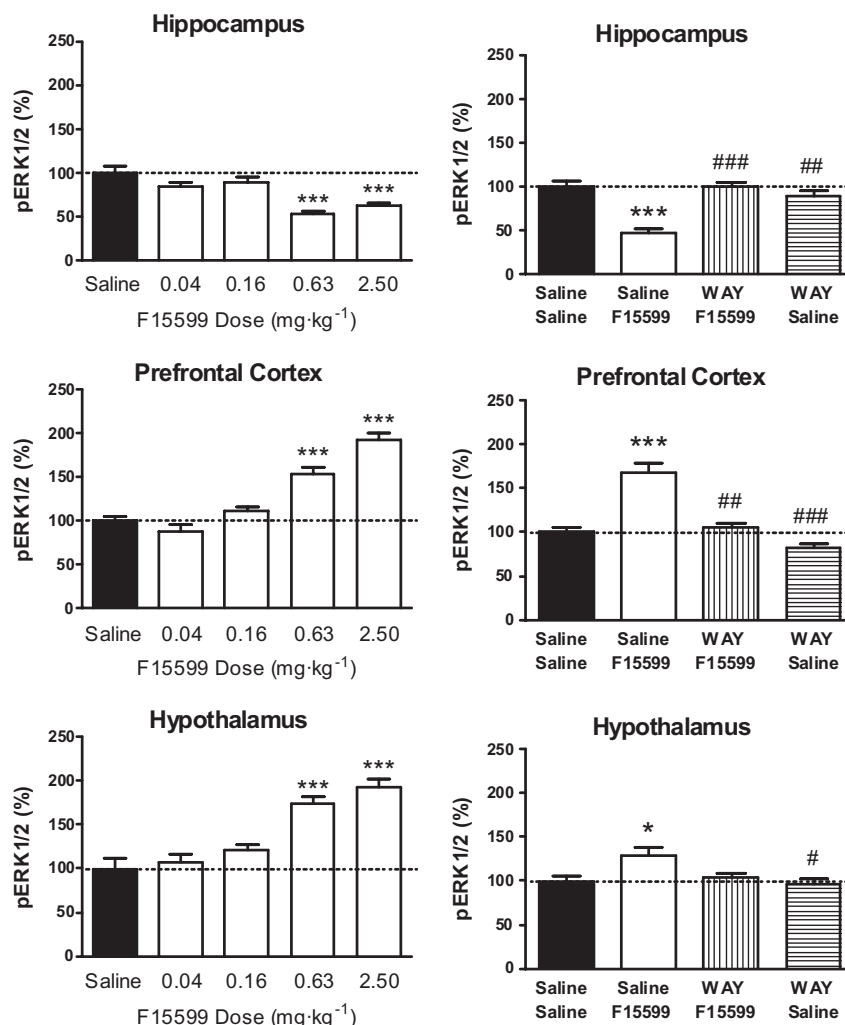


Figure 6 Influence of F15599 on phospho-ERK1/2 (pERK1/2) levels in rat hippocampus, prefrontal cortex and hypothalamus at 30 min post injection. F15599 was tested either alone (left-hand graphs) or following pre-treatment with WAY100635 (0.63 mg·kg⁻¹ subcutaneously; right-hand graphs). The mean value of the control group (saline-treated animals) was set at 100 (basal value) and all data were expressed as percentages relative to basal value (%). Data represent the mean \pm SEM ($n = 12$, two measures for each of six rats per group). ANOVA analyses were performed on raw data (see *Methods*): * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ as compared with saline/saline + saline group; # $P < 0.05$, ## $P < 0.01$ and ### $P < 0.001$ as compared with saline + F15599 group.

nitide of induction of c-fos mRNA expression by F15599 was highly diverse according to the brain area examined: a strong induction of c-fos was observed in prefrontal cortex, septum and hypothalamus (four- to five-fold induction relative to vehicle-treated animals) whereas a modest induction of c-fos was observed in dorsal raphe, entorhinal cortex, hippocampus and globus pallidus (2- to 2.5-fold, Figure 7).

Discussion

The main finding of this study is that F15599 is a highly selective, high-efficacy 5-HT_{1A} receptor agonist that exhibits distinctive signal transduction properties. F15599 is substantially less potent *in vitro* than a closely related analogue, F13714, or the prototypical agonist, (+)8-OH-DPAT, but potently occupies 5-HT_{1A} receptors *in vivo* and activates

ERK1/2 phosphorylation *ex vivo*. Notably, whereas F15599 exhibits little activation of c-fos expression in dorsal or median raphe, it potently stimulates c-fos in prefrontal cortex, hypothalamus and septum, suggesting preferential activation of 5-HT_{1A} receptors located in brain regions where those receptors are post-synaptic.

Affinity and selectivity of F15599 at 5-HT_{1A} receptors

F15599 exhibited exceptional selectivity for 5-HT_{1A} receptors. This was demonstrated in binding experiments where no interaction was detected with a large number of other receptors, binding sites and enzymes. The selectivity of F15599 was greater than 1000-fold for all sites examined. The selectivity of F15599 for 5-HT_{1A} was also greater than that of its congener, F13714 and of (+)8-OH-DPAT (Table 3). Notably, the latter has some affinity for 5-HT₇ receptors (Sprouse *et al.*, 2004), whereas F15599 did not interact with

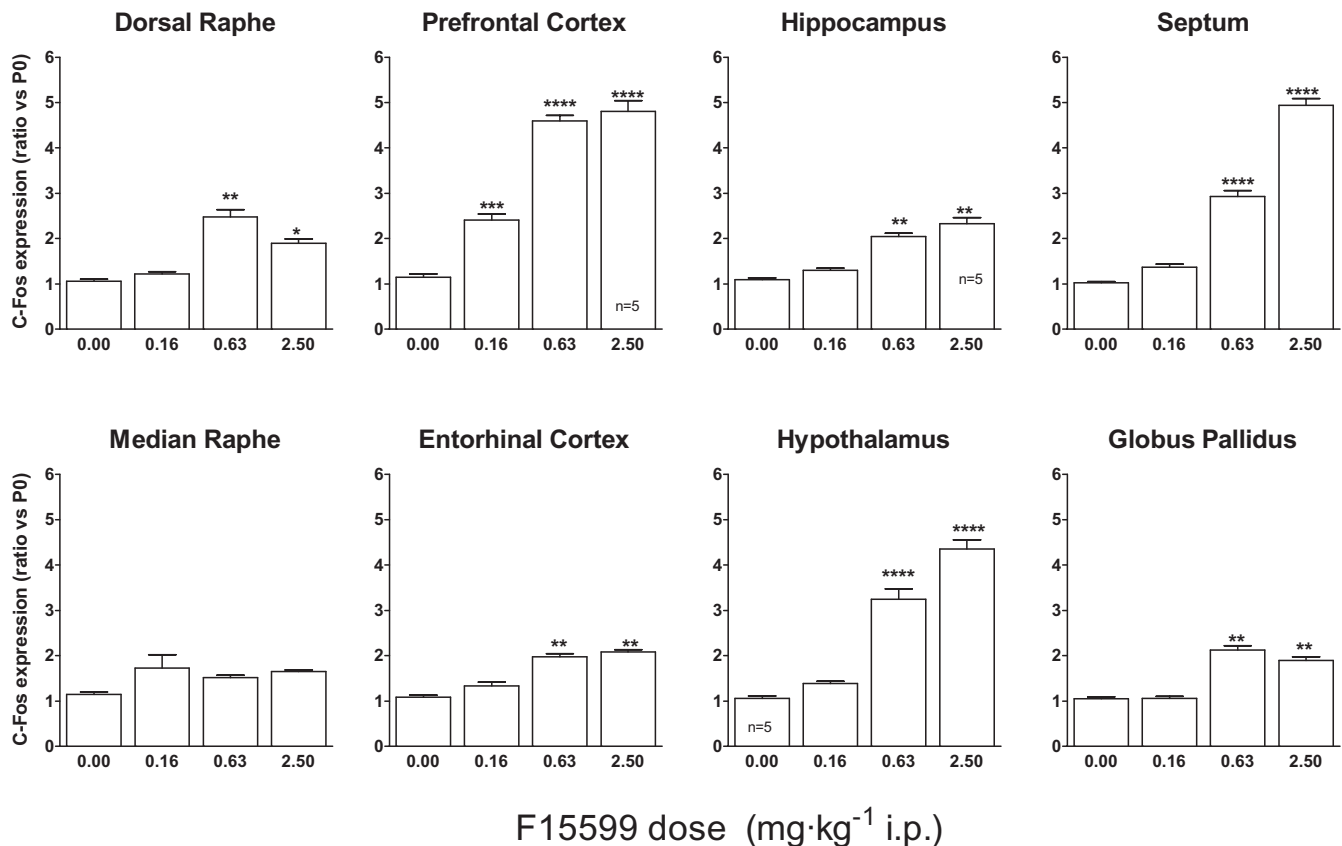


Figure 7 Influence of F15599 on c-fos mRNA induction by F15599 in different rat brain regions. The mean value of the control group (saline-treated animals) was set at 1 (basal value) using Arbp (i.e. P0) as the reference gene for normalization. All data were expressed as fold induction relative to basal value. Except where indicated otherwise, bars represent the mean \pm SEM of values from six animals. ANOVA with Tukey's test was used to evaluate differences in c-fos expression between treatment groups. The significance level is fixed at 5%.

this site. The selectivity of F15599 was supported by the observation that it stimulated G-protein activation in functional ($[^3S]$ -GTP γ S) autoradiography experiments in those brain regions that express 5-HT_{1A} receptors. Further, the selective 5-HT_{1A} receptor antagonist, WAY100635, abolished the effects of F15599 (Figure 4). These data are consistent with the observation that the neurochemical and behavioural effects of F15599 are all also abolished by 5-HT_{1A} antagonism, reflecting its remarkable selectivity (see *Introduction* and Assié *et al.*, 2006a; Auclair *et al.*, 2007; Celada *et al.*, 2007). It is interesting that the affinity of F15599 at h5-HT_{1A} receptors was 9- and 68-fold less than that of (+)8-OH-DPAT or F13714 (Table 3). This observation corresponds to *in vitro* measures of potency for signal transduction. However, such differences in potency are not observed for *in vivo* binding at mouse brain 5-HT_{1A} receptors. Thus, the ID₅₀ values determined for inhibition of the binding of the radiotracer (the selective 5-HT_{1A} receptor antagonist, [3 H]WAY100635) were less than threefold higher for F15599 than for F13714 (Figure 5). The unexpectedly potent *in vivo* affinity of F15599 cannot be readily attributed to a potential active metabolite arising from pharmacokinetic properties (in rat, F15599 possesses plasma half-life greater than 24 h, F. Sautel, unpubl. obs.), although other differences, such as lipophilicity, permeability and/or blood-brain barrier penetration, may play a role. Nevertheless, taken together with the frontal

cortex preference of F15599 seen in other models (see *Introduction*), the data suggest that the potent 5-HT_{1A} receptor occupancy observed for F15599 arises from its agonist-receptor interaction, possibly involving high affinity for receptor populations (not subtypes) coupled to specific G-protein subtypes. This conclusion is supported by the observation that the dose-response curve for F15599 was substantially shallower than that for F13714, suggesting that different conformations or affinity states of 5-HT_{1A} receptors may be distinguished by F15599 but not F13714.

Agonist efficacy of F15599 at human and rat 5-HT_{1A} receptors *in vitro*

In all the tests of signal transduction, F15599 displayed high agonist efficacy (see Tables 4–6) at both cloned human and native rat 5-HT_{1A} receptors. Indeed, the response to F15599 in all the systems tested is comparable with that observed for F13714 or (+)8-OH-DPAT [note: (+)OH-DPAT possesses approximately twofold greater efficacy than (–)8-OH-DPAT; Lejeune *et al.*, 1997]. F15599 is also much more efficacious than buspirone (Newman-Tancredi *et al.*, 2005), a clinically used anxiolytic. In contrast to its efficacy, the potency of F15599 in functional tests is generally modest, with pEC₅₀ values one to two orders of magnitude lower than for (+)8-OH-DPAT and F13714, consistent with the

lower binding affinity of F15599 (see above). Interestingly, F15599 exhibits a distinctive pattern of stimulation of signalling responses. Thus, F15599 was more potent for stimulation of ERK1/2 phosphorylation compared with G-protein activation, receptor internalization and adenylyl cyclase inhibition (Table 4). Indeed, each of the four agonists displayed a distinct order of potency for these signalling responses (Figure 2) and the more 'downstream' responses were not always more potently activated than activation of G-proteins or adenylyl cyclase inhibition. This indicates that there is not a systematic 'signal amplification' between receptor activation and intracellular signalling cascades and suggests that the agonists are preferentially directing responses to specific signalling cascades. Although the underlying mechanisms that produce the differential profiles of the 5-HT_{1A} agonists require further elucidation, the data suggest that F15599 and the other agonists exhibit functional selectivity (Kenakin, 2007). Given that all the second messenger responses are G-protein-mediated, the differences in signalling preference may result from activation of distinct G-protein subtypes. We therefore performed [³⁵S]-GTPγS binding experiments on membranes prepared from different brain regions (Table 5). The data showed that the potency (pEC₅₀ values) of the agonists vary according to brain region, with F15599 exhibiting greater potency in membranes from frontal cortex than from raphe. In addition, the slope values for the raphe were shallow (see legend of Table 5), an observation that is suggestive of G-protein heterogeneity. Thus, F15599 and the other agonists may differentially influence G-protein coupling in the different brain regions.

This hypothesis is supported by the observation that F15599 displayed enhanced activation of specific G-protein subtypes: its pEC₅₀ for G_{ai} activation (6.96) was nearly an order of magnitude greater than that for G_{ao} activation (6.07; Table 6). In contrast, 5-HT displayed similar potencies for both G_α subtypes while F13714 and (+)8-OH-DPAT had intermediate profiles (Figure 3). Taken together, these data suggest that F15599 may display signalling pathway specificity at 5-HT_{1A} receptors due to preferential activation of G_{ai} subunits. However, it should be noted that G_{ai} activation was determined in HeLa-h5-HT_{1A} cells because it is difficult to measure in brain tissue using the present antibody capture/SPA methodology, possibly because of low native G_{ai} expression levels. Further, we detected G_{ao} G-protein activation but did not differentiate between G_{ao} subtypes. Therefore, the latter's contribution to the actions of 5-HT_{1A} agonists remains to be evaluated. In addition, other parameters may influence G-protein activation, including RGS proteins, and other intracellular cofactors. Nevertheless, despite these caveats, the concept of G-protein-based 'functional selectivity' at 5-HT_{1A} receptors has been suggested previously: ipsapirone, but not rauwolscine, preferentially directs 5-HT_{1A} coupling to G_{ai3} rather than G_{ai2} (Gettys *et al.*, 1994) and pindolol preferentially activated G_{ai3} versus other G-proteins (Newman-Tancredi *et al.*, 2002). Functional selectivity at 5-HT_{1A} receptors is closely associated with the possibility of preferential activity in specific brain regions. Thus, although only a single molecular entity of 5-HT_{1A} receptors has been identified, the affinity of a series of 5-HT receptor agonists

was two- to fivefold lower at pre-synaptic 5-HT_{1A} receptors in the raphe than at post-synaptic 5-HT_{1A} receptors in the hippocampus (Johnson *et al.*, 1997), suggesting heterogeneity of 5-HT_{1A} receptor populations and potential for differential drug targeting.

Taken together, these data suggest that F15599 displays preferential activation of G_{ai} and that this is associated with a specific order of potency on intracellular signalling cascades. Such mechanisms would underlie the post-synaptic cortical preference of F15599 observed in neurotransmitter release and electrophysiological measures (Assié *et al.*, 2006a; Celada *et al.*, 2007).

Influence of F15599 on ERK1/2 phosphorylation in different brain regions ex vivo

In view of the potent *in vitro* stimulation of ERK1/2 phosphorylation by F15599 (Table 4), it was interesting to examine this response *ex vivo* in rat. Indeed, ERK1/2 is implicated in both long-term processes, such as neuronal plasticity/differentiation, and short-term influence on spatial memory and mood. 5-HT_{1A} receptors influence ERK1/2 phosphorylation both *in vitro* (Bruins Slot *et al.*, 2006) and *in vivo* (Chen *et al.*, 2002; Sullivan *et al.*, 2005; Crane *et al.*, 2007). Stimulation of ERK1/2 phosphorylation by F15599 was time, brain region and dose-dependent (Figure 6). In frontal cortex, ERK1/2 phosphorylation is controlled by activation of post-synaptic 5-HT_{1A} receptors. In contrast, inhibition of ERK1/2 phosphorylation in hippocampus by 5-HT_{1A} receptor agonists is likely to involve inhibition of 5-HT release consequent to activation of raphe-localized 5-HT_{1A} autoreceptors (Chen *et al.*, 2002). Thus, in *ex vivo* studies, F15599 (0.63 mg·kg⁻¹ i.p.) stimulated ERK1/2 phosphorylation in frontal cortex and reduced it in hippocampus, both of these responses being mediated by 5-HT_{1A} receptors, as shown by their reversal with WAY100635 (Figure 6). In comparison, F13714, but not (+)8-OH-DPAT, stimulated ERK1/2 phosphorylation in frontal cortex at 0.16 mg·kg⁻¹ but reduced ERK1/2 phosphorylation in hippocampus from 0.04 mg·kg⁻¹ i.p. (Buritova *et al.*, 2007), suggesting preferential activation of pre-synaptic 5-HT_{1A} receptors. Analysis of ERK1/2 in hypothalamus, another region expressing post-synaptic receptors, yielded similar tendency to that seen in frontal cortex but with reduced stimulation. Taken together, these data provide additional evidence for potent 5-HT_{1A} receptor activation by F15599 in rodents and support the notion that selective 5-HT_{1A} agonists can differentially regulate signal transduction in different brain regions. It is interesting that h5-HT_{1A} receptor-mediated ERK1/2 phosphorylation *in vitro* is mediated by G_{ai} subtypes (Lin *et al.*, 2002). Given the capacity of F15599 to preferentially activate G_{ai} versus G_{ao} (see above), this provides a mechanistic underpinning for its potent induction of ERK1/2 phosphorylation in rat, although a specifically designed study would be necessary to identify the precise G-protein subtypes involved.

Influence of F15599 on c-fos mRNA expression

Further evidence for a brain region-selective influence of F15599 was generated from c-fos mRNA expression studies

(Figure 7). These showed that induction of c-fos did not directly correlate with the known density of 5-HT_{1A} receptor throughout the rat brain. Thus, F15599 efficaciously stimulated expression of this immediate early gene in the frontal cortex, septum and hypothalamus (regions expressing post-synaptic 5-HT_{1A} receptors) but very little in median or dorsal raphe (the locus of 5-HT_{1A} auto-receptors) or in entorhinal cortex and hippocampus (which express high levels of post-synaptic 5-HT_{1A} receptors) or globus pallidus. In comparison, F13714 potently stimulated c-fos expression in both frontal cortex and also median and dorsal raphe whereas (+)8-OH-DPAT exhibited an intermediate profile (Buritova *et al.*, 2008). These data reinforce the conclusion that selective and efficacious 5-HT_{1A} receptor agonists can induce activation of 5-HT_{1A} receptor signalling in a brain region-dependent manner. In the case of F15599, its activity appears to be preferentially expressed at sub-populations of 5-HT_{1A} receptors found in brain regions with post-synaptic locations. In particular, the potent activation by F15599 of 5-HT_{1A} receptors in the frontal cortex (from 0.16 mg·kg⁻¹) is consistent with electrophysiological data showing that F15599 potently increases cortical pyramidal neuron cell firing in anaesthetized rats at doses 20-fold lower than those that inhibit dorsal raphe neuron firing (Celada *et al.*, 2007). In addition, F15599 also increases dopamine release in the frontal cortex (a post-synaptic 5-HT_{1A} receptor-mediated response) at doses about eightfold lower than those that inhibit 5-HT release in hippocampus (a pre-synaptic 5-HT_{1A} receptor-mediated response; Assié *et al.*, 2006a).

In summary, F15599 is a novel, highly selective and efficacious agonist at 5-HT_{1A} receptors. In a series of *in vitro* signal transduction assays, it induced a distinctive pattern of responses, unlike that of reference agonists. Although the agonist potency of F15599 *in vitro* was more modest than that of other agonists (Tables 4 and 5), its *in vivo* binding affinity was almost as great as that of its potent congener, F13714. F15599 was also remarkably potent for *ex vivo* stimulation of ERK1/2 phosphorylation, being active at low doses, comparable with those determined for 8-OH-DPAT. F15599, unlike F13714, was more potent for activation of G_o G-protein subunits than G_o, suggesting that it exhibited functional selectivity for 5-HT_{1A} receptor signalling. F15599 may, thus, preferentially activate some brain region-specific signal transduction pathways, a conclusion reinforced by the observation that it potently induces c-fos mRNA expression in frontal cortex, a region where 5-HT_{1A} receptors are post-synaptically located, but hardly at all in dorsal or median raphe, regions that express pre-synaptic receptors. Indeed, 5-HT_{1A} receptor signalling in these brain regions is mechanistically distinct, possibly involving different populations of G-protein subtypes. The present data provide a mechanistic basis for interpreting the profile of action of F15599 seen in microdialysis experiments and electrophysiological studies, where it shows marked preference for activation of cortical 5-HT_{1A} receptors and only little activity at pre-synaptic sites (see above). The preferential agonist properties at post-synaptic 5-HT_{1A} receptors displayed by F15599 are likely to underlie its favourable activity in rodent models of depression and cognitive deficits and would be of considerable promise for the treatment of depressive states and other psychiatric disorders.

Acknowledgements

All the authors are employees of Centre de Recherche Pierre Fabre. Nathalie Leduc, Nathalie Danty, Valérie Faucillon, Nathalie Consul-Denjean, Anne-Marie Ormière, Nathalie Delmas, Stéphanie Tardif, Marie-Christine Ailhaud, Géraldine Berrichon, Sabine Callewaert and Yann Leroy are thanked for technical assistance. Sophie Bréand, Jérôme Besse and Audrey Dos Santos are thanked for assistance with statistical analysis.

Conflicts of interest

All the authors are employees of Centre de Recherche Pierre Fabre.

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