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Antidepressant-like activity of VN2222, a serotonin reuptake inhibitor with high affinity at 5-HT_{1A} receptors

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Abstract

It has been suggested that drugs combining serotonin (5-hydroxytryptamine, 5-HT) transporter blockade and 5-HT_{1A} autoreceptor antagonism could be a novel strategy for a shorter onset of action and higher therapeutic efficacy of antidepressants. The present study was aimed at characterizing the pharmacology of 1-(3-benzo[b]tiophenyl)-3-[4-(2-methoxyphenyl)-1-piperazinyl]-1-propanol (VN2222) a new synthetic compound with high affinity at both the 5-HT transporter and 5-HT_{1A} receptors and devoid of high affinity at other receptors studied, with the only exception of α_1-adrenoceptors. In keeping with the binding affinity at the 5-HT transporter, VN2222 inhibited 5-HT uptake *in vitro* both in rat cortical synaptosomes and in mesencephalic cultures and also *in vivo* when administered locally into the rat ventral hippocampus. After systemic administration, VN2222 exhibited an inverted U-shape effect so the inhibition of [^{3}H]5-HT uptake *ex vivo* and the increase in 5-HT extracellular levels in microdialysis experiments was observed at low doses of 0.01–0.1 mg/kg whereas higher doses were ineffective. In studies related to 5-HT_{1A} receptor function, 0.01–0.1 μM VN2222 produced a partial inhibition of forskolin-stimulated cAMP formation behaving as a weak agonist of 5-HT_{1A} receptors. In body temperature studies, 5 mg/kg VN2222 produced a mild hypothermic effect in mice, suggesting a weak agonist activity at presynaptic 5-HT_{1A} receptors; much lower doses (0.01–0.5 mg/kg) partially antagonized the hypothermia induced by 8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT) possibly through 5-HT transporter blockade. In the learned helplessness test in rats, an animal model for antidepressants, 1–5 mg/kg VN2222 reduced significantly the number of escape failures. Consequently, VN2222 is a new compound with a dual effect on the serotonergic system, as 5-HT uptake blocker and 5-HT_{1A} receptor partial agonist, and with a remarkable activity in an animal model of depression with high predictive validity.

Keywords: Antidepressant; 5-HT (5-hydroxytryptamine, serotonin); Microdialysis; 5-HT (5-hydroxytryptamine, serotonin) transporter; 5-HT_{1A} receptor; Learned helplessness test

1. Introduction

It is well known that selective serotonin reuptake inhibitors are effective in the treatment of depression. A lag time of 2–4 weeks is however necessary between initiation of selective serotonin reuptake inhibitor therapy and the onset of beneficial effects (Asberg et al., 1986). This delay in the clinical action of selective serotonin reuptake inhibitors has been related to their limiting effects on forebrain 5-hydroxytriptamine extracellular levels. Acutely administered selective serotonin reuptake inhibitors preferentially increase extracellular levels of 5-HT in the raphe nuclei (Invernizzi et al., 1992; Hervás and Artigas, 1998) resulting in an activation of somatodendritic 5-HT_{1A} receptors that inhibit the firing activity of 5-HT neurons (Gartside et al., 1995; Blier et al., 1987). It has been repeatedly suggested that the lag time for the therapeutic effect of antidepressants is related, at least in part, to the functional desensitization of somatodendritic 5-HT_{1A} autoreceptors (Chaput et al., 1986; Moret and Briley, 1990; Kreiss and Lucki, 1994; Gardier et al., 1996). The higher extracellular 5-HT levels found in different forebrain regions after chronic but not acute administration of selective serotonin reuptake inhibitors support this hypothesis (Bel and Artigas, 1993; Kreiss and Lucki, 1994). Present strategies aimed at accelerating the onset of the antidepressant effect of selective serotonin reuptake

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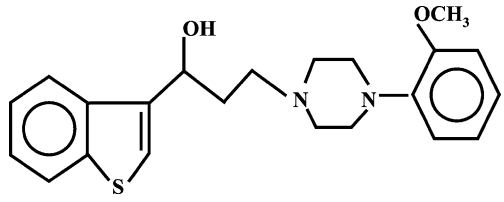

Fig. 1. Chemical structure of VN2222.

inhibitors are focused on shortening the time to produce an enhancement of 5-HT neurotransmission. Interestingly, it has been reported that the combination of drugs with 5-HT_{1A} receptor antagonist and 5-HT reuptake blocking properties results in a potentiation of the effects on forebrain extracellular 5-HT by preventing the self-limiting effect of 5-HT at the somatodendritic level. (Dreshfield et al., 1996). Recent clinical studies in which pindolol accelerated the antidepressant effect of selective serotonin reuptake inhibitors and improved the response to antidepressant treatment appear to confirm this assertion (Artigas et al., 1994; Pérez et al., 1997; Zanardi et al., 1997). So, it seems that a drug combining selective serotonin reuptake inhibition with 5-HT_{1A} autoreceptor antagonism could reach better its therapeutical goal.

1-(3-Benzo[b]tiophenyl)-3-[4-(2-methoxyphenyl)-1-piperazinyl]-1-propanol (VN2222; Fig. 1) was selected from a series of 86 new phenylpiperazine derivatives which were screened for their affinity at the 5-HT transporter and the 5-HT_{1A} receptor (Martínez-Esparza et al., 2001). Many of them showed a high/very high affinity at the 5-HT_{1A} receptor but only VN2222 was also endowed with a high affinity at the 5-HT transporter. Very low doses of VN2222 also reduced significantly the immobility time of mice in the forced swimming test, an animal model of depression (Martínez-Esparza et al., 2001). We herein report the further pharmacological characterization of this new compound.

2. Materials and methods

2.1. Animals

Male Swiss mice (23–28 g) housed in groups of 10 and male Wistar rats (260–280 g for microdialysis experiments and 180–220 g for all other studies) housed in groups of five were used. Animals were used only once and were kept in conditions of constant temperature (22 ± 1 °C) controlled lighting on a 12-h light/dark cycle and free access to food and water.

2.2. Monoamine uptake studies "in vitro" and "ex vivo"

These studies were performed as described (Cheng et al., 1993). Briefly, rats were killed and the cerebral cortex was dissected and homogenized in 15 volumes of 0.32 M ice-cold sucrose. The homogenates were centrifuged for 10 min at 1000 × *g* and 4 °C and the supernatants centrifuged again at 48 000 × *g* for 15 min. The resulting pellet was gently suspended in 0.27 M ice-cold sucrose. In this crude synaptosomal preparation, the protein concentration was 0.3–0.5 mg/ml. An aliquot (250 μl) of the synaptosomal preparation was added into 750 μl of Krebs buffer (pH 7.4) containing (mM): NaCl 115, KCl 4.97, $CaCl_2$ 1, $MgSO_4$ 1.22, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11.1, pargyline 0.01, ascorbic 1.7 and either [^{3}H]5-HT 1 nM or [^{3}H]noradrenaline 10 nM (for 5-hydroxytriptamine and noradrenaline uptake, respectively), in the absence (total uptake) or presence of competing compounds (six concentrations from 0.1 nM to 10 μM). After 6 min at 37 °C, the reaction was terminated by rapid filtration through Whatman GF/B filters.

For the measurement of serotonin uptake *ex vivo*, VN2222 or fluoxetine were administered i.p. or p.o. to rats 45 or 60 min, respectively, before killing the animals. [^{3}H]5-HT uptake was measured as above described.

2.3. Basal and K^+-evoked [^{3}H]5-HT release from cultured mesencephalic neurons

Cultures were prepared as described (Wichems et al., 1995) from the rostral portion of the rhombencephalon of fetal rat brains (E17). This region includes the 5-HT containing cell groups B7-9 which in the adult animal are located within the dorsal and median raphe nucleus. Cells were maintained in culture for 5–7 days before release experiments were performed. For these studies, medium was removed from the cultures which were rinsed once with preincubation buffer (138.75 mM NaCl, 2.6 mM KCl, 1 mM Na_2HPO_4, 0.75 mM $MgCl_2$, 2 mM $CaCl_2$, 6 mg/ml glucose, 37 °C, pH 7.4). Incubation buffer (preincubation buffer with the addition of 100 μM pargyline, 600 μM ascorbate and 1 mg/ml bovine serum albumin, 37 °C, pH 7.4) containing 50 nM [^{3}H]5-HT was then added. Thirty minues later, the medium was aspirated and replaced with fresh incubation medium at 5-min intervals for 20 min. After this washing period, 14 fractions (2 ml) were collected every 5 min. KCl (20 mM) was added in fractions 5 and 12 (S_1 and S_2, respectively). The effect on basal [^{3}H]5-HT outflow was determined by calculating the percentage change over basal efflux in two 5-min periods, i.e. before and after adding the drug. The effect of drugs on K^+-evoked [^{3}H]5-HT release was expressed as S_2/S_1 ratio. S_2 and S_1 were calculated as K^+-stimulated tritium increase on basal efflux.

2.4. Microdialysis experiments in freely moving rats

Male rats (260–280 g) were anaesthetised with 5 mg/kg ketamine and placed in a D. Kopf stereotaxic frame. A concentric microdialysis membrane (250 μM Cuprophan, 4 mm long outer diameter; Applied Neuroscience, UK) was

stereotaxically implanted into the right ventral hippocampus (AP-5.8, L+4.6 from bregma, 8.5 mm from dura; Paxinos and Watson, 1986). Twenty hours later, microdialysis experiments were initiated by perfusing the ventral hippocampus with artificial cerebrospinal fluid (composition in mM: NaCl 125, KCl 2.5, $CaCl_2$ 1.26, $MgCl_2$ 1.18; Adell and Artigas, 1998) through the probe. The probe was connected via a plastic tubing to a 2.5-ml syringe mounted on a microinfusion pump (mod. 100, CMA Microdialysis, Sweden) at a flow rate of 1 μl/min and, after a 2-h stabilization period, dialysates (20 μl) were collected every 20 min. After four basal samples were collected, drugs were administered either locally, dissolved in artificial cerebrospinal fluid, or i.p., and the response was followed for a further 3 h. At the end of each experiment, the brain was removed and the location of the microdialysis probe was verified by visual inspection, cutting the brains at the appropriate level. The concentration of 5-HT in the dialysate was determined by high performance liquid chromatography (HPLC) with coulometric detection (mod. 5200A Coulochem II, Chelmsford, USA) with a high sensitivity analytical cell (ESA mod. 5011). The first electrode was set at −150 mV and the second at +250 mV. The mobile phase consisted of 30 mM NaH_2PO_4, 0.48 mM octyl sulfonic acid, 0.5 mM ethylenediaminetetraacetic acid (EDTA), 200 μl triethylamine (pH 3.5 adjusted with phosphoric acid) plus 27% methanol and 4% acetonitrile and was delivered at a flow rate of 1 ml/min. Retention time for 5-HT was 5.5–6 min and the detection limit for 5-HT was 1–2 fmol/sample.

2.5. cAMP formation in HeLa cells transfected with the human 5-HT_{1A} receptor

2.5.1. Cell culture

A HeLa cell line permanently expressing the human 5-HT_{1A} receptor gene (kindly donated by Cajal Institute, Madrid) was cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 2 mM glutamine, 1 mM pyruvate and 10% heat-inactivated foetal calf serum. Subcultures were made by using 0.025% trypsin in PBS. Cultures were maintained at 37 °C in an air/CO_2 (95:5) water saturated atmosphere. cAMP experiments were carried out with cultures grown for 2–3 days in eight-well culture plates with 2 ml medium/well.

2.5.2. Forskolin-induced cAMP formation

Cultures (about 7.5×10^4 cells/well) were washed with PBS and incubated for 10 min with 1 ml of PBS containing 0.5 mM isobutylmethylxanthine and 10 μM forskolin in the presence or absence of test compounds. The medium was then aspirated and the reaction stopped by addition of 600 μl ice-cold ethanol. Two hours later, the extract was liofilized and the resulting pellet was resuspended in 100 μl of assay buffer (RPA 538, Amersham, UK) and cAMP was quantified by radioimmunoassay. To study the antagonism to the inhibition of forskolin-induced cAMP formation induced by 8-hydroxy-2-(di-*n*-propylamino) tetralin test compounds were preincubated 20 min before the addition of forskolin and 8-OH-DPAT.

2.6. 8-OH-DPAT-induced hypothermia

These studies were performed according to previously described methods (Bill et al., 1991). Body temperature was measured with a lubricated digital thermistor probe (pb0331, Panlab, Barcelona) inserted to a depth of 2 cm into the rectum of the mice. Temperature was measured immediately before drug injection and 15, 30 and 60 min later. To study the antagonism to 8-OH-DPAT-induced hypothermia, test compounds or vehicle was administered i.p. 30 min before the injection of 8-OH-DPAT (0.5 mg/kg s.c.). Results were expressed as a change in body temperature (Δt) over basal values.

2.7. Gross behavioural assessment

Different doses of VN2222, from 5 to 200 mg/kg i.p., were given to groups of five to seven mice and the behavioural state of the animals was subjectively evaluated at different times, from 1 to 24 h, after drug administration.

2.8. Learned helplessness test

The learned helplessness procedure was performed as described (Martin et al., 1992). The two phases of the test were the following.

2.8.1. Helpless induction

On the afternoon of day 1, male rats were placed individually in a Skinner box (Coulbourn, USA). Each rat was then exposed to inescapable electric foot shocks (1 mA, 10 s) every 30 s for 30 min.

2.8.2. Conditioned avoidance training

Animals were placed individually in a shuttle box (Letica, Spain) consisting of two equal size compartments separated by a door. The assay consisted in 30 stimulus-shock trials of 8 s with a 30-s resting period between trials. During the first 5 s of each trial, a light and sound conditioned stimulus was on. If the animal did not cross to the other compartment, the conditioned stimulus was followed by a shock (1 mA) of 3 s maximal duration. Avoidance sessions were performed for 3 consecutive days and the number of escape failures and of intertrial crossings were recorded.

Animals received either saline or test compounds throughout the 4-day period. On day 1, the drug was given 6 h after the exposure to inescapable shocks and on days 2–3 the drug was administered twice a day, 30 min before shuttle-box exposure and 6 h thereafter. On the last day, the drug was administered only once, 30 min before shuttle-box exposure. The antidepressants, amitriptyline and fluoxetine, were comparatively studied.

2.9. Light/dark exploration test in mice

This test was described by Crawley and Goodwin (1980). The apparatus was an open-topped rectangular box ($45 \times 27 \times 27$ cm high) divided into a small (2/5) black area and a large (3/5) white area. The floor of the white compartment was marked into 9-cm squares. Each mouse was placed individually in the centre of the white area and behaviour was recorded over a 5 min-period in which the percentage of time spent in the white area and the number of line crossings was measured. Mice were exposed to the test for two consecutive days, the first day without any treatment and the second day 30 min after drug administration. The percentage change in the time spent and in the number of line crossings in the white compartment after drug treatment was calculated.

2.10. Spontaneous locomotor activity in mice

Drugs or vehicle were injected i.p. and 30 min later mice were placed in a black wooden four-compartment box ($30 \times 30 \times 45$ cm high). Distance travelled in centimeter (locomotor activity) was recorded during a 30-min period using a digital VIDEOMEX-V system (Columbus Inst., USA) working with the appropriate computer program.

2.11. Drugs and chemicals

VN2222 was synthesized as described (Martínez-Esparza et al., 2001). The radiolabelled compounds were [^{3}H]5-HT, [^{3}H]noradrenaline and [^{125}I]cAMP RPA 538 kit (Amersham). The drugs used were 8-OH-DPAT (RBI, USA); 5-HT, amitriptyline, nomifensine (Sigma); diazepam (Roche); ketamine (Parke-Davis); paroxetine (Vita); fluoxetine and *N*-[2-(4-[2-methoxyphenyl]-1-piperazinyl)ethyl]-*N*-2pyridinylcyclohexanecarboxamide (WAY 100635) were a gift from Lilly and Wyeth, respectively. Inorganic salts and all other reagents were from Merck. DMEM, foetal calf serum and tissue culture plates were from Gibco (UK).

For in vitro studies, VN2222 was dissolved in dimethylsulphoxide, stored as frozen aliquots and diluted before each experiment. For in vivo studies, VN2222 (0.001–10 mg/kg) was suspended in saline with a drop of Tween-80 (Sigma). All other drugs were dissolved in saline.

3. Results

3.1. Monoamine uptake studies

Table 1 shows the K_i values of VN2222 for inhibition of [^{3}H]5-HT and [^{3}H]noradrenaline uptake by rat cortical synaptosomes. Paroxetine was a very potent and selective inhibitor of [^{3}H]5-HT uptake in vitro ($K_i = 1$ nM). VN2222 and fluoxetine were weaker 5-HT uptake inhibitors than paroxetine by at least one order of magnitude. In terms of selectivity, VN2222 was approximately 15 times more potent as a [^{3}H]5-HT uptake than as a [^{3}H]-noradrenaline uptake inhibitor.

Table 1
Inhibition by VN2222 and standard antidepressants (K_i, nM) of [^{3}H]5-hydroxytryptamine ([^{3}H]5-HT) and [^{3}H]noradrenaline ([^{3}H]NA) uptake by rat cortical synaptosomes

Drug	[^{3}H]5-HT uptake, K_i	[^{3}H] NA uptake, K_i	NA/5-HT
Paroxetine	1.0 ± 0.01	355 ± 14	355
Fluoxetine	15 ± 0.05	550 ± 60	55
Nomifensine	n.d.	3 ± 0.01	
VN2222	20 ± 0.05	275 ± 25.2	17.8

Values are means ± S.E.M. of three to five experiments, K_i values were calculated with the Cheng–Prusoff equation: $K_i = IC_{50}/(1+[L]/K_M)$, where IC_{50} is the molar concentration of the competing compound to inhibit 50% of the high affinity uptake, [L] is the molar concentration of [^{3}H]5-HT or [^{3}H]NA in the incubation medium and K_M is the Michaelis constant derived from previous saturation experiments. These constants were 112 nM for 5-HT and 98 nM for NA. nd: not determined.

The ex vivo effects of fluoxetine and VN2222 on [^{3}H]5-HT uptake were subsequently investigated. Fluoxetine caused a dose-related inhibition of [^{3}H]5-HT uptake ($ED_{50} = 5.6$ mg/kg i.p. and 10.5 mg/kg p.o.) with a maximal percentage inhibition of $83 \pm 1.7\%$ at 30 mg/kg, i.p. VN2222 exhibited an inverted U-shape activity following systemic administration so low doses (0.01–0.1 mg/kg) inhibited [^{3}H]5-HT uptake *ex vivo* with a maximal percentage inhibition of $65 \pm 2.6\%$ at 0.1 mg/kg i.p. while higher doses (1–10 mg/kg) were ineffective. Results are shown in Fig. 2.

3.2. Effect on tritium outflow in primary mesencephalic cultures

VN2222 and fluoxetine produced a significant concentration-dependent enhancement in the basal release of [^{3}H]5-HT from raphe primary cultures as shown by the percentage change in tritium outflow (Table 2). VN2222 enhanced the basal outflow of [^{3}H]5-HT by 32% and 391% at the concentrations of 1 and 10 μM, respectively, and the effect of fluoxetine at the same concentrations was slightly weaker. The selective 5-HT_{1A} receptor agonist 8-OH-DPAT (10 μM) also moderately enhanced the basal outflow of [^{3}H]5-HT (Table 2). However, neither VN2222 nor fluoxetine produced any apparent effect on K^+-evoked [^{3}H]5-HT release since the marked enhancement in the basal outflow of [^{3}H]5-HT by these two drugs precluded a precise estimation of their effects on depolarization-evoked [^{3}H]5-HT release (results not shown).

3.3. Microdialysis studies in freely moving rats

Basal extracellular 5-HT levels in the ventral hippocampus were 8.43 ± 0.23 fmol/20 μl (mean ± S.E.M.). Local administration of VN2222 and fluoxetine (10, 100 and 300 μM) significantly increased extracellular 5-HT levels in a

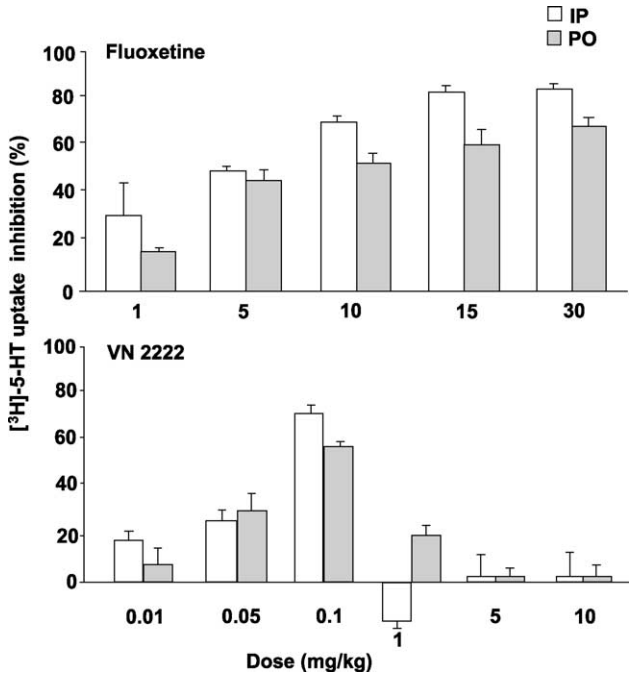

Fig. 2. *Ex vivo* inhibition by fluoxetine and VN2222 of [^{3}H]5-HT uptake by rat cortical synaptosomes. Compounds were administered i.p. or p.o. 45 or 60 min before the test, respectively. Values are means ± S.E.M. of three independent experiments.

concentration-dependent manner. The maximal increase in hippocampal extracellular 5-HT, approaching +360% and +300% over basal levels, was obtained after the higher concentration (300 μM) of VN2222 and fluoxetine, respectively. The EC_{50}'s for VN2222 and fluoxetine were 64 and 35 μM, respectively. There was no significant difference between these two drugs at any concentration tested.

Fluoxetine (10 mg/kg i.p.) caused a large increase (to 300% of baseline) in 5-HT levels in dialysates of the ventral hippocampus and this enhancement was significant ($P<0.01$) at all time points post-injection. VN2222 (0.01 mg/kg i.p.) also increased 5-HT levels in ventral hippocampus dialysates for 80 min. The maximal increase (over

Table 2
Effects of drugs on basal outflow of [^{3}H]5-HT from primary mesencephalic cultures

Drug	Concentration (μM)	[^{3}H]5-HT outflow (%)
Control	–	100
8-OH-DPAT	0.1	106 ± 8.9
	1	109 ± 2.9
	10	147 ± 8.5[a]
Fluoxetine	1	125 ± 31
	10	332 ± 33[b]
VN2222	1	132 ± 25
	10	491 ± 35[a]

Values (means ± S.E.M. of 5–10 experiments) indicate the percentage change over basal efflux. (One-way ANOVA followed by Student–Newman–Keuls test.)

[a] $P<0.05$ vs. control.

[b] $P<0.01$ vs. control.

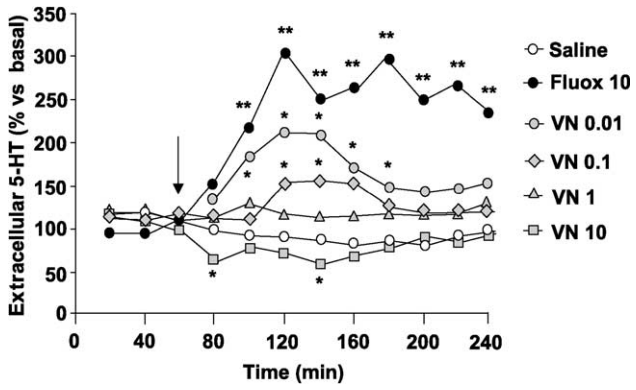

Fig. 3. *In vivo* effect of VN2222 (VN) and fluoxetine (doses in mg/kg i.p.) on extracellular 5-HT in rat ventral hippocampus. Each point is the mean ± S.E.M. ($n=6$) of the increase of 5-HT expressed as percentages of basal values. **$P<0.01$; *$P<0.05$ vs. the corresponding basal value (one-way ANOVA followed by Student–Newman–Keuls test).

200%) was observed at the 40 min time point. A ten-fold higher dose, 0.1 mg/kg, slightly increased 5-HT levels in dialysates. However, there was virtually no effect after 1 mg/kg and after the highest dose studied, 10 mg/kg, reduced extracellular 5-HT levels were observed. Results are depicted in Fig. 3.

3.4. cAMP assays

Forskolin (10 μM) enhanced cAMP formation in cells stably transfected with the 5-HT_{1A} receptor from 1.7 ± 0.2 to 22.3 pmol/well. The 5-HT_{1A} receptor agonist 8-OH-DPAT produced a concentration dependent inhibition of cAMP formation with an IC_{50} of approximately 42 nM. A weaker inhibition was found with the 5-HT_{1A} partial ago-

Table 3
Antagonism by WAY 100635 and VN2222 of 8-OH-DPAT-induced inhibition of forskolin-stimulated cAMP formation in HeLa cells expressing 5-HT_{1A} receptors

Treatment	Concentration (μM)	cAMP (pmol/well)	% Antagonism 8-OH-DPAT
Control (Forskolin 10 μM)	10	22.3 ± 0.7	
8-OH-DPAT	0.1	7.3 ± 0.4[a]	
	0.5	6.5 ± 0.5[a]	
	1	5.8 ± 0.4[a]	
Buspirone	1	11.6 ± 0.3[a]	
VN2222	0.01	12.2 ± 0.6[b]	
	0.1	13.8 ± 0.9[b]	
	1	20.7 ± 2.9	
WAY100635	1	22.8 ± 1.3	
WAY100635 + 8-OH-DPAT	1 + 0.1	17.5 ± 0.3[c]	78
VN2222 + 8-OH-DPAT	1 + 0.1	11.7 ± 1.4[d]	36

Data (pmol cAMP/well) are the means ± S.E.M. of five independent experiments. (One-way ANOVA followed by Student–Newman–Keuls test.)

[a] $P<0.01$ vs. forskolin.

[b] $P<0.05$ vs. forskolin.

[c] $P<0.01$ vs. 8-OH-DPAT.

[d] $P<0.05$ vs. 8-OH-DPAT.

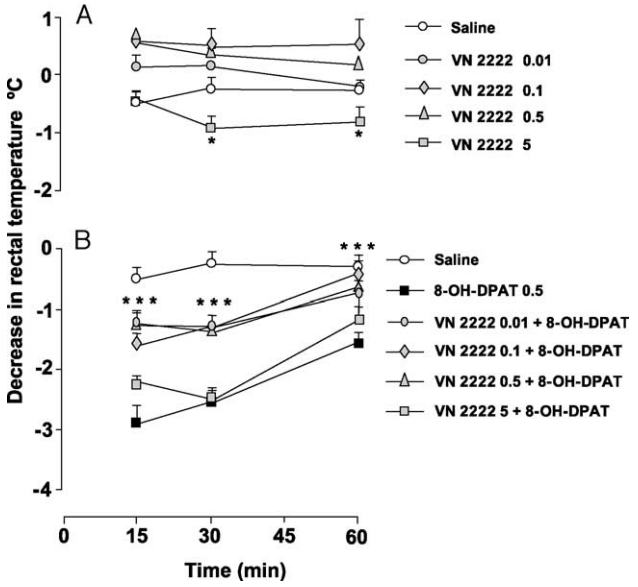

Fig. 4. Intrinsic effect of VN2222 on rectal temperature (A) and effect on the hypothermic response to 8-OH-DPAT in mice (B). Doses in mg/kg. Data are means ± S.E.M. of the changes in body temperature at different times after drug injection. * $P<0.05$; ** $P<0.01$ vs. vehicle; $^{+}P<0.05$ vs. 8-OH-DPAT (one-way ANOVA followed by Student–Newman–Keuls test).

nist, buspirone. VN2222 (0.01–0.1 μM) also produced a moderate inhibition of cAMP formation. No significant inhibition was however found at the higher 1 μM concentration. In antagonism studies, 1 μM WAY100635, which did not produce any intrinsic effect on its own, markedly prevented (−78%) the 8-OH-DPAT-mediated inhibition of forskolin-stimulated cAMP formation. VN2222 (1 μM) also prevented partially (−36%) the 8-OH-DPAT effect. The results are shown in Table 3.

3.5. Temperature measurements

The mean basal value of rectal temperatures in mice was 37.5 °C. There was no significant temperature difference

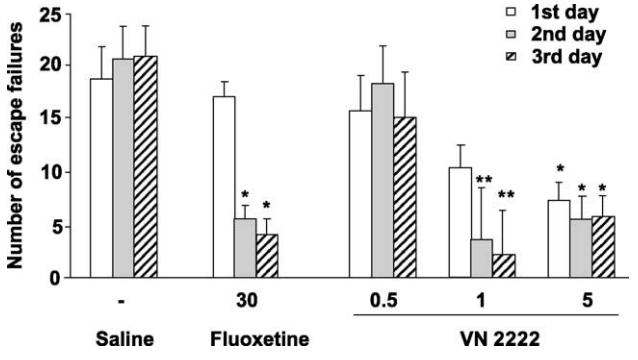

Fig. 5. Effect of fluoxetine and VN2222 (doses in mg/kg i.p.) in the learned helplessness test in rats. Data are means ± S.E.M. of escape failures in each of the three consecutive shuttle-box sessions (SB 1–3). Fluoxetine and VN2222 were given for four consecutive days, 6 h after inescapable shocks on day 1 and then twice a day, in the morning (30 min before shuttle-box sessions) and 6 h after. * $P<0.05$ (Mann–Whitney U-test).

Table 4
Effect of VN2222 and fluoxetine on the number of intertrial crossings during the three shuttle-box sessions

Drug	Repeated dose (mg/kg i.p.)	n	Mean number of intertrial crossings		
			First Session	Second Session	Third Session
Saline		25	6.0 ± 0.6	6.3 ± 0.05	6.3 ± 0.9
VN2222	0.5	7	6.2 ± 1.8	5.7 ± 1.6	6.4 ± 1.5
	1	6	7.8 ± 0.6	9.9 ± 0.9	10.8 ± 1.4[a]
	5	6	8.1 ± 0.7	8.8 ± 0.5	11.4 ± 1.7[a]
Fluoxetine	30	6	6.9 ± 0.9	10.0 ± 1.0	10.7 ± 0.9[a]

[a] $P<0.05$ vs. saline (Mann–Whitney U-test).

between groups before drug administration. The 5-HT_{1A} receptor agonist 8-OH-DPAT (0.5 mg/kg s.c.) produced a marked decrease in rectal temperature. The maximum hypothermic effect of 8-OH-DPAT was observed 30 min after injection. Low doses of VN2222 (0.01, 0.05 and 0.1 mg/kg i.p.) partially antagonised the hypothermic response to 8-OH-DPAT at different times after administration. A much higher VN2222 dose (5 mg/kg) produced however a significant hypothermic response in mice. The intermediate doses tested (0.01–1 mg/kg) neither produced an intrinsic hypothermic effect nor antagonized the effect of 8-OH-DPAT. The results are depicted in Fig. 4.

3.6. Gross behavioural effects

No behavioural changes, in particular changes in spontaneous motor activity, could be easily detected in mice 1–2 h after moderate VN2222 doses, 5–20 mg/kg i.p. The same doses did not produce any sign of sympathetic stimulation. Abnormal gait was found in some animals after 50–100 mg/kg. Clear signs of neurotoxicity, including occasional seizures, were observed after the higher dose. No lethal effect was produced by the used drug doses.

3.7. Learned helplessness test

The number of escape failures was high for control animals along the 3 days of the avoidance task (18.5 ± 2.7, 20.3 ± 3.1 and 20.6 ± 4.0 escape failures, respectively). A

Table 5
Effect of VN2222 and diazepam in the light/dark exploration test in mice

Drug	Dose (mg/kg, i.p.)	Time in white area (% change)	Locomotion in white area (% change)
Saline	–	56.4 ± 38.3	58.4 ± 13.6
Diazepam	1	106.1 ± 16.0[a]	106.8 ± 17.0[a]
VN2222	0.001	59.9 ± 12.3	47.0 ± 11.2
	0.01	51.7 ± 11.6	51.4 ± 10.5
	0.1	49.8 ± 16.1	55.6 ± 18.8
	1	41.4 ± 10.3	35.6 ± 8.1

Results (means ± S.E.M. of 9–11 mice) show the % change produced by drugs on the behavioural items recorded in the same animals 24 h before.
[a] $P<0.05$ vs. saline (Student's t test).

Table 6
Effect of VN2222 on spontaneous locomotor activity in mice

Drug	Dose (mg/kg, i.p.)	Distance (cm)			
		15 min	30 min	45 min	60 min
Saline	–	1875 ± 160	3490 ± 394	4553 ± 505	5924 ± 568
VN2222	0.01	1692 ± 112	3709 ± 279	4948 ± 510	6795 ± 627
	0.1	2089 ± 178	3659 ± 334	4580 ± 438	6315 ± 514
	1	1721 ± 228	2786 ± 357	3709 ± 506	5004 ± 717
	10	1512 ± 221	2865 ± 415	3878 ± 411	5852 ± 645

Drug given 30 min before the test. Locomotor activity recorded for 60 min. Values are means ± S.E.M. of eight animals per group.

moderate intertrial ambulatory activity was also observed in control animals. Repeated administration of 30 mg/kg fluoxetine significantly reduced the number of escape failures on the last 2 days of the avoidance task. VN2222, at repeated doses of 1 and 5 mg/kg, markedly reduced escape deficits on each of the three sessions. A lower dose (0.5 mg/kg) did not show a significant effect (Fig. 5).

Intertrial crossings were significantly increased by repeated administration of VN2222 (1–5 mg/kg) and fluoxetine (30 mg/kg). This increase was only significant on the third session (Table 4).

3.8. Light/dark exploration test

In this test for anxiolytic-like activity in mice, VN2222 (0.001–1 mg/kg i.p.) did not produce any significant change in the time spent in the illuminated area or in the number of line crossings (Table 5). The typical anxiolytic diazepam approximately doubled the time spent in the white area.

3.9. Spontaneous locomotor activity

VN2222 did not significantly modify spontaneous locomotor activity in mice over the wide range of doses studied, 0.001–10 mg/kg i.p. (Table 6).

4. Discussion

VN2222 is a new synthetic compound with high affinity at both the 5-HT transporter and the 5-HT_{1A} receptor. This compound was selected for further pharmacological development in light of current theories on the possible shorter onset of action of antidepressant drugs able to block both the 5-HT transporter and the somatodendritic 5-HT_{1A} receptors (see Section 1). In other receptor binding studies, VN2222 only showed a remarkable affinity at α_1-adrenoceptors, although it was almost two orders of magnitude weaker than the typical α_1-adrenoceptor antagonist, prazosin. No affinity was found at muscarinic receptors suggesting a lack of anticholinergic side effects which are prominent in most tricyclic antidepressants and much lower or inexistent in selective serotonin reuptake inhibitors (Martínez-Esparza et al., 2001).

Monoamine uptake studies in vitro showed that VN2222 caused a concentration-dependent inhibition of [^{3}H]5-HT uptake in rat cortical synaptosomes, with a potency similar to fluoxetine, and was much weaker as an inhibitor of [^{3}H]noradrenaline uptake. VN2222 was also studied *in vitro* using primary mesencephalic cultures preloaded with [^{3}H]5-HT. It was expected that, like other 5-HT uptake blockers, VN2222 would produce an apparent increase in 5-HT release which, in the present case, could be perhaps also affected by an additional effect on 5-HT_{1A} receptors. Indeed, both VN2222 and fluoxetine markedly enhanced the apparent basal outflow of [^{3}H]5-HT, VN2222 being slightly more potent. Since fluoxetine tended to be more potent than VN2222 as a 5-HT uptake blocker *in vitro*, a 5-HT releasing effect of VN2222 at high concentrations cannot be discarded. Even though VN2222 did not exhibit any effect on K^+-evoked [^{3}H]5-HT release, this effect was of doubtful significance due to the great apparent elevation in basal 5-HT outflow.

The functional characterization *in vitro* of the action of VN2222 at 5-HT_{1A} receptors was examined in a cell line stably transfected with this receptor by studying the ability to inhibit forskolin-stimulated cAMP formation or to antagonize 8-OH-DPAT-mediated inhibition of forskolin-stimulated cAMP formation, an index of agonist or antagonist action, respectively. The full agonist 8-OH-DPAT produced a concentration-dependent inhibition of cAMP formation and this effect was prevented by the *silent* 5-HT_{1A} receptor antagonist WAY100635, results in keeping with previous studies (e.g. Pauwels et al., 1993; Schoeffier et al., 1997). Low concentrations of VN2222 (0.01–0.1 μM) produced a partial inhibition of cAMP formation, whereas no inhibition was observed at the higher 1 μM concentration, which partially prevented 8-OH-DPAT-induced inhibition of forskolin-stimulated cAMP formation. It seems consequently that VN2222 behaves as a 5-HT_{1A} receptor partial agonist in this assay. *In vivo* functional characterization was performed by means of body temperature studies in mice. It is known that 8-OH-DPAT-induced hypothermia in mice is mediated, in principle, through activation of somatodendritic 5-HT_{1A} receptors (De Vry, 1995). VN2222 (0.01–1 mg/kg) did not show any intrinsic effect on core temperature and antagonized 8-OH-DPAT-induced hypothermia. A higher VN2222 dose (5 mg/kg) exhibited a hypothermic effect in mice, suggesting an agonist action at presynaptic 5-HT_{1A} receptors. It is possible that the antagonism by low VN2222 doses of 8-OH-DPAT-induced hypothermia may depend on 5-HT transporter blockade in raphe nuclei and not on an antagonistic action at presynaptic 5-HT_{1A} receptors. Indeed, such an antagonism to 8-OH-DPAT has been previously reported for different selective serotonin reuptake inhibitors (Bill et al., 1991). Consequently, *in vitro* and *in vivo* studies for 5-HT_{1A} receptor function rather appear to reflect a partial agonist profile of VN2222 at this 5-HT receptor subtype. The ultimate mechanism involved in the pharmacological effects of very low doses/concentrations of VN2222 is

however unclear since the affinity of this compound at 5-HT_{1A} receptors (K_i = 20 nM; Martínez-Esparza et al., 2001) was not strikingly high.

Microdialysis studies showed that local administration of both VN2222 and fluoxetine increased the extracellular concentration of 5-HT in rat ventral hippocampus dialysates in a concentration-dependent manner, with quite similar EC_{50} values. This study indicates that VN2222 is also able to block the 5-HT transporter in vivo when administered locally into a brain region. When VN2222 was given systemically, a biphasic effect was found in the microdialysis experiments. Low doses of VN2222 (0.01–0.1 mg/kg) increased extracellular 5-HT levels in the ventral hippocampus, the lower dose (0.01 mg/kg) being more effective. A much higher dose (10 mg/kg) reduced however extracellular 5-HT levels. These results were in line with 5-HT uptake studies *ex vivo*, in which VN2222 also exhibited a biphasic activity, inhibiting [^{3}H]5-HT uptake only in the low dose range, although the dose–response curve was not identical. The mixed pharmacological profile of VN2222 may not yield identical results in microdialysis experiments in freely moving animals and in 5-HT uptake studies *ex vivo*. In microdialysis experiments, reduced extracellular 5-HT levels after high VN2222 doses suggests an activation of somatodendritic 5-HT_{1A} receptors (cf. Sharp et al., 1997) which could mask the expression of the 5-HT reuptake blocking property of this drug. It is known that systemic administration of 8-OH-DPAT counteracts the increase of extracellular 5-HT levels in dorsal hippocampus produced by the administration of fluoxetine (Kreiss and Lucki, 1994). On the other hand, the high affinity of VN2222 at α_1-adrenoceptors, presumably acting as an antagonist (cf. Section 3.6), could influence the drug effect on extracellular 5-HT levels in the hippocampus. Other microdyalisis studies have shown that systemic administration of the α_1-adrenoceptor antagonist, prazosin, may produce a decrease of extracellular 5-HT levels in frontal cortex and hippocampus (Hjorth et al., 1995). In this regard, it is known that α_1-adrenoceptor antagonists reduce more markedly extracellular 5-HT in the hippocampus, brain region used in the present study, than in other regions, like the striatum (Rouquier et al., 1994). Dopaminergic agonists may also influence the activity of serotonergic neurons (Ferré et al., 1994). This possibility seems however unlikely for VN2222 since not only this drug was a rather weak dopamine D_2 receptor ligand (K_i = 150 nM) but also no trace of agonist activity at this dopamine receptor subtype could be inferred from the behavioural studies performed.

In our previous study (Martínez-Esparza et al., 2001), we had found that very low doses of VN2222 reduced significantly the immobility time of mice in the forced swimming test, an animal model widely used to test compounds with a potential antidepressant effect in humans, which in general is highly sensitive to 5-HT_{1A} receptor agonists (Borsini, 1995). In the present study, no effect was however found with VN2222 in the light/dark test in mice which is also known to be sensitive to the anxiolytic-like effect of 5-HT_{1A} receptor agonists/partial agonists (Misslin et al., 1990). Since VN2222 shows affinity at 5-HT_{1A} receptors and 5-HT transporter and selective serotonin reuptake inhibitors tend to be anxiogenic in this test (Artaiz et al., 1998), it is conceivable that the lack of effect of VN2222 in this anxiety model is a consequence of such a dual action. The antidepressant-like effect of VN2222 was confirmed in the learned helplessness test in rats, another animal model of depression with a very good predictive validity and also with a good face and construct validity (e.g. Willner, 1984). Yet, there was no good correlation between the effective doses of VN2222 in the forced swimming test in mice and in the learned helplessness test in rats, the doses being higher in the latter model, although still higher doses of fluoxetine were necessary to significantly reduce the number of escape failures. Obviously, the lack of cross-predictability between these two models suggests that they may reflect processes mediated by distinct neural mechanisms (Drugan et al., 1989), which occasionally may be operative in mice but not in rats or vice versa. In the learned helplessness test, both VN2222 and fluoxetine produced also an increase in the number of intertrial crossings on the third escape session, possibly reflecting a stimulant effect upon psychomotor retardation, as has been reported for other antidepressants (Besson et al., 1999).

In summary, VN2222 is a new compound with a dual action at 5-HT_{1A} receptors and 5-HT transporter. Such a pharmacological profile may perhaps account for the antidepressant-like activity found in animal models. Future studies will assess the real possibilities of VN2222 in eliciting a rapid and efficacious antidepressant response.

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References

Adell, A., Artigas, F., 1998. A microdialysis study of the in vivo release of 5-HT in the median raphe nucleus of the rat. Br. J. Pharmacol. 125, 1361–1367.

Artaiz, I., Zazpe, A., Del Río, J., 1998. Characterization of serotonergic mechanisms involved in the behavioural inhibition induced by 5-hydroxytryptophan in a modified light/dark test in mice. Behav. Pharmacol. 9, 103–112.

Artigas, F., Pérez, V., Alvarez, E., 1994. Pindolol induces a rapid improvement of depressed patients treated with serotonin reuptake inhibitors. Arch. Gen. Psychiatry 51, 248–251.

Asberg, M., Eriksson, B., Matenson, B., Traskman-Bendz, L., Wagner, A., 1986. Therapeutic effects of serotonin uptake inhibitors in depression. J. Clin. Psychiatry 47, 23–35.

Bel, N., Artigas, F., 1993. Chronic treatment with fluvoxamine increases extracellular serotonin in frontal cortex but not in raphe nuclei. Synapse 15, 243–255.

Besson, A., Privat, A.M., Eschalier, A., Fialip, J., 1999. Dopaminergic and opioidergic mediation of tricyclic antidepressants in the learned helplessness paradigm. Pharmacol. Biochem. Behav. 64, 541–548.

Bill, D.J., Knight, M., Forster, E.A., Fletcher, A., 1991. Direct evidence for an important species difference in the mechanism of 8-OH-DPAT-induced hypothermia. Br. J. Pharmacol. 103, 1857–1864.

Blier, P., de Montigny, C., Chaput, I., 1987. Modifications of the serotonin system by antidepressant treatment: implications for the therapeutic response in major depression. J. Clin. Psychopharmacol. 7, 24S–35S.

Borsini, F., 1995. Role of the serotonergic system in the forced swimming test. Neurosci. Biobehav. Rev. 19, 377–395.

Chaput, I., Blier, P., De Montigny, C., 1986. Effects of a selective 5-HT reuptake blocker, citalopram, on the sensitivity of 5-HT autoreceptors: electrophysiological studies in the rat brain. Naunyn-Schmiedeberg's Arch. Pharmacol. 333, 342–348.

Cheng, C.H.K., Costall, B., Naylor, R.J., Rudd, J.A., 1993. The effect of 5-HT receptor ligands on the uptake of [^{3}H]-5-HT into rat cortical synaptosomes. Eur. J. Pharmacol. 239, 211–214.

Crawley, J.N., Goodwin, F.K., 1980. Preliminary report of a simple animal behaviour model for the anxiolytic effects of benzodiazepines. Pharmacol. Biochem. Behav. 13, 167–170.

De Vry, J., 1995. 5-HT$_{1A}$ receptor agonists: recent developments and controversial issues. Psychopharmacology 129, 1–26.

Dreshfield, L.J., Wong, D.T., Perry, K.W., Engleman, E.A., 1996. Enhancement of fluoxetine-dependent increase of extracellular serotonin (5-HT) levels by (−)-pindolol, an antagonist at 5-HT$_{1A}$ receptors. Neurochem. Res. 21, 557–562.

Drugan, R.C., Skolnick, P., Paul, S.M., Crawley, J.N., 1989. A pretest procedure reliably predicts performance in two animal models of inescapable stress. Pharmacol. Biochem. Behav. 33, 649–654.

Ferré, S., Cortés, R., Artigas, F., 1994. Dopaminergic regulation of the serotonergic raphe-striatal pathway: microdialysis studies in freely moving rats. J. Neurosci. 14, 4839–4846.

Gardier, A.M., Malagie, I., Trillat, A.C., Jacquot, C., Artigas, F., 1996. Role of 5-HT$_{1A}$ autoreceptors in the mechanism of action of serotoninergic antidepressant drugs: recent findings from in vivo microdialysis studies. Fundam. Clin. Pharmacol. 10, 16–27.

Gartside, S.E., Umbers, V., Hajós, M., Sharp, T., 1995. Interaction between a selective 5-HT$_{1A}$ receptor antagonist and a SSRI in vivo: effects on 5-HT cell firing and extracellular 5-HT. Br. J. Pharmacol. 115, 1064–1070.

Hervás, I., Artigas, F., 1998. Effect of fluoxetine on extracellular 5-hydroxytryptamine in rat brain. Role of 5-HT autoreceptors. Eur. J. Pharmacol. 358, 9–18.

Hjorth, S., Bengtsson, H.J., Milano, S., Lundberg, J.F., Sharp, T., 1995. Studies on the role of 5-HT1 autoreceptors and α_1-adrenoceptors in the inhibition of 5-HT release: I. BMY7378 and prazosin. Neuropharmacology 34, 615–620.

Invernizzi, R., Belli, S., Samanin, R., 1992. Citalopram's ability to increase the extracellular concentrations of serotonin in the dorsal raphe prevents the drug effect in the frontal cortex. Brain Res. 584, 322–324.

Kreiss, D.S., Lucki, I., 1994. Differential regulation of 5-HT release in the striatum and hippocampus by 5-HT$_{1A}$ autoreceptors of the dorsal and median raphe nuclei. J. Pharmacol. Exp. Ther. 269, 1268–1279.

Martin, P., Gozlan, H., Puech, A., 1992. 5-HT$_3$ receptor antagonists reverse helpless behaviour in rats. Eur. J. Pharmacol. 212, 73–78.

Martínez-Esparza, J., Oficialdegui, A.M., Pérez-Silanes, S., Heras, B., Orús, L., Palop, J.A., Lasheras, B., Roca, J., Mourelle, M., Bosch, A., Del Castillo, J.C., Tordera, R., Del Río, J., Monge, A., 2001. New 1-(aryl)-3-[4-(aryl)piperazin-1-yl]-propane derivatives, with dual action at 5-HT$_{1A}$ serotonin receptors and serotonin transporter, as a new class of antidepressants. J. Med. Chem. 44, 418–428.

Misslin, R., Griebel, G., Saffroy-Spittler, M., Vogel, E., 1990. Anxiolytic and sedative effects of 5-HT$_{1A}$ ligands, 8-OH-DPAT and MDL 73005EF, in mice. NeuroReport 1, 267–270.

Moret, C., Briley, M., 1990. Serotonin autoreceptor subsensitivity and antidepressant activity. Eur. J. Pharmacol. 180, 351–356.

Pauwels, P.J., Gompel, P.V., Leysen, J.E., 1993. Activity of serotonin (5-HT) receptor agonists, partial agonists and antagonists at cloned human 5-HT$_{1A}$ receptors that are negatively coupled to adenylate cyclase in permanently transfected HeLa cells. Biochem. Pharmacol. 45, 375–383.

Paxinos, G., Watson, C., 1986. The Rat Brain in Stereotaxic Coordinates Academic Press, Sydney.

Pérez, V., Gilaberte, I., Faries, D., Alvarez, E., Artigas, F., 1997. Randomised, double blind, placebo-controled trial of pindolol in combination with fluoxetine antidepressant treatment. Lancet 349, 1594–1597.

Rouquier, L., Claustre, Y., Benavides, J., 1994. Alpha(1)-adrenoceptor antagonists differentially control serotonin release in the hippocampus and striatum: a microdialysis study. Eur. J. Pharmacol. 261, 59–64.

Schoeffier, P., Bobimac, I., Boddeke, E., Hoyer, D., 1997. Inhibition of cAMP accumulation via recombinant human serotonin 5-HT$_{1A}$ receptors: Considerations on receptor effector coupling across systems. Neuropharmacology 36, 429–437.

Sharp, T., Umbers, V., Gartside, S.E., 1997. Effect of a selective 5-HT reuptake inhibitor in combination with 5-HT$_{1A}$ and 5-HT$_{1B}$ receptor antagonists on extracellular 5-HT in rat frontal cortex in vivo. Br. J. Pharmacol. 121, 941–946.

Wichems, C.H., Hollingsworth, C.K., Bennett, B.A., 1995. Release of serotonin induced by 3,4-methylenedioxymethamphetamine (MDMA) and other substituted amphetamines in cultured fetal raphe neurones: further evidence for calcium-independent mechanisms of release. Brain Res. 695, 10–18.

Willner, P., 1984. The validity of animal models of depression. Psychopharmacology 83, 1–16.

Zanardi, R., Artigas, F., Franchini, L., Sforzini, L., Gasperini, M., Smeraldi, E., Perez, J., 1997. How long pindolol should be associated to paroxetine to improve the antidepressant response? J. Clin. Psychopharmacol. 17, 446–450.