Neuropsychopharmacology (2003) 28, 445–456

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Modulation of Serotonergic Function in Rat Brain by VN2222, a Serotonin Reuptake Inhibitor and 5-HT_{1A} Receptor Agonist

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VN2222 (1-(benzo[*b*]thiophen-3-yl)-3-[4-(2-methoxiphenyl piperazin-1-yl]propan-1-ol) is a potential antidepressant with high affinity for the serotonin transporter and 5-HT_{1A} receptors. Locally applied, VN2222 enhanced the extracellular 5-hydroxytryptamine (5-HT) concentration (5-HT_{ext}) in rat striatum to 780% of baseline whereas its systemic administration (1–10 mg/kg s.c.) reduced 5-HT_{ext}. In the presence of citalopram, 8-OH-DPAT or VN2222 applied in medial prefrontal cortex reduced 5-HT_{ext}. Fluoxetine, VN2222, and 8-OH-DPAT suppressed the firing rate of dorsal raphe 5-HT neurons (ED_{50}: 790, 14.9, and 0.8 μg/kg i.v., respectively). These effects were antagonized by WAY 100635. Administration of VN2222 for 2 weeks desensitized 5-HT_{1A} receptors as assessed by microdialysis and single-unit recordings (ED_{50} values for 8-OH-DPAT were 0.45 and 2.34 μg/kg i.v. for controls and rats treated with 6 mg/kg day VN2222). These results show that VN2222 is a mixed 5-HT reuptake inhibitor/5-HT_{1A} agonist that markedly desensitizes 5-HT_{1A} autoreceptors. These properties suggest that it may be a clinically effective dual action antidepressant drug.
Neuropsychopharmacology (2003) **28,** 445–456. doi:10.1038/sj.npp.1300062

Keywords: 5-HT_{1A} receptors; 5-hydroxytryptamine uptake; antidepressant drugs; dorsal raphe; selective serotonin reuptake inhibitors (SSRIs); frontal cortex

INTRODUCTION

The selective serotonin (5-hydroxytryptamine, 5-HT) reuptake inhibitors (SSRI) have become the most widely prescribed antidepressant drugs because of their relative absence of severe side effects. Their efficacy is comparable to that of tricyclic antidepressants (Tollefson *et al*, 1994), although the latter may be more effective in severely depressed inpatients (Danish University Antidepressant Group, 1986, 1990). The increase in the synaptic concentration of 5-HT that follows reuptake inhibition is limited by a negative feedback involving 5-HT_{1A} autoreceptors (Adell and Artigas, 1991; Artigas *et al*, 1996). Following repeated treatment, 5-HT_{1A} receptors desensitize (Blier and de Montigny, 1994; Invernizzi *et al*, 1994; Hervás *et al*, 2001). This effect results in a reduced efficacy of the negative feedback and therefore in an increase of the extracellular concentration of 5-HT (5-HT_{ext}) in the forebrain (Bel and Artigas, 1993; Invernizzi *et al*, 1994; Rutter *et al*, 1994; Kreiss and Lucki, 1995).

To overcome the above negative feedback and hasten the clinical action of SSRIs, the use of 5-HT_{1A} receptor antagonists, in combination with SSRI, was proposed (Artigas, 1993). In keeping with this hypothesis, selective (eg WAY 100635) and nonselective 5-HT_{1A} receptor antagonists have been shown to potentiate the increase in 5-HT_{ext} produced by antidepressant drugs in the rat brain (see Artigas *et al*, 1996 for review). At the clinical level, several open-label (Artigas *et al*, 1994; Blier and Bergeron, 1995; Bakish *et al*, 1997) and placebo-controlled trials (Maes *et al*, 1996; Pérez *et al*, 1997; Tomé *et al*, 1997; Zanardi *et al*, 1997, 1998; Bordet *et al*, 1998; Maes *et al*, 1999) have shown a faster action and/or greater efficacy of SSRIs when given in combination with the nonselective 5-HT_{1A} receptor antagonist pindolol. However, the efficacy of pindolol in chronically ill or treatment-resistant patients is questionable (Moreno *et al*, 1997; Tomé *et al*, 1997; Berman *et al*, 1999; Pérez *et al*, 1999).

In vivo, pindolol appears to display a preferential antagonism of pre- *vs* postsynaptic 5-HT_{1A} receptors in the rat brain (Romero *et al* (1996), Tada *et al* (1999), see however Corradetti *et al*, 1998). This may be related to its preferential occupancy of presynaptic 5-HT_{1A} receptors, as assessed by positron emission tomography scanning in rat

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Received 4 June 2002; revised 2 August 2002; accepted 7 August 2002
Online publication: 9 August 2002 at http://www.acnp.org/citations/Npp080902366

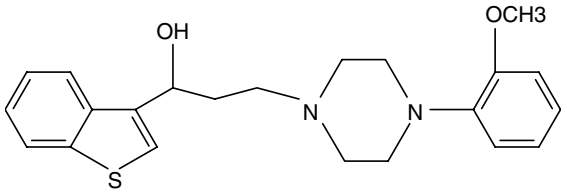

Figure 1 Chemical structure of VN2222, (±)1-(benzo[*b*]thiophen-3-yl)-3-[4-(2-methoxiphenyl piperazin-1-yl]propan-1-ol.

and human brains (Hirani *et al*, 2000; Rabiner *et al*, 2000; Martinez *et al*, 2000). However, pindolol displays higher affinity for β-adrenoceptors than for 5-HT_{1A} receptors, which has led to the development of pure 5-HT_{1A} antagonists or compounds with a dual 5-HT reuptake blockade/5-HT_{1A} receptor antagonist action. Yet, given the involvement of hippocampal postsynaptic 5-HT_{1A} receptors in the antidepressant effects of SSRIs (Blier and de Montigny, 1994; Haddjeri *et al*, 1998) there is some concern that the use of 5-HT_{1A} receptor antagonists not discriminating between pre- and postsynaptic 5-HT_{1A} receptors could cancel the benefits of enhancing presynaptically the 5-HT function. Moreover, recent data suggest that antidepressant drugs increase neurogenesis in the rat brain (Malberg *et al*, 2000), an effect that could be mediated by the activation of 5-HT_{1A} receptors (Gould, 1999). Therefore, an alternative approach would be the use of drugs with dual 5-HT reuptake blockade/5-HT_{1A} receptor agonist action. These could desensitize presynaptic 5-HT_{1A} receptors by virtue of their action at raphe 5-HT_{1A} autoreceptors. At the same time, they could enhance serotonergic transmission through the activation of postsynaptic 5-HT_{1A} receptors and, unlike selective 5-HT_{1A} receptor agonists, they could keep the tone on other postsynaptic 5-HT receptors because of their ability to inhibit 5-HT reuptake. The present study reports on the *in vivo* effects of one such compound (VN2222; (±)(1-(benzo[*b*]thiophen-3-yl)-3-[4-(2-methoxiphenylpiperazin-1-yl]propan-1-ol, Figure 1) (Martínez-Esparza *et al*, 2001) on the serotonergic system in rat brain using microdialysis and single-unit extracellular recordings.

MATERIALS AND METHODS

Animals

Male Wistar rats (Iffa Credo, Lyon, France) weighing 250–320 g at the time of experiments were used. Rats used in chronic treatments had a weight of 175 g at arrival and typically reached 250 g at the time of experiments. Animals were kept in a controlled environment (12 h light–dark cycle and 22 ± 2°C room temperature). Food and water were provided *ad libitum* before and during the experiments. Animal care followed the European Union regulations (OJ of EC L358/1 18/12/1986).

Drugs and Treatments

5-HT, 8-OH-DPAT [8-hydroxy-2-(di-n-propylamino) tetralin], DOI (1-[2,5-dimethoxy-4-iodophenyl-2-aminopropane]), fluoxetine, and WAY 100635 [*N*-(2-(4-(2-methoxyphenyl)-1-piperazinyl)ethyl)-*N*-(2-pyridyl) cyclohexanecarboxamide · 3HCl] were from RBI (Natick, MA). *p*-chlorophenylalanine methyl ester (PCPA) was from Sigma (St Louis, MO). Citalopram HBr was kindly provided by Lundbeck A/S. VN2222 was synthesized by VITA-INVEST (Sant Joan Despí, Barcelona, Spain). Concentrated stock solutions were prepared and aliquots were stored at −80°C. Working solutions were prepared daily by dilution. For local administration of drugs, these were dissolved in the dialysis fluid and applied by reverse dialysis at the stated concentrations (uncorrected for probe recovery). Systemic administration of VN2222 was performed s.c. at the stated doses in 10% Tween 80. 8-OH-DPAT and WAY 100635 were injected s.c. dissolved in saline. An additional experiment in which we examined the effects of the sustained administration of fluoxetine (3 mg/kg day, dissolved in 50% DMSO) and VN2222 (6 mg/kg day, dissolved in DMSO) was carried out in which these drugs were administered with minipumps following described procedures (Hervás *et al*, 2001). Control rats were treated with vehicle.

In electrophysiological experiments, 8-OH-DPAT, fluoxetine, WAY 100635, and VN2222 were administered i.v. through the femoral vein. In experiments assessing the changes in 5-HT_{1A} receptor sensitivity produced by the chronic treatment with VN2222, rats were injected twice daily (12 h apart) for 2 weeks with the drug (2 × 3 or 2 × 10 mg/kg s.c.) or vehicle. The same treatment schedule was used to examine the effects of 2-week administration of fluoxetine (3 mg/kg day). Microdialysis experiments were performed on the second day after the last drug injection to avoid residual effects of the drug. The sensitivity of 5-HT_{1A} receptors was also examined using single-unit recordings. These were performed 1 day after microdialysis (3-day washout) to assess the sensitivity of 5-HT_{1A} receptors by both techniques in the same rats. Yet, owing to the greater experimental requirements of electrophysiological *vs* microdialysis experiments, single-unit recordings could not be performed in all rats. We report on electrophysiological data from two complete groups (rats treated with vehicle and 6 mg/kg day VN2222, $n = 7$ and 5, respectively) and two rats treated with 20 mg/kg day VN2222.

Surgery and Microdialysis Procedures

Microdialysis procedures were performed essentially as described previously (see updated procedure in Adell and Artigas, 1998). In brief, anesthetized rats (pentobarbital 60 mg/kg i.p.) were stereotaxically implanted with I-shaped probes in the dorsal striatum or medial prefrontal cortex. The stereotaxic coordinates (in mm, with respect to bregma and duramater; Paxinos and Watson, 1986) were: prefrontal cortex (AP +3.4, L −0.8, DV −6.0, probe tip: 4 mm), dorsal striatum (AP +0.2, L −3.0, DV −8.0, probe tip 4 mm). Animals were allowed to recover from surgery for 20–24 h and then probes were perfused with artificial CSF (125 mM NaCl, 2.5 mM KCl, 1.26 mM $CaCl_2$, and 1.18 mM $MgCl_2$) pumped at 0.25 µl/min. When required by the experimental paradigm, the dialysis fluid was supplemented with 1 or 10 µM citalopram. Sample collection started 60 min after the beginning of perfusion. Dialysate samples were collected every 20 min (5 µl). Usually 5–6 fractions were collected before drug administration, of which four were used to obtain the individual basal values. At the end of the experiments, rats were killed by an overdose of anesthetic

and the placement of the dialysis probes was checked by perfusing Fast Green dye and visual inspection of the probe track after cutting the brain at the appropriate levels.

Biochemical Analyses

The concentration of 5-HT in dialysates was determined using a modification of an HPLC method previously described (Adell and Artigas, 1998). 5-HT was also determined in homogenates of frontal cortex of control rats and of rats treated with PCPA (350 mg/kg, 2 days before). Briefly, after removal of the brains from the skull, pieces of frontal cortex were rapidly dissected out on ice, weighed, and stored frozen (-80°C). Brain tissue was ultrasonically homogenized (15–20 s) in cold perchloric acid (0.4 mol/l) containing 0.1% sodium metabisulfite, 0.01% EDTA, and 0.01% cysteine. After centrifugation (10 min, 10 000 *g*) aliquots of supernatants were analyzed by HPLC. The composition of HPLC eluant was as follows: 0.15 M NaH_2PO_4, 1.3 mM octyl sodium sulfate, 0.2 mM EDTA (pH 2.8 adjusted with phosphoric acid), plus 27% methanol. 5-HT was separated on a 3 μm ODS 2 column (7.5 × 0.46 cm; Beckman, San Ramon, CA) and detected amperometrically with a Hewlett-Packard 1049 detector (oxidation potential +0.6 V). Retention time was 3.5–4 min. 5-HT values were calculated by reference to standard curves run daily.

Single-Unit Recordings

We examined the ability of VN2222 to suppress the firing activity of 5-HT neurons in the dorsal raphe nucleus in untreated rats. This was compared to that of the 5-HT_{1A} receptor agonist 8-OH-DPAT and the SSRI fluoxetine. The inhibitory effects of these agents on serotonergic firing rate were reversed by the administration of low doses of the 5-HT_{1A} receptor antagonist WAY 100635 (5–10 μg/kg i.v.). In chronic experiments, we assessed the sensitivity of 5-HT_{1A} receptors by comparing the ability of 8-OH-DPAT to suppress serotonergic cell firing in groups of rats pretreated with vehicle or VN2222 for 2 weeks after a washout period of 3 days.

Single-unit extracellular recordings were performed as previously described (Sawyer *et al*, 1985; Celada *et al*, 1996). Briefly, rats were anesthetized (chloral hydrate 400 mg/kg i.p.) and positioned in a stereotaxic apparatus. Additional doses of chloral hydrate (60 mg/kg) were administered i.v. Body temperature was maintained at 37°C throughout the experiment with a heating pad. All wound margins and points of contact between the animal and the stereotaxic apparatus were infiltrated with lidocaine solution (5%). In order to minimize pulsation, the atlanto-occipital membrane was punctured to release some CSF. For recordings in the dorsal raphe nucleus, a burr hole of approximately $4 \times 4\,mm^2$ was drilled over lambda and the sagital sinus was ligated, cut, and reflected. Single units in the dorsal raphe nucleus were recorded extracellularly with glass micropipettes pulled from 2.0-mm capillary glass (WPI, Saratosa, FL) on a Narishige PE-2 pipette puller (Narishige Scientific Instruments, Tokyo, Japan). Microelectrodes were filled with 2 M NaCl. Typically, impedance was between 4 and 10 MΩ. Descents were carried out along the midline. 5-HT neurons were recorded 5.1–5.8 mm below the brain surface and were identified according to electrophysiological criteria previously described (Wang and Aghajanian, 1977, 1982). They exhibited a regular spontaneous firing rate with frequencies of 0.4–2.0 Hz, and 2–5 ms bi- or triphasic extracellular waveform.

Single-unit potentials were amplified with a Neurodata IR283 (Cygnus Technology Inc., Delaware Water Gap, PA), postamplified, and filtered with a Cibertec amplifier (Madrid, Spain), and computed on-line using a DAT 1401plus interface system Spike2 software (Cambridge Electronic Design, Cambridge, UK). Data were also recorded on audiotape for off-line reanalysis if necessary. After recording stable baseline spontaneous activity for at least 5 min, 8-OH-DPAT, fluoxetine, or VN2222 were administered i.v. every 2 min. Only one neuron per rat was recorded.

Data and Statistical Analysis

Microdialysis results are expressed as fmol/fraction (uncorrected for recovery) and shown in figures as percentages of basal values (individual means of four predrug fractions). Statistical analysis of drug effects on dialysate 5-HT was performed using one- or two-way analysis of variance (ANOVA) for repeated measures of raw data with time as repeated factor and dose or pretreatment as independent factor. Student's *t*-tests have been used where appropriate. Changes in firing rate were quantified by averaging the values in the second minute after drug injection and expressed as percentage of baseline. EC_{50} and ED_{50} values were calculated with the GraphPad Prism program (GraphPad software, San Diego, CA). Data are expressed as the mean ± SEM. The number of animals in each group is given in figure legends. Statistical significance has been set at the 95% confidence level (two tailed).

RESULTS

In Vivo Effects of the Local and Systemic Administration of VN2222 on Extracellular 5-HT Concentration

Baseline 5-HT_{ext} values in striatum were 4.2 ± 0.2 fmol/fraction ($n = 26$). The local application of VN2222 (10–300 μM, uncorrected for recovery) significantly increased 5-HT_{ext} in dorsal striatum. The calculated EC_{50} value was 59 μM (Figure 2). The systemic administration of VN2222 (1, 3, and 10 mg/kg s.c.) induced a long-lasting and dose-dependent decrease of 5-HT_{ext} in dorsal striatum reaching a maximum of 50% of baseline at 10 mg/kg ($p < 0.000001$, time factor; $p < 0.000002$, time × dose interaction) (Figure 3a). Average values (fractions 7–13) were 101.2 ± 2.2, 92.7 ± 6.4, 72.6 ± 4.7, and 55.6 ± 2.6% of baseline for controls, 1, 3, and 10 mg/kg VN2222, respectively. In the presence of 1 μM citalopram (to block locally 5-HT reuptake) baseline 5-HT values were 21.0 ± 7.3 fmol/fraction ($n = 20$). In this experimental condition, the s.c. administration of VN2222 induced a greater reduction of 5-HT_{ext} (Figure 3b), which was also statistically significant ($p < 0.000002$, dose factor; $p < 0.000001$, time factor; $p < 0.000001$, time × dose interaction). The average values (fractions 7–13) were 104.6 ± 3.1, 90.0 ± 2.7, 70.2 ± 2.3,

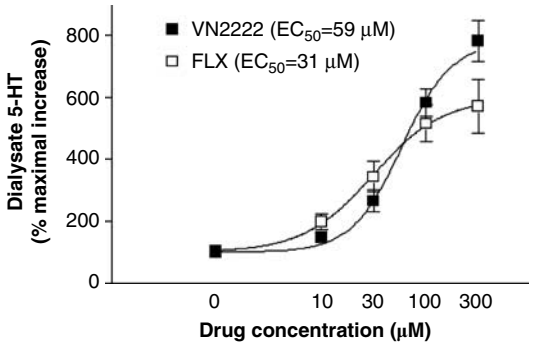

Figure 2 Increase of the extracellular 5-HT concentration in rat striatum produced by the local application of VN2222 using reverse dialysis ($n = 6$). Data points are means of the last two fractions at each concentration, expressed as percentage of baseline. For comparison, the effect of the local application of fluoxetine is also shown (adapted from Hervás and Artigas, 1998). The calculated EC_{50} values for VN2222 and fluoxetine were, respectively, 59 and 31 μM (uncorrected for recovery).

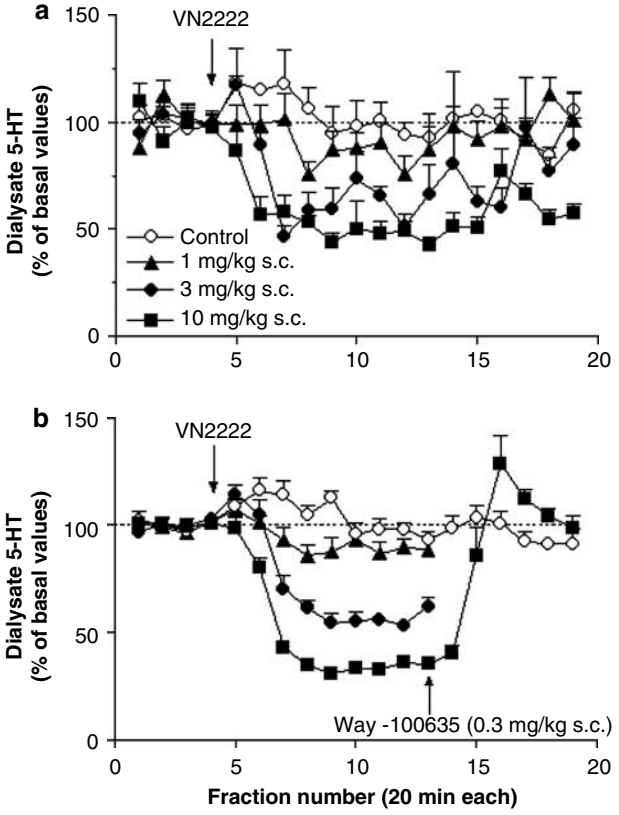

Figure 3 (a) Effect of the s.c. administration of 1, 3, and 10 mg/kg of VN2222 (arrow) on the extracellular 5-HT concentration in rat striatum ($n = 5$–6 rats/group). (b) Effect of the s.c. administration of 1, 3, and 10 mg/kg of VN2222 (first arrow) on the extracellular 5-HT concentration in rat striatum ($n = 4$–5 rats/group) using a perfusion fluid containing 1 μM of the reuptake inhibitor citalopram. The administration of the 5-HT_{1A} receptor antagonist WAY 100635 (0.3 mg/kg s.c.; second arrow) reversed the inhibition of 5-HT release elicited by VN2222 10 mg/kg s.c. See results for statistical analysis.

and 47.2 ± 0.9% of baseline for controls, 1, 3, and 10 mg/kg VN2222, respectively. The reduction in 5-HT_{ext} produced by 10 mg/kg VN2222 was fully counteracted by the administration of 0.3 mg/kg s.c. of the selective 5-HT_{1A} receptor antagonist WAY 100635 ($p < 0.000001$; Figure 3b).

Single-Unit Recordings in Dorsal Raphe Serotonergic Neurons

The i.v. administration of VN2222 reduced dose dependently the firing rate of identified 5-HT neurons in the dorsal raphe nucleus. Figure 4a shows an integrated firing rate histogram corresponding to the effects of cumulative doses of VN2222 (2–64 μg/kg i.v.). Neuronal activity ceased in most neurons examined at 64–128 μg/kg i.v. VN2222. The calculated ED_{50} was 14.9 μg/kg i.v. (Figure 4a). The effect of VN2222 was reversed by a low dose of WAY 100635 (5 μg/kg i.v.; Figure 4a). Owing to the high affinity of VN2222 for the 5-HT transporter and 5-HT_{1A} receptors, we also examined the effects of two reference compounds, fluoxetine and 8-OH-DPAT on 5-HT cell firing. Both inhibited the firing rate of dorsal raphe serotonergic neurons, with ED_{50} values of 790 and 0.76 μg/kg i.v., respectively (Figure 4b).

To examine whether VN2222 inhibited 5-HT neuronal firing by a direct action on 5-HT_{1A} receptors or indirectly, because of its ability to block 5-HT reuptake and increase 5-HT_{ext} in the raphe nuclei (as an SSRI), we examined its effects on rats pretreated with the 5-HT synthesis inhibitor PCPA (350 mg/kg, 2 days before). The 5-HT concentration in frontal cortex (wet tissue) was 764 ± 33 pmol/g ($n = 6$) in control rats and 56 ± 8 pmol/g ($n = 7$) in PCPA-treated rats (7% of controls). In the latter group, VN2222 inhibited 5-HT cell firing with an ED_{50} of 46.9 μg/kg. Two-way ANOVA analysis of the data revealed a significant effect of VN2222 on cell firing ($p < 0.0001$) and a significant VN2222 × PCPA interaction ($p < 0.03$) (Figure 4c). One neuron remained unaltered at a VN2222 dose of 64 μg/kg i.v. Omitting this neuron from the calculations resulted in an ED_{50} value of 27.1 μg/kg i.v. ($n = 4$) and a nonsignificant ($p = 0.25$) VN2222 × PCPA interaction.

Effects of VN2222 on Postsynaptic 5-HT_{1A} Receptors

In conditions of blockade of the 5-HT reuptake, the local application of potent 5-HT_{1A} receptor agonists, such as 8-OH-DPAT or BAY × 3702, in medial prefrontal cortex reduced 5-HT_{ext} (Casanovas *et al*, 1999a). This effect is attributable to an activation of postsynaptic 5-HT_{1A} receptors in this brain area since it was reversed by the coperfusion of WAY 100635 (Casanovas *et al*, 1999a). We used this experimental paradigm to assess the action of VN2222 at postsynaptic 5-HT_{1A} receptors. Given the 5-HT reuptake blocking properties of VN2222, a maximal concentration of citalopram (10 μM; Hervás *et al*, 2000) was used to block locally 5-HT reuptake. In this experimental condition, the application of 8-OH-DPAT (100 μM) significantly reduced 5-HT_{ext} in medial prefrontal cortex compared to control rats, perfused with the dialysis fluid supplemented with 10 μM citalopram ($p < 0.00001$, time effect; $p < 0.00001$, time × treatment interaction). The systemic administration of WAY 100635 0.3 mg/kg s.c. reversed the inhibition of 5-HT release produced by 8-OH-DPAT application ($p < 0.000001$) (Figure 5). In control rats perfused with citalopram 10 μM, WAY 100635 0.3 mg/kg s.c. increased 5-HT_{ext} above baseline ($p < 0.01$).

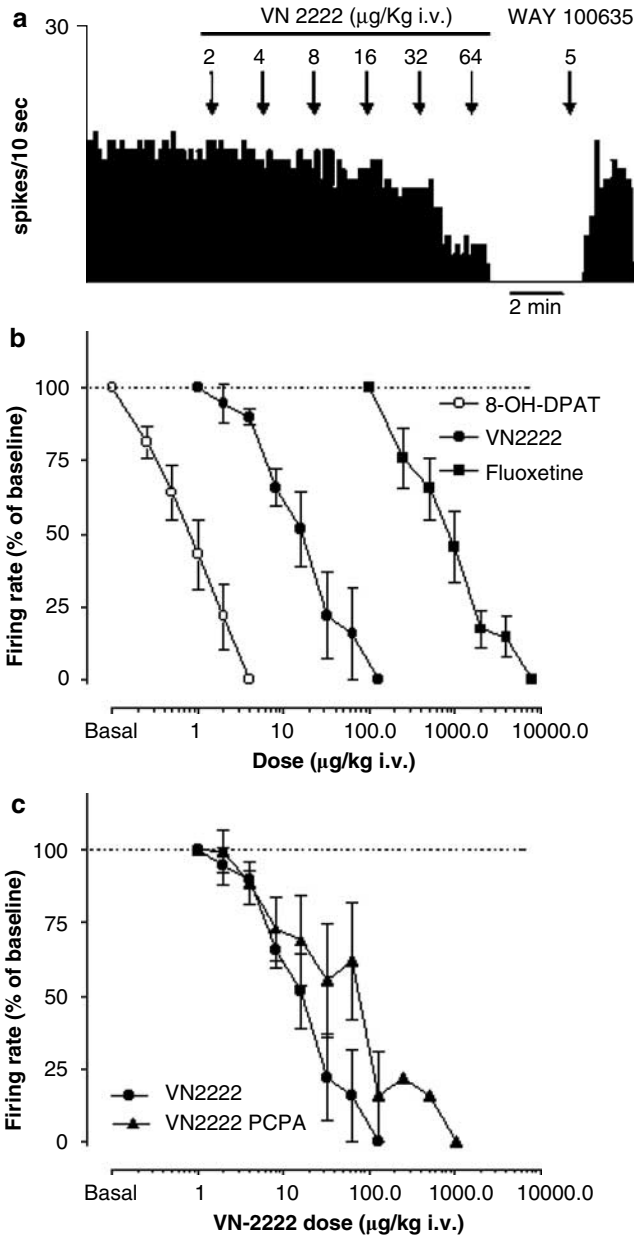

Figure 4 (a) Integrated firing rate histogram showing the effect of cumulative doses of VN2222 (arrows, up to 64 μg/kg i.v.) on the firing rate of a dorsal raphe 5-HT neuron. The suppression of the firing rate produced by VN2222 was reversed by the administration of a low dose of the selective 5-HT_{1A} receptor antagonist WAY 100635 (5 μg/kg i.v.). (b) Dose–response curves of 8-OH-DPAT, VN2222, and fluoxetine to suppress firing rate of 5-HT neurons in the dorsal raphe nucleus. The ED_{50} values were 0.76 [0.62–0.94], 14.9 [7.1–31.5], and 790 [590–1060] μg/kg i.v., respectively ($n = 6$–8 rats/group; 95% confidence intervals in brackets). (c) Comparison of the effect of VN2222 on the firing rate of dorsal raphe serotonergic neurons in untreated ($n = 7$) or rats depleted of 5-HT by the pretreatment with the 5-HT synthesis inhibitor PCPA ($n = 5$). See results for statistical analysis.

The application of VN2222 (300 μM) reduced 5-HT_{ext} in medial prefrontal cortex to an extent comparable to that produced by 100 μM 8-OH-DPAT ($p < 0.0001$, time effect; $p < 0.0002$, time treatment interaction). This effect was also attenuated by the s.c. administration of WAY 100635 ($p < 0.01$; Figure 5). In a small group of rats ($n = 3$), the

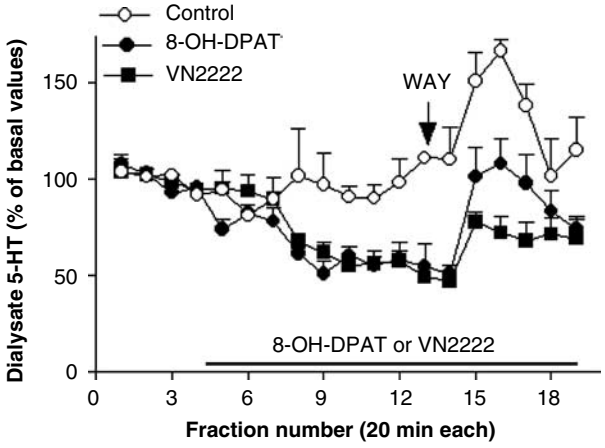

Figure 5 Effects of the local application of the selective 5-HT_{1A} receptor agonist 8-OH-DPAT (100 μM) and VN2222 (300 μM) on the extracellular 5-HT concentration in medial prefrontal cortex ($n = 4$ and 8, respectively). The 5-HT reduction elicited by 8-OH-DPAT and VN2222 was significantly counteracted by the s.c. administration of WAY 100635 (WAY; 0.3 mg/kg s.c.; arrow). Control rats ($n = 3$) were perfused with the dialysis fluid containing citalopram for the entire experiment and were also injected with WAY 100635, which significantly elevated 5-HT_{ext} over baseline. The bar shows the period of drug application. See results for statistical analysis.

local application of VN2222 counteracted the increase in local 5-HT release induced by the selective stimulation of 5-HT_{2A} receptors in medial prefrontal cortex produced by the 5-HT_2 agonist DOI 100 μM (DOI = 179 ± 26% of baseline, DOI+VN2222 = 122 ± 9% of baseline) (data not shown).

Chronic Administration of VN2222: Desensitization of 5-HT_{1A} Autoreceptors

We examined the ability of VN2222 to desensitize 5-HT_{1A} autoreceptors using two different experimental paradigms, *in vivo* microdialysis and single-unit recordings in the DR.

Rats treated with VN2222 (6 and 20 mg/kg day) had a weight gain that did not differ significantly from that of controls (mean values were: controls 74 g, VN2222 6 mg/kg day 83 g, VN2222 20 mg/kg day 85 g). On day 15, rats were implanted with dialysis probes in prefrontal cortex and allowed to recover from anesthesia for ca. 20–24 h, before microdialysis experiments began. Dialysis probes were perfused with 10 μM citalopram to block the 5-HT reuptake in medial prefrontal cortex. After collection of baseline values, rats were administered the selective 5-HT_{1A} receptor agonist 8-OH-DPAT (25 μg/kg s.c.). Baseline 5-HT_{ext} values did not significantly differ between groups (one-way ANOVA) although there was a tendency towards lower values in VN2222-treated rats: 13.1 ± 1.8 fmol/fraction in controls, 9.4 ± 1.8 fmol/fraction in rats treated with VN2222 6 mg/kg day, and 7.6 ± 1.2 fmol/fraction in those treated with VN2222 20 mg/kg day. One-way ANOVA did not show a significant difference between controls and VN2222-treated rats ($F_{2,20} = 2.55$; $p = 0.10$). The reduction in 5-HT_{ext} induced by 8-OH-DPAT was significantly less marked in rats pretreated with VN2222 than in controls (Figure 6). Two-way repeated measures ANOVA revealed a significant effect of time ($p < 0.000001$) and of the time × treatment interaction ($p < 0.002$). The injection of 8-OH-DPAT reduced 5-HT_{ext} maximally to 34 ± 6, 51 ± 8, and 55 ± 7% of

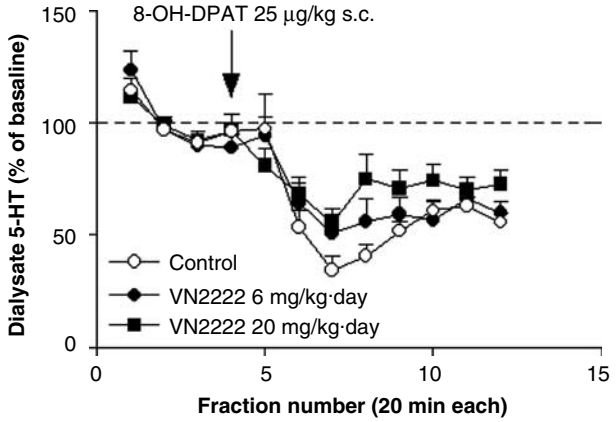

Figure 6 Effect of the administration of an 8-OH-DPAT challenge (25 μg/kg s.c.) on the dialysate 5-HT concentration in prefrontal cortex of rats pretreated with vehicle ($n = 12$), 2 × 3 mg/kg day VN2222 ($n = 5$) and 2 × 10 mg/kg day VN2222 ($n = 6$). Dialysis probes were perfused with artificial CSF containing 10 μM of the 5-HT reuptake inhibitor citalopram. See results for statistical analysis.

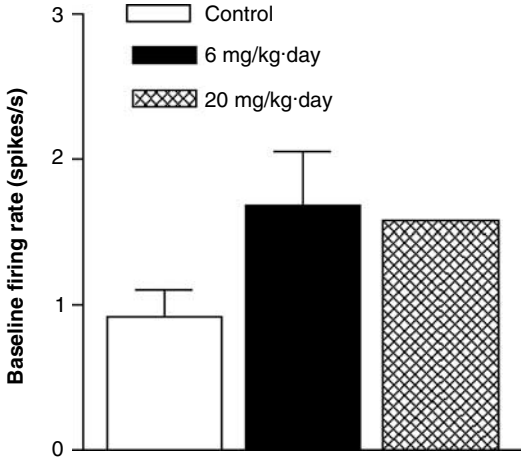

Figure 7 Baseline firing rate of dorsal raphe serotonergic neurons in rats treated with vehicle ($n = 7$), 6 mg/kg day VN2222 ($n = 5$) and 20 mg/kg day VN2222 ($n = 2$). No significant differences were found among groups (one-way ANOVA).

baseline for controls, VN2222 6 mg/kg day and VN2222 20 mg/kg day, respectively.

After microdialysis experiments, rats were kept in their cages and, on the following day, the effects of the pretreatment with vehicle or 6 mg/kg day VN2222 on 5-HT_{1A} receptor sensitivity were assessed by single-unit recordings of dorsal raphe 5-HT neurons. Once a stable recording was obtained for 5 min, cumulative doses of 8-OH-DPAT were injected (0.25–32 μg/kg i.v.) and individual dose–response curves were constructed (one neuron per rat). The baseline firing rate did not significantly differ among groups (Figure 7). This figure shows also the baseline firing of two neurons of rats treated with 20 mg/kg day VN2222. Figure 8 shows representative firing rate histograms of neurons corresponding to the effects of 8-OH-DPAT in controls and rats pretreated with 6 mg/kg day VN2222. The ED_{50} of 8-OH-DPAT for the vehicle-treated group was 0.45 μg/kg i.v. The corresponding value in rats treated with 6 mg/kg day VN2222 was 2.34 μg/kg i.v. Two-way ANOVA revealed a significant effect of the pretreatment (VN2222) ($p < 0.002$), dose (8-OH-DPAT) ($p < 0.0001$) and of the dose × pretreatment interaction ($p < 0.0001$) (Figure 8). In the two rats treated with 20 mg/kg day VN2222, 8-OH-DPAT elicited a moderate decrease of the firing rate at 32 μg/kg i.v. in one rat whereas in another rat, firing rate ceased at 4 μg/kg i.v. 8-OH-DPAT.

To further examine the desensitization of 5-HT_{1A} autoreceptors induced by the chronic administration of VN2222, two groups of rats were treated with 6 mg/kg day VN2222 (as above) or vehicle for 14 days and microdialysis experiments were conducted on the 15th day (15 h after last dose). Baseline 5-HT values were 2.6 ± 0.2 fmol/fraction in controls and 3.0 ± 0.3 fmol/fraction in rats treated with VN2222. The administration of a challenge dose of fluoxetine (10 mg/kg i.p.) elevated extracellular 5-HT significantly more in rats pretreated with VN2222 than in controls ($p < 0.039$, group effect; $p < 0.000001$, time effect; $p < 0.01$, time × group interaction; Figure 9).

In an additional experiment we examined the effect of the sustained administration of VN2222 (6 mg/kg day) and fluoxetine (3 mg/kg day) on extracellular 5-HT in prefrontal cortex using minipumps. Baseline 5-HT values were collected on the 14th day after implant, with the minipumps on board. The 5-HT values in the control groups for fluoxetine and for VN2222 did not differ significantly and were pooled to perform subsequent statistical analyses. Fluoxetine pretreatment increased dialysate 5-HT by 79% (7.5 ± 0.7 *vs* 4.2 ± 0.3 fmol/fraction; $p < 0.00001$) whereas VN2222 elicited a more moderate but still significant 33% increase (5.6 ± 0.5 *vs* 4.2 ± 0.3 fmol/fraction; $p < 0.015$) (Table 1).

DISCUSSION

VN2222 is a new serotonergic agent with putative antidepressant properties. In agreement with its *in vitro* receptor profile (Martínez-Esparza *et al*, 2001; Table 2), the *in vivo* pharmacological properties of VN2222 are consistent with a dual action at the 5-HT transporter and 5-HT_{1A} receptors. Thus, it inhibits 5-HT reuptake *in vivo* with a potency slightly lower than that of fluoxetine. On the other hand, its neurochemical and electrophysiological actions in rat brain are consistent with those of a pre- and postsynaptic 5-HT_{1A} receptor agonist, in the dorsal raphe nucleus and the medial prefrontal cortex, respectively.

VN2222 increased 5-HT_{ext} in the striatum when applied locally by reverse dialysis in the same concentration range as fluoxetine. The ratio between the respective EC_{50} values is ~2 (59 μM for VN2222, 28–31 μM for fluoxetine; Hervás and Artigas, 1998; Hervás *et al*, 2000). However, its systemic administration *reduced* 5-HT_{ext} in the same brain area. The striatum was chosen because (together with prefrontal cortex) it is one of the brain areas where the 5-HT release is more sensitive to the activation of 5-HT_{1A} autoreceptors by direct and indirect (eg SSRI) agonists (Kreiss and Lucki, 1994; Casanovas *et al*, 1997; Romero and Artigas, 1997; Casanovas *et al*, 2000). The discrepancy between the effects of the local (*5-HT increase*) and systemic administration (*5-HT decrease*) of VN2222 is likely accounted for by its

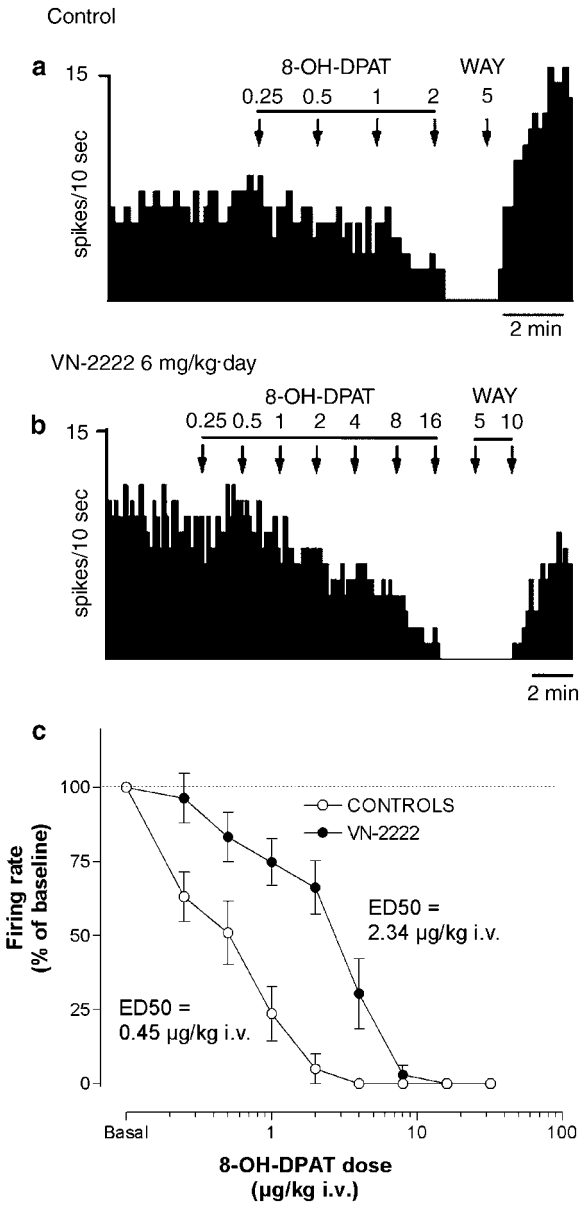

Figure 8 Integrated firing rate histograms corresponding to the effects of cumulative doses of 8-OH-DPAT (arrows, in µg/kg i.v.) on two representative dorsal raphe 5-HT neurons from rats treated with vehicle (a) and VN2222 (6 mg/kg day). (b) The suppression of serotonergic cell firing induced by 8-OH-DPAT was reversed by a low dose of WAY 100635 (WAY; 5–10 µg/kg i.v.). (c) Dose–response curves of the suppressing effect of 8-OH-DPAT on the firing of dorsal raphe 5-HT neurons in rats treated with vehicle ($n=7$) and 6 mg/kg day VN2222 ($n=5$). The calculated ED_{50} values were 0.45 [0.37–0.54] and 2.34 [1.87–2.92] µg/kg i.v., respectively (95% confidence intervals given in brackets).

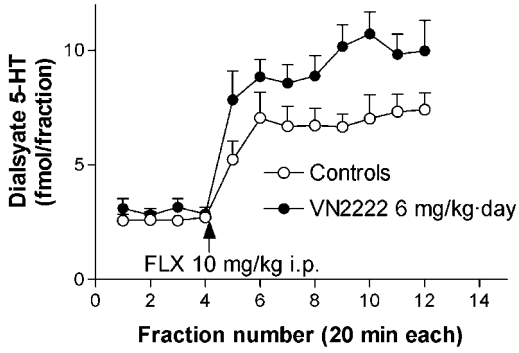

Figure 9 Effect of the administration of a fluoxetine challenge (10 mg/kg i.p., arrow) on the extracellular 5-HT in the medial prefrontal cortex of rats treated with vehicle (open circles; $n=7$) or VN2222 6 mg/kg day (filled circles; $n=8$). Two-way repeated measures ANOVA showed a significant effect of the group, time, and time × group interaction (see text for statistical details).

Table 1 Dialysate 5-HT Levels in the Prefrontal Cortex of Rats Treated with Fluoxetine 3 mg/kg day and VN2222 6 mg/kg day with Minipumps for 2 Weeks

Treatment	5-HT (fmol/fraction)
Vehicle (50% DMSO)	3.8 ± 0.3 (6)
Vehicle (DMSO)	4.5 ± 0.3 (7)
All controls	4.2 ± 0.3 (13)
Fluoxetine 3 mg/kg day (50% DMSO)	7.5 ± 0.3 (7)**
VN2222 6 mg/kg (DMSO)	5.6 ± 0.5 (8)*

Data are means ± SEM of the number of rats shown in brackets. *$p<0.05$ *vs* all controls ($p=0.09$ *vs* DMSO controls). **$p<0.0001$ *vs* all controls.

Table 2 Summary of the *In Vitro* Affinities of VN2222

Receptor	K_i (nM)
5-HT transporter	20 ± 2.3
5-HT_{1A}	20 ± 2.5
5-HT_{1D}	2650 ± 110
5-HT_3	>5000
5-HT_{2A}	250 ± 25
5-HT_{2c}	230 ± 24
Dopamine D_2	150 ± 10
Adrenoceptor α_1	35 ± 9.3
Adrenoceptor α_2	2000 ± 250
Adrenoceptor β	750 ± 56
Muscarinic	>5000

The values represent the mean ± SEM from at least three independent experiments (modified after Martínez-Esparza *et al*, 2001).

agonist effect at raphe 5-HT_{1A} autoreceptors. This action would reduce 5-HT_{ext} to an extent larger than the potential increase produced by systemic reuptake blockade. Indeed, the systemic administration of 5-HT uptake inhibitors activates a 5-HT_{1A} autoreceptor-mediated negative feedback that offsets the increase in forebrain 5-HT_{ext} produced by reuptake blockade (Adell and Artigas, 1991; Artigas *et al*, 1996). Hence, the systemic administration of SSRIs reduces 5-HT_{ext} when the 5-HT reuptake is locally blocked, an action that illustrates their 5-HT release-reducing properties (Rutter and Auerbach, 1993; Hjorth and Auerbach, 1994a; Romero and Artigas, 1997). This effect is because of the activation of raphe 5-HT_{1A} autoreceptors since it is antagonized by the local (in raphe) or systemic administration of WAY 100635 and other nonselective 5-HT_{1A} antagonists (Hjorth and Auerbach, 1994a; Romero and Artigas, 1997). For SSRIs, the balance between the two opposite factors controlling forebrain 5-HT_{ext} (inhibition of 5-HT reuptake *vs* activation of 5-HT_{1A} autoreceptors) is favorable to the former, whereas in the case of VN2222, the latter action predominates. Thus, high doses of SSRIs

increase 5-HT_{ext} after single treatment whereas VN2222 reduces 5-HT_{ext}.

In the presence of 1 μM citalopram in the dialysis fluid, VN2222 reduced 5-HT_{ext} slightly more than in standard dialysis conditions and this effect was counteracted by WAY 100635. This supports the exclusive involvement of 5-HT_{1A} receptors in the reduction of 5-HT release induced by VN2222. The dissimilar effects of SSRIs and VN2222 in the absence of citalopram, together with the fact that VN2222 reduced 5-HT_{ext} in both experimental conditions, suggest that VN2222 has direct agonist properties at 5-HT_{1A} autoreceptors. This view is supported by the results of single-unit recording experiments, in which VN2222 suppressed serotonergic cell firing and this effect was reversed by WAY 100635. The difference in the i.v. and s.c. doses of WAY 100635 used in electrophysiogical and microdialysis experiments of the present study is consistent with previous data showing antagonism of 5-HT_{1A} receptor-mediated effects (Forster *et al*, 1995) and reflects differences in the bioavailability by both routes. Thus, maximal effects of i.v. WAY 100635 take place in 1–2 min whereas they occur at 40–60 min by the s.c. route. An additional reason to use low i.v. doses of WAY 100635 was the observation that in some instances, higher doses suppressed serotonergic cell firing, in agreement with previous observations (Martin *et al*, 1999). Although VN2222 shows an appreciable affinity for α_1-adrenoceptors (Table 2) its suppressing effects on 5-HT release and cell firing do not appear to be mediated by a blockade of the α_1-adrenoceptor-mediated activation of 5-HT neurons, as these effects are fully counteracted by WAY 100635.

The affinity of VN2222 for 5-HT_{1A} receptors is lower than that of 8-OH-DPAT (Martínez-Esparza *et al*, 2001). In agreement, the calculated ED_{50} of VN2222 to suppress 5-HT cell firing (15 μg/kg i.v.) is greater than that of 8-OH-DPAT (0.76 μg/kg i.v.) but comparable or lower than that of other 5-HT_{1A} receptor agonists, such as buspirone (11 μg/kg i.v.; VanderMaelen *et al*, 1986), ipsapirone (30–125 μg/kg i.v.; Basse-Tomusc and Rebec, 1986; Cox *et al*, 1993), or flesinoxan (21–108 μg/kg i.v.; Gobert *et al*, 1995; Hadrava *et al*, 1995).

5HT_{1A} receptor agonists and 5-HT reuptake blockers inhibit directly and indirectly, respectively, the firing activity of serotonergic neurons (Scuvée-Moreau and Dresse, 1979; Quinaux *et al*, 1982; Sprouse and Aghajanian, 1986; Blier and de Montigny, 1987). Since VN2222 appears to display both activities *in vivo*, either could theoretically contribute to its firing-suppressant effects. However, the inhibition of 5-HT reuptake seems to play a minor role in this effect, since (a) the ED_{50} of VN2222 to suppress 5-HT cell firing is 53 times lower than that of fluoxetine, whereas its EC_{50} to inhibit locally 5-HT reuptake is double that of fluoxetine and (b) VN2222 inhibited the serotonergic cell firing in rats depleted of 5-HT by pretreatment with the 5-HT synthesis inhibitor PCPA. This drug abolishes the inhibitory activity of indirect 5-HT_{1A} receptor agonists, including 5-HT reuptake blockers and cocaine (Trulson and Crisp, 1986; Cunningham and Lakoski, 1990). There was some difference between the ED_{50} values in both situations but this was small and mostly because of the influence of one neuron that deviated from the average sensitivity and showed no inhibition at 64 μg/kg VN2222, which may be because of adaptive mechanisms in some 5-HT neurons after irreversible inhibition of tryptophan hydroxylase by PCPA.

The local application of 8-OH-DPAT and VN2222 in medial prefrontal cortex markedly reduced basal 5-HT_{ext}. These experiments were carried out in the presence of a maximal concentration of citalopram (10 μM; Hervás *et al*, 2000) to mask completely the 5-HT uptake inhibition elicited by VN2222. It was hypothetized that in this experimental condition we would observe the putative 5-HT_{1A} receptor agonist action of VN2222, because the 5-HT reuptake was completely blocked by citalopram. In agreement with previous observations (Casanovas *et al*, 1999a), WAY 100635 antagonized the reduction induced by both agents, which supports the involvement of postsynaptic 5-HT_{1A} receptors in these effects. Interestingly, WAY 100635 administration increased 5-HT_{ext} above baseline in control rats perfused with 10 μM citalopram. This suggests that the 5-HT elevation produced by this citalopram concentration was sufficient to activate postsynaptic 5-HT_{1A} receptors and self-attenuate 5-HT release. In the absence of citalopram, WAY 100635 administration does not increase 5-HT_{ext} in this and other forebrain areas of unanesthetized rats (Invernizzi *et al*, 1997; Romero and Artigas, 1997; Hervás *et al*, 2000; Romero *et al*, unpublished observations).

The reduction in 5-HT_{ext} in medial prefrontal cortex elicited by the local application of 5-HT_{1A} receptor agonists is because of the activation of postsynaptic 5-HT_{1A} receptors (Casanovas *et al*, 1999a; Celada *et al*, 2001). Pyramidal neurons in prefrontal cortex project to and control the activity of ascending 5-HT neurons in the dorsal raphe nucleus through the activation of 5-HT_{1A} and 5-HT_{2A} receptors (Sesack *et al*, 1989; Hajós *et al*, 1998; Peyron *et al*, 1998; Celada *et al*, 2001; Martín-Ruiz *et al*, 2001). Thus, the activation of 5-HT_{1A} receptors in pyramidal neurons results in neuronal hyperpolarization (Araneda and Andrade, 1991) and a reduction of the excitatory input onto ascending 5-HT neurons (Celada *et al*, 2001), which decreases firing-dependent 5-HT release. Thus, in common with selective 5-HT_{1A} agonists like 8-OH-DPAT or BAY × 3702, VN2222 displays agonist properties at postsynaptic 5-HT_{1A} receptors. Moreover, VN2222 counteracted the 5-HT_{2A} receptor-mediated elevation in 5-HT_{ext} induced by the local application of DOI, as observed previously for the potent 5-HT_{1A} receptor agonist BAY × 3702 (Martín-Ruiz *et al*, 2001). The agonism of VN2222 at cortical postsynaptic 5-HT_{1A} receptors may be important for its antidepressant effects in animal models of depression (Martínez-Esparza *et al*, 2001) because 5-HT_{1A} receptor agonists show anxiolytic/antidepressant properties (Lucki *et al*, 1994; de Vry, 1995). In addition, electrophysiological data support that the activation of hippocampal 5-HT_{1A} receptors may be important for the antidepressant action (Blier and de Montigny, 1994; Haddjeri *et al*, 1998). However, the present data cannot establish whether VN2222 acts as a full or partial agonist *in vivo* at cortical 5-HT_{1A} receptors, yet the similar reduction of 5-HT release elicited by VN2222 and 8-OH-DPAT suggests a substantial agonist effect of the former. Moreover, in hippocampal membranes, VN2222 inhibits the forskolin-induced cAMP formation with a potency lower than that of 8-OH-DPAT (Martínez-Esparza *et al*, 2001).

The repeated administration of VN2222 resulted in a functional desensitization of 5-HT_{1A} autoreceptors, as assessed by *in vivo* microdialysis and single-unit recordings of dorsal raphe 5-HT neurons. The five-fold shift of the sensitivity of 5-HT_{1A} receptors produced by 6 mg/kg day VN2222 is greater than that produced by maximal doses of SSRIs and selective 5-HT_{1A} receptor agonists (Haddjeri *et al*, 1999; Le Poul *et al*, 1995, 1999, 2000) and suggests a very efficient desensitization of 5-HT_{1A} autoreceptors controlling serotonergic cell firing. Interestingly, the baseline firing rate of rats treated chronically with VN2222 was higher, although not significantly different from controls, which suggests that 5-HT neurons had—at least—recovered their normal firing rate, in agreement with previous observations with other antidepressant drugs (Blier and de Montigny, 1994).

Likewise, in microdialysis experiments, the 8-OH-DPAT challenge reduced 5-HT release significantly less in rats pretreated with VN2222, further supporting the view that this agent desensitized 5-HT_{1A} autoreceptors. In microdialysis experiments, only one dose of 8-OH-DPAT was used. Therefore, we could not determine whether the difference between controls and VN2222-treated rats was because of a change in potency or in maximal effect. However, the electrophysiological data suggest a change in potency. Similarly, a challenge dose of fluoxetine was able to induce a greater increase in 5-HT_{ext} in rats pretreated with VN2222 than in controls, an observation that also supports the view that VN2222 effectively desensitized 5-HT_{1A} autoreceptors after repeated treatment for 2 weeks.

The increase in 5-HT_{ext} produced by minipump administration of fluoxetine 3 mg/kg day is in agreement with previous data from this laboratory (Hervás *et al*, 2000), although the effect size was slightly lower in the present experiments because of higher baseline 5-HT_{ext} values in controls. VN2222 produced a more moderate increase in 5-HT_{ext} which was, however, statistically significant (Table 1). Again, since acute treatment with this daily dose *reduced* 5-HT_{ext}, this is an additional argument in favor of an effective desensitization of 5-HT_{1A} receptors by VN2222.

Taken together, electrophysiological and microdialysis experiments suggest that a 2-week VN2222 treatment markedly reduced the effectiveness of 5-HT_{1A} receptors controlling presynaptic serotonergic function. Moreover, (a) postsynaptic 5-HT_{1A} receptors in cortico-limbic areas are involved in the effects of systemically administered 8-OH-DPAT (Ceci *et al*, 1994; Romero *et al*, 1994; Artigas *et al*, 1998; Hajós *et al*, 1999; Celada *et al*, 2001) and (b) these do not desensitize after prolonged treatments (Blier and de Montigny, 1994; Dong *et al*, 1997; Le Poul *et al*, 2000). Hence, it is possible that the actual sensitivity of raphe 5-HT_{1A} autoreceptors after repeated VN2222 treatment is actually lower than that determined in the present experiments using the systemic 8-OH-DPAT challenge.

The present data are in keeping with previous work showing that prolonged administration of 5-HT_{1A} receptor agonists and SSRIs desensitizes dorsal raphe 5-HT_{1A} autoreceptors (Blier and de Montigny, 1987, 1994; Hensler *et al*, 1991; Bohmaker *et al*, 1993; Invernizzi *et al*, 1994; Le Poul *et al*, 1995, 1999, 2000; Dong *et al*, 1997; Casanovas *et al*, 1999b; Hervás *et al*, 2000). However, some microdialysis studies have failed to observe such effects (Sharp *et al*, 1993; Hjorth and Auerbach, 1994b; Auerbach and Hjorth, 1995; Bosker *et al*, 1995; Invernizzi *et al*, 1995). The origin of these discrepancies is unclear and may involve differences in drug half-lives of the agents used, route of administration (eg repeated injections *vs* minipumps) and regional effects, since most of the latter studies were conducted in the hippocampus, mostly innervated by median raphe 5-HT neurons. Our own microdialysis data, obtained in prefrontal cortex, are consistent with single-unit recordings in the dorsal raphe indicating a desensitization of 5-HT_{1A} receptors. More importantly, some microdialysis and single-unit data were obtained in the same animals, which reinforces the association between both measures. It should be mentioned that the present observations have been obtained with a relative low dose of VN2222 (6 mg/kg day). This is equivalent to 3 mg/kg day fluoxetine in terms of *in vivo* reuptake blockade and lower than the standard 10 mg/kg day dose, which may appear too high compared with the standard clinical regimen (20 mg/day). It is thus possible that a more effective desensitization and/or greater 5-HT_{ext} increments could have been obtained after repeated treatment with larger doses of fluoxetine and VN2222.

In summary, the present results show that VN2222 inhibits the 5-HT reuptake and is a direct agonist at pre- and postsynaptic 5-HT_{1A} receptors *in vivo*. SSRIs possess significant clinical antidepressant activity and experimental data support the involvement of postsynaptic 5-HT_{1A} receptors in their clinical action. VN2222 shares both pharmacological activities *in vivo*, and in particular, it behaves as a cortical 5-HT_{1A} receptor agonist and effectively desensitizes 5-HT_{1A} autoreceptors. The therapeutic lag of SSRIs can be attributable to the 5-HT_{1A}-autoreceptor-mediated impairment of serotonergic activity that follows reuptake blockade in the raphe (presynaptic component) and to the absence of a sufficient tone on postsynaptic 5-HT receptors (postsynaptic component). As 5-HT_{1A} receptor desensitization progresses, the tone on postsynaptic receptors increases, which results in a therapeutic action. VN2222 shares with SSRIs the property of suppressing serotonergic activity (presynaptic component). Such a reduction may appear contradictory with the desired enhancement of the tone on postsynaptic 5-HT receptors demanded of antidepressant agents, which could potentially result in a greater latency to onset of action. However, recent evidence suggests that antidepressant drugs enhance neurogenesis in the rat hippocampus (Malberg *et al*, 2000), an effect possibly mediated by the activation of postsynaptic 5-HT_{1A} receptors (Gould, 1999). Moreover, blockade of 5-HT_{2A} neurotransmission by M100907 enhanced the antidepressant effects of fluoxetine in the DRL 72-S reinforcement schedule without concurrently increasing 5-HT_{ext} (Marek *et al*, 2001). Since 5-HT_{2A} colocalize with 5-HT_{1A} receptors in pyramidal neurons of medial prefrontal cortex (Martín-Ruiz *et al*, 2001) and both mediate opposing effect on pyramidal cell excitability (Araneda and Andrade, 1991) these behavioral results suggest that the superior antidepressant effect of fluoxetine+M100907 is because of an enhanced 5-HT_{1A}–mediated transmission. VN2222 may activate postsynaptic 5-HT_{1A} receptors during a time when SSRIs exert little or no activation of such receptors, an action that may result in a more rapid and/or effective antidepressant action.

ACKNOWLEDGEMENTS

Work supported by grants from the Fondo de Investigación Sanitaria (2001–1147) and Marató TV3. Financial support from VITA–INVEST SA is gratefully acknowledged. Llorenç Díaz–Mataix is recipient of a fellowship from the IDIBAPS. We thank Leticia Campa for her skillful technical assistance with HPLC analyses.

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