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RESEARCH PAPER

Reduced signal transduction by 5-HT$_4$ receptors after long-term venlafaxine treatment in rats

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BACKGROUND AND PURPOSE
The 5-HT$_4$ receptor may be a target for antidepressant drugs. Here we have examined the effects of the dual antidepressant, venlafaxine, on 5-HT$_4$ receptor-mediated signalling events.

EXPERIMENTAL APPROACH
The effects of 21 days treatment (p.o.) with high (40 mg·kg^{-1}) and low (10 mg·kg^{-1}) doses of venlafaxine, were evaluated at different levels of 5-HT$_4$ receptor-mediated neurotransmission by using *in situ* hybridization, receptor autoradiography, adenylate cyclase assays and electrophysiological recordings in rat brain. The selective noradrenaline reuptake inhibitor, reboxetine (10 mg·kg^{-1}, 21 days) was also evaluated on 5-HT$_4$ receptor density.

KEY RESULTS
Treatment with a high dose (40 mg·kg^{-1}) of venlafaxine did not alter 5-HT$_4$ mRNA expression, but decreased the density of 5-HT$_4$ receptors in caudate-putamen (% reduction = 26 ± 6), hippocampus (% reduction = 39 ± 7 and 39 ± 8 for CA1 and CA3 respectively) and substantia nigra (% reduction = 49 ± 5). Zacopride-stimulated adenylate cyclase activation was unaltered following low-dose treatment (10 mg·kg^{-1}) while it was attenuated in rats treated with 40 mg·kg^{-1} of venlafaxine (% reduction = 51 ± 2). Furthermore, the amplitude of population spike in pyramidal cells of CA1 of hippocampus induced by zacopride was significantly attenuated in rats receiving either dose of venlafaxine. Chronic reboxetine did not modify 5-HT$_4$ receptor density.

CONCLUSIONS AND IMPLICATIONS
Our data indicate a functional desensitization of 5-HT$_4$ receptors after chronic venlafaxine, similar to that observed after treatment with the classical selective inhibitors of 5-HT reuptake.

Abbreviations
R.O.D, relative optical density; SNRI, selective noradrenaline reuptake inhibitor; SSRI, selective serotonin reuptake inhibitor

Introduction

There is considerable evidence to support the idea that a deficit in 5-HT and noradrenaline neurotransmission is associated with depression (Schildkraut, 1965; Coppen, 1967; Lanni *et al.*, 2009). Accordingly, effective treatment of this condition is achieved with monoamine oxidase inhibitors, tricyclic antidepressants, selective 5-HT reuptake inhibitors (SSRIs) or 5-HT-noradrenaline reuptake inhibitors that

enhance either central 5-HT and/or noradrenaline neurotransmission (Vetulani and Nalepa, 2000; Schechter *et al.*, 2005). This increase in 5-HT and/or noradrenaline levels that occurs around 3–4 weeks after initiation of antidepressant treatment is mainly due to a functional desensitization of somatodendritic 5-HT_{1A} autoreceptors and presynaptic α_2-adrenoceptors (receptor nomenclature follows Alexander *et al.*, 2009) located on 5-hydroxytryptaminergic and noradrenergic neurons respectively (Blier and de Montigny, 1994; Le Poul *et al.*, 1995; Mateo *et al.*, 2001; Castro *et al.*, 2003; Invernizzi and Garattini, 2004; Parini *et al.*, 2005). In addition, other receptors such as 5-HT_2 and β-adrenoceptors have also been implicated in depression and in the mechanisms of action of antidepressants (see Brunello *et al.*, 2002; Adell *et al.*, 2005; Schechter *et al.*, 2005). However, although it is well established that chronic antidepressants produce a considerable functional desensitization of these receptors and of the reuptake sites for 5-HT and noradrenaline (Horschitz *et al.*, 2001; Benmansour *et al.*, 2004; Nadgir and Malviya, 2008), the role of other 5-HT receptors in the mechanisms of action of antidepressants involving both the 5-HT and noradrenergic systems, the so-called dual antidepressants, remains still unexplored.

Venlafaxine is a non-selective 5-HT-noradrenaline reuptake inhibitor that shows higher affinity for 5-HT than for noradrenaline reuptake (Muth *et al.*, 1986; Bolden-Watson and Richelson, 1993). Thus, at low doses, venlafaxine mainly acted as a 5-HT reuptake inhibitor alone whereas, only at high doses, was noradrenaline reuptake affected (Beiqüe *et al.*, 2000a,b).

Focusing on 5-hydroxytryptaminergic neurotransmission, 5-HT_4 receptors are widely distributed in the brain, in the basal ganglia, hippocampal formation, amygdala and cortex (Waeber *et al.*, 1994; Vilaró *et al.*, 1996; 2005). These 5-HT_4 receptors belong to the superfamily of G protein-coupled receptors that are positively coupled to adenylate cyclase (Hoyer *et al.*, 2002) promoting intracellular accumulation of cAMP. Activation of 5-HT_4 receptors also inhibits potassium channels, thus contributing to the neuronal excitability of pyramidal cells of hippocampus (Andrade and Chaput, 1991; Fagni *et al.*, 1992). In the CNS, 5-HT_4 receptors appear to modulate neurotransmitter (acetylcholine, dopamine, 5-HT and GABA) release and enhance synaptic transmission (Yamaguchi *et al.*, 1997; Bianchi *et al.*, 2002; Lucas and Debonnel, 2002; Alex and Pehek, 2007), and they may also play a role in memory, anxiety and depression (Bockaert *et al.*, 2004; 2008; see King *et al.*, 2008).

Over the last few years, fresh evidence indicates that 5-HT_4 receptors may represent a new target for antidepressant drugs. First, an increase of 5-HT_4 receptors in cortical and striatal areas was described in *post mortem* brain from depressed patients (Rosel *et al.*, 2004). Second, it has been described that 5-HT_4 receptors exert a facilitatory control on dorsal raphe nucleus 5-HT neuronal activity (Lucas *et al.*, 2005) whereas knockout mice of these receptors show a reduction in this firing (Conductier *et al.* 2006). Interestingly, it has recently been reported that two 5-HT_4 partial agonists, RS67333 and SL65.0155, show antidepressant properties comparable to those of SSRIs, with a faster onset of action (Lucas *et al.*, 2007; Tamburella *et al.*, 2009).

Although two studies have recently shown that long-term treatment with both fluoxetine and paroxetine decreased 5-HT_4 receptor density in the brain (Licht *et al.*, 2009; Vidal *et al.*, 2009), nothing is known about the regulation of this receptor by dual antidepressants. The goal of this study has been to evaluate the influence of chronic treatment with the dual antidepressant, venlafaxine, at different levels of the 5-HT_4 transductional pathway by using *in vitro* procedures. For comparative purposes, the effect of chronic reboxetine, a selective noradrenaline reuptake inhibitor, on 5-HT_4 receptor density was also analysed.

Methods

Animals

All animal care and experimental procedures were according to the Spanish legislation and the European Communities Council Directive on 'Protection of Animals Used in Experimental and Other Scientific Purposes' (86/609/EEC). Male Wistar rats weighing 200–250 g were group-housed and maintained at 21 ± 1°C on 12/12 h light/dark cycle, with access to food and water *ad libitum*.

Drug treatments

Rats were given venlafaxine (10 $mg \cdot kg^{-1}$ or 40 $mg \cdot kg^{-1}$), reboxetine (10 $mg \cdot kg^{-1}$) or saline, by gavage, once a day for 21 days. Drugs were administered at the same time each day, between 11 and 12 h a.m., and 24 h after the last dose, the animals were killed and their brains quickly removed: for *in situ* hybridization, autoradiographic and adenylate cyclase assays were frozen immediately in isopentane and then stored at –80°C until use. For electrophysiological studies, brains were placed in artificial cerebrospinal fluid (ACSF) (for composition see below).

In situ *hybridization*

Coronal sections of 20 µm thickness were cut at –20°C in a cryostat at the level of cortex, striatum and hippocampus according to the stereotaxic atlas of the rat brain (Paxinos and Watson, 1982). Sections were then thaw-mounted on slides and stored at –20°C until use.

Six different oligonucleotide probes were used simultaneously for the detection of 5-HT$_4$ receptor mRNA. They were complementary to the following bases of the rat 5-HT$_4$ receptor mRNA (Gerald *et al*. 1995) (base numbering corresponds to the sequence of the 5-HT$_{4(a)}$ splice variant, GenBank accession number U20906): 21–70, 258–307, 683–732, 741–790, 960–1009, 1029–1078. These regions of the mRNA are common to all four C-terminal splice variants cloned in the rat: r5-HT$_{4(a)}$, r5-HT$_{4(b)}$ (Gerald *et al*. 1995), r5-HT$_{4(e)}$ (Claeysen *et al*. 1999) and r5-HT$_{4(c1)}$ (Ray *et al*., 2009). Oligonucleotides were labelled at their 3'-end using [^{33}P] α-dATP (111 TBq·mmol^{-1}, Perkin Elmer, Waltham, MA, USA) and terminal deoxynucleotidyltransferase (Oncogene Research Products, San Diego, CA, USA). Labelled probes were purified from non-incorporated nucleotides with ProbeQuant G-50 micro columns (GE Healthcare, Little Chalfont, UK).

Tissues were treated before hybridization as described (Vilaró *et al*., 1992). They were air-dried, fixed by immersion for 20 min in a solution of 4% paraformaldehyde in phosphate-buffered saline (1 × PBS: 2.6 mM KCl, 1.4 mM KH_2PO_4, 136 mM NaCl, 8 mM Na_2HPO_4; pH 7.5), washed once in 3 × PBS, twice in 1 × PBS, 5 min each, and incubated in a freshly prepared solution of predigested pronase (Calbiochem, San Diego, CA) at a final concentration of 24 U·mL^{-1} in 50 mM Tris-HCl pH 7.5, 5 mM EDTA for 2 min at room temperature. Proteolytic activity was stopped by immersion for 30 s in glycine (2 mg·mL^{-1}) in PBS. Tissues were rinsed in PBS and dehydrated in a graded series of ethanol. For hybridization, labelled probes were diluted to a final concentration of approximately 2 × 10^7 cpm·mL^{-1} (0.3 nM each probe) in a solution containing 50% formamide, 4 × standard saline citrate (1 × SSC: 150 mM NaCl, 15 mM sodium citrate), 1 × Denhardt's solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin), 10% dextran sulphate, 1% Sarkosyl, 20 mM phosphate buffer pH 7.0, 250 µg·mL^{-1} yeast tRNA, 500 µg·mL^{-1} salmon sperm DNA (Vilaró *et al*., 1996). Tissues were covered with 70–80 µL of hybridization solution, overlaid with Nescofilm coverslips (Bando Chemical, Inc., Kobe, Japan) and incubated overnight at 42°C. Sections were washed four times (45 min each) in 600 mM NaCl, 10 mM Tris-HCl pH 7.5, 1 mM EDTA at 60°C, dehydrated and exposed to film (Biomax-MR, Kodak) for 2–3 weeks at –70°C.

[^{3}H]GR113808 receptor autoradiography

Sections were then thaw-mounted in gelatinized slides and stored at –20°C until use. 5-HT$_4$ receptor autoradiography was performed as previously reported by Waeber *et al*. (1994) using the 5-HT$_4$ antagonist [^{3}H]GR113808 as radioligand. Tissue sections, obtained as above, were pre-incubated at room temperature for 15 min in 50 mM Tris-HCl buffer (pH 7.5) containing $CaCl_2$ (4 mM) and ascorbic acid (0.1%). Sections were then incubated, at room temperature for 30 min, in the same buffer with 0.2 nM [^{3}H]GR113808. Non-specific binding was determined using 10 µM 5-HT. After incubation, sections were washed for 30 s in ice-cold buffer, briefly dipped in deionized water at 4°C, and then cold air-dried. Autoradiograms were generated by apposing the slides to Biomax MR film sheets (Kodak, Madrid, Spain) together with [^{3}H] labelled standards for 6 months at 4°C.

Adenylate cyclase assay

5-HT$_4$ receptor stimulated adenylate cyclase assays were carried out as previously described by Vidal *et al*. (2009). Frozen brain striata were homogenized (1:120 W/V) in 20 mM Tris-HCl, 2 mM EGTA, 5 mM EDTA, 320 mM sucrose, 1 mM dithiothreitol (DTT), 25 µg·mL^{-1} leupeptin, pH 7.4 and centrifuged at 500× *g* for 5 min at 4°C. The supernatants were pelleted at 13 000× *g* for 15 min at 4°C and resuspended in 20 mM Tris-HCl, 1.2 mM EGTA, 0.25 M sucrose, 6 mM $MgCl_2$, 3 mM DTT and 25 µg·mL^{-1} leupeptin. The membranes were used immediately after preparation.

Membrane suspensions were pre-incubated for 15 min on ice in reaction buffer (75 mM Tris-HCl pH 7.4, 5 mM $MgCl_2$, 0.3 mM EGTA, 60 mM sucrose, 1 mM DTT, 0.5 mM 3-isobutylmethylxanthine, 5 mM phosphocreatine, 50 U·mL^{-1} creatine phosphokinase and 5 U·mL^{-1} myokinase) and 25 µL of either water (basal activity) or zacopride (5-HT$_4$ receptor agonist; 10^{-3} M–10^{-8} M). The reaction was started by the addition of 0.2 mM Mg-ATP and incubated at 37°C for 10 min. The reaction was stopped by boiling the samples in water for 4 min and then centrifuged at 13 000× *g* for 5 min at 4°C. cAMP accumulation was quantified in 50 µL aliquots of supernatant by using a [^{3}H]cAMP commercial kit, based on the competition of a fixed amount of [^{3}H]cAMP and the unlabelled form of cAMP for a specific protein, achieving the separation of protein-bound nucleotide by adsorption on coated charcoal. (TRK 432, Amersham Pharmacia Biotech U.K.

Limited, Buckinghamshire, UK). Membrane protein concentrations were determined using the Bio-Rad Protein Assay Kit (Bio-Rad, Munich, Germany) using γ-globulin as the standard.

Hippocampal slice preparation and extracellular recording

After decapitation, the brain was quickly removed and placed in an ACSF consisting of 124 mM NaCl, 3 mM KCl, 1.25 mM NaH_2PO_4, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 26 mM $NaHCO_3$ and 10 mM glucose. Transverse slices, 400 µm thick, from hippocampus were obtained using a tissue slicer and were left to recover in ACSF for 1 h. A single slice was transferred to a recording chamber and continuously superfused at a rate of 1 mL·min^{-1} with ACSF saturated with 95% O_2, 5% CO_2 and maintained at 30°C. For extracellular recording of population spikes, a glass microelectrode filled with 3 M NaCl (1–4 MΩ) was positioned in the stratum pyramidalis of the CA1 area. A bipolar, tungsten electrode was placed in the stratum radiatum for stimulation of the Schaffer collateral-commissural pathway. Pulses of 0.05 ms duration were applied at a rate of 0.05 Hz. The population spike signals were amplified, bandpass-filtered (1 Hz–1 kHz) and stored in a computer using the Spike 2 program (Spike2, Cambridge Electronic Design, Cambridge, UK). On the basis of other studies (Tokarski and Bijak, 1996; Bijak *et al.*, 1997), half-maximum stimulation intensity was chosen to evaluate the effect of zacopride. After stabilization of the baseline response for at least 1 h (defined as no more than 10% variation in the median amplitude of the population spike or stable membrane potential), the slice was superfused for 10 min with zacopride (10 µM). Each slice in the extracellular recording was treated as an independent sample.

Data analysis

Autoradiograms were analysed and quantified (radioligand autoradiography) or semi-quantified (*in situ* hybridization) using a computerized image analysis system (Scion Image, Scion Corporation, Maryland, USA). In electrophysiological records, the effect of zacopride is expressed as mean (±SEM) percentage change of the baseline (pre-drug). E_{max} and EC_{50} values in both adenylate cyclase assays and electrophysiological recordings were calculated using the program GraphPad Prism program (GraphPad Software, GraphPad, San Diego, CA, USA). The statistical analysis of the results obtained following venlafaxine administration was performed using Student's *t*-test for *in situ* hybridization or one-way ANOVA followed by *post hoc* comparisons (Student Newman–Keuls test). Results from reboxetine administration (5-HT_4 receptor autoradiography) were analysed by Student's *t*-test. $P < 0.05$ was considered statistically significant.

Materials

[^{33}P] α-dATP (111 TBq·mmol^{-1}) was purchased from Perkin Elmer (Waltham, MA, USA). [^{3}H]GR113808 (specific activity 3.07 TBq·mmol^{-1}) was purchased from Amersham and venlafaxine HCl and reboxetine were kindly donated by FAES-Farma. 5-HT HCl was purchased from Sigma-Aldrich (Madrid, Spain). Zacopride ((R,S) 4-amino-5-chloro-2-methoxybenzamide) was obtained from RBI (Madrid, Spain). All other chemicals used were of analytical grade. Venlafaxine and reboxetine were dissolved in saline (0.9%) and given by oral administration (p.o.) in a volume of 5 mL·kg^{-1} body weight.

Results

Effect of chronic venlafaxine in mRNA 5-HT_4 expression

A specific distribution of the mRNA encoding for 5-HT_4 receptors was observed through different structures of the rat brain, in good agreement with previous studies. In vehicle treated rats, strong hybridization signals were observed in the hippocampus [film relative optical density (R.O.D.) = 80–135] and basal ganglia (R.O.D. = 35–55). Intermediate signals were also detected in superior colliculus whereas 5-HT_4 receptor mRNA expression in the frontal cortex was only moderately labelled (Figure 1). As shown in Figure 2, chronic administration of venlafaxine (40 mg·kg^{-1} p.o.) had no effect on 5-HT_4 mRNA expression at 24 h after the last administration of the antidepressant in any of the brain regions measured: frontal cortex, striatum or hippocampus.

Effect of chronic antidepressants on the density of 5-HT_4 receptors

To evaluate whether treatment with venlafaxine and reboxetine affects the density of 5-HT_4 receptors we measured the binding of the antagonist radioligand [^{3}H]GR113808 in rat brain sections. Only the high dose of venlafaxine tested produced a significant decrease in the density of 5-HT_4 receptors in caudate-putamen (% reduction = 26 ± 6; $P < 0.01$), hippocampus (% reduction = 39 ± 7% and 39 ± 8, for CA1 ($P < 0.01$) and CA3 ($P < 0.01$), respectively, and substantia nigra (% reduction = 50 ± 5; $P < 0.01$) when compared with vehicle treated rats. In contrast, neither dose of venlafaxine modified the density of 5-HT_4 receptors in the frontal cortex (Table 1 and Figure 3). On the other hand, chronic

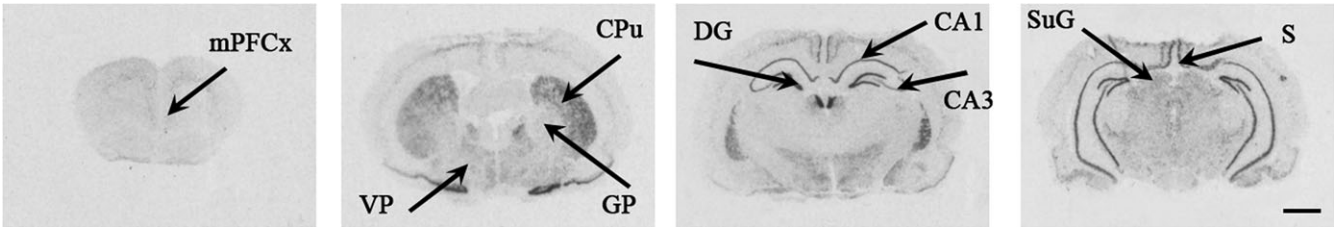

Figure 1

Representative autoradiograms showing the distribution of 5-HT$_4$ receptor mRNA in coronal sections of rats chronically treated with vehicle. mPFCx, medial prefrontal cortex; CPu, caudate-putamen; VP, ventral pallidum; GP, globus pallidus; CA1, CA1 field of hippocampus; CA3, CA3 field of hippocampus; DG, dentate gyrus; S, subiculum; SuG, superior colliculus. Bar: 2 mm.

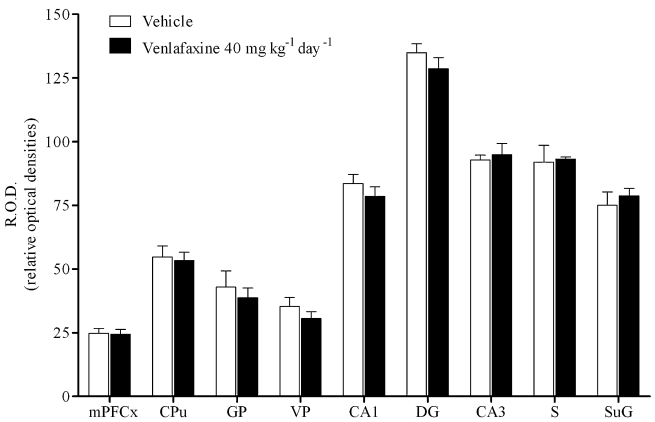

Figure 2

Effect of chronic venlafaxine on 5-HT$_4$ mRNA levels in rat brain measured as relative optical density (R.O.D.). R.O.D for background tissue signal was 47.16. mPFCx, medial prefrontal cortex; CPu, caudate-putamen; GP, globus pallidus; VP, ventral pallidum; CA1, CA1 field of hippocampus; DG, dentate gyrus of hippocampus; CA3, CA3 field of hippocampus; S, subiculum; SuG, superior colliculus. No significant differences were found between the experimental groups (Student's *t*-test, unpaired data; $n = 7$ rats per group).

reboxetine did not alter 5-HT$_4$ receptor binding in any of the brain areas analysed (Table 2).

Effect of chronic venlafaxine in zacopride-induced cAMP accumulation in rat striatum

Chronic venlafaxine did not alter the basal cAMP levels in rat striatum membranes although a tendency to the increase was observed after the dose of 40 mg·kg^{-1} (10.5 ± 3.0 and 17.5 ± 1.8 pmol·min^{-1}·mg·protein^{-1}, for vehicle and venlafaxine respectively). As shown in Figure 4, the agonist zacopride induced a concentration-dependent increase in cAMP production in the vehicle group, with EC_{50} = 2.9 ± 1.1 μM and an E_{max} = +46 ± 1% of stimulation over the basal value (100%). The treatment with the high dose of venlafaxine, administered for 21 days, induced a marked suppression of zacopride-stimulated cAMP accumulation yielding an E_{max} = +22 ± 1% of basal ($P < 0.05$ vs. vehicle). This reduction in the efficacy was also accompanied with an increase in EC_{50} (27 ± 1.2 μM). Nevertheless, chronic administration of venlafaxine at the dose of 10 mg·kg^{-1} did not significantly alter the cAMP accumulation induced by zacopride (Figure 4).

Effect of chronic venlafaxine on population spikes of CA1 field

According to previous reports from our group, the selective 5-HT$_4$ agonist zacopride induced a concentration-dependent increase of the population spike amplitude in the hippocampal CA1 field evoked by Schaffer collateral stimulation with a potency in the μM order (Vidal *et al.*, 2009). Taking into account this observation, we evaluated the effect of chronic treatment with venlafaxine on the stimulation of population spike induced by 10 μM zacopride. The effect of the application of zacopride was significantly reduced in slices obtained from rats treated with venlafaxine 10 mg·kg^{-1} (% reduction = 37 ± 8; $P < 0.05$). This decrease was even more pronounced with the dose of 40 mg·kg^{-1} (% reduction = 56 ± 12; $P < 0.01$) (Figure 5).

Discussion and conclusions

Dual antidepressant drugs affect both the 5-hydroxytryptaminergic and noradrenergic systems by inducing adaptive changes in several receptor subtypes in the brain. In the present study, we have found that a 21 day treatment with 40 mg·kg^{-1} of venlafaxine (high dose), induced a down-regulation of 5-HT$_4$ receptor density without altering the expression of its mRNA. It also resulted in an attenuation of the zacopride-stimulated adenylate cyclase. In contrast, 10 mg·kg^{-1} (low dose) had no significant effect on these neurochemical markers. Furthermore, both doses of this

Table 1

Effect of chronic venlafaxine on the specific binding of [^{3}H]GR113808 in coronal sections (20 µm) of rat brain

Area	Vehicle (10–12)	Venlafaxine (10 mg·kg^{-1}·day^{-1}) (7)	Venlafaxine (40 mg·kg^{-1}·day^{-1}) (6)
Medial prefrontal cortex	13.0 ± 1.1	12.7 ± 0.7	11.2 ± 1.3
Caudate-putamen	18.1 ± 0.6	17.9 ± 1.0	13.9 ± 1.1*†
Globus pallidus	17.2 ± 0.9	15.9 ± 0.9	12.8 ± 1.2*
CA1	14.7 ± 0.8	15.1 ± 0.6	8.9 ± 1.0**††
CA3	14.2 ± 1.0	13.7 ± 0.5	8.7 ± 1.2**††
Substantia nigra	14.6 ± 0.9	13.9 ± 0.6	7.4 ± 0.7**††

Values are expressed as the mean ± SEM of B_{max} (fmol·mg^{-1} tissue), assuming a K_D value of 0.2 nM for [^{3}H]GR113808. The number of determinations is shown in parenthesis, in each column heading.
*P < 0.05; **P < 0.01 different from vehicle and †P < 0.05; ††P < 0.01 different from 10 mg·kg^{-1} venlafaxine treated rats. One-way ANOVA followed by Student Newman–Keuls test.

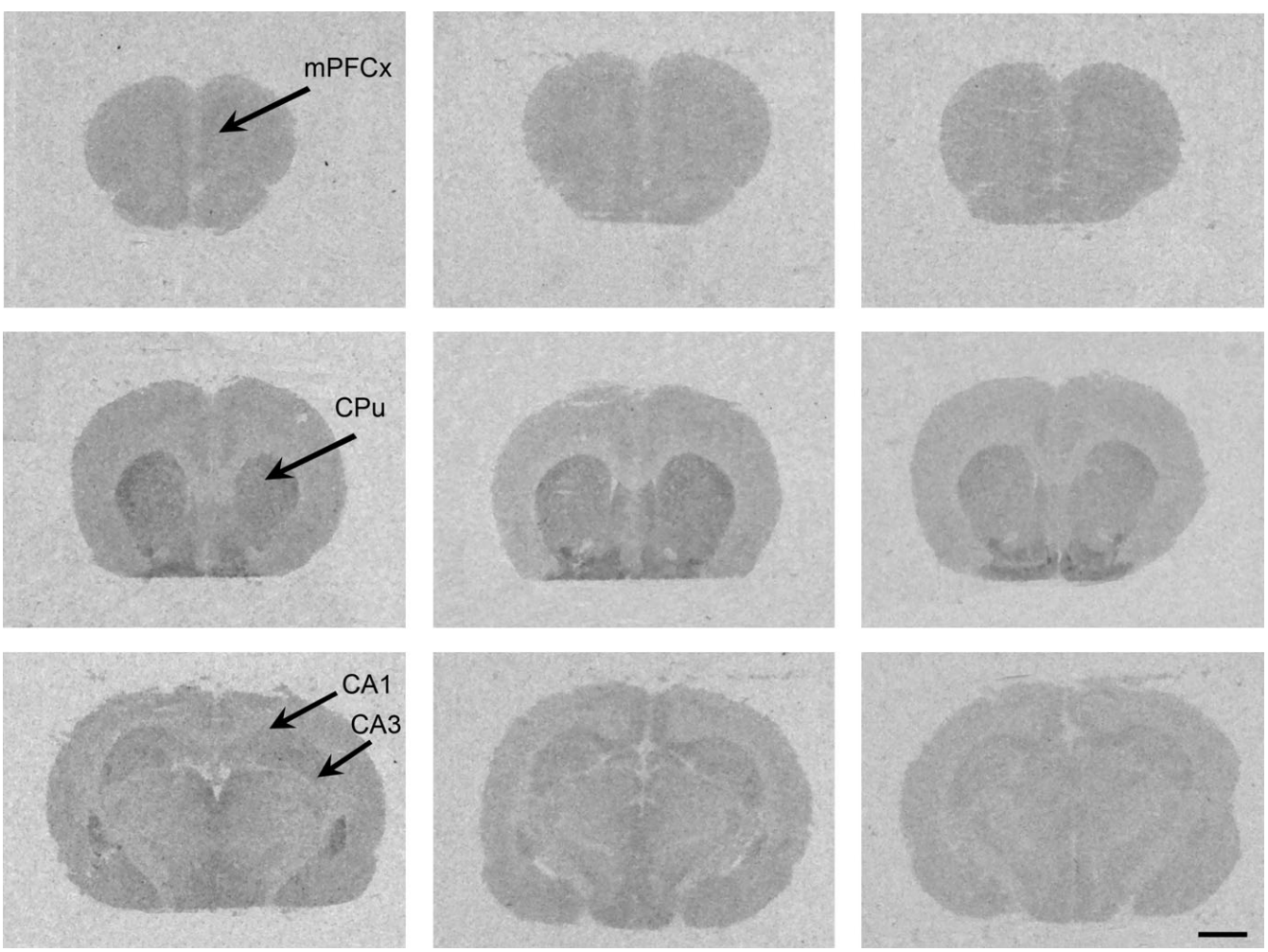

Figure 3

Representative autoradiograms of [^{3}H]GR113808 binding in rats chronically treated with vehicle (left), venlafaxine 10 mg·kg^{-1} (middle) and venlafaxine 40 mg·kg^{-1} (right) in coronal sections of frontal cortex, striatum and hippocampus. mPFCx, medial prefrontal cortex; CPu, caudate-putamen; CA1, CA1 field of hippocampus, CA3, CA3 field of hippocampus. Bar: 2 mm.

Table 2

Effect of chronic reboxetine on the specific binding of [^{3}H]GR113808 in coronal sections of rat brain

Area	Vehicle (10–12)	Reboxetine (10 mg·kg^{-1}·day^{-1}) (7)
Medial prefrontal cortex	13.9 ± 0.5	12.7 ± 0.8
Caudate-putamen	18.8 ± 0.8	18.5 ± 0.9
Globus pallidus	17.3 ± 0.5	16.7 ± 0.8
CA1	15.7 ± 0.9	15.2 ± 1.0
CA3	15.3 ± 0.9	14.6 ± 1.0
Substantia nigra	14.8 ± 0.7	13.9 ± 0.7

Values are expressed as the mean ± SEM of B_{max} (fmol·mg^{-1} tissue). The number of determinations is shown in parenthesis, in each column heading.

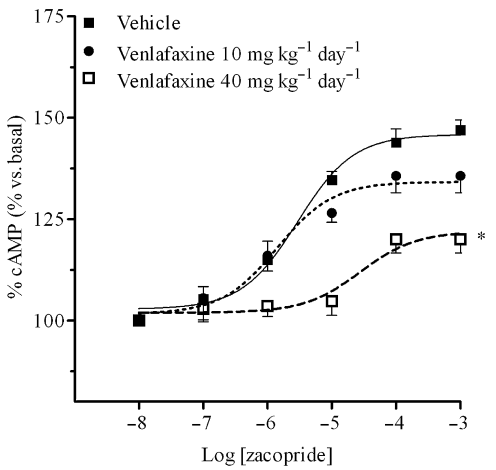

Figure 4

Concentration–response curves showing the effect of chronic venlafaxine on zacopride-induced accumulation of cAMP (expressed as mean ± SEM of the percentage of increase over basal values) in striatum membranes from vehicle and venlafaxine-treated rats. E_{max}: *P < 0.05 significantly different from vehicle-treated group by Student Newman–Keuls *post hoc* test. Six rats per experimental group were included.

antidepressant induced a desensitization of 5-HT$_4$ receptors in hippocampus, as evaluated by electrophysiological recordings of the neuronal activity controlled by this receptor subtype. Our results following chronic venlafaxine are in contrast with those obtained with the selective noradrenaline reuptake inhibitor reboxetine, with no significant modification of 5-HT$_4$ receptor density observed after its chronic administration.

To our knowledge this is the first preclinical report evaluating the modulation of the signalling cascades linked to 5-HT$_4$ receptors following a treatment with a 5-HT-noradrenaline dual reuptake inhibitor, venlafaxine. The present data show a down-regulation of 5-HT$_4$ receptors in striatum and hippocampus while the density in frontal cortex remains unaltered. This desensitization may not be explained by a direct effect of the drug as venlafaxine (Bymaster *et al.*, 2001; Artaiz *et al.* 2005) does not show a direct affinity for 5-HT$_4$ receptors (Bymaster *et al.*, 2001). These findings are in accordance with earlier reports of the effects of long-term administration of another class of antidepressants, the SSRIs, including fluoxetine (Vidal *et al.*, 2009) and paroxetine (Licht *et al.*, 2009). In contrast, Gobbi *et al.* (1997) failed to detect any significant changes on 5-HT$_4$ receptor density after chronic citalopram in substantia nigra.

It is important to note that the expression of mRNA for 5-HT$_4$ receptors remained unaltered by chronic venlafaxine. Taking into account this fact, it is unlikely that the down-regulation of 5-HT$_4$ receptors found in our study was a result of an alteration in their synthesis. The most feasible explanation indicates that this down-regulation reflects internalization and/or increased degradation as a consequence of prolonged exposure to either 5-HT or noradrenaline after chronic treatment with non-selective 5-HT and noradrenaline reuptake drugs. However, we cannot discount possible changes in 5-HT$_4$ receptor affinity after chronic venlafaxine, as we have used an antagonist of these receptors as the radioligand, thus assuming a similar affinity, for any different affinity states of the receptor that might be present. Further studies with an agonist radioligand should be carried out in order to clarify this point.

It is well known that the regulation of 5-HT receptors depends on several factors including the brain area examined (Castro *et al.*, 2003), signalling pathway (Berg and Clarke, 2001) or type of agonist used to evaluate the functional responses (Valdizán *et al.*, 2009). Similar to previous reports regarding chronic SSRIs (Licht *et al.*, 2009; Vidal *et al.*, 2009) the down-regulation of 5-HT$_4$ receptors induced by venlafaxine is region-dependent. As mentioned above, we found that the density of the receptor in medial prefrontal cortex was unaltered after long-term treatment with venlafaxine. Indeed, this differential regulation of 5-HT$_4$ receptors may be due to the higher density of 5-HT uptake sites observed in the hippocampus compared with the frontal cortex (Hrdina *et al.* 1990 and personal observation). Furthermore, the lack of down-regulation of 5-HT$_4$ receptors observed in frontal cortex could also be interpreted in the context of recent work suggesting that cortical 5-HT$_4$ receptors contribute to increase the firing activity of 5-HT neurons (Lucas and Debonnel, 2002; Lucas *et al.*, 2005). The evidence of

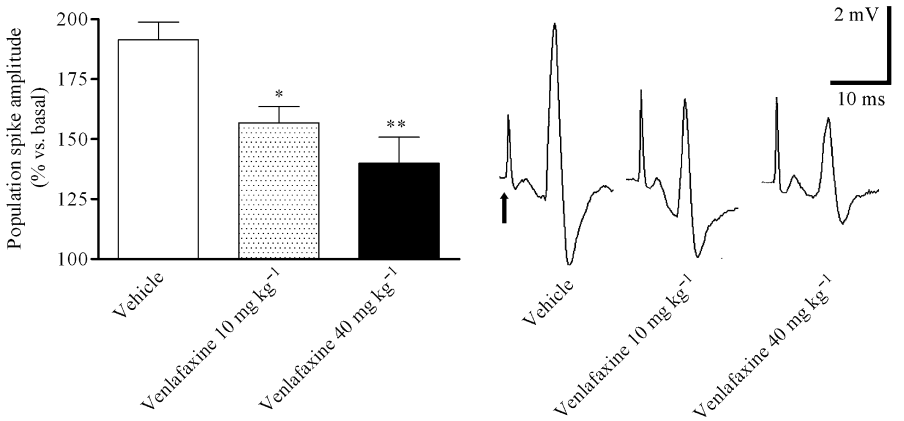

Figure 5

Left: Effect of repeated treatment with venlafaxine on the stimulatory action of zacopride on population spike amplitude. A population spike that was 50% of the maximum amplitude was chosen. $^{**}P < 0.01$; $^{*}P < 0.05$ vs. vehicle treated group (one-way ANOVA and Student Newman–Keuls *post hoc* test). (n = 8, 6 and 7 animals for vehicle, venlafaxine 10 and 40 mg·kg^{-1}·day^{-1} respectively). Right: Electrophysiological recordings of pyramidal cells during the perfusion of 10 μM zacopride after stimulation of the Schaffer collateral-commissural pathway in vehicle and venlafaxine-treated group. The arrow indicates the stimulus artifact.

opposite changes in 5-HT$_4$ receptor density (up-regulation) observed in frontal cortex and striatum in depressed suicide victims (Rosel *et al.*, 2004) corroborates the relevance of our findings.

In the last few years, the analysis of the mechanisms of action of antidepressants has moved from the receptor level to the intracellular signalling cascades. Thus, one element that is receiving special interest in depression as well as in the mechanism of action of antidepressant drugs is the adenylate cyclase system (Dowlatshahi *et al.*, 1999; Donati and Rasenick, 2003; Valdizán *et al.*, 2003). In this study we have found that long-term venlafaxine administration induced a functional desensitization of striatal 5-HT$_4$ receptors measured as zacopride-induced accumulation of cAMP, without changes in the basal levels. Interestingly only the 40 mg·kg^{-1} dose of chronic venlafaxine induced desensitization of striatal 5-HT$_4$ receptors while the lower dose had no effect. Thus, the modification of the sensitivity of this second messenger pathway could be attributable to the decrease in 5-HT$_4$ receptor density in striatum observed only at the 40 mg·kg^{-1} dose of venlafaxine. In addition, the decreased capacity of zacopride to induce accumulation of cAMP in the striatum may be also attributed to regulatory changes at the level of the G protein such as a decrease in the efficacy of coupling between the receptor and the heterotrimeric G$_S$ protein in response to receptor activation. Using [^{35}S]GTPγS, several studies have reported, for other 5-HT receptors, a desensitization at this coupling level after chronic antidepressants (Hensler, 2002; Pejchal *et al.* 2002; Castro *et al.*, 2003). Unfortunately, experimental limitations of the technique do not allow the visualization of the specific activation of G proteins for G$_s$-coupled receptors.

The last decade of research on the mechanisms underlying depression has lead to the accumulation of a large number of reports supporting the idea that the hippocampus may play an important role in this disease (see Frodl *et al.* 2008) and in the mechanism of action of antidepressant drugs (Duman *et al.* 2001; Drew and Hen, 2007; Mostany *et al.* 2008). In this context, our electrophysiological recordings also indicate that chronic venlafaxine modified, in a dose-dependent way, the sensitivity of postsynaptic 5-HT$_4$ receptors in the hippocampus as illustrated by attenuation of the zacopride-induced increase of the amplitude of population spike. The most plausible explanation for these results is that 5-HT$_4$ receptor desensitization may be a direct consequence of the decrease in 5-HT$_4$ receptor density in hippocampus. However, this functional desensitization was also observed after the administration of 10 mg·kg^{-1} of venlafaxine, a treatment that did not result in a significant modulation of 5-HT$_4$ receptor density. This finding suggests the involvement of other mechanisms, in addition to the modifications of the level of expression of the protein. In this way, similar findings have been reported after prolonged treatment with SSRIs in the regulation of other 5-HT receptors subtypes. Thus, the desensitization of 5-HT$_{1A}$ autoreceptors by chronic treatment with SSRIs (Blier and de Montigny, 1994; Le Poul *et al.*, 1995) is downstream of the receptor protein, in the intracellular signalling cascades and without changes in the receptor density (Hervás *et al.*, 2001; Hensler, 2002; Castro *et al.*, 2003; 2008).

On the other hand, *in vitro* and *in vivo* experiments in hippocampus using the same dose regimen of venlafaxine have shown a functional desensitization of the terminal 5-HT$_{1B}$ autoreceptor after chronic treatment with high but not low doses of chronic antidepressant (Beiqüe *et al.*, 2000a,b). Although the degree of modulation of extracellular 5-HT in hippocampus after chronic venlafaxine (40 mg·kg^{-1}) has not been reported yet, it is possible that the desensitization of 5-HT$_{1B}$ autoreceptors observed after the 40 mg·kg^{-1} dose would lead to higher synaptic 5-HT levels, compared with those after the 10 mg·kg^{-1} dose. Thus, this may account for the dose-dependent venlafaxine-induced desensitization of 5-HT$_4$ receptors as a more marked attenuation was observed following administration of the higher dose of venlafaxine. In line with our findings, using the same paradigm some groups have found that chronic SSRIs (imipramine, citalopram, fluoxetine), as well as repeated electroconvulsive shock, resulted in 5-HT$_4$ receptor desensitization in pyramidal cells of CA1 of hippocampus (Bijak *et al.*, 1997; 2001; Vidal *et al.*, 2009).

The higher dose of venlafaxine (40 mg·kg^{-1}) used in our study has been reported to modify noradrenaline uptake (Beiqüe *et al.*, 2000a,b). In this regard, although data about noradrenaline extracellular levels after this treatment are not currently available, an elevation in noradrenaline levels in frontal cortex has been reported following administration of a lower dose (10 mg·kg^{-1}) of the drug (Millan *et al.*, 2001). Interestingly, our results on the modulation of 5-HT$_4$ receptors by 40 mg·kg^{-1} of venlafaxine are quite similar to those previously reported for the chronic administration of fluoxetine (Vidal *et al.*, 2009), an antidepressant that does not affect noradrenaline neurotransmission. Then, it could be suggested that the noradrenergic component of venlafaxine does not play a relevant role in the changes induced in 5-HT$_4$ receptor function. Our results showing a lack of modifications on receptor density following chronic reboxetine (Invernizzi *et al.*, 2001; Parini *et al.*, 2005) further support the suggestion that the changes seen with venlafaxine were mainly due to modifications in 5-HT neurotransmission.

Our results, and those from other studies (Vidal *et al.*, 2009), showing a clear regulation of 5-HT$_4$ receptors following chronic treatment with antidepressants, are of special interest in view of the recent identification of these receptors as a direct target for a short-onset treatment of depression. It has been proposed that a 3 day treatment with the 5-HT$_4$ agonist RS67333 induces some antidepressant-like behavioural responses in animals (Lucas *et al.*, 2007). In this regard, data from our laboratory suggest that a short-term treatment with this agonist also results in neuroplastic and neuroproliferative changes, such as increase in bromodexoyuridine (BrdU) incorporation in dentate gyrus of hippocampus and increased expression of brain derived neurotrophic factor, quite similar to those observed after 2–3 weeks treatment with classical antidepressants (Pascual-Brazo *et al.*, 2009).

Taken together these results indicate an important role of 5-HT$_4$ receptors in the mechanism of antidepressant responses. The desensitization observed in our study, also reported for SSRIs and electroconvulsive shock, is probably a consequence of the sustained increase in 5-HT levels induced by antidepressants, which would result in the normalization of 5-hydroxytryptaminergic neurotransmission in the depressed patient. Whether or not the desensitization of 5-HT$_4$ receptors is also present after the short-term 'antidepressant' administration of 5-HT$_4$ agonists remains to be clarified.

In summary, long-term treatment with venlafaxine results in regulatory changes in 5-HT$_4$ signalling pathways particularly in striatum and hippocampus, similar to those observed after SSRIs. These changes appear to be mainly dependent on the increase in 5-HT levels.

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Conflicts of interest

Over the past 2 years, one of us (AP) has received compensation from FAES FARMA SA. The other authors declare no conflicts of interest.

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