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

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Short running title: 5-HT$_4$ receptors and chronic venlafaxine

Summary

Background and purpose: It has been recently described that 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-administration (p.o.) of 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* hibridization, 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: The administration of a high dose (40 mg kg^{-1}) of venlafaxine did not alter 5-HT_4 mRNA expression, but resulted in a significant decrease in the density of 5-HT_4 receptors in caudate-putamen (% reduction= 25.9 ± 5.6), hippocampus (% reduction=39.0 ± 6.8 and 39.0 ± 8.1 for CA1 and CA3, respectively) and substantia nigra (% reduction= 48.8 ± 4.5). Zacopride-stimulated adenylate cyclase activation was unaltered following the low dose treatment (10 mg kg^{-1}) while it was attenuated in rats treated with 40 mg kg^{-1} of venlafaxine (% reduction= 50.6 ±1.5). 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 with the classical SSRI drugs.

Keywords: Venlafaxine, *in situ* hybridization, adenylate cyclase, electrophysiology, 5-HT_4 receptors.

Abbreviations: relative optical density (R.O.D), 3'-5'-cyclic adenosine monophosphate (cAMP).

Introduction

Multiple evidences support the idea that a deficit in serotonin and noradrenaline neurotransmission is associated with depression (Schildkraut, 1965; Coppen, 1967; Lanni *et al.* 2009). In line with this concept, effective treatment of the disease is achieved with monoaminooxidase inhibitors (MAOI), tricyclic antidepressants, selective serotonin reuptake inhibitors (SSRIs) or serotonin-noradrenaline reuptake inhibitors that enhance either central serotonin and/or noradrenaline neurotransmission (Vetulani and Nalepa, 2000; Schechter *et al.* 2005). This increase in serotonin 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 receptors located on serotonergic and noradrenergic neurons respectively (Blier and de Montigny 1994; Le Poul *et al.* 1995; Mateo and Meana 2001; Castro *et al*, 2003; Invernizzi and Garattini, 2004; Parini *et al.* 2005). In addition, other serotonin and noradrenaline receptors such as 5-HT_2 and β-adrenoceptors have also been implicated in depression and in the antidepressant mechanisms of action (see Brunello, 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 as well as the reuptake serotonin and noradrenaline sites (Horschitz *et al.* 2001; Benmansour *et al.* 2004; Nadgir and Malviya, 2008), the role of other serotonin receptors in the mechanisms of action of dual antidepressants remains still unexplored.

Venlafaxine is a non selective serotonin-noradrenaline reuptake inhibitor that shows higher affinity for serotonin (5-HT) than for noradrenaline (NA) reuptake (Muth *et al.* 1986; Bolden-Watson and Richelson, 1993). In fact, it has been described that at low doses, venlafaxine mainly acts as a 5-HT reuptake inhibitor alone whereas only at high doses are noradrenergic reuptake properties affected (Beique *et al.* 2000a, 2000b).

Focusing on serotonergic neurotransmission, 5-HT_4 receptors are widely distributed in brain areas including basal ganglia, hippocampal formation, amygdala and cortex (Waeber *et al.* 1994; Vilaró *et al.* 1996; Vilaró *et al.* 2005). These 5-HT_4 receptors belong to the superfamily of G-protein coupled receptors which 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 central nervous system 5-HT_4 receptors appear to modulate neurotransmitter (acetylcholine, dopamine, serotonin and GABA) release and enhance synaptic transmission (Yamaguchi *et al.* 1997; Lucas and Debonnel, 2002; Bianchi *et al.* 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, novel 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 depressed brain (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 in a manner comparable with 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 decrease 5-HT_4 receptor density in the brain (Vidal *et al.* 2009; Licht *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 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 (SNRI), on 5-HT_4 receptor density was also analyzed.

Methods

Animals

Male Wistar rats weighing 200-250g were group-housed and maintained at 21±1°C on 12/12 h light/dark cycle, with access to food and water *ad libitum*. All experimental procedures were done 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).

Drug treatments

Rats were orally administered by gavage with venlafaxine (10 mg kg^{-1} and 40 mg kg^{-1}), reboxetine (10 mg kg^{-1}) or saline once a day for 21 days. Drugs were administered at the same time each day, between 11 -12 h a.m., and twenty-four h after the last administration 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 (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, 1986). 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 labeled at their 3'-end using [^{33}P] α-dATP (111 TBq $mmol^{-1}$, Perkin Elmer, Waltham, MA, USA) and terminal deoxynucleotidyltransferase (TdT) (Oncogene Research Products, San Diego, CA, USA). Labeled 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 x 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 x PBS, twice in 1 x 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 2 mg ml^{-1} glycine in PBS. Tissues were rinsed in PBS and dehydrated in a graded series of ethanol. For hybridization, labeled probes were diluted to a final concentration of approximately 2 x 10^7 cpm ml^{-1} (0.3 nM each probe) in a solution containing 50% formamide, 4 x standard saline citrate (1 x SSC: 150 mM NaCl, 15 mM sodium citrate), 1 x Denhardt's solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin), 10% dextran sulfate, 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 preincubated 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-hydroxytryptamine. After incubation, sections were washed for 30 s in ice-cold buffer, briefly dipped in deionized water at 4ºC, and then cold air-dried. Autoradiograms were generated by apposing the slides to Biomax MR film sheets (Kodak, Madrid, Spain) together with tritium labeled standards for 6 months at 4°C.

Adenylate cyclase assay

5-HT_4 stimulated adenylate cyclase procedure was 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 x g for 5 min at 4^{o}C. The supernatants were pelleted at 13000 x g for 15 min at 4^{o}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 (10^{-3} M- 10^{-8} M). The reaction was started by the addition of 0.2 mM Mg-ATP and incubated at 37ºC 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 4ºC. 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 artificial cerebrospinal fluid (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 1h. 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 30ºC. 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 (1Hz-1kHz) 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 analyzed 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 (predrug). E_{max} and ED_{50} values in both adenylate cyclase assays and electrophysiological recordings were calculated using the program GraphPad Prism program (GraphPad Software 1998). The statistical analysis of the results obtained following venlafaxine administration was performed using Student *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 analyzed by Student *t*-test. $P < 0.05$ was considered statistically significant.

Drugs and chemical reagents

All drugs and receptors nomenclature conforms to Alexander *et al.* (2008). [^{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-Hydroxytrytamine hydrochloride was purchased from Sigma-Aldrich (Madrid, Spain). 4-amino-5-chloro-2-methoxy-substituted benzamide (R,S) zacopride (zacopride) 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 = 25.9 ± 5.6; $P < 0.01$), hippocampus (% reduction = 39.0 ± 6.8 % and 39.0 ± 8.1, for CA1 ($P < 0.01$) and CA3 ($P < 0.01$), respectively and substantia nigra (% reduction = 49.8 ± 4.5; $P < 0.01$) when compared to 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 reboxetine did not alter 5-HT_4 receptor binding in any of the brain areas analyzed (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} = +45.9 ± 0.7 % 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} = +22.0 ± 1.4 ($P < 0.05$ *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 = 36.7 ± 7.6; $P < 0.05$). This decrease was even more pronounced with the dose of 40 mg kg^{-1} (% reduction =55.5 ± 12.2; $P < 0.01$) (Figure 5).

Discussion and conclusions

Dual antidepressant drugs affect both the serotonergic and noradrenergic systems by inducing adaptive changes in several receptor subtypes in the brain. In the present study, we have found that a 21-days treatment with 40 mg kg^{-1} of venlafaxine (high dose), leads to a down-regulation of 5-HT_4 receptor density without altering mRNA expression. It also results in an attenuation of zacopride-stimulated adenylate cyclase system. In contrast, 10 mg kg^{-1} (low dose) has no significant effect on these neurochemical markers. Furthermore, both doses of this antidepressant induce a desensitization of 5-HT_4 receptors in hippocampus 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 SNRI reboxetine, in which no significant modification of 5-HT_4 receptor density was observed after its chronic administration.

To our knowledge this is the first preclinical report evaluating the modulation of the signalizing cascades linked to 5-HT_4 receptors following a treatment with a 5-HT/NE 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 since 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 several data previously reported after long term administration of another class of antidepressants, SSRI drugs, 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 5-HT_4 mRNA expression remains 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 is a result of an alteration in the synthesis process. 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 NE after chronic no selective serotonin and noradrenaline reuptake drugs. However, we cannot discard possible changes in 5-HT_4 receptor affinity after chronic venlafaxine since we have used an antagonist as radioligand, thus recognizing with a similar affinity the different affinity states of the receptor. Further studies with an agonist radioligand should be carried out in order to clarify this point.

It is well known that the regulation of serotonin receptors depends on several factors including brain area expression (Castro *et al.* 2003), signalling pathway (Berg and Clarke, 2001) or type of agonist used to evaluate the functional responses (Valdizan *et al.* 2009). Similar to previous reports regarding chronic SSRIs (Vidal *et al.* 2009; Licht *et al.* 2009) the down-regulation of 5-HT_4 receptor induced by venlafaxine is region-dependent. As mentioned above, we found that the density of the receptor in medial prefrontal cortex remains unaltered by 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 to 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 works 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 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 interest about the mechanisms of action of antidepressants has moved from the receptor level to the intracellular signaling 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; Valdizan *et al.* 2003; Donati and Rasenick, 2003). In this work we have found that long term venlafaxine administration leads to 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 induces desensitization of striatal 5-HT_4 receptors while a lower dose had no effect. Thus, the modification on the sensitivity of this second messenger pathway could be attributable to the decrease in 5-HT_4 receptor density in striatum observed only at 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. In fact by using [^{35}S]GTPγS experimental procedures several studies have reported, for other serotonin 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 to visualize the specific activation of G proteins for Gs-coupled receptors.

The last decade of research on the mechanisms implicated in depression has lead to the accumulation of a large number of evidences 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 regard, the electrophysiological recordings also indicate that chronic venlafaxine modifies, in a dose-dependent way, the sensitivity of postsynaptic 5-HT_4 receptors in the hippocampus as illustrated by an attenuation of 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 fact 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 SSRIs (Blier and de Montigny 1994; Le Poul *et al.* 1995) takes place downstream the receptor protein, in the intracellular signalling cascades 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 high but not low doses of chronic antidepressant (Beiqüe *et al.* 2000a, 2000b). Although the degree of modulation of 5-HT extracellular levels in hippocampus after chronic venlafaxine (40 mg kg^{-1}) has not been reported yet, one could speculate that the desensitization of 5-HT_{1B} autoreceptors observed after 40 mg kg^{-1} dose leads to higher synaptic 5-HT levels compared to 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 a high dose of venlafaxine. In line with our findings, using the same paradigm some authors indicate that chronic SSRIs (imipramine, citalopram, fluoxetine) as well as repeated electroconvulsive shock also result in 5-HT_4 receptor desensitization in pyramidal cells of CA1 of hippocampus (Bijak *et al.* 1997; Bijak *et al.* 2001; Vidal *et al.* 2009).

The higher dose of venlafaxine (40 mg kg^{-1}) used in our study has been reported to modify NE uptake (Beicque *et al.* 2000a, b): in this regard, although data about NE extracellular

levels after this treatment are not currently available, an elevation in NE 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 which does not affect NE neurotransmission. Then, it could be suggested that the noradrenergic component of venlafaxine does not play a relevant role in the changes induced on 5-HT_4 receptors functionality. Our results showing a lack of modifications on receptor density following chronic reboxetine (Invernizzi *et al.* 2001; Parini *et al.* 2005) further support that the changes seen with venlafaxina are 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-days 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 (i.e. increase in BrdU incorporation in dentate gyrus of hippocampus, increased expression of BDNF) 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 serotonergic 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 pathway 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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Statement of Interest

The authors (RV, EMV, MTV, AP, EC) declare that, except for income received from the primary employer, no financial support or compensation has been received from any individual or corporate entity over the last two years for research or professional service and there are no personal financial holdings that could be perceived as constituting a potential conflict of interest. Over the past two years Angel Pazos has received compensation from FAES FARMA SA.

Tables

Table 1. Effect of chronic venlafaxine on the specific [^{3}H]GR113808 binding 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 ± S.E.M of B_{max} (fmol mg^{-1} tissue) considering a K_D value of [^{3}H]GR113808 0.2 nM. The number of determinations is shown in parenthesis, in each column heading. *$P < 0.05$; **$P < 0.01$ *versus* vehicle and $^{+}P < 0.05$; $^{++}P < 0.01$ *versus* 10 mg kg^{-1} venlafaxine treated rats. One-way ANOVA followed by Student Newman-Keuls test.

Table 2. Effect of chronic reboxetine on the specific [^{3}H]GR113808 binding 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 ± S.E.M of B_{max} (fmol mg^{-1} tissue). The number of determinations is shown in parenthesis, in each column heading.

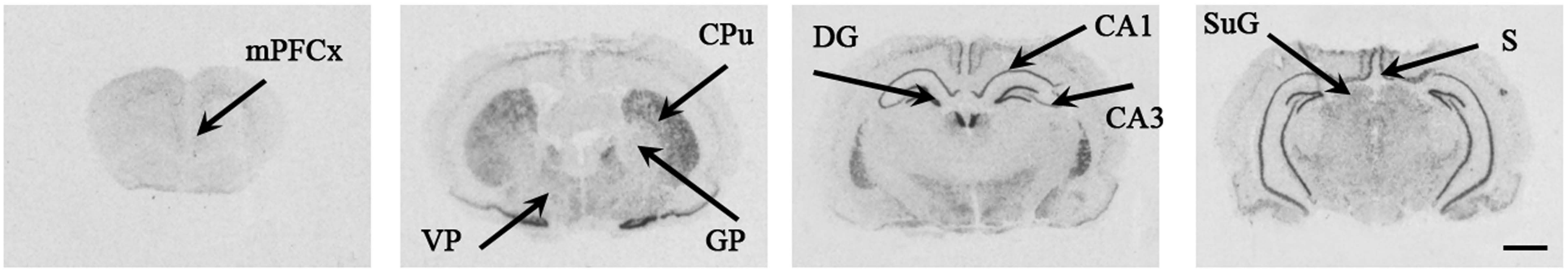
Figure 1. Representative autoradiograms showing the 5-HT_4 receptor mRNA distribution in coronal sections of rats chronically treated with vehicle. mPFCx: medial prefrontal cortex; CPu: caudate-putamen; VP: ventral pallidum; GP: globus pallidus; CA1: CA1 field of hippocampus; CA3: CA3 field of hippocampus; DG: dentate gyrus; S: subiculum; SuG: superior colliculus. Bar: 2 mm.

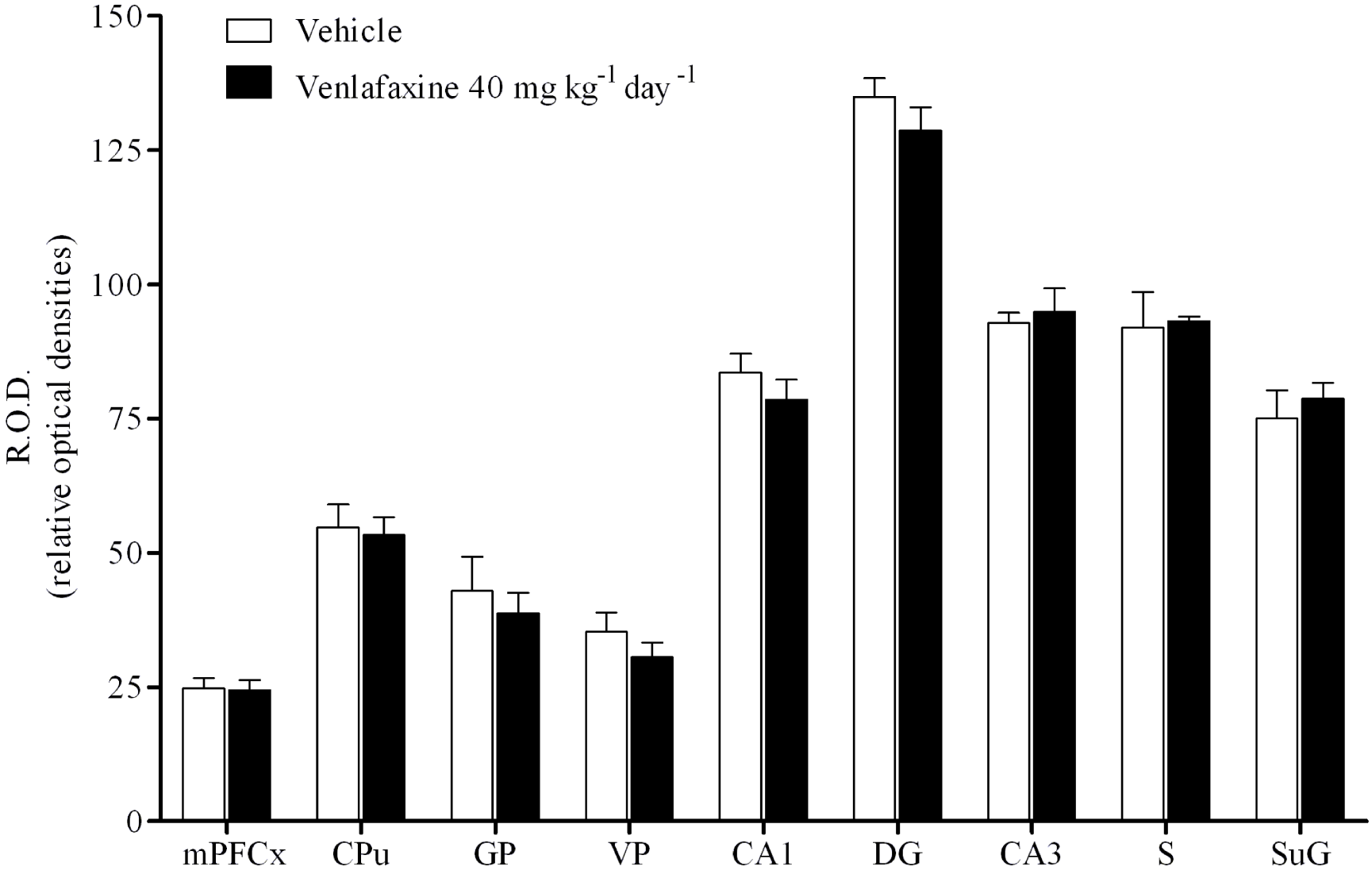
Figure 2. Effect of chronic venlafaxine on 5-HT_4 mRNA levels in rat brain measured as relative optical density (R.O.D.). R.O.D for background tissue signal was 47.16. mPFCx: medial prefrontal cortex; CPu: caudate-putamen; GP: globus pallidus; VP: ventral pallidus; CA1: CA1 field of hippocampus; DG: dentate gyrus of hippocampus; CA3: CA3 field of hippocampus; S: subiculum; SuG: superior colliculus. No significant differences were found between both experimental groups (Student t-test, unpaired data; n = 7 rats per group).

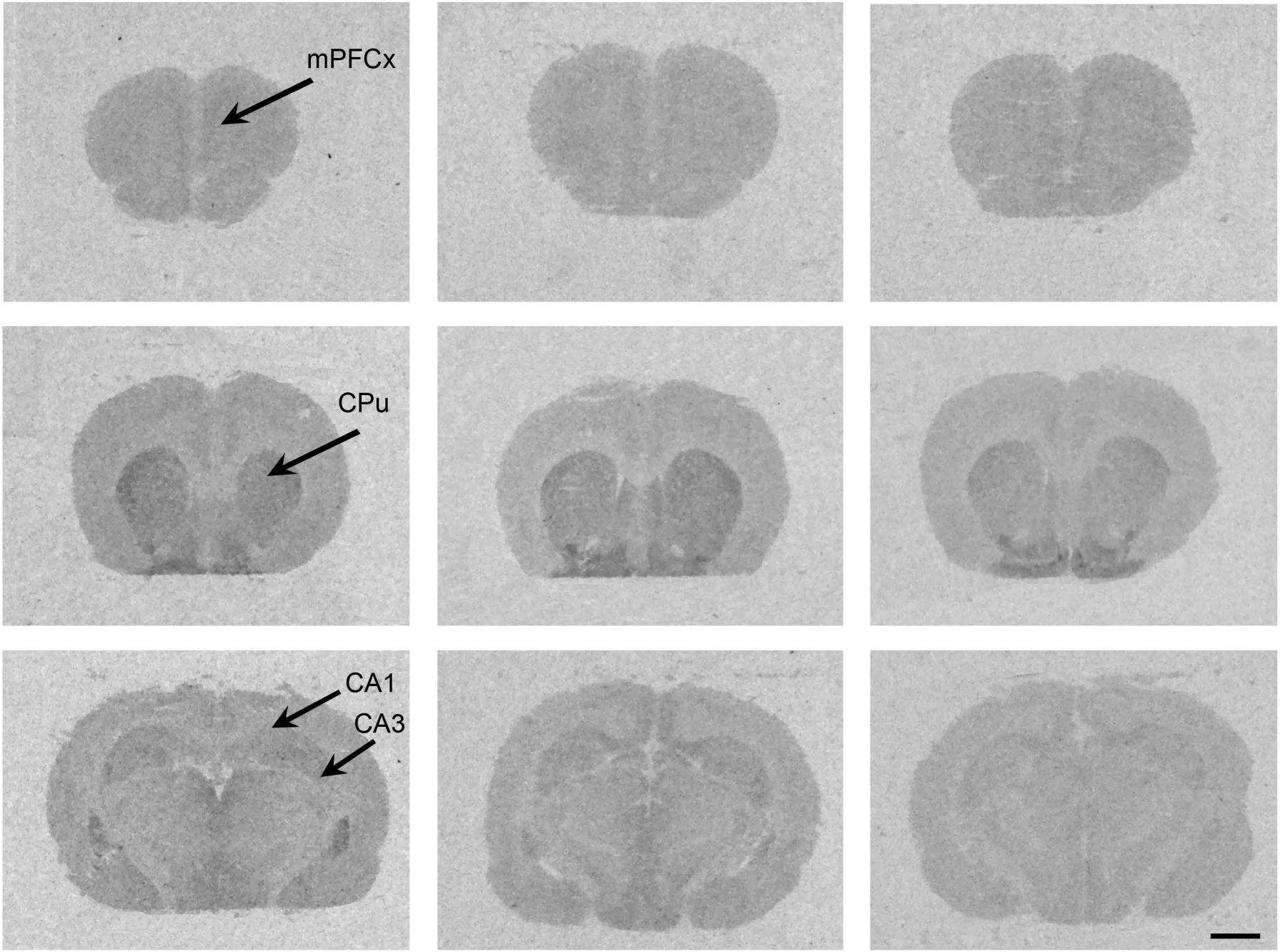
Figure 3. Representative autoradiograms of [^{3}H]GR113808 binding in rats chronically treated with vehicle (left), venlafaxine 10 mg kg^{-1} (middle) and venlafaxine 40 mg kg^{-1} (right) in coronal sections of frontal cortex, striatum and hippocampus. mPFCx: medial prefrontal cortex; CPu: caudate-putamen;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

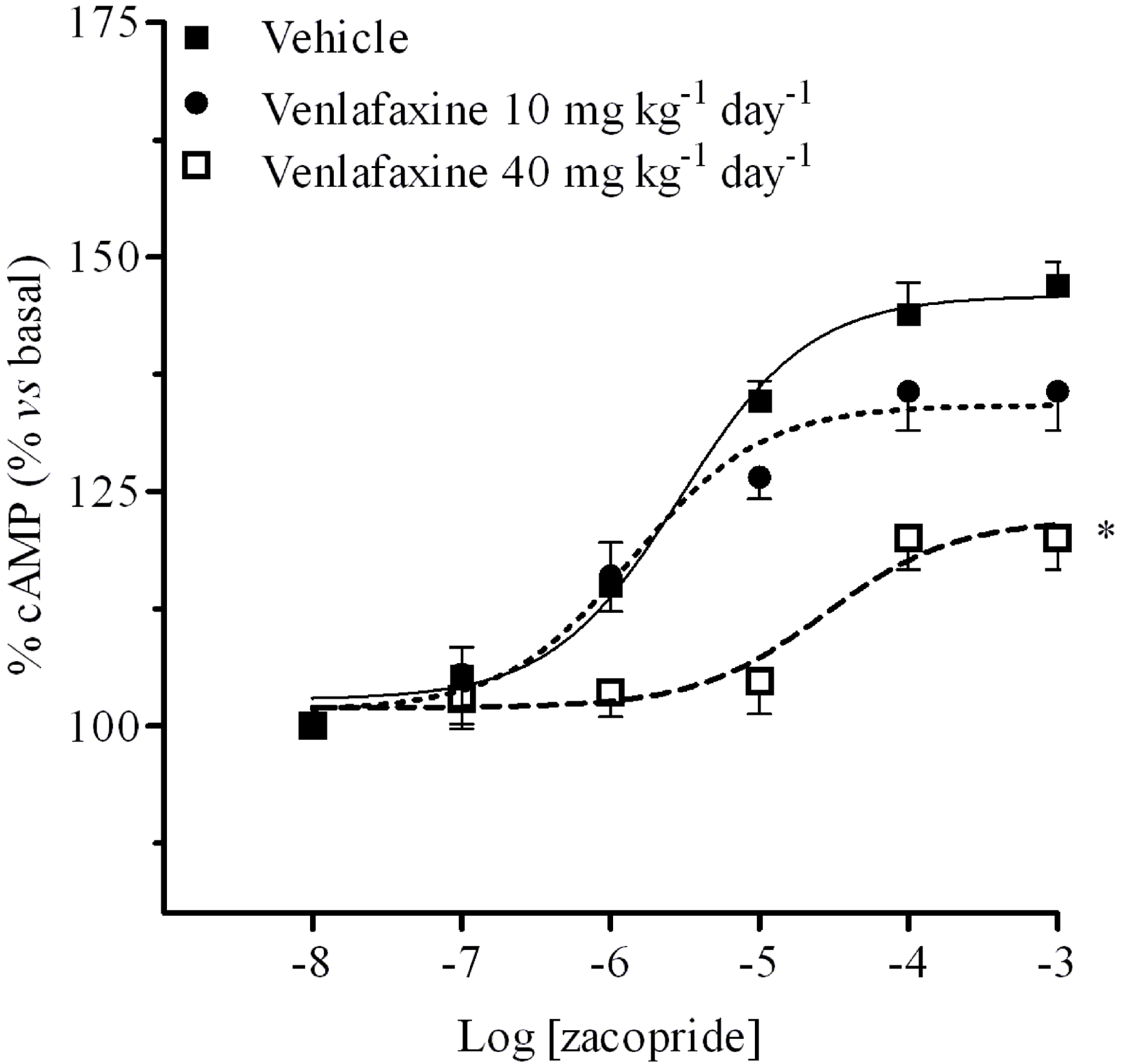
Figure 4. Concentration-response curves showing the effect of chronic venlafaxine on zacopride-induced accumulation of cAMP (expressed as mean ± SEM of the percentage of increase over basal values) in striatum membranes from vehicle and venlafaxine-treated rats. E_{max}: $^*P < 0.05$ significantly different from vehicle-treated group by Student Newman-Keuls *post hoc* test. Six rats per experimental group were included.

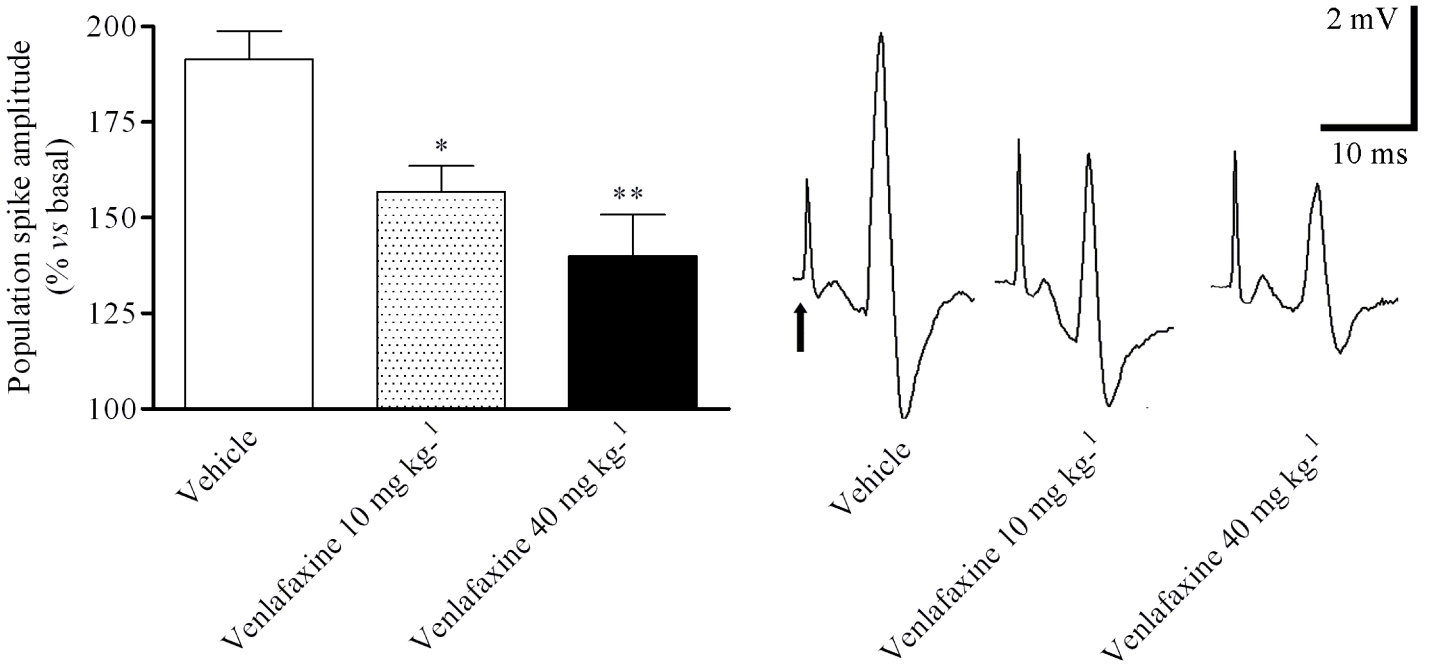
Figure 5. Left: Effect of repeated treatment with venlafaxine on the stimulatory action of zacopride on population spike amplitude. A population spike which was 50% of the maximum amplitude was chosen. $^{**}P < 0.01$; $^{*}P < 0.05$ *vs* vehicle treated group (One-way anova and Student Newman-Keuls *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.


mPFCx
CPu
VP
GP
DG
CA1
CA3
SuG
S


Vehicle
Venlafaxine 40 mg kg-1 day -1
R.O.D.
(relative optical densities)
150
125
100
75
50
25
0
mPFCx
CPu
GP
VP
CA1
DG
CA3
S
SuG


mPFCx
CPu
CA1
CA3


175
150
125
100
% cAMP (% vs basal)
Vehicle
Venlafaxine 10 mg kg-1 day-1
Venlafaxine 40 mg kg-1 day-1
*
-8
-7
-6
-5
-4
-3
Log [zacopride]


Population spike amplitude
(% vs basal)
200
175
150
125
100
*
**
Vehicle
Venlafaxine 10 mg kg-1
Venlafaxine 40 mg kg-1
2 mV
10 ms
Vehicle
Venlafaxine 10 mg kg-1
Venlafaxine 40 mg kg-1