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Long-term treatment with fluoxetine induces desensitization of 5-HT_4 receptor-dependent signalling and functionality in rat brain

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Abstract

The mode of action of antidepressant drugs may be related to mechanisms of monoamines receptor adaptation, including serotonin 5-HT_4 receptor subtypes. Here we investigated the effects of repeated treatment with the selective serotonin reuptake inhibitor fluoxetine for 21 days (5 and 10 mg/kg, p.o., once daily) on the sensitivity of 5-HT_4 receptors by using receptor autoradiography, adenylate cyclase assays and extracellular recording techniques in rat brain. Fluoxetine treatment decreased the density of 5-HT_4 receptor binding in the CA1 field of hippocampus as well as in several areas of the striatum over the doses of 5–10 mg/kg. In a similar way, we found a significant lower response to zacopride-stimulated adenylate cyclase activity in the fluoxetine 10 mg/kg/day treated group. Furthermore, post-synaptic 5-HT_4 receptor activity in hippocampus-measured as the excitatory action of zacopride in the pyramidal cells of CA1 evoked by Schaffer collateral stimulation was attenuated in rats treated with both doses of fluoxetine. Taken together, these results support the concept that a net decrease in the signalization pathway of 5-HT_4 receptors occurs after chronic selective serotonin reuptake inhibitor treatment: this effect may underlie the therapeutic efficacy of these drugs.

Keywords: 5-HT_4 receptors, adenylate cyclase and electrophysiology, autoradiography, fluoxetine, zacopride.

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Treatment with selective serotonin reuptake inhibitors (SSRIs) benefits many patients with major depression disorders. However, current antidepressant therapies need a sustained treatment of 2–4 weeks to be effective. In this regard, adaptive changes in both serotonergic and noradrenergic neurotransmission, through the activation of the different serotonin (5-HT) and norepinephrine receptor subtypes are believed to underlie the therapeutic efficacy of antidepressants drugs. Many research studies have been focused in the alterations of 5-HT and norepinephrine presynaptic reuptake sites, 5-HT_{1A}, 5-HT_2, β and α_2 receptors in both the pathogenesis of major depression (Klimek *et al.* 1997; Mann 1999; González-Maeso *et al.* 2002; Purselle and Nemeroff 2003; Valdizán *et al.* 2003; Parsey *et al.* 2006) and the antidepressants mechanisms of action (see Brunello *et al.* 2002; Adell *et al.* 2005; Schechter *et al.* 2005; Castro *et al.* 2008). However, despite numerous studies available in the literature, the role of the different neurotransmitter receptors in the mediation of the antidepressant effects of these drugs has not been clearly established. Moreover, except for 5-HT_{1A} and 5-HT_2 receptors, few data are available on the effect of chronic antidepressants administration on the other 5-HT receptor subtypes.

The actions of 5-HT are mediated by at least 14 receptor subtypes (Barnes and Sharp 1999; Hoyer *et al.* 2002) and their regulation by antidepressants is not yet fully understood. The 5-HT_4 receptor exhibits a wide distribution throughout the central nervous system. In the brain, this receptor is located post-synaptically primarily in the limbic areas (olfactory tubercule, prefrontal cortex, hippocampus and amygdala) and basal ganglia (caudate-putamen and ventral pallidum)

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Abbreviations used: 5-HT, serotonin; ACSF, artificial cerebrospinal fluid; DTT, dithiothreitol; SSRI, selective serotonin reuptake inhibitor.

(Waeber *et al.* 1994; Vilaró *et al.* 1996, 2005). 5-HT_4 receptors are coupled to G proteins and positively linked to the adenylate cyclase in the brain (Hoyer *et al.* 2002). The increase in cAMP levels leads to an activation of protein kinase A that mediates closure of potassium channels (Fagni *et al.* 1992; Ansanay *et al.* 1995). Thus, 5-HT_4 receptor contributes to the neuronal excitability of pyramidal cells of hippocampus (Chaput *et al.* 1990; Andrade and Chaput 1991). In addition to adenylate cyclase stimulation, a direct coupling to both voltage-sensitive calcium channels (Hoyer *et al.* 2002) and extracellular signal-regulated kinase pathway (Barthet *et al.* 2007) has also been proposed.

Neurochemical and behavioural studies indicate that 5-HT_4 receptor modulate neurotransmitter (acetylcholine, 5-HT, GABA and dopamine) release and enhance synaptic transmission in many brain areas (Yamaguchi *et al.* 1997; Bianchi *et al.* 2002; Lucas and Debonnel 2002; Alex and Pehek 2007) including those implicated in memory, anxiety, anorexia and depression (Matsumoto *et al.* 2001; Manuel-Apolinar *et al.* 2005; Jean *et al.* 2007; Lucas *et al.* 2007). Regarding depression, some findings suggest that 5-HT_4 receptors may have a potential interest in this illness. First, chronic antidepressant treatment has been proposed to induce subsensitivity to the 5-HT_4 receptor-mediated excitatory effects in the hippocampus (Bijak *et al.* 1997). Second, an increase in cortical and hippocampal 5-HT_4 receptor density has been reported in post-mortem brain samples of patients with major depression (Rosel *et al.* 2004). Finally, it has been recently reported that several 5-HT_4 receptor agonists show antidepressant-like effects in some acute and chronic animal models of depression (Lucas *et al.* 2007). Nevertheless, the information about modulation of 5-HT_4 receptors by antidepressants is still very limited.

In keeping with these observations it is not unlikely that some adaptive changes in 5-HT_4 receptors following chronic antidepressants drugs may occur. In the present study we have aimed to evaluate the effect of chronic treatment with the SSRIs fluoxetine at three different levels of 5-HT_4 receptor function (receptor number, regulation of adenylate cyclase activity and receptor functionality in rat brain).

Materials and methods

Animals

Male Wistar rats weighing 200–250 g were group-housed and maintained 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 treated by oral administration (p.o.) with saline or two doses of fluoxetine (5 and 10 mg/kg/day) once a day for 21 days. Six to thirteen animals per group were tested depending on experimental procedures. Drugs were administered at the same time each day, between 11 and 12 AM. The animals were killed 24 h after the last administration for all the experimental procedures. For autoradiographic and adenylate cyclase assays the brains were rapidly removed, frozen immediately in isopentane and then stored at −80°C until use.

[^{3}H]GR113808 autoradiography

For autoradiographic experiments, coronal sections of 20 µm thickness were cut at −20°C using a microtome cryostat and thaw-mounted in gelatinized slides and stored at −20°C until use. 5-HT_4 receptor autoradiography was carried out as previously described by Waeber (Waeber *et al.* 1994). The sections were pre-incubated at 25°C for 15 min in 50 mM Tris-HCl buffer (pH = 7.5) containing $CaCl_2$ 4 mM and ascorbic acid (0.1%). Two sections were then incubated, at 25°C for 30 min, in the same buffer with 0.2 nM of the selective 5-HT_4 antagonist [^{3}H]GR113808. In other consecutive section non-specific binding was determined using 10 µM 5-hydroxytryptamine (5-HT). Following 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 (Kodak, Madrid, Spain) with tritium labeled standards for 6 months at 4°C.

Adenylate cyclase assay

Frozen brain striata were homogenized (1 : 120 w/v) in ice cold buffer I, containing 20 mM Tris-HCl, 5 mM EGTA, 2 mM EDTA, 0.32 M sucrose, 1 mM dithiothreitol (DTT), 25 µg/mL leupeptin, pH = 7.4 and centrifuged at 500 *g* for 5 min at 4°C. The supernatants were pelleted 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 Cl_2 Mg, 3 mM DTT, 25 µg/mL 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-isobutylmethyl-xanthine, 5 mM phosphocreatine, 50 U/mL creatine phosphokinase and 5 U/mL myokinase) and 25 µL of either water (basal activity), 10^{-5} M GTPγS or zacopride (10^{-3} M–10^{-7} 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 supernatant aliquots 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 of 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 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 pyramidal of the CA1 (cornus ammonis 1, field of hippocampus). 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 others 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 different concentrations of zacopride alone or in the presence of the selective 5-HT_4 antagonist DAU 6285. Each slice in the extracellular recording was treated as an independent sample.

Data analysis and statistics

Autoradiograms were analyzed and quantified using a computerized image analysis system (Scion Image, Scion Corporation, Frederick, MD, 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 Inc., San Diego, CA, USA). The statistical analysis of the results was performed using One-way ANOVA followed by *post hoc* comparisons (Student Newman–Keuls test).

Drugs

[^{3}H]GR113808 (specific activity 83 Ci/mmol) was purchased from Amersham, DAU 6285 was generously donated by Boehringer-Ingelheim Pharma GmbH & Co. KG (Frankfurt, Germany) and fluoxetine-HCl was kindly donated by FAES FARMA S.A. (Lejona, Spain).

5-Hydroxytrytamine chlorhydrate 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 analytical grade. Fluoxetine was dissolved in saline (0.9%) and given by oral administration (p.o.) in a volume of 5 mL/kg body weight.

Results

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

Serotonin 5-HT_4 receptor binding sites were labeled with the selective 5-HT_4 receptor antagonist [^{3}H]GR113808 at a concentration close to the Kd value (0.2 nM). Autoradiogram of [^{3}H]GR113808 binding in vehicle and fluoxetine-treated rats at different rostral-caudal levels are shown in Fig. 1. Basal ganglia and hippocampal formation showed the highest levels of 5-HT_4 receptors in rat brain (Table 1 and Fig. 1a) whereas medial prefrontal cortex exhibited moderate densities of this receptor as previously reported (Vilaró *et al.* 1996). In rats treated with 10 mg/kg of fluoxetine a significant decrease in the density of specific [^{3}H]GR113808 binding was observed in caudate-putamen (% red = 16.0 ± 3.7), ventral pallidum (% red = 21.1 ± 3.5), CA1 field of hippocampus (% red = 38.5 ± 6.3) and substantia nigra (% red = 58.5 ± 2.4) (Fig. 1c). At the low dose of fluoxetine (5 mg/kg) a significant decrease in 5-HT_4 receptor density, compared to vehicle-treated rats, was only observed in caudate-putamen (% $red_{mean \pm SEM}$ = 13.0 ± 2.6%; $p < 0.05$). In contrast, chronic fluoxetine did not significantly alter the specific [^{3}H]GR113808 binding of 5-HT_4 receptors in the medial prefrontal cortex at any dose assayed, although a tendency to the decrease was observed (Table 1).

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

A slight tendency to the increase in basal cAMP levels (pmol/min/mg protein) in rat striatum homogenate membranes (11.7 ± 1.6 for vehicle, 12.4 ± 3.4 for fluoxetine 5 mg/kg and 22.7 ± 3.7 for fluoxetine 10 mg/kg) was observed, although it only reached statistical significance ($p < 0.01$) for the 10 mg/kg dose. In the vehicle group the incubation with zacopride resulted in a concentration-dependent increase of cAMP production yielding an E_{max} = 141.8 ± 3% stimulation. Figure 2 shows the effect of two doses of chronic fluoxetine on zacopride-induced accumulation of cAMP in rat striatum. Repeated fluoxetine induced an attenuation in zacopride-stimulated cAMP accumulation in homogenate membranes reaching the statistical significance only at the dose of 10 mg/kg/day (E_{max} = 28.0 ± 3.4% stimulation; $p < 0.05$) with no changes on potency (pEC_{50} = 6.1 ± 0.2 vs. pEC_{50} = 5.4 ± 0.2 for vehicle and fluoxetine group, respectively).

Effect of chronic fluoxetine on population spikes of CA1 field

The application of zacopride increases the population spike amplitude in the hippocampal CA1 field evoked by Schaffer collateral stimulation. As shown in Fig. 3(a), the excitatory effect of zacopride was concentration-dependent with an E_{max} = 205.2 ± 13.5% change (considering the basal amplitude value as 100%) and pEC_{50} = 5.7 ± 0.2. As illustrated in Fig. 3(b), this stimulation was significantly reduced by pre-perfusion with the selective 5-HT_4 antagonist DAU 6285 (5 μM) following a competitive pattern of antagonism. This shows the pharmacological specificity of this response. Two concentrations of zacopride (1 and 10 μM), around to its EC_{50} value, were chosen for chronic studies. For both concentrations of zacopride, a significant decrease in the

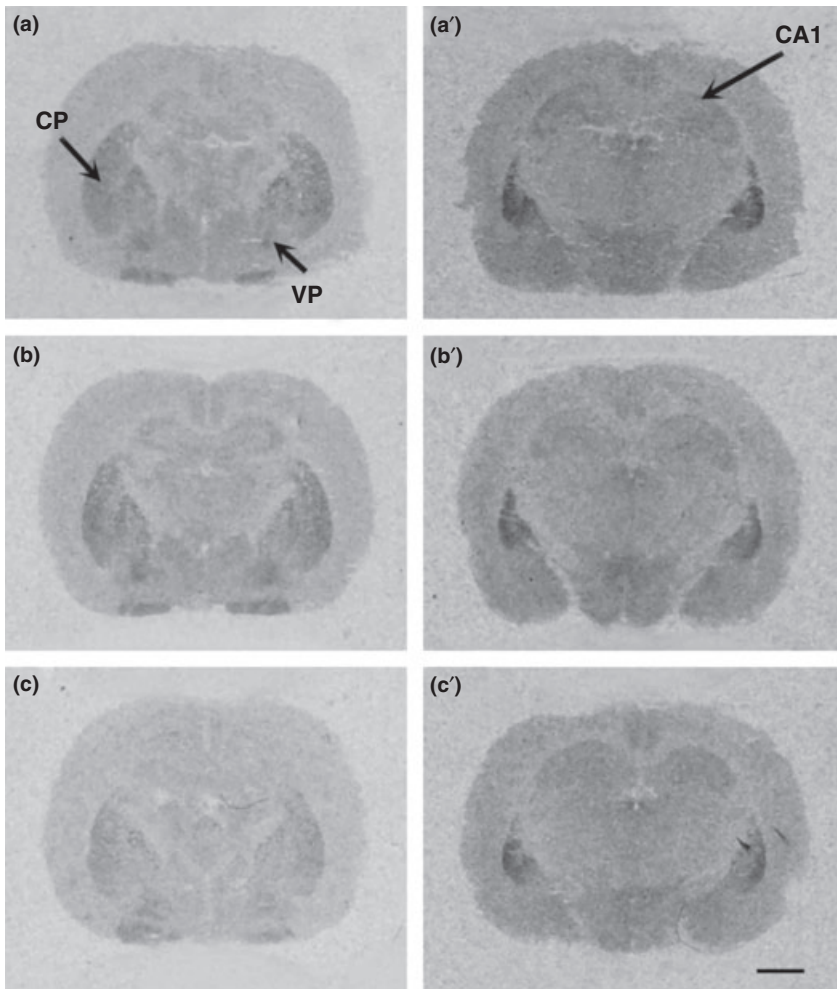

Fig. 1 Representative autoradiograms of [^{3}H]GR113808 binding in rats chronically treated with vehicle (a, a′), fluoxetine 5 mg/kg (b, b′) and fluoxetine 10 mg/kg (c, c′) at the levels of basal ganglia (left) and hippocampus (right). CP, caudate-putamen; VP, ventral pallidum; CA1, CA1 field of hippocampus. Bar = 2 mm.

Table 1 Effect of chronic fluoxetine on the specific [^{3}H]GR113808 binding in rat brain

Region	Vehicle (n = 13)	Fluoxetine (5 mg/kg/day) (n = 7)	Fluoxetine (10 mg/kg/day) (n = 6)
mPFCx	13.6 ± 1.0	11.6 ± 0.4	12.7 ± 0.3
Caudate-putamen	18.8 ± 0.7	16.3 ± 0.5*	15.8 ± 0.7*
VP	18.8 ± 0.8	16.3 ± 0.3	14.8 ± 0.7**
CA1, hippocampus	15.1 ± 0.8	14.2 ± 0.8	9.3 ± 0.9*
SN	15.1 ± 1.0	14.9 ± 0.8	6.3 ± 0.4**

Coronal sections of rat brain were incubated with [^{3}H]GR113808 (0.2 nM) and non-specific binding was defined in the presence of 10 μM 5-HT. Specific binding is expressed as fmol/mg tissue and the data are the mean ± SEM. *p < 0.05; **p < 0.01. One-way ANOVA followed by Student Newman–Keuls test. mPFCx, medial prefrontal cortex; VP, ventral pallidum; SN: substantia nigra.

excitatory action of zacopride was observed after 5 mg/kg of fluoxetine administration. This decrease was less pronounced with the higher dose of SSRI (Figs 3b and 4).

Discussion

Antidepressant treatments affect the serotonergic system in the brain by inducing adaptive changes in various 5-HT receptors subtypes (see Adell *et al.* 2005; Schechter *et al.* 2005). In the present work we have investigated the effect of repeated treatment with fluoxetine in the regulation of 5-HT$_4$ receptor-dependent signaling pathway. The main finding of this study is that chronic treatment with the antidepressant selectively decreased the density of 5-HT$_4$ receptors and resulted in both attenuated 5-HT$_4$ receptor-mediate adenylate cyclase activity and 5-HT$_4$-dependent neuronal excitability of CA1 neurons.

In order to evaluate the responses mediated by the stimulation of 5-HT$_4$ receptors we have used the 5-HT$_4$ receptor agonist zacopride (Bockaert *et al.* 2004) since it has shown good affinity for this receptor subtype. In addition, the few studies available focused on the functionality of 5-HT$_4$ receptors suggests that this agonist represent an adecuate pharmacological tool (Bijak *et al.* 1997, 2001). In the present study the potency of the 5-HT$_4$ agonist in both, stimulation of adenylate cyclase system and amplitude of population spike

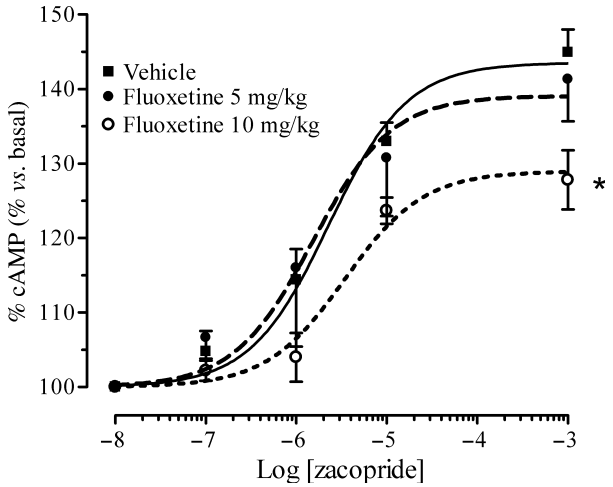

Fig. 2 Effect of increasing concentrations of zacopride on cAMP levels (expressed as mean ± SEM of the percentage of increase over the basal) in striatum membranes from vehicle and fluoxetine-treated rats. Six rats per experimental group were included. *$p < 0.05$ significantly different from vehicle by Student Newman–Keuls *post hoc* test.

assays, is around 1–2 μM. Although this potency is lower than that reported in binding assays (Wong *et al.* 1996), it is noteworthy that it is quite similar to the one previously reported in studies analyzing the excitatory action of zacopride on population spikes (Bijak *et al.* 1997, 2001). On the other hand, this is the first time that adenylate cyclase activation mediated by 5-HT_4 receptors in native tissue has been demonstrated: it is well established that the potency of agonists of different systems to induce modifications in adenylate cyclase activation is significantly lower than the radiometric affinity (Mato *et al.* 2002).

Our autoradiographic data show a significant decrease of striatal and hippocampal 5-HT_4 receptors density after repeated administration with the SSRI. To our knowledge, this is the first study measuring the density of this receptor after chronic fluoxetine treatment. Indeed, only one study has previously addressed the issue of antidepressants and 5-HT_4 receptor density, reporting no significant changes in substantia nigra after chronic citalopram administration (Gobbi *et al.* 1997) without any information about other brain areas. The apparent discrepancy between our data (down-regulation) and those previously reported by Gobbi (Gobbi *et al.* 1997) in substantia nigra may be related with the type of antidepressant (citalopram), the length of the treatment (14 vs. 21 days), the route of administration (i.p. vs. p.o.) or the radioligand ([^{125}I]SB207710) used to quantify the receptor density. In contrast with the clear reduction observed in striatum and hippocampus, the modifications in the density of 5-HT_4 receptors in medial prefrontal cortex after chronic fluoxetine did not reach statistical significance. This difference could be of relevance, since it has been described that cortical 5-HT_4 receptors may induce an

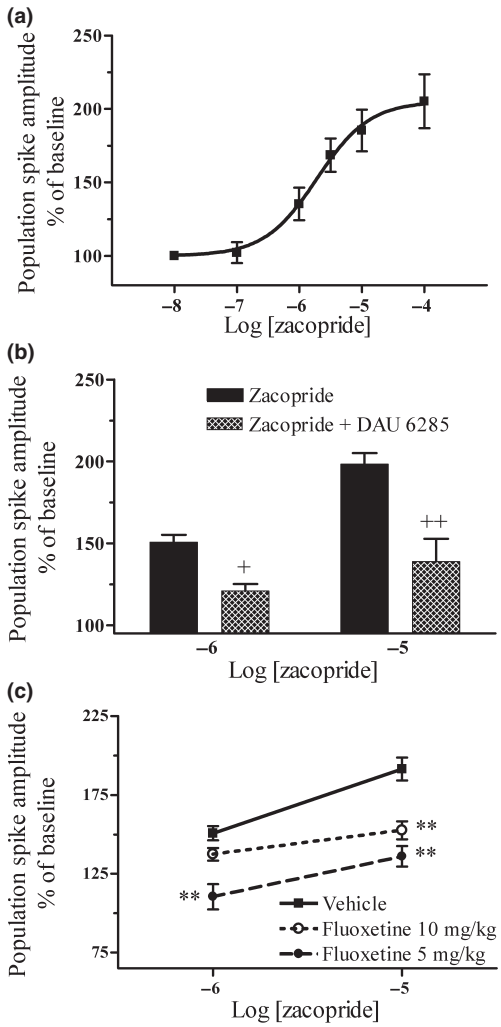

Fig. 3 (a) Dose-response curve of the zacopride-induced stimulation of population spike recorded in pyramidal cells of hippocampal slices ($n = 7$ rats). A population spike which was 50% of the maximum amplitude was chosen. (b) Administration of zacopride mediated stimulation of population spike in the presence of DAU6285 (5 μM) to block 5-HT_4 receptors ($n = 4$ rats). (c) Effect of chronic SSRI on the stimulatory action of zacopride on population spike in rats treated with vehicle ($n = 8$), fluoxetine (10 mg/kg, $n = 9$) and fluoxetine (5 mg/kg/day; $n = 7$). +$p < 0.05$; ++$p < 0.01$ from zacopride stimulations (Student's *t*-test paired data) and **$p < 0.01$ from vehicle treated group (One-way ANOVA and Student Newman–Keuls *post hoc* test).

increase in raphe nuclei 5-HT cell firing (Lucas and Debonnel 2002; Lucas *et al.* 2005): thus, a normosensitivity of cortical 5-HT_4 receptors could facilitate an antidepressant action. Anyway, it seems likely that the 5-HT_4 receptor down-regulation here reported may occur secondary to the antidepressant-induced increase of 5-HT within the synaptic cleft. Furthermore, in line with our results, an up-regulation of 5-HT_4 receptors has been described in depressed suicide victims, particularly in striatum and frontal cortex (Rosel *et al.* 2004). Thus, our results suggest that a down-regulation

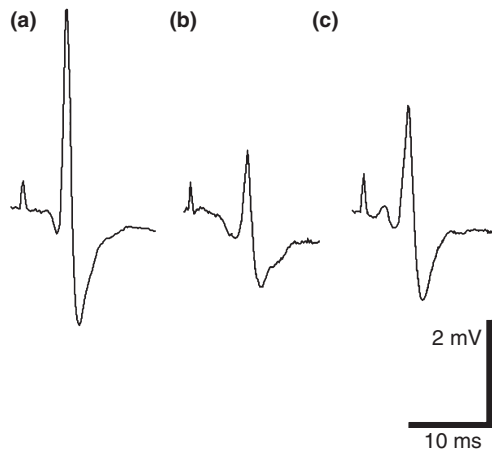

Fig. 4 Representative electrophysiological recordings of pyramidal cells during the perfusion of 10 μM of zacopride after stimulation of the Schaffer collateral-commissural pathway in vehicle (a), fluoxetine 5 mg/kg (b) and fluoxetine 10 mg/kg (c) treated groups.

of 5-HT_4 receptors induced by antidepressant may be a relevant therapeutic mechanism.

It has been proposed that the molecular basis of antidepressant action could be related to changes in the post-receptorial elements involved in cAMP production, such as alterations in the coupling between G proteins and the catalytic unit of adenylate cyclase (Donati and Rasenick 2003). In this regard, our group has observed an increase in basal cAMP levels after chronic fluoxetine-treatment in different brain regions such as hippocampus (Valdizán *et al.* 2002) and striatum (present study). This sensitization of adenylate cyclase might represent a cellular adaptive response to the chronic modification of neurotransmitter levels. The mechanism by which chronic antidepressants treatment alters basal cAMP values is currently under discussion. It has been suggested that some antidepressant facilitate the activation of adenylate cyclase by Gs (Chen and Rasenick 1995; Donati and Rasenick 2003) without changes in the amount of G-proteins (Chen and Rasenick 1995). A differential regulation of each adenylate cyclase isoform by Gαs protein subunits has also been implicated in the hererologous sensitization process (Watts 2002). Further studies should be carried out in order to clarify the exact role of basal cAMP in long-term administration with antidepressants. Although a direct relationship between the increased endogenous 5-HT tone on 5-HT_4 receptors and the cAMP-related molecular changes could be suggested, the modifications in the functionality of many other receptor subtypes following chronic antidepressants could be involved in the regulation of basal cAMP levels.

We have found that repeated treatment with fluoxetine causes a dose-dependent decrease in zacopride-stimulated cyclic AMP accumulation (E_{max} value). As previously reported for other 5-HT receptors, it is possible that the functional desensitization in 5-HT_4 receptors may be because of a change in G-protein level. Nevertheless, the data available in the literature about the regulation of G proteins by antidepressants have reported contradictory results. Several studies have reported a decrease in Gαs protein after repeated administration of antidepressants (Lesch *et al.* 1991, 1992) and electroconvulsive therapy (McGowan *et al.* 1996) in different areas of rat brain, although other studies have not confirmed these findings (Chen and Rasenick 1995; Emamghoreishi *et al.* 1996; Dwivedi and Pandey 1997); these differences may be due either the class of antidepressant or the duration of treatment. However, since it is well known that Gs proteins are coupled to 5-HT_4 receptor (see Barnes and Sharp 1999; Hoyer *et al.* 2002), the possibility that a modification in G_S expression contribute to this effect could not be ruled out. Fluoxetine-induced down-regulation of 5-HT_4 receptor-mediated cAMP stimulation could well be the consequence of a change in the coupling of the receptor to G-proteins, or in the modulation of a particular enzyme isoform (Watts 2002).

Several lines of evidence suggest that, in addition to other brain structures, the hippocampus play a relevant role in the mechanism of action of antidepressant drugs: the proposed relationship between antidepressants' responses and neurogenetic inputs strongly reinforces the role of hippocampus in the mediation of their effects (see Fujita *et al.* 2000; Schmidt and Duman 2007). Focusing on 5-HT neurotransmission, *ex vivo* electrophysiological studies have shown that endogenous 5-HT-mediated synaptic transmission in CA1 field of hippocampus is mediated by, at least, two 5-HT receptor subtypes: 5-HT_{1A} and 5-HT_4 receptors that exerts opposite effects on neuronal excitability (Mongeau *et al.* 1997). Our results indicate that chronic fluoxetine modify the sensitivity of post-synaptic 5-HT_4 receptors in the hippocampus as resulted in an attenuation of zacopride-induced increase of the amplitude of population spike. It is known that fluoxetine is a potent blocker of voltage-gated Ca^{2+} channels (Deak *et al.* 2000), Na^+ channels (Pancrazio *et al.* 1998) and K^+ channels (Yeung *et al.* 1999). However, it is unlikely that under our experimental conditions fluoxetine exert any effect in the electrical activity of CA1 pyramidal cells since the slices had been washed-out thoroughly for 90 min. The apparent decrease in sensitivity of post-synaptic hippocampal 5-HT_4 receptors induced by long-term administration of fluoxetine is also in agreement with the results of others groups (Tokarski and Bijak 1996; Bijak *et al.* 1997), who found a decrease in the response of CA1 pyramidal cells forebrain after administration of citalopram, paroxetine or imipramine for 14 days. The mechanism by which long-term antidepressants induce desensitization of the responsiveness of 5-HT_4 receptors in CA1 area may be complex, but is conceivable to involve increased levels of extracellular 5-HT. Nevertheless, it has been reported that forskolin, a direct activator of cAMP, also produce an increase in population spike and this effect is decreased after the administration of the tricyclic imipramine (14 days, twice daily, 10 mg/kg)

(Bijak 1997). Taking in account this observation, it seems likely that the functional desensitization of 5-HT_4 receptors by antidepressants on the membrane excitability observed in this report may implicate a mechanism involving adenylate cyclase system. In fact, the effects observed with the antidepressants in CA1 neurons excitability are correlated with those previously described in striatum membranes, thus pointing out that a possible desensitization of cAMP effector systems also taking place in hippocampus. Nevertheless, it is necessary to remark that caution is needed when extrapolating data from the striatum to the hippocampus, since different adenylate cyclase isoforms could be expressed depending on the brain area analyzed (see Hanoune and Defer 2001). It is possible that the desensitization in zacopride-mediated stimulation of population spike could be a direct consequence of the decrease in 5-HT_4 receptor density in hippocampus.

Our results have to be also analyzed with regard to the recent report of an antidepressant response for 5-HT_4 agonists. Short-term administration of these agonists could in fact result in a desensitization of 5-HT_4 receptors, similar to the one reported in this study following fluoxetine administration, although with an accelerated pattern of development. In this regard, it is noteworthy that a 3 days exposure to the selective 5-HT_4 receptor agonist RS67333 induces a rapid desensitization of 5-HT_{1A} autoreceptors in dorsal raphe nucleus (Lucas *et al.* 2007). Thus, the recent data suggesting a rapid antidepressant response induced by 5-HT_4 agonists, involving activation of hippocampal plasticity (Lucas *et al.* 2007) strongly support the relevance of our results.

In conclusion, the results of this study indicate that long-term fluoxetine administration produces important biological and physiological changes in 5-HT_4 receptors: down-regulates the density of 5-HT_4 receptors, reduces their ability to stimulate the activity of adenylate cyclase and produces a functional desensitization. All these findings provide strong evidence for 5-HT_4 receptors playing a relevant role for the mechanism of action of SSRIs reuptake inhibitors, contributing to the mediation of their clinical effects.

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