Title: Subchronic treatment with fluoxetine and the 5-HT\textsubscript{2A} antagonist ketanserin upregulates hippocampal BDNF and β-catenin in parallel with antidepressant-like effect.

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Running title: Subchronic SSRI + ketanserin raise neuroplasticity.
SUMMARY

Background and purpose: Serotonin 2A antagonists produce improved antidepressant responses when added to serotonin selective reuptake inhibitors (SSRIs) or tricyclic antidepressants. In this work we have studied the possible involvement of neuroplasticity pathways and/or serotonergic system in the antidepressant-like effect of this cotreatment, when subchronically administered.

Experimental approach: BDNF and TrkB expression, BrdU incorporation, and β-catenin protein expression in different cellular fractions, as well as 5-HT1A functionality were measured in the hippocampus of rats treated with fluoxetine, ketanserin and fluoxetine+ketanserin for seven days, followed by a forced swimming test (FST) to analyze antidepressant efficacy. Two-way ANOVA followed by Bonferroni posthoc test was used for statistical analysis.

Key results: BDNF mRNA was increased in the coadministration group in CA3 field (147±10%, \( p<0.05 \), vs vehicle) and dentate gyrus (179±14%, \( p<0.001 \)) of the hippocampus. β-catenin expression in total homogenate increased in the fluoxetine+ketanserin group (133±7%; \( p<0.001 \)), and in the membrane fraction (134±10%; \( p<0.01 \)), whereas it was unchanged in the nuclear fraction (95±4%). These effects were paralleled by a significant decrease in immobility time in the forced swimming test. There were no changes in BrdU incorporation, TrkB expression and 5-HT1A functionality in any of the groups studied.

Conclusions and implications: The antidepressant-like effect induced by subchronic coadministration of a SSRI and a 5-HT2A antagonist may be mainly due to modifications in hippocampal neuroplasticity (BDNF and membrane-associated β-catenin), without a significant role for other mechanisms involved in chronic antidepressant response, such as hippocampal neuroproliferation or 5-HT1A receptor desensitization in dorsal raphe nucleus.
Keywords: serotonin, 5-HT$_{2A}$ antagonist, SSRI, neuroplasticity, hippocampus, neutrophin, β-catenin, BrdU, behaviour

Abbreviations: 5-HT, serotonin; 8-OH-DPAT, 8-hydroxy-N,N-dipropyl-2-aminotetralin; BDNF, brain derived neurotrophic factor; BrdU, 5-bromo-2’-deoxyuridine; CA1 and CA3, CA1 and CA3 subfields of the hippocampus; DG, dentate gyrus of the hippocampus; DRN, dorsal raphe nucleus; FST, forced swimming test; $[^{35}\text{S}]$GTP$_{\gamma}$S, $[^{35}\text{S}]$guanosine 5’-O-[gamma-thio]triphosphate; SARI, serotonin 2A antagonists/reuptake inhibitor; SGZ, subgranular zone; SSRI, serotonin selective reuptake inhibitor; TrkB, tyrosine kinase B.
INTRODUCTION

Classically, the pathogenesis of depression has been explained by the monoamine hypothesis, involving a dysfunction of serotonergic, noradrenergic and/or dopaminergic systems (for review, Ressler and Nemeroff, 2000). More recently, a neurotrophic hypothesis has been proposed, on the basis of the neuroproliferative effects of antidepressants (Duman et al., 1997). It is noteworthy that most antidepressants as the selective serotonin reuptake inhibitors (SSRIs) need at least two-three weeks to show their therapeutic benefit.

The effect of the neurotransmitter serotonin (5-HT) is mediated by the 5-HT receptor family, formed by seven different subfamilies (5-HT\(_1\) to 5-HT\(_7\)) and 13 different subtypes (for example 5-HT\(_{2A/B/C}\)) (Alexander et al., 2009). 5-HT\(_{2A}\) receptors are present in dendrites and axons of several areas within the rat brain: cerebral cortex, septum, hippocampus, basal ganglia, amygdala, brain stem, etc (Pazos et al., 1985). The role of 5-HT\(_{2A}\) receptors is especially important in prefrontal cortex where the activation of this receptor subtype produces an increase of the excitability of pyramidal neurons (Aghajanian and Marek, 2000). Furthermore, 5-HT\(_{2A}\) receptors appear to be involved in psychiatric disorders: in fact, the antagonism of this receptor subtype is one of the mechanisms of action of atypical antipsychotic drugs (for review, Schmidt et al., 1995).

The role of 5-HT\(_{2A}\) receptors in depression is supported by several studies reporting changes at different levels in tissue samples from suicide victims. A downregulation of receptor protein (Rosel et al., 2000), together with an upregulation in G protein coupling (Rosel et al., 2000) and mRNA receptor expression (Pandey et al., 2002) has been reported in the hippocampus, although other studies have not found changes in this structure (Stockmeier et al., 1997). In contrast, an increase in 5-HT\(_{2A}\) receptor density and functionality has been consistently reported in frontal cortex (Pandey et al., 2002) and
platelets (Serres et al., 1999). In addition, antidepressant treatments have provided conflicting results: chronic SSRI s produce an up-regulation in 5-HT$_{2A}$ receptors (Massou et al., 1997), while tricyclic and/or monoamine oxidase inhibitors induce a down-regulation of this serotonin receptor subtype (Attar-Lévy et al., 1999).

Recently, it has been reported that 5-HT$_{2A}$ antagonists produce antidepressant-like effects (Marek et al., 2003; Pandey et al., 2010), acting through the blockade of the postsynaptic 5-HT$_{2A}$ receptors (Rosel et al., 2000). Since the activation of 5-HT$_{2A}$ receptor opposes the therapeutic effects of the SSRI s in major depression (Marek et al., 2003), the antidepressant effect of some selective serotonin reuptake inhibitors (SSRIs) appears to be potentiated by the coadministration of 5-HT$_{2A}$ subtype antagonists such as risperidone, olanzapine or M100907 (Marek et al., 2003), mainly by increasing serotonin, dopamine and norepinephrine release in medial prefrontal cortex (Huang et al., 2006). Drugs that mediate both serotonin reuptake inhibition and 5-HT$_{2A}$ blockade, are known as SARIs (Serotonin 2A antagonists/reuptake inhibitors), and are suggested in cases of treatment-resistant depression (Shelton et al., 2001; Marek et al., 2003; Adell et al., 2005).

The neurogenic hypothesis of depression is mainly supported by the fact that chronic antidepressant treatment produces an increase in cell proliferation in the subgranular layer of the dentate gyrus of the hippocampus (Duman et al., 1997; Malberg et al., 2000; Santarelli et al., 2003), as well as an increase in the expression of brain derived neurotrophic factor (BDNF) in hippocampus (Nibuya et al., 1995; Vaidya et al., 1999) and serum (Shimizu et al., 2003). It is noteworthy that BDNF is mainly involved in synaptic plasticity, rather than in neuron growth and survival (for review, Martinowich and Lu, 2008). The activation of 5-HT$_{2A}$ receptors increases BDNF levels in prefrontal cortex and decreases BDNF levels in the dentate gyrus of the hippocampus, effects mediated by glutamatergic and GABAergic neurons, respectively (Vaidya et al., 1999). The decrease in
BDNF mRNA expression in hippocampus in a stress model as the immobilization in rats is reversed at least in part by the antagonism of 5-HT$_{2A}$ receptors, thus suggesting the involvement of the 5-HT$_{2A}$ receptor subtype in the increased inhibitory control of the hippocampus and the stress-induced down-regulation of BDNF mRNA (Vaidya et al., 1999).

In the last years, it has been reported that chronic antidepressant treatments modulate the expression of β-catenin, a protein member of the canonical Wnt pathway (Madsen et al., 2003; Mostany et al., 2008), which is accumulated in the cytosol following GSK-3 inhibition, and translocates to the nucleus activating the transcription of genes associated to proliferation (for review, Wada, 2009). β-catenin is also associated to N-cadherin and α-catenin in the cell membrane, controlling the size of reserve vesicle pool in synapse development (Bamji et al., 2003), and providing a link between cadherin-mediated cell-cell adhesion and the F-actin cytoskeleton (Patapoutian and Reichardt, 2000).

In this work we have analyzed the effect of a seven days coadministration of the SSRI fluoxetine and the 5-HT$_{2A}$ antagonist ketanserin on cell proliferation (BrdU incorporation), expression of proteins involved in neuroplasticity (BDNF expression, β-catenin), and serotonergic markers classically involved in chronic antidepressant responses (5-HT$_{1A}$ functionality). These studies have been carried out in parallel with the analysis of antidepressant-like behavioural changes.

**METHODS**

*Animals*

Male Sprague-Dawley rats weighing 270-350 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

**Antidepressant treatment and BrdU administration**

Rats were divided in four groups, 7-12 rats per group, for each set of experiments, and administered via i.p. vehicle (0.9% NaCl solution), 5 mgkg⁻¹day⁻¹ fluoxetine (Fagron Iberica S.A.U., Barcelona, Spain), 0.1 mgkg⁻¹day⁻¹ ketanserin (Sigma, Madrid, Spain), and 5 mgkg⁻¹day⁻¹ fluoxetine + 0.1 mgkg⁻¹day⁻¹ ketanserin during 7 days or in an acute treatment. The dose of ketanserin used for this study is in accordance to previous reports using a similar dosage via i.p. (Andersson et al., 1988) or i.v. (Catafau et al., 2009). We have not used a higher dose of ketanserin, as reported by other authors (Jha et al., 2008; Pandey et al., 2010), in an attempt to avoid the effect over the 5-HT₂C receptor subtype.

For immunohistochemical analysis of cell proliferation, animals received 5-bromo-2’-deoxyuridine (BrdU; 4 x 75 mg/kg every 2 hours, i.p.; Sigma, Madrid, Spain) in sterile 0.9% NaCl solution the last day of antidepressant treatment and 24 hours prior killing. All other chemicals used were of analytical grade.

**Forced swimming test (FST)**

Rats were placed in swim tanks 18 cm in diameter and 40 cm tall. The tank was filled with enough water at 25°C, so that the rat could not touch bottom. The rat was placed in the swim tank for a single 5 min session approximately 24 h after the last treatment in the subchronic set of animals, and after 30 min to 1 h after an acute administration of ketanserin and/or fluoxetine. The seconds of immobility, swimming and climbing behaviour were scored by an observer blind to the treatment condition being tested (Overstreet et al., 2004; Cryan et al., 2005a). The behavioural effect is stronger in FST
performed within the first 5 min of the test, without performing pre-test induction (Cryan et al., 2005a). Antidepressant effect was defined as a decrease in time of immobility.

**In situ hybridization**

Rats were killed by decapitation and brains were quickly removed, frozen in dry ice and stored at -80°C until sectioning. Cryostat sections (20 µm) were thaw-mounted onto slides and pre-treated for in situ hybridization using a standard protocol previously published (Zetterström et al., 1998). Briefly, the tissues were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 5 min, acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine buffer for 10 min, dehydrated in a series of ethanol washes (70%, 80%, 95% and 100%), incubated in chloroform for 10 min, and finally rehydrated with 100% and 95% ethanol. The sections were air-dried and stored at -20°C until use.

Oligonucleotides complementary to mRNAs for BDNF (5' GGT CTC GTA GAA ATA TTG GTT CAG TTG GCC TTT TGA TAC CGG GAC 3', Zetterström et al., 1998) were 3’ end-labelled with [35S]dATP (PerkinElmer Inc., US-MA) using terminal deoxynucleotide transferase (TdT). The labelled oligonucleotide probe was purified and added to each section (250000 cpm/section) in the hybridization buffer (50% formamide (v/v), 4xSSC (saline sodium citrate buffer), 10 mM sodium phosphate, pH 7.0, 1 mM sodium pyrophosphate, 5x Denhardt’s Solution, 0.2 mg/ml salmon sperm DNA, 10% (w/v) dextran sulphate, 0.1 mg/ml polyadenylic acid, 0.12 mg/ml heparin and 20 mM DTT added fresh). After incubation at 42°C in humidity chambers for 16-20 hours, the slides were washed twice in 2x SSC containing 4 mM DTT, at 50°C followed by 5 min washes in 1x SSC, 0.1 x SSC, ethanol (80%) and 1 min ethanol (96%) at room temperature. Sections were then air-dried and exposed to films (Biomax MR, Kodak, Madrid, Spain) together
with $^{14}$C microscales (Amersham, Switzerland) at 4°C for 3 weeks. Controls included hybridization of sections with an excess of unlabelled probe (200x). The abundance of mRNA in hippocampus was analyzed and quantified using a computerized image analysis system (Scion Image, Scion Corporation, US-MD). Optical density values were calibrated to $^{35}$S tissue equivalents using $^{14}$C microscales (Amersham, Switzerland). The data are presented as a percentage of the mean of the saline group (100%).

**Immunohistochemistry**

Twenty-four hours after the last BrdU injection, rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.; Sigma, Madrid, Spain) and transcardially perfused with 4% paraformaldehyde in PBS. Brains were postfixed and cryoprotected with 30% sucrose. Serial coronal sections (40 µm) of the brains were obtained through the entire hippocampus. BrdU immunohistochemistry was performed as previously described (Mostany et al., 2008): sections were incubated for 2 h in 50% formamide/2x SSC at 65 ºC, followed by incubation in 2 N HCl for 30 min. Then sections were incubated for 10 min in 0.1 M borate buffer. After washing in PBS, sections were incubated in 1% H$_2$O$_2$ for 30 min, blocked with 5% goat serum (PBS-TS) for 30 min and then incubated with monoclonal mouse anti-BrdU (1:600; Roche Diagnostics, Barcelona, Spain) overnight at 4 ºC. Sections were washed in PBS-TS, and incubated with a biotinylated donkey anti-mouse IgG secondary antibody (1:200; Jackson ImmunoResearch Laboratories, Inc., US-PA) and amplified with avidin-biotin complex (Vector Laboratories, US-CA). BrdU positive (BrdU$^+$) cells were labeled using DAB + Ni as chromogen (Vector Laboratories).

For $\beta$-catenin immunohistochemistry sections were boiled at 90ºC in 10 mM citric acid, pH 6.0 for 20 min, blocked with 5% donkey normal serum and then, incubated overnight at 4ºC with an anti-$\beta$-catenin monoclonal IgG (1:500; Santa Cruz Biotechnology,
Inc., Heidelberg, Germany) and subsequently with a biotinylated donkey anti-mouse IgG (1:200; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) followed by ABC Vectastain Kit (Vector Laboratories). Finally, they were developed with DAB (Invitrogen, Barcelona, Spain).

For quantification of BrdU+ cells and β-catenin accumulates, every sixth section corresponding to interaural stereotaxic coordinates ranging 4.48-5.70 mm (Paxinos and Watson, 1998) throughout the hippocampus was processed and counted under a light microscope (Carl Zeiss Axioskop 2 Plus) at 40x and 100x magnification. The total number of BrdU+ cells or β-catenin+ aggregates per section were determined and multiplied by 6 to obtain the total number of BrdU+ cells or β-catenin+ aggregates per hippocampus.

**Western blot**

For Western blot analysis, animals were killed by decapitation, their brains removed, and the hippocampi dissected and stored at 80 °C. Each sample was homogenized and processed in order to obtain the total cell lysate (TCL), and membrane, cytosol and nuclear fractions as described by Mostany et al. (2008). Every sample was homogenized (1:15, 500 µl approx.) using a Potter homogenizer in homogenization buffer (HB: 10 mM Hepes, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl) containing protease and phosphatase inhibitors (PPI: 1 mM PMSF, 10 µl/ml aprotinin, 10 µg/ml leupetin, 10 µg/ml pepstatin A, 10 µg/ml antipain, 10 µg/ml chymostatin, 5 µg/ml trypsin inhibitor, 1 mM NaV, 1 mM NaF, 1 mM cantharidin and 10 µM E-64). After homogenization, 250 µl of homogenate were lysed in lysis buffer (HB containing 1% Igepal, 0.1% sodium deoxycholate, 0.2% SDS and 0.1% Triton X-100) 30 min on ice for the total cell lysate (TCL), and centrifuged at 14000 xg 10 min at 4°C. The supernatant (TCL) was alicuoted and conserved at -20°C. The remaining homogenate (250 µl) for subcellular fractionation
was centrifuged at 1000 xg for 10 min at 4 °C, and the resulting supernatant (S1) and pellet were (P1) separated. The S1 was ultra-centrifuged at 100000 xg for 15 min at 4 °C. The pellet P2 membrane fraction was resuspended in buffer containing detergents and PPI, incubated 30 min at 4°C, centrifuged for 10 min at 14000 xg and the supernatant was aliquoted as the membrane fraction (M). Nuclear proteins (N) were isolated by high salt extraction from P1 fraction. P1 fraction was homogenized in 20 mM Hepes pH 7.9, 0.45 M NaCl, 1 mM EDTA containing PPI and incubated in ice for 30 min. Solubilized proteins were recovered in the supernatant after centrifugation at 14000 x g for 10 min at 4 °C.

Protein quantification was performed according to the Lowry method (Lowry et al., 1951).

About 30-50 µg of protein were resolved on 12.5% or 15% SDS-PAGE and transferred to PVDF membranes. These membranes were incubated in mouse anti-β-catenin (1:1000), mouse anti-N-cadherin (1:1000), rabbit anti-BDNF (1:300), mouse anti-GAPDH (1:2000) and mouse anti-α-tubulin (1:20000) primary antibodies, from Santa Cruz Biotechnology, Inc. Heidelberg, Germany, overnight. After extensive washings in TBS-T (TBS, 0.05% Tween 20) membranes were incubated with horseradish peroxidase conjugated secondary antibodies. Secondary antibodies were detected with ECL Advance kit (GE Healthcare Europe GmbH, Munich, Germany). Blot quantitations were performed by densitometric scanning using Scion Image Software. The densitometry values were normalized with respect to GAPDH values for TCL and M fractions, and with respect to β-tubulin for N fraction. Data for every sample was the mean of at least two independent experiments.

5-HT1A functional autoradiography

Experiments were performed following modifications to a previously described protocol (Sim et al., 1997). Sections were pre-incubated for 30 min at 25°C in a buffer containing 50
mM Tris-HCl, 0.2 mM EGTA, 3 mM MgCl₂, 100 mM NaCl, 1 mM DTT and 2 mM GDP (pH 7.7), and subsequently incubated for 2 hours at 25°C in the same buffer containing 3 mU/mL adenosine deaminase and 0.04 nM [³⁵S]GTPγS (PerkinElmer Inc.). Consecutive sections were incubated with 10 µM (±)-8-OHDPAT (Sigma, Madrid, Spain) alone or in the presence of 10 µM WAY100635. Nonspecific binding was determined in the presence of 10 µM guanosine-5-O-(3-thio) triphosphate (GTPγS). After the incubation, the sections were washed twice for 15 min in 50 mM Tris-HCl buffer (pH 7.4) at 4°C, rinsed in distilled cold water and cold air dried. Sections were exposed to radiation-sensitive films (Hyperfilm™-βmax, Amersham, Switzerland) together with ¹⁴C-polymer standards (Amersham, Switzerland) for 2 days at 4°C.

Data analysis and Statistics

Data were expressed as % of vehicle-treated rats considering vehicle as 100%. Results represent mean ± S.E.M. Data were analyzed using a two-way ANOVA followed by a Bonferroni post hoc test to analyze the possible interaction between the antidepressant (fluoxetine) administration, and the 5-HT₂A blockade (administration of ketanserin) in the results obtained. Statistical significance was set at $p<0.05$.

RESULTS

Effect of fluoxetine, ketanserin and their coadministration on forced swimming test (FST)

In the forced swimming test (FST) the immobility time observed in the different treatment groups after subchronic (7 days administration) were: 135±11 s for vehicle, 106±11 s for fluoxetine, 164±17 s for ketanserin, and 85±8 s for the coadministration
A two-way ANOVA analysis showed a significant interaction in the immobility time between treatment with fluoxetine and 5-HT<sub>2A</sub> antagonism \[ F(1,35) = 56.23, p<0.001 \], and a significant main effect for the fluoxetine \[ F(1,35) = 204.9, p<0.001 \], while the group corresponding to 5-HT<sub>2A</sub> blockade alone did not show statistical changes \[ F(1,35) = 1.37, p=0.25 \] (Fig. 1A). In the swimming time, the two-way ANOVA analysis presented a significant interaction between 5-HT<sub>2A</sub> blockade and fluoxetine treatment \[ F(1,35) = 17.33, p<0.001 \], a significant main effect of fluoxetine treatment \[ F(1,35) = 23.16, p<0.001 \], and a significant main effect of 5-HT<sub>2A</sub> blockade \[ F(1,35) = 26.70, p<0.001 \] (Fig. 1B). Regarding climbing time, no significant interaction was observed, and a significant main effect of fluoxetine treatment \[ F(1,35) = 6.74, p<0.05 \] (Fig. 1C).

After an acute treatment, there were no significant changes between the different groups analyzed for the immobility time: 118±9 s vehicle group, 112±17 s fluoxetine group, 147±17 s ketanserin group, and 121±7 s coadministration group.

**Effect of fluoxetine, ketanserin and their coadministration on BDNF and TrkB expression**

Subchronic fluoxetine treatment induced a tendency to the increase in BDNF mRNA expression *versus* vehicle group in some regions such as the CA3 field (CA3) (133±14%) and the dentate gyrus of the hippocampus (DG) (126±16%) although without reaching statistical significance. After administering ketanserin, BDNF mRNA expression values were 105±7% and 108±5% for CA3 and DG, respectively, in line with those from vehicle group. However, the coadministration of fluoxetine+ketanserin for 7 days resulted in an increase of BDNF mRNA expression in several brain areas, as CA3 (147±10%), and DG (179±14%) (Fig. 2F and G). A two-way ANOVA analysis revealed a non significant interaction in the CA3 region of the hippocampus between the antidepressant treatment.
with fluoxetine and the 5-HT$_{2A}$ blockade group [F(1,44) = 0.20, \( p=0.66 \)], and the 5-HT$_{2A}$ antagonism [F(1,44) = 0.81, \( p=0.37 \)], while the antidepressant effect was significant [F(1,44) = 13.4, \( p<0.001 \)] (Fig. 2F). The DG of the hippocampus showed an interaction between the two variables studied [F(1,44) = 4.45, \( p<0.05 \)], the blockade of the 5-HT$_{2A}$ receptor [F(1,44) = 4.77, \( p<0.05 \)], and the antidepressant effect [F(1,44) = 17.66, \( p<0.001 \)] using a two-way ANOVA analysis (Fig. 2G). Other brain areas showing a tendency to the increase in BDNF mRNA expression in the coadministration group included amygdala (147±19%) and piriform cortex (151±21%), and areas such as the CA1 field of the hippocampus and medial prefrontal cortex did not present significative changes using the two-way ANOVA analysis following any drug treatment assayed (data not shown).

The analysis of BDNF protein levels in hippocampus after 7 days of treatment did not reveal significant changes in any of the experimental groups studied: vehicle 100±4%, fluoxetine 93±6%, ketanserin 80±10% and fluoxetine+ketanserin 90±6%; acute treatment with fluoxetine, ketanserin, or the association of both drugs also resulted in lack of significative changes: 100±8%, vehicle; 110±8%, fluoxetine; 102±9%, ketanserin, and 100±4%, coadministration group.

TrkB receptor expression was also measured by in situ hybridization. No significant modification was found for any of the experimental groups analyzed after 7 days of administration (Supplementary information; Fig. S1).

**Lack of effect of fluoxetine, ketanserin and their coadministration on BrdU incorporation**

The BrdU positive cells in the different experimental groups were 102±8%, 114±8% and 114±8%, for fluoxetine, ketanserin, and fluoxetine+ketanserin respectively (Fig. 3). The statistical analysis of the data using a two-way ANOVA showed no interaction...
between fluoxetine treatment and 5-HT$_{2A}$ blockade, and a significant main effect of ketanserin treatment \[F(1,24) = 34.60, p<0.001\].

**Effect of fluoxetine, ketanserin and their coadministration on $\beta$-catenin protein expression**

Western blot analysis of the total cell lysate (TCL) showed lack of changes in $\beta$-catenin protein levels in the fluoxetine (105±4% vs vehicle 100±3%), and ketanserin groups (98±5% vs vehicle), while a significant increase was observed in the fluoxetine+ketanserin group (133±7%; \(p<0.001\) fluoxetine and ketanserin vs fluoxetine+ketanserin group, Bonferroni post-hoc test) (Fig. 4A). The interaction between antidepressant administration and 5-HT$_{2A}$ blockade was statistically significant using a two-way ANOVA \[F(1,44) = 9.14, p<0.01\]; and there is a significant main effect of fluoxetine treatment \[F(1,44) = 15.55, p<0.001\], and serotonin 2A blockade \[F(1,44) = 6.77, p<0.05\]. In the membrane fraction there were no significant changes in $\beta$-catenin in the fluoxetine (106±2%), and ketanserin (102±6%) groups, while there was a significant increase of $\beta$-catenin labelling in the fluoxetine+ketanserin group (134±10%; \(p<0.01\) compared to fluoxetine and ketanserin groups; Bonferroni post-hoc test) (Fig 4B,D). The two-way ANOVA analysis showed a significant interaction of fluoxetine treatment and 5-HT$_{2A}$ blockade \[F(1,44) = 4.65, p<0.05\], a significant main effect of the fluoxetine treatment \[F(1,44) = 7.71, p<0.01\], and of the 5-HT$_{2A}$ antagonism \[F(1,44) = 6.23, p<0.05\]. No significant modifications in $\beta$-catenin nuclear fraction were observed in any of the experimental groups (vehicle, 100±4%; fluoxetine, 96±2%; ketanserin, 98±4%; fluoxetine+ketanserin, 95±4%) (Fig. 4C,D) using a two-way ANOVA analysis: \[F(1,44) = 0.061, p=0.806\] for the interaction between both drugs.
Acute drug administration did not significantly change β-catenin protein levels in hippocampal total homogenate, membrane or nuclear fraction in any of the different treatment groups (data not shown).

The β-catenin immunopositive cells in the different treated groups: vehicle 100±5% (mean±S.E.M.); fluoxetine 93±5%, ketanserin 89±4% and fluoxetine+ketanserin 105±6% (Fig. 5E,F), showed an interaction between fluoxetine administration and 5-HT2A antagonism: [F(1,24) = 36.28, p<0.001], and a significant main effect of the fluoxetine treatment [F(1,24) = 5.59, p<0.05], when using a two-way ANOVA test.

Effect of fluoxetine, ketanserin and their coadministration on N-cadherin protein expression

The analysis by western blot of the hippocampal cell lysate of the different experimental groups revealed a significant increase in N-cadherin protein levels in the association group (137±10%) vs fluoxetine (105±5%, p<0.01) and ketanserin (100±6%, p<0.001), groups (Bonferroni post-hoc test). A two-way ANOVA analysis showed significant fluoxetine x ketanserin 5-HT2A antagonism interaction [F(1,44) = 5.95, p<0.05], a significant main effect of the fluoxetine treatment [F(1,44) = 10.62, p<0.01], and a significant main effect of the ketanserin treatment [F(1,44) = 6.19, p<0.05] (Fig. 5A). N-cadherin protein levels in the membrane fraction were increased both in the fluoxetine (139±9% vs 100±4% in the vehicle group, p<0.01, Bonferroni post-hoc test), and in the coadministration group (176±13% vs ketanserin group, 97±7%, p<0.001; and vs fluoxetine group, p<0.05, Bonferroni post-hoc test). A significant interaction between antidepressant treatment and 5-HT2A antagonism was observed [F(1,44) = 4.79, p<0.05], and a significant main effect of the fluoxetine treatment [F(1,44) = 42.64, p<0.001], following a two-way ANOVA analysis (Fig. 5B). The analysis of N-cadherin protein levels in the nuclear
fraction showed a significant main effect of the ketanserin treatment \( [F(1,44) = 4.15, p<0.05] \), but not significant changes following a Bonferroni post-hoc test (Fig. 5C).

The acute administration of fluoxetine and ketanserin alone or combined did not changed the N-cadherin protein levels in hippocampal total homogenate (data not shown).

**Lack of effect of fluoxetine, ketanserin and their coadministration on 5-HT\textsubscript{1A} receptor functionality.**

Basal \(^{35}\text{S}\)GTP\(\gamma\)S binding and 8-OH-DPAT-mediated \(^{35}\text{S}\)GTP\(\gamma\)S stimulation were not significantly modified in the different brain structures studied in fluoxetine, ketanserin, and fluoxetine+ketanserin treatment groups.

The 8-OH-DPAT-mediated \(^{35}\text{S}\)GTP\(\gamma\)S stimulation was not significantly modified in any of the structures and treatments studied (i.e.: DRN: 154±8% and 176±14% of vehicle and fluoxetine+ketanserin groups, respectively; CA1 of the hippocampus: 126±6% and 138±7% of vehicle and fluoxetine+ketanserin groups, respectively; CA3 of the hippocampus: 122±8% and 137±7% of vehicle and fluoxetine+ketanserin groups, respectively; and DG of the hippocampus: 173±15% and 183±13% of vehicle and fluoxetine+ketanserin groups, respectively (Table 1).

**DISCUSSION AND CONCLUSIONS**

Current antidepressant treatments require several weeks to achieve therapeutic efficacy. Different strategies based on the pharmacological manipulation of monoaminergic systems have been developed in an effort to accelerate the onset of antidepressant action, including the combination of 5-HT reuptake blockers with antagonists of serotonin receptors (Adell *et al.*, 2005). In this sense, recent studies have reported a potentiation of the antidepressant effects by coadministration of drugs with a 5-HT\textsubscript{2A} antagonistic profile.
The aim of this study was to evaluate the effect of subchronic coadministration of the SSRI fluoxetine and the 5-HT$_{2A}$ antagonist ketanserin on hippocampal neurogenic markers, corroborating behavioural findings. The dose of ketanserin used (0.1 mg/kg/day) should result in a selective blockade of the 5-HT$_{2A}$ subtype (Hoyer et al., 2002). Our results show that the administration for 7 days of either fluoxetine or ketanserin did not significantly modify BDNF, β-catenin expression or depression-related behaviour. In contrast, coadministration of fluoxetine and ketanserin significantly increased those neurogenic markers, inducing an antidepressant-like response in FST.

To date, some reports indicate that the combination of 5-HT$_{2A}$ antagonists and tricyclic antidepressants (Pandey et al., 2010) or SSRIs (Marek et al., 2003) produces antidepressant-like effects in animals. More interestingly, this combination also increases antidepressant responses in humans, even in treatment-resistant cases (Shelton et al., 2001; Marek et al., 2003). Furthermore, the acute administration of 5-HT$_{2A}$ antagonists decreases immobility and increases the swimming behaviour in FST (Patel et al., 2004). In line with this, stimulation of 5-HT$_{2A}$ receptors results in depressogenic-like behaviour (Rajkumar et al., 2009). Our results in the coadministration group after 7 days of treatment show a decrease in immobility in the FST, a response previously reported to appear after 14 days of SSRI treatment (Cryan et al., 2005a), and an increase of the swimming time, which show statistical significance for the interaction of the fluoxetine treatment and the 5-HT$_{2A}$ blockade, which reflects an involvement of the serotonergic system in this antidepressant-like response (Cryan et al., 2005a; 2005b). In addition, the lack of changes in climbing time excludes a possible role of noradrenergic/dopaminergic mechanisms (Page et al., 1999; Cryan et al., 2005b). The acute administration of the different drugs alone or coadministered did not produce any change compared to vehicle. In this regard, our data are
in accordance with literature in which low doses of fluoxetine (8-20 mg/kg) (Castagné et al., 2006; Ulak et al., 2010), or the acute administration of ketanserin alone (Campos et al., 2005), or in combination with some antidepressant drugs (Campos et al., 2005) do not produce any significant change in immobility time in the FST.

Furthermore, we have not found any change associated to the functionality of the 5-HT$_{1A}$ receptor subtype, although a significant reduction of the basal values are observed in the basal values in the DRN between the fluoxetine and the coadministration groups, suggesting that the duration of the treatment (7 days) is not enough to desensitize 5-HT$_{1A}$ receptors in DRN, as occurs in chronic (14 days) treatments (Castro et al., 2003). Thus, a possible increased serotonergic activity might be a consequence of the influence of other regulatory mechanisms on DRN activity, such as GABAergic neurons (containing 5-HT$_{2A}$ receptors), and/or glutamate neurotransmission (for review, Celada et al., 2004), or other early neuroplastic changes, as it is discussed below.

BDNF expression is reduced in some brain areas (Duman et al., 1997) and serum (Shimizu et al., 2003) of depressed patients, as well as in hippocampus of some animal models of depression (Elfving et al., 2009). In this regard, the expression levels of this protein are increased after chronic antidepressant treatment in rat hippocampus (Nibuya et al., 1995; Larsen et al., 2007), human brain (Duman et al., 1997), and human serum (Shimizu et al., 2003). In the present work, no changes were observed on BDNF mRNA expression in hippocampus following subchronic independent fluoxetine or ketanserin administration, in agreement with other authors’ contribution (Larsen et al., 2007). However, after subchronic coadministration of fluoxetine and ketanserin, we found an increased expression of BDNF mRNA in CA3 subfield (CA3) of the hippocampus, reaching the statistical significance in the dentate gyrus (DG) of the hippocampus, an area clearly related to the pathogenesis of depression (for review, Lucassen et al., 2006). This
increase in BDNF mRNA expression is not accompanied by an increase in the expression of the protein: it has been reported that at least 3 weeks are required to show a significant increase in the hippocampus (De Foubert et al., 2004).

A relationship between 5-HT2A receptors and BDNF has been suggested by studies where activation of 5-HT2A receptors decreases hippocampal BDNF (Vaidya et al., 1999). Furthermore, studies in mice BDNF+/−, presenting a depression-like profile, show higher hippocampal 5-HT2A receptor levels, which are downregulated in hippocampal primary cultures and organotypic hippocampal slices after 7 days of incubation with BDNF (Trajkovska et al., 2009). It is noteworthy that antidepressant-like effects have been described 3-10 days after acute bilateral infusion of BDNF in CA3 and DG (Shirayama et al., 2002).

In contrast to the results corresponding to BDNF mRNA expression, TrkB mRNA expression was not modified after the seven days treatment with fluoxetine, ketanserin or the coadministration. An increase of TrkB mRNA expression after chronic antidepressant treatment (Nibuya et al., 1995) has been previously reported, although 21 days of administration appear to be necessary before a regulation of TrkB gene is achieved. The possibility exists that the regulation of TrkB receptor expression is slower than that of BDNF or that an increase of BDNF mRNA could lead to the activation of another signalling pathways, such as p75 neurotrophin receptor (Rantamäki et al., 2007).

The 5-HT2A receptor subtype is located postsynaptically (Peddie et al., 2008) in the pyramidal and granular cell layers within the hippocampus, and in GABAergic interneurons (Peddie et al., 2008), involved in the downregulation of BDNF expression (Zafra et al., 1991). Furthermore, specific 5-HT2A antagonists facilitate the induction of long term potentiation (LTP) in hippocampus (Wang and Arvanov, 1998). The increase in BDNF expression in CA3 and DG after subchronic treatment with fluoxetine and ketanserin may
be partially mediated by 5-HT2A subtype blockade, inhibiting GABAergic interneurons, and producing an increase in the activation of pyramidal neurons in hippocampus (Vaidya et al., 1999), thus modulating dendritic activation and synaptic plasticity (Gulyás et al., 1999).

The increase in β-catenin accumulation found in total hippocampal homogenates after subchronic coadministration of fluoxetine and ketanserin is comparable to that reported following electroconvulsive seizures (Madsen et al., 2003) and chronic antidepressant treatment (Mostany et al., 2008), not observing any change after acute administration. However, this increase in β-catenin is associated to the membrane fraction, not the nuclear one, in contrast to the results reported after chronic antidepressants (Mostany et al., 2008). This is further supported by the results from immunohistochemical labelling in the subgranular zone of the hippocampus, where those immunopositive cells are colocalized with progenitor cells (Mostany et al., 2008). β-catenin is an important regulator of synaptic plasticity and long term memory formation (Maguschak and Ressler, 2008); the cadherin-β-catenin complex localized in symmetrical synaptic junctions (Uchida et al., 1996) is involved in the recruitment and control of the size of the reserve vesicle pool (Bamji et al., 2003), as well as in synaptic targeting (Nishimura et al., 2002). The relevance of β-catenin as a part of the N-cadherin/β-catenin complex, in contrast to its role in the Wnt/β-catenin pathway, is supported by the parallel increase that is observed in N-cadherin protein attached to the membrane fraction, as a result of the coadministration of the antidepressant fluoxetine and the 5-HT2A antagonist ketanserin. The intracellular levels of cadherin/catenin complex are a limiting factor in dendritic morphogenesis: its overexpression increases dendritic branching (Yu and Malenka, 2003), and the association with cadherins effectively sequesters β-catenin from the cytoplasmic pool (Patapoutian and Reichardt, 2000). Regarding the factors regulating the interaction between Wnt/β-catenin signalling and cadherin-mediated adhesion, an increase in N-cadherin would result in the
inhibition of Wnt (for review, Nelson and Nusse, 2004), thus favouring the cell-cell adhesion role of β-catenin. In experiments deleting β-catenin in hippocampal pyramidal neurons, a reduction in the number of reserve pool vesicles per synapse and an impaired response to prolonged repetitive stimulation is observed (Bamji et al., 2003). The increase in synaptic activity is also associated with the redistribution of β-catenin into synapses, which depends on differential phosphorylation of this protein (for review, Nelson and Nusse, 2004), mediated by neurotrophic Trk receptors (David et al., 2008). Some authors indicate that β-catenin located in adherent junctions can also be released and translocated to the nucleus (Kam and Quaranta, 2009). Thus, the coadministration of fluoxetine and ketanserin could result in an increase of the role of β-catenin in the facilitation of synaptic plasticity.

We have not observed a significant interaction between both drug treatments in the modulation of hippocampal cell proliferation, although an increase was found following either ketanserin or fluoxetine+ketanserin administration. An increase in cell proliferation in the dentate gyrus of the hippocampus has been related to chronic antidepressant treatments, but it has not been reported following subchronic treatment with fluoxetine (Malberg et al., 2000). Regarding the effects of alone 5-HT$_{2A}$ antagonists, it depends on the duration of the treatment: a reduction in proliferation after an acute treatment (Jha et al., 2008), and an increase after 7 days administration (Jha et al., 2008) have been reported.

In summary, the subchronic (1 week) coadministration of a SSRI and a 5-HT$_{2A}$ antagonist is sufficient to increase some neuroplastic markers previously associated to chronic (2-3 weeks) antidepressant treatment, such as BDNF and β-catenin. In addition, this treatment does not induce the well characterized changes in monoaminergic neurotransmission following chronic antidepressants, such as 5-HT$_{1A}$ desensitization in DRN. However, this conjoint treatment is enough to promote antidepressant-like
behavioural changes with a shorter onset of action. Thus, we propose that the modifications in synaptic plasticity induced by this drug combination are enough to produce an antidepressant-like response, preceding the appearance of changes in cell proliferation and serotonergic markers. Although further experiments are needed to fully clarify the role of 5-HT$_{2A}$ receptors in this early antidepressant response, our results strongly support the interest of the pharmacological blockade of this receptor subtype as a promising target for the treatment of depression.

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FIGURES AND LEGENDS

Figure 1. Graphs showing immobility time (A), swimming time (B) and climbing time (C) in the forced swimming test (FST) for vehicle, fluoxetine, ketanserin and fluoxetine+ketanserin treatment groups. Data are expressed as seconds (s): mean ± S.E.M., n=8-10. Note that a two-way ANOVA analysis show significative changes in the immobility time: fluoxetine x ketanserin interaction [F(1,35) = 56.23, \( p<0.001 \)]; and in the swimming time: fluoxetine x ketanserin interaction [F(1,35) = 17.33, \( p<0.001 \)]. *** \( p<0.001 \) for the Bonferroni post-hoc test.

Figure 2. Autoradiograms illustrating BDNF in situ hybridization of vehicle (A), fluoxetine (B), ketanserin (C), and fluoxetine+ketanserin (D) treatments (upper images). Graphs show BDNF protein expression in hippocampus total cell lysate (E), and mRNA expression in both CA3 subfield (F) and dentate gyrus (DG) (G) of the hippocampus. Data expressed as % of vehicle-treated animals (0%). Data are expressed as mean ± S.E.M.; n=10-12. Two-way ANOVA analysis for BDNF mRNA in DG: fluoxetine x ketanserin interaction [F(1,44) = 4.45, \( p<0.05 \)]. * \( p<0.05 \), ** \( p<0.01 \), *** \( p<0.001 \) in the Bonferroni post-hoc test. Bar: 2 mm.

Figure 3. Graph showing BrdU+ immunolabelling in the subgranular zone (SGZ) of the dentate gyrus of the hippocampus for vehicle, fluoxetine, ketanserin, and fluoxetine+ketanserin treatment groups. Data are presented as percentage versus vehicle group. Data are expressed as mean ± S.E.M., n=7. ** \( p<0.01 \), *** \( p<0.001 \) using a two-way ANOVA analysis followed by a Bonferroni post-hoc test.
Figure 4. Effect of fluoxetine, ketanserin, and fluoxetine+ketanserin treatments on β-catenin level in rat hippocampus total cell lysate (A), membrane fraction (B), and nuclear fraction (C). Representative western blot of β-catenin protein expression in membrane and nuclear fraction from hippocampus (D). β-catenin immunohistochemical positive clusters in the SGZ of the hippocampus (E) and representative images showing β-catenin immunolabelling in SGZ of the hippocampus (F): i) vehicle, ii) fluoxetine, iii) ketanserin, and iv) fluoxetine+ketanserin. Western blot data are expressed as percentage of the relative optic density (OD), n=10-12, and data for immunolabelling are presented as β-catenin immunopositive groups SGZ, n=7 (vs vehicle-treated animals). Data are presented as mean ± S.E.M. Two-way ANOVA for the antidepressant fluoxetine x ketanserin interaction in total cell lysate [F(1,44) = 9.14, \( p<0.01 \)]; membrane fraction [F(1,44) = 4.65, \( p<0.05 \)]; and β-catenin immunolabelling [F(1,24) = 36.28, \( p<0.001 \)]. ** \( p<0.01 \), *** \( p<0.001 \) in the Bonferroni post-hoc test.

Figure 5. Parallel effect of fluoxetine, ketanserin, and fluoxetine+ketanserin treatments on N-cadherin protein levels in rat hippocampus total cell lysate (A), membrane fraction (B), and nuclear fraction (C). Western blot data are expressed as percentage of the relative optic density (OD) (vs vehicle-treated animals). Data are presented as mean ± S.E.M., n=10-12. Two-way ANOVA analysis for fluoxetine x ketanserin interaction in total cell lysate: [F(1,44) = 5.95, \( p<0.05 \)], and in membrane fraction: [F(1,44) = 4.79, \( p<0.05 \)]. * \( p<0.05 \), ** \( p<0.01 \), *** \( p<0.001 \) using a Bonferroni post-hoc test.
Table 1. Effect of 7-day fluoxetine, ketanserin and fluoxetine+ketanserin treatments on basal $[^{35}\text{S}]\text{GTP}_{\gamma}S$ binding and (±)-8-OH-DPAT stimulated $[^{35}\text{S}]\text{GTP}_{\gamma}S$ binding in some rat brain areas, reflecting 5-HT$_{1A}$ functionality. Values are presented as percentage of basal values (100%) (mean ± s.e.m.) for 8-OH-DPAT stimulated $[^{35}\text{S}]\text{GTP}_{\gamma}S$ binding. Two-way ANOVA followed no statistical differences. CA1: CA1 field of hippocampus; CA3: CA3 field of hippocampus; DG: dentate gyrus of hippocampus; DRN: dorsal raphe nucleus.

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STATEMENT OF CONFLICTS OF INTEREST

The authors declare that AP has received support for research from Faes Farma, S.A. FP-C and RV report no biomedical financial interests or potential conflicts of interest.

The present study is not related to any of these professional or collaboration relationships.