British Journal of Pharmacology

DOI:10.1111/j.1476-5381.2011.01516.x
www.brjpharmacol.org

RESEARCH PAPER

Subchronic treatment with fluoxetine and ketanserin increases hippocampal brain-derived neurotrophic factor, β-catenin and antidepressant-like effects

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Keywords
5-HT_{2A} receptor antagonist; SSRI; neuroplasticity; hippocampus; neutrophin; β-catenin; BrdU; behaviour

Received
24 September 2010
Revised
23 February 2011; 4 May 2011
Accepted
17 May 2011

BACKGROUND AND PURPOSE

5-HT_{2A} receptor antagonists improve antidepressant responses when added to 5-HT-selective reuptake inhibitors (SSRIs) or tricyclic antidepressants. Here, we have studied the involvement of neuroplasticity pathways and/or the 5-hydroxytryptaminergic system in the antidepressant-like effect of this combined treatment, given subchronically.

EXPERIMENTAL APPROACH

Expression of brain-derived neurotrophic factor (BDNF) and its receptor (TrkB), 5-bromo-2′-deoxyuridine (BrdU) incorporation, and β-catenin protein expression in different cellular fractions, as well as 5-HT_{1A} receptor function were measured in the hippocampus of rats treated with fluoxetine, ketanserin and fluoxetine + ketanserin for 7 days, followed by a forced swimming test (FST) to analyse antidepressant efficacy.

KEY RESULTS

mRNA for BDNF was increased in the CA3 field and dentate gyrus of the hippocampus by combined treatment with fluoxetine + ketanserin. Expression of β-catenin was increased in total hippocampal homogenate and in the membrane fraction, but unchanged in the nuclear fraction after combined treatment with fluoxetine + ketanserin. These effects were paralleled by a decreased immobility time in the FST. There were no changes in BrdU incorporation, TrkB expression and 5-HT_{1A} receptor function in any of the groups studied.

CONCLUSIONS AND IMPLICATIONS

The antidepressant-like effect induced by subchronic co-treatment with a SSRI and a 5-HT_{2A} receptor antagonist may mainly be because of 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-HT_{1A} receptor desensitization in the dorsal raphe nucleus.

Abbreviations

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; GSK, glycogen synthase kinase-3; [^{35}S]GTPγS, [^{35}S]guanosine 5′-O-[γ–thio]triphosphate; SARI, 5-HT_{2A} receptor antagonists/reuptake inhibitor; SGZ, subgranular zone; SSRI, 5-HT-selective reuptake inhibitor

Introduction

Classically, the pathogenesis of depression has been explained by the monoamine hypothesis, involving a dysfunction of 5-hydroxytryptaminergic, noradrenergic and/or dopaminergic systems (see 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 such as the selective 5-HT reuptake inhibitors (SSRIs) need at least 2–3 weeks to show their therapeutic benefit.

The effects of the neurotransmitter 5-HT are 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-$HT_{2A/B/C}$; nomenclature follows Alexander *et al.*, 2011). 5-HT_{2A} receptors are present in dendrites and axons of several areas within the rat brain, including the cerebral cortex, septum, hippocampus, basal ganglia, amygdala and brain stem (Pazos *et al.*, 1985). The role of 5-HT_{2A} receptors is especially important in the 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, and antagonism of this receptor subtype is one of the mechanisms of action of atypical antipsychotic drugs (see 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 down-regulation of the receptor protein (Rosel *et al.*, 2000), together with an up-regulation in G protein coupling (Rosel *et al.*, 2000) and expression of mRNA for the receptor (Pandey *et al.*, 2002) have 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 function has been consistently reported in the frontal cortex (Pandey *et al.*, 2002) and platelets (Serres *et al.*, 1999). In addition, antidepressant treatments have provided conflicting results, as chronic SSRIs produces 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 5-HT 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 SSRIs in major depression (Marek *et al.*, 2003), the antidepressant effect of some SSRIs appears to be potentiated by the co-administration of 5-HT_{2A} subtype antagonists such as risperidone, olanzapine or M100907 (Marek *et al.*, 2003), mainly by increasing 5-HT, dopamine and noradrenaline release in medial prefrontal cortex (Huang *et al.*, 2006). Drugs that mediate both 5-HT reuptake inhibition and 5-HT_{2A} receptor blockade are known as 5-HT_{2A} receptor antagonists/reuptake inhibitors (SARIs) and have been 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 (DG) 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 the 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 (see Martinowich and Lu, 2008). The activation of 5-HT_{2A} receptors increases BDNF levels in the prefrontal cortex and decreases BDNF levels in the DG of the hippocampus, effects mediated by glutamatergic and GABAergic neurons, respectively (Vaidya *et al.*, 1999). The decrease in expression of mRNA for BDNF in hippocampus, assessed in a stress model as immobility time, is reversed, at least in part by 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 constituent of the canonical Wnt pathway (Madsen *et al.*, 2003; Mostany *et al.*, 2008), which is accumulated in the cytosol following inhibition of glycogen synthase kinase-3 (GSK-3), and translocates to the nucleus, activating the transcription of genes associated with proliferation (see Wada, 2009). β-Catenin is also associated with N-cadherin and α-catenin in the cell membrane, controlling the size of the 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 the present experiments, we have analysed the effect of 7 days co-treatment with the SSRI fluoxetine and the 5-HT_{2A} receptor antagonist ketanserin on cell proliferation [5-bromo-2′-deoxyuridine (BrdU) incorporation], expression of proteins involved in neuroplasticity (BDNF expression, β-catenin), and 5-hydroxytryptaminergic markers classically involved in chronic antidepressant responses (5-HT_{1A} receptor function). These studies have been carried out in parallel with the analysis of antidepressant-like behavioural changes.

Methods

Animals

All animal care and experimental procedures complied with Spanish legislation and the European Communities Council Directive on 'Protection of Animals Used in Experimental and Other Scientific Purposes' (86/609/EEC). 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*.

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 $mg \cdot kg^{-1} \cdot day^{-1}$ fluoxetine (Fagron Iberica S.A.U., Barcelona, Spain), 0.1 $mg \cdot kg^{-1} \cdot day^{-1}$ ketanserin

(Sigma, Madrid, Spain) or the combination of 5 mg·kg^{-1}·day^{-1} fluoxetine + 0.1 mg·kg^{-1}·day^{-1} ketanserin for 7 days or acutely (single treatment). The dose of ketanserin used for this study was based on previous reports using similar i.p. (Andersson *et al.*, 1988) or i.v. doses (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 possible effects on the 5-HT$_{2C}$ receptors.

For immunohistochemical analysis of cell proliferation, animals received BrdU (4 × 75 mg·kg^{-1} every 2 h, i.p.; Sigma) in sterile 0.9% NaCl solution on the last day of antidepressant treatment and 24 h before killing. All other chemicals used were of analytical grade.

Forced swimming test (FST)

Rats were placed in swimming 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 the bottom. The rat was placed in the swimming tank for a single 5 min session approximately 24 h after the last treatment in the subchronic set of animals or after 30 min to 1 h, following an acute (single) administration of ketanserin and/or fluoxetine. The time spent in immobility, swimming and climbing behaviours were scored by an observer unaware of the treatments given (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 immobility time.

In situ *hybridization*

Rats were killed by decapitation, and the 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% paraformaldehide in 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 [^{35}S]dATP (PerkinElmer Inc., Waltham, MA, USA) using terminal deoxynucleotide transferase (TdT). The labelled oligonucleotide probe was purified and added to each section (250 000 cpm per section) in the hybridization buffer [50% formamide (v/v), 4× saline sodium citrate buffer (SSC), 10 mM sodium phosphate, pH 7.0, 1 mM sodium pyrophosphate, 5× Denhardt's Solution, 0.2 mg·mL^{-1} salmon sperm DNA, 10% (w/v) dextran sulphate, 0.1 mg·mL^{-1} polyadenylic acid, 0.12 mg·mL^{-1} heparin and 20 mM dithiothreitol (DTT) added freshly]. After incubation at 42°C in humidity chambers for 16–20 h, the slides were washed twice in 2× SSC containing 4 mM DTT, at 50°C followed by 5 min washes in 1× SSC, 0.1× 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, Buckinghamshire, UK) at 4°C for 3 weeks. Controls included hybridization of sections with an excess of unlabelled probe (200×). The abundance of mRNA in hippocampus was analysed and quantified using a computerized image analysis system (Scion Image, Scion Corporation, Maryland, MD, USA). Optical density values were calibrated to ^{35}S tissue equivalents using ^{14}C microscales (Amersham). The data are presented as a percentage of the mean of the saline group (set to 100%).

Immunohistochemistry

Twenty-four hours after the last BrdU injection, rats were anaesthetized with sodium pentobarbital (50 mg·kg^{-1}, i.p.; Sigma) and transcardially perfused with 4% paraformaldehyde in PBS. Brains were post-fixed and cryoprotected with 30% sucrose. Serial coronal sections (40 µm) of the brains were obtained through the entire hippocampus. BrdU inmunohistochemistry was performed as previously described (Mostany *et al.*, 2008); sections were incubated for 2 h in 50% formamide/2× SSC at 65°C, 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_2O_2 for 30 min, blocked with 5% goat serum in PBS (PBS-TS) for 30 min and then incubated with monoclonal mouse anti-BrdU (1:600; Roche Diagnostics, Barcelona, Spain) overnight at 4°C. Sections were washed in PBS-TS, and incubated with a biotinylated donkey anti-mouse IgG secondary antibody (1:200; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) and amplified with avidin–biotin complex (Vector Laboratories, Burlingame, CA, USA). BrdU-positive cells were labeled using diaminobenzidine (DAB) + Ni as chromogen (Vector Laboratories).

For β-catenin immunohistochemistry, sections were boiled at 90°C in 10 mM citric acid, pH 6.0, for 20 min, blocked with 5% donkey normal serum and then incubated overnight at 4°C with an anti-β-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.) followed by ABC Vectastain Kit (Vector Laboratories). Finally, they were developed with DAB (Invitrogen, Barcelona, Spain).

For quantification of BrdU-positive cells and β-catenin accumulation, every sixth section corresponding to interaural stereotaxic coordinates ranging from 4.48 to 5.70 mm (Paxinos and Watson, 1998) throughout the hippocampus was processed and counted under a light microscope (Carl Zeiss Axioskop 2 Plus) at 40× and 100× magnification. The total number of BrdU-positive cells or β-catenin positive aggregates per section were determined and multiplied by 6 to obtain the total number of BrdU-positive cells or β-catenin-positive 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 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 (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl) containing protease and phosphatase inhibitors (PPI; 1 mM PMSF, 10 μL·mL^{-1} aprotinin, 10 μg·mL^{-1} leupetin, 10 μg·mL^{-1} pepstatin A, 10 μg·mL^{-1} antipain, 10 μg·mL^{-1} chymostatin, 5 μg·mL^{-1} trypsin inhibitor, 1 mM NaV, 1 mM NaF, 1 mM cantharidin and 10 μM E-64). After homogenization, 250 μL of homogenate was 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 TCL, and centrifuged at 14 000× *g* 10 min at 4°C. Aliquots of the supernatant (TCL) were stored at –20°C. The remaining homogenate (250 μL) for subcellular fractionation was centrifuged at 1000× *g* for 10 min at 4°C, and the resulting supernatant (S1) and pellet were (P1) separated. The S1 fraction was centrifuged at 100 000× *g* for 15 min at 4°C, and the resulting pellet P2 membrane fraction was resuspended in buffer containing detergents and PPI, incubated 30 min at 4°C, centrifuged for 10 min at 14 000× *g*. Aliquots of this supernatant were stored as the membrane fraction (M). Nuclear proteins (N) were isolated by high salt extraction from the P1 fraction. The 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 14 000× *g* for 10 min at 4°C. Protein was quantified by the Lowry method.

About 30–50 μg of protein was 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 (1:20 000) primary antibodies,(Santa Cruz Biotechnology, Inc.) overnight. After extensive washings in 0.05% Tween 20 in Tris buffered saline, 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 (Scion Corporation). The densitometry values were normalized with respect to GAPDH values for TCL and M fractions, and with respect to β-tubulin for the N fraction. Data for every sample was the mean of at least two independent experiments.

Autoradiography for 5-HT$_{1A}$ receptor function

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 $MgCl_2$, 100 mM NaCl, 1 mM DTT and 2 mM GDP (pH 7.7), and subsequently incubated for 2 h at 25°C in the same buffer containing 3 mU·mL^{-1} adenosine deaminase and 0.04 nM [^{35}S]guanosine-5-*O*-(3-thio) triphosphate (GTPγS; PerkinElmer Inc.). Consecutive sections were incubated with 10 μM (±)-8-hydroxy-N,N-dipropyl-2-aminotetralin (8-OH-DPAT; Sigma) alone or in the presence of 10 μM WAY100635. Non-specific binding was determined in the presence of 10 μM 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) together with ^{14}C-polymer standards (Amersham) for 2 days at 4°C.

Data analysis

Data were expressed as percentage of values from vehicle-treated rats, with these values set to 100%. Results shown are means ± SEM. Data were analysed 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-HT$_{2A}$ receptor blockade (administration of ketanserin) in the results obtained. Statistical significance was set at $P < 0.05$.

Results

Effect of fluoxetine, ketanserin and their co-treatment on the FST

In the FST the immobility times observed in the different treatment groups after subchronic (7 days administration) are shown in Figure 1A. A two-way ANOVA analysis showed a significant interaction in the immobility time between treatment with fluoxetine and 5-HT$_{2A}$ receptor antagonist [$F(1,35) = 56.23$, $P < 0.001$], and a significant main effect for the fluoxetine [$F(1,35) = 204.9$, $P < 0.001$], while the group treated with ketanserin alone did not show statistical changes [$F(1,35) = 1.37$, $P = 0.25$]. In the swimming time, two-way ANOVA analysis presented a significant interaction between 5-HT$_{2A}$ 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$_{2A}$ blockade [$F(1,35) = 26.70$, $P < 0.001$] (Figure 1B). Regarding climbing time, no significant interaction was observed but there was a significant main effect of fluoxetine treatment [$F(1,35) = 6.74$, $P < 0.05$] (Figure 1C).

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

Effect of fluoxetine, ketanserin and their co-treatment on BDNF and TrkB expression

Subchronic fluoxetine treatment showed a trend towards increased expression of BDNF mRNA compared with the vehicle group in some regions such as the CA3 field (CA3) and the DG of the hippocampus, but this was not significant. After treatment with ketanserin alone, BDNF mRNA expression in the CA3 and DG regions, were also not different from those of the vehicle group. However, co-treatment with fluoxetine + ketanserin for 7 days increased BDNF mRNA expression in several brain areas, including CA3 and DG (Figure 2F,G). A two-way ANOVA revealed a non-significant interaction in the CA3 region of the hippocampus between the antidepressant treatment with fluoxetine and the 5-HT$_{2A}$ receptor antagonist group [$F(1,44) = 0.20$, $P = 0.66$], and the 5-HT$_{2A}$ receptor antagonist alone [$F(1,44) = 0.81$, $P = 0.37$], while the antidepressant effect was significant [$F(1,44) = 13.4$, $P < 0.001$] (Figure 2F). The DG of the hippocampus showed

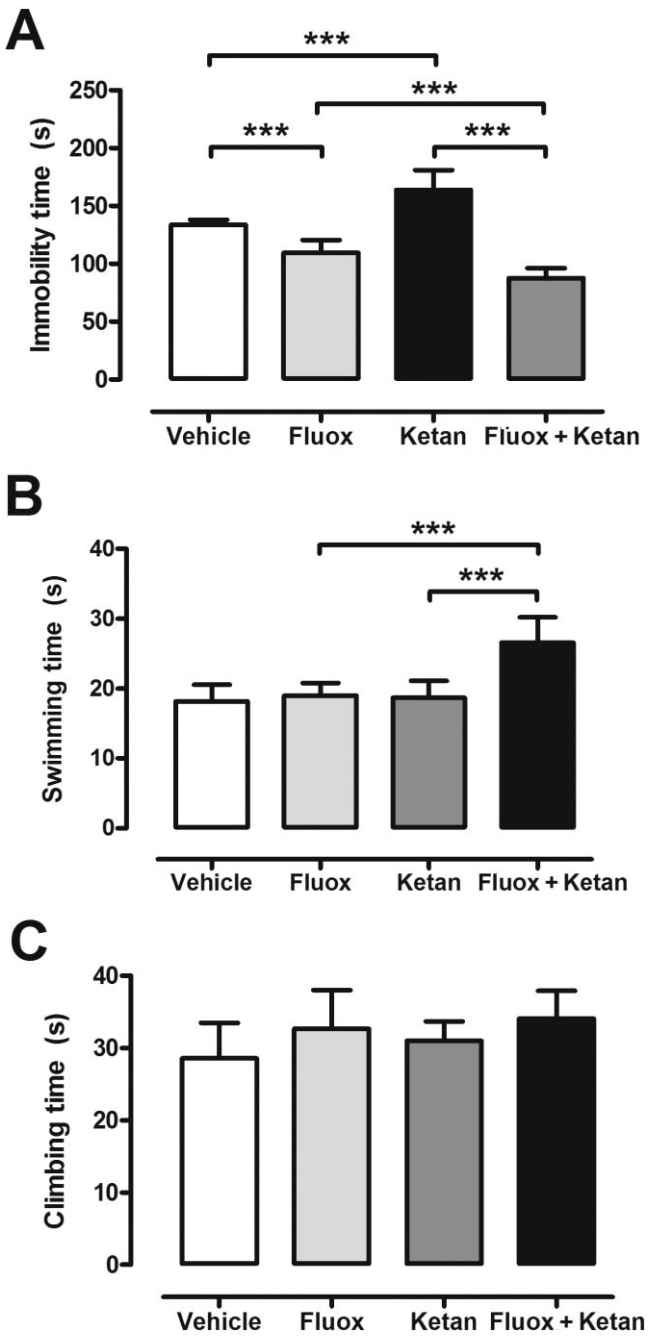

Figure 1

Graphs showing immobility time (A), swimming time (B) and climbing time (C) in the FST for vehicle, fluoxetine (Fluox), ketanserin (Ketan) and fluoxetine + ketanserin (Fluox + Ketan) treatment groups. Data are expressed in seconds (s) (mean ± SEM, n = 8–10). Note that a two-way ANOVA shows significant changes in the immobility time: fluoxetine × ketanserin interaction [$F(1,35) = 56.23$, $P < 0.001$]; and in the swimming time: fluoxetine × ketanserin interaction [$F(1,35) = 17.33$, $P < 0.001$]. ***$P < 0.001$ for the Bonferroni *post hoc* test.

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 (Figure 2G). Other brain areas showing a tendency towards increased in BDNF mRNA expression in the co-treatment group included the amygdala (147 ± 19%) and piriform cortex (151 ± 21%). Areas such as the CA1 field of the hippocampus and medial prefrontal cortex did not show significant changes using the two-way ANOVA 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 combination of both drugs also showed no changes: 100 ± 8%, vehicle; 110 ± 8%, fluoxetine; 102 ± 9%, ketanserin; and 100 ± 4%, co-treatment group.

TrkB receptor expression was also measured by *in situ* hybridization. No modification was found for any of the experimental groups analysed after 7 days of treatment (Supporting Information Figure S1).

Lack of effect of fluoxetine, ketanserin and the co-treatment on BrdU incorporation

The effects of the treatments (fluoxetine, ketanserin and fluoxetine + ketanserin) on the BrdU-positive cells are shown in Figure 3. Statistical analysis of the data using a two-way ANOVA showed no interaction between fluoxetine treatment and 5-HT_{2A} receptor blockade but a significant main effect of ketanserin treatment [$F(1,24) = 34.60$, $P < 0.001$].

Effect of fluoxetine, ketanserin and the co-treatment on β-catenin protein expression

Western blot analysis of the TCL showed no changes in β-catenin protein levels in the fluoxetine alone and ketanserin alone groups, with a significant increase in the fluoxetine + ketanserin group (Figure 4A). The interaction between antidepressant administration and 5-HT_{2A} receptor blockade was statistically significant using a two-way ANOVA [$F(1,44) = 9.14$, $P < 0.01$], and there was a significant main effect of fluoxetine treatment [$F(1,44) = 15.55$, $P < 0.001$] and for 5-HT_{2A} receptor blockade [$F(1,44) = 6.77$, $P < 0.05$]. In the membrane fraction, there were no changes in β-catenin in the fluoxetine and ketanserin groups, while there was a significant increase of β-catenin labelling in the fluoxetine + ketanserin group (Figure 4B,D). Two-way ANOVA 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} receptor antagonist [$F(1,44) = 6.23$, $P < 0.05$]. No significant modifications in β-catenin in the nuclear fraction were observed in any of the experimental groups (Figure 4C,D), using a two-way ANOVA [$F(1,44) = 0.061$, $P = 0.806$] for the interaction between both drugs.

Acute drug administration did not change β-catenin protein levels in hippocampal total homogenate, membrane or nuclear fraction in any of the different treatment groups (data not shown).

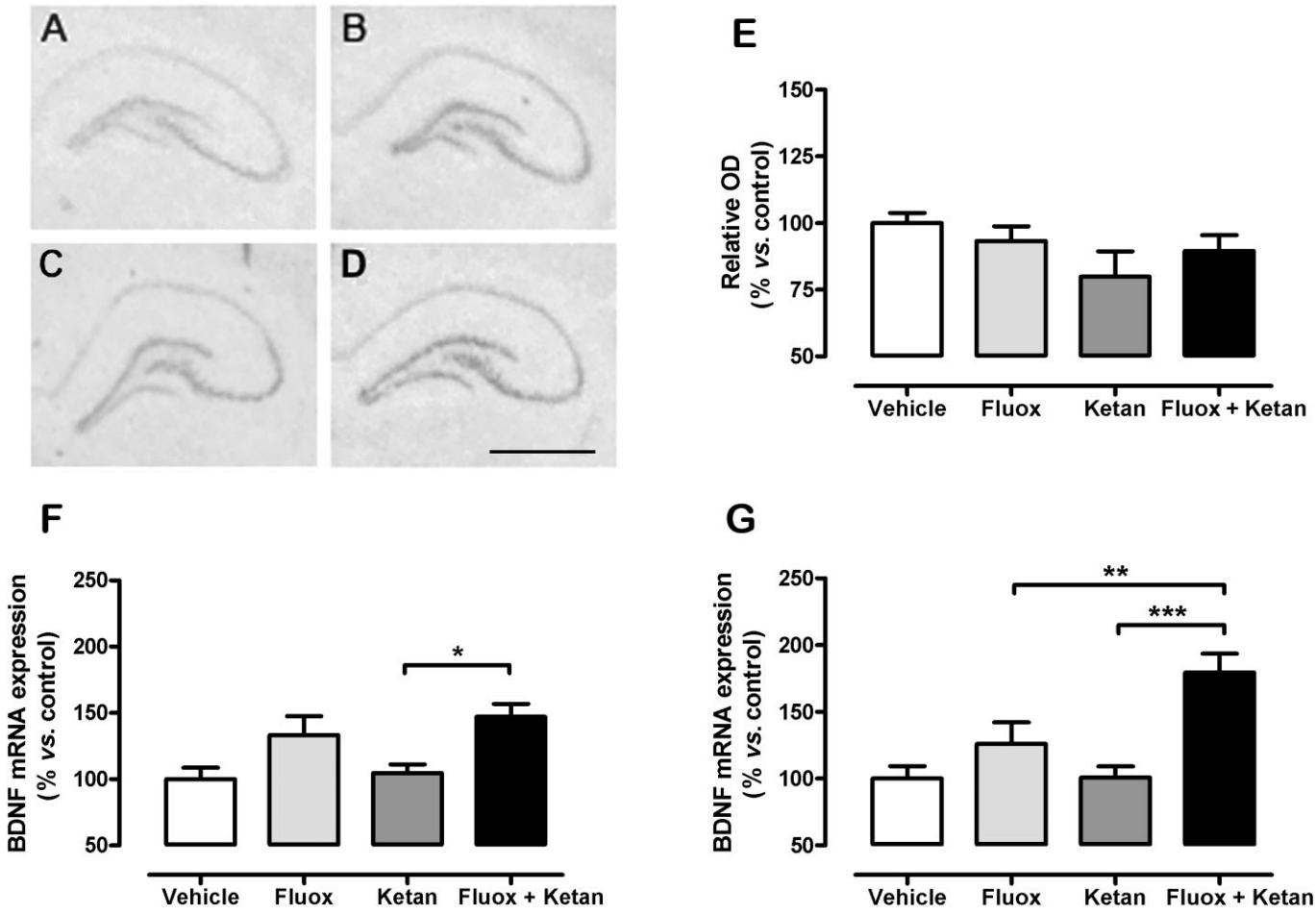

Figure 2

Autoradiograms illustrating BDNF *in situ* hybridization of vehicle (A), fluoxetine (Fluox; B), ketanserin (Ketan; C), and fluoxetine + ketanserin (Fluox + Ketan; D) treatments (upper images). Graphs show BDNF protein expression in hippocampus TCL (E), and mRNA expression in both CA3 subfield (F) and DG (G) of the hippocampus. Data expressed as percentage of vehicle-treated animals (100%). Data are expressed as mean ± SEM; n = 10–12. Two-way ANOVA for BDNF mRNA in DG: fluoxetine × 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. OD, optic density.

The β-catenin immunopositive cells in the different treatment groups (Figure 4E,F) showed an interaction between fluoxetine administration and the 5-HT_{2A} receptor antagonist, [$F(1,24) = 36.28$, $P < 0.001$] and a significant main effect of the fluoxetine treatment [$F(1,24) = 5.59$, $P < 0.05$] using a two-way ANOVA.

Effect of fluoxetine, ketanserin and the co-treatment on N-cadherin protein expression

The analysis by Western blot of the hippocampal cell lysate (TCL) of the different experimental groups revealed a significant increase in N-cadherin protein levels in the co-treatment group over those in the fluoxetine alone and ketanserin alone groups (Bonferroni *post hoc* test). Two-way ANOVA showed significant fluoxetine and ketanserin 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 [$F(1,44) = 6.19$, $P < 0.05$] (Figure 5A). N-cadherin protein levels in the membrane fraction (Figure 5B) were increased both in the fluoxetine and in the co-treatment groups. A significant interaction between antidepressant treatment and 5-HT_{2A} receptor antagonism was observed [$F(1,44) = 4.79$, $P < 0.05$], as was a significant main effect of the fluoxetine treatment [$F(1,44) = 42.64$, $P < 0.001$], following two-way ANOVA (Figure 5B). The analysis of N-cadherin protein levels in the nuclear fraction (Figure 5C) 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.

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

Lack of effect of fluoxetine, ketanserin and the co-treatment on 5-HT_{1A} receptor function

Basal [^{35}S]GTPγS binding and 8-OH-DPAT-mediated [^{35}S]GTPγS stimulation were not significantly modified in the different

brain structures studied in fluoxetine, ketanserin and fluoxetine + ketanserin treatment groups (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). Recent studies have reported a potentiation of the antidepressant effects by co-treatment with drugs with a 5-HT_{2A} receptor antagonist profile (Shelton *et al.*, 2001; Marek *et al.*, 2003; Celada *et al.*, 2004; Pandey *et al.*, 2010). The aim of this study was to evaluate the effect of subchronic co-treatment with the SSRI fluoxetine and the 5-HT_{2A} antagonist ketanserin on hippocampal neurogenic markers, along with behavioural findings. The dose of ketanserin used (0.1 $mg \cdot kg^{-1} \cdot day^{-1}$) 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, co-treatment with fluoxetine and ketanserin significantly increased those neurogenic markers, inducing an antidepressant-like response in the FST.

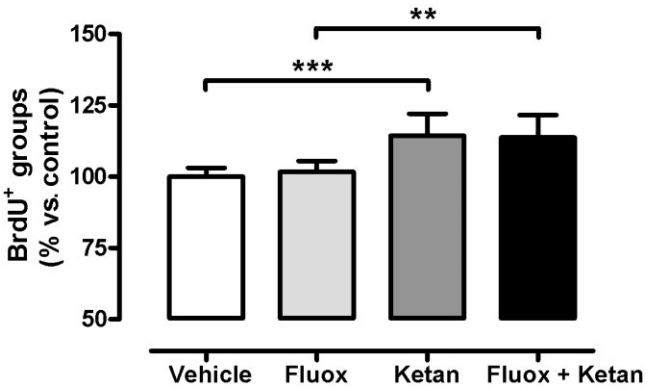

Figure 3

Graph showing BrdU immunolabelling ($BrdU^+$) in the SGZ of the DG of the hippocampus for vehicle, fluoxetine (Fluox), ketanserin (Ketan), and fluoxetine + ketanserin (Fluox + Ketan) treatment groups. Data are presented as percentage of vehicle group (100%). Data are expressed as mean ± SEM, $n = 7$. $^{**}P < 0.01$, $^{***}P < 0.001$ using a two-way ANOVA analysis followed by a Bonferroni *post hoc* test.

To date, some reports indicate that the combination of 5-HT_{2A} receptor 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} receptor antagonists decreases immobility and increases the swimming behaviour in the 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 co-treatment 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 shows statistical significance for the interaction of the fluoxetine treatment and 5-HT_{2A} receptor blockade, which reflects an involvement of the 5-hydroxytryptaminergic system in this antidepressant-like response (Cryan *et al.*, 2005a,b). In addition, the lack of changes in climbing time excludes a possible role of noradrenergic/dopaminergic mechanisms (Cryan *et al.*, 2005b). Acute administration of the different drugs alone or combined did not produce any change compared with vehicle. In this regard, our data are in accordance with others' in which low doses of fluoxetine (8–20 $mg \cdot kg^{-1}$) (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) did not produce any significant change in immobility time in the FST.

Furthermore, we have not found any change associated to the function of the 5-HT_{1A} receptor subtype, although a significant reduction of the basal values was observed in the basal values in the dorsal raphe nucleus (DRN) between

Table 1

Effect of 7 day treatment with fluoxetine, ketanserin or fluoxetine + ketanserin on basal [^{35}S]GTPγS binding and (±)-8-OH-DPAT-stimulated [^{35}S]GTPγS binding in some rat brain areas, reflecting 5-HT_{1A} function

	Vehicle	Fluoxetine	Ketanserin	Fluox + ketan
CA1	126 ± 6	122 ± 4	135 ± 8	138 ± 6
CA3	122 ± 8	124 ± 6	132 ± 7	137 ± 6
DG	173 ± 15	170 ± 5	192 ± 13	183 ± 13
DRN	154 ± 8	177 ± 8	168 ± 11	176 ± 11

Values are presented as percentage of basal values (100%) (mean ± SEM) for 8-OH-DPAT-stimulated [^{35}S]GTPγS binding. Two-way ANOVA showed no statistical differences.
CA1, CA1 field of hippocampus; CA3, CA3 field of hippocampus; DG, dentate gyrus of hippocampus; DRN, dorsal raphe nucleus.

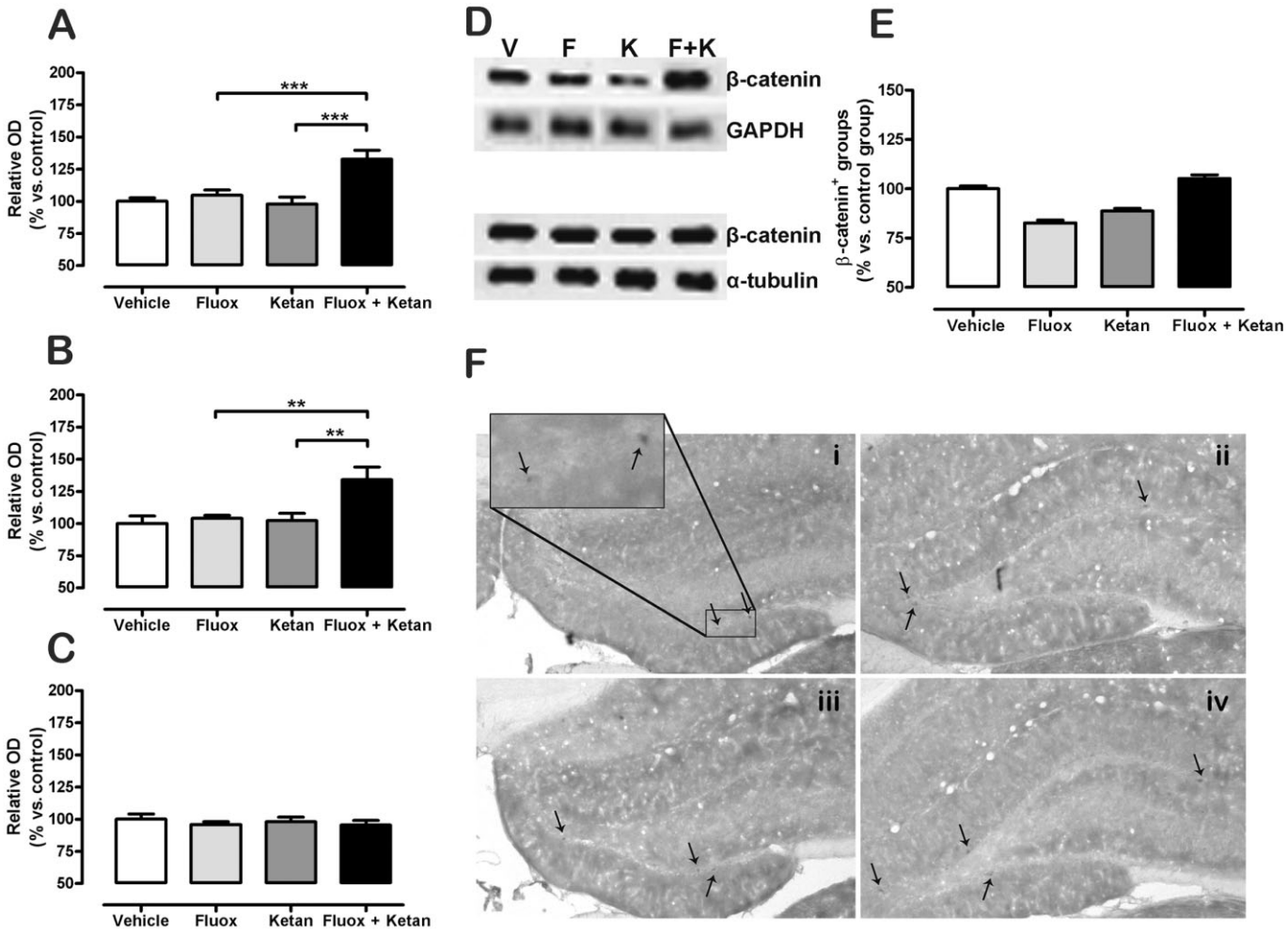

Figure 4

Effect of fluoxetine (Fluox), ketanserin (Ketan), and fluoxetine + ketanserin (Fluox + Ketan) treatments on β-catenin level in rat hippocampus TCL (A), membrane fraction (B) and nuclear fraction (C). Representative Western blots of β-catenin protein expression in membrane and nuclear fraction from hippocampus [vehicle (V), fluoxetine (F), ketanserin (K) and fluoxetine + ketanserin (F + K)] (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 optical density (OD) of the control, 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 ± SEM Two-way ANOVA for the antidepressant fluoxetine × ketanserin interaction in TCL [$F(1,44) = 9.14$, $P < 0.01$], 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.

the fluoxetine and the co-treatment groups, suggesting that the duration of the treatment (7 days) is not enough to desensitize 5-HT_{1A} receptors in the DRN, as occurs with chronic (14 days) treatments (Castro *et al.*, 2003). Thus, an increased 5-hydroxytryptaminergic 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 (see Celada *et al.*, 2004), or other early neuroplastic changes, as discussed later.

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 the hippocampus of some animal models of depression (Elfving *et al.*, 2009). The expression of this protein is 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 in BDNF mRNA expression in the hippocampus following subchronic treatment with fluoxetine or ketanserin alone, in agreement with previous work (Larsen *et al.*, 2007). However, after subchronic co-treatment with fluoxetine and ketanserin, we found an increased expression of BDNF mRNA in CA3, reaching statistical significance in the DG, an area clearly related to the pathogenesis of depression (see Lucassen *et al.*, 2006). This increase in BDNF mRNA expression was not accompanied by an increase in the expression of the protein. At least 3

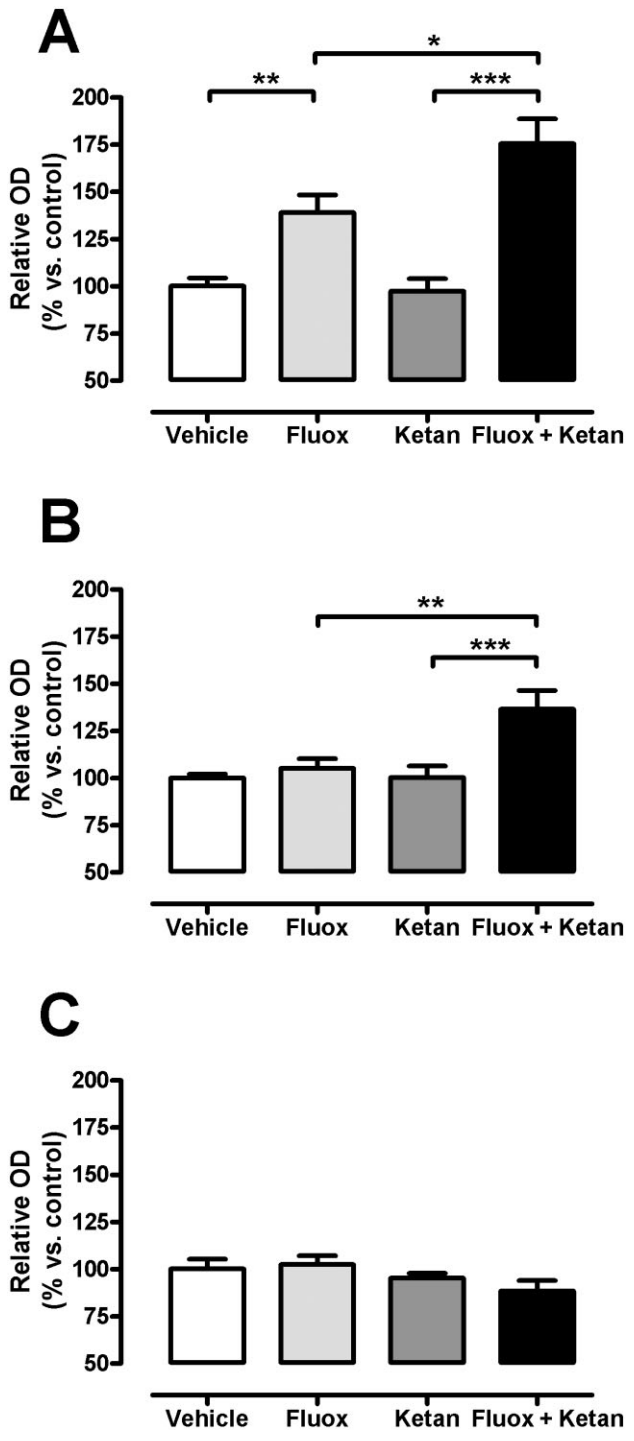

Figure 5

Parallel effect of fluoxetine (Fluox), ketanserin (Ketan), and fluoxetine + ketanserin (Fluox + Ketan) 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 optical density (OD) in the vehicle-treated animals (100%). Data are presented as mean ± SEM, n = 10–12. Two-way ANOVA analysis for fluoxetine × 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.

weeks are required to show a significant increase in the hippocampus (De Foubert *et al.*, 2004).

A relationship between 5-HT_{2A} receptors and BDNF has been suggested by studies where activation of 5-HT_{2A} receptors decreased hippocampal BDNF (Vaidya *et al.*, 1999). Furthermore, studies in mice with genetically low levels of BDNF ($BDNF^{+/-}$), which present a depression-like profile, show higher hippocampal 5-HT_{2A} receptor levels, which are down-regulated 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 our data on BDNF mRNA expression, TrkB mRNA expression was not modified after the 7 day treatment with fluoxetine, ketanserin or the co-treatment. An increase of TrkB mRNA expression after chronic antidepressant treatment (Nibuya *et al.*, 1995) has been previously reported, although 21 days of treatment appear to be necessary before an up-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 pathway, such as the p75 neurotrophin receptor (Rantamäki *et al.*, 2007).

The 5-HT_{2A} 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 down-regulation of BDNF expression (Zafra *et al.*, 1991). Furthermore, specific 5-HT_{2A} receptor antagonists facilitate the induction of long-term potentiation in the 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-HT_{2A} receptor blockade, inhibiting GABAergic interneurons and producing an increase in the activation of pyramidal neurons in the 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 co-treatment with fluoxetine and ketanserin was comparable with that reported following electroconvulsive seizures (Madsen *et al.*, 2003) and chronic antidepressant treatment (Mostany *et al.*, 2008), not observing any changes after acute administration. However, this increase in β-catenin was associated with the membrane fraction, not the nuclear fraction, 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 (SGZ) of the hippocampus where those immunopositive cells are co-localized 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 we observed in N-cadherin protein attached to the membrane fraction as a result of the co-treatment with the antidepressant fluoxetine and the 5-HT_{2A} receptor 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 (see 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 was 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 (see 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 co-treatment with 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 DG 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). The effects of 5-HT_{2A} receptor antagonists, given alone, depend on the duration of the treatment as a reduction in proliferation after an acute treatment (Jha *et al.*, 2008) and an increase after 7 days administration (Jha *et al.*, 2008) have both been reported.

In summary, the subchronic (1 week) co-treatment with an SSRI and a 5-HT_{2A} antagonist was sufficient to increase some neuroplastic markers previously associated with chronic (2–3 weeks) antidepressant treatment, such as BDNF and β-catenin. In addition, this treatment did not induce the well characterized changes in monoaminergic neurotransmission following chronic antidepressants, such as 5-HT_{1A} receptor desensitization in the DRN. However, this combined treatment was 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 5-hydroxytryptaminergic 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 pharmacological blockade of this receptor subtype as a promising target for the treatment of depression.

Acknowledgements

We wish to thank Drs Jesús Pascual-Brazo, Alvaro Díaz, Elena Castro and Elsa Valdizán for their scientific advice, and Ms Lourdes Lanza, Rebeca Madureira, Isabel Ruiz, Alicia Martin and Helena Blanco for their technical assistance. This work has been supported by Instituto de Salud Carlos III (Red Investigación de Enfermedades Mentales, REMTAP), Ministerio de Ciencia e Innovación SAF07-61862 and Fundación Alicia Koplowitz.

Conflicts of interest

The authors declare that AP has received support for research from Faes Farma, SA, 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 collaborative relationships.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Representative autoradiograms of TrkB *in situ* hybridization in vehicle (A), fluoxetine (B), ketanserin (C) and fluoxetine + ketanserine (D) treatment groups. Graphs showing TrkB mRNA expression in the different groups studied in two representative regions, as CA3 subfield (E) and dentate gyrus (DG) (F) of the hippocampus. TrkB expression is represented as percentage versus vehicle TrkB mRNA expression for each region. Data expressed as percentage of vehicle group. Data are expressed as mean ± SEM; n = 10–12. Two-way ANOVA followed by Bonferroni *post hoc* test showed no interaction between fluoxetine treatment and 5-HT_{2A} receptor blockade. Bar: 2 mm.

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