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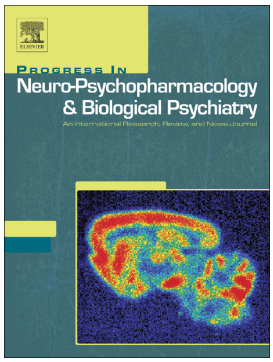

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SHORT TITLE: Behavioral effects of S 47445 in olfactory bulbectomized mice

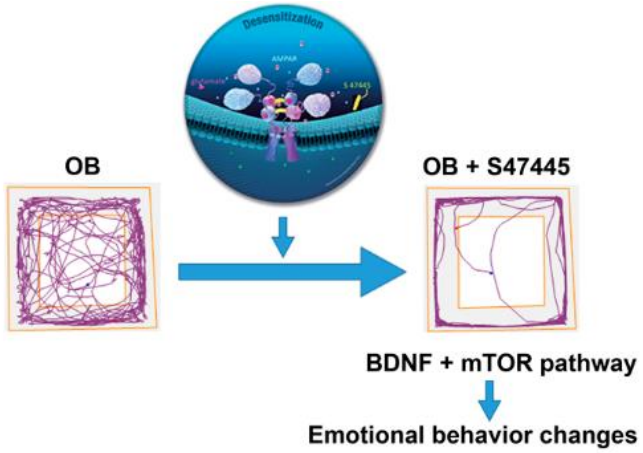
ABSTRACT

S 47445 is a positive allosteric modulator of glutamate AMPA-type receptors that possesses procognitive, neurotrophic and enhancing synaptic plasticity properties. Its chronic administration promotes antidepressant- and anxiolytic-like effects in different rodent models of depression.

We have evaluated the behavioral effects of S 47445 in the bilateral olfactory bulbectomy mice model (OB) and the adaptive changes in those proteins associated to brain neuroplasticity (BDNF and mTOR pathway). Following OB surgery, adult C57BL/6J male mice were chronically administered S 47445 (1, 3 and 10 mg/kg/day; i.p.) and fluoxetine (18 mg/kg/day; i.p.), and then behaviorally tested in the open field test. Afterwards, the expression levels of BDNF, mTOR, phospho-mTOR, 4EBP1 and phospho-4EBP1 were evaluated in hippocampus and prefrontal cortex.

Both drugs reduced the OB-induced locomotor activity, a predictive outcome of antidepressant efficacy, with a similar temporal pattern of action. S 47445, but not fluoxetine, showed an anxiolytic effect as reflected by an increased central activity. Chronic administration of S 47445 reversed OB-induced changes in BDNF and phopho-mTOR expression in hippocampus but not in prefrontal cortex.

The chronic administration of S 47445 induced antidepressant- and anxiolytic-like effects at low-medium doses (1 and 3 mg/kg/day, i.p.) associated with the reversal of OB-induced changes in hippocampal BDNF and mTOR signaling pathways.



HIGHLIGHTS

- S 47445 is an AMPA positive allosteric modulator.
- S 47445 has antidepressant-like effects in olfactory bulbectomized mice.
- S 47445 has anxiolytic-like effects in the olfactory bulbectomized mice.
- S 47445 modulates BDNF and mTOR in prefrontal cortex of bulbectomized mice.

KEYWORDS

AMPA positive allosteric modulator; depression; neuroplasticity; BDNF; mTOR pathway.

ABBREVIATIONS

Olfactory bulbectomy (OB), open field test (OF), fluoxetine (flx), S 47445 (S), vehicle (veh), AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid), AMPA-PAM (AMPA-Positive Allosteric Modulator), BDNF (brain derived neurotrophic factor), CUMS (chronic unpredictable mild stress), CMS (chronic mild stress), PMSF (phenylmethylsulfonyl fluoride).

1. INTRODUCTION

Major depression is a disorder with a high prevalence, and severe health and socioeconomic consequences (Kessler et al., 2003). Although a great effort has been done to unravel the molecular basis of the disease to develop more effective treatments, the currently prescribed antidepressant drugs present important limitations as the low rates of treatment response (Trivedi et al., 2006).

In the last years, new therapeutic strategies targeting BDNF and/or mTOR signaling pathways have been proposed since dysfunction of these neuroplasticity pathways have been linked to various psychiatric phenotypes, and clinically used antidepressants may require such signaling pathways to exert their therapeutic actions (Autry and Monteggia, 2012; Pilar-Cuéllar et al., 2013; Abelaira et al., 2014). Recently, these neuroplasticity-related actions have been also associated with the antidepressant effect of non-serotonergic compounds such as ketamine, a compound described as NR2B non-competitive NMDA antagonist that has demonstrated to induce effective and rapid effects at sub-anesthetic doses in clinical trials and in some rodent models (Zarate et al., 2006; Sanacora et al., 2008). Interestingly, BDNF release and activation of the mTOR signaling pathways induced by ketamine seems to be dependent on glutamate-AMPA receptor activation notably by involving the metabolite, (2R,6R)-hydroxynorketamine, which seems to act in a sustainable manner on AMPA receptors (Autry et al., 2001; Li et al., 2010; Lepack et al., 2014; Zanos et al., 2016).

Positive AMPA receptor modulators (AMPA-PAMs) have the ability to enhance AMPA receptor function without direct receptor activation (Arai and Kessler, 2007). AMPA receptor potentiating drugs are reported to increase LTP, learning and memory, and BDNF (Bliss and Collingridge, 1993; Arai et al., 2000; Arai and Kessler, 2007). Besides their procognitive effects, AMPA-PAMs have been also described as potential treatments for mood disorders, as

some of them act in a synergistic action with antidepressant drugs (Bai et al., 2003; Alt et al., 2005; O'Neill and Witkin, 2007; Damgaard et al., 2010). S 47445 (8-cyclopropyl-3-[2-(3-fluorophenyl)ethyl]-7,8-dihydro-3H-[1,3]oxazino[6,5-g][1,2,3] benzotriazine-4, 9-dione) is a selective AMPA-PAM (Bretin et al., 2017; Giralt et al., 2017) that has demonstrated an antidepressant-like effect in the corticosterone mice model and in chronic mild stress (CMS) rat model, a fast-acting effect on anhedonia, as well as an anxiolytic profile in different behavioral tests (Mendez-David et al., 2017).

Among the animal models that resemble a depression-like state with comorbid anxiety, the bilateral olfactory bulbectomy (OB), a recognized animal model of agitated hyposerotoninergic depression (Lumia et al., 1992; Wang et al., 2007), has been widely used for preclinical research in rodents. This model presents similarities of some of the behavioral, neurochemical, neuroendocrine and immunological changes, with those observed in depressive patients (Kelly et al., 1997; Song and Leonard, 2005). Olfactory bulbectomy leads to a typical hyperactive phenotype, increased exploratory behavior and also memory and learning deficits (Tadano et al., 2004; Zueger et al., 2005) that can be measured using some simple behavioral tests, thus allowing the characterization of the emotional and cognitive state of the animals. The chronic, but not acute, administration of clinically effective antidepressant drugs in OB animals reverse most of the behavioral, neurochemical and structural disturbances exhibited by OB animals (Song and Leonard, 2005; Jarosik et al., 2007; Sato et al., 2008), therefore representing an interesting tool for pharmacological research (Linge et al., 2016). Indeed, the reversal of OB-induced locomotor hyperactivity is considered a behavioral outcome for assessing antidepressant efficacy (Kelly et al., 1997; Song and Leonard, 2005).

Here we present the evaluation of the effect of the AMPA-PAM S 47445 in the olfactory bulbectomy model of depression in mice in comparison to a current marketed drug,

fluoxetine. In addition, we have evaluated the implication of the neurotrophic factor BDNF, and the mTOR pathway.

2. METHODS

2.1. Animals and OB surgery

Animals used were 2-3 month old male C57BL/6J mice weighing 25–30 g. All procedures were carried out with the previous approval of the Animal Care Committee of the University of Cantabria and according to the Spanish legislation (RD 53/2013) and the European Communities Council Directive (2010/63/UE) on "Protection of Animals Used in Experimental and Other Scientific Purposes. Animals were individually housed, in climate controlled rooms with 12 h light–12 h dark cycle, and provided with food and water *ad libitum*.

The OB and sham-operation (sham) procedure was performed as previously described (Linge et al., 2013; 2016). Mice were anesthetized with isoflurane (2%; Schering Plough, United Kingdom) to perform the bilateral olfactory bulbectomy. In brief, a midline sagital incision was made in the skin overlying the skull, and a burr hole was drilled through which both olfactory bulbs were bilaterally aspired using a suction pump. Finally, the hole was filled with bone wax to avoid bleeding. After bulbectomy/sham surgery, a four week period was waited for the animal recovery and the development of the OB syndrome before the initiation of drug treatment was confirmed by peripheral hyperactivity in the open field test. At the end of the study, animals were sacrificed and the lesions were verified to discard frontal pole lesions and/or incomplete removal of olfactory bulbs. Sham operations were done in the same way, although the bulbs were left intact.

2.2. Drugs and treatments

S 47445 (batch L 0041063) provided by Servier (Bretin et al., 2017) was administered at 1, 3 or 10 mg/kg/day i.p. for 4 weeks. The vehicle of S 47445 used was (HEC 1% and Tween 1%, i.p.). Fluoxetine (18mg/kg/day) i.p. for 4 weeks, using saline as vehicle. Both treatments started 1 month after OB surgery. Drugs were administered at 12 a. m. Vehicle group corresponded to the vehicle of S 47445 since no difference was previously observed between HEC 1% and Tween 1%, i.p. and saline (Mendez-David et al., 2017).

2.3. Behavioral testing: open field test (OF)

We evaluated the drug (S 47445 or fluoxetine) effects on the OB-induced hyperactivity (to assess antidepressant-like effects) and central ambulation (to assess anxiolytic-like effects) (Linge et al., 2016). The open field test was performed during the light phase (between 8 and 11 a. m.). Animals were transported to the experimental room 1 h before the start of the experiment to acclimatize, and 24 h after drug administration. The open field apparatus was a brightly lit (350 lx) white wooden box (50 cm × 50 cm × 30 cm) with white floor and bright walls. Mice were placed in the center of the apparatus for 5 min, and behavior was video-tracked using a computerized system (Any-maze Video-Tracking software, Stoelting Co., U.S.A.). Total, peripheral and central distances travelled were measured. The total and peripheral distance and the percentage of distance travelled in the center (30 cm × 30 cm) were used to evaluate drugs effects.

2.4. Western blot

Animals were killed by decapitation, their brains removed, and the hippocampi and prefrontal cortices dissected and stored at −80 ºC. Each sample was homogenized and processed in order to obtain the total cell lysate as described by Mostany et al. (2008). Each 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 (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 lysated in lysis buffer (homogenization buffer containing 1% Igepal, 0.1% sodium deoxycholate, 0.2% SDS and 0.1% Triton X-100) 30 min on ice and centrifuged at 14000 xg 10 min at 4ºC. The supernatant was aliquoted and conserved at -20ºC. Protein quantification was performed according to the Lowry method.

Protein preparations (45 µg per lane) were resolved on 8.5% or 15% SDS-PAGE gel electrophoresis and transferred to polyvinylidene difluoride (PVDF; non-phosphorylated proteins) or to nitrocellulose (phosphorylated proteins) membranes. Non-specific binding sites were blocked with 5% non-fat dry milk in TBS-T buffer (20 mM Tris, pH 7.2, 150 mM NaCl, and 0.05% Tween) for 60 minutes at room temperature. Membranes for determination of phosphorylated proteins were blocked in 3% non-fat dry milk in TBS-T buffer plus 1 mM sodium vanadate and 1 mM sodium fluoride. These membranes were incubated in rabbit anti-BDNF (1:500) from Santa Cruz Biotechnology, Inc. (USA); rabbit anti-mTOR (1:1000) and mouse anti-β-tubulin (1:20000) from Sigma-Aldrich (Spain); and rabbit anti-phospho-mTOR (1:500), rabbit anti-4EBP1 (1:500), rabbit anti-phospho-4EBP1 (1:500) from Cell Signaling Technology, Inc. (USA), overnight. After extensive washings in TBS-T (TBS, 0.05% Tween 20) membranes were incubated with horseradish peroxidase conjugated secondary antibodies. Specific signal was detected using ECL Advance kit (GE Healthcare Europe GmbH, Munich, Germany). Blots quantitation was performed by densitometric analysis using Scion Image Software. The densitometry values were normalized with respect to β-tubulin. Those values were used to calculate p-mTOR/mTOR and p-4EBP1/4EBP1 ratios.

2.5. Statistical analysis

All the values are expressed as mean ± standard error of mean (S.E.M). The data were statistically analyzed by a Student´s t-test or one-way ANOVA followed by a Student-Newman-Keuls posthoc test where appropriate. GraphPad Prism 5.01 (San Diego, CA, USA) was used for the statistical analysis. A p value < 0.05 was considered significant.

3. RESULTS AND STATISTICAL ANALYSES

3.1. Chronic S 47445 reverted OB-induced hyperactivity and anxiety

In this study, we have used the OB model of depression in C57BL/6J mice. OB-induced syndrome was confirmed 28 days post-surgery, and before the initiation of the chronic treatment with S 47445 (1, 3 and 10 mg/kg/day) and fluoxetine (18 mg/kg/day) (Figure 1).

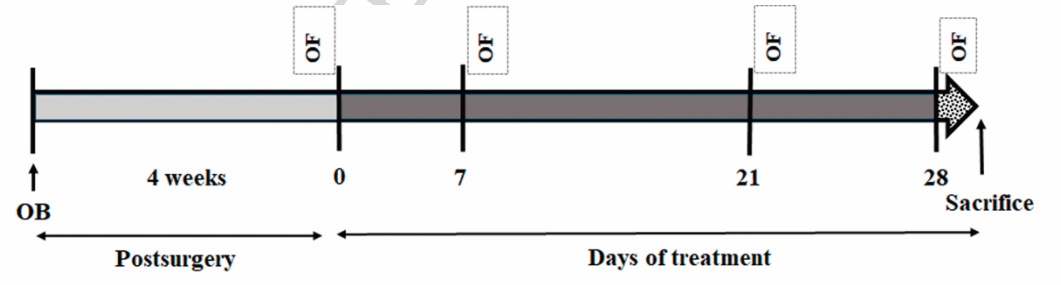


Figure 1. Experimental design. Time-line schedule for the olfactory bulbectomy and the behavioral testing. OB: olfactory bulbectomy; OF: open field test.

The typical locomotor hyperactivity of OB mice was evidenced by an increased total distance travelled (+105 % *vs* sham group, t=7.663, df=63, $p < 0.001$, Figure 2A, t=0), and an increase in the peripheral distance travelled (+132 % *vs* sham group, t=8.522, df=63, $p < 0.001$, Figure

2B, t=0). The OB-induced enhanced anxiety behavior was evidenced by a reduced central activity (-52 % *vs* sham group, t=6.906, df=63 $p < 0.001$, Figure 2C, t=0). The different groups of OB mice used for the drug treatments showed a similar OB-induced behavioral phenotype.

The behavioral effects of the chronic treatment with S 47445 (1, 3 and 10 mg/kg/day doses) and fluoxetine (18 mg/kg/day) were evaluated following 7, 21 and 28 days of administration. Regarding the total locomotor activity, a one-way ANOVA revealed an effect of treatment at 21-day ($F_{(4,46)}$=3,256, p = 0,020) and 28-day ($F_{(4,45)}$=4,658, p = 0,003) time points. *Post-hoc* analysis showed that treatment with S 47445 significantly reverted OB-induced hyperactivity after 28 days of treatment (1 mg/kg/day: -25 % *vs* OB-veh, $p < 0.05$; 3 mg/kg/day: -30 % *vs* OB-veh, $p < 0.05$). Chronic fluoxetine at the 18 mg/kg/day dose reduced total activity in OB mice (21-days: -26 % *vs* OB-veh, $p < 0.01$; 28-days: -34 % *vs* OB-veh, $p < 0.01$) (Figure 2A).

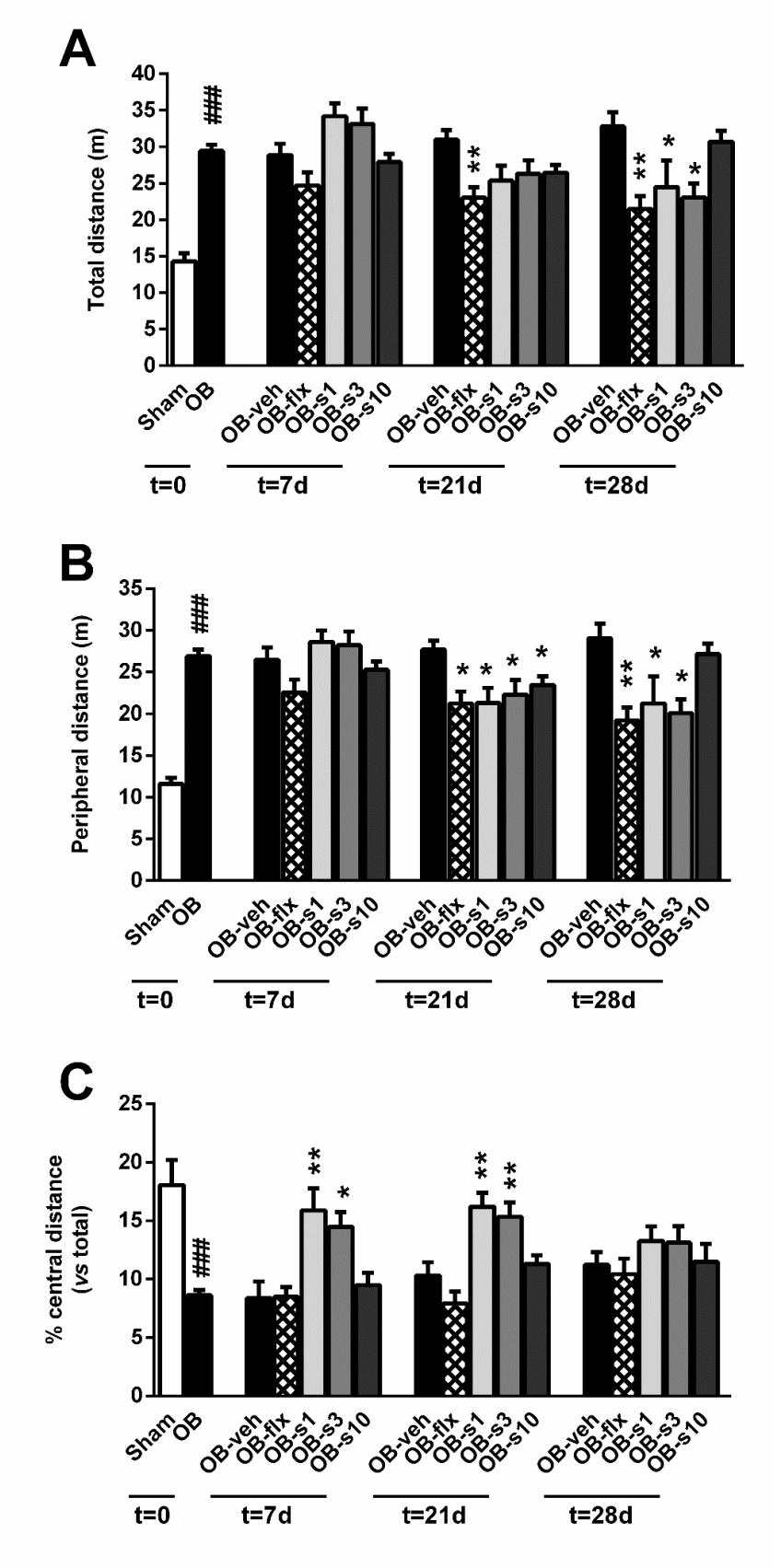


Figure 2. Effect of chronic administration of S 47445 and fluoxetine in OB mice in the open field test at different time points. Total distance travelled (A), peripheral deambulation (B) and percentage of central distance *versus* total (C). OB: olfactory bulbectomy mice; OB-flx corresponds to the OB mice treated with fluoxetine at the dose of 18 mg/kg/day, i.p.; OB-s1, s3 and s10 correspond to the OB mice treated with S 47445 at the doses of 1, 3 and 10mg/kg, i.p. respectively. Data are mean ± SEM. $^{###}$ $p < 0.001$ *vs* sham group (Student's t-test). * $p < 0.05$ and ** $p < 0.01$ *vs* OB-veh group (one-way ANOVA followed by Newman-Keuls post hoc test). n=10-11 animals per group.

The analysis of the peripheral distance travelled using a one-way ANOVA revealed an effect of treatment following 21 days ($F_{(4,46)}=3.369$, $p = 0.017$) and 28 days ($F_{(4,45)}=4.888$, $p = 0.002$) of administration. *Post-hoc* analysis showed that treatment with S 47445 significantly reverted OB-induced peripheral activity following 21 days (1 mg/kg/day: -23 % *vs* OB-veh, $p < 0.05$; 3 mg/kg/day: -19 % *vs* OB-veh, $p < 0.05$; 10 mg/kg/day: -15 % *vs* OB-veh, $p < 0.05$), and 28 days (1 mg/kg/day: -27 % *vs* OB-veh, $p < 0.05$; 3 mg/kg/day:-31 % *vs* OB-veh, $p < 0.05$) of administration. Chronic fluoxetine at the 18 mg/kg/day dose reduced peripheral activity in OB mice (21-days: -23 % *vs* OB-veh, $p < 0.05$; 28-days: -34 % *vs* OB-veh, $p < 0.01$) with a similar magnitude and temporal fashion than S 47445 (Figure 2B).

Regarding central activity, one-way analysis revealed a significant effect after 7-days ($F_{(4,49)}=7.031$, $p < 0.001$) and 21-days ($F_{(4,46)}=10.55$, $p < 0.001$) of treatment. *Post-hoc* analysis showed that treatment with S 47445 significantly increased open field central activity in OB mice following 7 days (1 mg/kg/day: +90 % *vs* OB-veh, $p < 0.01$; 3 mg/kg/day: +73 % *vs* OB-veh, $p < 0.05$) and 21 days (1 mg/kg/day: +57 % *vs* OB-veh, $p < 0.01$; 3 mg/kg/day:

+49 % *vs* OB-veh, $p < 0.01$) of treatment. However, fluoxetine treatment was devoid of any effect at all the time-points studied (Figure 2C).

3.2. BDNF in OB-mice: effect of S 47445 versus fluoxetine

The levels of mature BDNF, corresponding to the active form of the protein, were measured in the hippocampal and prefrontal cortex lysates from OB mice, and the effect of 4-week treatment with one active dose of S 47445 showing behavioral efficacy (3 mg/kg/day) and in comparison to fluoxetine (18 mg/kg/day) was evaluated. The olfactory bulbectomy tended to increase BDNF protein expression (+57 % *vs* sham-veh, t=1.349, df=18, $p = 0.19$, Student´s t test) in the hippocampus. A significant effect of drug treatment on BDNF protein expression was detected in this area in OB mice ($F_{(2,23)} = 4.395$, $p = 0.024$, one-way ANOVA). Chronic S 47445 significantly decreased hippocampal BDNF protein expression (-78 % *vs* OB-veh, $p < 0.05$) whereas chronic fluoxetine did not induce any significant change (- 1.4 % *vs* OB-veh) (Figure 3A).

The olfactory bulbectomy model did not induced any modification of the BDNF protein expression in prefrontal cortex. Drug treatments in this model did not present significant effect on BDNF protein expression, although a clear tendency was observed ($F_{(2,17)} = 2.862$, $p = 0.085$, one-way ANOVA) (Figure 3B).

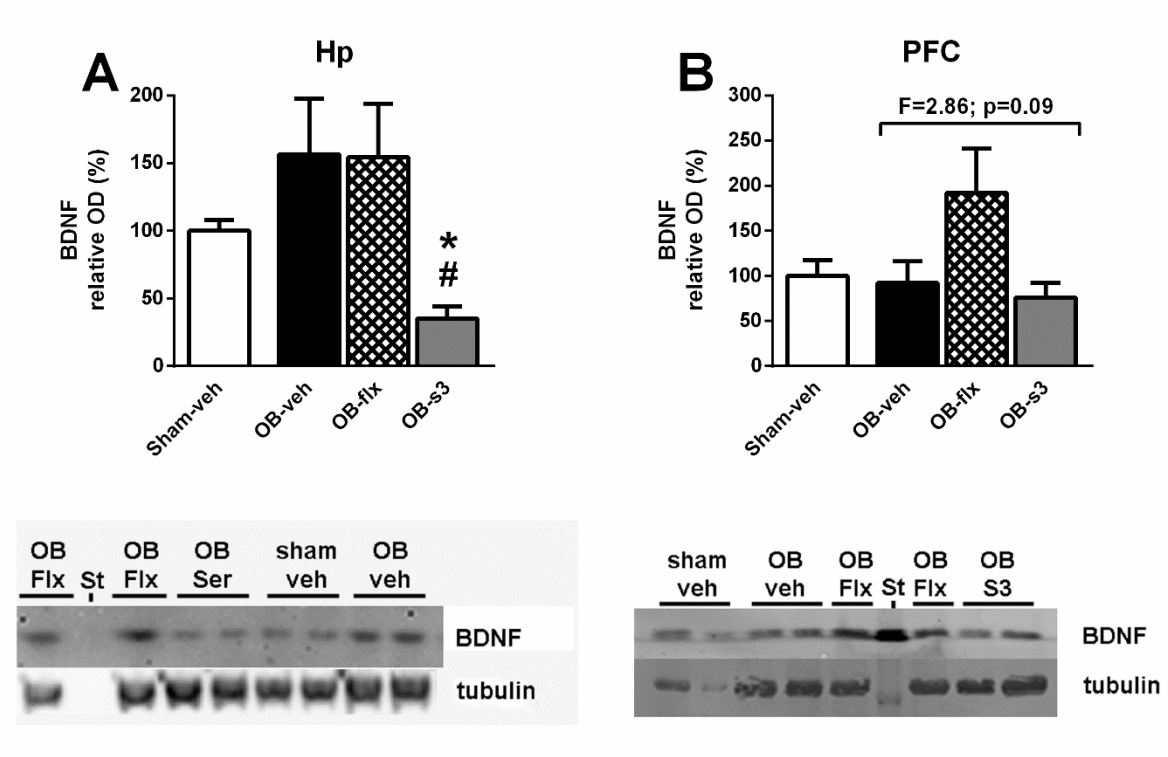


Figure 3. Expression of BDNF protein in hippocampus (A) and prefrontal cortex (B) from OB mice and the effect of chronic treatment (4 weeks) with S 47445 (3 mg/kg/day, i.p.) in comparison with fluoxetine (18 mg/kg/day, i.p.). Values represent mean ± SEM. Sham-veh, sham-operated mice treated with vehicle; OB-veh, OB-vehicle-treated; OB-flx, OB-fluoxetine-treated; OB-s3, OB mice treated with S 47445 at 3 mg/kg/day. * $p < 0.05$ *vs* OB-vehicle, and # $p < 0.05$ *vs* OB-fluoxetine; one-way ANOVA followed by Newman-Keuls post hoc test. n=7-10 animals per experimental group in duplicate for each sample.

3.3. mTOR signaling in OB mice: effect of S 47445 versus fluoxetine

We have evaluated the mTOR complex 1 signaling, since it has been described that ketamine antidepressant-like effects are dependent on the activation of this pathway (Li et al., 2010). Levels of mTOR signaling pathway proteins (mTOR/p-mTOR and 4RBP1/p-4EBP1) were measured in the hippocampal lysates from OB mice, and the effect of 4-week treatment with

S 47445 (3 mg/kg/day) versus fluoxetine (18 mg/kg/day) was evaluated. We did not detect significant changes in the expression of mTOR in OB-vehicle treated mice in comparison with sham-vehicle treated counterparts (-13 % in OB-veh vs sham-veh; t=0.920, df=13, p = 0.37, Student´s t test). Moreover, drug treatments did not modify the amount of mTOR protein in the hippocampus of OB mice (-1.3 % and +4.5% *vs* OB-veh for OB-flx and OB-s3, respectively; $F_{(2,17)}$ = 0.100, p=0.905, one-way ANOVA) (Figure 4A).

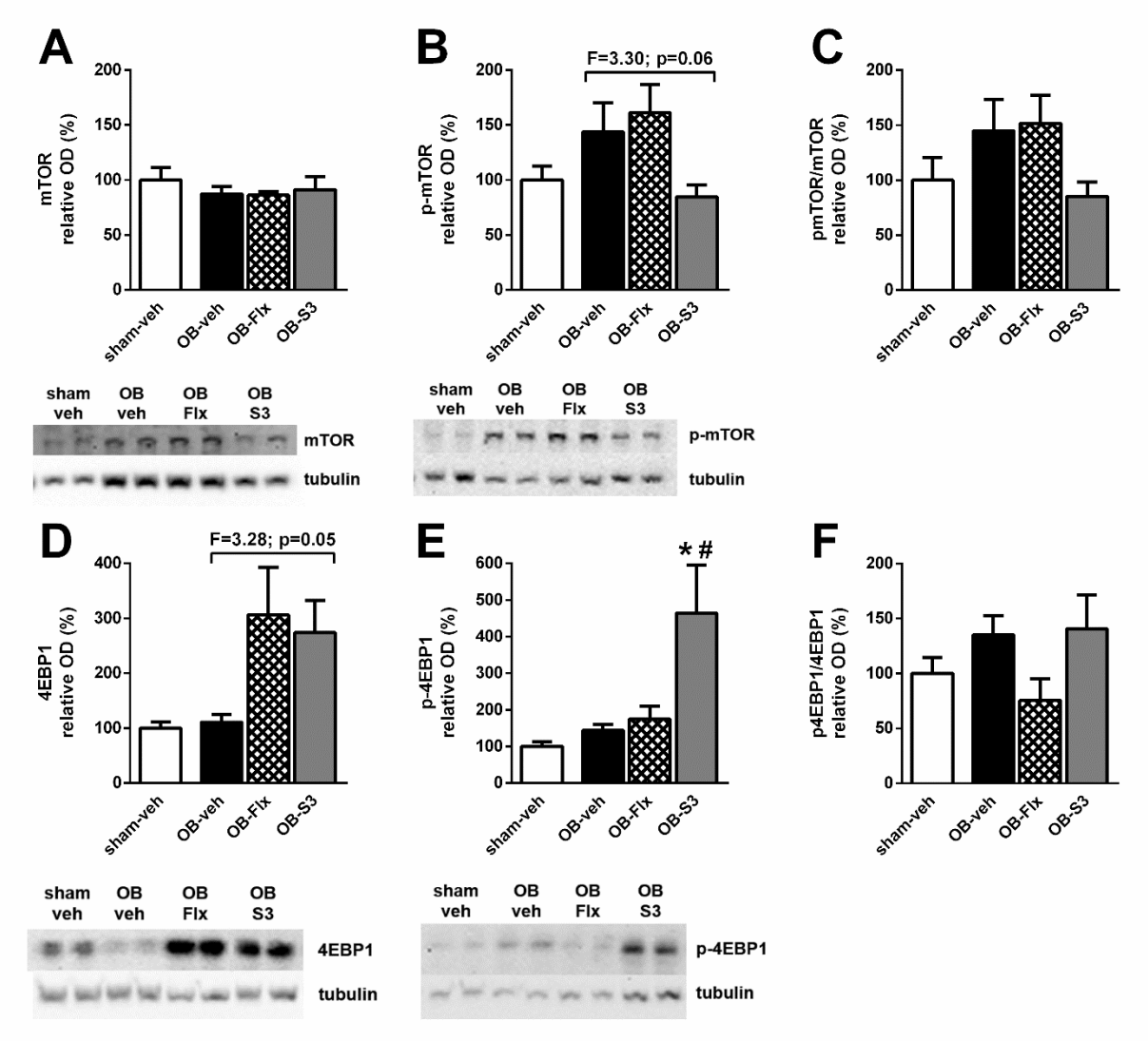


Figure 4. Expression of mTOR and p-mTOR, and 4EBP1 and p-4EBP1 in hippocampal total cell lysates from OB mice and the effect of chronic treatment (4 weeks) with S 47445 (3

mg/kg/day, i.p.) in comparison with fluoxetine (18 mg/kg/day, i.p.). A) mTOR protein, B) p-mTOR protein, C) p-mTOR/mTOR ratio, D) 4EBP1, E) p-4EBP1 and F) p-4EBP1/4EBP1 ratio. Values represent mean±S.E.M. Sham-veh, sham-operated mice treated with vehicle; OB-veh, OB-vehicle-treated; OB-flx, OB-fluoxetine-treated; OB-s3, OB mice treated with S 47445. * $p < 0.05$ *vs* OB-vehicle, and # $p < 0.05$ *vs* OB-fluoxetine; one-way ANOVA followed by Newman-Keuls post hoc test. n = 6-8 animals per experimental group in duplicate for each sample.

Regarding the expression of the functional mTOR protein (the phosphorylated mTOR, p-mTOR), OB surgery induced a trend to increased p-mTOR expression (+44% *vs* sham-veh; t=1.566, df=13, $p = 0.14$, Student´s *t* test). In contrast to fluoxetine, S 47445 decreased, though not significantly, p-mTOR expression in OB mice (+12% and -41% *vs* OB-veh for OB-flx and OB-s3, respectively; $F_{(2,18)} = 3.297$, $p = 0.060$, one-way ANOVA) (Figure 4B).

As shown in Figure 4C, OB induced a non-significant increase in the p-mTOR/mTOR ratio (+45 % *vs* sham-veh; t=1.310, df=13, $p = 0.21$, Student's *t* test). Chronic treatment with S 47445 or fluoxetine did not induce changes in the p-mTOR/mTOR ratio compared to the OB-vehicle group, although a tendency to a decrease was detected in the S 47445 group (+4,8% and -42% *vs* OB-veh for OB-flx and OB-s3, respectively; $F_{(2,18)}=2.465$, $p = 0.113$, one-way ANOVA).

The olfactory bulbectomy (Figure 4D) did not alter the expression of total 4EBP1 protein (+11% *vs* sham-veh; t=0.596, df=17, $p = 0.56$, Student´s *t* test). Following the chronic treatments with S 47445 and fluoxetine, an increase in the amount of 4EBP1 protein expression in the hippocampus of OB mice was detected (+76% and +47% *vs* OB-veh for OB-flx and OB-s3, respectively; $F_{(2,25)}=3.280$, $p = 0.054$, one-WAY ANOVA).

As shown in Figure 4E, OB surgery tended to increase the expression of the p-4EBP1 (+44% *vs* sham-veh; t=2.020, df=17, p = 0.06, Student´s t test). One-way analysis revealed a significant effect of treatment ($F_{(2,25)}$ = 4.654, p = 0.019). *Post-hoc* analysis showed that treatment with S 47445 significantly increased the expression of p-4EBP1 protein in the hippocampus of OB mice (+222% *vs* OB-veh, $p < 0.05$) in comparison with fluoxetine (+21% *vs* OB-veh, ns), and was significantly different from the effect of fluoxetine in OB mice ($p <$ 0.05, OB-3s *vs* OB-flx).

Regarding the ratio between functional (phosphorylated) 4EBP1 protein *vs* total 4EBP1 expression (p-4EBP1/4EBP1) (Figure 4F), no significant changes were observed following OB surgery (+35% vs sham-veh; t=1.503, df=17, p = 0.15, Student´s t test). Chronic treatment with fluoxetine or S 47445 did not induce significant changes in the p-4EBP1/4EBP1 ratio when compared to the OB-vehicle group value, although a tendency to a decrease was detected in the OB-fluoxetine group (-44% and +4.3% and *vs* OB-veh for OB-flx and OB-s3, respectively; $F_{(2,25)}$=2.092, p = 0.145, one way ANOVA).

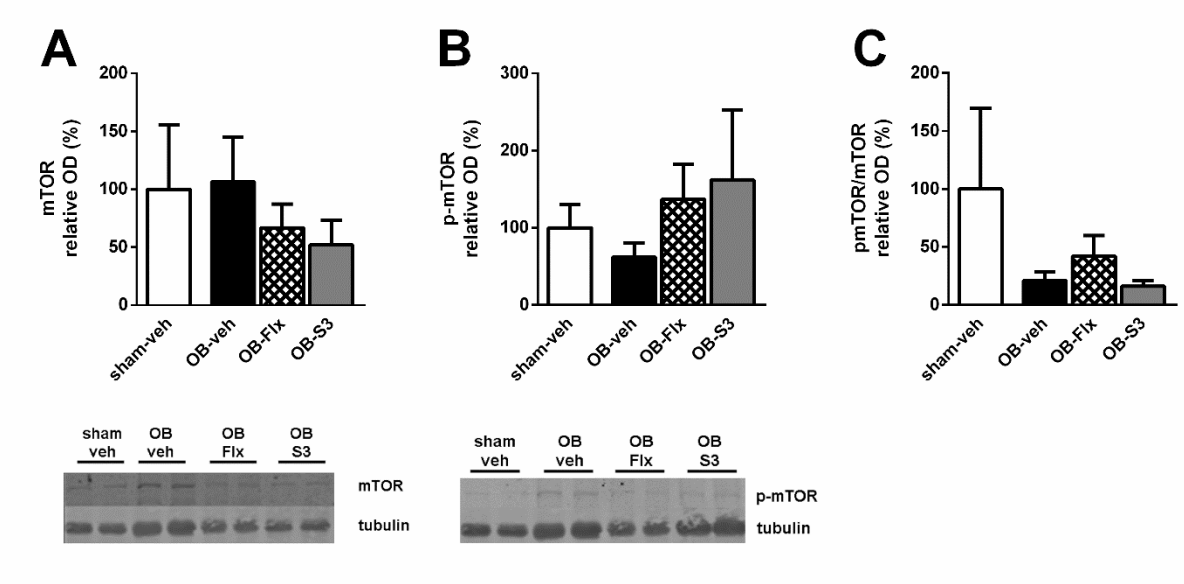


Figure 5. Expression of mTOR and p-mTOR, and 4EBP1 and p-4EBP1 in prefrontal cortex of OB mice and the effect of chronic treatment (4 weeks) with S 47445 (3 mg/kg/day, i.p.) in comparison with fluoxetine (18 mg/kg/day, i.p.). A) mTOR protein, B) p-mTOR protein, and C) p-mTOR/mTOR ratio. Values represent mean±S.E.M. Sham-veh, sham-operated mice treated with vehicle; OB-veh, OB-vehicle-treated; OB-flx, OB-fluoxetine-treated; OB-s3, OB mice treated with S 47445. n = 6-8 animals per experimental group in duplicate for each sample.

The mTOR signalling pathway was also evaluated in the prefrontal cortex. No significant differences were obtained in the total or activated mTOR protein in the olfactory bulbectomy model of depression, nor after the chronic treatment with fluoxetine or the compound S 47445 (Figures 5A, B and C).

4. DISCUSSION

Our results demonstrate the behavioral effects of S 47445 in an animal model that possesses a predictive validity. Indeed, most of the clinically effective antidepressants reverse many of the behavioral, neurochemical and structural disturbances exhibited by OB animals (Cryan et al., 1998; Song and Leonard, 2005; Jarosik et al., 2007; Sato et al., 2008). Locomotor hyperactivity is a core manifestation of OB animals since the olfactory bulbs belong to the limbic system and their removal affects extensive efferent networks including the striatum, which explain the impact of the olfactory bulbectomy on locomotor function (Song and Leonard, 2005). In our study, the chronic treatment with low-mild doses of S 47445 (1 and 3 mg/kg/day) reversed this typical locomotor hyperactivity (total distance travelled) that OB

mice exhibit when exposed to a brightly illuminated open field, a behavioral readout considered to reflect antidepressant efficacy (Kelly et al., 1997; Song and Leonard, 2005).

In our study, the reduction of hyperactivity elicited by the chronic administration of the compound S 47445 was similar to the SSRI antidepressant fluoxetine in the OB mouse model, and in good agreement with previous studies assessing clinically effective antidepressants in bulbectomized mice (Machado et al., 2012; Freitas et al., 2013), or in rats (Kelly et al., 1997; Song and Leonard, 2005; Rodríguez-Gaztelumendi et al., 2009). The choice of comparison of the effect of the drug with a current therapy (fluoxetine) was done in order to be more relevant for a future clinical use in Major Depressive patients. The antidepressant effect of S 47445 seems to be disease-dependent since this compound does not induce any impact on normal behavior as no changes in locomotor activity (Bretin et al., 2017), nor in general behavior over a dose range of 3 to 100 mg/kg in control mice (Bretin et al., 2018) were observed. Regarding the antidepressant effect of fluoxetine, we have already reported a decrease locomotor activity in OB mice, but a lack of changes in the total distance travelled in the open field test in their sham counterparts (Amigó et al., 2016). The antidepressant effect of S 47445 is in good agreement with the findings reported in other rodent models of emotional behavior since this AMPA-PAM also reversed the depressive-like state induced by chronic corticosterone model or CMS at the same range of doses, and presented anxiolytic behavior without altering locomotor activity in chronically-treated corticosterone mice (Mendez-David et al., 2017). Moreover, enhancement of AMPA signaling has been reported to result in antidepressant-like effects by other AMPA-PAM like LY392098 (Farley et al., 2010) or ampakines (Knapp et al., 2002). These drugs were effective in reducing the depressive-like manifestations in mice subjected to the chronic unpredictable mild stress (CUMS) and in rats subjected to the chronic-submissive behavior model, respectively.

Additionally, S 47445 had an early anxiolytic effect (after 7 and 21 days of treatment) on the attenuation of the enhanced anxiety response exhibited by OB mice, evidenced by an increase in central activity, together with reduced peripheral deambulation. However, the effect of S 47445 on central activity showed no significant effect following 28 days of treatment. Though it deserves further investigation, these changes might be dependent on molecular modifications leading to receptor desensitization/tolerance phenomena. In the literature, there are some evidences against and in favor of the potential of AMPA-PAMs as anxiolytic drugs at different doses. S 47445 presented anxiolytic effects from 1 to 10 mg/kg/day after 4 weeks of treatment when assessed in the elevated plus maze, but only at 1 mg/kg/day in the open field test (Mendez-David et al., 2017). The AMPA receptor potentiator LY392098 exhibited an antidepressant but not anxiolytic effect in the CUMS in mice (Farley et al., 2010). In our study, the SSRI fluoxetine was devoid of any anxiolytic-like effect. However, it is worth to mention that this SSRI induces paradoxical responses in C57BL/6J mouse strain, with no effect in the splash test, and even increasing the anxiogenic-like responses in the novelty suppressed feeding test in the CUMS model (Gosselin et al., 2017).

The higher efficacy of the low-mild doses of S 47445 (1 and 3 mg/kg/day) in decreasing the hyperactivity and anxious-like behavior contrasts with the lack of effect of the highest dose (10 mg/kg/day), since this dose was previously reported to be active in other emotional behavior models (corticosterone, CMS models or stress prenatal) (Bretin et al., 2016; Mendez-David et al., 2017). However, this pattern is quite similar to the biphasic and/or the U-shape dose-response described for the AMPA potentiators LY392098 (Li et al., 2001) in the FST paradigm in rats and mice.

The chronic administration of S 47445 (3 mg/kg/day) significantly reversed the increased BDNF hippocampal expression associated to OB syndrome. In OB mice, hippocampal mRNA (Amigó et al., 2016) and protein (Hellweg et al., 2007, Freitas et al., 2013) BDNF

levels are up-regulated, in line with our results. It is postulated that up-regulation of BDNF protein and other neuroplasticity proteins (i.e. pCREB and pERK) in the hippocampus of OB mice may represent an adaptive response to the model injury (Rodríguez-Gaztelumendi et al., 2009; Hendriksen et al., 2015). However, it is noteworthy to mention that the OB model presents opposite changes in rat (Hendriksen et al., 2012) and mice (Hellweg et al., 2007). These species-dependent differences regarding neurochemical and molecular changes, also affect to the response to drugs (Hendriksen et al., 2015). There are some evidences about the modulation of hippocampal BDNF expression by AMPA signaling. The antidepressant-like activity of magnesium in the OB model is associated with the AMPA/BDNF pathway (Pochwat et al., 2015), and AMPA-PAMs are reported to increase mRNA expression of BDNF and TrkB in rat hippocampus in a dose-and time-dependent manner (Mackowiak et al., 2002). Moreover, chronic treatment (2 weeks) with S 47445 was reported to increase or to reverse the decrease in both the mRNA and the protein levels of BDNF in the hippocampus of aged rats using procognitive and antidepressant active doses in rodents (Calabrese et al., 2017; Mendez-David et al., 2017). However, it has been reported that the antidepressant-like effects of ketamine and the AMPA-PAM LY451646 are preserved in bdnf $P^{+/-}$P heterozygous null mice contrary to biogenic amine-based drugs as imipramine (Lindholm et al., 2012). In contrast, the antidepressant-like effects of ketamine in the FST were abolished in conditional BDNF knockout homozygous mice (Autru et al., 2001). In our study, chronic administration of S 47445 reduced BDNF hippocampal expression, in parallel with the behavioral outcome, suggesting that the antidepressant-like effect induced by the compound would have compensated the dysregulation observed in the hippocampus of the OB mice. Chronic administration of fluoxetine did not modify BDNF expression in the hippocampus of OB mice, while a tendency to increase BDNF expression was observed in the prefrontal cortex, as described for other SSRIs (Alboni et al., 2010). The lack of effect of fluoxetine in

hippocampal BDNF expression is in contrast to Freitas´ study (Freitas et al., 2013). These discrepancies may be due to differences in the mouse strain and/or the gender (Freitas et al., 2013). It has been shown that some antidepressant-like effects of fluoxetine may be mediated through a neurogenesis non-dependent mechanism (David et al., 2009), which could also explain the lack of effect of this antidepressant on BDNF expression that we observe in our study.

In the present study, changes in phospho-mTOR and the ratio p-mTOR/mTOR in the hippocampus of OB mice were parallel to those observed for BDNF expression, which may be due to the direct correlation between both pathways (Autry et al., 2001; Duman et al., 2012). It is worth to mention that the chronic administration of S 47445 did not alter the expression of mTOR in the prefrontal cortex, as it has been reported for clinically used antidepressants (Liu et al., 2015). Regarding 4EBP1 hippocampal expression in OB mice, though the ratio phosphorylated/non-phosphorylated protein is not modified, both forms of 4EBP1 showed a clear tendency to the increase. In addition, a significant increment of the expression of both 4EBP1 and its phosphorylated forms was observed in OB mice treated with S 47445, though the ratio was unchanged. Although mTOR was not modified by the drug treatment, the phosphorylation of 4EBP1 is also induced by other signaling pathways as PI3K-Akt (Gingras et al., 1998; Kohn et al., 1998), ERK1/2 (Herbert et al., 2002), etc. This increase may be due to an attempt to maintain a correct transcription level, since the non-phosphorylated 4EBP1 acts as a repressor of the protein eukaryotic translation initiation factor (eIF) (Gringras et al., 1999). Moreover, previous studies suggest that the fast-acting antidepressant-like effects of ketamine are mediated by changes in the phosphatidylinositol 3-kinase (PI3K), Akt (protein kinase B, PKB), glycogen synthesis kinase 3 (GSK3), mammalian target of rapamycin (mTOR) and brain derived neurotrophic factor (BDNF) signaling pathway, which has been implicated in the adaptive response to stress and the development of

mood-related disorders (Liu et al., 2013; Abelaira et al., 2014; Park et al., 2014; Zhou et al., 2014). Moreover, studies in primary hippocampal cultures demonstrate the modulation of mTOR pathway by glutamatergic-, but not serotonergic-related antidepressants (Zhou et al., 2014). Regarding the modulation of mTOR signaling by fluoxetine treatment, recent studies report a lack of changes of this signaling pathway in hippocampus (Park et al., 2014; Liu et al., 2015), and in prefrontal cortex (Liu et al., 2015), in line with our findings. It should be considered that antidepressant-like effects of fluoxetine may be due not only to the modulation of neuroplasticity-related signaling pathways, but also to its capacity to modulate relevant neurotransmitter systems as the serotonergic (Riad et al., 2017) and the endocannabinoid (Rodríguez-Gaztelumendi et al., 2009) ones. Thus, the action of S 47445 on brain BDNF/mTOR/4EBP1signaling pathway in hippocampus could be related to its antidepressant-like effect in the OB model, and it appears to be distinctive to fluoxetine that presented no significant effect on the expression of any of these neuroplasticity-related proteins.

5. CONCLUSION

In conclusion, all the above findings demonstrate that S 47445 induces an anxiolytic effect after short-term administration, and antidepressant-like effects after chronic administration in the OB model of depression. These changes appear to be associated to a modulation of the hippocampal BDNF and mTOR expression, which may contribute to its behavioral effects.

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Conflict of interest: SB and EM are Servier employees.

AUTHOR INFORMATION

Author contributions:

FP-C designed and performed experiments, analyzed and interpreted data, and drafted the manuscript. EC performed experiments, analyzed and interpreted data. SB interpreted data and drafted the manuscript. EM performed the critical revision of the manuscript. AP performed a critical revision of the manuscript. AD designed experiments, analyzed, interpreted data, and drafted the manuscript.

Ethical statement:

Originality and plagiarism: we state that this work is original and it has not been submitted for publication, completely or in part, elsewhere and that all the authors have approved the attached manuscript.

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Author contributions: FP-C designed and performed experiments, analyzed and interpreted data, and drafted the manuscript. EC performed experiments, analyzed and interpreted data. SB interpreted data and drafted the manuscript. EM performed the critical revision of the manuscript. AP performed a critical revision of the manuscript. AD designed experiments, analyzed, interpreted data, and drafted the manuscript.

Bioethics: animals and experimental procedures were carried out with the previous approval of the Animal Care Committee of the University of Cantabria and according to the Spanish legislation (RD 53/2013) and the European Communities Council Directive (2010/63/UE) on "Protection of Animals Used in Experimental and Other Scientific Purposes.

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