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Modulation of neuroplasticity pathways and antidepressant-like behavioural responses following the short-term (3 and 7 days) administration of the 5-HT$_4$ receptor agonist RS67333

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Abstract

It has been recently suggested that activation of 5-HT$_4$ receptors might exert antidepressant-like effects in rats after 3 d treatment, suggesting a new strategy for developing faster-acting antidepressants. We studied the effects of 3 d and 7 d treatment with the 5-HT$_4$ receptor partial agonist RS67333 (1.5 mg/kg.d) in behavioural tests of chronic efficacy and on neuroplastic-associated changes, such as adult hippocampal neurogenesis, expression of CREB, BDNF, β-catenin, AKT and 5-HT$_4$ receptor functionality. RS67333 treatment up-regulated hippocampal cell proliferation, β-catenin expression and pCREB/CREB ratio after 3 d treatment. This short-term treatment also reduced immobility time in the forced swim test (FST), together with a partial reversion of the anhedonic-like state (sucrose consumption after chronic cortico-sterone). Administration of RS67333 for 7 d resulted in a higher increase in the rate of hippocampal cell proliferation, a significant desensitization of 5-HT$_4$ receptor-coupled adenylate cyclase activity and a more marked increase in the expression of neuroplasticity-related proteins (BDNF, CREB, AKT): these changes reached the same magnitude as those observed after 3 wk administration of classical antidepressants. Consistently, a positive behavioural response in the novelty suppressed feeding (NSF) test and a complete reversion of the anhedonic-like state (sucrose consumption) were also observed after 7 d treatment. These results support the antidepressant-like profile of RS67333 with a shorter onset of action and suggest that this time period of administration (3–7 d) could be a good approximation to experimentally predict the onset of action of this promising strategy.

Introduction

Depressive disorders are currently treated with drugs that mainly act by increasing brain monoamine levels (Adell *et al.* 2005). These drugs usually require 2–5 wk

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to improve the symptoms of the disease (Nierenberg *et al.* 2000). Thus, generating new drugs with a faster effect is a critical issue in order to avoid the dramatic consequences of this illness.

Antidepressant-like properties have been recently shown for the 5-HT$_4$ receptor partial agonist RS67333. This drug has been shown to be effective in the forced swim test (FST) (Lucas *et al.* 2007). After 3 d treatment in rats, this 5-HT$_4$ receptor agonist induced a powerful electrophysiological desensibilization of the 5-HT$_{1A}$ receptor in the dorsal raphe, as well as an increase in hippocampal cell proliferation and

CREB phosphorylation, in a similar manner to classical antidepressants (Lucas *et al.* 2007). Regarding other behavioural tests, 3 d treatment with RS67333 resulted in a partial abolishment of the hyper-locomotion induced by olfactory bulbectomy: the complete reversion was observed after 14 d RS67333 treatment. Furthermore, treatment with this agonist reached its maximal efficacy in the chronic mild stress model after 10 d administration. Therefore, it could be of interest to perform additional tests for indicators of antidepressant chronic response, in order to further characterize the pharmacological profile of this drug as an antidepressant with shorter onset of action than existing antidepressants. To this end we chose the novelty suppressed feeding (NSF) and the chronic corticosterone model as validated experimental paradigms to assess the chronic effects of antidepressants (Dulawa & Hen, 2005; Gourley *et al.* 2008*a*,*b*).

Moreover, several hippocampal neuroplastic changes, especially at the level of the dentate gyrus (DG), occur only after chronic treatment with classical antidepressants and are associated with antidepressant chronic efficacy (Adachi *et al.* 2008; Airan *et al.* 2007; Keith *et al.* 2007). It is now well accepted that antidepressants increase the rate of adult hippocampal proliferation after chronic but not acute treatment (Malberg *et al.* 2000; Santarelli *et al.* 2003). In addition, several neuroplasticity and proliferation related intracellular pathways appear to be involved in the mechanism of action of antidepressants, especially at the hippocampal level. They include BDNF (Bravo *et al.* 2009; Duman & Monteggia, 2006), CREB (Malberg & Blendy, 2005; Tardito *et al.* 2009), AKT (Mostany *et al.* 2008; Soumier *et al.* 2009) and β-catenin (Madsen *et al.* 2003; Mostany *et al.* 2008) pathways. Since chronic antidepressants have been reported to modulate 5-HT$_4$ receptor-coupled adenylate cyclase activity (Vidal *et al.* 2009, 2010), it would be of interest to analyse whether short-term administration of 5-HT$_4$ agonists results in the modification of this transductional signal.

The aim of this work was to study the time-course of the changes induced by short-term administration of RS67333 (3 d and 7 d) on behavioural responses and hippocampal neuroplasticity related to chronic antidepressant efficacy.

Method

For a complete description of methods, see Supplementary material (available online).

Animals and treatments

All experimental procedures were performed according to 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 weighting 270–320 g (except in the case of adult neurogenesis assessment that began with 350–400 g rats) were group-housed and maintained on 12-h light/dark cycle with food and water available *ad libitum*. Animals received 1.5 mg/kg.d RS67333 or vehicle subcutaneously (osmotic minipumps) for 3 d or 7 d, depending on the experimental process. In the case of fluoxetine, it was administered in drinking water (10 mg/kg.d) for 7, 14 or 21 d.

Immunohistochemistry

On the last day of antidepressant treatment, animals received BrdU (2×100 mg/kg every 12 h, i.p.) and were perfused with 4% paraformaldehyde. BrdU immunohistochemistry was performed in 40 μm sections as described previously by Mostany *et al.* (2008). The total number of cells with β-catenin cytoplasmatic accumulations in the subgranular zone of DG and the subtypes of adult neural progenitors and neuroblasts expressing β-catenin were quantified by immunohistochemistry and immunofluorescence co-localization with antibodies against β-catenin, GFAP, SOX2, BLBP and DCX. The different cell types were classified according to their morphology and the expression of phenotype markers. Quiescent neural progenitors (QNPs) present a triangular soma with processes that cross the granule cell layer and express GFAP, SOX2 and BLBP. However, amplifying neural progenitors (ANPs) express SOX2 and BLBP but not GFAP and have very short processes (Encinas *et al.* 2006). Immature neuroblasts are positive for DCX and their differentiated state was achieved by the length and arborization of their processes and SOX2 expression.

Western blot

The whole hippocampus or the different hippocampal regions were dissected as described by Newton *et al.* (2005) in order to obtain total cell lysate samples that were processed as previously described (Mostany *et al.* 2008). Membranes were incubated overnight in the following primary antibodies: anti-β-catenin, anti-GAPDH, anti-AKT1, anti-BDNF, anti-CREB and anti-pCREB. Secondary antibodies conjugated to horse-

radish peroxidase were detected with the ECL Advance kit (UK). Blot quantitations were performed by densitometric scanning using Scion Image Software (USA). The densitometry values were normalized against GAPDH values.

In-situ *hybridization*

Cryostat sections (20 μm) were thaw-mounted onto slides and pre-treated for *in-situ* hybridization. Oligonucleotides complementary to BDNF mRNAs (5′-GGT CTC GTA GAA ATA TTG GTT CAG TTG GCC TTT TGA TAC CGG GAC-3′ (Zetterstrom *et al.* 1998) were 3′-end-labelled with [^{35}S]dATP using terminal deoxynucleotide transferase and added to each section. After incubation, they were washed and exposed to film together with ^{14}C microscales at -20 °C for 3 wk (Vaidya *et al.* 2001). The abundance of mRNA in hippocampus was analysed and quantified using Scion Image Software. Optical density values were calibrated to ^{35}S tissue equivalents using ^{14}C microscales.

Adenylate cyclase assay

Hippocampi were homogenized (1:160, w/v) in ice-cold membrane buffer (MB), and centrifuged at 500 g for 5 min at 4 °C. The supernatant was centrifuged at 12 000 g for 5 min at 4 °C. The pellet was resuspended (1:160, w/v) in stimulation buffer (SB) (MB buffer containing 10 mM ATP magnesium salt). The protein concentration used per assay was 0.9–1 μg/μl. The incubation reaction consisted of 5 μl membrane homogenate and 5 μl SB alone (basal condition) or in the presence of different concentrations of the 5-HT$_4$ agonist zacopride (10^{-6} to 10^{-3} M). Measurement of cAMP was performed using an AlphaScreen cAMP Assay kit (PerkinElmer, USA). Protein concentration was determined using Bio-Rad Protein Assay kit (USA) with γ-globulin as standard.

Behavioural tests

Novelty-supressed feeding (*NSF*). The latency of each animal to eat a single food pellet placed in the centre of an open-field arena was measured (10-min session) after 24 h food deprivation. After the test, each animal was transferred to its home cage, and the individual latency to feed as well as home food consumption were measured during a 5-min session. If an animal did not eat during the open-field session, a latency value of 10 min was assigned. Those animals showing no feeding behaviour in the home cage were excluded from data analysis.

Forced swim test (*FST*). Rats were placed individually into a water-filled cylinder (25 cm diameter, 65 cm height; 45 cm depth; 23–25 °C). The total immobility time was measured during a 5-min period.

Sucrose consumption and preference after chronic corticosterone administration. Animals were chronically administered corticosterone in the drinking water for 20 d (Gourley & Taylor, 2009). Following treatment with either RS67333 or fluoxetine, they were habituated to sucrose for 2 d (5% in drinking water). Then, the amount of total intake (sucrose and water intake in ml), sucrose consumption (ml), and sucrose preference (percentage of sucrose intake *vs.* total intake) of each animal was measured during a 1-h period after 4, 14 and 19 h water deprivation. See experimental design in Fig. 1*c*.

Data and statistical analysis

Data are expressed as a percentage of vehicle group values (100%) and are presented as the mean ± S.E.M. Differences among the experimental groups were evaluated by unpaired Student's *t* test (when two experimental groups were included) or one-way ANOVA followed by Student–Newman–Keuls *post-hoc* test when appropriate (GraphPad Prism 5.01; USA). The level of significance was chosen at $p < 0.05$.

For specific analysis of data corresponding to the different experimental procedures see online Supplementary Material.

Results

Behavioural responses related to chronic efficacy

In the FST, a significant reduction in immobility time had been observed in animals treated for 3 d with RS67333 ($-24.7 \pm 2.7\%$, $p < 0.01$; Fig. 1*a*). This response was still present after 7 d treatment ($-40.5 \pm 5.6\%$, $p < 0.01$; Fig. 1*a*) compared to their respective vehicle groups. The lack of temporal parallelism between positive response in FST and the course of clinical efficacy for antidepressant drugs is well known. Thus, we undertook another well-validated paradigm to evaluate the antidepressant-like effects of drugs, i.e. sucrose intake in rats exposed to chronic corticosterone. As previously reported (Gourley & Taylor, 2009), corticosterone-treated rats showed an anhedonic behaviour, a pathognomonic feature of depression states, as they exhibited decreased sucrose preference (relative sucrose intake $\sim 50\%$) (Fig. 1*d*) compared to naive rats. The administration of RS67333 for 3 d induced a partial reversion of that
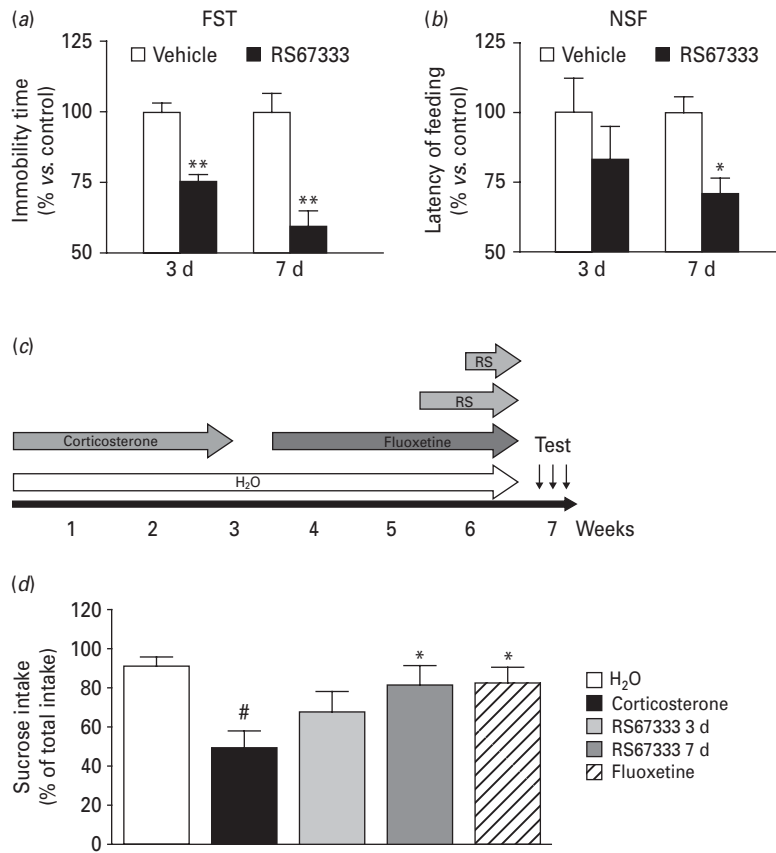
Fig. 1. Behavioural effects of 3-d and 7-d RS67333 treatment on (*a*) the forced swim test (FST) and (*b*) the novelty suppressed feeding (NSF) paradigm. (*c*) Experimental timeline on the sucrose-preference paradigm in rats exposed to chronic corticosterone, and (*d*) sucrose preference in the corticosterone model (% of sucrose intake *vs.* total intake). Note that 3-d RS67333 treatment attenuates the depressive-like effect, although after 7 d treatment the behavioural values are comparable to those observed with the classical antidepressant fluoxetine after its chronic administration (see online Supplementary Material). Data are mean ± s.e.m. of 8–9 animals per group. * $p < 0.05$ and ** $p < 0.01$ *vs.* vehicle group for FST and NSF (Student's *t* test). # $p < 0.05$ *vs.* vehicle group and * $p < 0.05$ *vs.* corticosterone-treated group for the sucrose preference test after chronic corticosterone administration (one-way ANOVA followed by Student–Newman–Keuls *post-hoc* test).

anhedonic-like state (relative sucrose intake ∼65%). However, the complete reversion was only observed after 7 d treatment, paralleling the effect of 21 d treatment with fluoxetine (Fig. 1*d*). When analysing sucrose consumption, there was a marked reduction in the corticosterone group compared to naive rats (6.7 ± 1.6 and 23.6 ± 1.5 ml, respectively, $p < 0.01$). The administration of fluoxetine for 21 d fully reversed that corticosterone-induced reduction in sucrose intake (22.3 ± 2.9 ml, $p < 0.01$ *vs.* corticosterone rats). Treatment with the 5-HT$_4$ receptor agonist RS67333 for 3 d significantly attenuated that anhedonic-like state, although the amount of sucrose intake did not reach the values of the naive group (15.5 ± 1.3 ml, $p < 0.05$ *vs.* corticosterone group and $p < 0.05$ *vs.* naive group). Interestingly, 7 d treatment with RS67333 resulted in

a complete reversion of sucrose consumption (23.0 ± 0.7 ml, $p < 0.01$ *vs.* corticosterone group). The amount of total intake (total ml of sucrose solution + water measured during a 1-h session) was significantly reduced in corticosterone-treated animals compared to naive rats (13.6 ± 2.5 and 25.2 ± 2.5 ml, respectively, $p < 0.01$). The total intake in the group of animals treated with RS67333 for 3 d and 7 d, and with fluoxetine was similar to that observed in naive animals and significantly higher than that measured in corticosterone-treated animals (3-d RS67333: 23.1 ± 2.0 ml; 7-d RS67333: 28.6 ± 2.7 ml; 21-d fluoxetine: 26.9 ± 1.5 ml; $p < 0.01$, $p < 0.001$, $p < 0.01$ *vs.* corticosterone group, respectively).

Moreover, we also assessed the chronic effects of drug treatments, in an anxiety-related test, the novelty

suppressed feeding (NSF). A slight reduction in the latency to feed was observed following 3 d treatment with RS67333 ($-16.7 \pm 11.7\%$; Fig. 1b), without reaching statistical significance. However, a significant reduction had been observed following 7 d RS67333 treatment compared to the vehicle-treated counterparts ($-29.0 \pm 5.3\%$, $p < 0.05$; Fig. 1b). No significant differences were detected in the latency to feed in the home cage (5-min session) between animals treated for 3 d (2.1 ± 0.7 min) or 7 d (1.9 ± 0.5 min) with RS67333 and vehicle groups (1.9 ± 0.5 and 2.0 ± 0.7 min for 3-d and 7-d vehicle groups, respectively). Further, there were no significant differences in home food consumption (tested in a 5-min session and following the open-field NSF) between animals treated for 3 d (1.7 ± 0.2 g) or 7 d (1.8 ± 0.4 g) with RS67333 and their respective vehicle groups (3 d: 1.5 ± 0.2 g; 7 d: 1.6 ± 0.3 g), thus discarding any effect of RS67333 on home food consumption. On the other hand, the SSRI fluoxetine, administered for 7 d in drinking water (10 mg/kg.d), did not induce any behavioural effect in NSF test compared to the vehicle group. In contrast, after 2 wk fluoxetine treatment, a significant reduction in the latency of feeding in the NSF was observed (see Supplementary Fig. S1, online) without affecting latency to feed and food consumption in the home cage (data not shown).

Hippocampal proliferation in β-catenin⁺ cells

The number of BrdU⁺ cells in the DG was significantly increased after 3 d administration of RS67333 ($119.3 \pm 5.7\%$, $p < 0.05$; Fig. 2a–d). Moreover, an increase of similar magnitude was observed in the number of β-catenin⁺ clusters in the subgranular zone of the DG ($122.6 \pm 6.7\%$, $p < 0.05$; Fig. 3a–d). Since classical antidepressants (e.g. fluoxetine) target ANPs (Encinas *et al.* 2006), we decided to explore if RS67333 treatment was targeting β-catenin in the same type of cells, addressing the co-localization of this protein with neural progenitor markers.

Regarding the cellular phenotype (see Methods, online), the majority of cells with β-catenin cytoplasmatic accumulations were ANPs and immature neuroblasts, with only a small portion of QNPs (Fig. 2i). The number of β-catenin-expressing ANPs (β-catenin⁺, Sox2⁺, GFAP⁻), were increased with respect to vehicle ($125.7 \pm 6.5\%$, $p < 0.05$; Fig. 2g, j) after 3-d treatment with the 5-HT₄ agonist. No differences were observed in QNPs (β-catenin⁺, Sox2⁺, GFAP⁺) positive for β-catenin ($105.1 \pm 6.0\%$; Fig. 2f) as well as in the total number of doublecortin immunopositive neurons ($107.7 \pm 8.7\%$; Fig. 2e), showing that RS67333

specifically targets ANPs. Moreover, when the immature neuroblasts (β-catenin⁺, Sox2⁺, DCX⁺) with very short processes expressing β-catenin were counted, an increase was observed ($120.5 \pm 5.8\%$, $p < 0.05$; Fig. 2h, k). These data suggest that the β-catenin pathway could be involved in the differentiation of ANPs to neurons induced by RS67333.

Treatment with RS67333 for 7 d increased the number of BrdU cells in the DG by almost 50% ($149.5 \pm 9.8\%$, $p < 0.01$; Fig. 2a, c, d) reaching the values observed after treatment with typical antidepressants. A similar increase in the total number of β-catenin⁺ clusters in the subgranular zone with respect to its vehicle ($143.5 \pm 9.9\%$, $p < 0.01$; Fig. 3a–d), was observed following 7 d administration.

β-catenin is not only expressed in neural progenitors, but also plays an important role in dendritic arborization and synapse formation in hippocampal adult neurons (Yu & Malenka, 2003). When β-catenin expression was measured by Western blot in hippocampal total cell lysates, significant higher levels of this protein were found after a 3-d RS67333 treatment ($129.9 \pm 8.4\%$, $p < 0.05$; Fig. 3e), this increase being maintained after the 7-d regimen ($146.5 \pm 4.6\%$, $p < 0.05$; Fig. 3e).

BDNF expression in DG

As shown in Fig. 4(a, b, e), 3-d RS67333 administration resulted in increased BDNF mRNA expression in the CA3 area ($128.2 \pm 5.6\%$, $p < 0.01$) but not in CA1 ($127.7 \pm 8.4\%$) or DG ($109.6 \pm 3.8\%$) of the hippocampus. However, after the 7-d regimen no differences were observed in the CA3 area ($113.5 \pm 5.1\%$) while an increase was found in CA1 ($172.6 \pm 19.9\%$, $p < 0.01$) and DG ($152.8 \pm 11.6\%$, $p < 0.01$) (Fig. 4c, d, f). When BDNF protein level was analysed by Western blot in the hippocampal total cell lysate after 3 d treatment, a clear tendency towards increase in the expression of mature BDNF was observed, although it did not reach statistical significance ($140.2 \pm 18.9\%$; Fig. 4g, h). One week was required to obtain a significant increase in BDNF protein levels ($147 \pm 13\%$, $p < 0.05$; Fig. 4g, h). In contrast, 7-d oral administration of fluoxetine did not significantly modify BDNF mRNA expression in the hippocampus (see Supplementary Fig. S2, online).

Hippocampal AKT and CREB expression

Immunoblot analysis of the expression of AKT/PKB in hippocampal homogenates showed a tendency to increase after 3 d treatment with the 5-HT₄ receptor agonist with respect to vehicle rats ($129.9 \pm 12.2\%$;
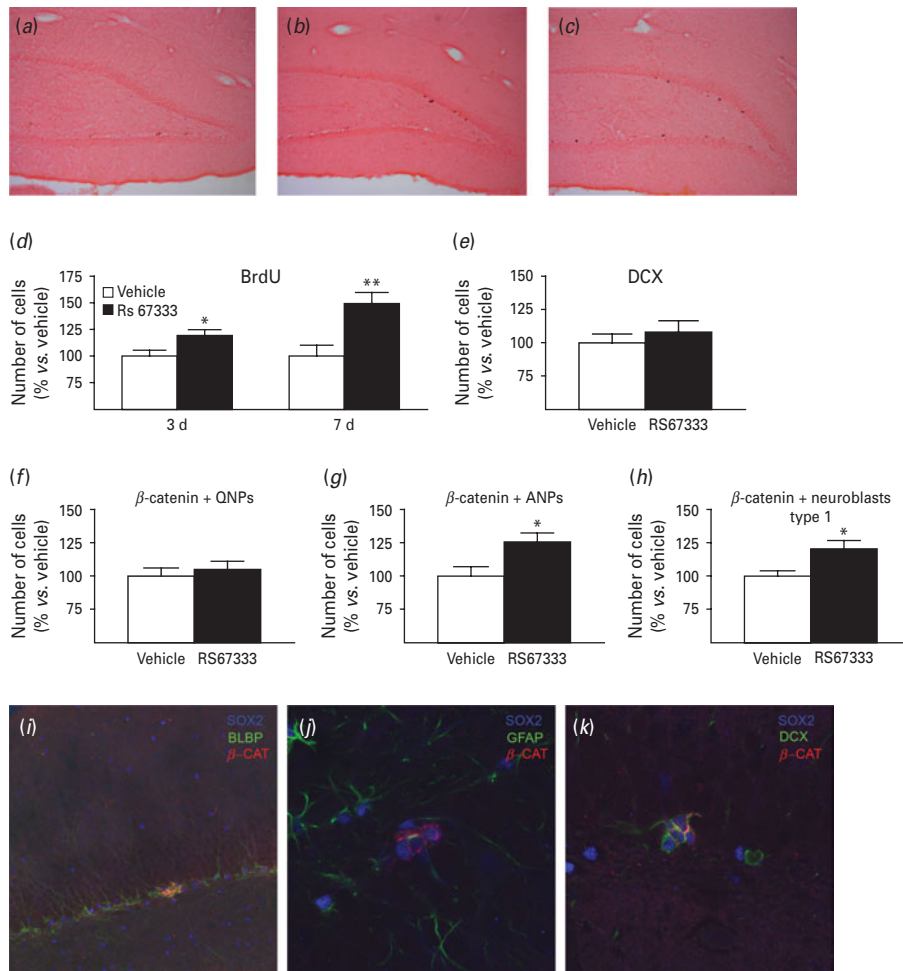
Fig. 2. Illustrations showing BrdU immunopositive cells and β-catenin-expressing cells in the dentate gyrus (DG) of rat hippocampus. Representative photomicrographs of BrdU$^+$ cells in animals treated with (*a*) vehicle, (*b*) 3-d RS67333, and (*c*) 7-d RS67333. (*d*) RS67333 increased the number of BrdU$^+$ cells in the adult DG after 3-d and 7-d treatment. Note that RS67333 does not alter either the total number of (*e*) DCX cells or (*f*) QNPs expressing β-catenin but increases the number of (*g*) ANPs and (*h*) Sox2-positive neuroblasts that express β-catenin. Photomicrographs illustrating β-catenin expression in (*i*) neural progenitors, (*j*) ANPs and (*k*) neuroblasts. Data are mean $\pm$ S.E.M. of six animals per group. * $p < 0.05$ and ** $p < 0.01$ *vs.* vehicle group by Student's *t* test.

Fig. 5*a, b*); however, 7 d treatment was necessary to reach statistical significance (140 ± 14%; Fig. 5*a, b*). Moreover, 3-d RS67333 treatment did not significantly modify the hippocampal expression levels of CREB (115.7 ± 23.6%; Fig. 5*a, b*) and its phosphorylated form (151.4 ± 32.5%; Fig. 5*a, b*) in spite of the tendency to increase. When the treatment was prolonged to 7 d, the expression of CREB (140 ± 16%, $p < 0.05$; Fig. 5*a, b*>) and its phosphorylated form (183 ± 38%, $p < 0.05$; Fig. 5*a, b*) were raised to statistically significant levels. Interestingly, a significant increase of the pCREB/CREB ratio (193.4 ± 33.5%, $p < 0.05$; Fig. 5*a, b*) was seen following 3 d treatment. This increase was

maintained following 7 d treatment (184 ± 21%, $p < 0.05$; Fig. 5*a, b*).

Desensibilization of 5-HT$_4$ receptor-mediated accumulation of cAMP

Incubation of hippocampal membranes with the 5-HT$_4$ agonist zacopride resulted in a concentration-dependent increase of cAMP for the vehicle and 3-d treatment experimental groups, while no modification of cAMP accumulation was observed following 7 d treatment, where cAMP levels were even below the basal values, although without reaching statistical
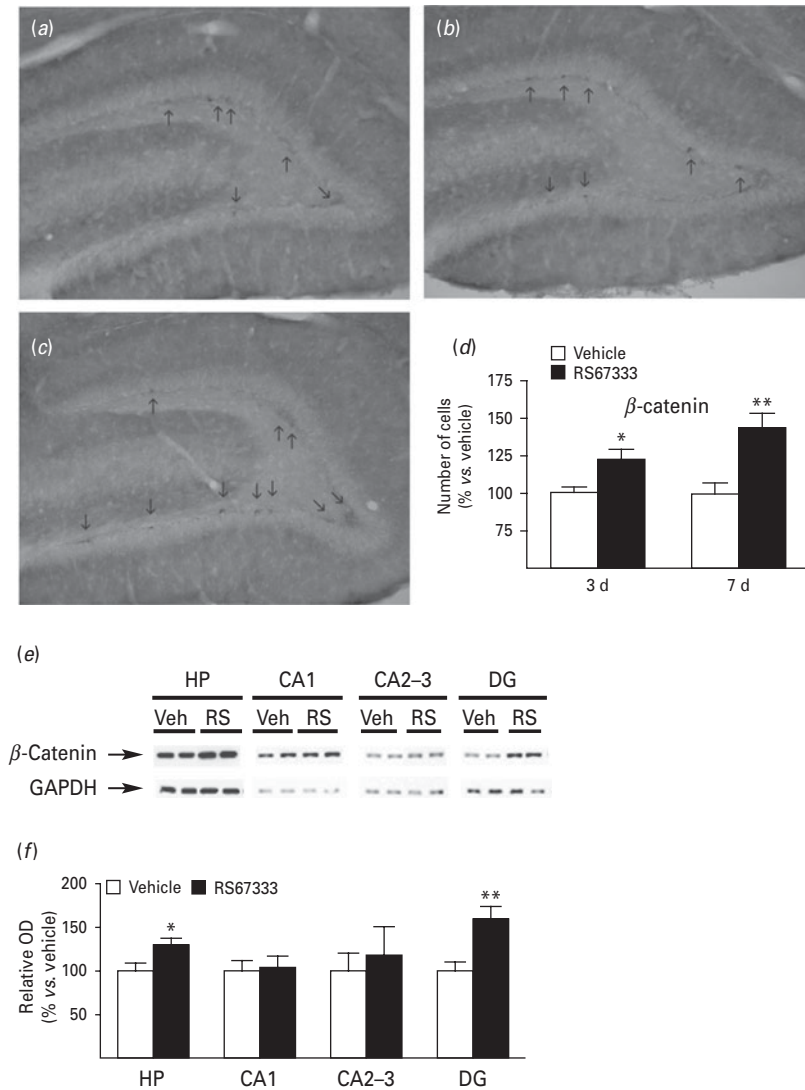
Fig. 3. Effect of RS67333 treatment on β-catenin expression in the rat hippocampus. Representative photomicrographs of hippocampal β-catenin clusters in animals treated with (*a*) vehicle, (*b*) 3-d RS67333, and (*c*) 7-d RS67333. (*d*) Treatment with this drug increased the number of cells with β-catenin accumulations in the subgranular zone of dentate gyrus (DG) after 3 d. (*e*) Representative Western blot of β-catenin protein expression in the whole hippocampus (HP). (*f*) RS67333 significantly increased β-catenin protein expression in whole hippocampus after 3-d and 7-d treatment. Data are mean $\pm$ S.E.M. of 5–7 animals *per* group. * $p < 0.05$ and ** $p < 0.01$ *vs.* vehicle group by Student's *t* test.

significance. There were significant differences between vehicle and RS67333 (7 d) treated groups with respect to E_{max} values (efficacy) ($p < 0.01$) (135.1 $\pm$ 12.9% *vs.* 84.1 $\pm$ 2.4%, respectively), but not between vehicle and RS67333 (3 d) groups (153.0 $\pm$ 19.7% *vs.* 135.5 $\pm$ 4.4%, respectively) (Fig. 6). The EC_{50} values (potency) were not modified in any of the different experimental groups (70.9 $\pm$ 49.5 μM *vs.* 20.8 $\pm$ 13.9 μM, vehicle and 3-d RS67333; and 16.7 $\pm$ 8.5 μM *vs.* 6.8 $\pm$ 5.2 μM, vehicle and 7-d RS67333; Fig. 6). No significant changes were observed either in potency and

efficacy of zacopride-induced cAMP accumulation between 3-d and 7-d vehicles.

Discussion

This work reports the modifications induced by the short-term administration (3 d and 7 d) of the 5-HT₄ receptor partial agonist, RS67333, on behavioural paradigms as indicators of antidepressant chronic efficacy, and several markers of hippocampal neuroplasticity previously reported to be modified by
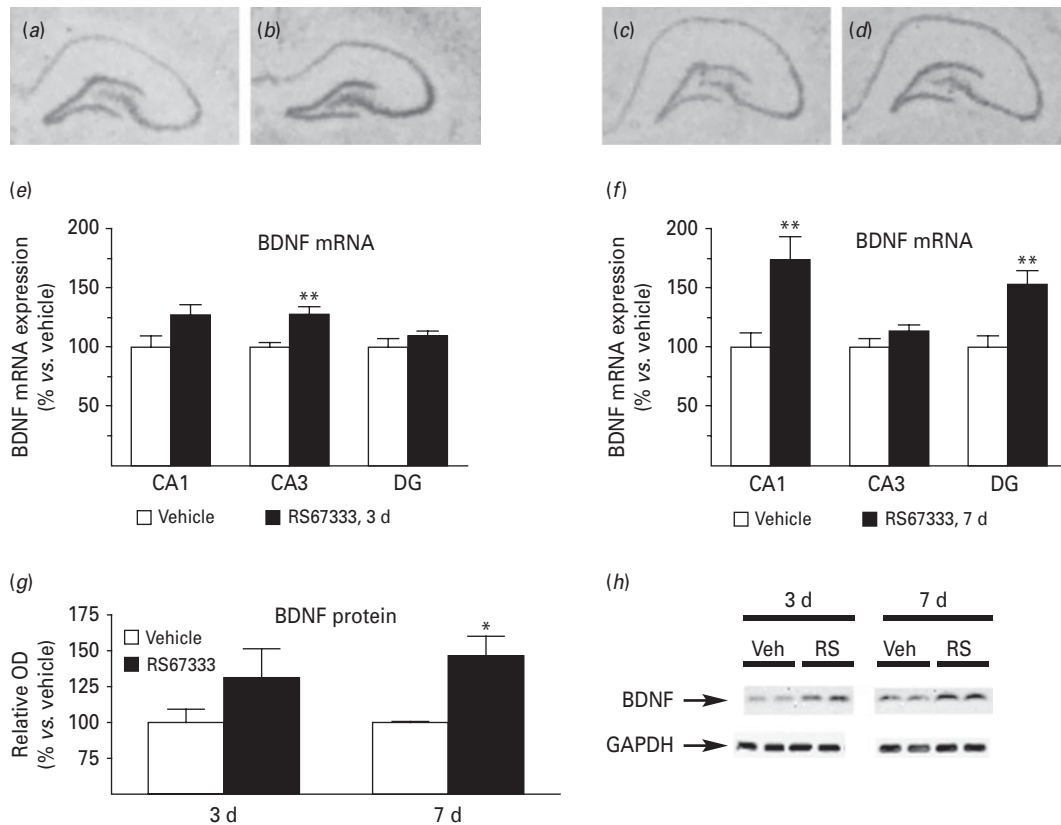
Fig. 4. Effect of RS67333 treatment on BDNF mRNA and protein expression in the rat hippocampus. Representative *in-situ* hybridization autoradiograms in animals treated for 3 d with (*a*) vehicle or (*b*) RS67333, and treated for 7 d with (*c*) vehicle or (*d*) RS67333. (*e*) RS67333 significantly increased BDNF mRNA expression in CA3 field after a 3-d regimen but (*f*) required 7 d to increase BDNF mRNA in CA1 field and dentate gyrus (DG). (*g*) Representative Western blot of BDNF protein expression in vehicle and RS67333 groups. (*h*) RS67333 significantly increased BDNF protein in total cell lysate from hippocampus at 7-d but not 3-d treatment. Data are mean ± S.E.M. of 5–7 animals per group. * $p < 0.05$ and ** $p < 0.01$ *vs.* vehicle group by Student's *t* test.

chronic administration of antidepressants. Regarding our results from behavioural paradigms, RS67333 was effective in the FST following 3 d administration, in line with previously reported results after acute administration (Lucas *et al.* 2007). Since a single administration of most antidepressants has been shown to induce a positive response in this paradigm, the FST is mostly considered as predictive of antidepressant efficacy (Cryan *et al.* 2005). Thus, we undertook another well-validated paradigm to evaluate antidepressant-like effects of drugs, the sucrose preference test, in which corticosterone-treated rats exhibit a decreased preference and consumption of sucrose that reflects an anhedonic behaviour, a pathognomonic feature of depression states (Gourley & Taylor, 2009). In our study, this anhedonic state was partially attenuated after 3-d RS67333 treatment, although 7 d were required to observe a complete reversion, similar to that induced by

21-d fluoxetine administration. It should be noted that subchronic fluoxetine is not able to reverse sucrose intake in this behavioural paradigm (Gourley *et al.* 2008*a, b*). The behavioural effect of RS67333 was also confirmed in the NSF, a test providing an anxiety-related measure that is sensitive to the effects of chronic, but not acute or subchronic antidepressant treatment, as demonstrated with most antidepressants currently available (Dulawa & Hen, 2005). In our case, RS67333 showed a tendency to reduce the latency to feed in NSF after 3 d treatment but 7 d were required to obtain a statistically significant effect in this test. In contrast, fluoxetine was only effective in this test when the administration was prolonged up to 28 d, as previously described (Marcussen *et al.* 2008). It is noteworthy that, in the pioneer study (Lucas *et al.* 2007), the administration of RS67333 for 3 d resulted in a partial reversion of the behavioural changes induced
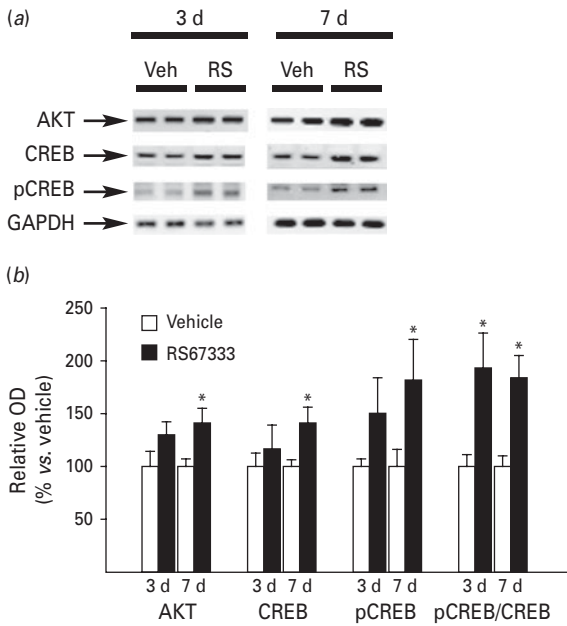
Fig. 5. Effect of RS67333 treatment on the expression of AKT and CREB in the rat hippocampus. (*a*) Representative Western blot of AKT, CREB and pCREB protein expression in total cell lysate from hippocampus. (*b*) Note that RS67333 significantly increased pCREB/CREB ratio after 3 d administration but 7 d were necessary to obtain significant changes in AKT, CREB and pCREB. Data are mean ± S.E.M. of 5–7 animals per group. * $p < 0.05$ *vs.* vehicle group by Student's *t* test.

Fig. 6. Concentration-response curves of zacopride-induced cAMP accumulation in hippocampal membrane homogenates from (*a*) vehicle (□) and RS67333 (■) animals treated for 3 d, and (*b*) vehicle (△) and RS67333 (▼) animals after 7 d treatment. Data are mean ± S.E.M. of 5–7 animals per group. ** $p < 0.01$ vehicle *vs.* 7-d RS67333, by Student's *t* test.

by olfactory bulbectomy, an animal model considered to reveal predictive capacity of chronic antidepressant response: the complete reversion of depressive-like phenotype was evident after 14 d treatment. It should be mentioned that the duration of treatment with classical antidepressants required for the full reversion of these behavioural changes varies from 2 to 4 wk (Rodriguez-Gaztelumendi *et al.* 2009; Willner *et al.* 1987). Taken together, our results show for the first time that a short-term (7 d) administration of a putative antidepressant induces a complete response in these paradigms.

In addition to behavioural data, several hippocampal neuroplastic changes have been related to antidepressant efficacy. In this regard, adult neurogenesis in the DG is increased after chronic but not acute administration of classical antidepressants (Malberg *et al.* 2000; Pilar-Cuéllar *et al.* unpublished observations), although there is some controversy about the relevance of this neurogenic change in the mediation of behavioural actions of antidepressants (Bessa *et al.* 2009; David *et al.* 2009; Surget *et al.* 2008). Our study shows that a 3-d regimen with RS67333
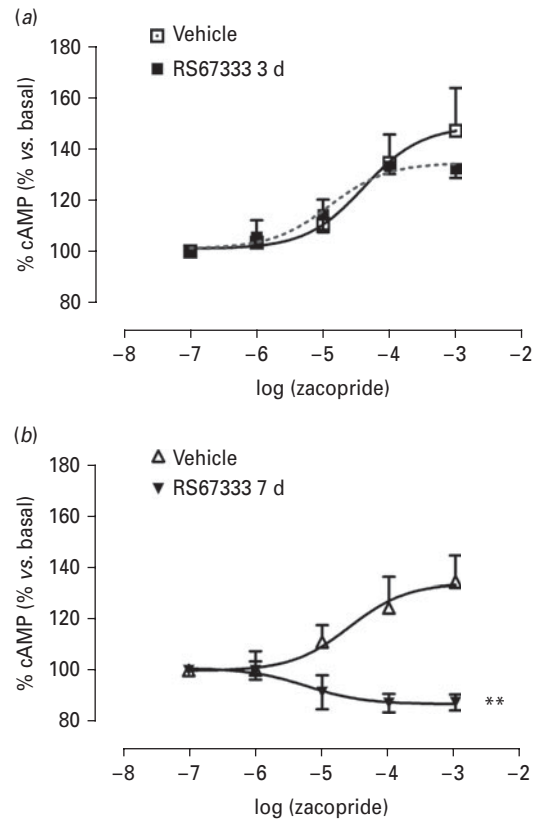
increased the number of new cells, in agreement with other authors (Lucas *et al.* 2007). Furthermore, the neurogenic changes induced by 7-d administration of RS67333 reached the level of increase previously reported for classical antidepressants after 2–3 wk treatment (Malberg *et al.* 2000; Warner-Schmidt & Duman, 2007). These data could suggest a possible relationship between cell proliferation in the adult DG and antidepressant response. Moreover, they also demonstrate for the first time that this increase is accompanied by the accumulation of β-catenin in ANPs promoting their differentiation to immature neuroblasts. Therefore, it can be suggested that the early mitogenesis induced by 3 d treatment with the 5-HT₄ partial agonist RS67333 is targeting the same type of cells as fluoxetine to generate new neurons (Encinas *et al.* 2006) and is modulating the canonical Wnt pathway, also targeted by chronic antidepressant therapies (Madsen *et al.* 2003; Mostany *et al.* 2008). It is of note

that the new cells generated after 3–7 d treatment with RS67333 would be still immature neuroblasts, as neural progenitors need at least 28 d to become mature (Encinas *et al.* 2006). Thus, it cannot be fully discounted that immature neuroblasts might play a role in antidepressant-like responses. These results also raise the question of whether the induction of adult neurogenesis by antidepressants is a requirement for the behavioural effects or just a good correlate of antidepressant efficacy. It has been suggested that both neurogenesis-dependent and -independent mechanisms contribute to the antidepressant response (David *et al.* 2009). However, our experimental design did not allow the clarification of this issue in animals treated with RS67333: because of the short duration of treatment, it was not be possible to study the integration of the new cells in the existing neuronal networks or to analyse their dendritic morphology.

Taking into account that currently used antidepressants induce hippocampal CREB and pCREB expression after chronic but not acute treatment (Nibuya *et al.* 1996; Thome *et al.* 2000), the increase observed in CREB and pCREB expression after RS67333 supports the view that short-term treatment could result in a fully antidepressant response. In a similar manner, 1 wk of treatment is enough to obtain significant modifications on the expression of AKT. On the other hand, 3-d RS67333 administration raised β-catenin accumulation in neural progenitors, in addition to increased expression in the whole hippocampal fraction. Since the activation of Wnt/β-catenin signalling pathway is associated with activity-related neural plasticity and remodelling (Peng *et al.* 2009; Yu & Malenka, 2003) and its up-regulation has been reported after chronic antidepressant (Mostany *et al.* 2008) and electroconvulsive seizure (ECS) treatments (Madsen *et al.* 2003), our results strongly support the relevance of this neural pathway in the mediation of the molecular actions of antidepressants. In general terms, in line with our results from neuroproliferative studies, a short-term treatment (3–7 d) with RS67333 reaches the level of up-regulation of these signalling pathways previously found following chronic (2–3 wk) administration of clinically used antidepressants.

Several studies have shown that antidepressants up-regulate hippocampal BDNF (Duman & Monteggia, 2006), although the temporal pattern of these changes is a matter of debate. While some authors have reported an induction of BDNF expression after subchronic treatment (Larsen *et al.* 2008; Musazzi *et al.* 2009), other studies only show up-regulation of BDNF after chronic antidepressant

administration (De Foubert *et al.* 2004; Nibuya *et al.* 1995). Here we report a tendency to increase in expression of BDNF after 3 d treatment, and a significant increase in the expression of BDNF protein in whole hippocampal homogenates following a 7-d regimen. In this regard, an increase in the expression of BDNF following acute administration of another 5-HT$_4$ agonist (SL65.0155) has been recently reported (Tamburella *et al.* 2009). Differences in the pharmacodynamic and/or kinetic properties might account for this apparent discrepancy. Previous studies suggest that the presence of BDNF in the DG is necessary for the behavioural effects of commonly prescribed antidepressants (Adachi *et al.* 2008); in a similar way, infusion of BDNF specifically into DG, but not CA1, induces antidepressant-like effects in the FST and learned helplessness test (Shirayama *et al.* 2002). Taken together, these data emphasize the relevance of BDNF expression in the DG for antidepressant responses. In this regard, our *in-situ* hybridization studies revealed no significant changes in BDNF mRNA levels either in DG or the CA1 field after 3-d RS67333 administration, while 7 d treatment appears to be necessary to increase BDNF mRNA in DG and CA1. It is of note that, in our hands, oral administration of fluoxetine for 3 d or 7 d did not significantly modify BDNF mRNA expression in DG (see Supplementary Fig. S2, online).

The second-messenger cAMP has also been strongly suggested to be involved in the mechanism of action of antidepressants (Donati & Rasenick, 2003). Interestingly, a significant decrease in 5-HT$_4$ receptor-dependent cAMP production has been reported after chronic treatment (3 wk) with fluoxetine (Vidal *et al.* 2009), venlafaxine (Vidal *et al.* 2010) or imipramine (Reierson *et al.* 2009), probably reflecting a desensitization process, due to a less efficient coupling to Gs proteins (Watts, 2002). In this regard, a decreased affinity of Gs proteins for guanine nucleotides (Manji *et al.* 1991), as well as a decrease in Gαs protein levels has been reported after antidepressant treatments (Lesch *et al.* 1991, 1992; McGowan *et al.* 1996). We did not find any significant difference in the maximal efficacy or potency of zacopride to increase cAMP levels after 3-d RS67333 administration, while the 7-d treatment significantly reduced zacopride-induced cAMP accumulation. The reduction in cAMP accumulation in this experimental group went below basal levels, although this difference did not reach statistical significance. Our results show that short-term administration (7 d) of RS67333 is required to fully desensitize the post-receptor signalling pathway associated to 5-HT$_4$ receptors, in agreement with the results obtained in behavioural tests predictive of chronic response.

In conclusion, our data show that, after 3 d administration, the 5-HT$_4$ receptor partial agonist RS67333 shows antidepressant-like positive responses in some behavioural tests, increases hippocampal cell proliferation in DG and up-regulates some neuroplasticity-related markers. Administration for 7 d results in a complete antidepressant response in those behavioural tests indicative of chronic efficacy, a more marked increase in the expression of neuroplasticity-related proteins and a 5-HT$_4$ receptor desensitization, in line with the modifications induced by clinically used antidepressants. It is noteworthy that this is the first study in which the administration of a putative antidepressant for 3 d and 7 d induces behavioural and neuroplastic changes in a magnitude fully comparable to that previously reported for chronic (2–3 wk) classical antidepressants. Although caution is needed when extrapolating results from animals to humans, our data allow predicting that activation of 5-HT$_4$ receptors provides an encouraging pharmacological strategy to obtain an early antidepressant response.

Note

Supplementary material accompanies this paper on the Journal's website (http://journals.cambridge.org/pnp).

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Statement of Interest

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