Neuropsychopharmacology (2014) 39, 1366–1378

www.neuropsychopharmacology.org

Rapid Anxiolytic Effects of a 5-HT_4 Receptor Agonist Are Mediated by a Neurogenesis-Independent Mechanism

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Selective serotonin reuptake inhibitors (SSRIs) display a delayed onset of action of several weeks. Past work in naive rats showed that 5-HT_4 receptor agonists had rapid effects on depression-related behaviors and on hippocampal neurogenesis. We decided to investigate whether 5-HT_4 receptor stimulation was necessary for the effects of SSRIs in a mouse model of anxiety/depression, and whether hippocampal neurogenesis contributed to these effects. Using the mouse corticosterone model of anxiety/depression, we assessed whether chronic treatment with a 5-HT_4 receptor agonist (RS67333, 1.5 mg/kg/day) had effects on anxiety- and depression-related behaviors, as well as on hippocampal neurogenesis in comparison with chronic fluoxetine treatment (18 mg/kg/day). Then, using our anxiety/depression model combined with ablation of hippocampal neurogenesis, we investigated whether neurogenesis was necessary for the behavioral effects of subchronic (7 days) or chronic (28 days) RS67333 treatment. We also assessed whether a 5-HT_4 receptor antagonist (GR125487, 1 mg/kg/day) could prevent the behavioral and neurogenic effects of fluoxetine. Chronic treatment with RS67333, similar to fluoxetine, induced anxiolytic/antidepressant-like activity and stimulated adult hippocampal neurogenesis, specifically facilitating maturation of newborn neurons. However, unlike fluoxetine, anxiolytic effects of RS67333 were already present after 7 days and did not require hippocampal neurogenesis. Chronic treatment with GR125487 prevented both anxiolytic/antidepressant-like and neurogenic effects of fluoxetine, indicating that 5-HT_4 receptor activation is necessary for these effects of SSRIs. 5-HT_4 receptor stimulation could represent an innovative and rapid onset therapeutic approach to treat depression with comorbid anxiety.
Neuropsychopharmacology (2014) **39,** 1366–1378; doi:10.1038/npp.2013.332; published online 22 January 2014

Keywords: 5-HT_4 receptor; anxiolytic; neurogenesis; fast onset

INTRODUCTION

Selective serotonin reuptake inhibitors (SSRIs) are the most commonly prescribed drugs for the treatment of depression and several anxiety disorders. Unfortunately, the onset of action of SSRIs is often delayed by 3–6 weeks (Artigas, 2013). The existence of this delayed action combined with the fact that one-third of patients do not respond to treatment emphasizes the need for faster acting and more effective antidepressants (Samuels *et al*, 2011).

It has been proposed that 5-HT_4 receptor agonists such as RS67333 may bring new hope for treating depression (Lucas, 2009; Lucas *et al*, 2005; Lucas and Debonnel, 2002; Lucas *et al*, 2010; Lucas *et al*, 2007). Indeed, administration of 5-HT_4 agonists induced similar molecular and behavioral changes as common antidepressants in rodents (Bockaert *et al*, 2008; Lucas *et al*, 2007; Pascual-Brazo *et al*, 2012). Depressed-like state in the olfactory bulbectomy or chronic mild stress model was completely abolished after 10–14 days of RS67333 treatment in rats, suggesting a more rapid response mechanism in comparison with classical antidepressants (Lucas *et al*, 2007). A positive behavioral response in the Novelty-Suppressed Feeding (NSF) test in rat, a complete reversion of anhedonic-like state (sucrose consumption), and an increase in swimming behavior in defeated mice in the forced swim test were also observed after a short period of RS67333 treatment (Gomez-Lazaro *et al*, 2012; Pascual-Brazo *et al*, 2012). In addition to behavioral data, and in agreement with a previous report from Lucas *et al* (2007), a recent study performed in naive rats confirmed that a short period of treatment with RS67333 increased the number of newborn cells in the dentate gyrus (DG) (Pascual-Brazo *et al*, 2012). These results are interesting because hippocampal neurogenesis has been implicated in some of the behavioral effects of antidepressants in adult rodents (David *et al*, 2009; Santarelli *et al*, 2003). However, no direct evidence has yet linked the antidepressant-like effects of 5-HT_4 receptor activation and its neurogenic effects. Finally, it has been

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Received 6 September 2013; revised 16 November 2013; accepted 20 November 2013; accepted article preview online 28 November 2013

suggested that SSRIs and 5-HT$_4$ receptor agonists share common mechanisms of action. Indeed, the 5-HT$_4$ receptor agonist, RS67333, augmented the acute effect of paroxetine on extracellular 5-HT levels in rat ventral hippocampus, and after only 3 days of administration it increased basal hippocampal 5-HT levels (Licht *et al*, 2010). The coadministration of the SSRI citalopram and RS67333 strongly potentiated the antidepressant-like properties of the latter in several electrophysiological, molecular, and behavioral paradigms (Lucas *et al*, 2010).

Although a number of studies have assessed the antidepressant-like activity of 5-HT$_4$ receptor agonists, none have so far evaluated their anxiolytic-like profile. It is noteworthy that some SSRIs are often prescribed for the treatment of anxiety disorders (Burghardt and Bauer, 2013). Anxiety disorders have a lifetime prevalence of over 25%, thus making them the most common psychiatric disorders (Kheirbek *et al*, 2012). Moreover, a comorbidity between depression and anxiety disorders is commonly observed. Thus, this study aimed to investigate both antidepressant and anxiolytic-like effects of either subchronic or chronic administration of a 5-HT$_4$ receptor agonist in a model of anxiety/depression based on the elevation of glucocorticoids in mice (CORT model) (David *et al*, 2009). Standard models of depression that rely on environmental stress manipulations such as learned helplessness or the chronic mild stress are hampered by protocol variability and reported difficulties in replication, thus highlighted the need for a reliable, easily replicable depression model (Nestler *et al*, 2002). The corticosterone model is a chronic exposure method optimized for use in modeling the persistent anxiety/depression-like state in rodents, allowing for multiple behavioral tests in the same animals using an etiologically relevant model of depression that is easily replicable between and within laboratories (David *et al*, 2009; David *et al*, 2010; Gould, 2011; Mendez-David *et al*, 2013).

We also assessed whether chronic 5-HT$_4$ receptor stimulation can affect proliferation of newborn cells and maturation of newborn neurons. Finally, using our mouse model of anxiety/depression combined with ablation of hippocampal neurogenesis by X-irradiation, we assessed whether the anxiolytic/antidepressant action of RS67333 after 7 and 28 days of treatment recruits a neurogenesis-dependent mechanism.

MATERIALS AND METHODS

Subjects

Adult male C57BL/6Ntac mice were purchased from Taconic Farms (Lille Skensved, Denmark and Germantown, NY, USA for the pharmacological and the X-irradiation studies, respectively). All mice were 7–8 weeks old, weighed 23–25 g at the beginning of the treatment, and were maintained on a 12 L:12 D schedule (lights on at 0600 hours). They were housed in groups of five. Food and water were provided *ad libitum*. All testing was conducted in compliance with the laboratory animal care guidelines and with protocols approved by the Institutional Animal Care and Use Committee (Council directive no. 87-848, October 19, 1987, Ministère de l'Agriculture et de la Forêt, Service Vétérinaire de la Santé et de la Protection Animale, permissions no. 92-256B to DJD).

Drugs

Corticosterone [4-pregnen-11b-DIOL-3 20-DIONE 21-hemisuccinate (35 μg/ml)] purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France) was dissolved in a vehicle (0.45% hydroxypropyl-β-cyclodextrin (β-CD); Sigma-Aldrich). Fluoxetine hydrochloride (160 μg/ml, equivalent to 18 mg/kg/day) was purchased from Anawa Trading, (Zurich, Switzerland) and dissolved in 0.45% β-CD/corticosterone solution. 1-(4-amino-5-chloro-2-methoxyphenyl)-3-(1-butyl-4piperidinyl)-1-propanone hydrochloride (RS67333), and 5-Fluoro-2-methoxy-[1-[2-[(methylsulfonyl) amino]ethyl]-4-piperidinyl]-1H-indole-3-methylcarboxylate sulfamate (GR125487) were purchased from Tocris Bioscience (Bristol, UK) and dissolved in 0.9% saline solution. RS67333 and GR125487 were chosen based on previous work (Cachard-Chastel *et al*, 2007; Lucas *et al*, 2007).

RS67333 shows high-binding affinity for the 5-HT$_4$ receptor with a pKi of 8.7 (Bockaert *et al*, 2004; Eglen *et al*, 1995). Except for the sigma receptors, which are bound at affinities comparable to 5-HT$_4$ (sigma 1: pKi = 8.9 and sigma 2: pKi = 8.0), RS67333 has a pKi of <6.7 for other neurotransmitter receptors including 5-HT$_{1A}$, 5-HT$_{1D}$, 5-HT$_{2A}$, 5-HT$_{2C}$, dopamine D$_1$, D$_2$, and muscarinic M$_1$–M$_3$ receptors. However, little is known about the function of sigma receptors'.

GR125487 is the most selective 5-HT$_4$ receptor antagonist with a pKi = 10.6 (Schiavi *et al*, 1994), presenting a selectivity more than 1000-fold over other 5-HT receptor (Gale *et al*, 1994). The dose of RS67333 and GR125487 used in this study were chosen based on previous works (Cachard-Chastel *et al*, 2007; Lucas *et al*, 2007).

Corticosterone Model and Treatment

Our model of elevated glucocorticoids (also named CORT model) is able to blunt the response of the hypothalamic–pituitary–adrenal axis as shown by the markedly attenuated stress-induced corticosterone levels observed in these mice (David *et al*, 2009). This is probably a consequence of the negative feedback exerted by corticosterone on the hypothalamic–pituitary–adrenal axis. This model displays hallmark characteristics of anxiety and depression.

The dose and duration of corticosterone treatment was selected based on previous studies (David *et al*, 2009; Rainer *et al*, 2011). Corticosterone (35 μg/ml, equivalent to about 5 mg/kg/day) or vehicle (0.45% β-CD) was available *ad libitum* in the drinking water in opaque bottles to protect it from light. Corticosterone-treated water was changed every 3 days in order to prevent any possible degradation. Thereafter, while administration with β-CD or corticosterone continued, mice were treated with vehicle (0.45% β-CD), fluoxetine, RS67333, GR125487 alone, or GR125487 in the presence of fluoxetine (Supplementary Figure 1). Both RS67333 and GR125487 were delivered by osmotic minipumps at a dose of 1.5 mg/kg/day and 1 mg/kg/day, respectively (Lucas *et al*, 2005). Fluoxetine (18 mg/kg/day) was delivered in the drinking water as previously described (David *et al*, 2009). Osmotic minipumps (42 days minipumps,

2006 model, Alzet, Cupertino, CA) were implanted subcutaneously under light anesthesia (ketamine/xylazine; (75/20 mg/kg) from Sigma-Aldrich. Control animals (vehicle/vehicle or corticosterone/vehicle groups) were also implanted with a minipump containing 0.9% saline (2006 model, Alzet). Treatment was always maintained until the end of the experiments. Corticosterone and fluoxetine dosages were calculated assuming an average fluid intake of about 5 ml/day (David *et al*, 2009).

Behavioral Tests

The same cohort of animals was tested in five different behavioral models of anxiety and depression. Each animal, over a week, was successively tested in the Open Field (OF), Elevated Plus Maze (EPM), NSF, Splash Test (ST), and Tail Suspension Test (Supplementary Material). Behavioral testing occurred during the light phase between 0700 and 1900 hours. Behavioral paradigms occurred after 7 or 28 days of drug treatment depending on the study (Supplementary Figure S1A and S1B).

Immunohistochemistry

The effects of chronic RS67333 treatment on cell proliferation or maturation of newborn neurons was assessed in corticosterone-treated animals. After anesthesia with ketamine and xylazine (100 mg/ml ketamine and 20 mg/ml xylazine), mice were perfused transcardially (cold saline for 2 min, followed by 4% cold paraformaldehyde at 4 °C). The brains were then removed and cryoprotected in 30% sucrose and stored at 4 °C. Serial sections (35 μm) were cryosectioned through the entire hippocampus and stored in PBS with 0.1% NaN_3.

Proliferation of newborn cells. We first looked at proliferation of newborn cells using Ki-67 immunohistochemistry as described previously (Xia *et al*, 2012). Sections were washed in PBS, blocked (PBS containing 0.3% Triton X-100 and 10% normal donkey serum (NDS)), and incubated with primary antibody overnight at 4 °C (Ki67 rabbit, 1 : 100, Vector, Burlingame, CA). Following washes in PBS, sections were incubated with fluorescence-coupled rabbit secondary antibody (Jackson ImmunoResearch, Beckman, France). Stereological quantification of Ki-67 labeling was performed using an Olympus BX51 microscope (Germany).

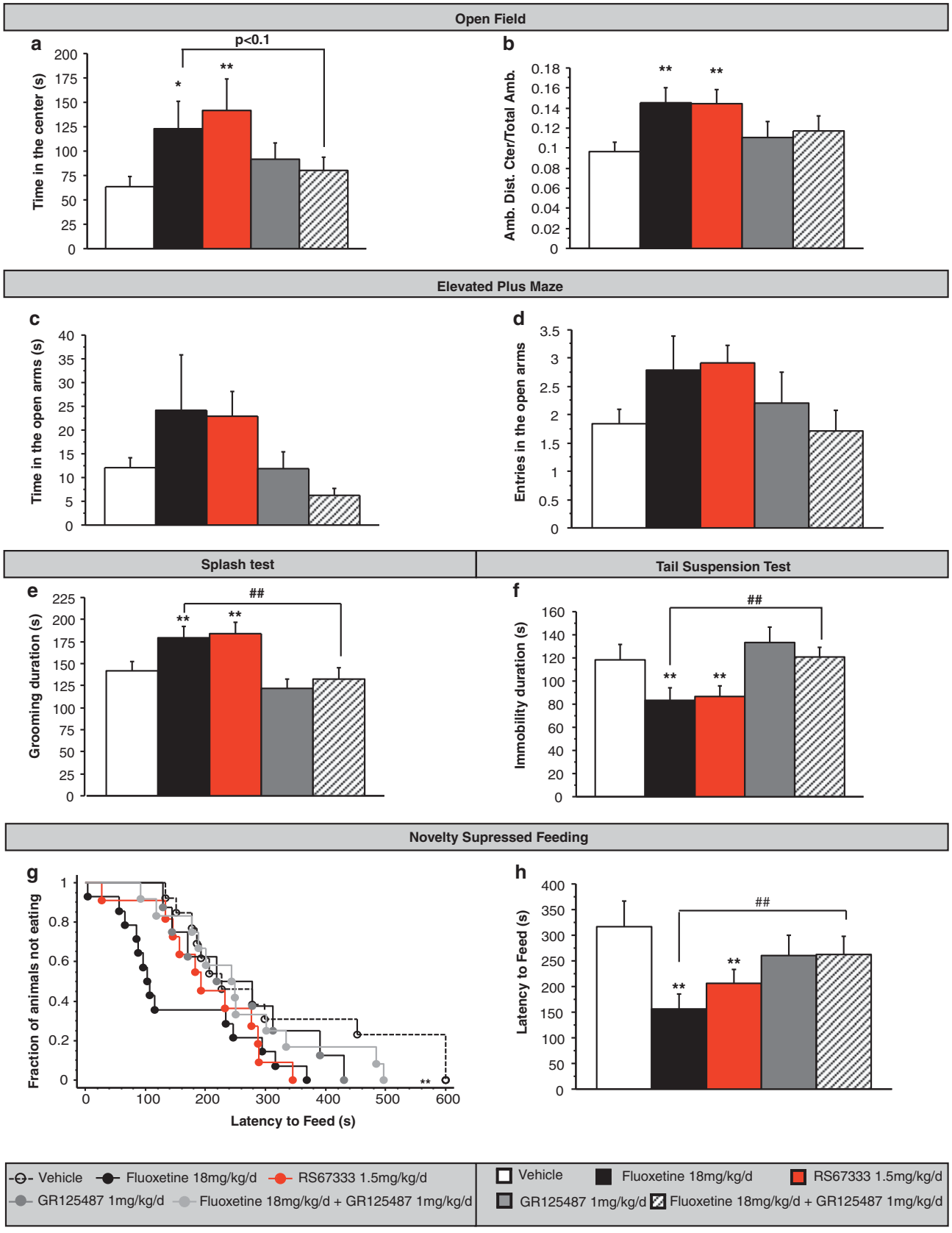
Maturation of newborn neurons. For doublecortin (DCX) staining, the procedure consisted of the following steps: 1 h incubation in 0.1 M TBS with 0.5% Triton X-100 and 10% NDS, followed by goat anti-DCX primary antibody (1 : 100) in TBS/Tx/NDS for 24 h at 4 °C. Biotinylated secondary donkey anti-goat antibody (1 : 500) in TBS/NDS for 1 h at room temperature was used, followed by a 1 h amplification step using an avidin–biotin complex (Vector). The immunohistochemistry protocol was adapted from David *et al* (2009). DCX-positive (DCX^+) cells were subcategorized according to their dendritic morphology: DCX^+ cells without and DCX^+ cells with tertiary (or higher order) dendrites. The maturation index was defined as the ratio of DCX^+ cells possessing tertiary dendrites to the total number of DCX^+ cells.

Sholl analysis. Sholl analysis was performed as described elsewhere (Guilloux *et al*, 2013). DCX^+ cells with tertiary or higher order dendrites were traced using Neurolucida software (MicroBrightField, Williston, VT) on an Olympus BX51 microscope equipped with a motorized stage device and ×100 immersion oil objective. Sholl analysis for dendritic complexity was performed using the accompanying software (NeuroExplorer; MicroBrightField, version 10) to determine dendritic length and number of intersections (branch points). One DCX^+ cell was traced for each 35-μm hippocampal slice; $n = 6$ cells/brain for DAB-stained sections).

X-irradiation

A separate batch of mice were anesthetized with ketamine and xylazine (75/20 mg/kg), placed in a stereotaxic frame, and exposed to cranial irradiation using a PXI X-RAD 320 X-ray system operated at 300 kV and 12 mA with a 2-mm Al filter. Animals were protected with a lead shield that covered the entire body, but left unshielded a 3.22 × 11-mm treatment field above the hippocampus (interaural 3.00 mm to 0.00 mm) exposed to X-ray, thus effectively preventing irradiation from targeting the rest of the brain (Santarelli *et al*, 2003). The corrected dose rate was ~0.95 Gy/min at a source to skin distance of 36 cm. The procedure lasted for 2 min and 39 s, delivering a total of 2.5 Gy. Three 2.5 Gy doses were delivered on days 1, 4, and 7 as previously described (Quesseveur *et al*, 2013). This 7.5 Gy cumulative dose was determined from prior pilot experiments to be the minimum dosage necessary to result in permanent ablation

Figure 1 Effects of chronic 5-HT$_4$ receptor stimulation (28 days) on the anxious/depressed-like phenotype induced by chronic corticosterone exposure. (a and b) Effects of chronic treatment with 5-HT$_4$ ligands or fluoxetine, starting after 4 weeks of corticosterone (35 μg/ml), on anxiety behaviors in the open field (OF) test. Anxiety is measured as mean time spent in the center in seconds (a) or the ratio of ambulatory distance in the center/total ambulatory distance (b). Values plotted are mean ± SEM ($n = 10$–15/group). *$p < 0.05$, **$p < 0.01$ *vs* corticosterone/vehicle group. (c and d) Effects of chronic treatment with 5-HT$_4$ ligands or fluoxetine, starting after 4 weeks of corticosterone (35 μg/ml), on anxiety behaviors in the elevated plus maze (EPM). Anxiety is expressed as mean time in (c) or entries into (d) the open arms. Values plotted are mean ± SEM ($n = 10$–15/group). (e) Effect of chronic treatment with 5-HT$_4$ ligands or fluoxetine on corticosterone-induced depression-related behaviors in the splash test (ST). Results are expressed as mean duration of grooming after receiving a 10% sucrose solution on the snout. Values plotted are mean ± SEM ($n = 10$–15/group). **$p < 0.01$, $^{\#\#}p < 0.01$ *vs* corticosterone/vehicle group and corticosterone/fluoxetine group, respectively. (f) Effect of chronic treatment with 5-HT$_4$ ligands or fluoxetine in the tail suspension test following chronic corticosterone. Results are expressed as mean of immobility duration in seconds. Values plotted are mean ± SEM ($n = 10$–15/group). **$p < 0.01$, $^{\#\#}p < 0.01$ *vs* corticosterone/vehicle group and corticosterone/fluoxetine group, respectively. (g and h) Effects of chronic treatment with 5-HT$_4$ ligands or fluoxetine on anxiety- and depression-like behaviors in the novelty-suppressed feeding (NSF) paradigm after chronic corticosterone. Values plotted are cumulative survival of animals that have not eaten over 10 min ($n = 10$–15/group) (g) or mean of latency to feed in seconds ± SEM (h). **$p < 0.01$, $^{\#\#}p < 0.01$ *vs* corticosterone/vehicle group and corticosterone/fluoxetine group, respectively.


Open Field
a
p<0.1
Time in the center (s)
b
Amb. Dist. Cter/Total Amb.
Elevated Plus Maze
c
Time in the open arms (s)
d
Entries in the open arms
Splash test
e
Grooming duration (s)
Tail Suspension Test
f
Immobility duration (s)
Novelty Supressed Feeding
g
Fraction of animals not eating
Latency to Feed (s)
h
Latency to Feed (s)
Vehicle
Fluoxetine 18mg/kg/d
RS67333 1.5mg/kg/d
GR125487 1mg/kg/d
Fluoxetine 18mg/kg/d + GR125487 1mg/kg/d

of adult-born neurons in the DG as assessed by expression of the immature neuronal marker DCX. The reason for using a fractionated paradigm rather than a single high dose of 7.5 Gy is that the ablation is not permanent after a single high dose. Histological staining for CD68 as a marker of inflammation throughout the brain revealed that irradiated mice were indistinguishable from sham animals 8 weeks post irradiation, indicating minimal nonspecific side effects of irradiation at time of behavioral testing (Meshi *et al*, 2006). Immunohistochemistry confirmed the ablation of adult hippocampal neurogenesis (Supplementary Figure 4).

Data Analysis and Statistics

Results from data analyses were expressed as mean ± SEM. Data were analyzed using StatView 5.0 software (SAS Institute, Cary, NC). For all experiments, one-way or two-way ANOVAs with repeated measures were applied to the data as appropriate. Significant main effects and/or interactions were followed by Fisher's PLSD *post hoc* analysis, unpaired *t*-tests. In the NSF test, we used the Kaplan–Meier survival analysis owing to the lack of normal distribution of the data. Mantel–Cox log rank test was used to evaluate differences between experimental groups. Statistical significance was set at $p<0.05$. A summary of statistical measures is included in Supplementary Tables S1–S6, available online.

RESULTS

5-HT$_4$ Receptor Stimulation Produces Anxiolytic-Like and Antidepressant-Like Effects in a Model of Anxiety/Depression

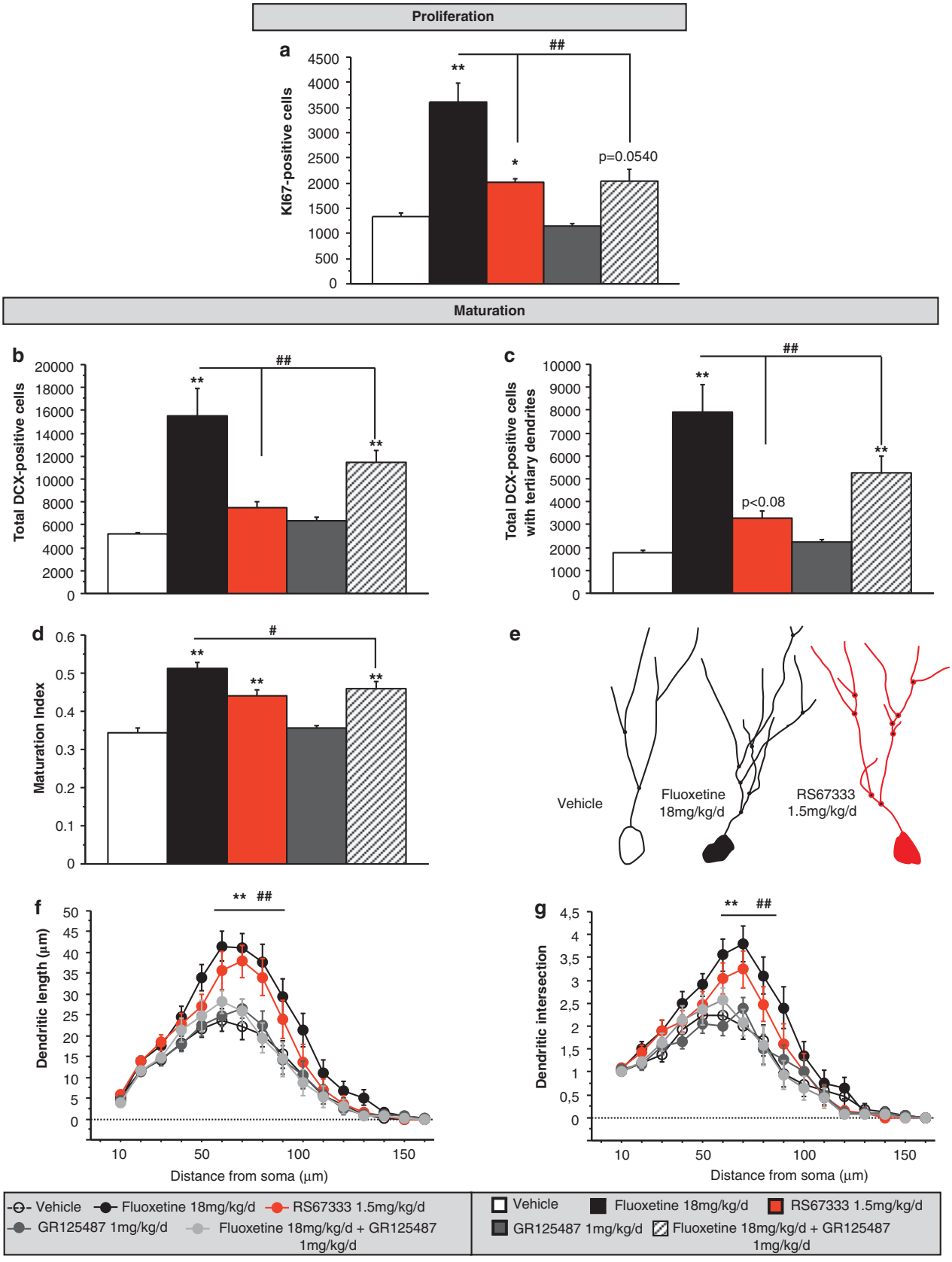
To induce an anxious/depressed-like state in C57BL/6Ntac mice, we administered a low dose of corticosterone (35 μg/ml) for 4 weeks as described in David *et al* (2009) ('CORT model'). After chronic corticosterone, we tested the effects of a 4-week treatment with the 5-HT$_4$ agonist RS67333 (1.5 mg/kg/day) in comparison with fluoxetine (18 mg/kg/day). To assess the selectivity of these effects, we also tested whether the 5-HT$_4$ antagonist GR125487 (1 mg/kg/day), alone or in combination with fluoxetine, affected the behavioral phenotype (see experimental design, Supplementary Figure S1). In the OF, the anxiety-like phenotype induced by chronic corticosterone was reversed by chronic fluoxetine and by the 5-HT$_4$ agonist RS67333 (one-way ANOVA, $*p<0.05$, Figure 1a). Indeed, chronic fluoxetine and RS67333 treatment increased time spent in the center (Figure 1a). A trend for an increase in the number of entries in the center was also observed with both compounds (Supplementary Figures S2A and B). It is unlikely that this effect was the consequence of a change in locomotor activity, as the total ambulatory distance was not affected and the ratio of ambulatory distance in the center divided by total distance was increased for both treatments (one-way ANOVA, $*p<0.05$, Figure 1b). Interestingly, while the 5-HT$_4$ antagonist GR125487 by itself did not affect any anxiety parameters, it prevented fluoxetine-induced anxiolytic-like effects. Indeed, the fluoxetine-induced increase in time spent in the center was prevented by chronic GR125487 administration. These data indicate that 5-HT$_4$ stimulation induces an anxiolytic-like effect and is necessary for the anxiolytic effect of chronic fluoxetine treatment.

To further validate these results, we next tested the effects of RS67333 and fluoxetine alone or in the presence of GR125487 in the same animals in another anxiety-related test, the EPM. We found that chronic RS67333 and fluoxetine induced a trend for an increase in time spent in and number of entries into the open arms, (Figure 1c and d). This anxiolytic-like effect of fluoxetine was completely abolished by treatment with the 5-HT$_4$ antagonist, GR125487.

We next assessed whether chronic treatment with the 5-HT$_4$ agonist RS67333 could also produce antidepressant-like effects. Thus, the same mice were tested in the ST and the tail suspension test. We observed that after squirting a 10% sucrose solution on the mouse's snout, increased grooming duration was observed in both the fluoxetine and the RS67333 groups (one-way ANOVA, $**p<0.01$, Figure 1e). Chronic treatment with the 5-HT$_4$ antagonist GR125487 prevented the antidepressant-like activity of chronic fluoxetine. Similarly, in the tail suspension test, both fluoxetine and RS67333 had antidepressant-like effects and these effects of fluoxetine were blocked by GR125487 (one-way ANOVA, $**p<0.01$; Figure 1f).

Finally, we tested these mice in the NSF test that is sensitive to both acute anxiolytics and chronic antidepressants (Guilloux *et al*, 2013) (Figure 1g and h). Similar to chronic fluoxetine, chronic RS67333 decreased the latency to feed (Kaplan–Meier survival analysis, Mantel–Cox log rank test, $**p<0.01$) without affecting the home-cage food consumption (Supplementary Figure S2C). Chronic treatment with the 5-HT$_4$ antagonist (GR125487) prevented the effect of fluoxetine. Altogether, these data indicate that 5-HT$_4$ receptor activation produces both anxiolytic-like and

Figure 2 Effects of chronic 5-HT$_4$ receptor stimulation (28 days) on proliferation and dendritic maturation of young neurons in the DG of the adult hippocampus. (a) Effect of chronic treatment with 5-HT4 ligands or fluoxetine, starting after 4 weeks of corticosterone (35 μg/ml), on cell proliferation. Cell proliferation is measured as mean number of Ki-67-positive cells (a). Values plotted are mean ± SEM ($n=3$–5/group). $*p<0.05$, $**p<0.01$, $^{\#\#}p<0.01$ *vs* corticosterone/vehicle group and corticosterone/fluoxetine group, respectively. (b) Effect of chronic treatment with 5-HT$_4$ ligands or fluoxetine on total number of doublecortin-positive cells (DCX$^+$; mean ± SEM; $n=4$–5 mice/group) was measured after chronic corticosterone. $**p<0.01$, $^{\#\#}p<0.01$ *vs* corticosterone/vehicle group, and corticosterone/fluoxetine group, respectively. (c and d) DCX$^+$ cells were categorized as to whether they exhibited tertiary dendrites. Effects of fluoxetine treatment on the DCX$^+$ cells with tertiary dendrites (c) and maturation (d) of newborn granule cells were measured after chronic corticosterone. Values are mean ± SEM ($n=4$–5/group). $**p<0.01$, $^{\#}p<0.05$, $^{\#\#}p<0.01$ *vs* corticosterone/vehicle group and corticosterone/fluoxetine group, respectively. (e) Representative image and traces from Sholl analyses of DCX$^+$ cells with tertiary branches after vehicle, chronic fluoxetine, chronic RS67333, and GR125487 in presence or not of fluoxetine in corticosterone-treated animals ($n=3$–4 mice/group, six cells/mouse). (f and g) Effects of chronic treatment with the 5-HT$_4$ ligands RS67333 or fluoxetine on the dendritic length (f) or the number of intersections (g) following a Sholl analysis. Values are mean ± SEM ($n=4$–5 mice/group). $**p<0.01$, $^{\#\#}p<0.01$ vs corticosterone/vehicle group and corticosterone/fluoxetine/GR125487 group, respectively.


Proliferation
a
Ki67-positive cells
4500
4000
3500
3000
2500
2000
1500
1000
500
0
**
*
##
p=0.0540
Maturation
b
Total DCX-positive cells
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
**
##
**
c
Total DCX-positive cells with tertiary dendrites
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
**
##
p<0.08
**
d
Maturation Index
0.6
0.5
0.4
0.3
0.2
0.1
0
**
**
#
**
e
Vehicle
Fluoxetine 18mg/kg/d
RS67333 1.5mg/kg/d
f
Dendritic length (µm)
50
45
40
35
30
25
20
15
10
5
0
** ##
10
50
100
150
Distance from soma (µm)
g
Dendritic intersection
4,5
4
3,5
3
2,5
2
1,5
1
0,5
0
** ##
10
50
100
150
Distance from soma (µm)
Vehicle
Fluoxetine 18mg/kg/d
RS67333 1.5mg/kg/d
GR125487 1mg/kg/d
Fluoxetine 18mg/kg/d + GR125487 1mg/kg/d
Vehicle
Fluoxetine 18mg/kg/d
RS67333 1.5mg/kg/d
GR125487 1mg/kg/d
Fluoxetine 18mg/kg/d + GR125487 1mg/kg/d

antidepressant-like effects comparable to those of fluoxetine in the chronic corticosterone model of anxiety/depression. Furthermore, we show that 5-HT$_4$ activation is necessary for the anxiolytic and antidepressant effects of fluoxetine in this model.

5-HT$_4$ Receptor Activation Facilitates the Maturation of Newborn Neurons in the Adult Hippocampus

To investigate the potential cellular mechanisms underlying the behavioral effects of the 5-HT$_4$ agonist RS67333, we next evaluated changes in adult hippocampal neurogenesis that may be relevant to antidepressant action (Surget *et al*, 2011).

In agreement with previous observations (David *et al*, 2009; Rainer *et al*, 2011), chronic fluoxetine exposure resulted in an increase in the number of dividing neural precursors as assessed by the number of Ki67-positive cells in the subgranular zone of the DG (one-way ANOVA, $^{*}p<0.05$, Figure 2a). The 5-HT$_4$ agonist, RS67333, also increased the number of neural precursors, but to a lesser extent than fluoxetine ($+51\%$ *vs* $+170\%$). The 5-HT$_4$ antagonist partially blocked the effect of chronic fluoxetine. These results suggest that 5-HT$_4$ receptors contribute to the effects of fluoxetine on proliferation, but that other 5-HT receptors are likely to be also involved.

We next assessed the number of young adult-born neurons in the DG that express DCX, a protein that is expressed for about a month after the birth of adult-born neurons (Couillard-Despres *et al*, 2005) (Supplementary Figure S3). We also subcategorized the DCX$^+$ cells according to their dendritic morphology: total number of DCX$^+$ cells and DCX$^+$ cells with complex, tertiary dendrites (Figure 2b–g). As previously described, chronic fluoxetine increased the number of DCX$^+$ cells with tertiary dendrites and the maturation index, defined as the ratio of DCX$^+$ cells possessing tertiary dendrites over the total DCX$^+$ cells in control animals (David *et al*, 2009) (one-way ANOVA, $^{**}p<0.01$, Figure 2b–d). Chronic treatment with RS67333 affected modestly both the total number of DCX$^+$ cells and also the number of DCX cells with tertiary dendrites.

The 5-HT$_4$ antagonist, GR125487, partially blocked the neurogenic effects of fluoxetine. However, while the effects of chronic fluoxetine on the number of DCX$^+$ cells with tertiary dendrites are larger than those of chronic 5-HT$_4$ receptor activation, the effect of these compounds on the maturation index is similar ($+51\%$ and 44% for fluoxetine and RS67333, respectively).

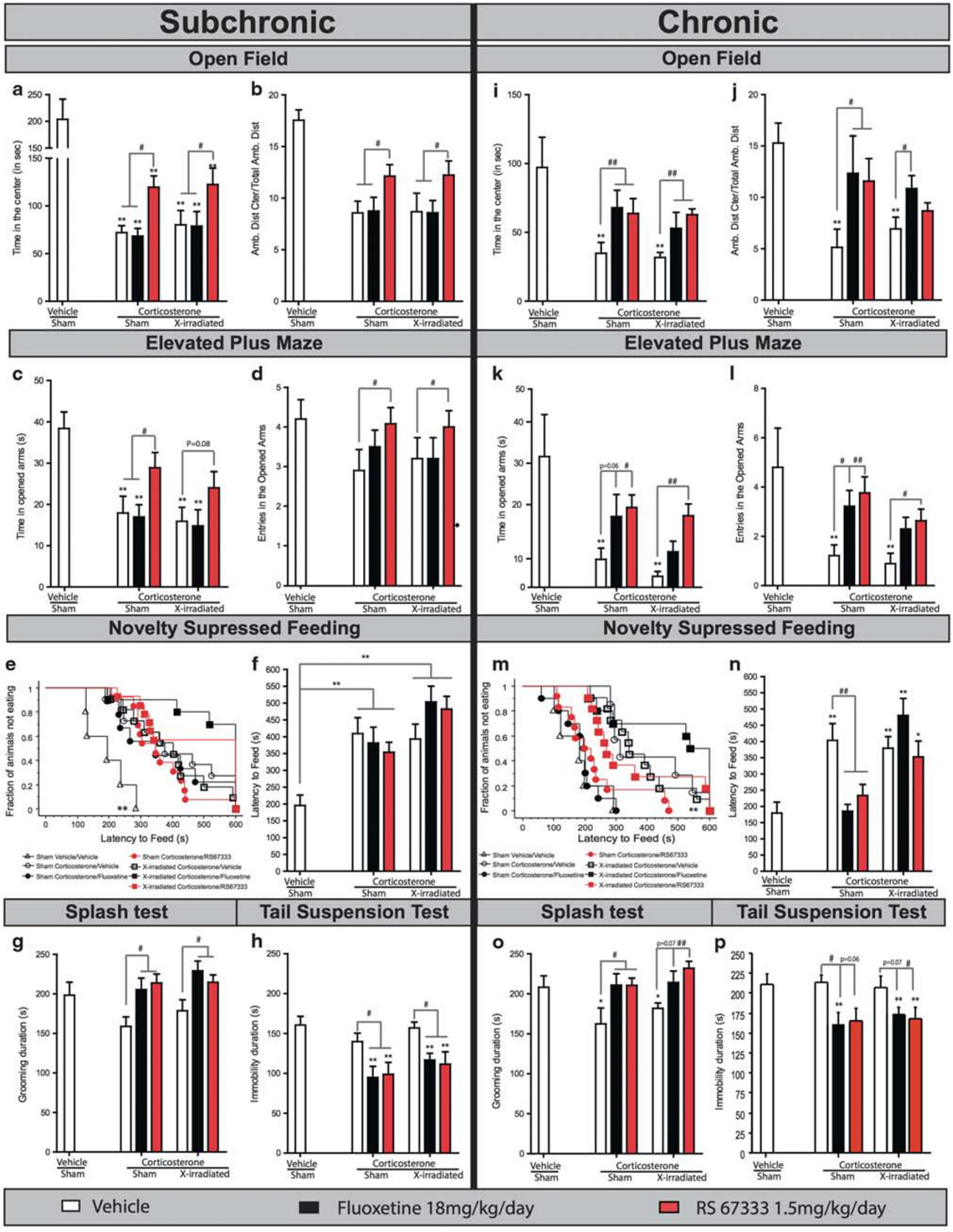
The dendrites of adult-born granule cells become progressively more complex during the first 4 weeks after their birth, a stage when the cells express DCX (Couillard-Despres *et al*, 2005). To further examine the effect of 5-HT$_4$ receptor activation on the dendritic morphology of newborn cells, we performed Sholl analyses on DCX$^+$ cells with tertiary dendrites. DCX$^+$ cells in chronic fluoxetine-treated and RS67333-treated animals displayed an increase in dendritic length (one-way ANOVA, $^{**}p<0.01$; Figure 2e and f) and in number of dendritic intersections (one-way ANOVA, $^{**}p<0.01$; Figure 2e and g). Fluoxetine-induced increase in dendritic complexity was abolished by a chronic treatment with the 5-HT$_4$ antagonist, GR125487.

Overall, these results suggest that 5-HT$_4$ receptor activation facilitates the maturation of newborn neurons in the adult hippocampus.

An Assessment of Causality Between the Neurogenic and Behavioral Effects of Short- and Long-Term Treatments with the 5-HT$_4$ Agonist in the Chronic CORT Model

As we have shown that long-term 5-HT$_4$ activation induced anxiolytic/antidepressant-like effects and facilitated maturation of newborn neurons, we decided to test the requirement of hippocampal neurogenesis for the emergence of behavioral changes after 5-HT$_4$ receptor activation in our CORT model. Moreover, a recent study in rats reported that the behavioral and neurogenic (proliferation of newborn cells) effects of the 5-HT$_4$ receptor agonist RS67333 occur after short-term administration (3–7 days depending on the paradigm) (Pascual-Brazo *et al*, 2012). Thus, we also investigated whether subchronic RS67333 treatment induced a rapid onset of behavioral effects. To address these points, mice were submitted to focal hippocampal

Figure 3 Neurogenesis-dependent and -independent effects of subchronic (7 days) or chronic (28 days) 5-HT$_4$ agonist treatment on corticosterone-induced behavioral changes in mice. (a and b/i and j) Effects of subchronic (a and b) or chronic (i and j) treatment with RS67333, a 5-HT$_4$ agonist, after focal X-irradiation of the mouse hippocampus on corticosterone-induced anxiety-like behaviors in the open field (OF) test. Anxiety is expressed as mean time spent in the center, in seconds, for the entire session (a or i), and also as the mean of percentage ambulatory distance in the center over total ambulatory distance traveled (b or j). Values are mean ± SEM ($n=9$–15 mice/group for corticosterone-treated animals and $n=5$ for vehicle/vehicle). $^{**}p<0.01$, $^{\#}p<0.05$, $^{\#\#}p<0.01$ *vs* control vehicle/vehicle group and corticosterone/RS67333 or corticosterone/vehicle group, respectively. (c and d/k and l) Effects of subchronic (c and d) or chronic (k and l) treatment with RS67333, a 5-HT4 agonist, after focal X-irradiation of the mouse hippocampus on corticosterone-induced anxiety-like behaviors in the elevated plus maze (EPM) paradigm. Anxiety is expressed as mean time in the open arms (c or k) and also as the mean entries in the open arms (d or l). Values are mean ± SEM ($n=9$–15 mice/group for corticosterone-treated animals and $n=5$ for vehicle/vehicle). $^{**}p<0.01$, $^{\#}p<0.05$ *vs* control vehicle/vehicle group and corticosterone/RS67333 or corticosterone/vehicle group, respectively. (e and f/m and n) Effects of subchronic (e and f) or chronic (m and n) treatment with RS67333, a 5-HT$_4$ agonist, after focal X-irradiation on corticosterone-induced anxiety- and depression-related behaviors in the novelty-suppressed feeding (NSF) paradigm. Results are cumulative survival of animals that have not eaten over 10 min (e or m) or mean ± SEM of latency to feed in seconds (f or n) ($n=9$–15 mice/group for corticosterone-treated animals and $n=5$ for vehicle/vehicle). $^{*}p<0.05$, $^{**}p<0.01$, $^{\#\#}p<0.01$ *vs* control vehicle/vehicle group and corticosterone/RS67333 or corticosterone/vehicle group, respectively. (g–o) Effects of subchronic (g) or chronic (o) treatment with RS67333, a 5-HT$_4$ agonist, after X-irradiation on behavior in the splash test (ST). Results are expressed as mean ± SEM duration of grooming after receiving a 10% sucrose solution on the snout ($n=9$–15/group for corticosterone-treated animals and $n=5$ for vehicle/vehicle). $^{*}p<0.05$, $^{\#}p<0.05$, $^{\#\#}p<0.01$, *vs* control vehicle/vehicle group and corticosterone/vehicle group, respectively. (h–p) Effects of subchronic (h) or chronic (p) treatment with RS67333, a 5-HT$_4$ agonist, after X-irradiation on behavior in the tail suspension test. Results are expressed as mean ± SEM immobility duration (in seconds) ($n=9$–15 mice/group for corticosterone-treated animals and $n=5$ for vehicle/vehicle). $^{**}p<0.01$, $^{\#}p<0.05$ *vs* control vehicle/vehicle group and corticosterone/vehicle group, respectively.


Subchronic
Chronic
Open Field
Open Field
Elevated Plus Maze
Elevated Plus Maze
Novelty Supressed Feeding
Novelty Supressed Feeding
Splash test
Tail Suspension Test
Splash test
Tail Suspension Test
Time in the center (in sec)
Amb. Dist Cter/Total Amb. Dist
Time in opened arms (s)
Entries in the Opened Arms
Fraction of animals not eating
Latency to Feed (s)
Grooming duration (s)
Immobility duration (s)
Vehicle
Sham
Corticosterone
Sham
X-irradiated
Vehicle
Fluoxetine 18mg/kg/day
RS 67333 1.5mg/kg/day

X-irradiation before the start of chronic corticosterone treatment alone or in combination with the 5-HT$_4$ agonist RS67333 (1.5 mg/kg/day) or fluoxetine (18 mg/kg/day) (Supplementary Figure S1B). These animals were subjected to anxiety- and depression-related tests first after 7 days of treatment and again after 28 days of treatment.

As previously described (David *et al*, 2009; Rainer *et al*, 2011), the chronic CORT paradigm resulted in an anxious/depressed-like phenotype. The efficacy of corticosterone model was assessed by comparing the behavioral phenotype of controls to corticosterone-treated mice (Figure 3). In anxiety-related tests, chronic corticosterone treatment had a marked effect on all anxiety parameters, resulting in decreased time spent in center and ratio of distance in center divided by total distance in the OF (one-way ANOVA, $*p<0.05$ or $**p<0.01$; Figure 3a, i and j), and in decreased time and entries in the open arms in the EPM (one-way ANOVA, $**p<0.01$, Figure 3c, k and l). In the ST, which is a depression-related test, chronic CORT resulted in a decrease in grooming (one-way ANOVA, $**p<0.01$; Figure 3o), and in the NSF test, which is related to both anxiety and depression, chronic CORT increased the latency to feed (Kaplan–Meier survival analysis, Mantel–Cox log rank test, $**p<0.01$; Figure 3e, f, m and n). As previously observed in a similar paradigm, the forced swim test (David *et al*, 2009; Rainer *et al*, 2011), chronic corticosterone treatment did not affect the immobility duration in the TST (Figure 3h and p), suggesting distinct underlying mechanisms between these tests and the OF, EPM, NSF, or ST.

The Rapid Anxiolytic and Antidepressant-Like Effects of a Subchronic 5-HT$_4$ Agonist Treatment do not Require Hippocampal Neurogenesis

A 7-day treatment with RS67333 produced anxiolytic and antidepressant-like effects in a battery of behavioral tests (Figure 3a–h). In the OF and EPM paradigms, all anxiety-related parameters were impacted. The time spent in the center (Figure 3a), the number of entries in the center (Supplementary Figure S5A), the ratio of center distance/total distance traveled (Figure 3b), the time spent in open arms (Figure 3c), and the number of entries in the open arms (Figure 3d) were increased after subchronic treatment with RS67333, regardless of whether the mice were exposed to X-irradiation or not (two-way ANOVA with significant treatment factor, $**p<0.01$; Figure 3a–c). In contrast, subchronic treatment with fluoxetine did not have an anxiolytic effect in the OF and EPM paradigms (Figure 3a–d). These results indicate that the anxiolytic effects of RS67333 have a faster onset than those of fluoxetine, and that these effects do not require adult hippocampal neurogenesis.

Interestingly, in the NSF test, neither subchronic RS67333 nor subchronic fluoxetine had an effect on latency to feed in both sham and X-irradiated groups (Figure 3e and f), indicating that in this test, the anxiolytic/antidepressant activity of RS67333 and fluoxetine require a longer treatment.

In the ST and TST (Figure 3g and h), after 11 and 12 days of administration, both RS67333 and fluoxetine increased grooming duration and decreased immobility duration, respectively (two-way ANOVA with significant treatment factor, $**p<0.01$). These antidepressant-like effects were not affected by focal hippocampal X-irradiation.

Altogether, these results demonstrate that, unlike fluoxetine, the 5-HT$_4$ agonist RS67333 elicits a rapid anxiolytic and antidepressant-like effect in all the paradigms tested (OF, EPM, ST, and TST) except the NSF. However, hippocampal neurogenesis is not required for these effects of RS67333.

In this study, we assessed the behavioral activity of RS67333 after both subchronic and chronic treatments. Thus, the same animals were tested after 7 and 28 days of treatment. It is therefore not surprising to observe changes in the basal values in control animals, as they have been exposed twice to behavioral tests. We have seen these effects of repeated testing routinely. For example, in the NSF paradigm, the latency to feed was decreased after a second exposure to the test (Wang *et al*, 2008). In the present study, we observed a decrease in the time spent in the center of the arena owing to the re-exposure to the test in all treated groups. However, the size of the anxiolytic-like effect of RS67333 remains the same between the first and the second exposure to the OF.

The Behavioral Effects of Long-Term 5-HT$_4$ Agonist Treatment are Mediated by Both Neurogenesis-Dependent and -Independent Mechanisms

As we previously demonstrated that chronic 5-HT$_4$ activation produced anxiolytic/antidepressant-like activity in the CORT model, we proceeded to investigate whether these behavioral effects require adult hippocampal neurogenesis.

The same battery of behavioral tests was performed again after 28 days of treatment with fluoxetine or RS67333 (Figure 3i–p). In the OF (Figure 3i and j) and the EPM (Figure 3k and l) paradigms, chronic RS67333 maintained the anxiolytic-like effect observed subchronically (two-way ANOVA with significant treatment factor, $*p<0.05$; Figure 3i–l). Chronic fluoxetine also elicited an anxiolytic-like effect, whereas it had no effect after subchronic treatment (two-way ANOVA with significant treatment factor, $*p<0.05$; Figure 3i and j). Moreover, these anxiolytic-like effects of RS67333 and fluoxetine were not affected by the ablation of adult hippocampal neurogenesis by X-irradiation.

In contrast, the anxiolytic/antidepressant-like effects of RS67333 and fluoxetine in the NSF paradigm were completely abolished by hippocampal X-irradiation (Figure 3m and n; Kaplan–Meier survival analysis, Mantel–Cox log rank test, $**p<0.01$, two-way ANOVA with significant interaction between irradiation and treatment, $**p<0.01$), indicating that these effects require adult hippocampal neurogenesis. Home-cage food consumption was not affected by drug treatment or irradiation (Supplementary Figure S5D).

In the ST and TST, long-term administration of RS67333 and fluoxetine induced an increase in grooming duration and a decrease in immobility duration that were not affected by focal X-irradiation (two-way ANOVA with significant treatment factor, $**p<0.01$ for both tests; Figure 3o and p).

Altogether, these results indicate that the effects of chronic treatment with RS67333 and fluoxetine in the

OF, EPM, ST, and TST are independent of hippocampal neurogenesis. In contrast, the anxiolytic/antidepressant-like effects of these compounds in the NSF test require neurogenesis.

DISCUSSION

Fast Anxiolytic Action of a 5-HT$_4$ Agonist

Most current antidepressant treatments are limited by a significant degree of nonresponsiveness among patients (Trivedi *et al*, 2006), delayed onset of therapeutic efficacy, and a number of side effects (Kato and Serretti, 2010). The development of new antidepressants is therefore of considerable importance (Wong *et al*, 2010), and understanding the mechanisms underlying the delayed onset should offer insights into new approaches. Recent studies as well as our present results indicate that 5-HT4 receptor agonists are faster acting than SSRIs (Lucas *et al*, 2007; Pascual-Brazo *et al*, 2012; Tamburella *et al*, 2009).

Although a 7-day treatment with fluoxetine or RS67333 induced antidepressant-like activity in the TST and ST, only the 5-HT$_4$ agonist RS67333 resulted in anxiolytic-like activity in the OF and the EPM. A longer duration of treatment (28 days) is required for fluoxetine to exert anxiolytic-like effects comparable to 7 days of RS67333 treatment. Although recent evidence indicates that 5-HT$_4$ receptors may represent a new target for antidepressant drugs (Bockaert *et al*, 2004; Lucas *et al*, 2007; Pascual-Brazo *et al*, 2012; Tamburella *et al*, 2009), the role of 5-HT$_4$ receptor ligands in anxiety is unclear. Discrepancies in results observed with 5-HT$_4$ receptor antagonists have been observed. In one study, the 5-HT$_4$ receptor antagonists, SDZ205-557, GR113808, and SB204070, administered acutely, failed to induce anxiolytic-like behavior in the light/dark choice test in mice (Costall and Naylor, 1997), whereas in two other studies, acute administration of the 5-HT$_4$ receptor antagonists, SB204070, GR113808 (Silvestre *et al*, 1996), and SB207266A (Kennett *et al*, 1997; Silvestre *et al*, 1996) had an anxiolytic-like effect in rats in the EPM. 5-HT$_4$ receptor knockout (KO) mice do not display an anxious or depressed-like phenotype, although an attenuated response to novelty may be relevant to mood disorders (Compan *et al*, 2004). In our hands, chronic treatment with GR125487 did not affect the anxiety-like phenotype induced by chronic corticosterone treatment. 5-HT$_4$ receptor agonists have mainly been tested in behavioral tests of antidepressant-like activity (see (Lucas, 2009) for review). Only one study investigated the effects of RS67333 in the OF paradigm over a 5-min period (Lucas *et al*, 2007). The authors showed that hyperlocomotion induced by olfactory bulbectomy was totally abolished after 14 days of RS67333 treatment in rats. To our knowledge, the present study is the first to clearly demonstrate fast anxiolytic-like activity of a 5-HT$_4$ receptor agonist in a mouse model of anxiety/depression. There is considerable evidence that RS67333 is a specific agonist of the 5-HT$_4$ receptor. Indeed, three studies have evaluated the effects of RS67333 in the presence of the selective 5-HT4 antagonist GR125487, and shown that the effects of RS67333 are blocked by GR125487. Lucas *et al* (2005) showed that the increase in 5-HT firing induced by RS67333 (1.5 mg/kg, acutely, during 3 or 21 days) is prevented by GR125487 administration. This effect of RS67333 on the firing of 5-HT neurons is likely to contribute to the phenotype we observe in the current study. Enhanced cognition induced by RS67333 was blocked by the 5-HT$_4$ receptor antagonist GR 125487 (1 mg/kg, intravenous) (Freret *et al*, 2012; Lamirault and Simon, 2001). Both studies tested the specificity of the effects of RS67333 in the Novel Object Recognition test by using the highly potent and selective 5-HT$_4$ receptor antagonist, GR125487. They found that 1 mg/kg of GR125487, which had no effect *per se* on the discrimination index, totally reversed the beneficial effects of RS67333, arguing thus that these effects of RS67333 are mediated by 5-HT$_4$ receptor.

Interestingly, in the NSF test, both fluoxetine and RS67333 had an anxiolytic/antidepressant-like effect only after chronic treatment, suggesting that the neurobiological mechanisms involved in this paradigm are different from those underlying the other tests (OF, EPM, TST, and ST). Indeed, we show that the effects of RS67333 and fluoxetine in the OF, EPM, TST, and ST are independent of hippocampal neurogenesis, whereas the effects of these compounds in the NSF test require neurogenesis. This is in agreement with our previous study showing both neurogenesis-dependent (NSF) and independent (OF, forced swim test) effects of fluoxetine in the CORT model (David *et al*, 2009). It is also noteworthy that the only test (NSF) that requires neurogenesis is also the one that requires a chronic administration. This observation is likely related to the fact that young adult-born neurons take several weeks to mature and that the critical period during which adult-born neurons contribute to behavior extends from 4 to 6 weeks after their birth (Denny *et al*, 2012). Interestingly, the effect of the 5-HT$_4$ agonist RS67333 on proliferation of neural precursors are weaker than those of fluoxetine, whereas the effects of RS67333 on the maturation of young neurons are similar to those of fluoxetine. Newborn neurons undergo an accelerated maturation after chronic fluoxetine treatment (Wang *et al*, 2008) and possibly also after 5-HT$_4$ receptor activation. These results suggest that the neurogenesis-dependent effect of RS67333 and fluoxetine in the NSF test is more likely to result from increased maturation than from increased proliferation.

The fast onset of action of the 5-HT$_4$ receptor agonist could be a consequence of an increase in serotonergic output to projection areas (Lucas *et al*, 2005; Lucas and Debonnel, 2002). Indeed, by measuring spontaneous electrical activity in mice lacking 5-HT$_4$ receptors, Conductier *et al* (2006) demonstrated that 5-HT$_4$ receptors exert a tonic positive influence on the firing activity of dorsal raphe nucleus 5-HT neurons, and previous studies have shown that 5-HT$_4$ receptor activation by selective agonists modulates central 5-HT neurotransmission, increasing the firing of dorsal raphe nucleus 5-HT neurons (Lucas and Debonnel, 2002). There is also accumulating evidence that cortical regions are involved in 5-HT4-induced anxiolytic/antidepressant-like activities (Lucas *et al*, 2005) (for review see also (Lucas, 2009)). 5-HT$_4$ receptors in the prefrontal cortex control the firing rate of midbrain serotonergic neurons via descending inputs (Lucas *et al*, 2005), and their activation leads to increases in serotonin release in projection sites including the hippocampus (Ge and Barnes, 1996).

Requirement of 5-HT$_4$ Receptors for the Behavioral and Neurogenic Effects of SSRIs

A blockade of 5-HT$_4$ receptors with the antagonist GR125487 prevented the anxiolytic and antidepressant-like effects of fluoxetine. These results show that 5-HT$_4$ receptor activation is necessary for the behavioral effects of SSRIs. Our results are consistent with a previous study showing a specific induction of 5-HT$_4$ expression after SSRI treatment (Schmidt *et al*, 2012). SSRIs are thought to act as indirect agonists of 5-HT$_4$ receptors rather than direct agonists. Using the NIMH Psychoactive Drug Screening Program database, we did not find any study looking at the binding affinity of fluoxetine at the mouse 5-HT$_4$ receptor. The only study looking at binding affinities of fluoxetine for serotoninergic receptor demonstrated negligible binding of fluoxetine to the 5-HT$_4$ receptor in pig striatal membranes (Lucchelli *et al*, 1995). In addition, quantitative autoradiography revealed that the binding of the 5-HT$_4$ receptor ligand [^{3}H]GR113808 was not significantly changed in fluoxetine-treated mice (Kobayashi *et al*, 2012). Thus, in the present study, the anxiolytic/antidepressant-like effects of fluoxetine likely resulted from an indirect activation of the 5-HT4 receptor through an increase in endogenous 5-HT levels in the synaptic cleft following the blockade of the selective serotonin transporter.

However, it is unlikely that 5-HT$_4$ receptor activation alone can be responsible for all SSRIs-mediated anxiolytic/antidepressant-like activity. Among the 14 known 5-HT receptor subtypes, the 5-HT$_{1A}$ receptor has been prominently implicated in the modulation of mood and anxiety-related behaviors (Santarelli *et al*, 2003). 5-HT$_{1A}$ receptor KO mice were insensitive to the behavioral effects of chronic fluoxetine, suggesting that activation of 5-HT$_{1A}$ receptors is also a critical component in the mechanism of action of SSRIs. Recent data also suggest a potential non-cell autonomous mechanism by which serotonin regulates neurogenesis and the response to antidepressants through 5-HT$_{1A}$ receptor (Samuels, personal communication). However, we cannot rule out adaptive changes in the serotoninergic system, including variations in 5-HT$_4$ receptor levels, which could explain the absence of behavioral effects of fluoxetine in 5-HT$_{1A}$ receptor KO mice. Indeed, decreases in the density of the serotonin transporter (5-HTT) were measured in several brain regions of these 5-HT$_{1A}$ mutant mice (Ase *et al*, 2001), and a recent study described that variation in serotonin transporter expression could cause adaptive changes in 5-HT$_4$ receptor levels in serotonin transporter overexpressing mice (Jennings *et al*, 2012).

SSRIs are potent stimulators of adult hippocampal neurogenesis (Klempin *et al*, 2010; Santarelli *et al*, 2003). However, the role of each serotoninergic receptor in the neurogenic effects of SSRIs is still a matter of investigation. We have showed that the 5-HT$_4$ agonist, RS67333, increased neurogenesis (proliferation and maturation) to a lesser extent than fluoxetine, and that the 5-HT$_4$ antagonist, GR125487, partially blocked neurogenic effects of chronic fluoxetine. These results suggest that 5-HT$_4$ receptors contribute to the effects of fluoxetine on proliferation and maturation of newborn neurons, but that other 5-HT receptors are likely to be involved. Pharmacological manipulations suggested that 5-HT$_{1A}$ receptors are involved in proliferation of precursor cells, whereas 5-HT$_2$ receptors affect both proliferation and promote neuronal differentiation (Klempin *et al*, 2010). Moreover, fluoxetine had no effect on neurogenesis (proliferation and survival) in 5-HT$_{1A}$ KO mice (Santarelli *et al*, 2003).

These results suggest that both 5-HT$_4$ and 5-HT$_{1A}$ receptors contribute to the effects of SSRIs on behavior and neurogenesis. Interestingly, both receptors are expressed in the DG, which may be the site responsible for their effects on neurogenesis. Recently, it has been suggested that 5-HT$_4$ receptor activation may also be involved in antidepressant-induced dematuration of mature dentate granule cells (Kobayashi *et al*, 2010). The exact mechanisms underlying in this phenomenon still needs further investigations. However, our results also show that most effects of SSRIs and 5-HT$_4$ agonists do not require hippocampal neurogenesis. Examining effects of tissue-specific manipulations of these receptors will be important to identify the circuits responsible for their fast acting anxiolytic and antidepressant actions.

CONCLUSIONS

Taken together, our results show, for the first time, in a mouse model of anxiety/depression, that a 5-HT$_4$ receptor agonist may be a fast-acting anxiolytic agent, and that 5-HT$_4$

Table 1 Neurogenesis-dependent and Independent Mechanism Involved in the Behavioral Effects of Subchronic and Chronic 5-HT$_4$ Agonist Treatment

Fluoxetine (18 mg/kg/day)		RS67333 (1.5 mg/kg/day)	
Subchronic	**Chronic**	**Subchronic**	**Chronic**
Open Field			
ϕ	+	+	+
/	Neurogenesis-independent	Neurogenesis-independent	Neurogenesis-independent
Elevated Plus Maze			
ϕ	+	+	+
/	Neurogenesis-independent	Neurogenesis-independent	Neurogenesis-independent
Novelty Suppressed Feeding			
ϕ	+	ϕ	+
/	Neurogenesis-dependent	/	Neurogenesis-dependent
Tail Suspension Test			
+	+	+	+
Neurogenesis-independent	Neurogenesis-independent	Neurogenesis-independent	Neurogenesis-independent
Splash Test			
+	+	+	+
Neurogenesis-independent	Neurogenesis-independent	Neurogenesis-independent	Neurogenesis-independent

Summary of effects seen in multiple behavioral tests throughout the study: ϕ, no effect; +, anxiolytic/antidepressant-like effects.

stimulation is necessary for the behavioral and neurogenic effects (proliferation and maturation) of fluoxetine, a classic SSRI antidepressant. Furthermore, we showed that, with the exception of the NSF test, the anxiolytic and antidepressant-like effects of the 5-HT$_4$ agonist were independent of hippocampal neurogenesis (Table 1).

The present study is encouraging for the development of RS-67333 as an anxiolytic/antidepressant compound for use in patients. However, the use of the 5-HT$_4$ receptor as a novel antidepressant target may be hampered by the fact that it also has important roles outside the central nervous system, for example, in the heart, gastrointestinal tract, adrenal gland, and urinary bladder (Tonini and Pace, 2006), which may prevent its development as an effective anxiolytic/antidepressant drug (Bockaert *et al*, 2004, 2008). Thus, signaling molecules that interact with the 5-HT$_4$ receptor such as P11 (Egeland *et al*, 2011; Warner-Schmidt *et al*, 2009) may represent novel targets for fast-acting anxiolytic/antidepressant treatments. There is indeed recent evidence that cortical neurons that express both P11 and 5-HT$_4$ receptors are involved in the behavioral effects of SSRIs (Schmidt *et al*, 2012), and that chronic treatment with fluoxetine results in an increase in 5-HT$_4$ receptor expressions in cortical neurons (Schmidt *et al*, 2012).

FUNDING AND DISCLOSURE

This work was also supported by Young Investigator Award to DJD (The Brain & Behavior Research Foundation). Dr DJD currently received investigator-initiated research support from Lundbeck and served as a consultant in the areas of target identification and validation and new compound development to Lundbeck, Roche, and Servier. Dr RH receives compensation as a consultant for Roche, Lundbeck and Servier in relation to the generation of novel antidepressants.

ACKNOWLEDGEMENTS

This work was supported by the technical assistance of Valerie Dupont-Domergue and staff from the animal care facility of the Institut Federatif de Recherche-IFR141 of the Paris-Sud University. We also thank Dr Jean-Philippe Guilloux for his help designing Figure 2.

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Supplementary Information accompanies the paper on the Neuropsychopharmacology website (http://www.nature.com/npp)