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Original Paper

Effects of [Nphe^1, Arg^{14}, Lys^{15}] N/OFQ-NH$_2$ (UFP-101), a potent NOP receptor antagonist, on molecular, cellular and behavioural alterations associated with chronic mild stress

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Abstract

The present study investigated the effect of [Nphe^1] Arg^{14}, Lys^{15}-N/OFQ-NH$_2$ (UFP-101), a selective NOP receptor antagonist, in chronic mild stress (CMS) in male Wistar rats. NOP receptor antagonists were reported to elicit antidepressant-like effects in rodents. Our aim was to investigate UFP-101 effects on CMS-induced anhedonia and impairment of hippocampal neurogenesis. UFP-101 (10 nmol/rat intracerebroventricularly) did not influence sucrose intake in non-stressed animals, but reinstated basal sucrose consumption in stressed animals from the second week of treatment. UFP-101 also reversed stress effects in forced swimming test and in open field. Fluoxetine (10 mg/kg intraperitoneally) produced similar effects. Moreover, we investigated whether UFP-101 could affect CMS-induced impairment in hippocampal cell proliferation and neurogenesis, and in fibroblast growth factor (FGF-2) expression. Our data confirm that CMS reduced neural stem cell proliferation and neurogenesis in adult rat hippocampus. Chronic UFP-101 treatment did not affect the reduced proliferation (bromodeoxyuridine-positive cells) observed in stressed animals. However, UFP-101 increased the number of doublecortin-positive cells, restoring neurogenesis. Finally, UFP-101 significantly increased FGF-2 expression, reduced by CMS. These findings support the view that blockade of NOP receptors produces antidepressant-like effects in CMS associated with positive effects on neurogenesis and FGF-2 expression. Therefore, NOP receptors may represent a target for innovative antidepressant drugs.

Keywords

UFP-101, depression, chronic mild stress, hippocampal neurogenesis, FGF-2

Introduction

The opioid peptidergic system, consisting of nociceptin/orphanin FQ (N/OFQ) and its receptor (NOP), modulates several high functions including response to stress, anxiety and depression. In particular, the blockade of NOP receptors, using different compounds, has been shown to evoke antidepressant-like actions in various species and assays (Asth et al., 2016; Gavioli et al., 2003, 2004; Holanda et al., 2016; Medeiros et al., 2015; Post et al., 2016; Rizzi et al., 2007). A novel NOP receptor antagonist, LY2940094, recently showed antidepressant-like efficacy in patients with major depressive disorders (Post et al., 2016).

Electrophysiological, immunohistochemical and neurochemical studies point to an important role played by monoaminergic systems, particularly the 5-hydroxy-tryptamine (5-HT) one, in mediating the antidepressant-like properties of NOP antagonists. However, other mechanisms of action have been hypothesized, including modulation of the hypothalamic–pituitary–adrenal axis (HPA), of the circadian rhythm and of the neuroendocrine-immune control (Gavioli and Calò, 2013).

Genetic evidence also indicates that endogenous N/OFQ-NOP receptor signalling is crucially involved in despair-like behaviour in rodents. Mice and rats knockout for the NOP receptor gene display an antidepressant-like phenotype in specific tests for depression, NOP receptor antagonist effects

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being mediated by the NOP receptor (for a review see Gavioli and Calò, 2013).

Prolonged exposure of experimental animals to mild stressors brings about changes in animal behaviour, including anhedonia (Willner et al., 1992, 1997). The chronic mild stress (CMS) model has been extensively used to study behaviours associated with depression and antidepressant effects of drugs (Wiborg, 2013; Willner, 2005) and possesses face, construct and predictive validity. All the major classes of antidepressant drugs, when chronically administered, reverse reward deficits in this model (Abelaira et al., 2013). In a previous pivotal experiment we have submitted rats to CMS and then to a chronic treatment with N/OFQ (Arletti et al., 2005). N/OFQ displayed no effect in sucrose preference test, body weight gain and behavioural (forced swimming test (FST) or open field) tests if compared with stressed non-treated rats. We have also previously shown that the N/OFQ-NOP system plays an important role in mood control via modulation of central 5-HT neurotransmission: repeated administration of a NOP antagonist, [Nphe^{1}] Arg^{14}, Lys^{15}-N/OFQ-NH$_2$ (UFP-101), restored behavioural and neurochemical alterations evoked by CMS in a manner similar to the reference antidepressant imipramine (Vitale et al., 2009). UFP-101 is a fairly potent (pA2: 7.0–7.5) and highly selective NOP receptor antagonist (Calò et al., 2002), and one of the most widely used NOP receptor ligands (Calò et al., 2005; Toll et al., 2016).

In the present work, we used osmotic minipumps to ensure constant, reproducible and long-term delivery of UFP-101. Compared with repeated injections, minipumps have the advantages of reducing animal stress and diminishing the risk of infections. We submitted rats to multiple behavioural tests (sucrose consumption, FST and open field) to evaluate the degree of depression-like effects and their reversal by the tested drugs. Moreover, we measured corticosterone (CORT) serum levels. Corticosteroids can mediate a variety of effects on neuronal excitability, neurochemistry and structural plasticity (De Kloet et al., 1998; McEwen, 1999; Sapolsky, 2000). Stress and glucocorticoid hormones produce short- and long-term effects on brain function, which can involve the regulation of specific neurotrophic factors. Prolonged exposure to stress hormones can damage specific brain structures, like the hippocampus, thus determining persistent functional deficits (McEwen and Sapolsky, 1995). Most of these events occur in the context of ongoing neuronal activity (McEwen, 1999).

It is well known that the therapeutic effect of antidepressant treatments requires weeks to become clinically detectable, whereas an increase in monoamine levels occurs within hours of administration. A proposed explanation for this delay may be that the initial effects on monoamines trigger a time-dependent cascade of effects leading to modulation of adult hippocampal neurogenesis (Abrous et al., 2005), which contributes to functional plasticity (Lledo et al., 2006) under both physiological and pathological conditions. Thus, we examined the effects of CMS on neural stem cell proliferation and neuronal differentiation in the hippocampus of adult rats after continuous intracerebroventricular (i.c.v.) infusion of UFP-101. Bromodeoxyuridine (BrdU), a thymidine analogue, was used as a marker of mitotic cells to determine proliferation, whereas differentiation and survival of newborn cells was estimated using doublecortin (DCX), an early marker of commitment to neuronal differentiation of immature neurons. Fluoxetine was used as a positive control antidepressant, which is well known to be active at a dose of 10 mg/kg (intraperitoneally (i.p.)) in this model (First et al., 2011; Ge et al., 2015; Perez-Caballero et al., 2014; Zhao et al., 2015a, 2015b).

Changes in neurogenesis may depend on alterations in the expression and availability of specific neurotrophic factors (NTFs). To begin exploring the possibility that UFP-101 may mediate its effects through modulation of NTFs, we examined hippocampal fibroblast growth factor (FGF-2) expression. FGF-2 is the most potent agent capable of inducing proliferation of hippocampal progenitors (Becq et al., 2005; Nakatomi et al., 2002).

FGF-2 stimulates neonatal and adult neurogenesis and can reactivate a latent neurogenic programme in neural stem cells from different regions of the adult central nervous system (Kuhn et al., 1997; Palmer et al., 1999). In line with these findings and relevant to the present work, decreased hippocampal FGF-2 levels and depressive-like responses have been reported in rodents after prolonged chronic restraint stress (Cheng et al., 2015).

Materials and methods

Animals

In vivo studies have been reported according to the ARRIVE guidelines (Kilkenny et al., 2010). Protocols were approved by Ethic Committees for Animal Use of the University of Modena-Reggio and the Italian Ministry of Health. Forty-eight male Wistar rats, weighing 180–200 g at the beginning of the experiments, were housed in conventional cages in groups of four under controlled standard conditions (free access to food and water; 12-h dark/light cycle; temperature 22±1°C; humidity 60%). Ethical guidelines for investigation of experimental pain in conscious animals were followed; all experiments were conducted in conformity with the European Directive (EEC No. 86/609) and the Italian D.L. 27/01/1992, No.116.

Chronic mild stress procedure and sucrose intake

The experimental protocol employed in this study is shown in Figure 1(a). After one week of adaptation, animals were placed in a soundproof room in single cages and subjected to the behavioural experiments according to our previously validated method (Vitale et al., 2009). Following the baseline tests (week –1), animals were divided into six groups (eight rats per group) matched on the basis of their mean sucrose intake so that the starting means ± SEM of sucrose intake were not significantly different among the different groups. Four groups of rats were exposed to CMS, whereas two groups were not stressed except for the food and water deprivation that preceded each sucrose preference test (for stress protocol details see Table 1 in Vitale et al., 2009). Sucrose intake was calculated as the percentage of consumption of the sucrose solution over the average value of its intake by the same animal, during the baseline pre-tests. Body weight was monitored once a week, every Monday morning.

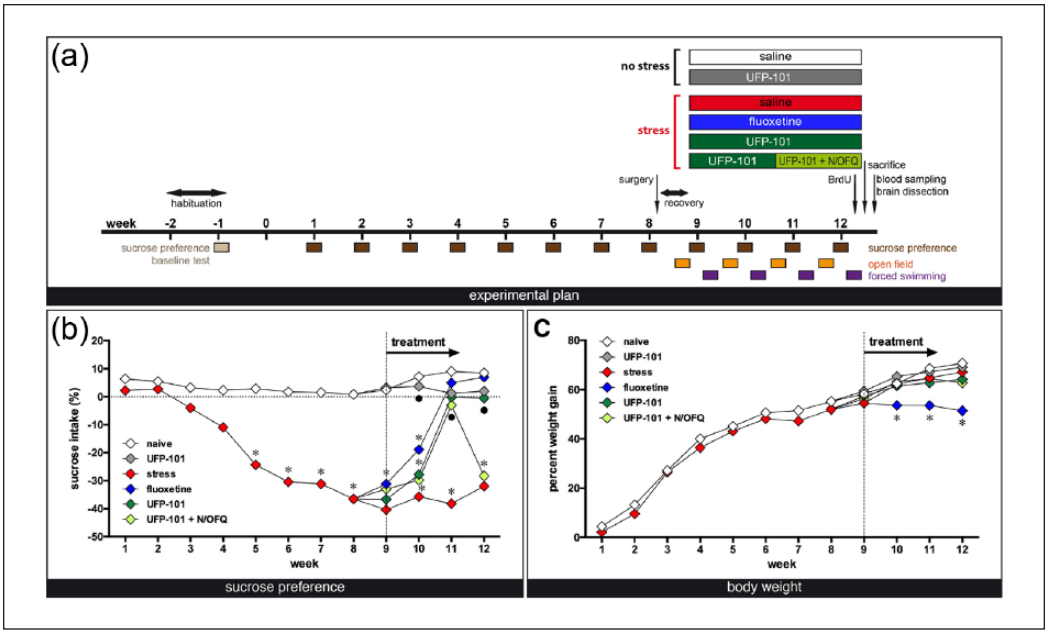

Figure 1. **(a)** Experimental plan. See text for details. Note that non-stressed (defined as naïve) animals received only two of the four treatments, namely saline or UFP-101. **(b)** Sucrose solution intake in rats exposed to chronic mild stress. Treatments started after nine weeks of stress exposure. Non-stressed and stressed animals received saline. Fluoxetine was administered at a dose of 10 mg/kg (i.p.), UFP-101 (10 nmol/rat) was continuously infused i.c.v. N/OFQ (5 nmol/rat) was daily injected i.c.v. together with UFP-101 in the last 10 days only, that is, starting 14 days after treatment with UFP-101 alone. For each group, values represent the per cent ratio of sucrose solution consumption over the average value of the sweetened solution intake by the same animal during the baseline pre-tests (see panel (a)). Data are means of eight animals per group. For clarity, SEM bars, always < 10% of the mean, have been omitted. Data from the non-stressed and stressed subgroups, foregoing the onset of treatment, have been pooled together. *p < 0.05 compared with non-stressed + saline, same week; p < 0.05 compared with stressed + saline, same week. ANOVA followed by the LSD multiple comparison test. **(c)** Per cent increase in body weight in rats exposed to CMS. Data are means of eight animals per group. For clarity, SEM bars, always < 10% of the mean, have been omitted. Data from the non-stressed and stressed subgroups that preceded the onset of treatment have been pooled together. *p < 0.05 compared with non-stressed + saline, same week. ANOVA followed by the LSD multiple comparison test.

UFP-101: [Nphe^1] Arg^{14}, Lys^{15}-N/OFQ-NH_2; i.p.: intraperitoneal; i.c.v.: intracerebroventricular; N/OFQ: nociceptin/orphanin FQ; ANOVA: analysis of variance; LSD: least significant difference; CMS: chronic mild stress

Surgery

After eight weeks of continuous exposure to the unpredictable sequence of mildly stressful situations, when sucrose consumption was significantly reduced in all groups of stressed animals, all rats (stressed and non stressed) were implanted with properly activated Alzet osmotic minipumps (Alzet, Cupertino, CA, USA) in order to allow continuous i.c.v. drug administrations. Rats were anaesthetized with ketamine and xylazine (i.p.; 115 + 2 mg/kg; Farmaceutici Gellini, Aprilia, Italy and Bayer, Milan, Italy) and positioned in a stereotaxic apparatus. Stainless-steel guide cannulae were implanted in the right lateral ventricle (from the bregma: AP = −0.8 mm; L = 1.4 mm; V = 3.25 mm) and secured to the skull using acrylic dental cement and one screw. Minipumps were then subcutaneously implanted in the dorsal area and connected with the brain infusion kit II (Alzet) to allow a constant flow of 0.25 µL/h, which provided a dosage of 10 nmol per day. A second guide cannula (Plastics One Inc., Roanoke, VA, USA) was symmetrically inserted in the left lateral ventricle to permit double treatments (saline or N/OFQ). We have chosen saline as vehicle for either continuous or intermittent infusion, as previously experimented, and not the artificial cerebrospinal fluid (preferred for i.c.v. infusions) due to the nature of the peptides dissolved and to the critical issues of maintaining drug homogeneity, compatibility and stability throughout the whole duration of the minipump delivery.

A two/three day recovery period was allowed before any further procedure.

Treatments

On week 9, the six groups of stressed (undergoing the CMS procedure) or non-stressed rats were assigned in a randomized manner to one of the following treatments:

Stressed rats: (1) sterile saline (continuous i.c.v. infusion) for 24 days; (2) fluoxetine (10 mg/kg i.p.) for 24 days; (3) UFP-101 (10 nmol i.c.v.) for 24 days; (4) UFP-101 (10 nmol i.c.v.) for 14 days, then co-administration of UFP-101 and N/OFQ (5 nmol i.c.v.) for the subsequent 10 days.

Non-stressed rats: (5) sterile saline (continuous i.c.v. infusion) for 24 days; (6) UFP-101 (10 nmol i.c.v.) for 24 days.

The sucrose preference tests were performed at day –1 (baseline) and 7, 14 and 21 days after the beginning of the treatments. Non-stressed rats continuously treated with UFP-101 were used for evaluation of possible primary effects of the

drug; the dose of 10 nmol was chosen on the basis of our previous results (Vitale et al., 2009) and literature data (Gavioli and Calò, 2006). Non-stressed rats continuously treated with saline represent the reference group to evaluate stress-induced effects. The double treatment with N/OFQ was continued for 10 days to ensure reversal of UFP-101 effects (Vitale et al., 2009).

On day 24 from the beginning of the treatments control and CMS rats were sacrificed by decapitation. Blood was collected for CORT assay and brains were rapidly removed, formalin fixed and paraffin-embedded for histological analysis.

Drugs

The heptadecapeptide N/OFQ was purchased from Bachem (Merseyside, UK). [Nphe^{1}] Arg^{14}, Lys^{15}-N/OFQ-NH_2 (UFP-101) was synthesized and purified as previously described (Guerrini et al., 2005). Fluoxetine hydrochloride and all reagents were from Sigma Chemicals Co. (Milan, Italy). Peptides and fluoxetine were dissolved in sterile saline.

FST

The FST was performed on days 2, 9, 16 and 23 after the beginning of the treatments. The FST (Porsolt et al., 1977, 1978) is commonly used to assess antidepressant-like activity of new compounds. A 15-min training session was followed, after 24 h, by a 5-min test session only before the first exposure to the test, according to the Porsolt method version for repeated analyses. Three behavioural parameters, previously shown to be reliable and validated for detection of antidepressant drug effect (Detke et al., 1995), were scored during the 5-min test period: 1) immobility time (i.e. the time spent floating in the water without struggling, making only those movements necessary to keep the head above the water); 2) swimming time (i.e. the time spent making active swimming motions to move around in the cylinder); 3) climbing time (i.e. the time spent making active movements with forepaws in and out of the water, directed to the cylinder wall). Increased passive behavioural responses in FST are thought to be indicative of depressive-like symptomatology (Detke et al., 1997; López-Rubalcava and Lucki, 2000).

Open field test

The open field test was performed on days –2, 6, 13 and 20 with respect to the beginning of the treatments. Rats were transferred to the test room 1 h before testing for acclimatization. One rat at a time was introduced into the arena (50 cm × 50 cm × 30 cm) and its behaviour was recorded for 5 min. Horizontal (the number of total floor sections crossed) and vertical (rearing) activities were recorded (Overstreet, 2012).

Serum corticosterone assay

All blood samples were collected from the trunk after rat decapitation and processed as previously described (Vitale et al., 2009). Taking into account the circadian rhythm of CORT, all sacrifices were carried out between 12:00 h and 14:00 h, that is, during the diurnal period. Assessment of serum CORT was performed by means of enzyme immunoassay using a commercially available kit (DetectX Arbor Assays, MI, USA) which uses a microplate reader set at 450 nm. Serum samples were diluted 1:100 in appropriate assay buffer in order to fall within the calibration curve range and assayed in duplicate. The detection limit of the assay was 16.9 pg/mL; intra- and inter-assay coefficients of variations were 5.2% and 7.9% respectively.

BrdU in vivo labelling and immunohistochemistry

On the last day of treatment, rats were administered a series of four BrdU injections (50 mg/kg i.p., dissolved in propylenic glycol water; Boehringer Mannheim, Germany), every 2 h over a period of 6 h. Animals were sacrificed 24 h after the last BrdU injection. After sacrifice, brains were rapidly removed, immersed in 10% formalin and then paraffin embedded. Coronal sections (10 μm thick) were cut at the level of the dorsal hippocampus and mounted into poly-lysine-coated slides.

BrdU immunohistochemistry was performed according to Zucchini and coworkers (2008). Briefly, sections were deparaffinized (2 × 10-min washes in xylol, 5 min in 100% ethanol, 5 min in 95% ethanol) and then rehydrated in distilled water for 5 min and in 1× phosphate-buffered saline (PBS) for 10 min. DNA was denatured by incubating tissue sections in 2 M HCl for 30 min at 37°C and then rinsed three times for 5 min each in 1× PBS and treated with H_2O_2 3V to block endogenous peroxidase. They were then washed in 1× PBS and incubated with Ultra V Block (Ultra Vision Detection System; Lab Vision Corporation, Fremont, CA, USA) for 5 min at room temperature. Sections were incubated with a primary antibody (diluted in PBS 1×) for BrdU (mouse monoclonal, 1:100; Boehringer Mannheim, Monza, Italy) overnight at room temperature in a humid atmosphere. After 5-min rinses in PBS, sections were incubated with biotinylated secondary antibody (Ultra Vision Detection System; Lab Vision Corporation) for 10 min, washed again in PBS for 5 min, and then incubated for 10 min in streptavidin peroxidase. The reaction product was detected as a brown substrate using a 3,3-diaminobenzidinetetrahydrochloride (Sigma Chemicals Co.) in a solution containing PBS 1× and H_2O_2 24V. Finally, sections were washed three times in PBS 1× (5 min each), counterstained with hematoxylin for 2 min and washed again in PBS 1× (5 min). Coverslips were mounted using Gel/mount (Biomeda Corp., Foster City, CA, USA). The specificity of immunolabelling was verified in all experiments by controls in which the primary antibody was omitted.

Quantification of BrdU immunohistochemistry experiments was performed in a blinded manner by two investigators, counting the number of BrdU-positive cells in the granular layer and in the hilus of the dorsal hippocampus dentate gyrus (Paradiso et al., 2009). Paraffin-embedded brains were cut in successive 10 μm sections across the entire dorsal hippocampus (i.e. 260 sections, corresponding to 2600 μm) (Paxinos and Watson, 2005). One section every 52 was used for BrdU immunohistochemistry, that is, five sections per animal were examined using a Leica microscope (DMRA2, Leica, Wetzlar, Germany). An estimate of the total BrdU-positive cells was obtained by multiplying the number of BrdU-positive cells in each section by 52 and summing the five resulting counts.

DCX immunofluorescence

Sections adjacent to those used for BrdU immunostaining were deparaffinized, rehydrated and unmasked as described above. After washing in PBS 1×, they were incubated with Triton (0.3% in PBS 1×, room temperature, 10 min), washed twice in PBS 1× and incubated with 5% serum of the species in which the secondary antibody was produced, for 30 min. They were then incubated with an anti-DCX (goat anti-rat) primary antibody diluted 1:25 in PBS 1× overnight at 4°C (Millipore, Billerica, MA, USA). The following day, sections were washed twice for 5 min in PBS 1×, incubated with Triton 0.3% for 30 min, washed in PBS 1×, and incubated with a Texas Red-conjugated donkey anti-goat secondary antibody (1:25 dilution; Jackson ImmunoResearch, West Grove, PA, USA) at room temperature for 3.5 h. After staining, sections were washed in PBS 1× for 10 min, counterstained with 0.0001% 4′,6-diamidino-2-phenylindole for 15 min, and washed again. Coverslips were mounted using Gel/Mount (Biomeda Corp. Foster City, CA, USA). The quantification of the number of DCX-positive cells in the dentate gyrus was performed as described above for BrdU.

FGF-2 immunohistochemistry

The FGF-2 immunohistochemistry protocol was followed as previously described (Zucchini et al., 2008). Sections were deparaffinized and then rehydrated as described above, and the FGF-2 antigen was unmasked using a commercially available kit (Unmasker, Diapath, Martinengo, BG, Italy), according to the manufacturer's instructions. After washing in PBS 1× for 5 min, sections were incubated overnight at 4°C in humid atmosphere with the primary antibody for FGF-2 (mouse monoclonal, 5 μg/mL in PBS 1×; BD Transduction Laboratories, San Jose, CA, USA). After rinsing in PBS 1×, they were incubated with biotinylated goat anti-polyvalent serum (Ultra Vision Detection System; Lab Vision Corporation, Fremont, CA, USA) at room temperature for 10 min, washed in PBS 1× for 5 min and then incubated in streptavidine peroxidase following the above-mentioned immune-histochemical protocol. The specificity of immunolabelling was verified in all experiments by controls in which the primary antibody was omitted.

Image analysis was conducted using the DMRA2 Leica microscope equipped with the Metamorph Image Analysis software (Universal Imaging Inc., Downingtown, PA, USA). The expression levels of FGF-2 were measured using a thresholding approach (Mazzuferi et al., 2010) by investigators that were blind to the group to which the rats belonged. Images of the hippocampus were captured using a Hamamatsu C11440 camera (Hamamatsu, Japan), in 2^{16} grey levels. Using the Metamorph software, the area of the hippocampus was selected as the region of interest (ROI) and the minimum and average grey levels within the ROI were calculated. FGF-2-positive pixels were identified by thresholding at the grey level corresponding to the mean plus the difference between average and minimum. Using this approach, only those pixels that were significantly above background (i.e. FGF-2-positive) were selected. Data were then expressed as percentage of positive pixels over total hippocampal pixels. As stated above, one section every 52 was examined, that is, five sections per animal. Numbers from these five sections were used as quintuplicates, that is, the average was used for statistical analysis.

Statistical analysis

Data were analysed using a repeated (two-way) analysis of variance (ANOVA). On the basis of the statistically significant interactions revealed by ANOVA, separate evaluations were performed to reveal specific differences among groups. Following ANOVA analyses, the least significant difference of means (LSD) post hoc test was used for sucrose intake and body weight data, while the Student–Newman–Keuls post hoc test was used to analyse FST, open field test, CORT and histological data. The significance level was set at $p < 0.05$. Statistical analysis was performed using GraphPad (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Sucrose preference test

Figure 1(b) shows baseline sucrose solution intake in the control and CMS rats. Preference for sucrose remained constant throughout the experiment in non-stressed rats, whereas it was significantly decreased ($p < 0.001$) from week 5 and to the end of the experiment (week 12) in stressed rats. Before the beginning of the treatment (from week 1 to week 8) all data from the main subgroups (non-stressed and stressed rats) were pooled together, as no difference in the behavioural measures was detected. From week 1 to week 8 the repeated ANOVA revealed statistically significant effects of stress ($F_{(1,46)} = 187.38$; $p < 0.01$), time ($F_{(7,322)} = 19.13$; p 0.01) and stress × time interaction ($F_{(7,322)} = 12.41$; $p < 0.01$).

Treatment with UFP-101 or fluoxetine, which started on week 9, reinstated sucrose consumption from the third week of administration (corresponding to week 11 of the protocol). The ANOVA (from week 9 to week 12) revealed significant effects of treatment ($F_{(5,42)} = 155.08$; $p < 0.01$), time ($F_{(3,126)} = 63.17$; $p < 0.01$) and interaction time × treatment ($F_{(15,126)} = 14.2$, $p < 0.01$). UFP-101 treatment significantly increased sucrose preference in comparison with the stressed group at week 11 and the effect was maintained until the end of the experiment (LSD, $p < 0.001$ for either week). Fluoxetine treatment also restored sucrose intake from week 11 to the end of the experiment (LSD, $p < 0.001$ for week 11 or 12). N/OFQ significantly reversed the effect of UFP-101 at week 12 (LSD, $p < 0.001$), decreasing sucrose consumption to values not different from those of the stress + saline group. UFP-101 had no effect on sucrose intake in non-stressed animals in any week of treatment ($p > 0.05$ for weeks 9–10–11–12).

Body weight

As previously found (Vitale et al., 2009), CMS did not affect body weight, nor did UFP-101 treatment. Before the beginning of treatments, no body weight differences in the subgroups of non-stressed and stressed rats were observed; thus, all data from subgroups were pooled together (Figure 1(c)). Weight gain was not reduced by CMS. Repeated ANOVA, performed from week 1 to week 8, revealed a significant time effect ($F_{(7,322)} = 26.58$; $p < 0.01$) and no stress or stress × time interaction effect. Repeated ANOVA for the time-period from week 9 to week 12 indicated a statistically significant time effect ($F_{(3,126)} = 9.09$; $p < 0.05$), treatment effect ($F_{(5,42)} = 8.13$; $p < 0.05$) but not

interaction time × treatment. There was no difference in weight gain between the treatment groups from week 9 to 12, except for the stressed group treated with fluoxetine, which interrupted weight gain from the 10th week (LSD $p < 0.05$ compared with non-stressed + saline-treated or with stressed + saline-treated animals from week 10 to week 12). This finding is consistent with evidence that fluoxetine, by inhibiting 5-HT reuptake, can reduce food intake and, consequently, body weight (McGuirk et al., 1992).

FST

CMS caused an increase in immobility time and a decrease in climbing time in the FST, a typical pattern of depressive-like behaviour. As shown in Figure 2(a) and (b), stressed rats floated more and spent less time in climbing compared with control rats. Two-way repeated ANOVAs for immobility and climbing times revealed significant effects of time ($F_{(3,126)} = 20.02$; $p < 0.01$; $F_{(3,126)} = 43.73$; $p < 0.01$, respectively), treatment ($F_{(5,42)} = 35.14$; $p < 0.01$; $F_{(5,42)} = 38.71$ $p < 0.01$, respectively) and significant interaction of time × treatment ($F_{(15,126)} = 24.24$; $p < 0.01$; $F_{(3,126)} = 12.63$; $p < 0.01$, respectively).

A nine-day fluoxetine or UFP-101 treatment was needed to reduce the immobility time and to increase the climbing time to values significantly different from those of saline-treated stressed rats (as indicated by post hoc analysis, $p < 0.05$). No significant effect was observed at day 2. Interestingly, both treatments increased climbing time above control values in stressed animals at days 9, 16 and 23. Moreover, they decreased immobility time below the control values at day 23. UFP-101 treatment reduced the immobility time also in non-stressed animals after a 23-day treatment, as revealed by the Student–Newman–Keuls post hoc test ($p < 0.05$ *vs*. non-stressed + saline). A prolonged administration of N/OFQ in UFP-101-treated animals reversed its effect, bringing back immobility and climbing times to those of saline-treated stressed rats ($p > 0.05$ for immobility and climbing): this effect was detected after 23 but not 16 days. The post hoc test showed no differences in any treatment or CMS as regards swimming time (data not shown). These data support a NOP-mediated, time-dependent anti-depressant effect of UFP-101.

Open field test

As expected (D'Aquila et al., 2000; First et al., 2011), CMS caused a decrease in the number of crossings (i.e. decreased locomotor activity) in the open field test, another typical pattern of depressive-like behaviour (Figure 2(c)). The treatment with fluoxetine or UFP-101 led to a reversal of this effect in about two weeks (normalization of crossing counts after 13, but not six, days of treatment). These observations were confirmed by two-way repeated ANOVA that revealed only an effect of treatment ($F_{(5,42)} = 13.75$; $p < 0.01$) in the chronically stressed groups; UFP-101 treatment did not influence total crossings in non-stressed rats. The effect induced by UFP-101 administration was reversed by the co-administration of N/OFQ by day 20, as shown by the Student–Newman–Keuls test ($p > 0.05$ *vs*. stressed + saline). None of the groups showed any significant difference in total rearing duration (data not shown).

Serum CORT levels

Increased CORT levels were observed following CMS (Figure 3). This effect was fully reversed by fluoxetine and UFP-101 treatment, and the effect of UFP-101 was in turn counteracted by N/OFQ In non-stressed rats. UFP-101 treatment did not modify serum CORT levels, while it reduced CMS-induced increase in CORT to values comparable to those of controls. Indeed, in the CMS rats treated with UFP-101, two-way ANOVA showed significant stress effect ($F_{(1,28)} = 22.72$; $p < 0.01$), treatment effect ($F_{(1,28)} = 18.31$; $p < 0.01$) and stress × treatment interaction ($F_{(1,28)} = 31.79$; $p < 0.01$) on serum CORT levels. Fluoxetine also significantly reversed the elevated CORT levels caused by CMS compared with saline-treated CMS rats (post hoc test: $p < 0.01$). A significant increase in CORT levels, similar to values measured in stressed saline-treated rats, was observed after co-administration of UFP-101 and N/OFQ ($p > 0.05$ *vs*. stressed non-treated rats) (Figure 3). These results confirm those of our previous study (Vitale et al., 2009).

Neural stem cell proliferation

To confirm the reduction of cell proliferation induced by experimental models of stress (Gould et al., 1997, 1998; Tanapat et al., 1998) and to examine whether the administration of UFP-101 can modulate neural stem cell proliferation, we measured the number of BrdU+ cells in the subgranular zone of the dentate gyrus (Figure 4(a)). This analysis revealed that stress induced a significant decrease in cellular proliferation ($F_{(1,28)} = 17.24$; $p < 0.01$). At the tested doses, UFP-101 was not able to recover cell proliferation, whereas fluoxetine, in agreement with literature data (Santarelli et al., 2003), increased BrdU+ cell number reinstating a proliferation level similar to that in control rats (Figure 4(b)).

Neuronal differentiation

To determine whether the UFP-101 administration led to changes in neurogenesis, we employed DCX, an early marker of neuronal differentiation (Figure 4(c)). Two-way ANOVA showed significant difference only on stress × treatment interaction effect ($F_{(1,28)} = 16.08$; $p < 0.01$). Stressed rats were not significantly different from controls but when treated with UFP-101 or fluoxetine had a significant increase in the number of DCX+ cells compared with control values. This increase in DCX+ cells was not observed in stressed rats treated with UFP-101 + N/OFQ ($p < 0.05$ *vs*. stressed + UFP-101-treated rats) indicating an effective N/OFQ antagonism (Figure 4(d)).

Expression of FGF-2 in the hippocampus

To begin exploring the mechanism of UFP-101 effects, we then used immunohistochemistry to evaluate whether the modulation of cell proliferation was accompanied by changes in FGF-2 expression, a neurotrophic factor that plays a key role in neural stem cell proliferation and differentiation. A typical expression pattern has been observed in control rats: in coronal sections of the dorsal hippocampus, FGF-2 was mainly localized in pyramidal CA2 cells and, more diffusely, in other cells, presumably astrocytes (Figure 5(a)). As

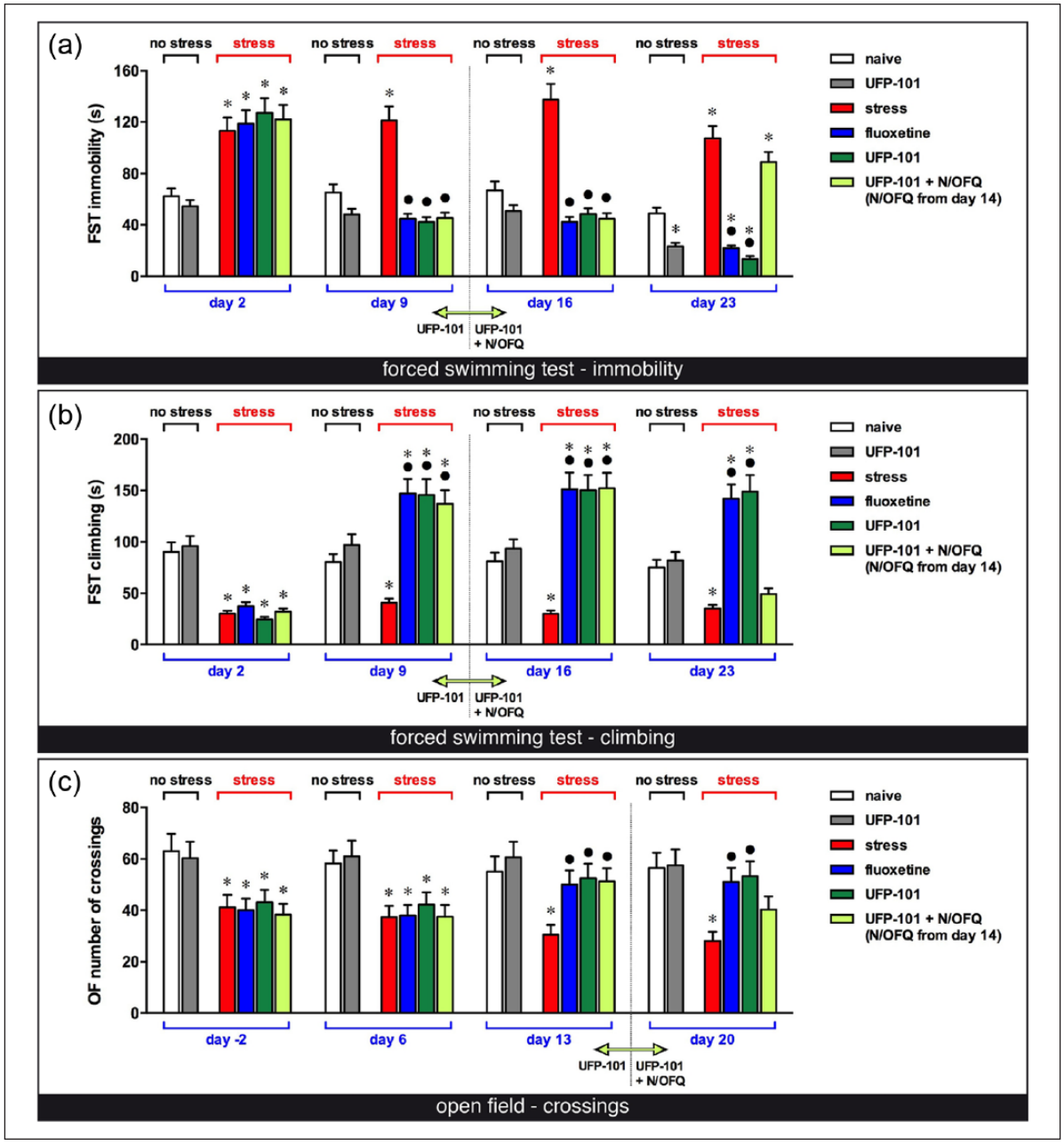

Figure 2. Effect of CMS on **(a)** immobility and **(b)** climbing duration in the forced swim test on days 2, 9, 16 and 23 after onset of treatments. See Figure 1 for specifications on treatments. The light green arrow in the centre is to emphasize that N/OFQ is added to UFP-101 only for the last 10 days of treatment. Data are means ± SEM of eight animals per group. *$p < 0.05$ compared with non-stressed + saline, same session; $p < 0.05$ compared with stressed + saline, same session. ANOVA followed by the Student–Newman–Keuls test. **(c)** Effect of CMS on total crossings in the open field before the beginning of treatments (–2 days) and on days 6, 13 and 20 after the onset of treatments. See Figure 1 for specifications on treatments. The light green arrow in the centre is to emphasize that N/OFQ is added to UFP-101 only for the last 10 days of treatment. Data are means ± SEM of eight animals per group. *$p < 0.05$ compared with non stressed + saline, same session; $p < 0.05$ compared with stressed + saline, same session. ANOVA followed by the Student–Newman–Keuls test.

UFP-101: [Nphe1] Arg14, Lys15-N/OFQ-NH2; N/OFQ: nociceptin/orphanin FQ; FST: forced swim test; ANOVA: analysis of variance; OF: open field; CMS: chronic mild stress

expected, a significant reduction of FGF-2 expression levels was detected in CMS compared with control animals (Figure 5(b)). This reduction was abolished by treatment with UFP-101 (Figure 5(c)) or fluoxetine. Two-way ANOVA indicated only a significant treatment effect ($F_{(1,28)} = 13.78$, $p < 0.01$) for FGF-2 expression levels (Figure 5(d)). FGF-2 expression remained significantly higher also in the group of UFP-101 + N/OFQ-treated rats ($p < 0.05$ *vs.* stressed rats) (Figure 5(d)).

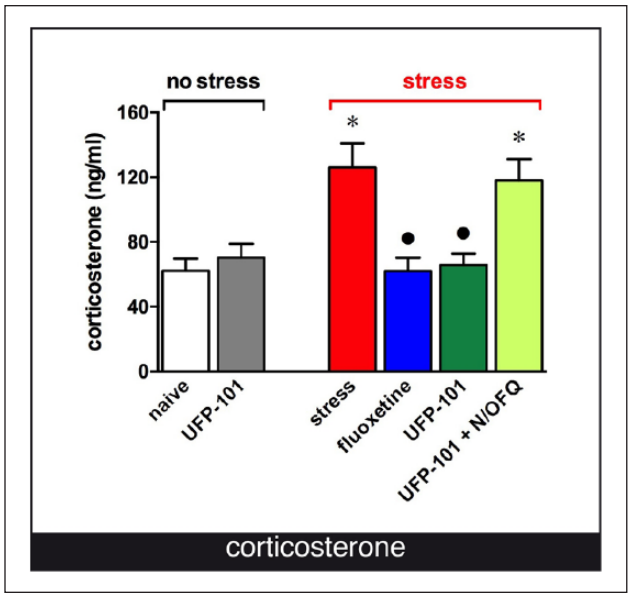

Figure 3. Effects of UFP-101 (or fluoxetine) treatment on serum corticosterone levels in the non-stressed and CMS rats. See Figure 1 for specifications on treatments. Data are means ± SEM of eight animals per group. •$p < 0.05$ compared with non-stressed + saline; $p < 0.05$ compared with stressed + saline. ANOVA followed by the Student–Newman–Keuls test.
UFP-101: [Nphe^1] Arg^{14}, Lys^{15}-N/OFQ-NH$_2$; N/OFQ: nociceptin/orphanin FQ; CMS: chronic mild stress; ANOVA: analysis of variance

Discussion

The present study shows that the NOP antagonist UFP-101 reverses CMS-induced depression-like behavioural and biological effects: not only the alterations in sucrose intake, behaviour in the FST and in the open field tests and serum CORT levels, but also the dampening in the neuronal differentiation of neural stem cells as well as in the expression of the neurotrophic factor FGF-2.

Behavioural and biochemical effects

Our modified CMS protocol was able to induce a significant reduction in sucrose consumption five weeks after the beginning of stress exposure. UFP-101, continuously i.c.v. infused via minipumps, rapidly produced a reversal of the anhedonia-like conditions in stressed rats. Similarly, a two-week treatment with fluoxetine restored sucrose consumption in stressed rats to baseline levels. The use of osmotic minipumps allowed a constant and continuous infusion of the peptide, avoiding daily administration and discharge of rats from the experiment due to loss of the cannulae.

CMS-induced depressive-like behaviour was also reversed by chronic treatment with fluoxetine. This finding is in line with previous reports. Indeed, many preclinical studies have demonstrated fluoxetine antidepressant effects in animal models of depression including CMS (First et al., 2011; Zhao et al., 2015a, 2015b). In particular, anhedonia, a core symptom of major depression disorders, was reversed after chronic fluoxetine administration (Grippo et al., 2006). We have previously used imipramine as a reference drug in our CMS protocol (Vitale et al., 2009), and fluoxetine was found here to behave similarly, in particular being effective only after a two-week treatment. This is in line with other studies comparing fluoxetine with imipramine, which demonstrated that treatment of CMS with either antidepressant induced significant alterations in biochemical profiles as well as in behaviours and body weight (Zhao et al., 2015a).

The reduced body weight gain that we observed with chronic fluoxetine treatment is consistent with data in humans (Domecq et al., 2015). In animal studies, this parameter was found to vary in different CMS experimental schedules (First et al., 2011; Gamaro et al., 2008; Li et al., 2009). It is difficult to interpret these discrepancies, which likely depend on specifics of the experimental design. Anyway, this possible side effect was not observed with UFP-101.

The changes in behavioural parameters in the FST and open field test were completely normalized by either UFP-101 or fluoxetine, suggesting an antidepressant activity of UFP-101. Surprisingly, also the non-stressed group displayed an antidepressant-like effect in FST (but not in open field test) after a 23-day UFP-101 treatment; this could be due to a long-term blockade of the tonically active NOP receptor system, since antagonists' actions derive entirely from the blockade of synaptic receptors and require an active state of the system (Gavioli and Calò, 2006, 2013).

Not only serotonin-selective reuptake inhibitors like fluoxetine, but also various other classes of antidepressants can reduce immobility time during the FST while increasing the swimming and/or climbing time.

Our pivotal findings (Arletti et al., 2005) indicate that a chronic N/OFQ administration is ineffective per se, in the CMS paradigm, in inducing behavioural changes, this datum being supported by other evidences regarding N/OFQ or other NOP receptor agonists (Gavioli and Calò, 2003; Witkin et al., 2014). On the contrary, repeated co-administration of N/OFQ with UFP-101 prevented the behavioural actions of UFP-101 alone. This outcome supports the hypothesis that the action of NOP receptor antagonists occurs specifically at receptor level, with the blockade of N/OFQ signalling. Probably the chronic stressful conditions determine an endogenous release of N/OFQ contributing to the depressive state which is counteracted by UFP-101 administration.

One of the most consistent changes found in depression is the dysregulation of the HPA axis (Holsboer, 2000; Schuld et al., 2003). High levels of adrenocorticotropic hormone and CORT are commonly observed in depressed patients and accompanied by the impairment of negative feedback of the HPA axis (Pariante and Lightman, 2008). We also found that rats exposed to CMS have increased serum CORT levels. In line with other studies demonstrating that antidepressant therapy, in particular with fluoxetine (Khemissi et al., 2014), restores the HPA axis function (Pariante and Lightman, 2008), in the present work, we demonstrated that fluoxetine can normalize serum CORT levels. Moreover, also UFP-101 produces the same effect while N/OFQ co-administration counteracts CORT reduction induced by UFP-101 and reinstated its levels to those of stressed rats.

To reinforce the hypothesis of a specific implication of NOP receptors in the antidepressant action of UFP-101, in the present study we confirm our previous findings (Vitale et al., 2009) that repeated co-administration of N/OFQ with UFP-101 is able to reverse either the behavioural or the biochemical (corticosterone) effects, reinstating the values to those of stressed rats.

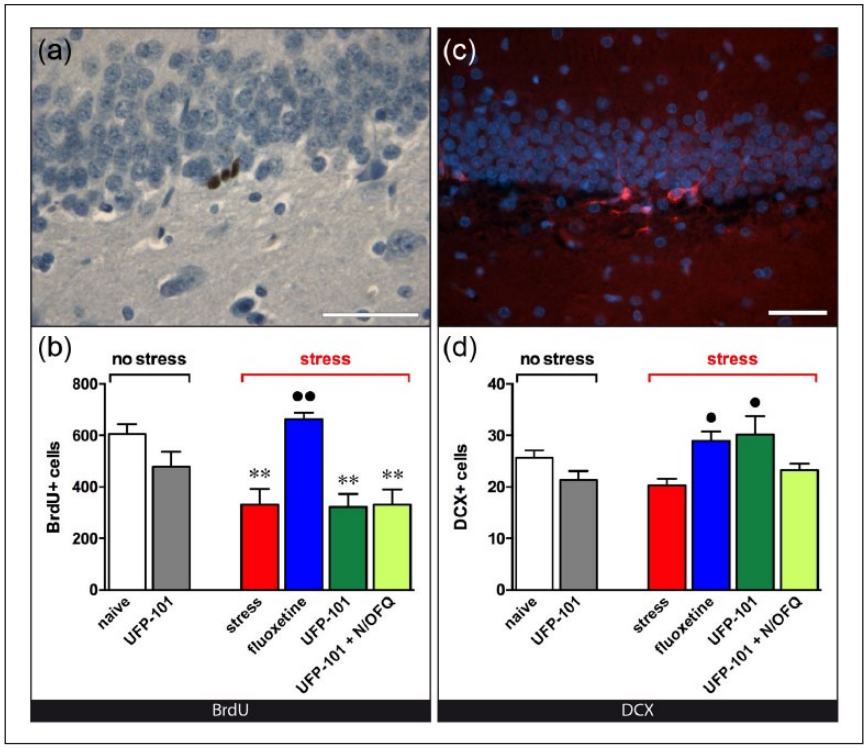

Figure 4. (a) and (b) Effects of UFP-101 or fluoxetine on cell proliferation in the dentate gyrus of the dorsal hippocampus, as evaluated using BrdU. A representative section from a naïve animal is shown in (a) to illustrate the method of analysis. Data quantification is shown in (b). Data are means ± SEM of eight animals per group. $^{**}p < 0.01$ compared with naïve; $^{**}p < 0.01$ compared with stressed. ANOVA followed by the Student–Newman–Keuls test. **(c) and (d)** Effects of UFP-101 or fluoxetine on the number of DCX expressing cells in the dentate gyrus of the dorsal hippocampus. A representative section from a naïve animal is shown in (c) to illustrate the method of analysis. Data quantification is shown in (d). Data are means ± SEM of eight animals per group. $^{\bullet}p < 0.05$ compared with stressed. ANOVA followed by the Student–Newman–Keuls test. Horizontal bars in (a) and (c) = 50 µm.
UFP-101: [Nphe1] Arg14, Lys15-N/OFQ-NH2; N/OFQ: nociceptin/orphanin; BrdU: bromodeoxyuridine; DCX: doublecortin; ANOVA: analysis of variance [AQ: 4]

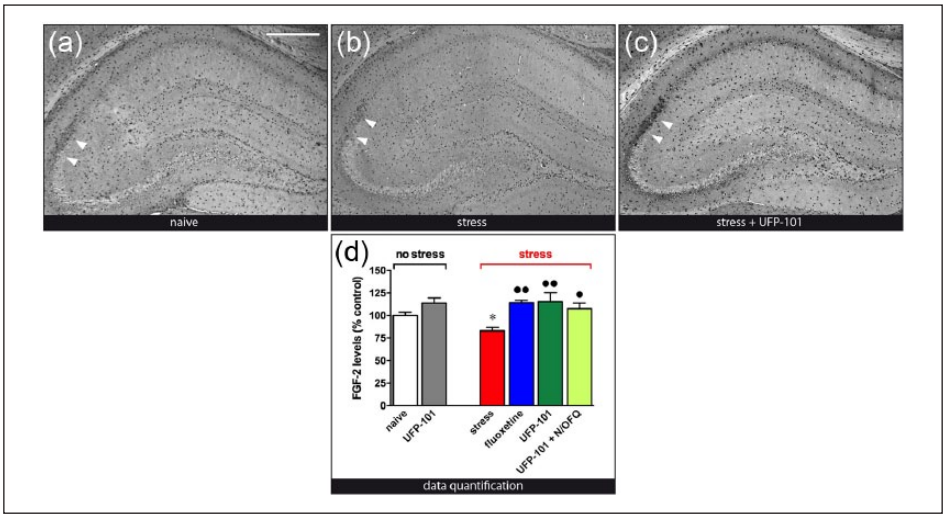

Figure 5. Effects of UFP-101 or fluoxetine on FGF-2 immunoreactivity. **(a) to (c)** Representative sections taken across the dorsal hippocampus of a naïve (a), a stressed (b) and a stressed animal treated with UFP-101 (c). Note the reduced levels of immunoreactivity associated with stress and the effect of UFP-101, which abolishes this reduction and even increases FGF-2 levels above those observed in controls. These effects are particularly evident in the CA2 pyramidal layer (arrowheads). These 'representative' sections will not fully correlate with the mean FGF-2 levels shown in (d) because of slight differences in expression in the eight animals of each group. **(d)** Data quantification. Data are means ± SEM of FGF-2 expression (per cent of control values in naïve animals) in eight animals per group. See Figure 1 for specifications on treatments. $^{*}p < 0.05$ compared with naïve; $^{\bullet}p < 0.05$ compared with stressed; $^{\bullet\bullet}p < 0.01$compared with stressed. ANOVA followed by the Student–Newman–Keuls test. Horizontal bar = 500 µm.
UFP-101: [Nphe1] Arg14, Lys15-N/OFQ-NH2; N/OFQ: nociceptin/orphanin; FGF-2: fibroblast growth factor; ANOVA: analysis of variance

In summary, we observed that the profile of action and the time-course of UFP-101 effects in behavioural tests of depression and on serum CORT levels are similar to those of fluoxetine. This suggests that there may be some commonality of mechanisms of action: in fact, the serotonergic neurotransmission has been hypothesized to have an important role in the antidepressant-like activity of UFP-101 (Vitale et al., 2009).

Effects on neurogenesis

We also examined whether the development and remission from depressive-like symptoms after UFP-101 (or fluoxetine) treatment can be associated with effects on cell proliferation, neurogenesis or NTF expression. Neuroplasticity, neurodegeneration and neurogenesis are currently viewed as key elements that underlie the pathophysiology of depression as well as the action of antidepressant drugs (Malberg and Schechter, 2005).

These events have been reported to occur primarily in the hippocampus. Stress is believed to be the most significant environmental factor in the aetiology of depression, and neurons in the hippocampal formation are known to be hypersensitive to stress (Gould et al., 1997, 1998; Tanapat et al., 1998). Furthermore, a reduced hippocampal volume has been observed in depressed patients (Bremner et al., 2000; Sheline et al., 2003) while chronic treatment with different classes of antidepressants reverses stress-induced inhibition of neurogenesis in the hippocampal formation (Alonso et al., 2004; Czeh et al., 2001; Fuchs et al., 2004; Malberg and Duman, 2003; Malberg et al., 2000; Santarelli et al., 2003; van der Hart et al., 2002).

We found that CMS causes a reduction in the proliferation of neural stem cells in the dentate gyrus of the dorsal hippocampus, as measured using BrdU. As expected (Alboni et al., 2015; Santarelli et al., 2003), this effect was reversed by fluoxetine but, surprisingly, not by UFP-101. This negative finding should be taken with caution, because, in this study, we explored a single time point at the end of the experimental procedure. As stated, however, the effect of fluoxetine was fully evident at this time point, arguing that, unlike other antidepressants, the behavioural anti-anhedonic effects of UFP-101 may be independent of modulation of neural stem cell proliferation.

However, antidepressants influence not only proliferation of neural stem cells, but also their survival and neuronal differentiation (Keilhoff et al., 2006; Klomp et al., 2014; Malberg et al., 2000; Marcussen et al., 2008; Pinnock et al., 2009; Possamai et al., 2015; Santarelli et al., 2003). In order to explore these other possibilities, we measured DCX-positive cells in the dentate gyrus of the dorsal hippocampus. DCX labels precursor cells still proliferating but already committed towards the neuronal lineage of differentiation (Couillard-Despres et al., 2005), thus providing an indication of generation and survival of new neurons. Stress was reported to reduce the density of DCX-positive cells in previous studies (de Andrade et al., 2013; Murata et al., 2015; Yun et al., 2016). We also observed a tendency to this reduction, which, however, did not reach statistical significance. A possible explanation for this failure can be due to several parameters: the animal model used, the severity of the stress regime applied and the statistical test utilized. Moreover, the part of the hippocampus investigated (i.e. ventral *vs*. dorsal) can be critical for differential results in the developmental progress of hippocampal immature neurons (Jayatissa et al., 2008). This notwithstanding, interestingly, the density of DCX-positive cells in stressed animals was increased not only by fluoxetine, but also by UFP-101. The apparent discrepancy between the effects of UFP-101 on neural stem cell proliferation and on survival/differentiation of newborn neurons may be due to our experimental conditions. As stated above, BrdU provides an estimate of the proliferative state in the 24 h before sacrifice, whereas DCX evaluates the number of new neurons generated and surviving in a time period of several days before sacrifice (Plümpe et al., 2006).

A first explanation could be that cell proliferation may contribute to the number of DCX-positive cells in an earlier period of time, compared with that of sacrifice, as well as to the survival and differentiation of the previously generated cells. An alternative hypothesis may be that UFP-101 produces a specific effect on survival and/or differentiation, in the absence of significant effects on proliferation. Indeed, N/OFQ contrasts the effects of the neurotrophin BDNF, a key factor in the promotion of neuronal differentiation (Alder et al., 2013). The NOP antagonist UFP-101 may therefore promote BDNF activation and prompt neuronal differentiation of newborn cells. A specific profile of actions on adult neurogenesis for UFP-101 would not be an exception, since it is known that different classes of antidepressants have distinct actions on this parameter. For example, it has been suggested that imipramine, at variance with fluoxetine, ameliorates anxiety and cognitive deficits induced by stress, independently of the effects on neurogenesis (Bessa et al., 2013).

The molecular mechanisms underlying the effects of antidepressants on neurogenesis are still largely obscure, even if there is evidence that they may involve NTFs like FGF-2. FGF-2 not only exerts neuroprotective effects directly on neurons or indirectly via glial cells (Turner et al., 2006) but is also the most potent agent capable of inducing proliferation of hippocampal progenitors (Becq et al., 2005; Nakatomi et al., 2002). Indeed, levels of FGF-2 and its receptors are downregulated in several brain regions in depressed subjects (Gaughran et al., 2006); furthermore, the antidepressant treatment opposes these effects and upregulates FGF-2 in humans (Evans et al., 2004) and in rodent models (Bachis et al., 2008). Moreover, FGF-2 blocks CMS-induced inhibition of neural stem cell proliferation while fluoxetine induction of proliferation requires FGF receptor signalling (Elsayed et al., 2012). Coherently with these and other reports (Mallei et al., 2002; Nibuya et al., 1995), we found here that CMS significantly reduced the FGF-2 expression with respect to non-stressed rats, while fluoxetine increased its levels. This effect was also observed with UFP-101, suggesting that modulation of FGF-2 signalling may be part of the mechanism of its antidepressant effect. A more extensive and systematic study of the modulation of NTF signalling by NOP antagonists will be needed to refine and strengthen this concept.

Conclusive concluding remarks

In conclusion, UFP-101 is effective in reversing behavioural, cellular and molecular effects of the exposure to a non-predictable sequence of mild stressful events in the rat. Notably, UFP-101 seems to mediate its effects by increasing neuronal differentiation and survival of newborn neurons, possibly via activation of NTF (FGF-2) expression. Therefore, NOP receptors may represent a target for innovative antidepressant drugs.

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Declaration of conflicting interests

The authors declare that there is no conflict of interest.

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References

Abelaira HM, Réus GZ and Quevedo J (2013) Animal models as tools to study the pathophysiology of depression. *Rev Bras Psiquiatr* 35: S112–S120.

Abrous DN, Koehl M and Le Moal M (2005) Adult neurogenesis: From precursors to network and physiology. *Physiol Rev* 85: 523–569.

Alboni S, van Dijk RM, Poggini S, et al. (2015) Fluoxetine effects on molecular, cellular and behavioral endophenotypes of depression are driven by the living environment. *Mol Psychiatry* 1–10. doiDOI: 10.1038/mp.2015.191. [AQ: 2]

Alder J, Kallman S, Palmieri A, et al. (2013) Neuropeptide orphanin FQ inhibits dendritic morphogenesis through activation of RhoA. *Dev Neurobiol* 73: 769–784.

Alonso R, Griebel G, Pavone G, et al. (2004) Blockade of CRF1 or V-1b receptors reverses stress induced suppression of neurogenesis in a mouse model of depression. *Mol Psychiatry* 9: 278–286.

Arletti R, Vitale G and Ruggieri V (2005) Nociceptin/Orphanin FQ and chronic mild stress: Effect of acute and chronic treatment in the rat. *32° Congresso Nazionale della società Italiana di Farmacologia*, Naples, Italy, 1–4 June, p.134. [AQ: 3]

Asth L, Ruzza C, Malfacini D, et al. (2016) Beta-arrestin 2 rather than G protein efficacy determines the anxiolytic-versus antidepressant-like effects of nociceptin/orphanin FQ receptor ligands. *Neuropharmacology* 105: 434–442.

Bachis A, Mallei A, Cruz MI, et al. (2008) Chronic antidepressant treatments increase basic fibroblast growth factor and fibroblast growth factor-binding protein in neurons. *Neuropharmacology* 55: 1114–1120.

Becq H, Jorquera I, Ben-Ari Y, et al. (2005) Differential properties of dentate gyrus and CA1 neural precursors. *J Neurobiol* 62: 243–261.

Bessa JM, Morais M, Marques F, et al. (2013) Stress-induced anhedonia is associated with hypertrophy of medium spiny neurons of the nucleus accumbens. *Transl Psychiatry* 3: e266.

Bremner JD, Narayan M, Anderson ER, et al. (2000). Hippocampal volume reduction in major depression. *Am J Psychiatry* 157: 115–117.

Calò G, Guerrini R, Rizzi A, et al. (2005) UFP-101, a peptide antagonist selective for the nociceptin/orphanin FQ receptor CNS. *Drug Rev* 11: 97–112.

Calò G, Rizzi A, Rizzi D, et al. (2002) [Nphe^{1},Arg^{14},Lys^{15}]nociceptin-NH_2, a novel potent and selective antagonist of the nociceptin/orphanin FQ receptor. *Br J Pharmacol* 136: 303–311.

Cheng Y, Rodriguiz RM, Murthy SR, et al. (2015) Neurotrophic factor-α1 prevents stress-induced depression through enhancement of neurogenesis and is activated by rosiglitazone. *Mol Psychiatry* 20: 744–754.

Couillard-Despres S, Winner B, Schaubeck S, et al. (2005) Doublecortin expression levels in adult brain reflect neurogenesis. *Eur J Neurosci* 21: 1–14.

Czeh B, Michaelis T, Watanabe T, et al. (2001) Stress-induced changes in cerebral metabolites, hippocampal volume, and cell proliferation are prevented by antidepressant treatment with tianeptine. *Proc Natl Acad Sci USA* 98: 12796–12801.

D'Aquila PS, Peana AT, Carboni V, et al. (2000) Exploratory behaviour and grooming after repeated restraint and chronic mild stress: Effect of desipramine. *Eur J Pharmacol* 399: 43–47.

de Andrade JS, Céspedes IC, Abrão RO, et al. (2013) Chronic unpredictable mild stress alters an anxiety-related defensive response, Fos immunoreactivity and hippocampal adult neurogenesis. *Behav Brain Res* 250: 81–90.

De Kloet ER, Vreugdenhil E, Oitzl MS, et al. (1998) Brain corticosteroid receptor balance in health and disease. *Endocr Rev* 19: 8269–8301.

Detke MJ, Johnson J and Lucki I (1997) Acute and chronic antidepressant drug treatment in the rat forced swimming test model of depression. *Exp Clin Psychopharmacol* 5: 107–112.

Detke MJ, Rickels M and Lucki I (1995) Active behaviors in the rat forced swimming test differentially produced by serotonergic and noradrenergic antidepressants. *Psychopharmacology (Berl)* 121: 66–72.

Domecq JP, Prutsky G, Leppin A, et al. (2015) Clinical review: Drugs commonly associated with weight change: a systematic review and meta-analysis. *J Clin Endocrinol Metab* 100: 363–370.

Elsayed M, Banasr M, Duric V, et al. (2012) Antidepressant effects of fibroblast growth factor-2 in behavioral and cellular models of depression. *Biol Psychiatry* 72: 258–265.

Evans SJ, Choudary PV, Neal CR, et al. (2004) Dysregulation of the fibroblast growth factor system in major depression. *Proc Natl Acad Sci USA* 101: 15506–15511.

First M, Gil-Ad I, Taler M, et al. (2011) The effects of fluoxetine treatment in a chronic mild stress rat model on depression-related behavior, brain neurotrophins and ERK expression. *J Mol Neurosci* 45: 246–255.

Fuchs E, Czeh B and Flugge G (2004) Examining novel concepts of the pathophysiology of depression in the chronic psychosocial stress paradigm in tree shrews. *Behav Pharmacol* 15: 315–325.

Gamaro GD, Prediger ME, Lopes J, et al. (2008) Fluoxetine alters feeding behavior and leptin levels in chronically-stressed rats. *Pharmacol Biochem Behav* 90: 312–317.

Gaughran F, Payne J, Sedgwick PM, et al. (2006) Hippocampal FGF-2 and FGFR1 mRNA expression in major depression, schizophrenia and bipolar disorder. *Brain Res Bull* 70: 221–227.

Gavioli EC and Calo' G (2006) Antidepressant- and anxiolytic-like effects of nociceptin/orphanin FQ receptor ligands. *Naunyn Schmiedebergs Arch Pharmacol* 372: 319–330.

Gavioli EC and Calo' G (2013) Nociceptin/orphanin FQ receptor antagonists as innovative antidepressant drugs. *Pharmacol Ther* 140: 10–25.

Gavioli EC, Marzola G, Guerrini R, et al. (2003) Blockade of nociceptin/orphanin FQ-NOP receptor signalling produces antidepressant-like effects: Pharmacological and genetic evidences from the mouse forced swimming test. *Eur J Neurosci* 17: 1987–1990.

Gavioli EC, Vaughan CW, Marzola G, et al. (2004) Antidepressant-like effects of the nociceptin/orphanin FQ receptor antagonist UFP-101: New evidence from rats and mice. *Naunyn Schmiedebergs Arch Pharmacol*. 369: 547–553.

Ge L, Zhu MM, Yang JY, et al. (2015) Differential proteomic analysis of the anti-depressive effects of oleamide in a rat chronic mild stress model of depression. *Pharmacol Biochem Behav* 131: 77–86.

Gould E, McEwen BS, Tanapat P, et al. (1997) Neurogenesis in the dentate gyrus of the adult tree shrew is regulated by psychosocial stress and NMDA receptor activation. *J Neurosci* 17: 2492–2498.

Gould E, Tanapat P, McEwen BS, et al. (1998) Proliferation of granule cell precursors in the dentate gyrus of adult monkeys is diminished by stress. *Proc Nat Acad Sci USA* 95: 3168–3171.

Grippo AJ, Beltz TG, Weiss RM, et al. (2006) The effects of chronic fluoxetine treatment on chronic mild stress-induced cardiovascular changes and anhedonia. *Biol Psychiatry* 59: 309–316.

Guerrini R, Caló G, Lambert DG, et al. (2005) N- and C-terminal modifications of nociceptin/orphanin FQ generate highly potent NOP receptor ligands. *J Med Chem* 48: 1421–1427.

Holanda VA, Medeiros IU, Asth L, et al. (2016) Antidepressant activity of nociceptin/orphanin FQ receptor antagonists in the mouse learned helplessness. *Psychopharmacology* 233: 2525–2532.

Holsboer F (2000) The stress hormone system is back on the map. *Curr Psychiatry Rep* 2: 454–456.

Jayatissa MN, Bisgaard CF, West MJ, et al. (2008) The number of granule cells in rat hippocampus is reduced after chronic mild stress and re-established after chronic escitalopram treatment. *Neuropharmacology* 54: 530–541.

Keilhoff G, Becker A, Grecksch G, et al (2006) Cell proliferation is influenced by bulbectomy and normalized by imipramine treatment in a region-specific manner. *Neuropsychopharmacology* 31: 1165–1176.

Khemissi W, Farooq RK, Le Guisquet AM, et al. (2014) Dysregulation of the hypothalamus-pituitary-adrenal axis predicts some aspects of the behavioral response to chronic fluoxetine: Association with hippocampal cell proliferation. *Front Behav Neurosci* 8: 340.

Kilkenny C, Browne WJ, Cuthill IC, et al. (2010) Improving bioscience research reporting: The ARRIVE guidelines for reporting animal research. *J Pharmacol Pharmacother* 1: 94–99.

Klomp A, Václavu L, Meerhoff GF, et al. (2014) Effects of chronic fluoxetine treatment on neurogenesis and tryptophan hydroxylase expression in adolescent and adult rats. *PLoS One* 9: e97603.

Kuhn HG, Winkler J, Kempermann G, et al. (1997) Epidermal growth factor and fibroblast growth factor-2 have differ neural progenitors in the adult rat brain. *J Neurosci* 17: 5820–5829.

Li YC, Wang FM, Pan Y, et al. (2009) Antidepressant-like effects of curcumin on serotonergic receptor-coupled AC-cAMP pathway in chronic unpredictable mild stress of rats. *Prog Neuropsychopharmacol Biol Psychiatry* 33: 435–449.

Lledo PM, Alonso M and Grubb MS (2006) Adult neurogenesis and functional plasticity in neuronal circuits. *Nat Rev Neurosci* 7: 179–193.

López-Rubalcava C and Lucki I (2000) Strain differences in the behavioral effects of antidepressant drugs in the rat forced swimming test. *Neuropsychopharmacology* 22: 191–199.

Malberg JE and Duman RS (2003) Cell proliferation in adult hippocampus is decreased by inescapable stress: Reversal by fluoxetine treatment. *Neuropsychopharmacology* 28: 1562–1571.

Malberg JE and Schechter LE (2005) Increasing hippocampal neurogenesis: A novel mechanism for antidepressant drugs. *Curr Pharm Des* 11: 145–155.

Malberg JE, Eisch AJ, Nestler, et al. (2000) Chronic antidepressant treatment increases neurogenesis in adult rat hippocampus. *J Neurosci* 20: 9104–9110.

Mallei A, Shi B and Mocchetti I (2002) Antidepressant treatments induce the expression of basic fibroblast growth factor in cortical and hippocampal neurons. *Mol Pharmacol* 61: 1017–1024.

Marcussen AB, Flagstad P, Kristjansen PEG, et al. (2008) Increase in neurogenesis and behavioural benefit after chronic fluoxetine treatment in Wistar rats. *Acta Neurol Scand* 117: 94–100.

Mateus-Pinheiro A, Pinto L, Bessa JM, et al. (2013) Sustained remission from depressive-like behavior depends on hippocampal neurogenesis. *Transl Psychiatry* 3: e210.

Mazzuferi M, Palma E, Martinello K, et al. (2010) Enhancement of GABA(A)-current run-down in the hippocampus occurs at the first spontaneous seizure in a model of temporal lobe epilepsy. *Proc Natl Acad Sci USA* 107: 3180–3185.

McEwen BS (1999) Stress and hippocampal plasticity. *Annu Rev Neurosci* 22: 105–122.

McEwen BS and Sapolsky RM (1995) Stress and cognitive function. *Curr Opin Neurobiol* 5: 205–216.

McGuirk J, Muscat R and Willner P (1992) Effects of chronically administered fluoxetine and fenfluramine on food intake, body weight and the behavioural satiety sequence. *Psychopharmacology (Berl)* 106: 401–407.

Medeiros IU, Ruzza C, Asth L, et al. (2015) Blockade of nociceptin/orphanin FQ receptor signaling reverses LPS-induced depressive-like behavior in mice. *Peptides* 72: 95–103.

Murata Y, Yanagihara Y, Mori M, et al. (2015) Chronic treatment with tandospirone, a serotonin 1A receptor partial agonist, inhibits psychosocial stress-induced changes in hippocampal neurogenesis and behavior. *J Affect Disord* 180: 1–9.

Nakatomi H, Kuriu T, Okabe S, et al. (2002) Regeneration of hippocampal pyramidal neurons after ischemic brain injury by recruitment of endogenous neural progenitors. *Cell* 110: 429–441.

Nibuya M, Morinobu S and Duman RS (1995) Regulation of BDNF and trkB mRNA in rat brain by chronic electroconvulsive seizure and antidepressant drug treatments. *J Neurosci* 15: 7539–7547.

Overstreet DH (2012) Modeling depression in animal models. *Methods Mol Biol* 829: 125–144.

Palmer TD, Markakis EA, Willhoite AR, et al. (1999) Fibroblast growth factor-2 activates a latent neurogenic program in neural stem cells from diverse regions of the adult CNS. *J Neurosci* 19: 8487–8497.

Paradiso B, Marconi P, Zucchini S, et al. (2009) Localized delivery of fibroblast growth factor-2 and brain-derived neurotrophic factor reduces spontaneous seizures in an epilepsy model. *Proc Natl Acad Sci USA* 106: 7191–7196.

Pariante CM and Lightman SL (2008) The HPA axis in major depression: Classical theories and new developments. *Trends Neurosci* 31: 464–468.

Paxinos G and Watson C (2005) *The Rat Brain in Stereotaxic Coordinates*, 5th ed. New York: Academic Press.

Perez-Caballero L, Torres-Sanchez S, Bravo L, et al. (2014) Fluoxetine: A case history of its discovery and preclinical development. *Expert Opin Drug Discov* 9: 567–578.

Pinnock SB, Lazic SE, Wong HT, et al. (2009) Synergistic effects of dehydroepiandrosterone and fluoxetine on proliferation of progenitor cells in the dentate gyrus of the adult male rat. *Neuroscienc*e 158: 1644–1651.

Plümpe T, Ehninger D, Steiner B, et al. (2006) Variability of doublecortin-associated dendrite maturation in adult hippocampal neurogenesis is independent of the regulation of precursor cell proliferation. *BMC Neurosci* 7: 77.

Porsolt RD, Anton G, Blavet N, et al. (1978) Behavioural despair in rats: A new model sensitive to antidepressant treatments. *Eur J Pharmacol* 47: 379–391.

Porsolt RD, Le Pichon M and Jalfre M (1977) Depression: A new animal model sensitive to antidepressant treatments. *Nature* 266: 730–732.

Possamai F, dos Santos J, Walber T, et al. (2015) Influence of enrichment on behavioral and neurogenic effects of antidepressants in Wistar rats submitted to repeated forced swim test. *Prog Neuropsychopharmacol Biol Psychiatry* 58: 15–21.

Post A, Smart TS, Krikke-Workel J, et al. (2016) A selective nociceptin receptor antagonist to treat depression: Evidence from preclinical and clinical studies. *Neuropsychopharmacology* 41: 1803–1812.

Rizzi A, Gavioli EC, Marzola G, et al. (2007) Pharmacological characterization of the nociceptin/orphanin FQ receptor antagonist SB-612111 [(-)-cis-1-methyl-7-[[4-(2,6-dichlorophenyl)piperidin-1-yl]methyl]-6,7,8,9-tetrahydro-5H-benzocyclohepten-5-ol]: In vivo studies. *J Pharmacol Exp Ther* 321: 968–974.

Santarelli L, Saxe M, Gross C, et al. (2003) Requirement of hippocampal neurogenesis for the behavioral effects of antidepressants. *Science* 301: 805–809.

Sapolsky RM (2000) Glucocorticoids and hippocampal atrophy in neuropsychiatric disorders. *Arch Gen Psychiatry* 57: 925–935.

Schuld A, Schmid DA, Haack M, et al. (2003) Hypothalamo–pituitary–adrenal function in patients with depressive disorders is correlated with baseline cytokine levels, but not with cytokine responses to hydrocortisone. *J Psychiatr Res* 37: 463–470.

Sheline YI, Gado MH and Kraemer HC (2003) Untreated depression and hippocampal volume loss. *Am J Psychiatry* 160: 1516–1518.

Tanapat P, Galea LA and Gould E (1998) Stress inhibits the proliferation of granule cell precursors in the developing dentate gyrus. *Int J Dev Neurosci* 16: 235–239.

Toll L, Bruchas MR, Calò G, et al. (2016) Nociceptin/orphanin FQ receptor structure, signaling, ligands, functions, and interactions with opioid systems. *Pharmacol Rev* 68: 419–457.

Turner CA, Akil H, Watson SJ, et al. (2006) The fibroblast growth factor system and mood disorders. *Biol Psychiatry* 59: 1128–1135.

Van der Hart MGC, Czeh B, de Biurrun G, et al. (2002) Substance P receptor antagonist and clomipramine prevent stress-induced alterations in cerebral metabolites, cytogenesis in the dentate gyrus and hippocampal volume. *Mol Psychiatry* 7: 933–941.

Vitale G, Ruggieri V, Filaferro M, et al. (2009) Chronic treatment with the selective NOP receptor antagonist [Nphe^{1}, Arg^{14}, Lys^{15}]N/OFQ-NH_2 (UFP-101) reverses the behavioural and biochemical effects of unpredictable chronic mild stress in rats. *Psychopharmacology (Berl)* 207: 173–189.

Wiborg O (2013) Chronic mild stress for modeling anhedonia. *Cell Tissue Res* 354: 55–69.

Willner P (1997) Validity, reliability and utility of the chronic mild stress model of depression: A 10-year review and evaluation. *Psychopharmacology* 134: 319–329.

Willner P (2005) Chronic mild stress (CMS) revisited: Consistency and behavioural-neurobiological concordance in the effects of CMS. *Neuropsychobiology* 52: 90–110.

Willner P, Muscat R and Papp M (1992) Chronic mild stress-induced anhedonia: A realistic animal model of depression. *Neurosci Biobehav Rev* 16: 525–534.

Witkin JM, Statnick MA, Rorick-Kehn LM, et al. (2014) The biology of Nociceptin/Orphanin FQ (N/OFQ) related to obesity, stress, anxiety, mood, and drug dependence. *Pharmacol Ther* 141: 283–299.

Yun S, Donovan MH, Ross MN, et al. (2016) Stress-induced anxiety- and depressive-like phenotype associated with transient reduction in neurogenesis in adult nestin-CreERT2/Diphtheria toxin fragment a transgenic mice. *PLoS One* 11: e0147256.

Zhao J, Jung YH, Jang CG, et al. (2015a) Metabolomic identification of biochemical changes induced by fluoxetine and imipramine in a chronic mild stress mouse model of depression. *Sci Rep* 5: 8890.

Zhao L, Xiong Z, Lu X, et al. (2015b) Metabonomic evaluation of chronic unpredictable mild stress-induced changes in rats by intervention of fluoxetine by HILIC-UHPLC/MS. *PLoS One* 10: e0129146.

Zucchini S, Buzzi A, Barbieri M, et al. (2008) FGF-2 overexpression increases excitability and seizure susceptibility but decreases seizure-induced cell loss. *J Neurosci* 28: 13112–13124.