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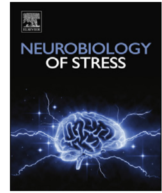

Corticosterone exposure augments sensitivity to the behavioral and neuroplastic effects of fluoxetine in C57BL/6 mice

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ABSTRACT

Both genetic background and pre-existing stress play critical roles in the effects of antidepressant drugs. The current studies showed this principal by demonstrating that exposure to the stress hormone corticosterone (CORT) allowed behavioral and neurogenic effects to emerge following chronic treatment with fluoxetine of C57BL/6 mice, a strain ordinarily resistant to these effects. Adult male mice were implanted subcutaneously with 21-day slow-release CORT pellets (10 mg) or placebo and then co-treated with 5 mg/kg fluoxetine (b.i.d., i.p.) or saline for 14 days. Animals were then assessed for approach behavior in the novelty-induced hypophagia (NIH) test, hippocampal cell proliferation, corticosteroid receptor expression, and CORT plasma levels. Co-treatment of CORT with fluoxetine significantly reduced approach behavior in the novel environment of the NIH test and increased hippocampal cell proliferation whereas fluoxetine given alone was ineffective. CORT given alone did not alter approach behavior in the novel environment and caused a smaller increase of cell proliferation. The CORT effect was blocked by adrenalectomy and was likely due to increased adrenal feedback. Cell proliferation in CORT-treated animals was associated with reduced mineralocorticoid, but not glucocorticoid, receptor mRNA expression. Although the pellets were advertised to release CORT for 21 days, plasma CORT levels were increased at 1 day after implantation but were not sustained when measured at 7 days or longer intervals. Nevertheless, the transient CORT increase was sufficient to induce long-lasting behavioral and molecular changes when followed by fluoxetine treatment. These studies warrant further investigation into the role of glucocorticoids and environmental stress as adjunctive facilitators of the response to antidepressants, especially for treatment-resistant patients.

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1. Introduction

Major Depressive Disorder (MDD) is one of the most common psychiatric disorders, with a lifetime prevalence of 17% in the United States and 4% worldwide (Eaton et al., 2008; Kessler et al., 2005). In terms of years lost to disability, MDD is considered one of the most disabling medical conditions and is predicted to become a leading contributor to the worldwide burden of disease (Mathers and Loncar, 2006). The majority of pharmacotherapies developed for the treatment of MDD target brain monoamine systems, primarily serotonin (5-HT), norepinephrine, and dopamine. The most common of these, the selective serotonin reuptake inhibitors (SSRIs) and selective norepinephrine reuptake inhibitors (SNRIs), comprise a large proportion of pharmaceutical sales and are considered first line treatments for MDD. Unfortunately, an estimated 40% of patients fail to respond to these therapies (Cipriani et al., 2009; Culpepper, 2010). Further insight into the neurobiological mechanisms underlying antidepressant response is needed for the development of more efficacious antidepressant regimens.

The combination of genetic vulnerabilities and environmental factors, such as stress, are thought to be significant contributors to the onset of depression in humans (Charney and Manji, 2004). The likelihood of experiencing a depressive episode is greatly increased following a stressful life event or after accumulation of chronic minor stresses (Caspi et al., 2003; Harkness and Monroe, 2006). Moreover, many patients suffering from depression exhibit signs of dysfunctional hypothalamic-pituitary-adrenal (HPA) axis activity,

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as demonstrated by elevated basal cortisol levels and resistance to dexamethasone, an exogenous steroid that suppresses cortisol in healthy individuals (Gillespie and Nemeroff, 2005; Pariante and Miller, 2001). Interestingly, successful antidepressant treatment is often associated with restored suppression of HPA axis response (Schule, 2007). Together, these findings suggest a potential role of stress hormones, such as cortisol (corticosterone (CORT) in rodents), in the pathology and treatment of depression.

CORT produces its effects in the central nervous system *via* activation of glucocorticoid (GR) and mineralocorticoid (MR) receptors. Though these receptors are ubiquitous throughout the brain, they are highly abundant in the hippocampus, where they provide crucial inhibitory feedback signals to the HPA axis (Jacobson and Sapolsky, 1991; Sapolsky et al., 1984). A reduction or absence of these inhibitory signals can promote hyperactivation of the axis and augmented secretion of glucocorticoids (Anacker et al., 2011; McEwen et al., 2012). In a healthy individual, elevated corticosteroid activity helps facilitate the physiological and behavioral adaptations required to appropriately respond to stressors and reinstate homeostasis. However, prolonged exposure to CORT can inhibit the proliferation and survival of adult-born hippocampal neurons, which have been shown to play an important role in the behavioral and neuroendocrine components of stress responses in rodents (Gould and Tanapat, 1999; Snyder et al., 2011). Conversely, chronic treatment of normal rodents with SSRIs, such as fluoxetine, increases hippocampal neurogenesis and neurotrophins such as brain derived neurotrophic factor (BDNF) (Duman and Monteggia, 2006; Krishnan and Nestler, 2008; Schmidt and Duman, 2006). Increased hippocampal neurogenesis is associated with behavioral indications of antidepressant efficacy in rodents, such as reduced hyponeophagia in the novelty-induced hypophagia (NIH) test and performance in the forced swim test (Dranovsky and Hen, 2006).

Not all strains of mice respond to the behavioral and neurogenic effects of antidepressant treatments. For example, normal C57BL/6 mice are unresponsive to the behavioral effects of chronic fluoxetine treatment, measured in the NIH test, and do not exhibit increased hippocampal cell proliferation (Balu et al., 2009a). Rodent strains that are unresponsive to antidepressants could provide information about treatment resistance. However, the effects of antidepressants may be altered after exposure to stress. CORT is a vital component of the central nervous system's stress response circuitry. Although corticosteroids alone do not encompass all aspects of stress exposure (Belzung, 2014), previous studies have shown that chronic CORT exposure can induce a depressive-like motivational state in rodents that is similar to that produced by a chronic mild stress paradigm (Gourley et al., 2008). Moreover, CORT treatment alone is sufficient to alter molecular targets that are implicated in depression and antidepressant efficacy, such as hippocampal neurogenesis (Bilsland et al., 2006; Gourley and Taylor, 2009). In a small clinical study Dinan et al. (1997), found that 4-day dexamethasone therapy significantly enhanced antidepressant response to SSRIs in treatment-resistant patients. Therefore, we hypothesized that activation of stress circuitry might be important to reveal the behavioral and neurogenic effects of the SSRI fluoxetine in C57BL/6 mice, a non-responsive mouse strain.

In the current study we investigated the effects of exposure to commercial CORT pellets for 21 days in augmenting fluoxetine's behavioral and proliferative effects in C57BL/6 mice. The results of this study showed that chronic fluoxetine produced behavioral effects in the NIH test only in mice exposed to CORT. Furthermore, CORT administration with fluoxetine co-treatment augmented hippocampal cell proliferation, an effect potentially mediated by alterations in hippocampal corticosteroid receptor expression. Interestingly, analysis of plasma at the end of treatment revealed a paradoxical decrease in CORT levels in animals treated with the pellets, suggesting that the CORT pellets did not work as advertised. Adrenalectomized animals implanted with CORT pellets revealed a sharp drop in CORT plasma levels by day 7 of treatment, indicating that this method of CORT exposure produced transiently elevated, but not sustained, CORT levels. Nevertheless, these experiments revealed the important finding that CORT exposure potentiates the behavioral and neurogenic effects of chronic fluoxetine administration in a mouse strain that is otherwise non-responsive to this antidepressant treatment.

2. Materials and methods

2.1. Animals

Intact and adrenalectomized male C57BL/6J, 7–8 weeks old upon arrival, were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were housed in groups of 4 (except for those used in the NIH test whom were housed in pairs) in polycarbonate cages and maintained on a 12 h light–dark cycle (lights on at 0700 h) in a temperature (22 °C)- and humidity-controlled environment. Food and water were available ad libitum. All experiments were approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania.

2.2. Experimental design

2.2.1. Experiment 1

Intact animals were implanted with CORT pellets (10 mg) or placebo pellets. Beginning on day 7 of CORT treatment, animals were dosed with either fluoxetine (5 mg/kg b.i.d., i.p.) or saline daily for the remaining 14 days of the experiment. Cohort 1: Animals were tested in the NIH and home cage test on the last two days of drug treatment ($n = 8–10$ per group). Cohort 2: Animals received a single injection of BrdU on the last day of drug treatment and were sacrificed 24 h later. In these animals hippocampal tissue was dissected and analyzed for BrdU positive cells and corticosteroid mRNA expression. Trunk blood was collected at time of sacrifice and analyzed for plasma CORT levels ($n = 15–19$ per group).

2.2.2. Experiment 2

Adrenalectomized animals were implanted with CORT pellets (10 mg) or placebo pellets and received chronic fluoxetine treatment as described in Experiment 1. All mice received additional CORT replacement through the drinking water (25 μg/ml in 0.9% saline) to prevent the loss of electrolyte homeostasis (Funder, 2006) and eliminate the confounding effects of adrenalectomy alone on neurogenesis (Cameron and Gould, 1994). Animals received a single injection of BrdU on the last day of drug treatment and were sacrificed 24 h later. Hippocampal tissue was dissected and analyzed for BrdU positive cells ($n = 7–10$ per group).

2.2.3. Experiment 3

Intact animals were implanted with CORT pellets (2.5 mg) or placebo pellets and received chronic fluoxetine treatment as described in Experiment 1. Animals received a single injection of BrdU on the last day of drug treatment and were sacrificed 24 h later. Hippocampal tissue was dissected and analyzed for BrdU positive cells ($n = 9–10$ per group).

2.2.4. Experiment 4

Adrenalectomized animals were implanted with CORT pellets (10 mg) or placebo pellets and then sacrificed 1, 7, 14, or 21 days after implantation. Trunk blood was collected at time of sacrifice and analyzed for plasma CORT levels ($n = 5–6$ per group).

2.3. Drug formulation

CORT pellets (2.5 mg and 10 mg, 21 day release, Innovative Research of America, Sarasota, FL, USA) were composed of a proprietary matrix of cholesterol, cellulose, lactose, phosphates and stearates designed to facilitate continuous and sustained diffusion of CORT over a period of 21 days. Placebo pellets consisted of the same matrix without the active product. Fluoxetine hydrochloride (5 mg/kg; Anawa, Zurich) was dissolved in distilled water and delivered by intraperitoneal (i.p.) injection in a volume of 10 ml/kg. Fluoxetine was administered twice daily because, due to its half-life, this dosing strategy results in relatively stable plasma levels (Hodes et al., 2010) and occupation of brain serotonin transporters (Hirano et al., 2004). Control animals received saline (0.9% NaCl). 5-Bromo-deoxyuridine (BrdU; Roche Applied Sciences Indianapolis, IN) was dissolved in warm saline at a dose of 200 mg/kg and administered i.p. in a volume of 10 ml/kg.

2.4. NIH test

Mice were pair housed and trained to eat a palatable food (three peanut butter chips presented in a small, clear petri dish) in a home cage environment. Animals were trained daily in 15-min sessions until they met the criteria of three consecutive days with approach latencies of 30 s or less. Opaque, black, plastic dividers were placed inside each cage to separate the mice during training of home cage training sessions. Mice were allowed to habituate to the dividers for 1 h before the start of the training session. Once all animals had met criteria, training sessions were suspended and drug treatments were initiated. Three days before novel testing all animals were re-exposed to the peanut butter chips through additional training sessions. For novel cage testing, peanut butter chips were presented in the center of an empty, clear polycarbonate cage (25.5 × 46 × 20 cm) with bright lighting (60 W light bulb) and scented with lemon (20% Lemon Joy solution). Novel cage testing was videotaped. Mice were placed into the test cage and the latency to approach during the 15-min test session was measured. The approach latency was defined as the time to ingestion. There was no food deprivation or habituation period prior to the novel cage test. All behavioral testing took place during the light phase. The home cage test was performed the day after the novel cage test.

2.5. BrdU incorporation using flow cytometry

Flow cytometry is a frequently used method for analyzing newly dividing cells in the hippocampus. This method has been previously validated by our lab and others, and compared to results obtained from immunostaining (Balu et al., 2009b; Bilsland et al., 2006; Spoelgen et al., 2011). BrdU labeling was measured in cells displaying the nuclear marker 7-aminoactinomycin D (7-AAD) by flow cytometry as previously described (Balu et al., 2009b). Briefly, mice were decapitated 24 h following BrdU injection, their brains quickly removed, and the hippocampus dissected. Hippocampal tissue was manually minced, digested using an enzymatic mixture (1 mg/ml papain, Roche Applied Science; 0.1 M L-cysteine, Sigma–Aldrich, St. Louis, MO), and then mechanically triturated to form a single cell suspension. Cells were fixed, permeabilized, and stained using the fluorescein isothiocyanate (FITC) BrdU Flow Kit (BD Biosciences, San Jose, CA). Data were collected on the same day using a BD FACS Canto System (BD Biosciences) at the University of Pennsylvania Flow Cytometry Core Facility. Background signals were controlled for by collecting data from a BrdU-free control. All data were analyzed using BD FACSDiva Software (BD Biosciences).

2.6. Analysis of corticosteroid receptor expression using quantitative real-time polymerase chain reaction (qRT-PCR)

RNA was extracted with Trizol reagent (Gibco BRL, Life Technologies, NY) and purified using the RNeasy Mini Kit (Qiagen, Valencia, CA) following the manufactures' instructions. RNA concentrations were measured and 300 ng/µl RNA was used as a template to synthesize c-DNA using the Superscript Vilo c-DNA Synthesis kit (Invitrogen, Carlsbad, CA, USA). All reactions were performed with a master mix of SYBR green (Applied Biosystems, Austin, TX) and 300 nM primers (final concentration). Quantitative real-time polymerase chain reactions (qRT-PCR) were run using the Stratagene MX3000 and MXPro QPCR software. Cycling parameters were as follows: 95 °C for 10 min, 40 cycles at 95 °C (30 s) and 60 °C (1 min), ending with a melting curve analysis to control for amplification. All reactions were performed in triplicate and the mean cycle threshold was used for analysis. The mRNA levels of target genes were normalized to the house-keeping gene TATA binding protein (TBP) using the $2^{\Delta ct}$ method. Primer sequences are available upon request.

2.7. Analysis of plasma CORT using enzyme-linked immunosorbent assay (ELISA)

Trunk blood was collected at time of sacrifice, which occurred between 8 and 10 am for all experiments. Blood was stored in 0.5 mL heparin and centrifuged at 3000 rpm for 20 min. Plasma was removed and stored frozen (−80 °C) until analysis. The amount of CORT in the plasma from each sample was measured in duplicate by ELISA following the manufactures instructions (Immunodiagnostic Systems, Fountain Hills, AZ). Intra-assay variability for the CORT kit ranged from 5.9% to 7.0%, inter-assay variability ranged from 8.2 to 8.9%; mean assay sensitivity was 0.17 ng/mL.

2.8. Data analysis

One-way and two-way ANOVA were performed to examine the significance of differences between treatments. Significant overall main effects ($p < 0.05$) or interactions showing a trend ($p < 0.10$) were followed by Tukey or Bonferroni post-hoc tests. For all follow-up tests, $p < 0.05$ was considered statistically significant. Data are expressed as mean ± SEM.

3. Results

3.1. The effects of 10 mg CORT pellet exposure and fluoxetine treatment on behavior

Mice were randomly assigned to either placebo or CORT pellet exposure, and then further separated into either saline or fluoxetine treatment groups. As seen in Fig. 1A, a two-way repeated measures ANOVA revealed a significant interaction [$F(9,102) = 3.761$, $p < 0.001$] and main effect [$F(3,102) = 12.68$, $p < 0.001$] of time on body weight during drug treatment. Placebo-treated animals exhibited significant weight gain by day 14 ($p < 0.05$). Although CORT-exposed animals failed to gain weight within the initial 7 days of treatment, animals subsequently treated with fluoxetine showed significant weight gain by day 21 ($p < 0.001$) whereas saline treated animals continued to show inhibited weight gain. The overall change in body weight (from day 1 to day 21) shown in Fig. 1B illustrates a significant main effect of treatment with fluoxetine [$F(1, 34) = 8.830$, $p < 0.01$].

The behavioral effects of CORT and fluoxetine treatment were then measured in the NIH test. Exposure to a novel environment increased approach latency [$F(1,65) = 972.4$, $p < 0.0001$] and

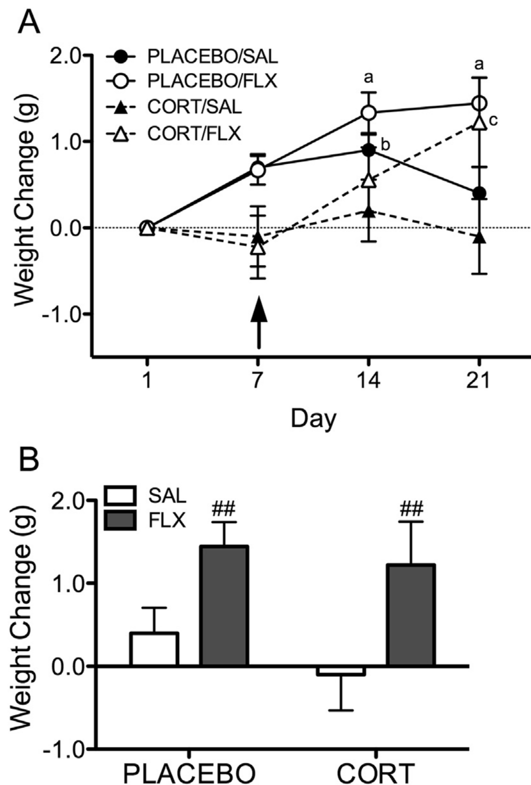

Fig. 1. Effects of 10 mg CORT pellet and fluoxetine treatment on weight gain. SAL = Saline, FLX = Fluoxetine, CORT = Corticosterone. Arrow denotes start of fluoxetine treatment. (A) Weight change over time in each treatment group. Placebo treated animals gained weight over time. CORT/SAL animals displayed inhibited weight gain whereas CORT/FLX animals showed normal weight gain after beginning FLX treatment. Symbols represent significant differences compared to day 1: a (PLACEBO/FLX, $p < 0.05$) b (PLACEBO/SAL, $p < 0.01$) c (CORT/FLX, $p < 0.01$) (B) Overall weight change (from day 1 to day 21) showed that fluoxetine treatment increased weight gain in both placebo and CORT treated animals (n = 9–10 per group). Data is depicted as mean ± SEM. ##$p < 0.01$ within placebo or CORT treated groups.

reduced the amount of food consumed [$F(1,68) = 136.0$, $p < 0.0001$] compared to home cage in all treatment groups. There was a significant interaction between CORT exposure and fluoxetine treatment in approach latencies in the novel environment [$F(1,33) = 8.041$, $p < 0.01$]. CORT-exposed animals treated with fluoxetine displayed significantly lower approach latencies compared to CORT-exposed animals treated with saline. Moreover, fluoxetine treatment had no effect on approach latency in placebo-treated animals in the novel environment (Fig. 2A). There were no significant differences in food consumption in the novel environment between drug treatment groups (Fig. 2B). In the home cage, CORT treatment significantly reduced latency to approach [$F(1, 33) = 4.772$, $p < 0.05$] and increased the amount of food consumed compared to placebo treated animals [$F(1, 34) = 4.956$, $p < 0.05$] (Fig. 2C and D).

3.2. *The effects of 10 mg CORT pellet exposure and fluoxetine treatment on hippocampal cell proliferation, CORT plasma levels and corticosteroid receptor expression*

In a separate cohort, animals received a single injection of BrdU on the last day of drug treatment and were sacrificed 24 h later. Hippocampal tissue was analyzed for BrdU positive cells and corticosteroid mRNA expression. Additionally, trunk blood was collected at time of sacrifice and analyzed for plasma CORT levels. As seen in Fig. 3A, flow cytometric analysis of hippocampal tissue revealed that in placebo treated animals, fluoxetine had no effect on hippocampal cell proliferation. Interestingly, CORT-exposure significantly increased hippocampal cell proliferation compared to placebo treated animals [$F(1,59) = 50.87$, $p < 0.001$]. Moreover, there was a significant interaction between CORT exposure and fluoxetine treatment on neurogenesis [$F(1, 59) = 6.702$, $p < 0.05$]. Post-hoc multiple comparisons revealed that CORT-exposed animals treated with fluoxetine displayed significantly higher hippocampal cell proliferation compared to CORT-exposed animals treated with saline.

Analysis of circulating CORT levels at the time of sacrifice revealed that exposure to CORT pellets significantly reduced CORT plasma levels in both saline and fluoxetine treated animals by approximately 50% when measured on day 21 [$F(1, 66) = 36.06$, $p < 0.001$] (Fig. 3B). Fluoxetine treatment did not alter CORT levels.

Glucocorticoid (GR) and mineralocorticoid (MR) receptor transcription was examined in the hippocampus as a potential molecular mechanism underlying the CORT-induced neurogenic response to fluoxetine in C57BL/6 mice. There was no significant effect of CORT or fluoxetine on GR mRNA expression (Fig. 3C). However, exposure to CORT significantly reduced hippocampal MR mRNA expression in both saline and fluoxetine treated animals [$F(1,68) = 4.276$, $p < 0.05$] (Fig. 3D).

3.3. *The effects of adrenalectomy on 10 mg CORT pellet exposure induced hippocampal cell proliferation*

To investigate the mechanisms underlying the increase in hippocampal cell proliferation by CORT pellets, adrenalectomized animals were used to examine the effects of 10 mg CORT pellet exposure and fluoxetine treatment on hippocampal neurogenesis in the absence of adrenal feedback. There was a significant main effect of CORT on cell proliferation [$F(1, 30) = 5.298$, $p < 0.05$] and a trend towards an interaction [$F (1, 30) = 3.372$ $p = 0.08$]. As illustrated in Fig. 4A, adrenalectomized CORT-exposed animals treated with fluoxetine, but not saline, displayed a significant two-fold increase in cell proliferation compared to placebo treated animals ($p < 0.05$). However, CORT treatment did not increase cell proliferation in adrenalectomized animals.

3.4. *The effects of 2.5 mg CORT pellet exposure and fluoxetine treatment on hippocampal cell proliferation*

We next examined whether a lower dose of CORT pellet exposure combined with fluoxetine treatment would induce an increase in hippocampal neurogenesis in intact animals. There was a significant main effect of CORT pellet exposure [$F(1, 35) = 6.477$, $p < 0.05$] and a significant interaction between CORT pellet exposure and fluoxetine treatment [$F(1, 35) = 4.705$, $p < 0.05$] on hippocampal cell proliferation. As shown in Fig. 4B, CORT-exposed animals treated with saline exhibited a significant increase in cell proliferation compared to placebo treated animals, as in prior studies. In contrast, the lower dose of CORT pellet was incapable of increasing hippocampal cell proliferation when combined with fluoxetine treatment.

3.5. *Evaluation of the sustained effects of 10 mg CORT pellet treatment on plasma CORT levels*

To determine whether 10 mg CORT pellets maintain elevated plasma CORT levels for the advertised duration, adrenalectomized animals were implanted with 10 mg CORT pellets on day 0 and, CORT plasma levels were assessed on day 1, 7, 14, and 21 post-implantation. As shown in Fig. 5, plasma CORT levels changed dramatically over time ($F(3,19) = 16.18$, $p < 0.01$), and were no longer in the supraphysiological range by the seventh day of CORT

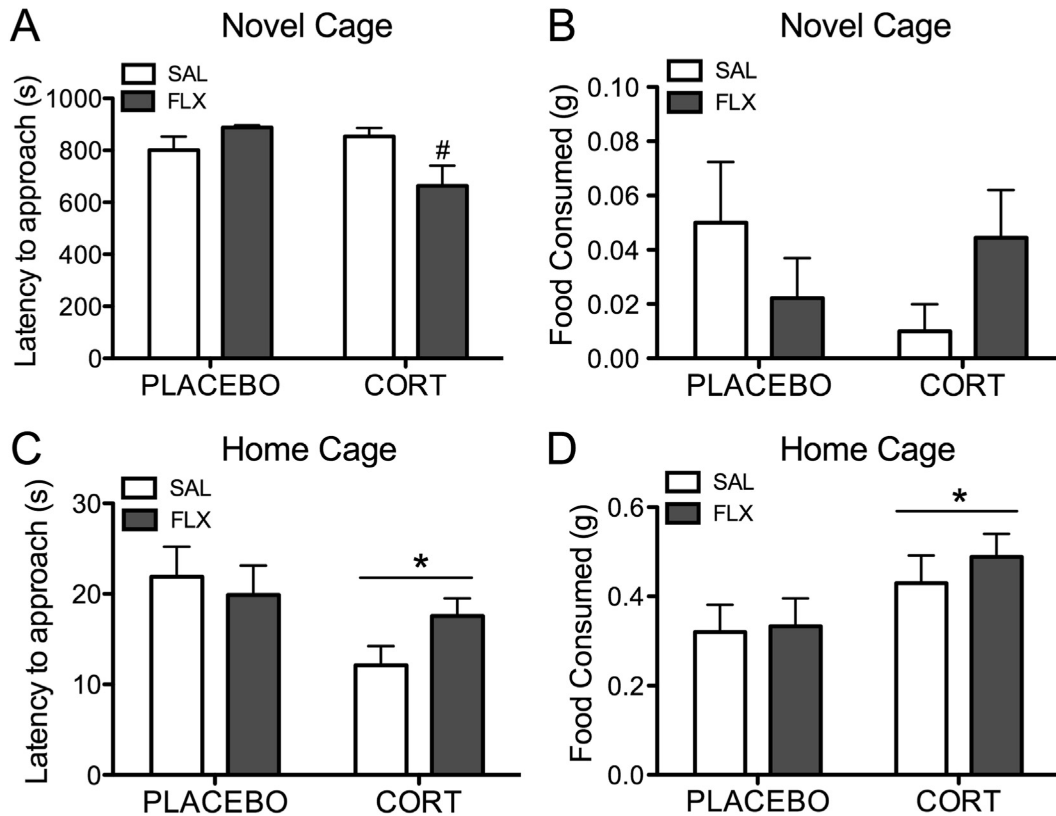

Fig. 2. Effects of 10 mg CORT pellet and fluoxetine treatment in the novelty induced hypophagia test. SAL = Saline, FLX = Fluoxetine, CORT = Corticosterone. A) Fluoxetine significantly reduced approach latency in the novel arena in animals exposed to CORT, but not placebo. B) There was no effect of treatment on amount consumed. C) In the home cage, CORT exposure significantly reduced latency to approach and D) increased the amount of food consumed (n = 8–10 per group). Data is depicted as mean ± SEM. #$p < 0.05$ within CORT treated groups, *$p < 0.05$ between placebo and CORT treated groups.

pellet exposure (Fig. 5).

4. Discussion

Activation of stress circuitry from implanted CORT pellets produced behavioral and neurogenic effects from chronic fluoxetine treatment in a strain of mice that would otherwise have been unresponsive to the effects of the antidepressant. Specifically, co-treatment of CORT with fluoxetine significantly reduced the effects of novelty stress measured on approach behavior in the NIH test and increased hippocampal cell proliferation. These two effects of antidepressant treatment have been linked together because the behavioral response to fluoxetine is blocked in mice that cannot increase hippocampal cell proliferation (Sahay and Hen, 2007). Although treatment with CORT alone unexpectedly increased cell proliferation to a lesser extent, this effect was absent in adrenalectomized mice while the augmented combination treatment effect was preserved. Measurement of plasma CORT levels revealed that the CORT pellet did not maintain elevated levels for more than a few days, even though it was expected to be active for 21 days, suggesting that the impact of the CORT treatment was likely the after-effect resulting from the supraphysiological levels of acute exposure. Overall, these findings reveal potential neurobiological mechanisms underlying effective antidepressant response in a unique model of treatment resistance.

Hyponeophagia, the unconditioned suppression of feeding in a novel environment, is a behavioral measure of stress that may be sensitive to the anxiolytic effects of chronic, but not acute, antidepressant treatment with SSRIs (Bechtholt et al., 2007; Bodnoff et al., 1988; Bodnoff et al., 1989; Dulawa and Hen, 2005; Dulawa et al., 2004). Fluoxetine's effect of reducing approach latency to food in a novel environment is abolished after focal irradiation of the hippocampus or genetic deletion of hippocampal precursor cells, indicating that hippocampal neurogenesis is a necessary component of this behavioral antidepressant response (David et al., 2009; Santarelli et al., 2003; Surget et al., 2008; Wang et al., 2008). Intriguingly, unlike other mouse strains, C57BL/6 mice did not exhibit reduced hyponeophagia or increased hippocampal neurogenesis following chronic fluoxetine treatment (Balu et al., 2009a). However, in the present study, we showed that CORT-exposure via pellet implantation induced a behavioral response to fluoxetine in the NIH test in this unresponsive strain. Moreover, CORT-exposure in combination with fluoxetine treatment produced a robust increase in hippocampal neurogenesis that was not seen in placebo treated animals. Although correlative, the increased behavioral response to chronic fluoxetine treatment in CORT-treated mice could be attributed to heightened hippocampal cell proliferation.

Stress is a well-established robust inhibitor of adult neurogenesis (Gould and Tanapat, 1999; McEwen et al., 2012). Similarly, CORT exposure alone has been shown to be a negative regulator of hippocampal neurogenesis (Bilsland et al., 2006; Brummelte and Galea, 2010; Cameron and Gould, 1994; Murray et al., 2008; Wong and Herbert, 2004). Reduced hippocampal cell proliferation typically coincides with increased plasma CORT levels, signifying that circulating CORT levels at the time of testing underlie CORT-induced changes in proliferation (Wong and Herbert, 2006). Paradoxically, we observed dramatically reduced plasma CORT levels in all CORT-exposed animals following the 21-day pellet treatment,

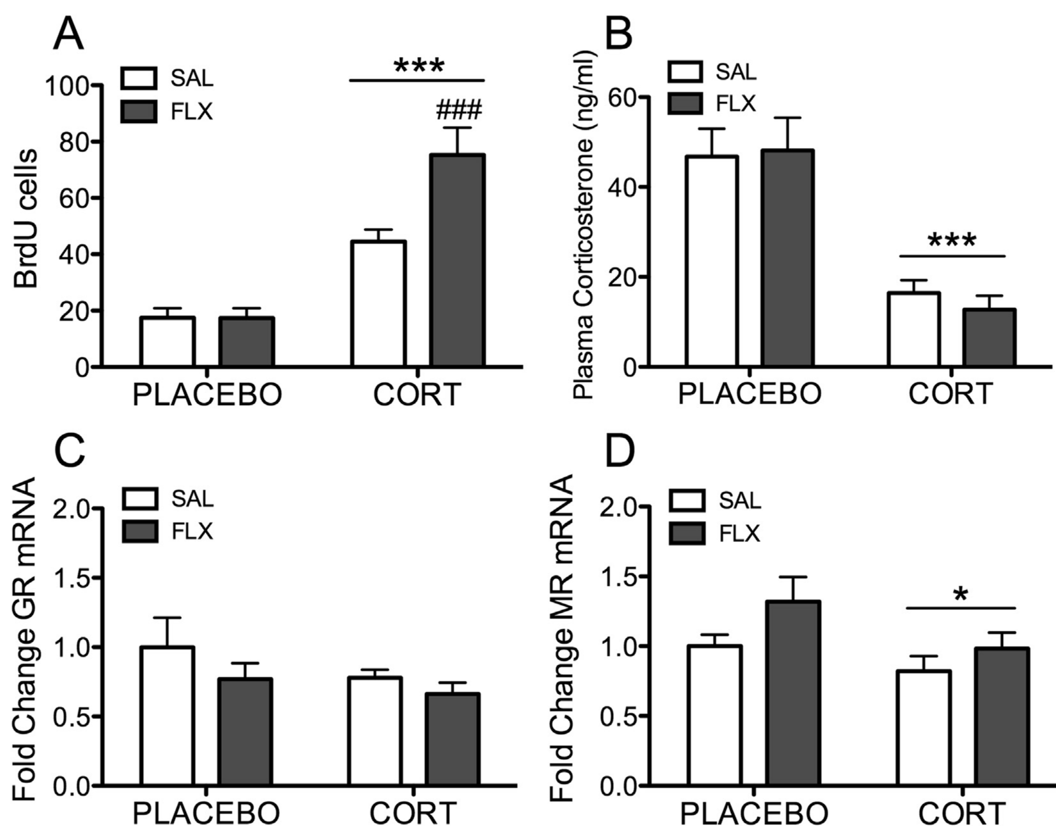

Fig. 3. Effects of 10 mg CORT pellet and fluoxetine treatment on hippocampal cell proliferation, CORT plasma levels, and hippocampal corticosteroid receptor expression. SAL = Saline, FLX = Fluoxetine, CORT = Corticosterone. A) Values are expressed as the number of BrdU-positive cells per 10,000 7-AAD events. Intact animals exhibited a significant increase in hippocampal cell proliferation after treatment with CORT. This effect was further augmented in CORT-exposed animals treated with fluoxetine. Fluoxetine had no effect in placebo treated animals (n = 14–18 per group). B) CORT treatment significantly reduced plasma CORT levels. Fluoxetine had no additive effect on CORT levels (n = 15–19 per group). C) CORT had no effect on hippocampal GR mRNA expression but (D) reduced hippocampal MR mRNA expression (n = 15–19 per group). Data is depicted as mean ± SEM. ###p < 0.001 within CORT treated groups, ***p < 0.001, *p < 0.05 between placebo and CORT treated groups.

while hippocampal cell proliferation was increased. We suspected this might have been due to unanticipated changes in adrenal function. The adrenals operate through an inhibitory feedback system in which increased circulating CORT levels serve as a signal for reduced synthesis and secretion from the adrenals (Herman and Cullinan, 1997; Sapolsky et al., 1984). CORT pellet treatment may have increased internal negative feedback to the point of adrenal inactivation, resulting in reduced endogenous circulating CORT levels and disinhibition of cell proliferation. To test for this we examined the effects of CORT pellet and chronic fluoxetine co-treatment on hippocampal cell proliferation in adrenalectomized animals. Notably, in the absence of adrenal feedback, CORT-exposed animals treated with saline did not demonstrate increased neurogenesis whereas those given fluoxetine still exhibited a proliferative response. Therefore, the mechanisms underlying the augmented neurogenic effect seen in combination treated animals cannot be attributed to artifacts of adrenal feedback. It is important to note, however, that adrenalectomized animals in all treatment groups were supplemented with a low dose CORT-treatment (25 μg/ml) delivered via drinking water. This mode of delivery produces small rhythmic changes in plasma CORT levels, which have been shown to be necessary for fluoxetine-stimulated neurogenesis in rats (Huang and Herbert, 2006). It is possible, then, that rhythmic fluctuations in CORT levels modulate sensitivity to the proliferative effects of fluoxetine. In spite of this, adrenalectomized placebo animals treated with fluoxetine did not exhibit increased proliferation, demonstrating that supplemental CORT alone was not sufficient to induce a neurogenic response to fluoxetine.

To determine whether a lower dose of CORT could elicit a neurogenic response in the presence of fluoxetine without increasing proliferation on its own, we evaluated hippocampal cell proliferation in intact animals treated with 2.5 mg CORT pellets. Similar to the 10 mg CORT pellet treatment, exposure to 2.5 mg CORT pellet treatment produced elevated cell proliferation. However, there was no additional effect in the presence of fluoxetine, suggesting that this dose is sufficient to produce CORT-induced increases in neurogenesis, but not sufficient to elicit an augmented proliferative response when combined with fluoxetine. This finding is in contrast to David et al. (2009) who showed a low dose of 5 mg/kg/day CORT treatment to be effective in reducing hippocampal cell proliferation alone and stimulating proliferation when paired with fluoxetine treatment in C57BL/6 mice. However, whereas the current study utilized a three-week CORT pellet treatment, David and colleagues had CORT delivered though drinking water and animals were treated for a substantially longer period of time (7 weeks). Therefore, rhythmic low dose CORT treatment over a longer period of time may be sufficient to increase neuronal sensitization to fluoxetine in this strain.

Hippocampal MRs and GRs play a vital role in mediating stress responsiveness. Altered corticosteroid activity can dysregulate the stress response system and enhance the risk of development of stress-related disorders (Groeneweg et al., 2012). On the other hand, synergistic interactions between hippocampal corticosteroid receptors and serotonergic signaling pathways may mediate the effects of CORT exposure on enhancing the neurogenic responses to

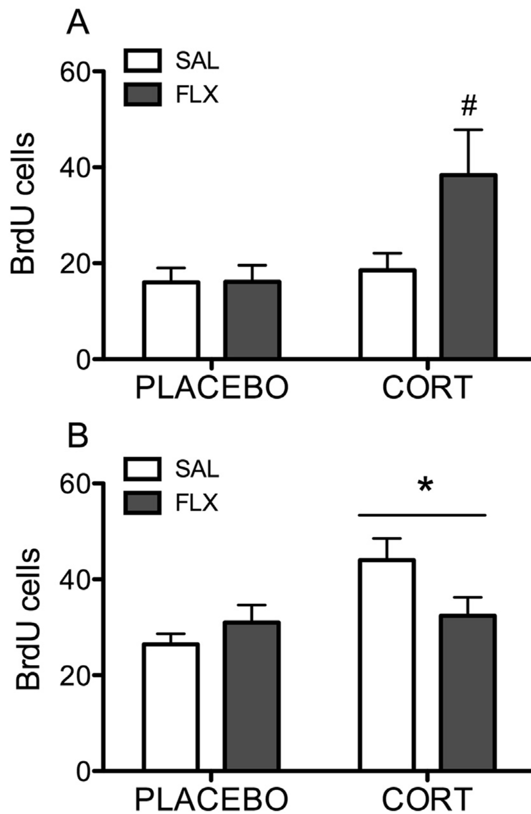

Fig. 4. Effects of adrenalectomy or low dose CORT exposure on hippocampal cell proliferation. SAL = Saline, FLX = Fluoxetine, CORT = Corticosterone. A) Values are expressed as the number of BrdU-positive cells per 10,000 7-AAD events. In adrenalectomized animals exposed to 10 mg CORT pellets, fluoxetine produced an increase in proliferation (n = 7–9 per group. B). In intact animals exposed to 2.5 mg CORT pellets, hippocampal cell proliferation was increased, but not further augmented by fluoxetine (n = 9–10 per group). Data is depicted as mean ± SEM. #$p < 0.05$ within CORT treated groups, *$p < 0.05$ between placebo and CORT treated groups.

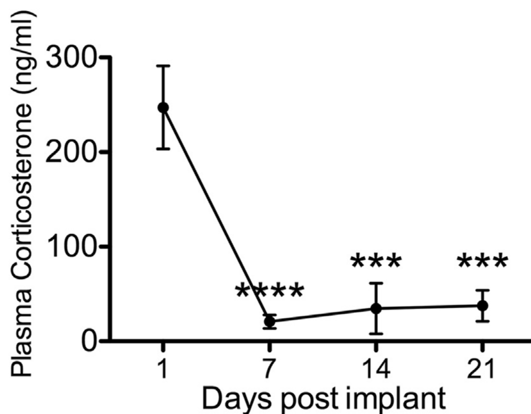

Fig. 5. Effects of CORT pellet on plasma CORT levels over time. Adrenalectomized animals implanted with 10 mg CORT pellets displayed significantly reduced CORT plasma levels after 7 days of treatment (n = 5–6 per group). Data is depicted as mean ± SEM. Absolute mean values are: 247.2 ± 43.92, 20.8 ± 7.02, 34.7 ± 24.44 and 37.6 ± 16.32 ng/ml ****$p < 0.0001$, ***$p < 0.001$ compared to day 1.

fluoxetine in C57BL/6 mice. For example, CORT treatment has been shown to facilitate fluoxetine-induced enhancement of dopaminergic modulation at the mossy fiber synapse (Kobayashi et al., 2013).

Consistent with recent findings, CORT treatment reduced hippocampal MR mRNA expression while having no effect on GR mRNA expression (Saenz del Burgo et al., 2013), suggesting that MR mRNA expression is more sensitive to the effects of CORT exposure. This may be due to the fact that MRs exhibit 10-fold higher affinity for CORT compared to GRs (Joels et al., 2008). Hippocampal MRs selectively contribute to neuronal stability and excitatory tone and have been shown to mediate behavioral reactivity to a novel environment (Berger et al., 2006; Oitzl et al., 1994), hence changes in MR expression and function are likely to impact both hippocampal plasticity and associated behaviors. Interestingly, in the current study, MR expression was similar between CORT-exposed animals treated to either saline or fluoxetine treated, indicating that variations in expression alone cannot explain the augmented behavioral and neurogenic responses seen in CORT-exposed animals treated with fluoxetine. However, it is important to note that MR and GR expression exhibit a diurnal regulation that is modulated by circulating CORT levels (Herman et al., 1993; Holmes et al., 1995). Since exogenous CORT treatment has been reported to flatten the natural circadian rhythm of plasma CORT (Leitch et al., 2003), CORT pellet exposure could potentially alter rhythmic MR and GR occupancy throughout the day. Thus, the observed neurogenic effects of CORT and fluoxetine treatment might be mediated by changes in circadian expression of MR and GRs. Further studies are needed to confirm this.

A major caveat of this study is the lack of sustained CORT release from the pellet treatment. CORT pellets have been used to model chronically elevated CORT levels, a physiological indicator of dysregulated HPA axis functioning and risk factor for the onset of MDD (Goodyer et al., 2010; Owens et al., 2014). On the contrary, we found that CORT plasma levels dropped precipitously between day 1 and day 7 of CORT pellet treatment. Rather than a sustained release, the CORT pellets produced a rapid, but short-lived, elevation of CORT during the initial days of exposure and then became inactive, resulting in CORT levels falling to the normal physiological range at the time of the experimental studies. Similar findings were reported in another study evaluating the performance of pellets designed to release CORT for 7 days in birds. Muller et al. (2009) found that CORT plasma levels peaked 1–2 days after pellet implantation and reached placebo levels by day 3. The authors posited that the pellets, being originally designed for rodents, are not as effectively metabolized in other species. However, our data corroborate the findings that the CORT pellets do not reliably produce sustained CORT release for the indicated length of treatment. In light of this, slow-release CORT pellets are not appropriate for modeling prolonged elevated CORT levels. Instead, these pellets may more closely model the effects of exposure to a strong acute stressor, as with post-traumatic stress disorder. Interestingly, the initial surge in CORT levels during the first few days of treatment was sufficient to induce long lasting molecular and behavioral changes in treated animals, suggesting that alterations in CORT levels, not necessarily at a pathological level, can impact the efficacy of fluoxetine.

In conclusion, this study found that exposure to exogenous CORT increases behavioral and neurogenic sensitivity to chronic fluoxetine treatment in C57BL/6 mice, a typically non-responsive strain of mice. These data recapitulate the general findings that genetic background and environment play a fundamental role in antidepressant response. Although slow-release CORT pellets did not model the effects of sustained elevated CORT exposure as anticipated, these studies effectively indicate that CORT exposure is sufficient to reveal the anxiolytic and neuroplastic effects of chronic fluoxetine treatment in a typically unresponsive strain and could model an augmentation strategy for treatment-resistant patients. These findings implicate corticosteroid receptor activity and modulation as a potential variable in the stratification of antidepressant

response in patients with MDD and possibly as a mediator of the effects of environmental stress on the effects of antidepressants.

Conflict of interest

The authors have no conflict to disclose.

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