Psychoneuroendocrinology 87 (2018) 74–82

Contents lists available at ScienceDirect

Psychoneuroendocrinology

journal homepage: www.elsevier.com/locate/psyneuen

Molecular changes associated with escitalopram response in a stress-based model of depression

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ARTICLE INFO

Keywords:
Treatment-resistant depression
Escitalopram
Hypothalamus
Corticosterone
HPA axis
Neuroinflammation

ABSTRACT

Converging evidence points at hypothalamus-pituitary-adrenal (HPA) axis hyperactivity and neuroinflammation as important factors involved in the etiopathogenesis of major depressive disorder (MDD) and in therapeutic efficacy of antidepressants. In this study, we examined the molecular effects associated with a response to a week-long treatment with escitalopram in the chronic escape deficit (CED) model, a validated model of depression based on the induction of an escape deficit after exposure of rats to an unavoidable stress. We confirmed our previous result that a treatment with escitalopram (10 mg/kg) was effective after 7 days in reverting the stress-induced escape deficit in approximately 50% of the animals, separating responders from non-responders. Expression of markers of HPA axis functionality as well as several inflammatory mediators were evaluated in the hypothalamus, a key structure integrating signals from the neuro, immune, endocrine systems. In the hypothalamus of responder animals we observed a decrease in the expression of CRH and its receptors and an increase in GR protein in total and nuclear extracts; this effect was accompanied by a significant decrease in circulating corticosterone in the same cohort. Hypothalamic IL-1β and TNFα expression were increased in stressed animals, while CXCL2, IL-6, and ADAM17 mRNA levels were decreased in escitalopram treated rats regardless of the treatment response. These data suggest that efficacy of a one week treatment with escitalopram may be partially mediated by a decrease HPA axis activity, while in the hypothalamus the drug-induced effects on the expression of immune modulators did not correlate with the behavioural outcome.

1. Introduction

Understanding the neurobiological basis of major depressive disorder (MDD) and the mechanisms behind the efficacy of antidepressant strategies is a pressing need for the scientific community worldwide.

One of the most reliable reported neurobiological alterations in MDD is impaired hypothalamus-pituitary-adrenal (HPA) axis functionality: HPA axis hyperactivity, glucocorticoid (GC) insensitivity and CRH overexpression in the hypothalamus or the cerebrospinal fluid have been reported in depressed patients (Sanders and Nemeroff, 2016), and similar effects were observed in preclinical studies in animal models of depression (Wang et al., 2008). Chronically elevated glucocorticoids may exert detrimental effects on the central nervous system (CNS) functionality, cause atrophy and disruption of connectivity, especially in the hippocampus and prefrontal cortex, and can also increase the number of inflammatory cells and the production of pro-inflammatory cytokines both centrally and in the periphery (Himmerich et al., 2013). Elevated levels of inflammatory markers have been reported in peripheral blood and spinal fluid of depressed patients (Köhler et al., 2017), while a variety of pro- and anti-inflammatory cytokines were altered in the frontal cortex of subjects with MDD (Shelton et al., 2011). HPA axis hyperactivity may be associated as well with neuroinflammatory process: cytokines, in fact, can activate the HPA axis and can impair glucocorticoid receptor (GR) functioning at multiple levels (Zunszain et al., 2011): by inhibiting GR translocation to the nucleus, GR-mediated gene transcription or by stimulating GRβ, an inactive form of GR (Anacker et al., 2011).

Accumulating evidence, clinical and preclinical, has been reported that the efficacy of antidepressant treatments may rely on normalization of hypothalamic function, by restoring GR mediated feedback inhibition of HPA axis activity (Anacker et al., 2011; Funato et al., 2006), and of cytokine production and plasma levels (Kenis and Maes,

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http://dx.doi.org/10.1016/j.psyneuen.2017.10.011
Received 20 June 2017; Received in revised form 15 September 2017; Accepted 12 October 2017
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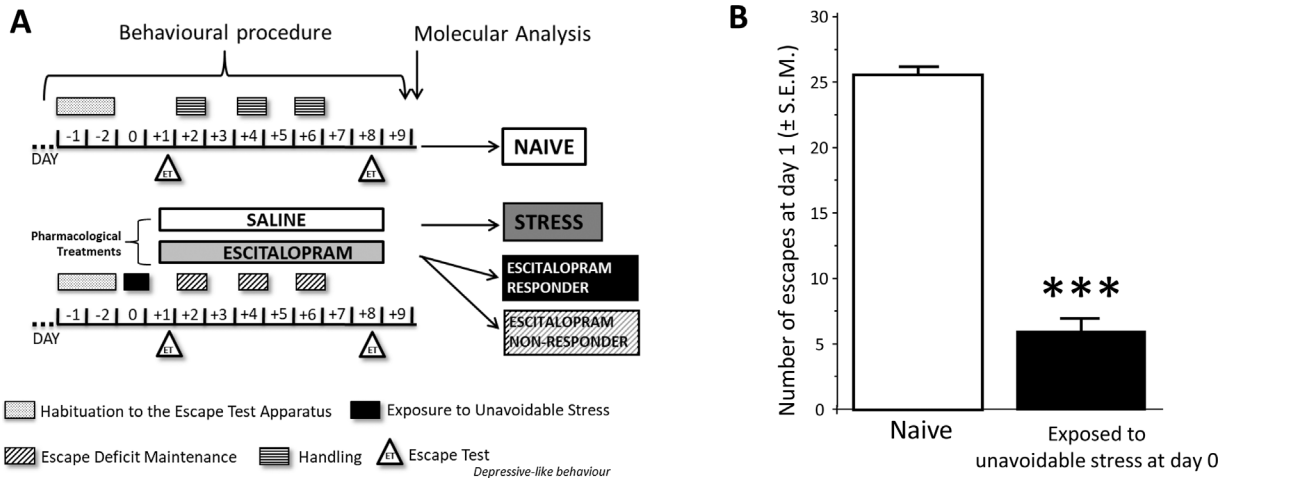

Fig 1. Flowchart of the experimental procedure (A). Induction of the acute escape deficit (B): the unavoidable stress exposed group (n = 85) underwent exposure to an acute unavoidable stress procedure and was tested for escape deficit 24 h later together with the naive group (n = 15) that was not exposed to the unavoidable stress procedure; scores are expressed as the mean number of escapes ± S.E.M. in a test session consisting of 30 consecutive electric shocks, every 30 s. Comparisons were made by one way analysis of variance (ANOVA), *** $p < 0.0001$ vs naive group (See materials and methods for a detailed description of the behavioural procedure).

2002).

However, the efficacy of currently available antidepressants is still limited by a significant delay between start of treatment and onset of beneficial effects and by high rates of treatment resistance (Willner and Belzung, 2015). Chronic behavioural models possess a great potential to help elucidate and overcome these important limitations of antidepressant treatment because on one side they more closely mimic the delayed pharmacological response observed in patients (O'Leary and Cryan, 2013), and in some of them, like the chronic unpredictable stress and chronic social defeat, is possible to separate rodents into bimodal subpopulations that respond or not to traditional antidepressant treatments (Willner and Belzung, 2015).

Along with these paradigms, also the chronic escape model of depression (CED), a valid and straightforward model, that is based on the induction of an escape deficit after exposure of rats to unavoidable stress (Benatti et al., 2012; Gambarana et al., 2001), can be used to study pharmacological antidepressant responsiveness. In fact, we previously demonstrated that: 1. Combination of acetylsalicylic acid with fluoxetine (FLX) accelerates and potentiates the effect of the antidepressant alone (Brunello et al., 2006); 2. After 7 days of treatment, escitalopram (ESC) (10 mg/kg) is already effective in restoring the natural tendency to avoid a noxious stimulus in about 50% of stressed rats developing an escape deficit (Benatti et al., 2014); 3. Co-administration of aspirin with ESC increases the treatment response rate to escitalopram at about 75% (Brunello et al., 2007).

To investigate the molecular mechanism behind the therapeutic efficacy of antidepressants, we examined the different molecular effects associated with a response to a week-long treatment with escitalopram in the CED model of depression on two key elements known to be altered in MDD: HPA axis functionality and cytokine production within the CNS. We focused on the hypothalamus, the neuro-endocrine interface in the brain and a key station for central circuits to orchestrate the maintenance of body homeostasis or allostasis, that is highly responsive to immune signals as well (MDAlboni et al., 2017a,b).

For this purpose, animals developing an escape deficit were treated for a week with escitalopram, tested for their ability to avoid a noxious stimulus and divided in responder and non-responder as previously reported (Benatti et al., 2014). Then, we evaluated the effects of escitalopram on expression of CRH, its receptors (CRHR1 and CRHR2), and heat shock protein 70 (HSP70), and also on glucocorticoid receptor (GR) mRNA and protein levels in the hypothalamus and on circulating corticosterone. We also measured in our model changes in hypothalamic expression of pro-inflammatory cytokines: Interleukin (IL-) 1β, Tumour Necrosis Factor (TNF) α, Interferon (IFN) γ, IL-6 (and its system [IL-6R and gp130, SOCS3, ADAM10 and ADAM17]), IL-18, rat homologues of IL-8 (CXCL1 and CXCL2), and two anti-inflammatory cytokines (IL-10, IL-4).

2. Methods

2.1. Animals

Experiments were performed on male Sprague-Dawley albino rats (Charles River Laboratories, Calco, Italy), weighing 150–175 g at their arrival. Animals were housed in polycarbonate cages (38 × 15 × 22 cm; 2 per cage) with ad libitum access to food and tap water throughout the study, and maintained under a 12 h inverted light-dark cycle (lights on at 6.00 p.m.) at the ambient temperature of 21 + 3 °C and relative controlled humidity. Animals were left undisturbed for 3 weeks before beginning any behavioural procedure. Experiments were carried out under a red light. Animals were handled and weighed daily, from the day before the beginning of the behavioural procedure throughout the whole experiment. The procedures used in this study were in strict accordance with European legislation on the use and care of laboratory animals (EU directive 2010/63/EU), with the guidelines of the National Institutes of Health on the use and care of laboratory animals (NIH Publications No.8023), and had the approval of the Ministry of Health and of the local Ethical Committee. All efforts were made to minimize animal suffering and to reduce the number of animals used in this study.

2.2. Behavioural procedures and pharmacological treatments

Animals were exposed to an unavoidable stress (US) session for the induction of an escape deficit. The US session consists of 50 min of immobilization in flexible wire nets and exposure to 80 electric shocks (1,5 mA x 7 s, one every 30 s), through an electrode applied to the distal third of the tail and connected to an S48 Grass stimulator as already described (Benatti et al., 2012).

Twenty-four hours later (Day 1), rats exposed to the US and a group of animals not exposed to the US (Naive), were tested for their reactivity towards an avoidable noxious shock in an escape-test apparatus, divided by a sliding door into two chambers one of which was connected to an electrode applied to the tail of the rat through a stimulator. All tested animals were allowed to explore the apparatus for at least 20 min/day in the 3 days preceding the test (Fig. 1A). The test

session consists of 30 consecutive electric shocks (1.2 mA × 3.5 s), every 30 s, starting after a 5 min habituation period. Behaviour was labelled "escape" when an animal moved to the neutral chamber of the apparatus within this 3.5 s period (Brunello et al., 2006; Gambarana et al., 2001).

Naive rats scored between 20 and 30 escapes out of 30 trials [mean of the naive group at day 1 ± S.E.M. = 25.8 ± 0.60; n = 15]. Approximately 75% of rats exposed to the unavoidable stress developed an escape deficit and showed a significantly lower mean of escapes with respect to the naive group [6.24 ± 0.94; n = 85; ANOVA univariate; F (1.98) = 73.51725; p < 0.0001; Fig. 1B]. After the test, animals scoring 0–9 escapes were randomly divided in two groups and received for 1 week an i.p. injection of either saline (1 mL/kg) (n = 14; stress) or escitalopram (10 mg/kg/day, kindly provided by H. Lundbeck A/S. Copenhagen-Valby, Denmark) (n = 25; escitalopram). The escape deficit was maintained by repeated exposure to mild unavoidable stress on alternate days as previously described (Brunello et al., 2006; Gambarana et al., 2001). Saline and escitalopram treated animals were tested at day 8 with a group of naive rats (n = 15; naive) that did not receive any treatment and were handled on alternate days (Fig. 1A). All stress procedures and the escape tests were conducted during the dark phase (9.00 a.m and 3 p.m.), while the pharmacological treatments were performed before the beginning of the light phase (5:30 p.m.).

Rats were sacrificed by decapitation 18 h after the last injection on day 9; hypothalamus was dissected as previously described (Alboni et al., 2013), immediately frozen on dry ice and stored at −80 °C for further molecular analysis.

2.3. Corticosterone serum levels

Blood samples were collected from the rat trunk after decapitation. To improve serum separation from whole blood, samples were allowed to clot at room temperature 15 min and 1 h on ice before centrifugation (1000*g* for 15 min). Serum was transferred into clean tubes and stored at −80 °C until the assay. All sacrifices were carried out between 12.00 and 15.00 p.m. (lights off). Assessment of serum corticosterone levels was done by means of enzyme immunoassay (EIA) using a commercially available kit (Arbor Assays, Ann Arbor, MI, USA), which utilizes microplate reader set at 450 nm following the manufacturer's instructions. Serum samples were diluted 1 : 150 in appropriate assay buffer and assayed in duplicate. The detection limit of the assay was 16.9 pg/mL; intra- and inter-assay coefficients of variations were, respectively, 10.45 and 11.50%.

2.4. Total RNA extraction, reverse transcription, and real time polymerase chain reaction

RNA extraction and DNAse treatment were performed as previously described (Alboni et al., 2013) using GenElute™ Mammalian Total RNA Miniprep Kit and DNASE70-On-Column DNase I Digestion Set (Sigma Aldrich ®, Milan, Italy). Two μg of total RNA were reverse transcribed with High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA USA) and Real Time PCR was performed, as previously described, in ABI PRISM 7900 HT (Thermo Fisher Scientific, Waltham, MA USA) using Power SYBR Green mix (Thermo Fisher Scientific, Waltham, MA USA) and specific forward and reverse primers at a final concentration of 150 nM (see Supplementary Table 1). Ct (cycle threshold) value was determined by the SDS software 2.2.2 (Thermo Fisher Scientific, Waltham, MA USA), mRNA expression was calculated with the ΔΔCt method with glyceraldehydes-3-phosphate dehydrogenase (GAPDH) as endogenous control.

2.5. Protein extraction

For protein extraction, hypothalami were homogenized by potter (12 strokes at 600 rpm) in lysis buffer containing Hepes 10 mM, EGTA 0.1 mM, Sucrose 0.28 M, 1X Complete protease Inhibitor Cocktail (Roche, Mannheim, Germany), $Na_4P_2O_7$ 5 mM, NaF 20 mM, Na_3VO_4 1 mM. After homogenization a fraction of the lysate was collected (total extract) and cytosolic fraction and nuclear enriched extract were obtained as previously described (Alboni et al., 2014). Protein concentration of the extracts was determined using standard protocol Coomassie® reagent (Thermo Fisher Scientific, Waltham, MA USA).

2.6. Western blotting

Western blots were carried out on total extracts (40 μg) for IL-6 detection, GR protein analysis was performed on (16 μg) total, cytosolic and nuclear enriched extracts. Electrophoresis was performed as previously described (Alboni et al., 2014). Membranes were incubated with primary antibodies: anti-GR dil 1:1000 (anti-GR rabbit polyclonal antibody, Santa Cruz M-20, sc-1004), anti-IL-6 dil. 1:1500 (anti-IL-6 rabbit polyclonal antibody, Abcam®, #ab6672) and anti-β-tubulin dil. 1:5000 (β-tubulin mouse monoclonal antibody, Santa Cruz D-10, sc-5274) and then with Anti-rabbit IgG-HRP-linked Cell Signalling®, #7071 (dil. 1:7500) as previously described (Alboni et al., 2014). Bands were detected using Immobilon Western Chemiluminescent HRP (Merck Millipore). The levels of protein were calculated by measuring the peak densitometric area of the autoradiography analysed with an image analyser (GS-690 BIORAD). Each experiment was performed twice and the mean of the OD ratios (target/internal standard) was analysed.

2.7. Statistical analysis

Behavioural data were expressed as the mean number of escapes ± S.E.M. (Standard Error of the Mean) in a test session consisting of 30 consecutive electric shocks, every 30 s.

For gene expression analysis, the mRNA levels of each target were normalized to the endogenous control, GAPDH. Endogenous control mRNA levels were not affected by any treatment (p > 0.05, One-way ANOVA). For quantitative evaluation of changes the comparative ΔΔCt method was performed, using as calibrator the average levels of expression of naive animals.

For protein analysis, the optical densities (OD) of IL-6 and GR signals were normalized according to the OD of β-tubulin. Ratios were expressed as percentage of naive ± S.E.M.

Statistical analyses were performed using an analysis of variance (ANOVA). Significant changes were determined by Tukey post-hoc test (with p < 0.05 significance level). Effect sizes for significant results were calculated by Cohen's *f*; Cohen's $f = 0.10$ was considered as small effect size, Cohen's $f = 0.25$ was considered as medium effect size, Cohen's $f = 0.40$ was considered as large effect size (Lakens, 2013; Thompson, 2007). Analyses were conducted using SPSS for Windows® v.23 (SPSS Inc., Chicago, USA).

3. Results

3.1. Seven days of treatment with escitalopram (10 mg/kg) reverted the stress-sustained escape deficit condition in about 50% of the animals

To evaluate the effect of a 7-day exposure to escitalopram (10 mg/kg/mL) on the escape deficit, animals were tested after one week of treatment, with naive animals [mean of the naive group ± S.E.M. = 23.73 ± 1.16; n = 15]. One-way ANOVA showed a significant difference among the groups [ANOVA univariate; F (3.53) = 98.708, p < 0.0001, $f = 1.652$; Fig. 2].

As already reported for this model, about the 50% of escitalopram treated-animals (n = 25) were responsive to the treatment and were divided according to their performance in the escaper test into responders (n = 13) and non-responders (n = 12) (Benatti et al., 2014).

In particular the mean of escapes of escitalopram non-responder

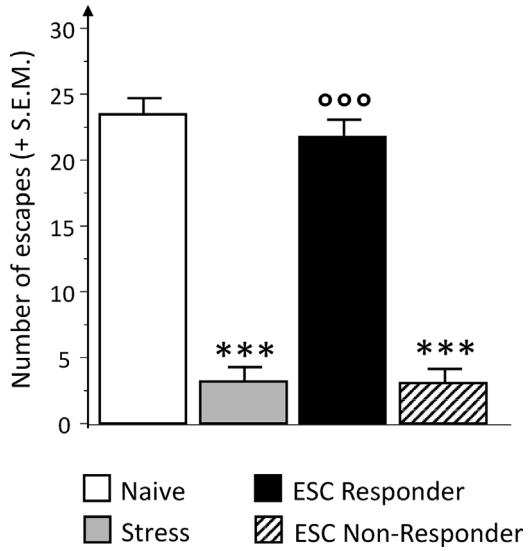

Fig. 2. A 7-day treatment with escitalopram reverted the stress-induced escape deficit in approximately 50% of the animals. The escitalopram non-responder (n = 12) group scored less than 9 escapes, while escitalopram-responder rats performed more than 15 escapes (n = 13) in the escape test. Animals were treated for 7 days with either saline (1 mL/kg) (Stress; n = 14) or escitalopram 10 mg/kg (ESC; n = 25), exposed to mild unavoidable stress on alternate days, and then tested for escape ability with the naive group (n = 15). Scores are expressed as the mean number of escapes ± S.E.M. out of 30 consecutive electric shocks. Comparisons were made by one way analysis of variance (ANOVA), followed by Tukey post-hoc test ***p < 0.0001 vs naive group; °°°p < 0.0001 vs stress group.

animals [3.33 ± 1.02; n = 12] was significantly lower than that of the naive group (p < 0.0001), but was not different from saline exposed stressed counterparts (p > 0.05). On the other hand, the number of escapes of responders to escitalopram [22.00 ± 1.28; n = 13] was not different from naive animals (p > 0.05) while being significantly higher than the score of the stress group (p < 0.0001) (Fig. 2).

3.2. Expression of hypothalamic CRH, CRHRs and serum corticosterone were decreased in escitalopram-receiving rats that responded to the treatment in the CED model of depression

CRH within the brain binds mainly to two receptors CRHR1 and CRHR2 (Sanders and Nemeroff, 2016). One-way ANOVA revealed a main effect on expression levels of CRH [F (3;23) = 5.639, p = 0.006, f = 0.516] (Fig. 3A) and its receptors CRHR1 [F (3;24) = 5.271, p = 0.007, f = 0.476] and CRHR2 [F (3;23) = 4.786, p = 0.011, f = 0.460] (Fig. 3A). In particular, post-hoc analysis showed a significant decrease in hypothalamic mRNA levels of CRH and its receptors only in animals that responded to a 7 day treatment with escitalopram with respect to naive animals (p < 0.05) (Fig. 3A).

Expression levels of GR mRNA in the hypothalamus were not altered in our experimental conditions [F (3;24) = 1.184, p = 0.340] (Fig. 3A). GR protein levels in animals that responded to a 7 day treatment with escitalopram were increased in total hypothalamic extracts with respect to all the other groups [F (3;25) = 6.220, p = 0.003, f = 0.473]. We observed an increase of GR levels in the nuclear fraction [F (3;25) = 4.864, p = 0.009, f = 0.421] with respect to naive and saline-receiving stressed animals (Fig. 3B). On the other hand, not responding animals receiving ESC presented an increase in GR protein levels solely in the cytosol, with respect to the naive group and their counterpart that responded to the treatment [F (3;25) = 4.329, p = 0.014, f = 0.364] (Fig. 3B).

Serum corticosterone levels showed a clear decrease in stressed animals responders to escitalopram with respect to naive animals, while in non-responder animals no statistically significant effect was observed [F (3;36) = 3.434, p = 0.028, f = 0.245] (Fig. 3C).

We also evaluated HSP70 expression, this protein is required for GR heterocomplex assembly and maturation of the receptor's hormone binding ability (Yu et al., 2010). We found that HSP70 mRNA levels were significantly increased in animals that after 7 days of treatment with escitalopram were considered non-responder [F (3;24) = 4.960, p = 0.009, f = 0.456] with respect to their responder counterparts, stressed animals receiving saline, and naive animals (Fig. 3A).

3.3. mRNA levels of IL-1β and TNF-α were increased in the hypothalamus of stressed rats in the CED model of depression

Increased pro-inflammatory cytokines have been reported in several stress-based rodent models of depression (Kenis and Maes, 2002; You et al., 2011). One-way ANOVA revealed a main effect for IL-1β and TNF-α expression [F (3;23) = 3.506, p = 0.034, f = 0.367 and F (3;23) = 3.814, p = 0.027, f = 0.406 respectively] (Fig. 4A), post-hoc revealed a significant increase in hypothalamic mRNA levels of both pro-inflammatory cytokines in stressed animals treated for 7 days with saline with respect to the naive group, while stressed animals receiving escitalopram for 7 days were not different from naive animals. One-way ANOVA failed to reveal a main effect in our experimental condition on expression levels of IFN-γ and IL-18 in the rat hypothalamus [F (3;23) = 1.256; p = 0.316 and F (3;24) = 0.893; p = 0.461 respectively] (Fig. 4A).

CXCL1 (also known as keratinocyte-derived chemokine, KC) and CXCL2 (macrophage inflammatory protein, MIP-2), in rodents perform the same functions as human CXCL8 (also known as IL-8) (Alboni et al., 2013). CXCL1 mRNA levels were not affected in our experimental conditions [F (3;24) = 1.742; p = 0.189], while CXCL2 expression was decreased in rats exposed to escitalopram irrespective of the behavioural outcome [F (3;22) = 4.484; p = 0.015, f = 0.456] (Fig. 4B).

No effect was observed in our experimental condition on the hypothalamic expression levels of the anti-inflammatory cytokines IL-4 and IL-10 [F (3;24) = 1.802; p = 0.178 and F (3;22) = 0.490; p = 0.693 respectively] (Fig. 4C).

3.4. Hypothalamic IL-6 and ADAM17 mRNAs were decreased by a 7-day treatment with escitalopram in the CED model of depression

IL-6 appears to be one of the most reliable markers of inflammation associated with mood and anxiety disorders (Köhler et al., 2017). IL-6 mRNA was significantly decreased in the hypothalamus of rats exposed to the unavoidable stress and treated for 7 days with escitalopram irrespective of the treatment outcome: both responder and non-responder to the drug showed lower levels of expression of this pleiotropic cytokine [F (3;23) = 3.884, p = 0.024, f = 0.396] (Fig. 5A). Protein levels of IL-6 did not differ between naive animals and stressed animals receiving saline or escitalopram [F (3.25) = 1.295; p = 0.298] (Fig. 5C).

We also investigated the main effect on the hypothalamic expression levels of several other members of IL-6 system in our experimental conditions. No effect on expression levels of both subunits of IL-6 receptor: IL-6 receptor (IL-6R), gp130 (signal-transducing receptor) and IL-6 responsive suppressor of cytokine 3 (SOCS3) were observed in the rat hypothalamus in our experimental conditions. Neither exposure to stress nor a 7-day treatment with the antidepressant affected mRNA levels of these targets [F (3;21) = 1.332; p = 0.295 for IL-6R, F (3;21) = 1.084; p = 0.377 for gp130, F (3;22) = 0.394; p = 0.759 for SOCS3] (Fig. 5A).

The classic IL-6 signalling is mediated by the complex IL-6/IL-6R/gp130, while IL-6 trans-signalling is mediated by a soluble form of IL-6R (sIL-6R), generated either by alternative splicing or by proteolytic cleavage mediated mainly by two specific members of the ADAM (*a disintegrin and metalloproteinase*) family, ADAM17 and ADAM10 (Rose-John, 2012). Considering that neurons and astrocytes express high levels of gp130 and low levels of IL6R, sIL-6R may be crucial for IL-6-mediated neuroinflammatory effects on these cells (Maes et al.,

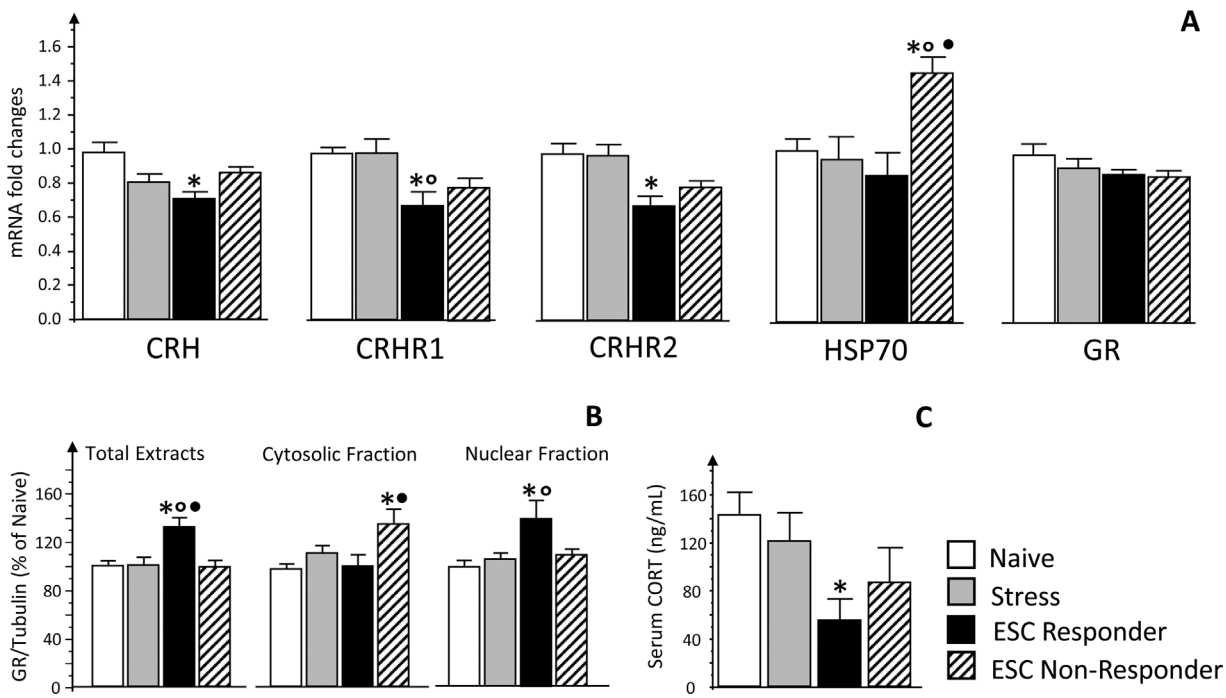

Fig. 3. Hypothalamic CRH and CRHRs mRNA levels and serum corticosterone were decreased in escitalopram-receiving rats that responded to the treatment. Animals were treated for 7 days with either saline (1 mL/kg) (Stress; n = 7-10) or escitalopram (ESC) 10 mg/kg, exposed to mild unavoidable stress on alternate days, and then tested for escape ability with the naive group (n = 7-10). Escitalopram-receiving rats were divided in responder (n = 5-10) and non-responder (n = 4-10) according to their performance. CRH, CRHR1, CRHR2, Heat Shock protein 70 (HSP70), GR hypothalamic mRNA expression (A), with GAPDH as endogenous control, were measured by Real-time PCR. Hypothalamic GR protein levels (B) were determined by western blotting in total extracts, cytosolic and nuclear fractions. Serum corticosterone (CORT) was measured by EIA (C). Data are represented as means ± S.E.M. and were analysed with ANOVA followed by Tukey (*$p < 0.05$ is significant difference with respect to naive group; °$p < 0.05$ is significant difference with respect to stress group; •$p < 0.05$ is significant difference with respect to ESC-responder).

2014). The trans-signalling pathway is considered pro-inflammatory and is implicated in several chronic inflammatory diseases (Burton et al., 2011).

While no main effect was revealed for ADAM10 [$F(3;22) = 0.321$, $p = 0.810$], ADAM17 mRNA levels were significantly decreased in rats treated with escitalopram for 7 days irrespective of the outcome at the escape test with respect to both naive untreated animals and the stress group receiving saline [$F(3;23) = 8.306$, $p = 0.001$, $f = 0.667$] (Fig. 5B).

4. Discussion

In this study we employed the chronic escape deficit model of depression, an animal model in which a stress-induced behavioural change can be restored by an antidepressant treatment (Brunello et al., 2006; Gambarana et al., 2001; Raone et al., 2007). This model allowed us to study the different effects of a therapeutic treatment in antidepressant responders and non-responders, in fact here we confirmed that a 7-day treatment with escitalopram restored the natural tendency to avoid a noxious stimulus in about 50% of stressed rats, in agreement with our previous results (Benatti et al., 2014) and results obtained with other behavioural paradigms (Jayatissa et al., 2006). At the molecular level, first we demonstrated that the treatment outcome was associated with decreased expression of CRH, CRHRs and increased GR translocation in the hypothalamus, and decreased circulating corticosterone. Secondly, we confirmed that escitalopram exerted an inhibiting effect on IL-6 and CXCL2 hypothalamic transcription, in both responders and non-responders.

Converging evidence points at HPA axis hyperactivity and neuroinflammation as important factors involved in the etiopathogenesis of major depressive disorder (MDD) and in therapeutic efficacy of antidepressant drugs (Benatti et al., 2016).

In the CED model of depression, animals responding to a week-long escitalopram treatment showed a significant decrease in circulating levels of corticosterone with respect to naive animals, while serum levels of corticosterone of the group not responding to escitalopram were not different from unstressed rats. Other groups demonstrated that a 4 week exposure to escitalopram was able to decrease circulating corticosterone levels in control animals (Doron et al., 2014; Flandreau et al., 2013), in our condition this effect was present only when the drug restored in the animals the ability to avoid noxious stimuli. No difference was observed in serum corticosterone levels between naive and animals stressed for a week, while an increase in plasma corticosterone levels was induced in this model after a 4-week unavoidable stress procedure (Raone et al., 2007). In these conditions Raone and co-workers reported also higher CRH levels and decreased GR in the hypothalamus of stressed animals (Raone et al., 2007). Acute and chronic stress have been shown to cause an increase in CRH hypothalamic expression (de Andrade et al., 2014) and increased CRH concentrations were reported in the cerebrospinal fluids of depressed subjects. Chronic AD treatment was able to attenuate both the stress-induced increase in CRH expression in the PVN of rats (Stout et al., 2002) and also in patients with MDD (Veith et al., 1993). CRH actions are mediated by its receptors CRHR1 and CRHR2: CRHR1 is able to recruit anxiety-like behaviour, blocking CRHR1 can reverse or inhibit the stress-induced behavioural alterations in several animal models of depression (Jutkiewicz et al., 2005). However the role of CRHR2 in stress response and depressive-like behaviour remains unclear and may vary according to brain region or the preclinical model (Sanders and Nemeroff, 2016).

Here we reported that hypothalamic expression of CRH and both its receptors were decreased in animals that responded to a week-long treatment with escitalopram. Consistently with our result, it has been proposed that downregulation of CRH activity may be a common pathway of antidepressant therapeutic effect (Licinio et al., 2004). In control, unstressed, animals we failed to observe a reduction in hypothalamic CRH expression following a chronic treatment with

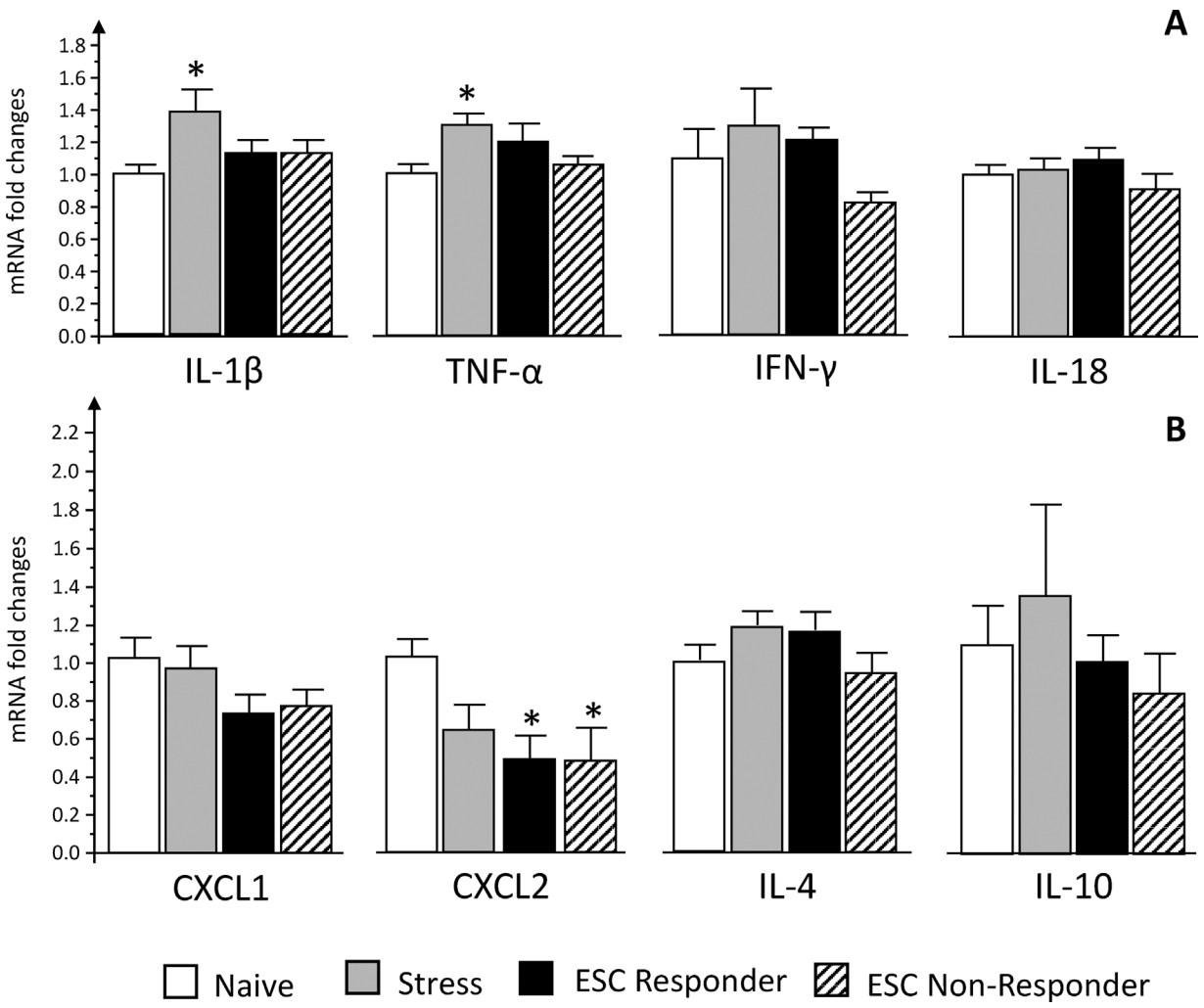

Fig. 4. Effect of a 7 day treatment with escitalopram on hypothalamic expression of the main pro- and anti-inflammatory cytokines in the CED model of depression. Animals were treated for 7 days with either saline (1 mL/kg) (Stress; n = 7) or escitalopram (ESC) 10 mg/kg, exposed to mild unavoidable stress on alternate days, and then tested for escape ability with the naive group (n = 7). Escitalopram-receiving rats were divided in responder (n = 5) and non-responder (n = 5) according to their performance in the test. Interleukin (IL-) 1β, Tumour Necrosis Factor (TNF) α, Interferon (IFN) γ, and IL-18 (A), CXCL1 and CXCL2 (B), IL-4 (H) and IL-10 (C), hypothalamic mRNA expression, with GAPDH as endogenous control, were measured by Real-time PCR. Data are represented as means ± S.E.M. and were analysed with ANOVA followed by Tukey (*$p < 0.05$ is significant difference with respect to naive group).

different classes of antidepressants (unpublished observation), suggesting that only the combination of the stressful procedure with escitalopram exposure is able to affect CRH gene expression. The molecular mechanism underlying the reduced transcription of CRH and its receptor in the hypothalamus of animals responding to escitalopram may be mediated by an increase in GR feedback sensitivity or by altering transcription of GR-responsive genes. After 7 days of treatment GR protein, but not mRNA, levels were significantly increased in the hypothalamus of escitalopram responders, in particular in total extracts and in the nuclear-enriched fraction, this effect was not present in non-responder animals, where a 7-day exposure to escitalopram induced an accumulation of glucocorticoid receptors in the cytosol. GRs once translocated in the nucleus exert a negative regulation on transcription of several genes, CRH itself, mostly in the hypothalamus (Yamamori et al., 2007). A GR-mediated decrease of CRH transcription coupled with a decrease in CRH protein levels could result in a decreased activation of the HPA axis, this chain of events could underlie the decrease of corticosterone serum levels in escitalopram treated rats that responded to the treatment.

We observed also an upregulation in HSP70 hypothalamic transcription specifically in non-responder animals. HSP70 controls the activation of glucocorticoid receptor, and its expression appears to be regulated by several stimuli, including stress (Pae et al., 2007); future studies will be needed to further understand the biological consequences of an increase in HSP70 mRNA levels in animals not responding to escitalopram, especially because this family of proteins is proposed to play a role in the response and efficacy of antidepressant treatments (Wang et al., 2008).

Major depression may be associated with immune activation, and chronic stress-induced GC resistance may dampen anti-inflammatory processes and induce prolonged production of pro-inflammatory mediators (Joana et al., 2016). In fact, numerous acute and chronic stress paradigms are able to induce the expression of pro-inflammatory markers, including TNF-α, IL-1β, and IL-6. Here, we demonstrated a general increase in IL-1β and TNFα expression in the hypothalamus of stressed animals in which the natural tendency to avoid a noxious stimulus is disrupted, this effect was not observed in their counterparts that received a 7-day treatment with escitalopram, irrespective of the outcome. Expression levels of other pro-, such as IL-18 or IFN-γ, or anti-inflammatory, IL-4 and IL-10, cytokines were not altered in this behavioural paradigm. These data confirm the idea that an increase in the expression of pro-inflammatory mediators is not a universal response to all stressors (Blandino et al., 2009), in fact a stimulatory threshold for each cytokine to be increased within the CNS, and a specific time frame

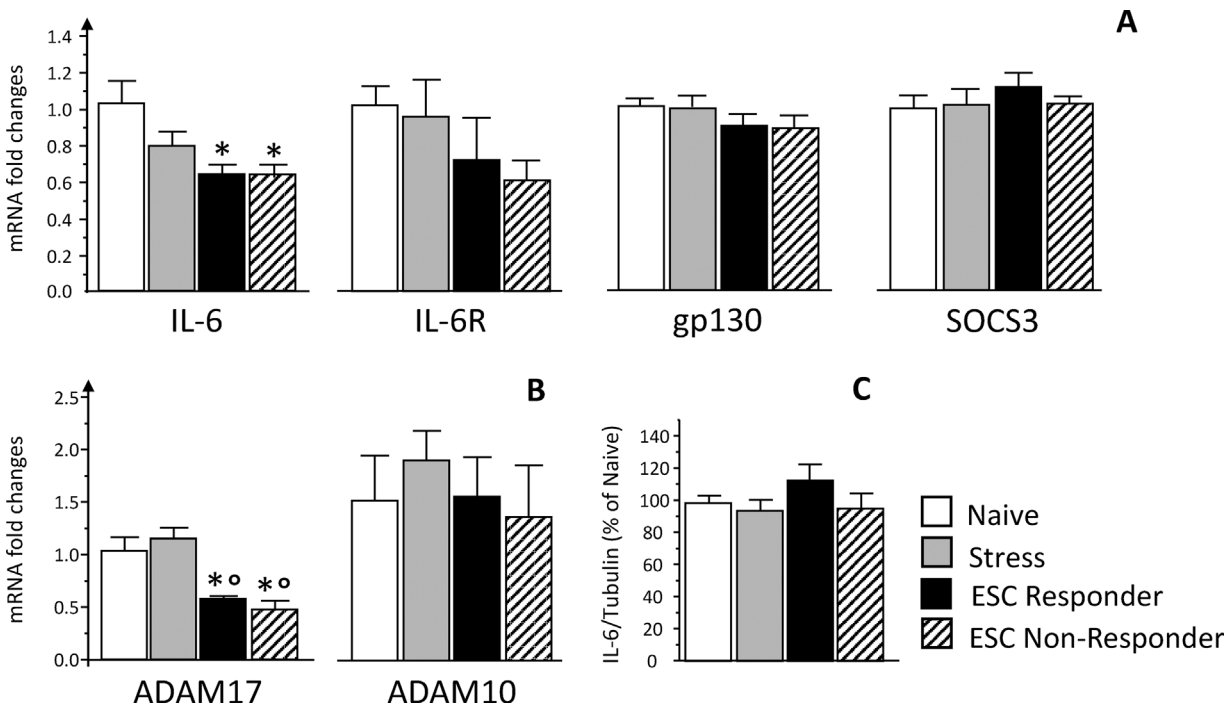

Fig. 5. A 7-day treatment with escitalopram decreased hypothalamic IL-6 and ADAM 17 expression in the CED model of depression. Animals were treated for 7 days with either saline (1 mL/kg) (Stress; n = 6-7) or escitalopram (ESC) 10 mg/kg, exposed to mild unavoidable stress on alternate days, and then tested for escape ability with the naive group (n = 6-7). Escitalopram-receiving rats were divided in responder (n = 5-7) and non-responder (n = 4-5) according to their performance in the test. IL-6, IL-6R, gp130, and SOCS3 (A), ADAM17 and ADAM10 (B) hypothalamic mRNA expression, with GAPDH as endogenous control, were measured by Real-time PCR. IL-6 protein levels (C) in the hypothalamus were determined by western blotting in total extract. Data are represented as means ± S.E.M. and were analysed with ANOVA followed by Tukey (*$p < 0.05$ is significant difference with respect to naive group; °$p < 0.05$ is significant difference with respect to stress group).

for detecting these changes may be necessary (Deak et al., 2005).

The unavoidable stress that induces the behavioural alteration in the CED model is a combination of restraint and electric shocks, increased hypothalamic IL-1 has been observed after exposure to immobilization (Suzuki et al., 1997), and footshock (Blandino et al., 2009). Data on TNF-α and stress are less consistent: studies reported an hyperproduction of TNF-α induced by acute and chronic stress paradigms or in animal models of depression (Himmerich et al., 2013), as well as a decrease in its expression in diverse stress situations (Joana et al., 2016). Also IL-6 can be considered a stress-responsive cytokine (Jankord et al., 2010), but we failed to observe any difference in hypothalamic levels of mRNA or protein for this cytokine between stressed and naive animals. The lack of protein increase is consistent with observation from other group who reported changes in protein in the CUS model after 14 days, but not after 7 days while they observed an increase in mRNA levels following several chronic stressors (Girotti et al., 2013). It is possible that the induction occurred at a different time point or that the translated protein was released, in fact IL-6 can act within the hypothalamic nuclei or be secreted from neural lobe (Jankord et al., 2010).

Hypothalamic CXCL2, one of the rodent analogues of IL-8, was significantly decreased in escitalopram treated rats regardless of the treatment response. Data on the association between the chemokine IL-8 and depression are inconsistent (Kenis and Maes, 2002; Young et al., 2014), however we have previously demonstrated a decrease in CXCL2 hypothalamic transcription in control animals exposed to a chronic treatment with the SSRI fluoxetine, but not the tricyclic antidepressant imipramine (Alboni et al., 2013). In depressed patients, IL-8 serum changes were reported during escitalopram monotherapy regardless of treatment response (Eller et al., 2009). Given that IL-8 may have both pro and anti-inflammatory properties and that CXCL1 and CXCL2 may act as neuroprotective or neurotrophic agents, its exact involvement in antidepressant response requires further investigation.

In our experimental conditions, IL-6 expression is decreased in the hypothalamus of CED-animals following escitalopram exposure for 7 days irrespective of the treatment outcome. Molecular effects common to responders and non-responders may be ascribed to the exposure to the drug itself and may not likely participate in mediating its therapeutic outcome. In accordance, a downregulation of hypothalamic IL-6 mRNA was observed in control, unstressed rats after a chronic exposure with either fluoxetine or imipramine (Alboni et al., 2013). The time taken by escitalopram or fluoxetine to downregulate IL-6 (and CXCL2) in the hypothalamus is consistent with the treatment duration that resulted effective in the CED and other behavioural models of depression (Benatti et al., 2014; Brunello et al., 2006; Gambarana et al., 2001; Reed et al., 2009).

Interestingly, we have previously reported that IL-6 expression was decreased as well in the hippocampus of CED animals following a week-long escitalopram treatment, but only in the group that responded to the treatment with respect to naive controls (Benatti et al., 2014). Hippocampus and hypothalamus are known to be involved in mood and neuroendocrine regulation (Girotti et al., 2013; Han et al., 2005), with the hippocampus extensively projecting to the hypothalamus and governing HPA axis activity (Surget et al., 2011). Given their different roles and functions, numerous data from the literature concur that molecular impacts of stress and antidepressants are brain region-specific, with different therapeutic targets for each brain area (Alboni et al., 2010; Alboni et al., 2017a,b; Surget et al., 2009). Area specific effect on IL-6 transcription may affect different functions (Aniszewska et al., 2015), however the decrease in IL-6 mRNA was not complemented by detectable changes in IL-6 receptors and SOCS3, a STAT3 target gene or in IL-6 protein levels (Girotti et al., 2013).

Interestingly, depression is not only associated with changes in serum levels of IL-6 but also of the soluble IL-6R (sIL-6R) (Maes et al., 2014). IL-6 trans-signalling pathway, mediated by sIL-6, is considered pro-inflammatory and is implicated in several chronic inflammatory diseases (Burton et al., 2011). We observed a down regulation in IL-6R cleaving enzyme ADAM17, but not ADAM10, mRNA levels following

escitalopram exposure. Evidence suggests a different role for these two enzymes: ADAM10 is known to mediate the constitutive cleavage, while ADAM17 is responsible for the inducible cleavage (Chalaris et al., 2010), it is possible that ADAM17 is the master regulator for the generation of the soluble IL-6R. So far, no data on the effect of antidepressants on ADAM17 expression are available, while higher baseline levels were demonstrated in the hippocampus of aged mice.

5. Conclusion

The CED model allowed us to evaluate the molecular effects elicited by a seven-day exposure to escitalopram in two populations that responded to or not to the treatment. Our results strengthened the assumption that escitalopram mediated reversion of stress- induced impaired behaviour is associated with a modulation of the HPA functionality in the hypothalamus. In fact, here we demonstrated that an effective treatment with escitalopram caused a general decrease in circulating corticosterone, and that this peripheral effect was correlated with a modulation of CHR/CRHRs transcription and GR activity in the hypothalamus, a key area involved in central regulation of the HPA axis. On the other hand, escitalopram appears to modulate hypothalamic transcription of inflammatory mediators regardless of the behavioural outcome. Future studies aimed at unravelling the separate and interacting roles of the HPA axis and immune systems in key areas of the CNS such as hypothalamus, hippocampus or frontal cortex in the CED model will advance our comprehension of responsiveness to therapeutic treatment and help to identify and isolate the molecular mechanisms most rigorously sustaining the therapeutic efficacy.

Contributions

AS, **BC**, **TF**, and **BN** were responsible for the study concept. **AS**, and **BC** performed behavioural and molecular experiments. **BC** analysed molecular and behavioural data. **BC** drafted the article. **BC** and **BJMC** performed statistical analysis. **AS**, **TF**, **BJMC**, **MJ**, and **BN** provided feedback on the manuscript. All authors have reviewed the manuscript and approved the final version submitted for publication.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Conflict of interest

None declared.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.psyneuen.2017.10.011.

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