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TITLE PAGE

VENLAFAXINE REDUCES THE STRIATAL IL6/IL10 RATIO AND INCREASES HIPPOCAMPAL GR EXPRESSION IN FEMALE MICE SUBJECTED TO CHRONIC SOCIAL INSTABILITY STRESS

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RUNNING TITLE

Effects of venlafaxine in stressed females

ABSTRACT

Women are twice as likely as men to develop depression and antidepressant treatment is more frequent in females. Moreover, neuroinflammatory changes related to affective disorders differ in accordance with sex. Despite this evidence, female populations have been largely omitted from preclinical experiments studying antidepressants. The aim of this work is to analyze the potential restorative effect of venlafaxine on an animal model of depression.

Female CD1 mice were subjected to chronic social instability (CSI) stress for 7 weeks, and were administered venlafaxine during the last 3 weeks of the stress period. Behavioral and physiological parameters were then analyzed.

Stressed mice showed a decreased sucrose preference and increased whisking behavior, and had a lower body weight, higher plasma corticosterone levels and increased hypothalamic GR expression. They also had lower levels of 5-HT, 5-HIAA and NA and a higher KYN/TRYP ratio in the hippocampus. Moreover, CSI increased striatal IL-6 mRNA expression levels. Venlafaxine treatment reduced the striatal IL-6/IL-10 ratio and increased hippocampal GR expression, although it did not reverse stress-induced behavioral changes.

In conclusion, seven weeks of exposure to CSI produced depressive-like alterations in female mice. The venlafaxine treatment regimen was found to have a modest anti-inflammatory effect in the striatum and increased hippocampal GR mRNA, although it failed to redress stress-induced behavioral disturbances.

Key words: social instability stress, female, venlafaxine, HPA axis, monoamines, depressive-like behavior

MAIN TEXT

1. INTRODUCTION

The prevalence of depression is twice as high in women as in men, and antidepressant treatment is more frequent in the female population (Albert., 2015). Sex-specific behavioral and physiological symptoms, along with different pharmacological responses, have been reported in connection with stress-related disorders (Bangasser and Wicks, 2017). For instance, the hypothalamic-pituitary-adrenal (HPA) axis operates in a sex dependent manner, and it has been suggested that this difference contributes to the failure of novel HPA axis-based antidepressants in clinical trials (Kokras et al., 2019). Similarly, sex-specific serotonergic modulation of antidepressants has been observed in animal models of depression (Goel et al., 2014; Pitychoutis et al., 2012), and the sex differences found in the neuroimmune consequences of stress should also be taken into account when searching for effective treatments. However, females have been largely omitted as experimental subjects from both preclinical and clinical studies, and a remarkable male bias characterizes many animal models of human disorders (Bekhbat and Neigh, 2018).

The primary sources of chronic stress in humans are social in nature, being linked to relationships and conflicts with other people (Kessler, 1997; Slavich et al., 2010). The animal model of chronic social instability (CSI) stress consists of alternating isolation and crowding phases with membership rotation, since this disrupts the social networks which are especially important to females, who benefit from the “tend-and-befriend” strategy when coping with stress (Taylor et al., 2000). Indeed, the CSI model has resulted in reiterated behavioral and physiological changes in female rodents that resemble certain characteristics of human depression, such as anhedonia (Dadomo et al., 2017; Labaka et al., 2017), social withdrawal (Baranyi et al., 2005; Saavedra-Rodríguez and Feig, 2013), high corticosterone levels (Baranyi et al., 2005; Herzog et al., 2009; Jarcho et al., 2016; Labaka et al., 2017; Schmidt et al., 2010), hippocampal inflammatory imbalance and enhanced serotonergic and noradrenergic activity in the same structure (Labaka et al., 2017). Studying the mechanisms by which antidepressants can reverse

the intricate sequence of changes triggered by stress is a challenging undertaking. Selective serotonin and noradrenaline reuptake inhibitors (SNRI) are a major class of antidepressants, and their therapeutic effect is generally attributed to an increase in the availability of monoamines in the synapses between neurons (Hamon and Blier, 2013). This mechanism has been shown to restore corticosterone and peripheral cytokine levels, neuroinflammation and central monoamine levels (Bharti et al., 2020; Chen et al., 2018; Kaminska et al., 2014; Xing et al., 2013).

Thus, the present study aims to determine whether treatment with the SNRI venlafaxine reverses the changes induced by a seven-week CSI stress on behavior, the HPA axis, central cytokine levels and central monoaminergic activity in female mice.

2. MATERIAL AND METHODS

2.1 Animals

Ninety-six 8-week-old female outbred mice (Janvier Labs, France), a mouse strain commonly used in behavioral studies (de Sá-Calçada et al., 2015), were housed in groups of three for 7 days in transparent plastic cages measuring 24.5 cm × 24.5 cm × 15 cm provided by sawdust (LIGNOCEL BK 8/15 from Safe). Food (2018 Teklad global 18 % protein rodent diets from Envigo) and water were available *ad libitum*. The holding room was maintained at a constant temperature of 20 °C with a reversed 12-h light/dark cycle (white lights on from 20:00 h to 08:00 h), to enable mouse manipulation during the animals' active phase (dark). European regulations for the care and treatment of experimental animals were followed, and the procedures were controlled and approved by the *Diputación Foral de Guipúzcoa* (Guipúzcoa Provincial Council), Spain, in compliance with the European Directive (2010/63/EU) on the protection of animals used for scientific purposes (22 September 2010). The procedures were approved by the University of the Basque Country's Ethical Committee for Animal Welfare (CEBA).

2.2 Experimental procedure

The experiment began after a 7 day adaptation period (Fig. 1). Two groups were randomly established: a control group (n=48) and a group of socially stressed mice

(n=48). Chronic social instability stress was applied to the stress group for 49 days. Controls remained in same conditions as during the adaptation period. Half of the subjects from the control and stress groups were administered the antidepressant drug venlafaxine from day 29 to day 49. Remaining subjects received a physiological saline solution as vehicle. Blood was collected via submandibular vein puncture on days 1 and 29 (immediately prior to treatment onset) for all subjects (N = 96) to determinate plasma corticosterone levels. Body weight was measured at the start of the stress period (day 1), inmediatelly prior to treatment onset (day 29) and on the day following the end of the stress period (day 50). On day 50, twelve subjects from each subgroup (control-venlafaxine, control-saline, stress-venlafaxine and stress-saline) were sacrificed by cervical dislocation. Blood was immediately collected by cardiac puncture to determinate plasma corticosterone concentrations, a whisking assessment was performed, the brain was removed, and the whole hypothalamus and hippocampus were dissected and analyzed, along with both striata. All dissections were performed under sterile conditions with stereomicroscopic observation with reference to the mouse brain atlas (Paxinos and Franklin, 2004). The samples were stored at – 80 °C until used for biological measurements. The remaining mice, twelve from each subgroup, were exposed to the sucrose preference (SPT) and open field (OFT) tests, on days 51 and 53, respectively, before being sacrificed. Whiskers were not assessed in this case, since isolation prevents whisking behavior.

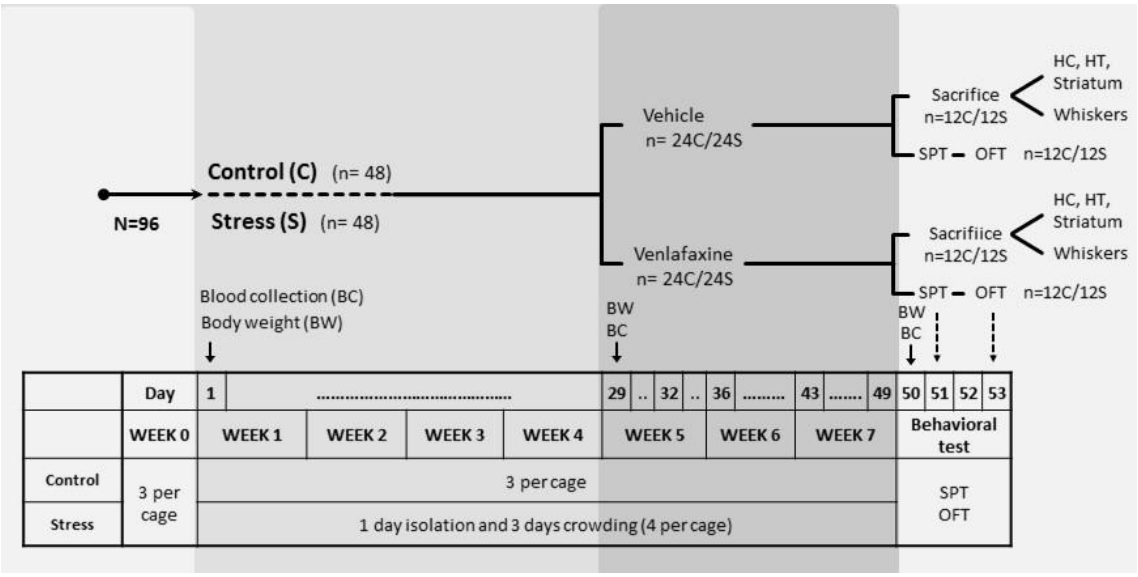

***Fig. 1**. Experimental procedure. Notes: C = control; S = stress; BC = blood collection; BW = body weight; HC = hippocampus; HT = hypothalamus; SPT = sucrose preference test; OFT = open field test.*

2.3 Stress procedure

The female mice were stressed using the chronic social instability stress model, which was adapted and modified from a protocol described by Haller et al. (1999) in rats and by Schmidt et al. (2010) in mice. Briefly, the mice were exposed to a highly unstable social situation with alternating phases of isolation (1 day) and crowding (4 per cage, for 3 days) over a 49–day period. During each crowding phase, we ensured that four different mice that had had no previous contact were placed together in a new clean cage. Meanwhile, during this period control mice were allocated in stable groups of 3 mice.

2.4 Venlafaxine treatment

The dose for venlafaxine in this study was determined according to published reports (Liu et al., 2019; Venzala e al., 2012). We dissolved 20 mg/kg of venlafaxine (Sygma Aldrich, Madrid, Spain) in saline (0.9 %) and administered it intraperitoneally once daily. The treatment lasted 3 weeks, from day 29 to day 49. We administered the venlafaxine solution to the treatment group and saline to the vehicle group between 9:00 and 10:00 am. No toxic effects were observed.

2.5 Behavioral assessment

2.5.1 Sucrose preference test (SPT)

The control and stressed mice that were assigned to the behavioral tests were individually housed and were offered a free choice between two bottles for 24 h; one bottle contained a 0.8% sucrose solution, and one bottle contained water. To prevent the possible effects of a side preference in drinking, the position of the bottles was counterbalanced. The animals were not deprived of food or water before the test. The consumption of the sucrose solution and water was measured by weighing the bottles at the beginning and end of the test. The consumption of sucrose was expressed in

relation to body weight. The sucrose preference was calculated as percentage of sucrose consumption vs. sucrose plus water consumption.

2.5.2. Open field test (OFT)

The OFT was performed to assess anxiety-like behavior. Mice were placed in the center of a black Plexiglas OFT arena (40 cm × 40 cm x 30 cm) and were recorded for 5 min with video cameras (GZ-MG773; JVC, Yokohama, Japan) for subsequent behavioral assessment using the ANY-maze© version 4.96 video-tracking software (Stoelting Europe, Dublin, Ireland). Virtual lines divided the floor into two areas, center (20 cm x 20 cm) and periphery. Horizontal locomotor activity (distance travelled in cm), time spent in each area, number of entries and immobility time were measured. The apparatus was cleaned with a solution of 0.5% acetic acid between tests in order to hide animal clues.

2.5.3 Whisking assessment

As the mice showed no traces of barbering on any body parts other than the snout, we specifically examined whisking signs at the moment of sacrifice. Whisking is a type of heterobarbering in which the so-called barber holds a cage mate down and cuts its vibrissae with its incisors (Kalueff et al., 2006). Whisker length was assessed by a trained observer and classified using the following two-point scale: (1) intact whiskers—for no deterioration of the vibrissae and (2) shortened whiskers—for shortening or removal of the vibrissae.

2.6 Physiological determinations

2.6.1. Blood collection and plasma isolation

Blood was collected from all subjects (N = 96) by submandibular vein puncture on days 1 and 29 between 9:00 and 10:00 a.m. for corticosterone measurement. This method, developed by Golde et al. (2005), allows investigators to obtain a sufficient volume of blood from the submandibular vein in a short time, simply while holding the mouse, without the use of anesthesia. On day 50, blood was collected by cardiac puncture from those mice that were not scheduled to perform behavioral tests (n = 48).

The blood was stored in heparinized containers and then centrifuged at 1800 x g for 15 min at 4 °C. The resulting plasma was collected and stored at −70 °C until analysis.

2.6.2. Determination of plasma corticosterone concentrations

Plasma corticosterone concentrations (ng/ml) were determined using a commercially available enzyme immunoassay kit (Assay Designs, Ann Arbor, MI, USA) and a Synergy HT microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). The sensitivity of the assay was 5 pg/ml, and the intra- and inter-assay variation coefficients were 7 % and 8 %, respectively.

2.6.3. Real-Time polymerase chain reaction (RT – PCR) measurements of mRNA expression

Real-Time RT –PCR measurements of mRNA expression in the hypothalamus, hippocampus and striatum were carried out as described previously (Labaka et al., 2017) for the target genes included in Appendix A. Both hypoxanthine phosphoribosyl transferase (HPRT) and glyceraldehyde-6- phosphate dehydrogenase (GAPDH) were used as reference genes.

2.6.4. Determination of hippocampal and striatal monoamines, their metabolites and amino acids using high-performance liquid chromatography (HPLC)

To determine monoaminergic activity in the hippocampus and striatum, dopamine (DA), noradrenaline (NA) and serotonin (5-HT) levels were analyzed, as well as their respective metabolite levels, including: 3,4-dihydroxyphenylacetic acid (DOPAC), 3-Methoxy-4-hydroxyphenylglycol (MHPG) and 5-hydroxyindoleacetic acid (5-HIAA). The serotonin precursor tryptophan (TRYP), kynurenine (KYN) and 3-hydroxykynurenine (3-HK) were also determined. All the analytes were analyzed using HPLC. The procedure was carried out as described in our previous work (Labaka et al., 2017). The final data were expressed as pg/mg wet tissue.

2.7 Statistical analysis

All statistical analyses were performed using SPSS 22 for Windows (SPSS Inc., Chicago, Ill., USA) with the level of significance set at $p < 0.05$. Normality and homogeneity criteria were respected. Outlier values were detected in accordance with

the boxplot outlier labeling rule (Tukey, 1977), considering outlier any value that will be more than the upper limit, Q3 + (2,2 * (Q3-Q1)), or lesser than the lower limit, Q1 - (2,2 * (Q3-Q1)), and replaced by winsorized mean (Rivest, 1994). The behavioral and physiological variables were analyzed with a one-tailed one-way or two-way ANOVA. The corticosterone and body weight-derived data were analyzed with two-way (time (from day 1 to day 29 measures) and group) and three-way (time (from day 29 to day 50 measures), group and treatment) linear mixed model ANOVAs. When the interaction between two factors was significant, specific comparisons were made using post hoc Bonferroni tests. Whisking behavior was analyzed using Fisher's exact test.

3. RESULTS

3.1 Sucrose Preference Test

According to the two-way ANOVA, stressed mice showed a weaker sucrose preference ($F_{[1,45]} = 6.41$; $p = 0.015$) and consumed less sucrose solution in relation to body weight than the control group ($F_{[1,45]} = 7.58$; $p = 0.009$) (Fig 2a). We also found that venlafaxine subjects had a weaker sucrose preference and lower consumption ($F_{[1,45]} = 8.44$; $p = 0.006$ and $F_{[1,45]} = 19.54$; $p < 0.001$) than vehicle subjects (Fig 2b). No interactions were observed between the *stress* and *drug* factors in any of the above variables.

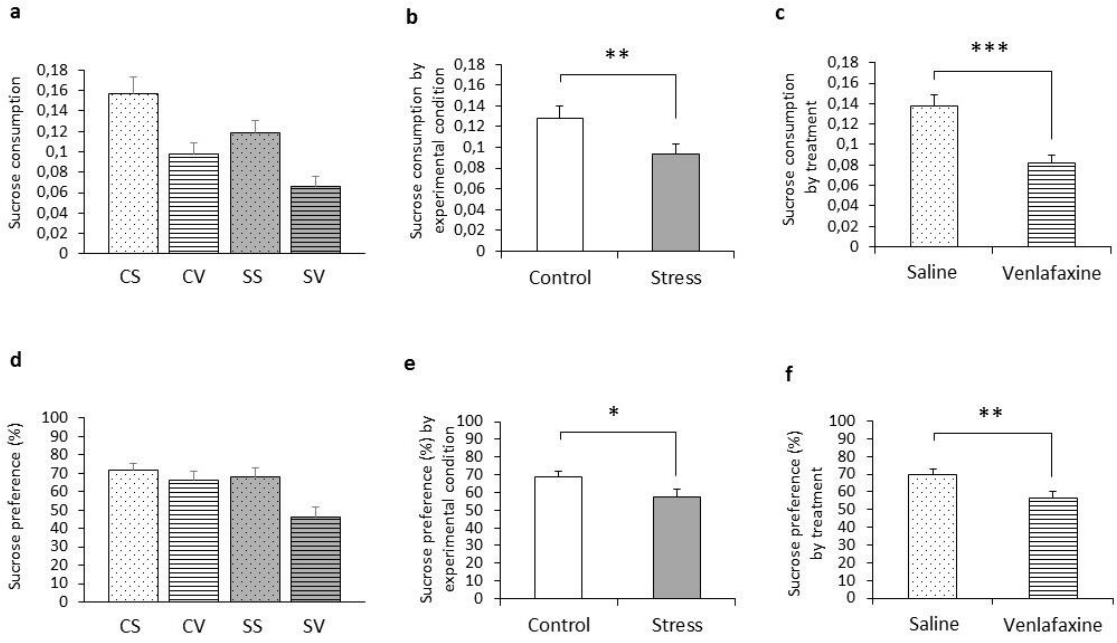

Fig. 2. *Sucrose preference test. Relative sucrose consumption (sucrose g/body weight g) for (a) the control-saline (CS), control-venlafaxine (CV), stress-saline (SS) and stress-venlafaxine (SV) groups (n = 12 in each group); (b) for the control (n = 24) and stress groups (n = 24); and (c) for the saline (n = 24) and venlafaxine (n = 24) groups. Sucrose preference percentage (sucrose g/total volume g) x100 for (d) CS, CV, SS and SV groups (n = 12 in each group); (e) for the control (n = 24) and stress (n = 24) groups and (f) for the saline (n = 24) and venlafaxine (n = 24) groups. Data are expressed as the mean + SEM (standard error of the mean). **p < 0.01 and ***p < 0.001.*

3.2 Open Field Test

We found no differences between the control and stressed groups in terms of immobility time, distance walked, time spent in the center of the Open Field or entries. Treatment with venlafaxine was not observed to affect these variables either.

3.3 Whisking behavior

Fisher's exact test revealed that CSI affected whisker length. In the stress group, 64 % of subjects had shortened whiskers, in comparison with only 36 % in the control group (p = 0.044) (Fig. 2c,d). Venlafaxine did not change the distribution of this variable.

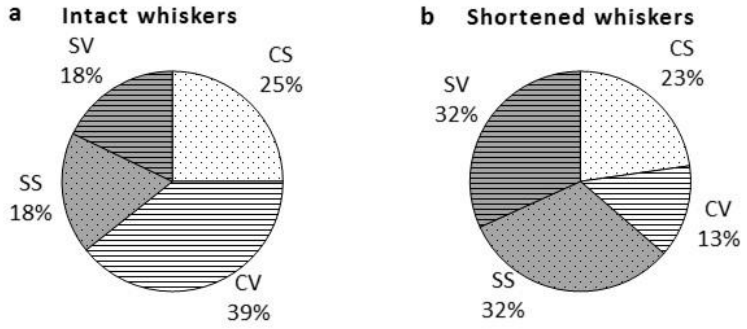

Fig. 3. *Whisking behavior. Distribution of intact whiskers (a) and shortened whiskers (b) for the control-saline (CS, n = 12), control-venlafaxine (CV n = 12), stress-saline (SS n = 12) and stress-venlafaxine groups (SV n = 12).*

3.4 Body weight

A two-way mixed model ANOVA (*time (day 1 and day 29) and group*) revealed significant differences in the *time factor* ($F_{[1,97]}$ = 27.47; p < 0.001) *and time* x *group* interaction ($F_{[1,97]}$ = 109.58; p < 0.001). Specifically, in the first 4 weeks of stress procedure, stressed mice showed a body weight reduction, meanwhile control mice increased their body weight (see Fig. 4). The three-way mixed model ANOVA *(time (day 29 and day 50), group and treatment)* revealed significant effect in the *time factor* ($F_{[1,94]}$ = 48.48; p < 0.001) and *time x group* interaction ($F_{[1,94]}$ = 37.20; p < 0.001). In this case, from day 29 to the end of the experiment, stressed mice increased their body weight, while control group kept their weight. Despite this, stressed mice showed a lower body weight on day 50 in comparison to control group according to the one-way ANOVA ($F_{[1,96]}$ = 34.29; p < 0.001). No effect of venlafaxine was observed in body weight (Fig. 4).

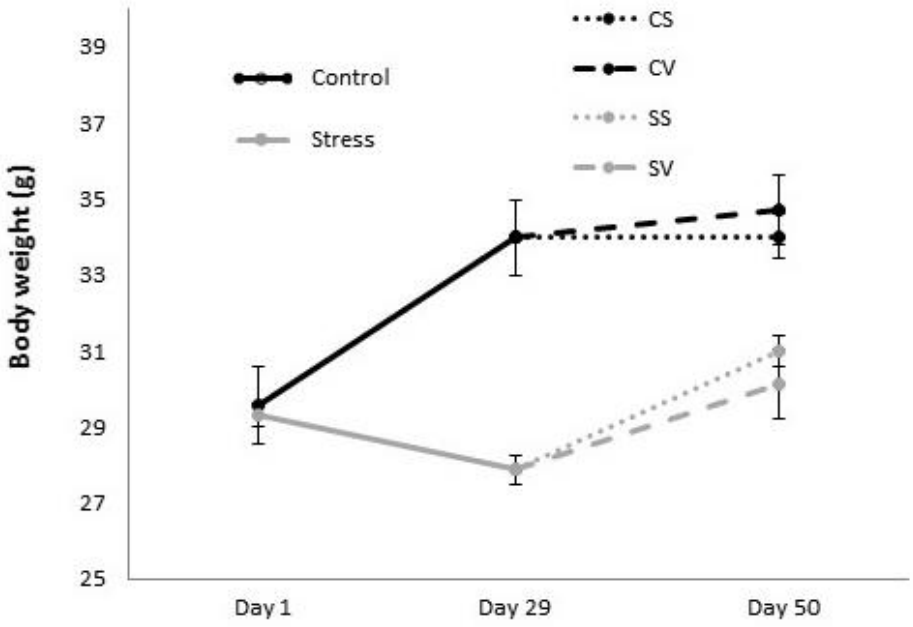

Fig. 4. *Body weight evolution between days 1 – 29 for control (n = 48) and stress (n = 48) groups, and between days 29-50 for the control-saline (CS), control-venlafaxine (CV), stress-saline (SS) and stress-venlafaxine (SV) groups (n = 24 in each group). Data are expressed as the mean ± SEM.*

3.5 Plasma corticosterone levels and relative hypothalamic and hippocampal glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) mRNA expression

A two-way mixed model ANOVA (*time (day 1 and day 29) and group*) revealed significant differences in the *time* x *group* interaction ($F_{[1,46]}$ = 14.32; p < 0.001). Specifically, the corticosterone levels of the stress group increased specifically from day 1 to day 29 in comparison with controls. In the three-way mixed model ANOVA *(time (day 29 and day 50), group and treatment)* a significant effect for time factor was observed ($F_{[1,43]}$ = 38.74; p < 0.001), indicating that both control and stress groups increase the corticosterone levels from day 29 to day 50. Nevertheless, stressed mice showed higher corticosterone levels on day 50 in comparison to control group according to the one-way ANOVA ($F_{[1,45]}$ = 4.47; p = 0.04). No effect of venlafaxine was observed in corticosterone levels (Fig. 5).

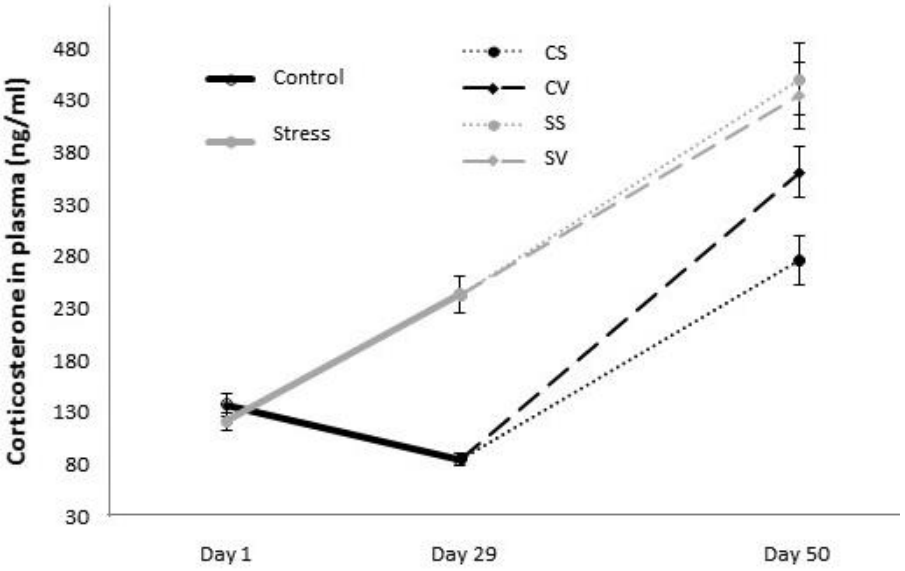

Fig. 5. *Plasma corticosterone concentrations on days 1 - 29 for control (n = 24) and stress (n = 24) groups, and concentrations on days 29 - 50 for the control-saline (CS), control-venlafaxine (CV), stress-saline (SS) and stress-venlafaxine (SV) groups (n = 24 in each group). Data are expressed as the mean ± SEM.*

A two-way ANOVA revealed that stressed subjects had higher levels of GR mRNA expression in the hypothalamus than controls ($F_{[1,46]}$ = 5,030; p = 0.030) (Fig. 4b). We found no changes in either MR mRNA expression (Fig. 4c) or the interaction between stress and treatment, and no differences were observed in the GR/MR ratio (Fig. 4d).

Venlafaxine subjects had increased hippocampal GR mRNA expression in comparison with saline subjects ($F_{[1,46]}$ = 6.55; p = 0.014) (Fig. 4e), while no changes were observed in MR mRNA expression (Fig. 4f). Consequently, venlafaxine subjects had a lower MR/GR ratio ($F_{[1,46]}$ = 6.289; p = 0.016) (Fig. 4g).

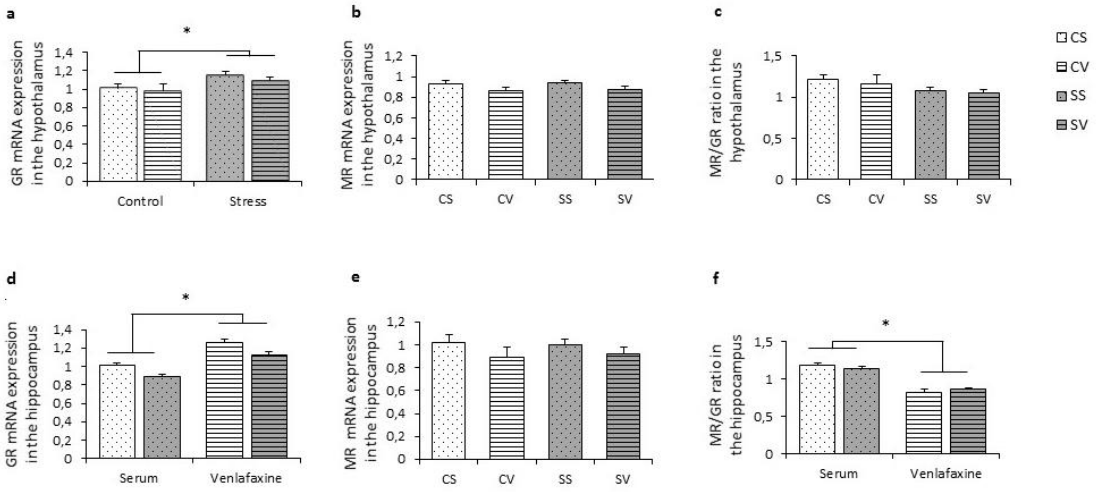

Fig. 6. *GR and MR mRNA expression. (a) hypothalamic GR mRNA expression, (b) hypothalamic MR mRNA expression and (c) hypothalamic MR/GR ratio. (d) Hippocampal GR mRNA expression, (e) hippocampal MR mRNA expression and (f) hippocampal MR/GR ratio. CS (control-saline, n = 12); CV (control-venlafaxine, n = 12); SS (stress-saline, n = 12) and SV (stress-venlafaxine, n = 12) groups. Data are expressed as the mean + SEM. *p < 0.05.*

3.6 Relative pro- and anti-inflammatory cytokine mRNA expression levels in the hippocampus and striatum

Stress did not change hippocampal cytokine expression, while venlafaxine increased interleukin 1β (IL-1β) expression ($F_{[1,46]}$ = 5.19; p = 0.027) (Fig. 5a). We found no interaction between the two factors, or any differences in the pro-/anti-inflammatory ratio (Fig. 5b).

Stress increased both interleukin 6 (IL-6) mRNA expression ($F_{[1,48]}$ = 4,64; p = 0.037) (Fig. 5c) and the IL-6/interleukin 10 (IL-10) ratio ($F_{[1,48]}$ = 4,04; p = 0.050) in the striatum. In contrast, venlafaxine treatment reduced the IL-6/IL-10 ratio ($F_{[1,48]}$ = 10,32; p = 0.002), and the *stress*treatment* interaction was significant in this same ratio ($F_{[1,48]}$ = 5,26; p = 0.026). Specifically, the stress-saline (SS) group had a higher IL-6/IL-10 ratio in comparison with the control-saline (CS) (p = 0.029), control-venlafaxine (CV) (p = 0.002) and stress-venlafaxine (SV) (p = 0.003) groups (Fig 5d).

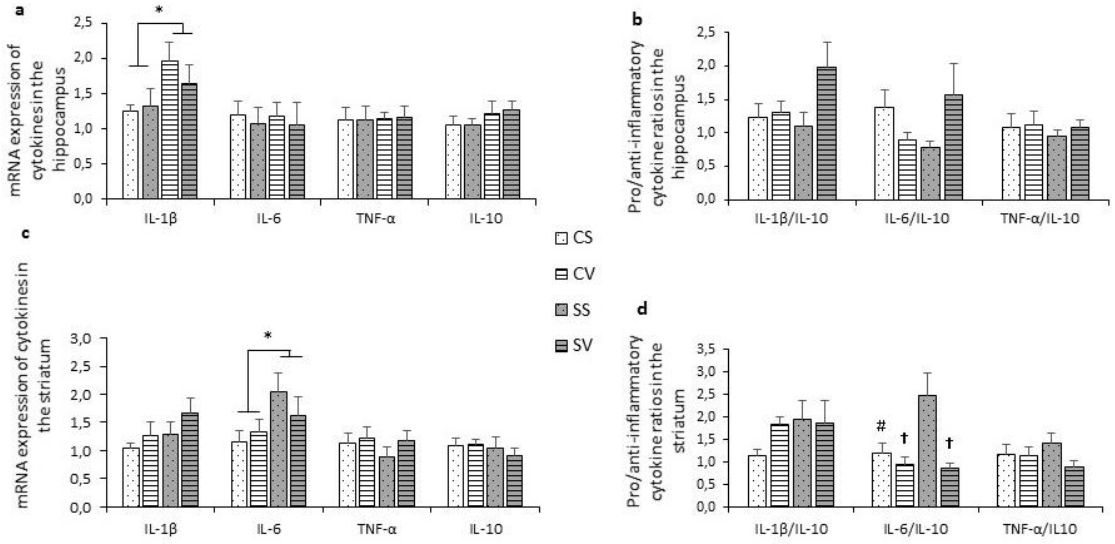

Fig. 7*. Central cytokines' mRNA expression. (a) Relative IL-1β, IL-6, TNF-α and IL-10 mRNA expression levels, and (b) the pro- inflammatory versus anti-inflammatory cytokine ratios in the*

*hippocampus. (c) Relative IL-1β, IL-6, TNF-α and IL-10 mRNA expression levels, and (d) the pro-inflammatory versus anti-inflammatory cytokine ratios in the striatum. CS (control-saline, n = 12); CV (control-venlafaxine, n = 12); SS (stress-saline, n = 12) and SV (stress-venlafaxine, n = 12) groups. Data are expressed as the mean + SEM. *p < 0.05. #p < 0.05 and †p < 0.01 in comparison with the stress-saline group (Bonferroni test).*

3.7 Central monoamine, metabolites and amino acids.

The two-way ANOVA revealed that stressed mice had lower levels of both 5-HT $F_{[1,46]} = 6.869$; $p = 0.012$) and 5-HIAA ($F_{[1,46]} = 4.386$; $p = 0.042$) in the hippocampus (Fig 6a). NA levels were also lower in the stressed group ($F_{[1,46]} = 11.097$; $p = 0.002$) (Fig 6b), while no changes were observed in MHPG levels. Regarding the KYN/TRYP ratio, stressed mice had a higher ratio than controls ($F_{[1,46]} = 7.621$; $p = 0.008$) (Fig. 6c). We found no significant difference in the striatum in terms of the variables measured using HPLC.

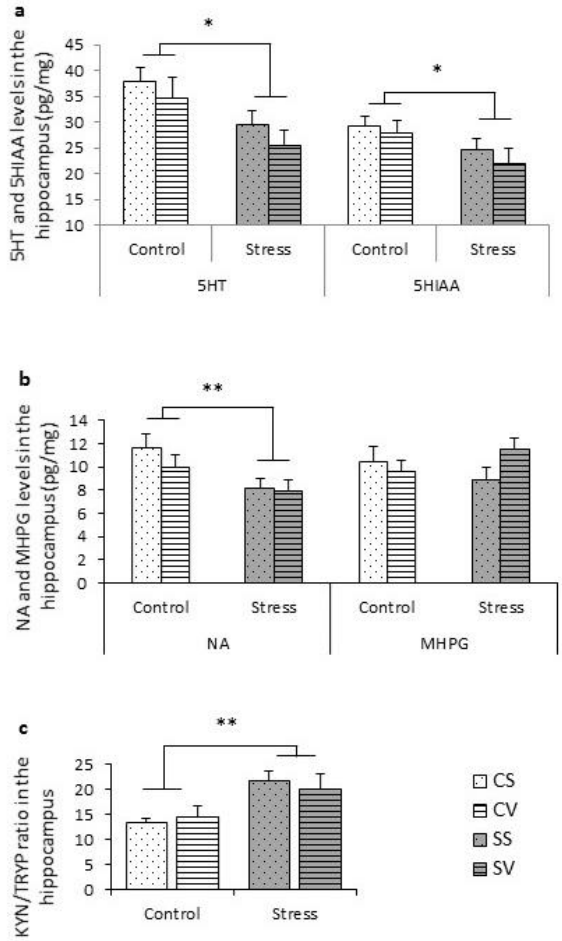

***Fig. 8**. Monoamine, metabolite and amino acid levels in the hippocampus. (a) 5HT and 5HIAA levels, (b) NA and MHPG levels and (c) the KYN/TRYP ratio. CS (control-saline, n = 12); CV (control-*

*venlafaxine, n = 12); SS (stress-saline, n = 12) and SV (stress-venlafaxine, n = 12) groups. Data are expressed as the mean + SEM. *p < 0.05. **p < 0.01.*

4. DISCUSSION

The results of this study indicate that seven weeks of exposure to CSI has a detrimental effect on female mice's adaptability, producing depressive-like behavioral and physiological changes.

The corticosterone increase observed from day 1 to day 29 indicates that CSI affected the HPA axis in the stress group. Curiously, the rise in corticosterone levels from day 29 to day 50 in stressed mice was accompanied by a parallel increase in controls. This may be a response to the manipulation involved in daily vehicle injection. Alternatively, it is possible that mice were undergoing an age-related physiological increase in corticosterone levels, as previously reported in CD1 female mice (Kolbe et al., 2015). Despite this, the increase in corticosterone levels from basal day to day 50 was significantly steeper in the stressed group.

The hypothalamic GR mRNA expression increase observed in stressed group may reflect an attempt to regulate HPA axis hyperactivity, since some studies have reported that augmented GR levels reduce corticosterone levels (Reichardt et al., 2000; Zhang et al., 2009). However, in our sample, the increase in GR expression was not accompanied by any such decrease in corticosterone levels. Interestingly, female-specific allostatic mechanisms such as deletion of exchange proteins and reduction in methylation have been found to act on GR functioning following chronic stress (Aesoy et al., 2018; Rowson et al., 2019). Furthermore, in female rats, Bourke et al. (2013) found that chronic stress exposure resulted in attenuated hippocampal GR translocation and a subsequent delay in the cessation of corticotropin-releasing hormone (CRH) transcription, and therefore in the activity of the HPA axis.

Unlike in the hypothalamus, stress did not change hippocampal GR expression, while venlafaxine increased it. Hippocampal GR increase is a glucocorticoid regulating mechanism that is well-known in antidepressant, and has been reported also with venlafaxine treatment. Specifically, 20 mg/kg of venlafaxine administered for 2 weeks has been found to mitigate chronic mild stress-induced hippocampal GR decrease in

male rats (Briones et al., 2012). GR signaling has been described as a prerequisite for successful antidepressant treatment (Ising et al., 2019), but the translocation resistant phenomenon and subsequent continuity of CRH transcription cited above may preclude such signaling in females.

Regarding behavioral changes, we found that 7 weeks of CSI induced a decrease in both sucrose preference and sucrose consumption in female mice. This is indicative of anhedonia, a core symptom in animal studies aimed at emulating human depression (Berridge and Kringelbach, 2008). In the OFT, no differences were observed between mice in accordance with stress or venlafaxine treatment. This is consistent with that reported by Schmidt et al. (2010) and Jarcho et al. (2016), who found no changes in the duration of time spent by female mice in the center or perimeter of the open field after exposure to CSI. Conversely, Saavedra-Rodríguez and Feig (2013) found increased locomotor activity in female mice after a seven-week exposure to CSI. This inconsistency may be attributable to the age difference, since the subjects in the study by Jarcho et al. (2016) were 4 weeks old at the start of the stress regime, and puberty is known to be an especially vulnerable period in terms of coping with stress (McCormick and Green, 2013).

Consistently with our previous findings (Labaka et al., 2017), we found that whisking behavior was more frequent among female stressed mice. Whisking is considered a natural behavior included in the repertory of social interaction behaviors in male and female mice, and has been linked to the establishment of social hierarchies (Kalueff et al., 2006). Moreover, whisking has been interpreted as an anxiety-like behavior triggered by stress (Kalueff et al., 2006; Labaka et al., 2017). This finding suggests that, even though no anxiety symptoms were observed in the OFT, the stress model used in this study likely produces anxiety-like behavior in the social networks domain by preventing the establishment of group hierarchies.

The seven-week CSI period resulted in a lower body weight gain among stressed mice than among controls. This is consistent with that reported by some previous studies that used CSI in females (Baranyi et al., 2005; Dadomo et al., 2017), although other studies found no differences between groups (Jarcho et al., 2016; Labaka et al., 2017; Schmidt et al., 2010). This discrepancy may be the result of differences in the

characteristics of the stress protocol. Interestingly, even though Schmidt et al. (2010) also used CD1 female mice and a seven-week stress period, they found no weight differences between groups. This may be due to the stress regime, since these authors did not include isolation periods. According to Herzog et al. (2009), the alternation of the two phases makes this model more robust, as isolation or crowding *per se* may be insufficient to induce a stress response in females.

Stress was found to increase IL-6 cytokine expression in the striatum, as well as the IL-6/IL-10 ratio. Interestingly, a correlation between depressive-like behavior and IL-6 has been found in both male and female rodents (Hodes et al., 2016). However, the sensitivity of females to cytokines seems to be greater in some behavioral aspects, such as sexual activity and reward-response to sucrose (Avitsur et al., 1997; Merali et al., 2003). Thus, the increase in IL-6 and the ratio found in the striatum, a structure whose involvement in the reward processes is known (Wise, 2008), could be responsible for the reduction in sucrose preference observed in the presence of stress. This is in agreement with several studies that indicate that animals and humans with an immunological challenge, either with LPS or inflammatory cytokines, present anhedonia, together with a decrease in the response to reward of the ventral striatum (Nunes et al., 2014; Eisenberger et al., 2010). However, it should be noted that in the present work the levels of cytokines were measured in the entire striatum and not specifically in the ventral striatum main substructure that regulates hedonic behavior, while the dorsal striatum facilitates action-related behavior (Floresco 2015; Der-Avakian and Markou 2012).

Venlafaxine reversed the stress-induced increase of the IL-6/IL-10 ratio in the striatum, in line with the anti-inflammatory effect reported in antidepressants (Chen et al., 2018). However, it did not reverse the anhedonic behavior, perhaps due, in accordance with the above, to the fact that the reversal of the ratio observed in the entire striatum structure was not significant in the ventral striatum. On the other hand, it must be considered that the treatment did not reverse the increase in IL-6 levels, which may also explain the presence of anhedonic behavior in venlafaxine-administered mice, since a maternally separated model also resulted in an increase in striatal IL-6

levels, accompanied by a drop in sucrose consumption, both of which were reversed when fluvoxamine reduced striatal IL-6 (Dallé et al., 2017).

The seven-week CSI regime did not affect the hippocampal proinflammatory cytokines IL-1β, tumor necrosis factor α (TNF-α) and IL-6, or the anti-inflammatory cytokine IL-10. Bearing in mind the reduced hippocampal IL-10 mRNA expression we observed previously in mice stressed for 4 weeks, the present results may indicate that cytokine production patterns change depending on the challenge and demand of the stress period. Interestingly, Bollinger et al. (2017) found that acute and chronic stress differentially affected the microglial morphological activation state in male and female rats across several brain regions. Moreover, Liu, Li, Su, Wang and Jiang (2019) found decreased hippocampal IL-10 levels after a 4week exposure to chronic unpredictable mild stress in female mice, suggesting that females may exhibit a pro-/anti-inflammatory switching balance in microglia while males' microglia is activated mainly towards the pro-inflammatory phenotype. For its part, venlafaxine treatment increased IL-1β levels in the hippocampus. Although this drug has also been reported to decrease plasma IL-1β levels in depressed humans (Chen et al., 2018), increased cytokine levels have previously been found after antidepressant treatments (Munzer et al., 2013). Interestingly, IL-1β has been attributed a key role in mediating the stress response, since constitutive levels of the IL-1 family of proinflammatory cytokines are necessary for cognition, learning, memory (Singhal et al., 2014) and HPA axis sensitivity regulation (Eyre and Baune, 2012).

The seven-week stress period reduced hippocampal 5-HT, 5-HIAA and NA levels, consistently with the findings of another study that analyzed depressive-like changes in female mice (Avraham et al., 2017). Given the increased hippocampal noradrenergic and serotonergic activity we found previously after 4 weeks of exposure to CSI, it can be inferred that monoaminergic hyperactivity becomes monoaminergic depletion when stress is maintained over time. In line with the serotoninergic deficit, the stressed mice in our study also had a higher KYN/TRYP ratio in the hippocampus, suggesting that tryptophan had been degraded in the kynurenine pathway, reducing subjects' ability to synthesize serotonin. Although in our study we did not find the elevated levels of proinflammatory cytokines required to activate the indoleamine 2,3-dioxygenase (IDO)

enzyme, there is an alternative pathway for kynurenine degradation through glucocorticoid-induced tryptophan 2,3-dioxygenase (TDO) enzyme activation (O'Farrell and Harkin, 2017). However, it does not seem that kynurenine was metabolized via the neurotoxic pathway, since no differences were observed in hippocampal 3-HK levels. It should be considered that glucocorticoids (corticosterone) can alter the expression of tph2, the rate-limiting enzyme for brain serotonin synthesis (Donner et al., 2012). Although the mechanism of this action is not well understood, GR receptors have been shown to inhibit the expression of this gene in the dorsal nucleus of the raphe, which supplies many of the serotonergic projections to brain regions relevant to anxiety and depressive-like behavior (Fu et al., 2010; Vincent et al, 2018) and, also in the hippocampus, along with a decrease in serotonin levels in this same structure (Wang et al., 2019).

We found that venlafaxine exerted a modest antidepressant effect by reducing the IL-6/IL-10 ratio and increasing hippocampal GR expression. However, our results do not coincide with those reported by other studies, which found that venlafaxine reversed the stress-induced changes in monoaminergic synthesis and metabolism, corticosterone levels and behavior (Liu et al., 2019; Xing et al., 2013). Notwithstanding, methodological differences may have contributed to these discrepancies. Xing et al. (2013) started the 10 mg/kg administration of venlafaxine at the same time as the chronic mild stress, and the 21 day-long treatment was found to reduce stress-induced high corticosterone levels in both male and female rats, although it was less effective in females. Liu et al. (2019) also began the venlafaxine treatment (20 mg/kg·day for 28 days) in male rats at the same time as the stress regime, finding that the drug improved both central monoaminergic enzyme activity and locomotor activity. In humans, antidepressants are administered once the symptoms have appeared. In order to mimic clinical recovery rather than the preventive use of antidepressants, we started venlafaxine administration after four weeks of stress, once stress had increased corticosterone levels. In addition, previous works have found behavioral changes in females subjected to CSI in such time period (Dadomo et al., 2017; Labaka et al., 2017). Responsiveness to antidepressants varies depending on whether the drugs are administered before or after the detrimental onset of stress, given that chronic stress

can damage the neuroplasticity required for a variety of drugs to reduce depressive-like behavior in rodents (Surget et al., 2011), and poorer antidepressant efficacy has been related to higher baseline levels of oxidative stress markers (Lindqvist et al., 2017).

In conclusion, the seven week CSI stress period induced anxiety/depressive-like behaviors in female mice, as reflected in lower sucrose consumption in the SPT, more whisking behavior, HPA axis deregulation and a hippocampal monoaminergic deficit. Venlafaxine treatment was found to have a modest anti-inflammatory effect and to modify HPA axis-related variables, although it did not reverse stress-induced behavioral changes. These alterations may trigger subtle changes in other systems, rendering subjects more susceptible when re-exposed to stress (Rowson et al., 2019).

One limitation of the present study is that we only measured mRNA, not protein levels of cytokines, GR or MR receptors in the brain. Confirming that the observed changes in transcript levels translate into changes in protein levels would have enabled results that are more precise. Moreover, while the KYN/TRYP ratio gives some indication of kynurenine pathway activity, we were unable to detect other revealing metabolites, such as neuroprotectant kynurenic acid (Meier et al., 2017), in the samples.

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DECLARATION OF INTEREST

The authors have no conflicts of interest to declare.

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