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Research report

Impact of environmental enrichment on the GABAergic neurons and glucocorticoid receptors in the hippocampus and nucleus accumbens of Wistar rats: Pro-resilient effects

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ABSTRACT

The unpredictable chronic mild stress (UCMS) model has been used to induce depressive-like symptoms in animal models. Our work aims to evaluate the impact of environmental enrichment on male Wistar rats in an animal model for depression. For this purpose, we aim to assess changes in GR and GABAergic (PV+) density in cerebral regions related to cognitive-affective processes associated with depressive disorder, such as the dorsal-ventral hippocampus and accumbens nuclei.

Three groups of rats were used: UCMs (unpredictable chronic mild stress), EE+ UCMs (enrichment + stress) and CONT (behavioral tests only). Hedonic responses elicited by sucrose solution were examined by licking behavior analysis; the anxiety level was evaluated using the elevated zero maze and the forced swimming (passive coping) tests. The environmental enrichment reduced the effects of chronic stress, promoting greater resilience. Thus, the UCMs group showed an anhedonia response, more anxiety and immobility behavior than either the control or the EE+ UCMs groups. Regarding immunochemistry results, there was a reduction in GABAergic activity coupled with increased activation of GR in UCMs in the dorsal hippocampus, but there were no differences between groups in the ventral hippocampus.

These results suggest environmental enrichment could enhance greater resilience, reducing the vulnerability of the subjects to develop disorders such as depression and anxiety.

1. Introduction

Chronic stress is a key factor for the development of disorders such as anxiety and depression. The World Health Organization (WHO,2017) estimates that depressive disorder affects 3.8 % of the population. This percentage is even higher when reported in samples of adults and adults over 60 years, reaching 5 % and 5.7 %, respectively. Subjects with MDD show despair and difficulties in concentration and attention. They also present sleep and body weight alterations, and Type II diabetes. Among all the symptoms, anhedonia, which refers to the loss of the ability to feel pleasure, is considered a core feature of depressive disorders (Höflich et al., 2018; Coccurello, 2019).

Not every subject who experiences adverse situations develops depressive symptoms, with resilience being a protective variable. For this reason, neurobiological studies are currently focused on understanding the mechanisms that favor resilience (Pascual-Leone and Bartres-Faz, 2021). Chronic exposure to moderate and unpredictable stressors (UCMS) is a chronic-stress paradigm based on the successive and repeated application of different stressors that would not necessarily involve risk to subjects when presented individually but can induce

Abbreviations: CRH, corticotropin-releasing hormone; DA, dopamine; dCA1, dorsal Cornu Ammonis 1; dCA3, dorsal Cornu Ammonis 3; dDG, dorsal Dentate Gyrus; EE, Environmental Enrichment; EZM, Elevated Zero Maze; FST, Forced Swimming test; GR, Glucocorticoid Receptor; HPA, Hypothalamic Pituitary Adrenal; mPFC, medial Prefrontal Cortex; MDD, Mayor Depressive Disorder; MR, Mineralocorticoid Receptor; NAc, Accumbens nucleus; OD, Optical Density; PV^{+}, Parvalbumin neurons; UCMS, Unpredictable Chronic Mild Stress.; vCA1, ventral Cornu Ammonis 1; vCA3, ventral Cornu Ammonis 3; vGD, ventral Dentate Gyrus; VTA, Ventral Tegmental Area.

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long-lasting brain and behavioral alterations when combined. In this case, physical (water and food deprivation, for example) and psychological stressors (overcrowding or isolation) are combined in a variable and unpredictable way (Atrooz et al., 2021). Thus, the UCMS protocol is meant to reflect the continuous pressures humans are exposed to in everyday life, modeling MDD with good face validity (Willner, 2017a, 2017b).

Among the neurobiological mechanisms involved in the pathophysiology of depression, the regulation of the Hypothalamic-Pituitary-Adrenal (HPA) axis plays a central role (Gold, 2015). This regulation involves limbic system regions such as the hippocampus prefrontal cortex (PFC) that exert negative feedback control over the HPA. But the amygdala nucleus induces hyperactivity of this axis, with an increase of cortisol in the blood. Preclinical studies in rodents have reported atrophy of neurons in the hippocampus and PFC in chronic stress models (Duman et al., 2019; McEwen, 2017). Neuroimaging studies in humans confirm a reduction in the hippocampal volume in chronically depressed patients (Malhi and Mann, 2018).

Other brain regions have been included in the control of HPA axis activity. Acute stress can trigger the release of corticotropin-releasing hormone (CRH) into the ventral Tegmental area (VTA) to stimulate dopamine (DA) into the accumbens nucleus (NAc). This response is associated with increased reward-seeking behaviors that help mitigate the negative effects produced by stressors (Dallman et al., 2005). However, chronic stress exposure can lead to alterations in this system that produce behavioral deficits linked to depression as an anhedonic response. In addition, the NAc maintains connections with the ventral hippocampus, thus participating in the control of the stress response (Muir et al., 2020). At the brain level, cortisol acts through mineralocorticoid (MR) and glucocorticoid receptors (GR) (Meijer et al., 2019). The formers have a lower distribution in the brain, being mainly present in the hippocampus, compared to GR, which are distributed more extensively. MR acts in the presence of cortisol basal levels, while the GR plays a role when the cortisol level is high, and its elevated levels are maintained continuously. Thus, high levels of glucocorticoids activate the lower-affinity GR. Therefore, this receptor could be related to greater vulnerability or resilience to affective disorders. Therefore, it is important to assess the role of GR in stress control, considering behavioral and neurobiological variables in the analysis of subjects under situations of chronic stress.

The GABAergic system is also involved in the regulation of the HPA axis activation under conditions of chronic stress. CRH neurons of the hypothalamic paraventricular nucleus are regulated by GABAergic inhibition (Mody and Maguire, 2012). Studies using proton magnetic resonance spectroscopy reveal a significant reduction in the GABAergic activity of the prefrontal cortex of MDD patients, showing a deficit in GABA concentration in brain, alteration of the GABAergic interneurons, and dysfunction of $GABA_A$ receptors (Luscher and Fuchs, 2015). The reduction in GABAergic activity would induce greater resistance to treatments and reduce the efficacy of these interventions (O'Leary and Cryan, 2019). The "monoaminergic hypothesis" has been proposed to explain MDD pathophysiology and validated with the development of monoaminergic antidepressants. Most antidepressant treatments, such as selective serotonin and norepinefrine reuptake inhibitors, are effective in relieving symptoms in a significant percentage of patients (Pitsillou et al., 2020). However, clinical studies show how many patients with MDD fail to respond to these treatments (Guerrera et al., 2020). Therefore, new pharmacological proposals emphasize the role of GABAergic activity in the treatment of depressive disorder.

Interventions such as environmental enrichment (EE) could act on GABAergic activity favoring greater resilience. SERT -/- rodents show improvement in anxiety and depression-like behaviors after one month of exposure to EE, and this improvement is accompanied by a normalization of GAB 65 and GAB 67 levels in the prefrontal cortex (Sbrini et al., 2020). This beneficial effect of EE is even observed in old rats subjected to EE for two months, as they presented increased expression of Parvalbumin-positive (PV^+) cells in the medial prefrontal cortex (mPFC) and reduced anxiety (Sampedro-Piquero et al., 2016).

We aim to analyze whether EE prior to UCMS can attenuate the negative consequences of stress in rats, favoring greater resilience. In relation to the cellular mechanisms underlying depression-like behaviors versus resilience, we have examined the functional states of GABAergic neurons and GR activation in the hippocampus and NAc for resilience versus vulnerability.

We hypothesize that subjects exposed to UCMs would show changes in the GR and lower hippocampal GABAergic activity as compared to subjects having environmental enrichment prior to the UCMS treatment. The EE should act increasing resilience and inducing high GABAergic activity. In addition, we hope that the EE treatment alleviates the negative impact of UCMS on hedonic response to flavors during voluntary consumption.

2. Material and methods

2.1. Subjects

Twenty-six adult male rats supplied by the vivarium of the University of Seville (Spain) were used. They were 8 weeks old at the start of the experiment and had a mean weight of 200–250gr. Upon arrival, the rats were accommodated in the animal facilities of the University of Oviedo with a pattern of 12 h of light and dark cycle (8:00–20:00 light/20:00–8:00 dark), relative humidity of 65–70 %, and a temperature of 20 ± 1ºC. All the procedures were carried out during the day and followed the Directive 2010/63/EU of the European Parliament and the Spanish regulation for the protection of animals used in experimentation. The different procedures were implemented once the weight of the animals had stabilized after arrival at the laboratory to 250–300gr and the subjects were 12 weeks old (PROAE 13–2019).

The animals were randomly assigned to three different groups: Control (CONT, n:8), housed under normal conditions without any intervention and submitted to behavioral tests (sucrose preference, anxiety, and forced swimming task); Unpredictable Chronic Mild Stress (UCMs, n:8), submitted to a UCMS protocol and the behavioral tasks discussed above; Environmental Enrichment in Unpredictable Chronic Mild Stress group (EE+ UCMs, n:8), exposed to EE prior to the UCMS protocol and behavioral tests. The total number of subjects necessary to reach a test power of 1- β:0.95 was calculated with G-power software. Groups of subjects within each condition were randomly divided into three cages (55 cm × 20 cm x 35 cm) of five animals each. They had unlimited access to both food and water.

2.2. Behavioral procedures

2.2.1. Environmental enrichment (EE)

EE is an experimental paradigm used to explore how a complex, stimulating environment can impact global health. In laboratory animal experiments, EE housing conditions typically include larger-than-standard cages, abundant bedding, running wheels, mazes, toys, and shelters, which are rearranged regularly to further increase novelty and exploratory behavior.

The EE protocol was applied for 4 consecutive weeks. For this purpose, the animals were grouped in a cage (100 cm × 95 cm x 54 cm) that contained different types of ropes, wooden platforms, plastic tubes, and objects of different shapes. Once a week, the arrangement of these items was changed, and some of them were replaced by new ones to ensure the presence of novel stimuli. The EE group consists of 8 animals all in a bigger cage. The rest of the groups were distributed in 4 animals per home cage.

2.2.2. Unpredictable chronic mild stress

During the four consecutive weeks in which the UCMS protocol was carried out, rats were exposed to different stressors: (a) abnormal cage

inclination (45º for 6 h), (b) wet sawdust (300 ml of water poured on the sawdust for 6 h), (c) food deprivation (total deprivation for 24 h), d) water deprivation (total deprivation for 5 h + 1 h with empty bottle), e) crowding (grouping of 7–8 animals in the same cage, 6 h), f) immobilization (immobilization in a restraint tube for 5–30 min), g) flashing light (300 flashes/second for 6 h). Each day there were one or two different stressors depending on the duration of each. In the case of restraint in each presentation, the time was extended from 5 min to 20 min last week. Each week the sequence of stressors applied varied.

Then, the UCMs and EE+UCMs groups are exposed for four weeks to a chronic stress protocol. Once the UCMS protocol is finished, the sucrose consumption test was performed again. The next day, the zero-maze test is carried out in a single session of 5 min. The forced swimming test was performed in a single session of 5 min the next day; after completing this test, the brains will be processed for study.

2.2.3. Sucrose consumption test (SCT)

In the test of sucrose consumption in the pre-exposure phase, the general consumption pattern in all groups is recorded. To do this, the animals are deprived of water 4 h before starting the test. There is no deprivation of food. Each daily session lasts 10 min. The anhedonia test took place in a dimly lit room containing 8 custom-made drinking boxes measuring 42 × 25 x 20 cm, with acrylic walls, steel mesh flooring, and wire mesh lids. Drinking bottles (50 ml) with metal spouts could be inserted at one end of each box. A contact-sensitive lickometer registered the licks made by rats to the nearest 0.01 s, and MED-PC software (Med Associates, Fairfax, VT) controlled the equipment. The anhedonia protocol involves analyzing the microstructure of licking behavior during voluntary consumption (Dwyer, 2012). The mean number of licks per cluster (lick cluster size) provides a measure of palatability that is dissociable from consumption levels and directly related to the nature and concentration of the ingested solution. Lick cluster size monotonically increases with the concentration of palatable sweet solutions, whereas it decreases monotonically with the increasing concentration of unpalatable solutions (Davis and Smith, 1992; Spector et al., 1998). In our study, this experimental procedure consisted of two phases. Briefly, after habituation (2 days with water), the rats receive a 4 % sucrose solution (3 consecutive days). In the second phase consists of exposure (2 consecutive days-sessions) after the stress protocol. The following procedure is detailed in Begega et al. (2023). Total consumption(ml), total number of licks, and lick cluster size were recorded across the experimental sessions (Fig. 1).

2.2.4. Anxiety test: elevated zero maze

All subjects were assessed in an Elevated Zero Maze (EZM). The EZM consists of a circular platform constructed in Perspex, 6.1 cm wide and 40 cm in diameter, elevated 72.4 cm from the ground, and divided into four quadrants of the same size.This test is carried out in a single session of 5 min.

The test session was carried out one week after passing the preference test. It was recorded with a video camera connected to a computer equipped with a video tracking program (Ethovision Pro, Noldus Information Technologies, Wageningen, The Netherlands). The following variables were recorded: a) number of entries into the open area, b) time spent in each area, c) latency of entry into the open area and d) time by entries in the open area (TbE). This measure is a correction of the time spent by the animal in the open arm considering the level of activity. For the calculation of this index, the duration is taken into account as well as the number of entries. So, it may happen that the duration in the open arm is greater but because it has a greater number of entries to the open arm. In this case, it would not reflect a reduction in anxiety, but an increased hyperactivity. High TbE rating is indicative of low levels of anxiety, while low TbE rating is indicative of high levels of anxiety (Heredia et al., 2012).

2.2.5. Behavioral despair: forced swim test

All groups were assessed in the forced swim test (FST) in a single session of 5 min duration that was carried out the day after being evaluated in the EZM. Rats were placed in an upright cylinder (20 cm diameter) filled with warm water (20 ± 1 °C) up to 5 cm below its opening. This container was refilled with fresh water between each test. At the end of the swimming session, the animals were removed from the cylinder and dried with towels. Each rat was judged to be immobile when it ceased struggling and remained floating motionless in the water and making only those movements necessary to keep its head above water. The lack of struggling was considered a state of behavioral

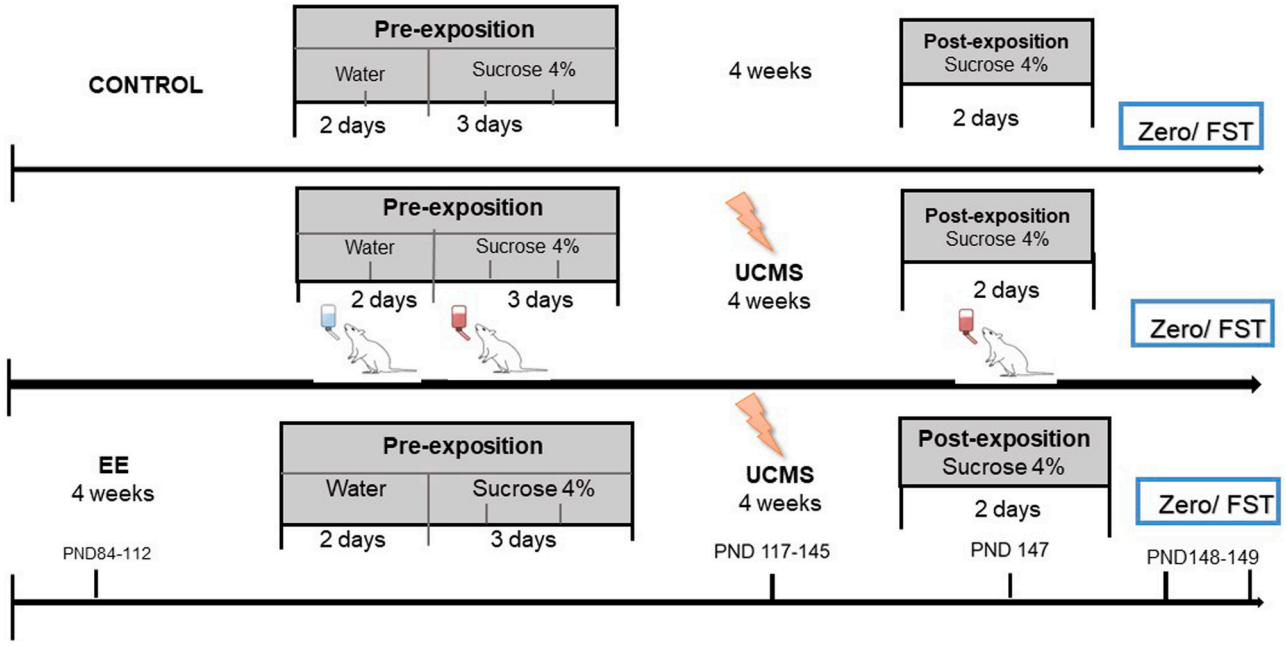

Fig. 1. Graphic illustration of experimental design. Experimental groups of male Wistar (CONT: control, UCMs: Stress, and EE+ UCMs: Environmental enrichment +Stress). Pre-phase of sucrose consumption. Unpredictable chronic stress paradigm (four weeks); post-phase of sucrose consumption, zero maze, forced swimming task (FST) and sacrifice.

despair. In this task, frequency, duration, and latency were recorded in immobility, mobility, and high mobility behaviors. These behaviors were recorded with the Ethovision 3.5 (Noldus Information Technologies, Wageningen, The Netherlands).

2.3. Immunohistochemistry assessment of GABA PV^+ neurons and GR

Once the forced swimming test is over, twenty-six subjects were deeply anaesthetized (Ketamine 0.4 ml and Xylazine (0.2 ml) and perfused transcardially with 0.9 % saline (20 min).

The brains were postfixed with paraformaldehyde at 4 % (0.1 M; pH 7.4) for 3 h, and then transferred into PBS (pH: 7,4; 0,1 M). Systematic cuts (50 μm^2) were performed with vibratome (VT1000S, Leica Biosystems) which include the NAc (+3.72 to −1.44 mm from bregma), and the dorsal and ventral hippocampus (dentate gyrus (DG), CA3 and CA1) (−3.30 to −6.04 mm from bregma).

Floating sections were incubated for 45 min in the dark to inactivate endogenous peroxidases (10 % methanol, 10 % hydrogen peroxide, 80 % PBS). Then, the sections were preincubated with donkey serum (S30 – 1 ml, Merck,) for 45 min. Then, sections were incubated with rabbit polyclonal antibody diluted in PBS at 1:800, both anti-glucocorticoid receptors (M-20, Santa Cruz Biotechnology) or anti-PV^+ at 1:800 (Ref. Swan: Switzerland) overnight at 4ºC. The second day, the sections were incubated for 90 min with a secondary anti-rabbit polyclonal antibody (31820, Invitrogen) diluted in 0.25 % serum albumin bovine (BSA) (1:1000). They were washed in PBS and incubated with the buffered aqueous solution of ExtrAvidin-Peroxidase (2886 – 1 ml, Sigma, USA) for 45 min in the dark. Sections were developed using diaminobenzidine (DAB) as a chromogen. Finally, the sections were mounted on slides, dehydrated through a series of graded alcohols, cleared with xylene and coverslipped with Entellan. The counting was conducted in one out of every six Section (1/6). For GR quantification, density was measured using ImageJ software (U.S. National Institutes of Health, Maryland, USA) and expressed as average optical density (OD). Photographs at 10x were captured with a digital camera coupled to the microscope (Leica Microsystems DFC490, Switzerland) to measure the density of GR in each region. Background intensity was subtracted from the OD of each section by taking the mean of 10 random measurements of the background. The results were converted to percentage of OD with respect to the Control. Quantification PV^+ neurons was done with Leica LAS X Software (Leica Microsystems, Switzerland) using 10x magnification through a microscope (Leica Microsystems DFC490). We counted the number of positive cells expressed as the average number of cells per mm^2 of area.

2.4. Statistical analysis

Data were analyzed with SPSS 23 (SPSS Inc. Chicago, USA).

In the anhedonia test, we analyzed mean lick cluster size. A cluster was defined as a series of licks separated by pauses of no more than a 0.5-s interval, a criterion used in our previous studies examining flavor aversion by licking analysis (Begega et al., 2023; Dwyer, 2012). Data from consumption, total licks, and lick cluster size during Prep (3 days) and Post (2 days) phases were analyzed by mixed analyses of variance (ANOVAs) with Group as a between-subject factor and sessions as a within-subject factor. Data obtained in the EZM and FST were analyzed by multivariate analyses of variance (MANOVA). Data of GR density and the number of PV^+ neurons in dorsal - ventral hippocampus (GD, CA1, CA3) and NAc were analyzed by a one-way ANOVA. In all cases, the Bonferroni test was used for post hoc analyses. The results were presented graphically with SigmaPlot 12.0 (Systat, Richmond, EEUU).

3. Results

3.1. Sucrose consumption test

The mixed ANOVAs performed with the data from the Pre phase (sessions Pre-1–3) revealed significant differences between groups in sucrose intake, $F(2,22) = 5.70$, $p = 0.010$, and number of licks, $F(2,22) = 4,36$, $p = 0.025$, but not in lick cluster size ($F(2,22) = 0.834$, $p = 0.447$). There was also a significant effect of session for consumption, $F(2,44) = 8.09$; $p = 0.001$) and number of licks $F(2,44) = 8.28$, $p = 0.001$), but not for cluster size data ($F(2,44) = 0.43$, $p = 0.64$). The interactions sessions x groups showed not statistical differences. The post hoc Bonferroni confirmed that UCMs showed less consumption on session 1–2 ($p = 0.001$ and $p = 0.05$) than EE+ UCMs group. In addition, the UCMS group shows a lower number of licks on session 1 than CONT($p = 0.04$) and on session 2, the UMCs showed a lower total lick than the EE+ UCMs group($p = 0.045$). But the three groups did not differ in session 3 ($p = 0.117$). The pairwise comparisons performed with the cluster size data revealed no differences between the groups in any of the sessions ($p = 0.37$). These results indicate that there was no evidence of differential hedonic valuation of the sucrose solution during the Pre phase despite differences in consumption and number of licks in sessions 1–2.

The analysis of the data from Post phase (sessions Post 1–2) showed significant differences between groups in sucrose consumption, $F(2,22) = 10.02$, $p = 0.001$, number of licks, $F(2,22) = 8.10$, $p = 0.002$, and cluster size $F(2,22) = 7,15$; $p = 0.004$. But there was not effect of session for consumption ($F1,22 = 2,95$; $p = 0.10$). However, there were significant differences of session in total licks ($F(1,22) = 76,98$; $p = 0.001$ and cluster size ($F(1,22) = 7,18$, $p = 0.014$. The group UCMs displayed less consumption than CONT ($p = 0.02$) and EE+ UCMs ($p = 0.001$) and less total licks than EE + UCMs($p = 0.002$). Moreover, the UCMs had a lower cluster size than CONT ($p = 0.045$) and EE+ UCMs ($p = 0.004$). This reduction is observed on session 1 UCMs vs CONT($p = 0.002$) and UCMs vs EE+ UCMs($p = 0.041$) and it is maintained on session 2 UCMs vs EE+ UCMs($p = 0.046$). Data from the anhedonia test are shown in Fig. 2.

These data suggest that the environmental enrichment treatment attenuated the negative impact of the stress manipulation on the hedonic valuation of the sucrose solution.

3.2. Anxiety test: elevated zero maze

We found significant differences in the number of entries into the open area between the groups in the EZM $F(2,24) = 11.66$; $p = 0.001$). UCMs and EE+ UCMs presented a higher number of entries into the open zone compared to CONT ($p = 0.004$ and $p = 0.001$, respectively). There were group differences in the time spent in the open zone ($F(2,24) = 4.60$, $p = 0.02$). The EE+ UCMs spent more time in this zone compared to the rest of the groups. Furthermore, EE+ UCMs showed a shorter latency to enter this area than the rest ($F(2,24) = 5519$; $p = 0.011$). The time-by-entry index shows group differences ($F(2,24) = 10.698$; $p = 0.001$). For this reason, although the EE+ UCMs and UCMs presented a similar frequency of entries into the open zone, the EE+ UCMs explored the open arm for more time than the UCMs, showing less anxiety and more exploration than the UCMs. Data from this task are shown in Fig. 3.

3.3. Behavioral despair: forced swim test

The analysis of the behavioral data in the FST showed statistically significant group differences in duration ($F(2,21) = 12.63$; $p = 0.001$) and latency to immobility ($F(2,22) = 2.72$; $p = 0.001$). In the duration of immobility, the Control Group maintains immobility for less time than UCMs($p = 0.001$) and EE+UCM($p = 0.023$). Both groups subjected to stress show no statistically significant differences($p = 0.15$).

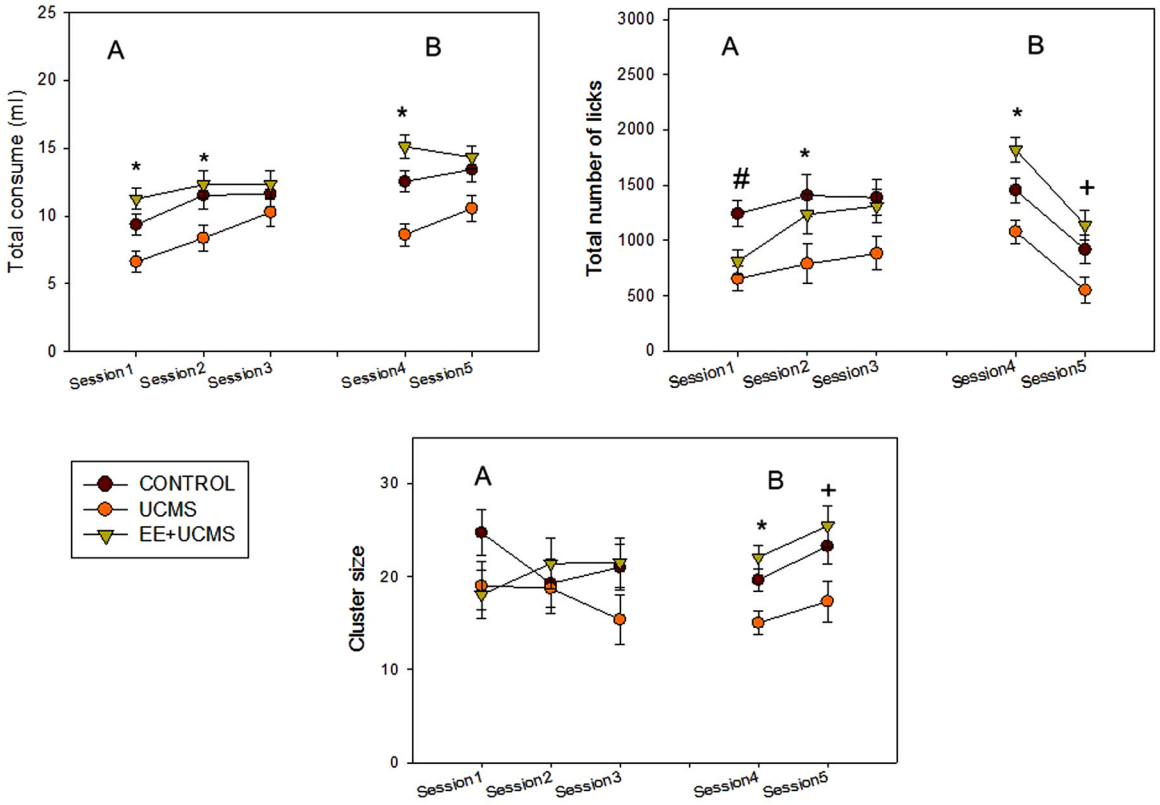

Fig. 2. Results of sucrose consumption task. Mean and ± SEM of the two phases over the days. (A) Pre exposition phase: * Represents the statistically significant differences ($p \leq 0.05$). The UMCS group showed a lower consumption than EE+ UCMs group in session 1 and 2. In the total number of licks, UCMs showed a lower number of licks than the CONT on session 1(# $p = 0.04$) and EE+ UCMs on session 2 (* $p = 0.045$). (B) Post exposition phase: * Represent the statistically significant differences($p \leq 0.05$). The group UCMs displayed less consumption, and lower licks than the rest of the groups in session 4. In session 5, UCMs group maintained this reduction compared to the EE+UMs group ($^{+}$ $p = 0.002$). Moreover, the UCMs had a lower cluster size than CONT and EE+ UCMs on session 4. In session 5, UCMs group maintained this reduction compared to the EE+UMs group ($^{+}p = 0.04$). N = 8 subjects each group.

EE+ UCMs took longer to show immobility compared to the CON ($p = 0.001$) and UCMs ($p = 0.001$). Groups differed in high mobility ($F(2,21) = 6.24$; $p = 0.007$). The Control group showed higher frequency of this behavior than the EE+ UCMs ($p = 0.023$) and UCMs ($p = 0.014$). Fig. 4.

3.4. Immunohistochemistry of GABA PV^{+} neurons and GR density in the hippocampus and NAc

In general, there were statistically significant group differences in GR density in the dorsal hippocampus. UCMs presented a higher density of GR in all dorsal hippocampal regions compared to CONT and EE+ UCMs. Thus, dDG ($F(2,21) = 12$; $p = 0.001$), dCA1($F(2, 21) = 16,58$; $p = 0.001$) and dCA3 ($F(2,21) = 31,56$; $p = 0.001$) regions showed statistically significant group differences. GR density showed no group differences in ventral hippocampal regions vDG ($F(2,17) = 1.38$; $p = 0.28$), vCA1 ($F(2,17) = 3.22$; $p = 0,06$), or vCA3 ($F(2,17) = 0.90$; $p = 0.42$). In the case of NAc, there were statistically significant group differences ($F(2,23) = 5.65$; $p = 0.01$). UCMs showed lower GR density than the CONT ($p = 0.036$) and EE + UCMs ($p = 0.018$) in NAc. No differences were found in NAc GR density between Control and EE+ UCMs ($p = 0.76$) (Fig. 5).

Quantification of GABAergic PV+ neurons revealed statistically significant group differences in all dorsal hippocampal subregions (dDG ($F(2,21) = 12.01$; $p = 0.001$); dCA1 ($F(2,21) = 16.58$; $p = 0.001$) and dCA3 ($F2,21 = 31.56$; $p = 0.001$). UCMs showed lower PV+ density than the EE+ UCMs ($p = 0.001$) and CONT ($p = 0.006$) groups in dDG. The same pattern of results was found in dCA1 and dCA3, where the UMCs group presented a lower density of GABAergic PV+ neurons than the EE+ UCMs (dCA1p=0.012 and dCA3 $p = 0.001$) and CONT (both dCA1and dCA3; $p = 0.001$) groups. The three groups did not differ in their density of GABAergic PV+ neurons along the vDG ($F(2,17) = 0.30$; $p = 0.74$). However, there were statistically significant group differences in vCA1 ($F(2,17) = 6.17$; $p = 0.011$ and vCA3 $F(2,17) = 4.93$; $p = 0.023$, respectively). In both regions, EE+ UCMs showed a higher number of PV^{+}neurons than UCMS (vCA1 $p = 0.042$; vCA3, $p = 0.033$). In general, the UCMs presented lower expression of PV^{+} neurons in the dorsal hippocampus. Therefore, previous exposure to an EE reduced these effects of stress on GABAergic functioning of the dorsal hippocampus. This same effect was observed only in vCA1 and vCA3. In the case of NAc, there were no statistically significant group differences in the density of GABAergic PV+ neurons ($F(2,20) = 1.634$; $p = 0.22$) (Fig. 6).

4. Discussion

The objective of this study was to investigate the mechanisms involved in the negative consequences of chronic stress and the beneficial effects of environmental enrichment. to prevent UCMS dysfunctions. Specifically, we evaluated the impact of UCMS alone and UCMS preceded by EE exposure on anhedonia, anxiety, and despair, as well as on GABAergic activity and GR density in the hippocampus and NAc. For this purpose, the comparison between EE + UCMs and UCMs (without pre-exposure to EE) would show us the effects of EE in responses of anhedonia, anxiety, and hopelessness. As both groups were subjected to the same conditions of chronic stress and behavioural tests, the differences between the two groups would be associated with EE. These pro-resilient effects have been observed with the application of different EE

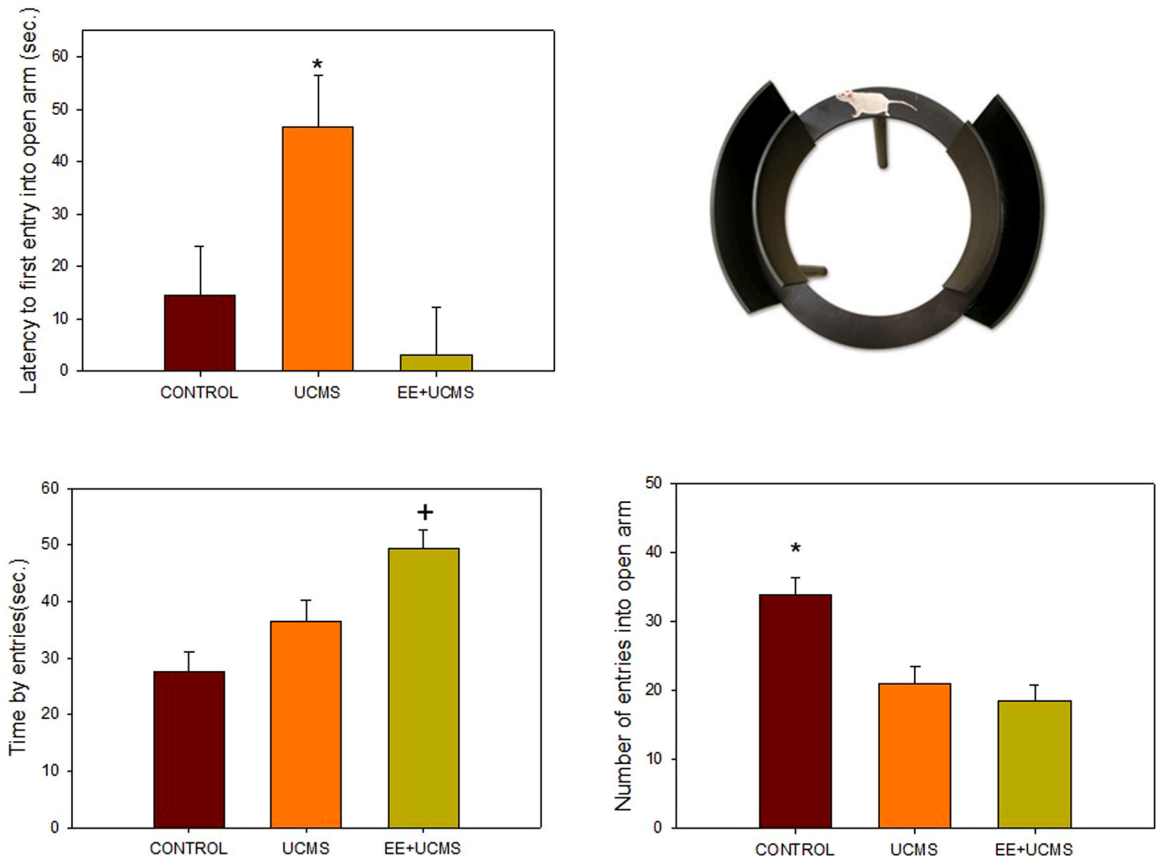

Fig. 3. Mean and ± SEM of latency to entry, frequency of entries and time spent on open arm. * Represent statistical differences ($p \leq 0.05$). The UCMs shows a higher latency to enter the arm than the rest of the groups. + Represent statistical differences ($p \leq 0.001$) in the time-by-entry index. he EE+ UCMs spent more time in the open arm than the rest of the groups. The EE+ UCMs explored the open arm for more time than the UCMs, showing less anxiety and more exploration than the UCMs. However, the control group showed a higher frequency of entries to the open arm (* $p \leq 0.05$). N = 8 subjects each group.

conditions, e.g.: intermittent (3 h per day)(Sampedro-Piquero et al., 2013) continuous (24 h for approx. two months) (Sampedro-Piquero et al., 2014), and even with old rats (18 mmonths) (Sampedro-Piquero et al., 2016). In general, our study shows that previous EE buffers the effects on anhedonia and anxiety associated with stress. These effects are accompanied by a lower density of GR and greater PV+ activity, especially in the dorsal hippocampus in the EE+ UCMs.

Behaviorally, the symptoms that accompany MDD disorder include emotional-cognitive alterations such as loss of interest, despair, anxiety, and difficulties in concentration and attention. Among all of them, anhedonia has been considered key in depressive symptomatology and a substantial predictor of suicide (Scheggi et al., 2018). In our study, the 4-week UCMS protocol induced low hedonic assessment of sucrose. However, when EE preceded the UCMS protocol, the anhedonic effect was prevented, as reflected in that the groups EE+ UCMs and CONT displayed a similar high fluid consumption and lick cluster size.

Therefore, the EE + UCMs showed a hedonic response similar to the Control, reflecting that previous exposure to EE reduces the effects of stress on hedonic assessment, promoting greater resilience. On the other hand, food and drink deprivation clearly influences the consumption of sucrose, interfering with the interpretation of anhedonia (Forbes et al., 1996). In prolonged periods of food deprivation— e.g., 21 h—, it has been shown that in addition to weight loss, there is also a lower sucrose consumption. This response has not been associated with a decrease in the hedonic valuation of sucrose but with a reduced metabolic need for sucrose consumption.

To avoid this interference in our work, there are no periods of food deprivation or weight loss in the experimental groups. We designed a short period of water deprivation (4 h) to induce the consumption response while avoiding interference with the physiological control mechanisms of thirst.

In the pre-exposure phase, we have observed an increase in the consumption and number of licks in the EE group pre-exposed. Although it may be surprising, this same effect was found in another research. Mileva and Bielajew, 2015 evaluated sucrose consumption, anxiety and depressive- like behaviors, as in FST in Wistar and Wistar Kyoto subjects (Mileva and Bielajew, 2015). In this study, a previous EE, for four weeks, caused a higher consumption of sucrose at 1 % than the standard and isolated groups. This effect of EE could be associated with physical exercise. This is an important component in the environmental enrichment protocol. The practice of exercise induces greater hippocampal plasticity and neurogenetic response in DG accompanied by an increase in BDNF (Phillips C, 2017). It should be noted that these effects associated with the practice of physical exercise have an antidepressive effect.

Taking this into account, in our case, the consumption pattern shown by the previous EE group could be related to these processes. But, although consumption and the number of licks increased in the pre-exposure to stress phase, this effect was reduced, showing the same consumption pattern between the groups on the third day. On the other hand, the cluster size showed no group differences in the pre-exposure phase. The mean number of licks per cluster (lick cluster size) provides a measure of the hedonic value of the fluid, which is dissociable from consumption levels and directly related to the nature and concentration of the ingested solution. Lick cluster size monotonically increases with the concentration of palatable sweet solutions, whereas it decreases monotonically with the increasing concentration of unpalatable quinine solutions (Davis and Smith, 1992; Spector et al., 1998). However, to determine whether exercise is crucial in this response,

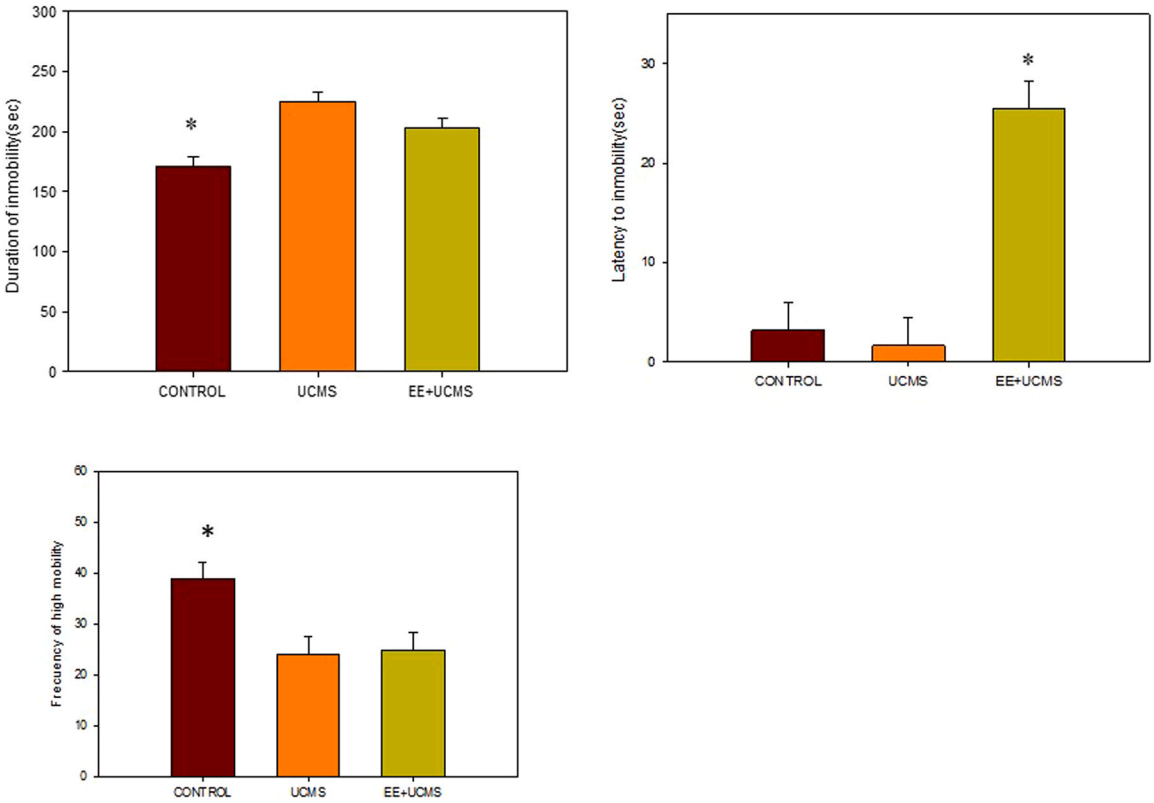

Fig. 4. Result of Forced swimming task. Mean and ± SEM of duration, latency to immobility behaviour and frequency of high mobility. * Represent statistically significant differences in duration of immobility. Control maintains immobility for less time than UCMs and EE+UCMs. Both groups subjected to stress show no statistically significant differences. * Represent statistically differences ($p = 0.001$) in latency to immobility. EE+ UCMs took longer to show immobility compared to the CON($p = 0.001$) and UCMs ($p = 0.00$). In the case frequency high mobility * Represents the statistically significant differences ($p \leq 0.05$). The Control group showed higher frequency of high mobility than the EE+ UCMS and UCMs. N = 8 subjects each group.

studies are needed in which only exercise and its behavioral effects are evaluated in different environments.

Circulating glucocorticoid levels are associated inversely with increased consumption of the artificial sweetener saccharin (Packard et al., 2016). Pleasurable experiences reduce stress responses and increase exploratory behavior (Ulrich-Lai et al., 2010).

Neurobiologically, our study demonstrates that the NAc showed low density of GR after the UCMS protocol. Therefore, in chronic stress, the anhedonia response could be produced by the dysfunction of brain regions involved in reinforced processing. The NAc, along with the VTA, takes part in this reinforcement assessment circuit and has been related to the presence of anhedonia (Yang et al., 2018).

Regarding anxiety responses, the UCMs showed high anxiety levels, as they spent less time in the open zone, despite showing a high frequency of entries. This could reflect high mobility between the closed and open zones, preventing continuous exploration of the open area. The EE+ UCMs explored the open zone for more time than the UCMs because although the number of entries in this zone was similar to the UCMs, the EE+ UCMs remained for more time in this open area than the UCMs.The control group showed a higher frequency of entries into the open arm. Although it may seem strange, this pattern may respond to increased hyperactivity with further exploration in this group. It should be noted that the number of entries is recorded when the centre point of the animal is located in the arm, not when the animal remains with its whole body in the open arm. This can confuse the interpretation of the data. Therefore, the time index is considered a more representative measure of anxiety, because it considers the duration in the open arm and the frequency of visits. However, this pattern must be analysed in detail. It should be noted that Group EE + UCMs did not show a higher frequency of entries, but it remained a longer time in the open arm, as indicated by the time per entry. As Heredia et al. (2012) mention, it is advisable to consider this anxiety index because subjects with high mobility may seem to spend a lot of time in the open arm, but they move a lot (a greater frequency of entries). This index is calculated by taking into account the time spent in the open arm and the frequency of entries.

Taking into account the time index, EE + UCMs did not show a greater frequency of entries, remaining longer in the open arm. These results show an anxiolytic effect that could be associated with the hypothesis of EE as a stress inoculator.

In the FST, UCMs and EE+UCMs groups showed a similar pattern, although the EE + UCMs took longer for immobilization to appear compared to the rest. The duration of immobilization did not show statistically significant group differences. The UMs showed a decrease in the immobilization latency compared to EE+UCMs. So, Immobilization and floating behavior were exhibited first in UCMs. The interpretation of the results is complex. FST has been widely used to evaluate hopelessness in animal model. However, there are different interpretations of the response to this task. In this situation, the animal shows greater hopelessness because it is impossible to escape from the situation. However, this greater immobilization could be considered adaptive because it reduces energy expenditure in an inescapable situation. Therefore, this test is also considered to inform about the strategy employed by the animal (Commons et al., 2017). This immobilization is considered choosing a passive coping strategy that can be beneficial and not reflecting hopelessness. And finally, another possible interpretation is to consider it great immobility related to anxiety (Anyan and Amir, 2018). The FST is a very stressful test because the animal is tired and cannot escape from it, which raises its level of anxiety. Hence, the subject displays a freezing-immobilization response. In recent studies, people with a depressive disorder of 12 months also show anxiogenic symptoms.

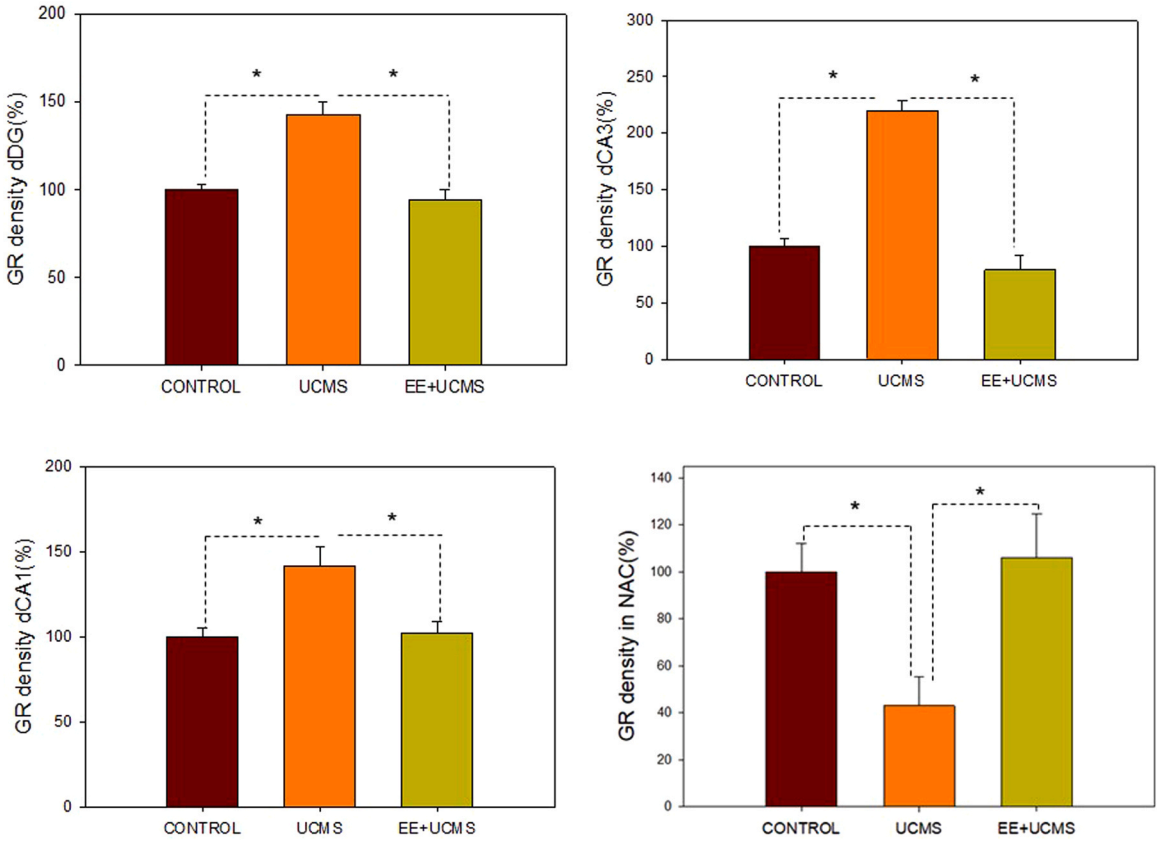

Fig. 5. Result of GR density in hippocampal regions and Accumbens nuclei. The UCMs group showed an increase of GR density than CONT and EE+ UCMs in all hippocampal subregions. However, UCMs showed lower GR density than the CONT ($p = 0.036$) and EE + UCMs ($p = 0.018$) in NAc. No differences were found in NAc GR density between Control and EE+ UCMs ($p = 0.76$). PN = 8 subjects each group.

Clinical studies showed that this comorbidity could represent 45 % of the cases analyzed in clinical population (Kalin, 2020). Taking into account that one of the responses associated with fear is freezing, greater immobilization in both groups would clearly reflect a high level of fear.

In our study, the UCMs showed immobilization from the very first moment of the test and less active escape responses from the adverse situation than the rest of groups. Thus, we would assume a clear recognition of the inescapable consequences of this situation by this group. Probably, in the case of the control and EE+ UCMs, greater mobility could reflect an active coping, trying to escape from the situation more persistently, but no less adaptively. To analyze in future studies whether or not the most frequent and lasting behavioral strategy in each group is adaptive, it would be appropriate to consider the effects of prior exposure to this test on subsequent days.

In our study, UCMs showed anhedonia and immobilization than the rest of the groups. These same results have been observed in mice studies where the presence of anhedonic responses was related to greater immobilization (Strekalova et al., 2004). These responses are considered a good index of these negative emotions frequently presented in depressive states. Although these two responses involve different processes. In general, anhedonia is related to the processes of assessment and evaluation of a pleasant situation, whereas the immobilization response in FST has to do with hopelessness. However, Brenes Sáenz et al. (2006), applying confirmatory factor analysis, grouped the two responses into a factor called "behaviour despair" (Brenes Sáenz et al., 2006). The authors indicate that greater immobilization is positively related to higher sucrose consumption (Brenes and Fornaguera, 2008; Brenes Sáenz et al., 2006). In our case, the relationship between the two responses was negative. Thus, the UCMS group showed a higher anhedonia response (with lower consumption) and a rapid appearance of immobilization in the FST. Factors such as the concentration of sucrose used, and the duration of deprivation may explain these discrepancies.

In the study of GABAergic activity and GR density in hippocampus and accumbens nuclei, we have used ketamine and xylazine (KX) for perfusion procedure. Even though the use of ketamine and xylazine (KX) solution is extended, being one of the main anesthetics used in animal research, it is true that in the recent years there is increasing evidence in neuroscience regarding its impact on the brain, and particularly, on the HPA axis and excitatory-inhibitory balance. This could be an indirect effect of the well-known ketamine NMDARs inhibition in key brain regions, showing that even low doses could have an antidepressant effect (Gold and Kadriu, 2019; Zanos and Gould, 2018). However, the specific effect on the HPA axis is not yet clear. For example, Pereira et al. (2021) found increased corticosterone levels after KX. Other authors reported a decrease (Saha et al., 2005) or nonsignificant changes in these levels (Arnold and Langhans, 2010). These contrasting findings probably depend on the dosage and exposure time. In addition, the specific KX impact on specific parvalbumin+ cells must still be studied. On the one hand, ketamine anesthesia can increase cortical glutamate and suppress GABAergic interneurons (Deane et al., 2020). On the other hand, KX is still commonly used in experiments that aim to study the physiology of PV+ neurons (Kuki et al., 2015; Vo et al., 2023). Furthermore, it is relevant to note that these divergent findings are not only due to differences in doses but also in the timing of the procedure itself. For example, the modulatory effects of KX could be higher if the anesthetic is applied at the beginning of stereotaxic surgery and the rodents continue to perform the experiment (Moreno-Fernández et al., 2020). However, in our case, given that each subject of each group in the experiment was submitted to this procedure in the final context of perfusion, we believe that the long-term modulation of glucocorticoid receptors or PV interneurons in the brain is avoided, and the short-term effect has thus been controlled for in each group and brain area measured.

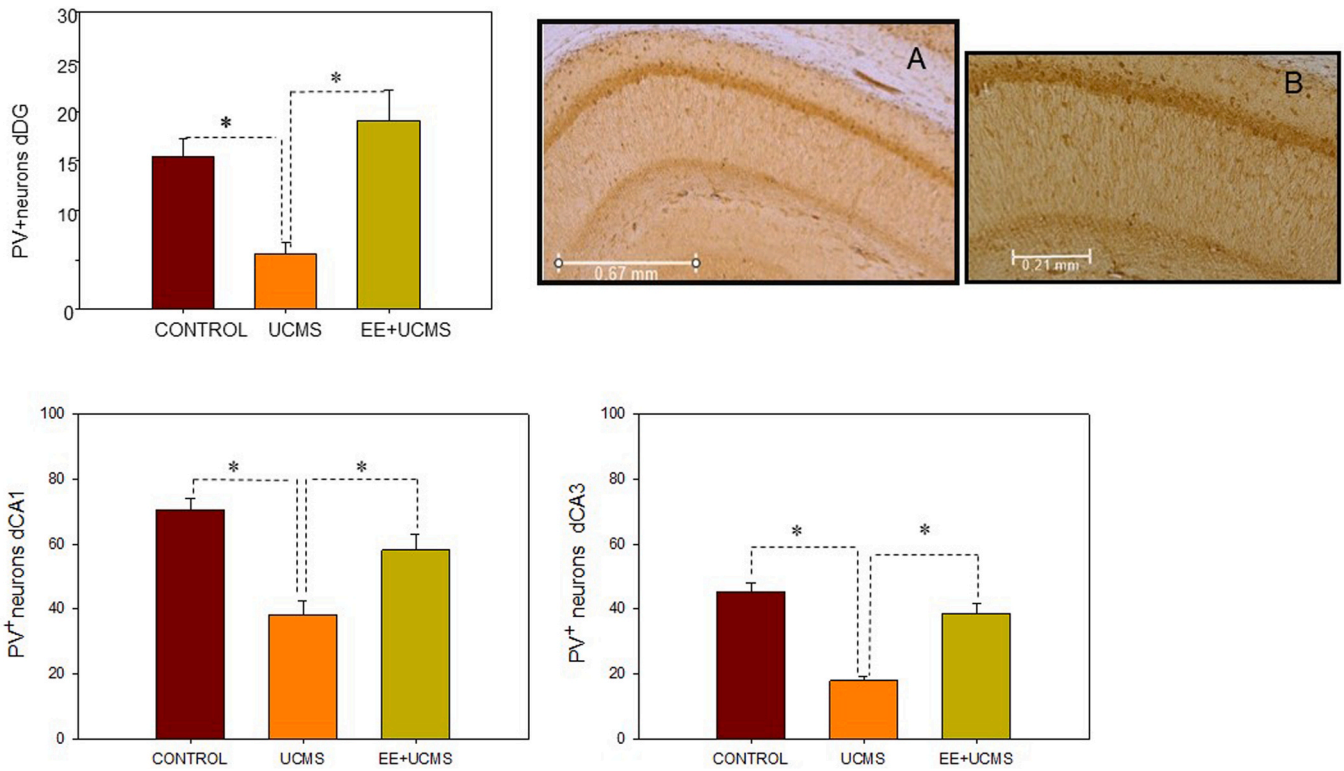

Fig. 6. Results of immunohistochemistry PV^{+}neurons in dorsal Hippocampus subregions. * Represents significant statistical differences between groups. UCMs showed lower PV+ density than the EE+ UCMs ($p = 0.001$) and CONT ($p = 0.006$) groups in dDG. The same pattern of results was found in dCA1 and dCA3, where the UMCs group presented a lower density of GABAergic PV+ neurons than the EE+ UCMS (dCA1p=0.012 and dCA3 $p = 0.001$) and CONT (both dCA1and dCA3; $p = 0.001$) groups. The photomicrographs immunopositivity PV+ neurons in dorsal hippocampus were made at A (x 2,5 objetive) and B (× 5objetive). N = 8 subjects each group.

With regard to GR expression in the hippocampal regions, our data show a higher density in UCMs compared to the rest of the groups. GR plays a relevant role in the control of HPA axis activity, and GR responds mainly to situations of lasting stress, contrary to MR, which are involved in shorter stress situations. In our study, the greater density of GR in the hippocampus of UCMs subjects could indicate a response to sustained hyperactivity of the HPA axis associated with chronic stress. This GR response could be displayed as a consequence of a higher allostatic load induced by the chronic stress situation produced by the UMCS protocol. The long-lasting hyperactivity of the HPA axis is accompanied by increased secretion of CRH in patients with depressive symptoms (Packard et al., 2016). Both clinical and preclinical studies demonstrate that GR antagonists show antidepressant effects, diminishing immobilization in the FST and preventing the reduction of sucrose preference, thus diminishing anhedonia responses (Wulsin et al., 2010). In our study, we observed that the highest GR density affected all regions of the dorsal hippocampus, while the ventral hippocampal regions presented this pattern exclusively in the CA1 and CA3 regions. This difference could be due to an unequal distribution of GR along the dorsal-ventral hippocampal poles. The ventral hippocampus shows a higher expression of MR than GR, while the dorsal hippocampus shows the opposite pattern (Shirazi et al., 2015). The chronic stress affected mainly the dorsal hippocampus in our study due to a greater presence of GR receptors in these regions. Thus, the greater GR density in the hippocampal formation in the UCMs could indicate vulnerability to depressive-like disorders. However, previous EE exposure prevented this effect, as the EE+ UCMs showed lower density of hippocampal GR compared to the UCMs. Also, the administration of a GR antagonist has an antidepressant effect in the case of mice subjected to chronic social defeat (Moldow et al., 2005). However, a reduction in the expression of GR has been observed in the hippocampus of stressed subjects when compared with resilient and control mice, in agreement with many other studies showing that chronic exposure to various stressors or glucocorticoid exposure can down-regulate GR mRNA and/or protein binding activity in the brain (Wang et al., 2019). For this reason, it is necessary to continue investigating the mechanisms associated with GR activity in regions involved in emotional-cognitive control in resilient versus vulnerable subjects.

Regarding the GABAergic activity of PV+ neurons, chronic stress reduced the number of PV+ -immunoreactive cells and altered rhythmic inhibitory postsynaptic currents originating from the PV+ neurons (Fogaça & Duman, 2019). Hu et al. (2010) showed that 3 weeks of restraint protocol induces a reduction of PV + neurons in ventral hippocampus (Hu et al., 2010). Our stress protocol did not induce changes in the entire ventral hippocampus because PV+ neurons in the vDG did not differ between groups, but we found a clear reduction of PV+ neurons in all regions of the dorsal hippocampus. In the hippocampus, the PV+ neurons receive most excitatory inputs from granular neurons. Sustained excitation of these neurons induces a greater vulnerability in PV+ neurons to the deleterious effects of stress (Zaletel et al., 2016). As a consequence of this, the inhibitory control by PV+ over the excitatory granular neurons would be reduced and, as a consequence of this disinhibition, the excitation-inhibition balance is disrupted, causing overexcitation and greater vulnerability to cellular neurotoxicity. PV+ interneurons play an important role in the control and functioning of glutamatergic pyramidal neurons. This requires a high expenditure of energy and optimal mitochondrial functioning. Throughout this process, a large amount of mitochondria-reactive oxygen species (ROS) is generated, which must be controlled by the mechanisms of the antioxidant system. The beneficial effects of EE appear to be related to a better antioxidative response because EE has increase oxidate marked as superoxide anion and elevated superoxide dismutase (Marcon et al., 2018). This effect has been observed in rat models to induce stroke. In addition, the application of EE reduces neuroinflammation, the

oxidative stress response and reduces PV+ alterations induced by oxidative deficits (Dwir et al., 2021). Similar results are shown when applying EE for 28 days in a zebrafish model, which increased ROS levels in the stressed group, but not in the enrichment group (Marcon et al., 2018). In our case, the beneficial effects of previous EE could be associated with better control of oxidative stress and maintenance of GABAergic activity by the hippocampal PV+ interneurons. In the UCMs group, this loss of PV+ is accompanied by a greater number of immunopositive cells for GRs. These GR receptors participate in a continuous elevation of cortisol, as in chronic stress. An increase of immunopositive cells for GRs in the prefrontal cortex, paraventricular nucleus of the hypothalamus and amygdala after alcohol consumption in adolescence was observed(Sampedro-Piquero et al., 2022). Another study shows that after applying EE for approximately two months in adult rats, there is an increase in the number of immunopositive cells for GRs in the dorsal hippocampus, in addition to a reduction in anxiety in the zero maze and better execution in the hole board compared to the control group (Sampedro-Piquero et al., 2014). This damage of PV+ neurons due to sustained activation of non-genomic GR induces greater glutamatergic excitatory activity (Hu et al., 2010). Western blotting analysis established a positive relationship between overexpression of glutamate receptors and GR activity in the hippocampus, both in chronic mild stress for 8 weeks and in acute stress. Susceptible rats show anhedonia and overexpression of glutamatergic receptors in the hippocampus compared to resilient rats. The administration of a GR antagonist normalizes glutamate receptors' expression and decreases anhedonia in stressed groups (Larosa and Wong, 2022). In fact, MDD patients and chronically stressed animals show reduced GABA and glutamic acid decarboxylase 67 levels in the brain, decreased expression of GABAergic interneuron markers, and alterations in $GABA_A$ and $GABA_B$ receptor levels (Fogaça & Duman, 2019). Therefore, more research is needed to determine the role of GR and the relevance of PV+ interneurons in resilience.

The NAc also presented compromised GABAergic activity in depressive disorder. After examining the effect of chronic stress for 3 weeks in mice, Zhu et al. (2017) observed a reduction of GABAergic activity in this nucleus in stressed mice (Zhu et al., 2017). In our case, the presence of anhedonia in the UCMs could suggest a GABAergic alteration of this region. However, this group presented only a reduction of GR receptors with no alteration of PV+ density. Adult male rats subjected to 6 weeks of chronic mild stress presented an anhedonia response, which is associated with hypertrophy in GABAergic neurons without neuronal number alteration (Bessa et al., 2013). In our case, there were no changes in the density of PV+ , but this does not directly imply an absence of functional impairment of these neurons. The functionality of the PV+ neurons in NAc could be altered without affecting their number.

Taking into account the behavioral consequences and neurobiological mechanisms related to continued stress exposure, new interventions such as EE have been proposed. This intervention would act by reducing the harmful effects of glucocorticoids on brain function, facilitating better neuroprotection (Queen et al., 2020). In fact, it is proposed that EE could act as prophylactically in preparation for future stressors, and as a means of minimizing the impact of previously experienced stressors (Smail et al., 2020). Given this, preserving, or maintaining GABAergic function would be necessary to promote resilience. Finally, in the case of studies with humans, activities such as meditation, mindfulness, cognitive reappraisal and aerobic exercise, and performing activities in green environments are believed to exert adaptive effects on emotion regulation by enhancing limbic and brainstem systems (Rojas-Carvajal et al., 2022). All this improves individuals' health. Therefore, both studies in animal models and in humans support the idea that these interventions could be an additional complement to pharmacological treatments. Its cost is low and its implementation simple. In addition, it is possible to implement these programs in people's daily lives, favoring greater resilience of people in the face of adversity.

Finally, authors such as Rojas-Carvajal et al. (2022) analyze the influence of different environmental conditions on the well-being of subjects. Although the group of 2–3 animals per cage has been considered as a standard and control group, it is observed that this group shows some deficits that prevent its optimal development. Therefore, it is considered that studies on the effects of EE should include a new control group. This group would be formed by 4–6 subjects, in addition to including objects and material that allow the animals to play and hide. All this, to create a more natural and adequate environment to promote the well-being of the subjects. Therefore, a limitation of our work is that the group considered control was formed by 4 subjects, but they did not have these objects and therefore lacked the environmental conditions that will reflect a semi-natural situation. The inclusion of a control group with the above environmental conditions should be considered in future research.

It should be mentioned that it would be necessary to evaluate whether the effects on GABAergic activity in the dorsal hippocampus are maintained over time in adulthood. In this case, the reduction of inhibitory Hippocampal activity by chronic stress is linked to mitochondrial dysfunction and increase in oxidative stress, impairs the function of PV-positive interneurons. This damage not only induces increased vulnerability to depressive and anxiety disorders, but also to the development of neurodegenerative diseases.

5. Conclusions

The current study shows that the environmental enrichment (EE) manipulation prior to the administration of the stress treatment (UCMS) alleviates the negative impact of stressors on hedonic responses and anxiety, promoting a greater resilience. These effects are accompanied by a lower density of GR and greater PV+ activity, especially in the dorsal hippocampus in the EE+ UCMs.

CRediT authorship contribution statement

AB: Conceptualization, Methodology, Formal analysis, behavioral and brain protocols, manuscript preparation and funding; **CJ**: behavioral and quantification processes, data analysis. ML: Conceptualization, behavioral protocol, data analysis manuscript preparation and **RM:** brain analysis and data collection.

Declaration of Competing Interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

Data Availability

Data will be made available on request.

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