Effects of the combination of chronic unpredictable stress and environmental enrichment on anxiety-like behavior assessed using the elevated plus maze in Swiss male mice: Hypothalamus-Pituitary-Adrenal Axis-mediated mechanisms

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Highligths

Combination of CUS and EE may induce an anxiety-like behavior in the plus-maze test (EPM)

Metyrapone prevents anxiety-like behavior in EE mice exposed to CUS

CUS increases ACTH levels in EE mice

Metyrapone reverses the ACTH increase in EE-CUS mice

Metyrapone increases corticosterone levels in EE-CUS mice

Abstract

Environmental enrichment (EE) is a paradigm that offers the animal a plethora of stimuli, including physical, cognitive, sensory, and social enrichment. Exposure to EE can modulate both anxiety responses and plasma corticosterone. In this study, our objective was to explore how chronic unpredictable stress (CUS) impacts anxiety-related behaviors in male Swiss mice raised in EE conditions. Additionally, we investigated corticosterone and adrenocorticotropic hormone (ACTH) levels to assess the involvement of the hypothalamic-pituitary-adrenal (HPA) axis in mediating these responses. Mice were housed under either EE or standard housing conditions for 21 days. Afterward, they were exposed to 11 days of CUS while still reared in their distinct housing conditions, with half of the mice receiving daily pretreatment with the vehicle and the other half receiving daily metyrapone (MET) injections, an inhibitor of steroid synthesis, 30 mins before CUS exposure. Blood samples were obtained to assess plasma corticosterone and ACTH levels. The 11-day CUS protocol induced anxiety-like phenotype and elevated ACTH levels in EE mice. Chronic MET pretreatment prevented anxiety-like behavior in the EE-CUS groups, by mechanisms involving increased plasma corticosterone levels and decreased ACTH. These results suggest a role of the HPA axis in the mechanism underlying the anxiogenic phenotype induced by CUS in EE mice and shed light on the complex interplay between environmental factors, stress, and the HPA axis in anxiety regulation.

Keywords: environmental enrichment, stress, metyrapone, HPA axis, corticosterone

Introduction

Anxiety disorders are among the most prevalent psychiatric disorders globally, representing approximately 3.6 % of the global population according to World Health Organization (WHO, 2017). A study on the Global Burden of Diseases in 2019 further underscored their significance, ranking anxiety disorders among the top 25 leading causes of global health burden (GBD, 2019). Moreover, the year 2020 witnessed a significant surge in the prevalence of both

anxiety and major depressive disorders, primarily attributable to the far-reaching impact of the COVID-19 pandemic. Data reveals a striking uptick of 26% in anxiety disorders and a substantial 28% increase in major depressive disorders within the span of just one year (WHO, 2022).

Chronic repeated stressful situations are known risk factors triggering anxiety in rodents and humans (Hammels et al. 2015) leading to the development of neuropsychiatric disorders (see review by Marcolongo-Pereira et al. 2022). A common model used in rodents to simulate the variability of stressors encountered in daily life (construct validity) is the unpredictable chronic stress (CUS) model (Papp et al.1996; Santos-Rocha et al. 2018; Malta et al. 2021), which has been used to study behavioral and physiological stress responses, such as anxiety and corticosterone/cortisol (CORT) levels (McEwen, 2007; Santos-Rocha et al. 2018). Usually, the CUS model continuously raises the activity of the hypothalamic-pituitary-adrenal (HPA) axis, making it challenging for animals to adapt to aversive conditions (Willner et al. 1987; Franco et al. 2016). While most researchers employing the CUS model adhere to fundamental principles with regards to protocols, such as exposing animals to a variety of unpredictable stressors over a prolonged period, the exact nature and sequence of stressors employed can differ between research groups.In general, CUS is a well-accepted experimental model of stress-induced mood disorders (Monteiro et al. 2015), however, it does not always necessarily elicit anxiety-like behavior (Malta et al. 2021), which depends on multiple variables (Monteiro et al. 2015; Malta et al. 2021), such as genetic factors, duration, intensity, among others (Franklin et al., 2012; Monteiro et al., 2015).

The HPA axis comprises important components involved in the stress response. These include corticotropin-releasing hormone (CRH) neurons located in the paraventricular nucleus, which play a crucial role in initiating the stress response by triggering the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary. ACTH circulates in the bloodstream and acts on the adrenal cortex, stimulating the secretion of glucocorticoids. The primary glucocorticoid in rodents is corticosterone, which exerts feedback control by signaling back to the brain and pituitary, effectively shutting off the stress response (Rivier and Vale, 1981). Metyrapone, an inhibitor of glucocorticoid synthesis, is a valuable tool that can modulate the HPA axis activity by blocking the production of glucocorticoids

and is frequently used to assess the integrity of the HPA axis (Fiad et al. 1994; Schimmer and Parker, 1996).

Several rodent studies have demonstrated a relationship between elevated plasma corticosterone levels and the development of anxiety-like behaviors (Adams et al. 2003; Aguilar-Valles et al. 2005; Aisa et al. 2007; Heim et al. 2008). For example, Mitra and Sapolsky (2008) observed an anxiogenic effect in rats treated with corticosterone. These findings align with clinical studies that have established dysregulated hyperactivity of the HPA axis in patients diagnosed with anxiety disorders (Buchanan et al. 2004; Kukolja et al. 2008). Thus, both preclinical and human investigations suggest a positive correlation between stress and high plasma glucocorticoid levels (Alfarez et al. 2003; Choy et al. 2008). However, it is important to note that changes in anxiety phenotype and in glucocorticoid levels can vary depending on several factors such as type and duration of stressors, as well as the species under investigation (Taraborelli et al. 2011; Monteiro et al., 2015).

On the other hand, environmental enrichment (EE) is an experimental protocol employed in rodents that acts as a protective factor against stressful stimuli. EE entails a stimulating environment that exposes animals to inanimate objects with various colors and textures, such as exercise wheels, tunnels, and stairs besides social enrichment (Nithianantharajah and Hannan, 2006). EE can confer a resilient phenotype on rodents subjected to chronic stress, such as social defeat (Lehmann and Herkenham, 2011), maternal separation (Francis et al. 2002), or acute restraint stress (Novaes et al. 2017).

Most studies tend to emphasize the impact of CUS either before, in-between or after EE (Zeeni et al., 2015; Shen et al., 2019 Costa et al., 2021). Our objective in this study was to assess the effects stemming from the combination of two distinct stimuli: EE and CUS - an ethological model for investigating the consequences of psychological stressors (Willner, 2017a) - in Swiss mice, an outbred strain. We evaluated the impact of CUS in male mice reared in EE on anxiety-like behavior, corticosterone, and ACTH levels. The plus-maze test served as a behavioral measure to examine anxiety-like responses in this context. To gain a deeper understanding of the underlying mechanisms, we used metyrapone as a pretreatment, which acts as an inhibitor of corticosterone, to assess the functionality of the HPA axis.

MATERIAL AND METHODS

Experimental Animals

Seventy-four male Swiss mice were used from the Animal Facility of the Department of Pharmacology of the Institute of Biomedical Sciences at the University of São Paulo (ICB-USP). The animals arrived in the experimental facility on postnatal day (PND) 49, and were housed in polycarbonate cages in groups of 5 with water and feed *ad libitum* with controlled conditions of temperature (23 ± 2 ° C) and light (12-hour light/ dark cycle, with the start of the light phase at 7 AM). All care, maintenance and treatment procedures were approved by the Ethics Committee on the Use of Animals (CEUA) of the Institute of Biomedical Sciences of University of São Paulo (protocol CEUA nº 1186020719). Given the complexity and variability in the literature regarding the effects of estrous cycle stages on EPM performance in mice, we opted to limit our study to male mice to minimize potential confounding factors related to hormonal fluctuations associated with the estrous cycle (Lovick and Zangrossi, 2021).

Experimental Design

The experimental design is depicted in Fig. 1. The animals were acclimatized for two weeks in the experimental facility, before starting the experiments. Mice were randomly distributed into 8 groups: a) non-enriched (NE), non-stressed (NCUS), vehicle (VEH) (n = 7); b) NE, stressed (CUS), VEH (n = 8); c) NE, NCUS, metyrapone (MET) (n = 11); d) NE, CUS, MET (n = 11); e) enriched (EE), NCUS, VEH (n = 8); f) EE, CUS, VEH (n = 8); g) EE, NCUS, MET (n = 11); h) EE, CUS, MET (n = 10).

Figure 1. Experimental design

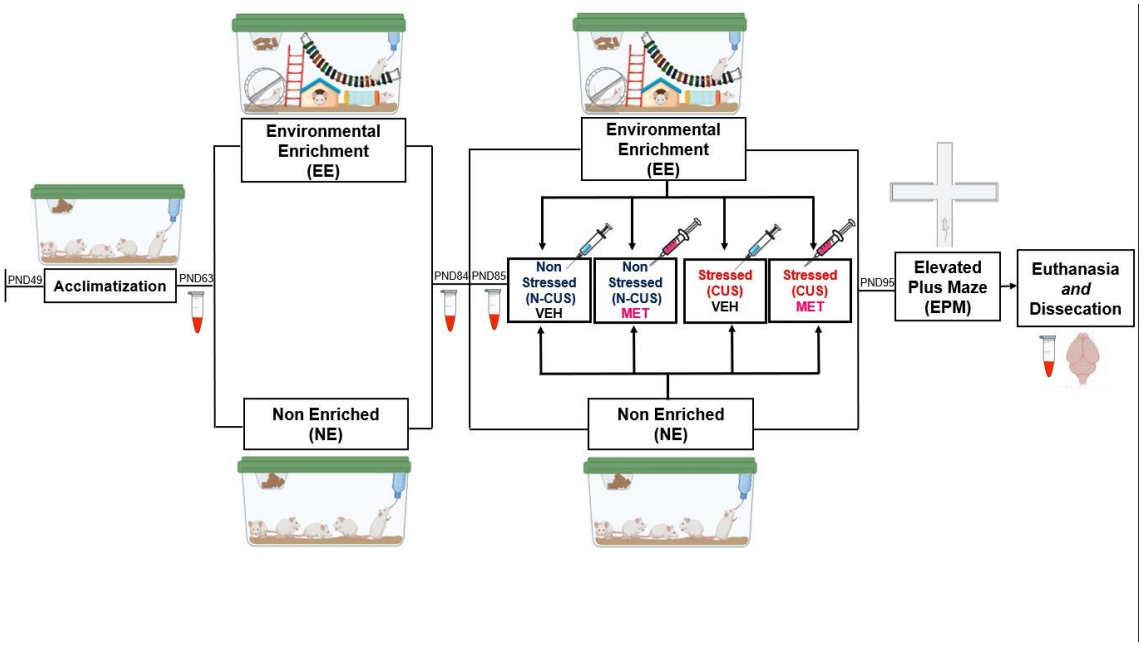

Fig 1. Experimental design illustrating the behavioral protocols in which mice were exposed to different conditions: EE (environmental enrichment), NE (non enriched), CUS (chronic unpredictable stress), NCUS (non stressed) and submitted to treatment with MET (metyrapone) or VEH (vehicle). EE and NE reared animals exposed or not to CUS were daily pretreated with vehicle (VEH) or metyrapone (MET) during the 11 days of stress period. There were 8 groups of animals. a) non-enriched (NE), non-stressed (NCUS), vehicle (VEH) (n = 7); b) NE, stressed (CUS), VEH (n = 8); c) NE, NCUS, metyrapone (MET) (n = 11); d) NE, CUS, MET (n = 11); e) enriched (EE), NCUS, VEH (n = 8); f) EE, CUS, VEH (n = 8); g) EE, NCUS, MET (n = 11); h) EE, CUS, MET (n = 10).The behavioral test to evaluate anxiety-like phenotypes is described as EPM (elevated plus maze). The red microtubes represent the collection of blood samples on days PND63, PND84, PND85 and PND95.

To prevent potential circadian disruptions caused by a large number of groups, the experiment was conducted in multiple runs. Each run included members from all groups, which resulted in different sample sizes for each group.

The animals in the EE and NE groups were exposed to the enriched environment or standard housing conditions, respectively for a total of 32 days, with the last 11 days overlapping with the CUS protocol in the corresponding groups. Control group did not receive CUS. Half of the animals were treated with 50 mg/kg MET and the other half with vehicle receiving the i.p. injections 30

minutes before each stress session during the 11-day CUS protocol. For the non stressed groups, MET or vehicle was injected at the same time points as the stress exposed groups. Twenty-four hours after the last stress session, all animals were tested in the EPM to assess behavioral parameters related to anxiety. The mice were euthanized 10 minutes after the elevated plus maze (EPM) test. Blood samples were collected from the trunk to measure corticosterone and ACTH levels.

Environmental enrichment (EE)

The EE included the provision of various objects in a large cage (42 cm long x 28 cm wide x 21.5 cm high), which were changed and cleaned twice a week following a predetermined protocol that defined the type and arrangement of the objects in the cage. The objects included a voluntary exercise wheel, stairs, plastic houses, hanging ropes, colorful tunnels of different sizes, ramps, and balls (Marianno, Abrahão and Camarini, 2017). The floor of the cage was covered with soft wood shavings and changed twice a week, always at 7 AM, just after the start of the light period. The animals were housed in groups of five to maintain consistent social interaction between the animals, similar to those in the standard cages, and to avoid any variations in behavior due to population size. For the control group of mice, standard cages were employed, measuring 27.5 cm in length, 16.5 cm in width, and 13 cm in height.

Chronic Unpredictable Stress (CUS)

Following 21 days of EE or NE housing from PND63 to PND84, mice were exposed to 11 days CUS protocol. The 11-day CUS protocol, which involved alternating different types of stress, was adapted from the protocol developed by Willner et al. (1987) and modified by Santos-Rocha et al. (2018) (Table 1). Twenty-four hours after the last stress session, animals were tested in the plus-maze. Blood was collected for analyses of corticosterone on PND63 (basal levels), PND84 (after 21 days of EE), PND85 (after the first stress) and PND95 (after euthanasia, on the following day after the last stress session) and for analyses of adrenocorticotrophic hormone (ACTH) on PND95. The details are found in the item **Euthanasia and Collection of Biological Material.**

Table 1. Chronic Unpredictable Stress protocol

Protocol Day	Daytime Stress	Overnight Stress
1	Physical Restraint (1h) 8-9am	-
2	-	Wet Bed (12h)
3	Cold Isolation (45min)	Overnight Illumination (12h)
4	Lights Off (3h) 8-11am Forced Swim (4min) 2pm	-
5	Wet Bed (12h)	Food and Water deprivation (12h)
6	Forced Swim (4min) 2pm	Isolation (12h)
7	Cold Isolation (15min) 8am Lights Off (2h) 2pm	-
8	-	Wet Bed (12h) Overnight Illumination (12h)
9	Isolation (12h) Food and Water Deprivation (12h)	-
10	Physical Restraint (1h) 2pm	Overnight Illumination (12h)
11	Physical Restraint (1h) 8-9am Forced Swim (4min) 12noon	-

Daytime: Light Cycle (7am-7pm); Dark Cycle (7pm-7am).

Elevated Plus Maze (EPM)

The EPM consists of an apparatus with four arms, two open arms (33.5 cm long x 7 cm wide), surrounded by a 0.5 cm high wall to prevent the animals

from falling; two closed ones (33.5 cm long x 7 cm wide, with 20 cm high walls), and a central platform (13.5 cm x 10 cm).

Before starting the test, the animals were acclimatized in an anteroom with adequate lighting (30 ± 5 lux) for 30 minutes. The apparatus was raised 50 cm from the floor in a room with appropriate lighting (Open Arm: 90 ± 5 Lux; Closed Arm: 10 ± 5 Lux; Center: 75 ± 5 Lux). Each mouse was placed on the central platform facing the open arm opposite to the experimenter and had its behavior recorded for 5 minutes. During the intervals between each tested animal, the apparatus was cleaned with 5% ethanol.

The test was conducted the following day, after the last stress session between 9am and 12 noon and was recorded by a video camera (Intelbras®) fixed above the maze. The videos were analyzed by a blind experimenter for group allocation, using a manual counting program (Plus MZ®). The criterion for considering entries in the compartment was when the four legs of the animal were inside it. The parameters analyzed were: total number of entries in the closed arms, percentage of entries in the open arms; and percentage of time spent in open arms.

Inhibition of Corticosterone Synthesis by Metyrapone

Metyrapone (MET; 2-Methyl-1,2-di-3-pyridyl-1-propanone; Sigma-Aldrich®) was administered daily, 30 minutes before each stress session (i.e., once or twice a day, depending on the protocol of the stress session). On the days when the animals were exposed to two types of stress, the interval between stress sessions was at least 3 hours, since this is the MET half-life. The dose used was 50 mg/kg, administered intraperitoneally (i.p.), based on a previous study (Perusine et al. 2016). The vehicle was prepared with 0.9% saline plus 5% ethanol. MET solution was initially diluted in 100% ethanol and subsequently diluted 20x in saline.

Euthanasia and Collection of Biological Material

The blood was collected at four different time points: at basal levels (before manipulations), after 21 days of EE/NE, and on the 22nd day, 5 minutes after the

end of the first stressor. The blood was collected between 9 and 10am. The first stressor (physical restraint) was conducted from 8 – 9am. On the 33rd day, 10 min after exposure to the EPM, the final collection was taken after decapitation (between 9am and 12 noon, on the following day after the last stressor). For the first three collections, 250µL of blood was collected from the tail using heparinized microtubes (500U/ml in 10% of the total blood collected) for corticosterone measurement. For the final collection, blood was collected from the trunk in heparinized tubes for measurements of corticosterone and ACTH levels. The tubes were centrifuged at 5000 g at 4ºC for 15 minutes to separate the plasma, which was stored in a freezer at -80ºC for subsequent quantifications of corticosterone and/or ACTH.

Plasma corticosterone levels - ELISA

For the quantification of plasma corticosterone levels, the Corticosterone EIA kit (Enzo Life Sciences, Inc, Farmingdale, NY) was used. Plasma samples were diluted 40 times in an assay buffer provided by the kit and the assays were run in duplicate, according to the manufacturer recommendations, and the results were averaged. The samples were thawed immediately prior to the analysis and kept on ice.

Adrenocorticotrophic Hormone (ACTH) levels - ELISA

For the quantification of plasma ACTH concentrations, we used the ACTH Extraction-Free EIA kit (Phoenix Pharmaceuticals, Inc., Burlingame, CA). The plasma samples were diluted 1: 1 in the dilution buffer provided by the kit and the assays were carried out in duplicate, according to the manufacturer recommendations, and the results were averaged.

Statistical analysis

All results are presented as mean ± standard error. Multifactorial analysis of variance (ANOVA) was employed to assess the contribution of each variable (environment/housing, stress/non stress exposure, treatment with metyrapone/vehicle), as well as the interactions among them. The anxiety

parameters determined in the plus-maze task (% open arm entries, % open arm time and closed arm entries, distance travelled, head dips) were analyzed by a three-way ANOVA that included the following factors: environment (non-enriched, NE or enriched, EE), exposure or not to stressful unpredictable conditions (stressed, CUS or non-stressed, NCUS) and treatment with vehicle or metyrapone (VEH or MET) (Fig. 2). An ANCOVA was conducted to examine the impact of the covariate "entries in closed arms" on the open arm entries of association between the grouping variable (EE, stress and MET variables) and the outcome variable. The analysis of ACTH levels was similar to those specified above. The speed data in the EPM were analyzed using the Kruskal-Wallis ANOVA by Ranks and Median Test because they did not meet the assumption of normality.

For the plasma corticosterone levels, first, the one-way ANOVAs were used to assess differences in the baseline corticosterone levels and the effects of exposure to environmental enrichment "per se" during the first phase of the experiment to check potential differences among the groups. As no differences were found, we pooled the results to increase the power of statistical analyses and performed an unpaired Student t-test to compare non-enriched and enriched groups (as illustrated in Fig. 3A). Our first aim was to evaluate whether 21 days of enrichment would change corticosterone levels, which was analyzed by a Student t-test (Fig. 3B). A four-way ANOVA with repeated measures on the factor of days was conducted to analyze the effects of EE, stress and treatment with metyrapone at different time points on corticosterone levels (environment X stress X treatment X time) with time as repeated measures (Fig. 4). The Levene test was used to test the homogeneity of variance and the normal distribution was tested with the Shapiro-Wilks test. When significant effects or interactions were observed, post-hoc comparisons were performed with Tukey HSD or Newman-Keuls for analysis of repeated measures. A $p < 0.05$ was considered statistically significant. The results were submitted to statistical analysis using the Statistica program, version 7.0. All main effects and interactions are described in Table 1 (supplementary material).

RESULTS

CUS exposure precipitates an anxiogenic phenotype in EE in the plus-maze test but not standard housed mice, an effect prevented by metyrapone pretreatment

The analysis of the percentage of entries in the open arms revealed a significant three-way interaction between environment, stress and treatment (Fig. 2A, $F_{1,66} = 5.64$; $p < 0.05$; $\eta^2p = 0.08$). Tukey's HSD post-hoc analyses revealed a near significant decrease in the percentage of entries in the open arms in enriched mice subjected to stress (EE-CUS-VEH) compared to non-enriched mice subjected to stress (NE-CUS-VEH) (trend observed, $p = 0.051$) as well as both non-enriched control groups (NE-NCUS-VEH, $p = 0.01$) and enriched non stressed control group (EE-NCUS-VEH, $p = 0.04$). This reduction was prevented by treatment with MET (EE-CUS-VEH significantly different from EE-CUS-MET, $p = 0.003$).

The analysis of the percentage of time spent in the open arms revealed a significant three-way interaction between environment, stress, and treatment (Fig. 2B, $F_{1,66} = 11.46$; $p < 0.01$; $\eta^2p = 0.15$). Tukey HSD test revealed that stress reduced the percentage of time spent in open arms in enriched mice (EE-CUS-VEH) compared to the non-enriched control (NE-NCUS-VEH, $p = 0.01$) and a trend was found related to enriched control (EE-NCUS-VEH, $p = 0.055$), indicating an anxiety-like phenotype. Treatment with MET significantly prevented this effect (EE-CUS-VEH differed from EE-CUS-MET, $p = 0.007$). EE-CUS-VEH also differed from their counterparts NE-CUS-VEH ($p = 0.002$). EE-NCUS-MET exhibited reduced time in the open arms compared to NE-NCUS-MET ($p = 0.03$) and EE-CUS-MET ($p = 0.016$).

Interestingly, CUS did not change the percentage of time or entries in the open arms in NE mice. Taken together, these results suggest an increase in anxiety levels in enriched mice exposed to CUS in the plus-maze test, which was reduced by treatment with the glucocorticoid synthesis inhibitor.

A three-way ANOVA on number of entries in the closed-arm indicated a significant environment X stress X treatment interaction (Fig. 2C, $F_{1,66} = 7.10$, $p < 0.05$; $\eta^2p = 0.09$). However, due to the greater sensitivity of ANOVA compared to pairwise test sensitivity, *post hoc* Tukey analysis did not find differences among groups. The analysis of the percentage of entries in the open arms by ANCOVA,

as closed-arm as covariate, showed that only the grouping variable “EE” demonstrated statistical significance ($p = 0.01$), suggesting that the covariate does not significantly adjust the association between the predictor and the outcome variable. No significant statistical differences were found for the other variables, i.e., entries in closed arms ($p = 0.65$), CUS ($p = 0.55$) or MET ($p = 0.52$). In other words, the results retain its significance in predicting the outcome even after accounting for the covariate.

Analysis of distance travelled in the EPM by a three-way ANOVA yielded a significant environment X stress X treatment interaction (Fig. S1, supplementary material; $F_{1,66} = 5.77$, $p < 0.05$; $\eta^2p = 0.08$). Pairwise comparisons did not find any differences between NE and respective EE groups. An interaction effect between environment X stress ($F_{1,66} = 9.28$, $p < 0.01$; $\eta^2p = 0.12$] indicated that stress increased the distance travelled of NE-CUS and EE-CUS groups compared to the NE-NCUS groups. Moreover, a stress X MET interaction ($F_{1,66} = 7.51$, $p < 0.01$; $\eta^2p = 0.102$; Fig.1, supplementary material) revealed that the MET groups (NCUS-MET) exhibited lower distance travelled compared to the NCUS-VEH, CUS-VEH and CUS-MET groups. These results suggest that MET and stress have contrasting effects on distance travelled.

The assessment of the animal mean speed (m/s) was conducted as it provides a more sensitive measure of alterations in locomotor activity compared to the entries in the closed entries (Dawson et al., 1995) (Fig. S2, supplementary material). Kruskal Wallis revealed significant differences among groups (chi-square = 33.893, df = 7, $p < 0.01$). Non-stressed NE and EE animals treated with MET exhibited a decreased mean speed in the maze. The observed differences were between NE-NCUS-MET and NE-CUS-VEH, as well as between EE-NCUS-MET and EE-NCUS-VEH.

Head dips were assessed as a measure of risk evaluation, representing an exploratory behavior where the animal scans the periphery of the maze, directing its gaze towards the floor (Shepherd et al., 1994). A 3-way ANOVA revealed a significant environment X stress X treatment interaction (Fig. S3, supplementary material; $F_{1,66} = 65.068$, $p < 0.01$; $\eta^2p = 0.49$). The EE group exposed to CUS and MET exhibited higher frequency of head dips than all the other groups (Tukey test).

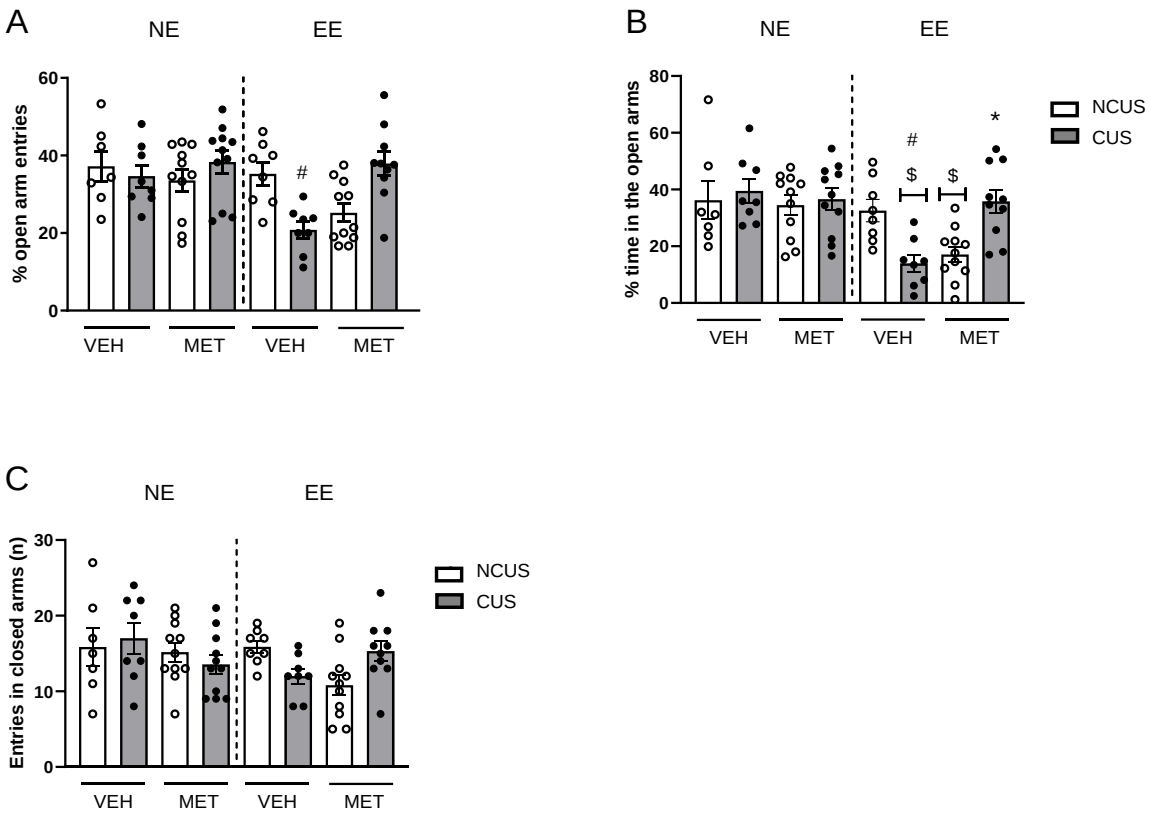

Figure 2. Effects of chronic unpredictable stress (CUS) exposure on anxiety-like behavior in mice reared in EE conditions and its modulation by metyrapone (MET). Anxiety-like behavior was measured using the elevated plus-maze (EPM). Mice were housed in either enriched (EE) or standard (NE) conditions for 21 days, followed or not (NCUS) by exposure to CUS for 11 days. During the stress protocol, mice received daily repeated injections of either vehicle (VEH) or metyrapone (MET). The data represent mean ± SEM. (A) Percentage of entries in open arms; (B) Percentage of time spent in open arms; (C) Total number of entries in the closed arms. The data were analyzed by three-way-ANOVA, followed by the Tukey HSD post-test. $^{\#}p < 0.05$, vs NE-NCUS-VEH, EE-NCUS-VEH and EE-CUS-MET; $^{\$}p < 0.05$ vs all 4 NE groups; $^{*}p < 0.05$ vs EE-NCUS-MET (n = 7-11/group). The arrow divides NE from EE mice.

Metyrapone pretreatment prevents acute stress-induced enhancement of corticosterone levels irrespective of housing conditions but increases corticosterone levels in EE reared mice exposed to CUS

For plasma corticosterone concentrations, the basal levels are shown in Fig.3A. After a period of twenty-one days of EE, plasma was collected to evaluate corticosterone levels (n = 37/group; total = 74; Fig. 3B). As there were only two groups at this stage (NE and EE) and the animals had not yet been exposed to CUS, we combined the samples and conducted a Student's t-test, which revealed

no significant differences between NE and EE groups (t= 1.25, df = 72, p = 0.21; t = 0.95, df = 72, p = 0.35; Figs. 3A and 3B, respectively), suggesting no differences in basal levels and that EE did not have a significant impact on corticosterone levels and unlikely to affect corticosterone responses following CUS.

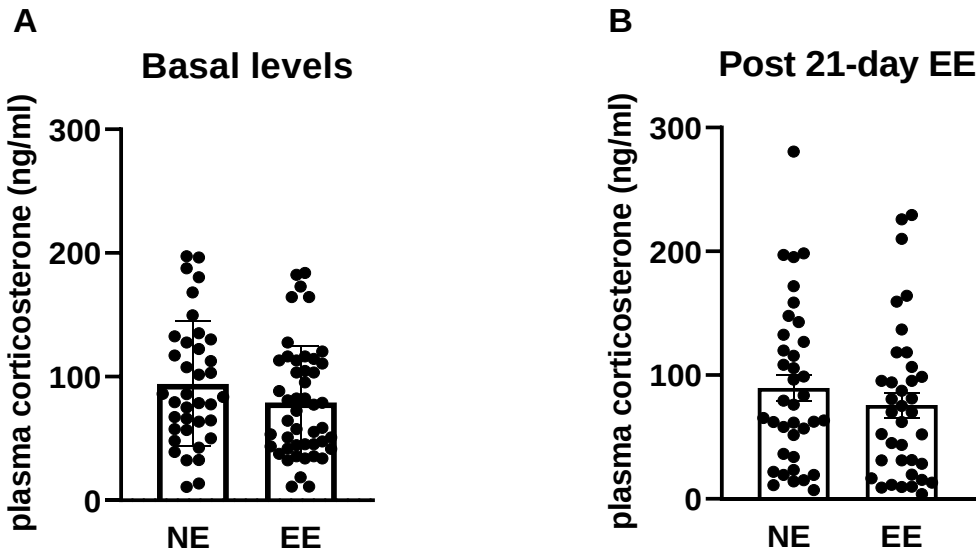

Figure 3. Effects of environmental enrichment (EE) on corticosterone levels in male Swiss mice. Blood samples were collected at baseline and after 21 days of EE or NE (non environmental enrichment). The data represent mean ± SEM. A) Basal plasma corticosterone levels B) Plasma corticosterone levels in mice exposed to EE or NE for 21 days (n = 37/group).

Plasma corticosterone levels were also assessed on the 22nd day (PND85), 5 minutes after the conclusion of the initial stressor (restraint stress), and on the 33rd day (on the following day after last stress, PND95), after the EPM. We aimed to investigate the impact of MET on corticosterone levels following acute stress and CUS. The corticosterone levels from days 22 and 33 were analyzed by a 4-way ANOVA analysis for repeated measures.

We conducted a four-way multifactorial ANOVA to assess hormone level variations over time and within each day of test, including responses to acute and chronic stress, as well as the effect of MET pre-treatment in both EE and NE mice

(Fig. 4). On the day 22, 1 sample is missing (n = 73) and on the day 33, 4 samples are missing (n = 70), due to insufficient sample volume or technical issues. Several main effects and interactions were found to reach significance (supplementary material), including a 4-way interaction between environment, stress, treatment, and time [$F_{1,62} = 5.97$; $p < 0.05$; $\eta^2p = 0.09$]. Newman-Keuls *post-hoc* test revealed that repeated exposure to stress resulted in decreased corticosterone levels in the NE-CUS-VEH ($p = 0.0001$) and EE-CUS-VEH groups ($p = 0.001$). Exposure to EE in combination with stress and MET (EE-CUS-MET, $p = 0.0001$) increased corticosterone levels (see left vs right panels) across days, suggesting a hyperactivation of HPA axis following chronic stress only in that specific group.

Newman-Keuls test also showed that exposure to the first stress (acute, restriction) resulted in significant increase in corticosterone levels in both environmental conditions (NE-CUS-VEH vs NE-NCUS-VEH, $p = 0.0002$; EE-CUS-VEH vs NE-NCUS-VEH, $p = 0.0001$ and vs EE-NCUS-VEH, $p = 0.0001$; Fig 4A). MET treatment was effective in decreasing the corticosterone levels in both NE-CUS and EE-CUS mice ($p = 0.0001$ for both; Fig 4A). Interestingly, an acute MET injection increased corticosterone levels in enriched mice, as compared to their NCUS vehicle controls (EE-NCUS-MET vs EE-NCUS-VEH, $p = 0.001$; Fig 4A).

Combination of EE and stress increased corticosterone levels compared to the respective NE groups, i.e., EE-CUS-VEH and EE-CUS-MET compared with NE-CUS-VEH ($p = 0.004$) and NE-CUS-MET ($p = 0.000$), respectively. Moreover, the EE group exposed to CUS and MET exhibited higher corticosterone levels than all the other groups.

Taken together, although repeated exposure to stress has led to decreased corticosterone levels in both the NE and EE compared to acute stress, in general, the exposure to EE resulted in HPA hyperactivity in those mice exposed to stimuli (CUS and MET) compared to the respective NE groups.

The average percentage coefficient of variation (%CV) between duplicate measurements of corticosterone levels was calculated for ELISA analysis. Inter-assay variability was assessed to determine the consistency across different assay runs (please find this information in the supplementary material).

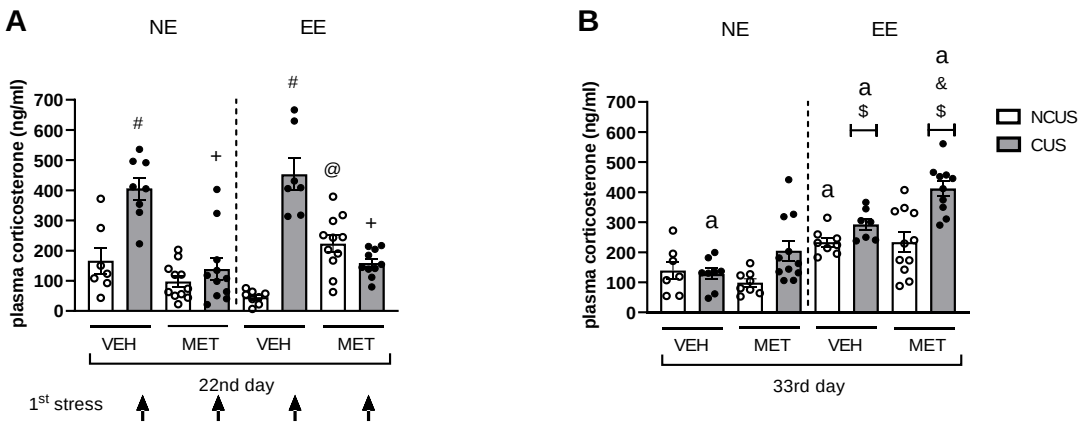

Figure 4. Effects of chronic unpredictable stress (CUS) on corticosterone levels in mice reared in EE conditions and its modulation by metyrapone (MET) prestress pretreatment. Mice were housed in either enriched (EE) or standard (NE) conditions for 21 days, followed or not (NCUS) by exposure to CUS for 11 days. During the stress protocol, mice received either vehicle (VEH) or MET. The data represent mean ± SEM. The Figure 4A presents the corticosterone levels of mice after 22 days of EE or NE, 5 minutes after the first stress exposure; and Figure 4B, corticosterone levels after 33 days of EE or NE (including 11 days of CUS). The data were analyzed using a 4-way ANOVA for repeated measures, followed by Newman-Keuls *post-hoc* test. $^{a}p < 0.05$, represents differences within the same groups (22nd vs 33rd day); $^{\#}p < 0.05$, compared to the respective controls (NCUS-VEH) within the same day; $^{+}p < 0.05$, compared to the respective CUS-VEH group within the same day; $^{@}p < 0.05$, compared to the respective control (EE-NCUS-VEH) within the same day; $^{\$}p < 0.05$, compared to the respective NE groups, within the same day; $^{\&}p < 0.05$, compared to all other groups, within the same day (n = 7-11/group). The arrow divides NE from EE mice.

CUS increases ACTH levels in EE but not standard housed mice, an effect which is reversed by metyrapone pretreatment

The ACTH levels were measured on the 33rd day, on the following day after the last stressor. Due to an insufficient amount of blood, the number of samples had to be reduced in certain groups (n=7-8/group). A three-way ANOVA yielded a significant environment X stress X treatment interaction (Fig. 5, $F_{1,55} = 9.32$, $p < 0.01$; $\eta^2p = 0.14$). *Post hoc* test revealed an increase in ACTH levels in EE mice exposed to CUS (EE-CUS-VEH) compared to all of the other groups, which was reversed by MET.

The average percentage coefficient of variation (%CV) between duplicate measurements of ACTH levels was calculated for ELISA analysis. Inter-assay variability was assessed to determine the consistency across different assay runs (please find this information in the supplementary material).

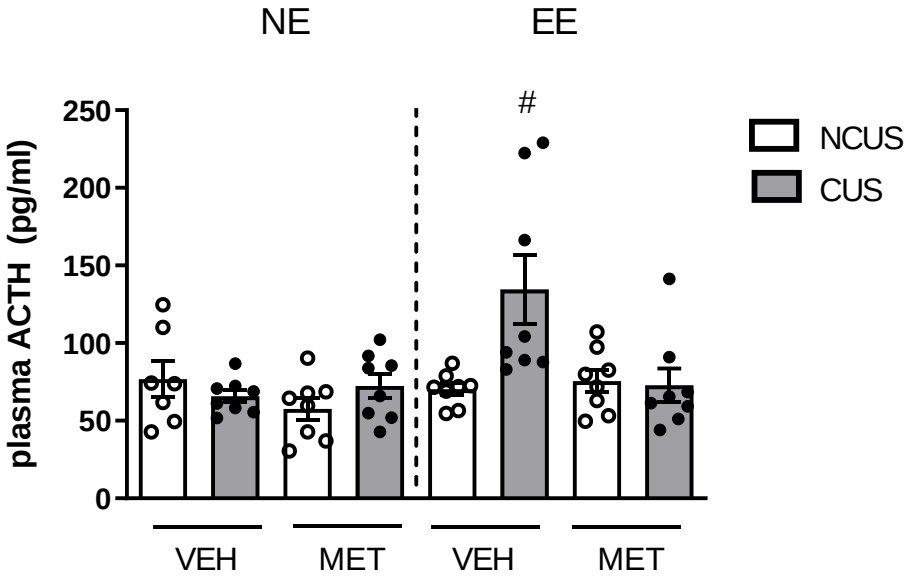

Figure 5. Effects of environmental enrichment (EE) on adrenocorticotrophic hormone (ACTH) levels in mice following exposure to chronic unpredictable stress (CUS) or non-stressed (NCUS) conditions. Mice were housed in either enriched (EE) or standard (NE) conditions for 21 days, followed by exposure to CUS or non-stress (NCUS) conditions for 11 days. During the stress protocol, mice received either vehicle (VEH) or metyrapone (MET). Data was analyzed by three-way ANOVA, followed by the Tukey post-test. Values expressed as mean ± SEM, with $^{\#}p < 0.05$ compared to all the other groups. (n = 7-8/group). The arrow divides NE from EE mice.

DISCUSSION

The main findings of this study revealed a precipitation of an anxiety-like phenotype in EE mice exposed to CUS, which was effectively prevented by daily pretreatment with MET.

An important aspect to be considered in this discussion is the interplay between EE and CUS, considering Crofton et al. (2015) “inoculation stress”

theory of EE and its impact on stress resilience. In light of this theory, the overlapping of a mild stressor (EE) with CUS may have promoted a state of stress overload. It is known that EE may promote resilience to acute stress (Novaes et al., 2017) or predictable chronic stress (Zanca et al., 2015), but when combined with chronic unpredictable stressors, it appears to lower the stress threshold, making it harder for the animal to cope with the stress. In this context, it is interesting to note that EE blocked the rewarding effects of cocaine, but when EE animals were transferred to an NE environment, they exhibited an exacerbated response to the conditioned preference to cocaine (Nader et al., 2012), indicating that EE animals may be vulnerable to environmental changes. At first, our hypothesis was that EE would prevent anxiety-like behavior induced by stress, as evidenced by studies conducted by our laboratory and others (Chapillon et al., 1999; Francis et al., 2002; Schrijver et al., 2002; Lehmann et al., 2011; Peña et al., 2009; Marianno et al., 2017; Rae et al., 2018; Novaes et al., 2017). However, previous research findings regarding this hypothesis have not been consistently aligned (Pietropaolo et al., 2014). EE housing paradigms are typically linked to favorable outcomes, but it is important to acknowledge that negative and neutral results have also been documented. These findings highlight the need for cautious evaluation of the limitations and potential drawbacks when implementing EE as a model of stress resilience. For instance, there have been reports of heightened aggression in male rodents (Haemisch et al., 1994; Loo et al., 2002).

Moreover, EE appears to influence novelty preference and may increase anxiety, as highlighted by Dickson and Mittleman (2021), suggesting that exposure to novelty may induce a mild level of stress. This observation offers a potential explanation for the anxiety-enhancing effect observed in EE mice subjected to CUS during the plus-maze test. Given their upbringing in an enriched environment, EE mice may perceive the CUS-induced novel environment as aversive, potentially contributing to the gradual development of anxiety. Consequently, when exposed to the plus-maze test, this unfamiliar environment might evoke heightened aversion in EE mice. Conversely, standard-housed animals exposed to CUS encountered a new environment that is less dissimilar to their familiar cage conditions compared to EE-reared counterparts. Consequently, these standard-housed animals exhibit less aversion to the novel

environment, as it aligns more closely with their regular living conditions. Studies have highlighted a connection between individual variations in novelty reactivity and differences in the activity of the HPA axis. In our study, CUS seems to induce a desensitization in the HPA axis, as further explained below. Interestingly, it was observed elevated corticosterone levels in individuals identified as lower responders to novelty in the open-field test (Cavigelli et al., 2007). Thus, it is important to note that neurobiological or behavioral changes induced by CUS or EE also rely on genetics and individual susceptibility to stressors (Castro et al., 2012; Nasca et al., 2015; Dickson and Mittleman, 2021).

The impact of EE on anxiety can vary depending on several factors, including the specific experimental design, duration and intensity of the enrichment and stress, species or strain of animals, and individual differences in response to environmental stimuli. Interestingly, studies examining the effects of EE on stress-induced anxiety, such as maternal separation (Francis et al., 2002) and social defeat (Lehmann and Herkenham, 2011), have found differential effects depending on the testing apparatus used, with alternative apparatuses like the open field or light-dark box showing effects, while the classic plus-maze test did not demonstrate significant effects despite its widespread use for anxiety measurement. It's essential to acknowledge that this discrepancy in the effectiveness of different testing apparatuses could be a potential limitation in our study. Moreover, although EE was able to mitigate the impact of maternal separation on anxiety-like response, it did not reverse the effects on CRF gene expression (Francis et al., 2002). As discussed in Hendershott et al. (2016), the observed anxiolytic behavior on the elevated plus maze test in the EE group may be attributed to alterations in activity rather than a reduction in anxiety-like behavior; however, it is worth noting that we did not find differences in locomotor activity in the open arm entries in the EE control group (EE-NS-VEH) as compared to the NE control, neither we observed significant differences in entries into the closed arms in any group. Thus, the heightened anxiety observed in EE mice exposed to CUS in not likely linked to motor alterations. It is important to emphasize that our study focused on assessing anxiety in EE-reared mice exposed to CUS. It is worth noting that the behavioral outcomes could differ if our aim were to evaluate anxiety in CUS mice exposed to EE. It is plausible to

hypothesize that the transition from an EE to a stressful one may exert a greater anxiogenic effect in the EE + CUS group. The findings indicate that prior exposure to EE does not confer resilience in the context of CUS.

In line with the findings of Malta et al. (2022), our study did not find anxiety-like behavior in animals exposed to CUS, despite previous studies reporting such an anxiety-like phenotype (Ortolani et al., 2014; Chaby et al., 2015; Monteiro et al., 2015). This discrepancy might be attributed to variations in the duration and intensity of the CUS protocol, as shorter-term exposure may not consistently elicit an increase in anxiety-like behavior (Vyas et al., 2002; Mitra et al., 2005; Matuszewich et al., 2007). The point at which stress responses shift from being adaptive to detrimental is influenced by a range of factors, including neuroendocrine, neurochemical, and genetic elements, which collectively contribute to individual variability in stress perception and response. In fact, Monteiro et al. (2015) reported that C57BL/6 mice displayed differences in anxiety-like behavior only after 8 weeks of CUS exposure. Additionally, Henry et al. (2006) found that this behavior was specifically induced under high-stress conditions but not in lower-stress environments. It is possible that our NE animals did not exhibit an anxiety response due to the relatively limited extent and intensity of stress they were exposed. In this regard, the EPM test may not possess sufficient sensitivity to detect anxious behavior in animals exposed to multiple stressors. In designing our study, we chose to implement stress protocols that fall within the category of mild stress. When employing more robust paradigms, such as daily restraint stress for several hours, defeat, and inescapable aggression, which are recognized as more potent stressors (Franklin et al., 2012), observable signs of anxiety-like behavior become evident. In a study comparing different protocols used across various laboratories, Willner (2017b) reported problems with the reproducibility of depression indices following the CUS protocol. These methodological discrepancies may stem from factors such as individual differences, age, sex, variations in protocols, and specific tests employed (Willner et al., 1987). It is important to note that our primary objective was to investigate the behavioral response of EE animals exposed to CUS, rather than the inherent anxiogenic effects of the CUS protocol itself.

In the present study, EE alone did not alter anxiety level, which is consistent with reports by Costa and Pimentel (2017), nor did it alter the

corticosterone levels. However, the combination of factors affecting the HPA axis, such as EE combined with CUS or EE combined with MET, increased susceptibility to an anxiogenic phenotype. However, administering MET before each stressor (EE-CUS-MET) effectively prevented the expression of anxiety, which is consistent with findings by Calvo et al. (1998). In line with Conrad, Mauldin-Jordain, and Hobbs (2001), rats injected with MET, regardless of stress condition, exhibited reduced freezing behavior to the tone during the testing day of a fear-conditioning tests using foot shock compared to their baseline levels observed during the training day. While MET has been shown to affect locomotion in rats at high doses, such as 150mg/kg (Canini et al., 2009; Drouet et al., 2011), it is important to note that in the current study, MET administered at 50mg/kg did not alter either the number of entries in the closed arms. This finding is consistent with results from other studies in mice (Calvo et al., 1988; Lorivel et al., 2010), suggesting that the behavioral effects of MET was not attributed to changes in exploratory activity. The number of number of entries into closed arms is considered as a more pertinent index of activity than open arms entries (Cruz et al., 1995). However, it is important to emphasize that while there were no observed alterations in number of entries into closed arms among all groups, we found a reduction in distance travelled and speed in MET groups (supplementary material), regardless of the environment, which could be a confounding factor.

The combination of EE-CUS-MET increased the frequency of head dips in the EPM (supplementary material). This effect aligns with effects typically observed with benzodiazepines, which not only increase the percentage of time spent in the open arms but also augments the occurrence of head dipping (Shepherd et al., 1994). Chemical inactivation of the amygdala induced an anxiolytic effect in mice, characterized by increased time spent in the distal areas of the open arms and total head-dips (Sorregotti et al., 2018). Thus, our findings suggest that MET can prevent anxiety-like behaviors.

In our model, 22 days of EE alone did not affect plasma corticosterone levels, but comparisons between 22 and 33 days of EE (EE-NCUS-VEH) showed a gradual increase in corticosterone levels. Studies have shown no alterations (Leal-Galicia et al., 2007; Prendergast and Bardo, 2011) higher (Marashi et al., 2003; Moncek et al., 2004) or lower (Konkle et al., 2010; Grégoire et al., 2014) corticosterone levels in EE mice compared to controls. The adaptation of plasma

corticosterone response to various stimuli may be attributed to a mechanism that involve feedback inhibition caused by elevated circulating corticosterone levels. The gradual elevation of corticosterone levels in EE may play a crucial role in programming changes observed in the social domain following stress.

After being exposed to the first stressor (a restraint stress), corticosterone levels increased in both NE and EE groups, which is consistent with literature findings (Sarnyai, Shaham, and Heinrichs, 2001). Since the activation of the HPA axis is a normal response to acute stress (Herman et al., 2003), EE did not prevent the expected increase in stress-induced corticosterone rise. Administration of MET before the stressor prevented this increase in both EE and NE mice, indicating that there was indeed pharmacological inhibition of corticosterone synthesis and a subsequent reduction in plasma concentrations, regardless the environment. In fact, MET suppressed plasma corticosterone levels in animals exposed to acute restraint stress (Moldow et al., 2001) or repeated social defeated stress (Niraula et al., 2018). The finding supports the idea that MET could be a potent regulator of the HPA axis in response to acute stress, regardless of the environmental context.

It is worth noting that both EE groups exposed to 11-day CUS (EE-CUS-VEH and EE-CUS-MET) showed elevated corticosterone levels compared to the corresponding NE groups, indicating a dissociation between the anxiety phenotype and plasma corticosterone concentration. Notably, previous research by Mesa-Gresa et al., (2016) similarly demonstrated that the concurrent experience of chronic social stress and EE resulted in heightened corticosterone levels compared to EE group (Mesa-Gresa et al., 2016), emphasizing the potentiation of plasma corticosterone levels when chronic stress and EE co-occur. Furthermore, repeated administration of MET increased plasma corticosterone levels in EE mice exposed to CUS, which is particularly interesting given the lack of chronic effects of MET in NE mice. While MET has prevented the acute corticosterone response to stress, elevated basal plasma corticosterone concentrations appeared after 11 days of stress only in EE mice (EE-CUS-MET), indicating persistent activation of the hypothalamic-pituitary-adrenal axis in this group.

We hypothesize that the frequent and chronic suppression of negative feedback on the hypothalamus and pituitary during CUS sessions by MET may

have induced a rebound effect and hyper-activated the HPA axis in EE mice, as result of a neuroplasticity of the HPA axis. It also seems that corticosterone response to acute stress and its feedback are not necessary for the HPA axis to remain activated in EE mice. Notably, Kennedy et al. (2020) found that chronic administration of MET increased mineralocorticoid receptor (MR) and glucocorticoid receptor (GR) binding to glucocorticoid response elements (GREs). The authors attributed this upregulation to an accumulation of the corticosterone precursor (11-deoxycorticosterone), which has a high affinity for MR.

A dissociation between corticosterone peaks and the enhancement of anxiety has already been described (Moss et al., 2023). In our study, MET seemed to cancel out the disturbing agent (stress) in favor of increased corticosterone synthesis and release.

We have also observed that repeated exposure to stress resulted in decreased corticosterone levels in both NE and EE groups compared to acute stress, suggesting a potential dysregulation of the HPA axis following chronic stress. The intricacy of the HPA axis response to stress seems to involve various factors, including the stressor nature, time intervals between exposures, and stress severity, all contributing to long-term desensitization or sensitization effects. Prolonged exposure to elevated corticosterone levels due to frequent stressors can lead to metabolic dysregulation associated with stress, as discussed below. As examples, in male rats, chronic stress sensitized the HPA axis, making it more responsive to stressors. This sensitization appeared unrelated to impaired rapid glucocorticoid feedback at the hypothalamus or pituitary level but rather stemmed from heightened synaptic excitability of paraventricular nucleus corticotropin-releasing hormone (CRH) neurons and increased CRH sensitivity of the pituitary (Franco et al., 2016). In addition, chronic corticosterone exposure has been linked to reduced cortical glucocorticoid receptors and blunted corticosterone responses to stressors, as seen in responses to swim stress and dexamethasone (Ago et al., 2008). This suggests that mice exposed to chronic corticosterone become less responsive to stressors over time. Furthermore, exposure to a single session of severe emotional or systemic stressors can induce long-lasting desensitization of the HPA response when subsequently exposed to the same homotypic stressor (Armario et al.,

2004). This long-term desensitization not only affects peripheral HPA hormones like ACTH and corticosterone but also extends to central components of the HPA axis, impacting gene expression in the paraventricular nucleus of the hypothalamus (PVN). Interestingly, these long-term stress-induced changes may not primarily originate in the PVN itself but rather in other brain nuclei exhibiting synaptic plasticity and potentially regulating the HPA axis and other physiological responses (Armario et al., 2004). However, it is important to emphasize that the corticosterone levels following CUS, on the 33rd day of experiment, represent the hormone levels measured on the following day after the final stressor. Studies in birds have also revealed a dampening of the stress response in the face of chronic stress, indicating that elevated corticosterone levels do not invariably accompany prolonged stress experiences (Rich and Romero, 2005).

Taking everything into consideration, the findings suggest that while repeated exposure to stress led to decreased corticosterone levels in both the non-enriched and enriched groups, the overall impact of EE resulted in hyperactivity of the HPA axis in mice exposed to multiple stimuli such as CUS or MET treatment. This indicates that the combination of EE and these stimuli elicited a heightened HPA axis response. Our results suggest that both behavioral and physiological responses are valuable indicators of stress; however, the behavioral phenotype cannot be solely relied upon as a direct surrogate for glucocorticoid levels.

The noteworthy finding here is the elevated ACTH concentration in the EE-CUS-VEH group, that was mitigated by repeated MET treatment. This raises the question of why ACTH increased, as it is expected that EE should reduce this hormone in response to stressors. Notably, studies on patients with post-traumatic stress disorder, such as Heim et al. (2000), indicate heightened ACTH levels, particularly in response to psychosocial stressors, as noted by Rasmusson et al. (2001). It's plausible that the combination of multiple stressors has led to a sustained sensitization of the HPA axis. The hormonal results suggest that the anxiogenic phenotype observed in EE mice exposed to CUS may be mediated through the HPA axis, as indicated by ACTH levels, although the role of corticosterone appears more intricate. For instance, an extensive review by Dickens and Romero (2013) indicates there is a lack of consensus when it comes to identifying chronically stressed animals based on endocrine profiles. While it

has been commonly assumed that measures of glucocorticoid function would increase in chronically stressed animals, the collected literature paints a more complex picture. Factors such as the specific stressors used to induce chronic stress, the potential for habituation to these stressors, and the species under study play crucial roles in shaping glucocorticoid responses.

The reduction in corticosterone levels in the EE-CUS-VEH after 11-day CUS may have interfered with the negative pituitary feedback loop, leading to an increase in ACTH levels. Conversely, the EE-CUS-MET group exhibited elevated corticosterone levels, potentially strengthening negative feedback and resulting in reduced ACTH concentrations. Interestingly, while repeated stress also decreased corticosterone levels in the NE mice, chronic MET treatment did not disrupt the HPA axis, as observed in the EE group. These findings suggest that mice exposed to EE may display a higher susceptibility to neuroplastic changes in the HPA axis compared to NE mice (Larsson et al., 2002; Mejier et al., 2007; Hutchinson et al., 2012). However, whether this response represents an attempt to counteract the anxiety-like state remains an unanswered question.

Limitations of the study

Previous research on the specific mechanisms under investigation has predominantly used male animal models, allowing for better comparability and alignment with existing literature. However, we recognize the significance of understanding potential sex differences and the necessity for comprehensive investigations that encompass both male and female subjects. This limitation has been explicitly addressed in the methods to emphasize the importance of extending future research to include a more balanced representation of both sexes.

Conclusions

In short, EE modifies the neuroendocrine regulation, making the HPA axis response more adaptive. The results obtained in this work raises the question: are EE prepared to exposure to chronic unpredictable stressors? Our findings demonstrate that exposure to CUS in mice reared in an enriched environment triggers a variety of behaviors related to anxiety in the EPM, and that the HPA axis may play a role in modulating this response. Additionally, our findings suggest that MET may represent a potential therapeutic option for anxiety disorders, even in the presence of high corticosterone levels.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Belo-Silva conducted the behavioral and biochemical experiments and contributed to the data analysis. Silva, Marianno, Costa and Da-Rovare assisted with the behavioral and biochemical experiments. Camarini, Novaes and Bailey designed the experiments, supervised the study, and participated in paper writing. Munhoz contributed to the data analysis and paper writing. Funding for this study was provided by grants #2018/05038-0 and #2021/04816-1, São Paulo Research Foundation (Fundação de Amparo à Pesquisa do Estado de São Paulo - FAPESP) to Camarini. Camarini and Munhoz are Research Fellows of National Council for Scientific and Technological Development (Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq). This study was also financed in part by the Coordination for the Improvement of Higher Education Personnel (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - CAPES) – offered to Belo-Silva. Figure 1 was created with BioRender.com

REFERENCES

Adams, F.; Grassie, M.; Shahid, M.; Hill, D.R.; Henry, B. 2003. Acut oral dexamethasone administration reduces levels of orphan GPCR glucocorticoid-induced receptor (GIR) mRNA in rodent brain. *Molecular Brain Reseasch,* 117(1):39-46.

Ago Y, Arikawa S, Yata M, Yano K, Abe M, Takuma K, Matsuda T. 2008. Antidepressant-like effects of the glucocorticoid receptor antagonist RU-43044 are associated with changes in prefrontal dopamine in mouse models of depression. Neuropharmacology. 55(8):1355-63.

Agrawal, A.; Jaggi, A. S.; Singh N. 2011. Pharmacological investigations on adaptation in rats subjected to cold water immersion stress. *Physiology & Behavior,* 103 (3-4): 321-9.

Aguillar-Valles, A.; Sanchez, E.; De Gortari, P.; Balderas, I.; Ramirez-Amaya, V.; Bermudez-Rattoni, F. 2005. Joseph-Bravo, P. Analysis of the stress response in rats trained in the water-maze: differential expression of corticotropin-release hormone, CRH-R1, glucocorticoids receptors and brain-derived neurotrophic factor in limbic regions. *Neuroendocrinology,* 82(5-6): 306-319.

Aisa, B.; Tordera, R.; Lasheras, B.; Del Rio, J.; Ramirez, M. J. 2007. Cognitive impairment associated to HPA axis hyperactivity after maternal separation in rats. *Psychoneuroendocrinology,* 32(3): 256-266.

Alfarez, D. N.; Joel, M.; Krugers, H.J. 2003. Chronic unpredictable stress impairs long-term potatiation in rat hippocampal CA1 area and dentate gyrus in vitro. *European Journal of Neuroscience,* 17(9): 1928-1934.

Armario, A.; Gavalda, A.; Marti, J. 1995. Comparison of the behavioural and endocrine response to forced swimming stress in five inbred strains of rats. *Psychoneuroendocrinology*, 20(8): 879-90.

Armario A, Vallès A, Dal-Zotto S, Márquez C, Belda X. 2004. A single exposure to severe stressors causes long-term desensitisation of the physiological response to the homotypic stressor. *Stress*. 7(3):157-72

Buchanan, T.W.; Kern, S.; Allen, J.S.; Tranel, D.; Kirshbaum, C. 2004. Circadian regulation of cortisol after hippocampal damage in humans. *Biological Psychiatry,* 56(9): 651-656.

Calvo, N.; Martijena, I.D.; Molina, V.A.; Volosin, M. 1998. Metyrapone pretreatment prevents the behavioral and neurochemical sequelae induced by stress. *Brain Research*, 800(2):227-35.

Canini, F., Brahimi, S., Drouet, J.-B., Michel, V., Alonso, A., Buguet, A., Cespuglio, R., 2009. Metyrapone decreases locomotion acutely. Neuroscience letters 457, 41-44.

Castro, J.E.; Diessler, S.; Varea, E.; Márquez, C.; Larsen, M.H.; Cordero, M.I.; Sandi, C. 2012 Personality traits in rats predict vulnerability and resilience to developing stress-induced depression-like behaviors, HPA axis hyper-reactivity and brain changes in pERK1/2 activity. *Psychoneuroendocrinology,* 37(8):1209-23.

Cavigelli, S.A.; Stine, M.M.; Kovacsics, C.; Jefferson, A.; Diep, M.N.; Barrett, C.E. (2007). Behavioral inhibition and glucocorticoid dynamics in a rodent model. *Physiology & Behavior*. 92(5):897-905

Chaby, L.E.; Cavigelli, S.A.; Hirrlinger, A.M.; Caruso, M.J.; Braithwaite, V.A. 2015. Chronic unpredictable stress during adolescence causes long-term anxiety. *Behavioural Brain Research*, *278*: 492-495.

Choy, K.H.; De Visser, Y.; Nichols, N.R.; Van Den, B.M. 2008. Combined neonatal stress and young-adult glucocorticoid stimulation in rats reduce BDNF expression in hippocampus: effect on learning and memory. *Hippocampus,* 18(7): 655-667.

Conrad, C.D.; Mauldin-Jourdain, M.L.; Hobbs, R.J. 2001. Metyrapone reveals that previous chronic stress differentially impairs hippocampal-dependent memory. *Stress*, 4(4): 305-318.

Costa, J.C.; Pimentel, B. N. 2017. Effects of environmental enrichment on behavioral and anxiety at *Status Epilepticus. ConScientiae Saúde,* 16(2): 249-258.

Costa, R.; Carvalho, M.S.M.; Brandão, J.D.P.; Moreira, R.P.; Cunha, T.S.; Casarini, D.E.; Marcondes, F.K. 2021. Modulatory action of environmental enrichment on hormonal and behavioral responses induced by chronic stress in rats: hypothalamic renin-angiotensin system components. *Behavioral. Brain Research* 397:112928

Crofton, E.J,; Zhang, Y.; Green, T.A. 2015. Inoculation stress hypothesis of environmental enrichment. *Neurosci Biobehav Rev*. 49:19-31

Cruz, A.P.; Frei, F.; Graeff, F.G. 1994. Ethopharmacological analysis of rat behavior on the elevated plus-maze. *Pharmacology, Biochemistry, and Behavior*.49:171-6.

Dawson, G.R., Crawford, S.P., Collinson, N., Iversen, S.D., Tricklebank, M.D. 1995. Evidence that the anxiolytic-like effects of chlordiazepoxide on the elevated plus maze are confounded by increases in locomotor activity. Psychopharmacology (Berl). 118:316-23.

Dickens, M.J., Romero, L.M. (2013). A consensus endocrine profile for chronically stressed wild animals does not exist. *Gen Comp Endocrinol*. 191:177-89.

Fiad, T.M., Kirby, J.M., Cunningham, S.K., McKenna, T.J. 1994. The overnight single-dose metyrapone test is a simple and reliable index of the hypothalamic–pituitary–adrenal axis [see comments]. *Clin Endocrinol* 40: 603–609.

Francis, D.D.; Diorio, J.; Plotsky, P.M.; Meaney, M.J. 2002. Environmental enrichment reverses the effects of maternal separation on stress reactivity. *Journal of Neuroscience*, *22*(18): 7840-7843.

Franco, A.J.; Chen, C.; Scullen, T.; Zsombok, A.; VEIahudeen, A.A.; Di, S.; Herman, J.P.; Tasker, J.G. 2016. Sensitization of the hypothalamic-pituitary-adrenal axis in a male rat chronic stress model. *Endocrinology*, *157*(6): 2346-2355.

Franklin, T.B.; Saab, B.J.; Mansuy, I.M. 2012. Neural mechanisms of stress resilience and vulnerability. *Neuron,* 75:747–61.

Global Burden Disease (GBD). 2020. Global burden of 369 diseases and injuries in 204 countries and territories, 1990–2019: a systematic analysis for the Global Burden of Disease Study, *Lancet*; 396: 1204-1222

Grégoire, C.A..; Bonenfant, D.; Le Nguyen, A.; Aumont, A.; Fernandes, K.J. 2014. Untangling the influences of voluntary running, environmental complexity, social housing and stress on adult hippocampal neurogenesis. *PloS one*, *9*(1).

Haemisch, A.; Voss, T.; Gärtner, K. 1994. Effects of environmental enrichment on aggressive behavior, dominance hierarchies, and endocrine states in male DBA/2J mice. *Physiology & Behavior*, *56*(5): 1041-1048.

Hammels, C., Pishva, E., De Vry, J., Van den Hove, DL., Prickaerts, J., Van Winkel, R. 2015. Defeat stress in rodents: from behavior to molecules, *Neurosci. Biobehav. Rev.,* 59: 111-140

Heim, C.; Newport, D.J.; Heit, S.; Graham, Y.P.; Wilcox, M.; BonVEIl, R.; Miller, A.H.; Nemeroff, C.B. 2000. Pituitary-adrenal and autonomic responses to stress in women after sexual and physical abuse in childhood. *Jama*, *284*(5): 592-597.

Heim, C.; Newport, D.J.; Mletzko, T.; Miller, A.H.; Nemeroff, C.B. 2008. The link between childhood trauma and depression: insights from the HPA axis studies in humans. *Psychoneuroendocrinology,* 33(6): 693-710.

Herman, J.P. 2013. Neural control of chronic stress adaptation. *Frontiers in Behavioral Neuroscience,* 7(61):1-12.

Herman, J.P. 2013. Role of prefrontal cortex glucocorticoid receptors in stress and emotion. *Biological Psychiatry,* 74(9): 672-9.

Hutchinson, K.M.; McLaughlin, K.J.; Wright, R.L.; Ortiz, J.B.; Anouti, D.P.; Mika, A.; Diamond, D.M.; Conrad, C.D. 2012. Environmental enrichment protects against the effects of chronic stress on cognitive and morphological measures of hippocampal integrity. *Neurobiology of Learning and Memory*, 97(2): 250-260.

Issa, A.M.; Rowe, W.; Gauthier, S. 1990. Meaney, M.J. Hypothalamicpituitary-adrenal activity in aged, cognitively impaired and cognitively unimpaired rats. *Journal of Neuroscience,* 10:3247 – 3254.

Kennedy, C.L.; Carter, S.D.; Mifsud, K.R.; Reul, J.M. 2020. Unexpected effects of metyrapone on corticosteroid receptor interaction with the genome and subsequent gene transcription in the hippocampus of male rats. *Journal of Neuroendocrinology*, *32*(2): 12820.

Konkle, A.; Kentner, A.C.; Baker, S.L.; Stewart, A.; Bielajew, C. 2010. Environmental-enrichment–related variations in behavioral, biochemical, and physiologic responses of Sprague–Dawley and Long Evans rats. *Journal of the American Association for Laboratory Animal Science*, 49(4): 427-436.

Kukolja, J.; Thiel, C.M.; Wolf, O.T.; Fink, G.R. 2008. Increased cortisol levels in cognitively challenging situations are beneficial in young but not older subjects. *Psychopharmacology*, *201*(2): 293-304.

Larsson, F., Winblad, B., Mohammed, AH. 2002. Psychological stress and environmental adaptation in enriched vs impoverished housed rats. *Pharmacol Biochem and Behav*, 73:193–207.

Leal-Galicia, P.; Saldívar-González, A.; Morimoto, S.; Arias, C. 2007. Exposure to environmental enrichment elicits differential hippocampal cell proliferation: role of individual responsiveness to anxiety. *Developmental Neurobiology*, 67(4): 395-405.

Lehmann, M.L.; Herkenham, M. 2011. Environmental enrichment confers stress resiliency to social defeat through an infralimbic cortex-dependent neuroanatomical pathway. *Journal of Neuroscience,* 31: 6159-73.

Lorivel, T., Gras, M., Hilber, P. 2010. Effects of corticosterone synthesis inhibitor metyrapone on anxiety-related behaviors in Lurcher mutant mice. *Physiology & Behavior*, 101(2):309-14.

Lovick, T.A., Zangrossi, H. Jr. 2021. Effect of Estrous Cycle on Behavior of Females in Rodent Tests of Anxiety. *Front Psychiatry*. 12:711065.

Malta, MB., Martins, J., Novaes, LS., Dos Santos, NB., Sita, L., Camarini, R., Scavone, C., Bittencourt, J., Munhoz, CD. 2022. Norepinephrine and Glucocorticoids Modulate Chronic Unpredictable Stress-Induced Increase in the Type 2 CRF and Glucocorticoid Receptors in Brain Structures Related to the HPA Axis Activation. *Mol Neurobiol,* 58(10):4871-4885. doi: 10.1007/s12035-021-02470-2.

Marashi, V.; Barnekow, A.; Ossendorf, E..; Sachser, N. 2003. Effects of different forms of environmental enrichment on behavioral, endocrinological, and immunological parameters in male mice. *Hormones and Behavior*, 43(2): 281-292.

Marcolongo-Pereira, C., Castro, FCAQ., Barcelos, RM., Chiepe, KCM., Rossoni Junior, JV., Ambrosio, RP., Chiarelli-Neto, O., Pesarico, AP. 2022. Neurobiological mechanisms of mood disorders: Stress vulnerability and resilience. *Front Behav Neurosci*, 28;16:1006836. doi: 10.3389/fnbeh.2022.1006836

Marianno, P.; Abrahao, K.P.; Camarini, R. 2017. Environmental enrichment blunts ethanol consumption after restraint stress in C57BL/6 mice. *PloS one*, *12*(1).

Matuszewich, L.; Karney, J.J.; Carter, S.R.; Janasik, S.P.; O'Brien, J.L.; Friedman, R.D. 2007. The delayed effects of chronic unpredictable stress on anxiety measures. *Physiology & Behavior*, 90(4): 674-681.

McEwen, B.S. 2007. Physiology and neurobiology of stress and adaptation: central role of the brain. *Physiological Reviews*, *87*(3): 873-904.

Mikics, É.; Barsy, B.; Barsvári, B.; Haller, J. 2005. Behavioral specificity of non-genomic glucocorticoid effects in rats: effects on risk assessment in the elevated plus-maze and the open-field. *Hormones and Behavior*, 48(2):152-162.

Mitra, R.; Jadhav ,S.; McEwen, B.S.; Vyas, A.; Chattarji, S. 2005. Stress duration modulates the spatiotemporal patterns of spine formation in the basolateral amygdala. *Proceedings of the National Academy of Sciences*, 102:9371–9376.

Mitra, R.; Sapolsky, R.M., 2008. Acute corticosterone treatment is sufficient to induce anxiety and amygdaloid dendritic hypertrophy. *Proceedings of the National Academy of Sciences*, *105*(14): 5573-5578.

Moncek, F.; Duncko, R.; Johansson, B.B.; Jezova D. 2004. Effect of environmental enrichment on stress related systems in rats. *Journal of Neuroendocrinol*, 16:423-31.

Monteiro, S.; Roque, S.; de Sá-Calçada, D.; Sousa, N.; Correia-Neves, M; Cerqueira, J.J. 2015. An efficient chronic unpredictable stress protocol to induce stress-related responses in C57BL/6 mice. *Frontiers in Psychiatry*, 6:6.

Moss, EM., Mahdi, F., Worth, CJ., Paris JJ. 2023. Physiological Corticosterone Attenuates gp120-Mediated Microglial Activation and Is Associated with Reduced Anxiety-Like Behavior in gp120-Expressing Mice. *Viruses* ;15(2):424. doi: 10.3390/v15020424.

Nader, J.; Claudia, C.; Rawas, R.E.; Favot, L.; Jaber, M.; Thiriet, N.; Solinas, M. 2012. Loss of environmental enrichment increases vulnerability to cocaine addiction. *Neuropsychopharmacology*, 37: 1579-1587.

Nasca, C.; Bigio, B.; Zelli, D.; Nicoletti, F.; McEwen, B.S. 2015. Mind the gap: glucocorticoids modulate hippocampal glutamate tone underlying individual differences in stress susceptibility. *Mol Psychiatry*. 20(6):755-63.

Niraula, A., Wang, Y., Godbout, JP., Sheridan, JF. 2018. Corticosterone Production during Repeated Social Defeat Causes Monocyte Mobilization from the Bone Marrow, Glucocorticoid Resistance, and Neurovascular Adhesion Molecule Expression. *J Neurosci*. 38(9):2328-2340. doi: 10.1523/JNEUROSCI.2568-17.2018.

Nithianantharajah, J.; Hannan, A.J. 2006. Enriched environments, experience-dependent plasticity and disorders of the nervous system. *Nature Reviews Neuroscience,* 7:697-709.

Novaes, L.S.; Dos Santos, N.B.; Batalhote, R.F.; Malta, M.B.; Camarini, R.; Scavone, C.; Munhoz, C.D. 2017. Environmental enrichment protects against stress-induced anxiety: Role of glucocorticoid receptor, ERK, and CREB signaling in the basolateral amygdala. *Neuropharmacology*, 113:457-466.

Ortolani, D.; Garcia, M.C.; Melo-Thomas, L.; Spadari-Bratfisch, R.C. 2014. Stress-induced endocrine response and anxiety: the effects of comfort food in rats. *Stress*, 17(3): 211-218.

Papp, M.; Moryl, C. 1996. Antidepressant-like effects of 1-aminocyclopropanecarboxylic acid and D-cycloserine in an animal model of depression. *European Journal of Pharmacology,* 316: 145-151.

Papp, M.; Moryl, E.; Willner, P. 1996. Pharmacological validation of the chronic mild stress model of depression. *European Journal of Pharmacology*, *296*(2): 129-136.

Peña, Y.; Prunell, M.; Rotllant, D.; Armario, A.; Escorihuela, R.M. 2009. Enduring effects of environmental enrichment from weaning to adulthood on pituitary-adrenal function, pre-pulse inhibition and learning in male and female rats. *Psychoneuroendocrinology*, 34(9):1390-1404.

Perusine, J.N.; Meyer, E.M.; Long, V.A.; Rau, V.; Nocera, N.; Avershal, J.; Maksymetz, J.; Spigelman, I.; Fanselow, M.S. 2016. Induction and Expression of Fear Sensitization Caused by Acute Traumatic Stress. *Neuropsychopharmacology*, 41(1):45-57.

Rae, M. B; Zanos, P.; Georgiou, P.; Chivers, P.; Bailey, A.; Camarini, R. 2018. Environmental enrichment enhances conditioned place preference to ethanol via an oxytocinergic-dependent mechanism in male mice. *Neuropharmacology*, 138:267-274.

Rasmusson, A.M.; Lipschitz, D.S.; Wang, S.; Hu, S.; Vojvoda, D.; Bremner, J.D.; Southwick, S.M.; Charney, D.S. 2001. Increased pituitary and adrenal reactivity in premenopauVEI women with posttraumatic stress disorder. *Biological Psychiatry*, 50(12): 965-977.

Reis, F.M.; Albrechet-Souza, L.; Franci, C.R.; Brandao, M.L. 2012. Risk assessment behaviors associated with corticosterone trigger the defense reaction to social isolation in rats: role of the anterior cingulate cortex. *Stress*, *15*(3):318-328.

Rich EL, Romero LM. 2005 Exposure to chronic stress downregulates corticosterone responses to acute stressors. *Am J Physiol Regul Integr Comp Physiol*. 288:R1628-36.

Rivier, C., Vale, W. 1983. Modulation of stress-induced ACTH release by corticotropin-releasing factor, catecholamines and vasopressin. *Nature.* 305:325–327.

Santos-Rocha, JB., Rae, M., Teixeira, AMA., Teixeira, SA., Munhoz, CD., Muscará, MN., Marcourakis, T., Szumlinski, KK., Camarini, R. 2018. Involvement of neuronal nitric oxide synthase in cross-sensitization between chronic unpredictable stress and ethanol in adolescent and adult mice. *Alcohol.* 68:71-79.

Sarnyai, Z.; Shaham, Y.; Heinrichs, S.C. 2001. The role of corticotropin-releasing factor in drug addiction. *Pharmacology Reviews,* 53 (2): 209-43.

Schimmer, B. P.; Parker, K. L. Hormônio Adrenocorticotrófico, esteróides adrenocorticais e seus análogos sintéticos, inibidores da síntese e das ações dos hormônios adrenocorticais. As Bases Farmacológicas da Terapêutica. 9ªed. Mc Graw Hill Interamericana, p. 1082-1102, 1996.

Schrijver, N.C.; Bahr, N.I.; Weiss, I.C.; Würbel H. 2002. Dissociable effects of isolation rearing and environmental enrichment on exploration, spatial learning and HPA activity in adult rats. *Pharmacology, Biochemistry & Behavior,* 73: 209-24.

Shen, J., Li, Y., Qu, C., Xu, L., Sun, H., Zhang, J. 2019. The enriched environment ameliorates chronic unpredictable mild stress-induced depressive-like behaviors and cognitive impairment by activating the SIRT1/miR-134 signaling pathway in hippocampus. *J. Affect. Disord*., 248: 81-90.

Shepherd, J.K., Grewal, S.S., Fletcher, A. et al. 1994. Behavioural and pharmacological characterisation of the elevated “zero-maze” as an animal model of anxiety. Psychopharmacology 116: 56–64.

Sorregotti, T., Cipriano, A.C., Cruz, F.C., Mascarenhas, D.C., Rodgers, R.J., Nunes-de-Souza, R.L. 2018. Amygdaloid involvement in the defensive behavior of mice exposed to the open elevated plus-maze. Behav Brain Res. 338:159-165.

Veenit, V., Cordero, MI., Tzanoulinou, S., Sandi, C. 2013. Increased corticosterone in peripubertal rats leads to long-lasting alterations in social exploration and aggression. *Front Behav Neurosci*. 4;7:26. doi: 10.3389/fnbeh.2013.00026.

Vogel, J. R.; Beer, B.; Clody, D. E. 1971. A simple and reliable conflict procedure for testing anti-anxiety agents. *Psychopharmacologia*, *21*(1):1-7.

Vyas, A.; Mitra, R.; Shankaranarayana, R.B.S.; Chattarji, S. 2002. Chronic stress induces contrasting patterns of dendritic remodeling in hippocampal and amygdaloid neurons. *Journal of Neuroscience,* 22: 6810-6818.

Willner, P. 2017a. The chronic mild stress (CMS) model of depression: History, evaluation and usage. Neurobiol. Stress, Vol. 6 (2017), pp. 78-93,

Willner, P. 2017b. Reliability of the chronic mild stress model of depression: a user survey. *Neurobiology of Stress,* 6: 68-77.

Willner, P.; Towell, A.; Sampsons, D.;Sophokleous, S.; Muscat, R. 1987. Reduction of sucrose preference by chronic unpredictable mild stress and its restoration by a tricyclic antidepressant. *Psychopharmacology,* 93:358-364.

World Health Organization. (2017). Depression and other common mental disorders: global health estimates. World Health Organization, Geneva.

World Health Organization. (2022). Mental Health and COVID-19: Early evidence of the pandemic's impact. World Health Organization, Geneva

Zanca, RM., Braren, SH., Maloney, B., Schrott, LM., Luine, VN., Serrano, PA. 2015. Environmental Enrichment Increases Glucocorticoid Receptors and Decreases GluA2 and Protein Kinase M Zeta (PKMζ) Trafficking During Chronic Stress: A Protective Mechanism? *Front Behav Neurosci*.;9:303. doi: 10.3389/fnbeh.2015.00303.

Zeeni, N., Bassil, M., Fromentin, G., Chaumontet, C., Darcel, N., Tome, D., Daher, CF. 2015. Environmental enrichment and cafeteria diet attenuate the response to chronic variable stress in rats. Physiol. Behav., 139 (2015), pp. 41-49.