

# Long-term Effects of Maternal Separation on Anxiety-Like Behavior and Neuroendocrine Parameters in Adult Balb/c Mice

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## Abstract

**Introduction:** Disruption of maternal care using maternal separation (MS) models has provided significant evidence of the deleterious long-term effects of early life stress. Several preclinical studies investigating MS showed multiple behavioral and biomolecular alterations. However, there is still conflicting results from MS studies, which represents a challenge for reliability and replicability of those findings. **Objective:** To address that, this study was conducted to investigate whether MS would affect anxiety-like behaviors using a battery of classical tasks, as well as central and peripheral stress-related biomarkers. **Methods:** Male Balb/c mice were exposed to MS from postnatal day (PND) 2 to 14 for 180-min per day. Two independent cohorts were performed to evaluate both baseline and anxiety-like behavior responses to MS at PND60. We performed composite scores to evaluate MS effects on anxiety and risk assessment phenotypes. Also, we assessed mRNA gene expression in the medial pre-frontal cortex (mPFC) of glucocorticoid and mineralocorticoid receptors (GR and MR) using real-time PCR and peripheral corticosterone levels (CORT) to investigate possible neurobiological correlates to anxiety behaviors. **Results:** We found increased anxiety-like behavior and decreased risk assessment and exploratory behaviors in MS mice. The animals exposed to MS also presented a decrease in MR mRNA expression and higher levels of CORT compared to controls. **Conclusions:** Our findings reinforce the body of evidence suggesting that long-term MS induces effects on anxiety and risk assessment phenotypes following the exposure to a standardized MS protocol. Moreover, MS affected the expression of MR mRNA and induced significant changes on CORT response. This data highlights that the reprogramming MS effects on HPA axis could be mediated by MR gene expression in mPFC and chronic overactivity of peripheral CORT levels.

## Keywords

early life stress, anxiety-like behavior, HPA axis, mPFC, neuroendocrine

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## Introduction

Exposure to adverse experiences during early postnatal period is considered an environmental risk factor for the development of multiple psychiatric conditions later in life, especially those involving mood and anxiety disorders.<sup>(1–3)</sup> Previous preclinical studies have reported that rodents exposed to early-life stress (ELS) paradigms present higher levels of depressive and anxiety-like behaviors, as well as increased fear and stress responsiveness.<sup>(4,5)</sup>

These ELS-induced behavioral alterations are known to be partially mediated by chronic activations of the hypothalamic-pituitary-adrenal (HPA) axis, especially through the regulatory role of glucocorticoid and mineralocorticoid

receptors (GR and MR respectively).<sup>(6,7)</sup> Both receptors are largely expressed in a range of brain regions that have modulatory roles over HPA axis functioning.<sup>(8–11)</sup> However, there are still numerous inconsistencies between preclinical studies,

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especially regarding the effectiveness of classical paradigms chosen to mimic postnatal stress effects on behavioral and biomolecular outcomes.<sup>(12–14)</sup>

Between all models of ELS in rodents, maternal separation (MS) is the well-known and most used protocol. MS is suggested to induce disruption of the dam-pup relationship, which could alter maternal care.<sup>(14)</sup> Recent systematic and meta-analytic reviews have argued that a significant number of studies have failed, or were underpowered, in the effort to replicate previous findings elicited by MS.<sup>(15–19)</sup> MS commonly evokes debates regarding the accurate procedures for postnatal separation and methodological issues that might interfere with the outcomes evaluated.<sup>(12)</sup> The absence of detailed information and appropriate description of each protocol used are crucial factors involved in both data reliability and replicability.<sup>(20)</sup>

Classical behavioral tasks commonly employed to assess anxiety phenotypes are the elevated plus maze (EPM), the light dark (LD) and the open field (OF) tests.<sup>(21–23)</sup> These tasks have been suggested as able to evaluate traditional parameters referred as anxiety-like behavior measures, as well as other complementary parameters that could reflect exploratory behaviors and risk assessment (RA).<sup>(4,24–29)</sup> Nevertheless, what is still observed is that a significant proportion of behavioral data is derived from the interpretation of one specific anxiety-like evaluation task, which could increase the risk of task-dependent results, since adequate anxiety-like assessment should derive from more than one single task evaluation.<sup>(30–34)</sup>

Considering the inconsistencies regarding MS protocols and outcomes evaluation, one of the goals of this study was to properly describe the MS protocol, and to evaluate the long-term effects of MS on anxiety-like behavioral phenotypes in a battery of classical behavioral tasks (OF, LD and EPM). In addition, it is known that the presence of anxiety-like phenotype in response to ELS in mice is related to alterations in corticosterone (CORT) plasmatic levels, as well as GR and MR expression.<sup>(8,10,12,35–37)</sup> Given the importance of establishing a relation between behavioral and neurobiological alterations, the present study also aimed to explore the effects of MS on GR and MR gene expression in the medial prefrontal cortex (mPFC), and plasmatic CORT levels of adult Balb/c mice.

## Methods

This study was conducted with male Balb/c mice ( $n = 34$ ) from the Center for Experimental Biological Models (CeMBE) at the Pontifical University of Rio Grande do Sul (PUCRS), Brazil. All animals were maintained in standard cages (22cm X 16cm X 14cm) under controlled temperature ( $21 \pm 1$  °C.), humidity ( $55 \pm 5\%$ ) and ventilation. The lighting conditions were maintained on a 12-h light-dark cycle (lights on at 6 AM and off at 6 PM). All animals received *ad*

*libitum* water and food (Nuvilab CR-1, Colombo, Paraná, Brasil). Cage cleaning procedures were weekly done.

Breeding procedures were carried out by placing two females and one male in the same cage for a period of 24h. The day in which pups were found was considered the postnatal day (PND) 1. Cross-fostering was performed, and litter control was performed when necessary, respecting the limit of 5 to 7 pups per dam. After cross-fostering, the dams were randomly assigned to different experimental conditions: animal facility rearing (AFR) or maternal separation (MS). AFR animals were left undisturbed from PND1 until weaning day. On PND21 all animals were weaned and housed by sex in groups of 2 to 3 animals per cage.

We performed two independent cohorts for this study, the first cohort was used to assess baseline neurobiological parameters and the second cohort was used to investigate the behavioral phenotype. The experimental designs are described in details in Figure 1. All the procedures were approved by the Ethics Committee on Animal Use (CEUA) of PUCRS (registration number #14/00421) and were conducted accordingly with the National Institute of Health (NIH) guidelines for laboratory animal use and care and the International Council for Laboratory Animal Science (ICLAS).

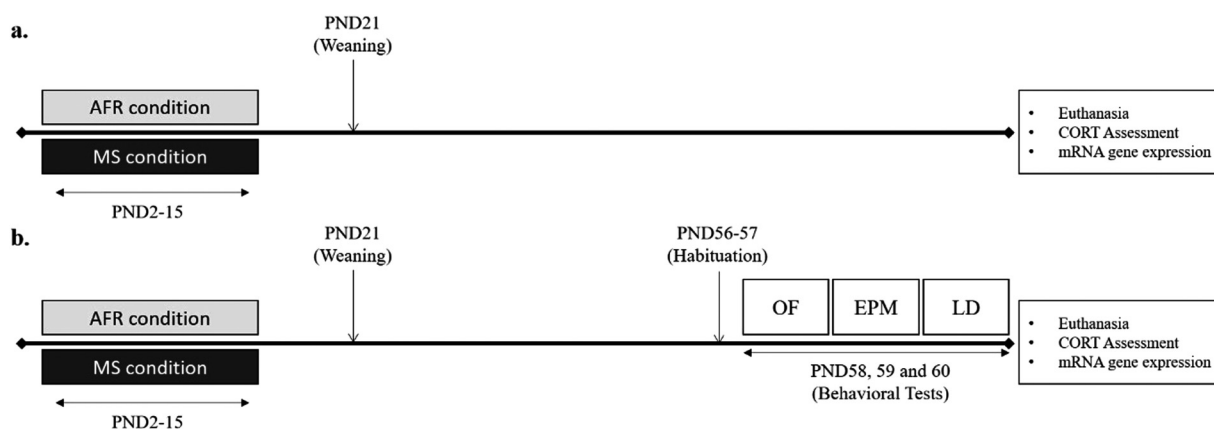
## Maternal Separation

The protocol consisted of daily separation for 180 min, during the first two weeks of life (PND2 to PND15), from 4 PM to 7 PM. The protocol followed previous standardized recommendation for MS models in mice<sup>(12)</sup> and is detailed in Figure 2. First, MS consisted on reallocating the dams into a similar cage and then transferring them to a different room in order to avoid any kind ultrasonic vocalization.<sup>(38–40)</sup>

Pups were carefully relocated individually into small transparent plastic box containing a small amount of clean bedding. This procedure induced an isolation and exposure to a new environment (without any sensory cues). All these small boxes were distributed over an electronic heating pad with temperature set between  $32$  °C  $\pm$   $2$  °C, in order to avoid any stress-induced hypothermia. After MS, the pups were returned to the cages followed by the dam. The AFR offspring were kept undisturbed, with the exception of cleaning procedures.

## Anxiety-like Behavioral Tasks

The behavioral tasks were performed in three consecutive days (between PND58 and PND60) with a 24h test interval, always from 6 PM onwards (dark phase of light cycle). Animals were habituated with the experimenter and with the experimentation room for 2 days before testing. The apparatus were cleaned with isopropyl alcohol between animals. All tasks were analyzed using tracking software AnyMaze (Stoelting CO, Wood Dale, IL, USA). Only



**Figure 1.** Experimental design for the two independent cohorts. (a) Baseline cohort, for evaluation of mRNA gene expression and CORT levels without behavioral task effect; (b) Anxiety cohort, for evaluation of anxiety phenotype and potential changes on mRNA gene expression and CORT levels. AFR = Animal Facility Rearing, MS = maternal separation, OF = Open Field Test, EPM = Elevated Plus Maze Test, LD = Light Dark Test.

animals that completed all tasks were included in behavioral analyses.

## Open-field Task

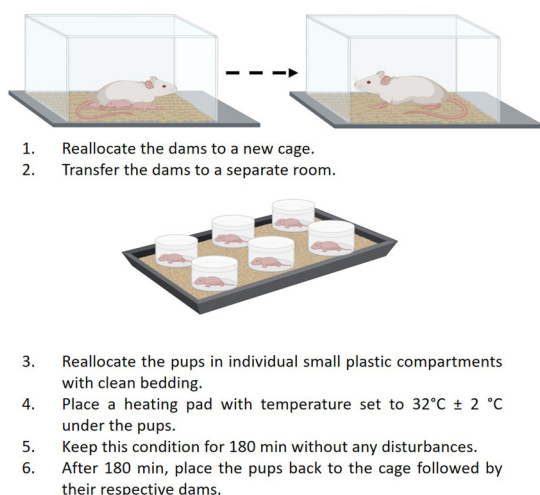
OF is a recognized and vastly used task to evaluate the exploratory activity and anxiety-like behavior in mice.<sup>(21,41)</sup> OF task consisted in placing the animal in the center of a square and transparent apparatus (33cm x 33cm wide x 30cm), leaving the mice free to explore it for a period of 20 min.<sup>(42)</sup> We used a longer duration of time for OF test to better explore possible behavioral changes across the time and reduce the effects of stress from exposure to a new environment for the first time,<sup>(43,44)</sup> considering that the OF was the first task to be performed. The intensity of the lighting in the experiment room was setup to 140 lux. Exploratory and anxiety-

like behavioral parameters were evaluated from the total distance travelled during the task, total time spent in the central zone of the apparatus, total number of rearing behavior (vertical exploration), total number of stretching behavior (horizontal exploration), and the quantity of fecal bolus produced.<sup>(21,25,41)</sup>

## Elevated Plus Maze

EPM is a classical paradigm used to evaluate anxiety like behaviors.<sup>(23,45)</sup> The EPM apparatus, as described previously<sup>(37)</sup> consisted of two open arms (30 cm x 5 cm) and two closed arms (30 cm x 5 cm x 15 cm) connected via a central platform (5 cm x 5 cm). The apparatus is 40 cm elevated above the ground.<sup>(46)</sup> The EPM uses the paradigm of the natural aversion to open spaces and exploratory drive of mice to assess fear and anxiety-like behavioral phenotypes, whereas the avoidance of the open arms indicates the presence of such phenotypes. Since closed arms represent safety spots without further stimuli, it's possible to see how anxiogenic exploration becomes after interventions, such as ELS exposure.<sup>(47,48)</sup>

The task consisted in placing the animal in the central area of the apparatus (neutral zone) with the head facing to one of the open arms. The animals were allowed to explore the apparatus for the period of 10 min. The following parameter were measured: time in the open and closed arms, number of entries in open and closed arms, total number of stretch behavior, total number of head dips, and quantity of fecal bolus. Besides that, avoidance index was calculated, as proposed by Trullas and Skolnick<sup>(49)</sup>:  $100 - [(\% \text{ time spent in the open arms} + \% \text{ entries in the open arms}) / 2]$ . This index considers the ratio between time spent in the open arm and total testing time x 100 (% time spent in open arms) and the ratio between the number of entries in the open arms and the number of total entries (% entries in the open arms) x 100.



**Figure 2.** Maternal separation procedure and recommendations.

In addition, ethological parameters were measured in order to evaluate complementary behaviors such as RA behaviors and information gathering. These indexes take into account the proportion of RA behaviors in relation to the decision of entering in the open arm. The indexes were calculated based on: (A) ratio between the number of head dips and the number of entries in the open arms (head dips /  $n^\circ$  of entries in the open arms) and (B) ratio between the number of stretch and the number of entries in the open arms (stretch /  $n^\circ$  of entries in the open arms).

### Light Dark Test

LD test is a frequently used task for evaluation of anxiety-like behavior and is based on a conflict between the innate animal tendency to explore new environments versus the innate animal aversion to bright environments.<sup>(22)</sup> The apparatus used for this test consisted in a rectangular box (21cm X 42cm X 25cm) divided in two compartments of the same size, with one small open door which allows the animals to freely explore both environments. The animals were placed on the light compartment, and allowed to explore both compartments for 10 min. Before the test, all animals were kept in a dark room for 1h for habituation. We measured the time spent on light and dark compartments, number of entries in each compartment, total number of RA behaviors, total number of rearing behaviors on the light compartment, and total number of fecal boluses. Furthermore, evaluated the avoidance index was calculated following proposition for such parameter in EPM:  $100 - [(\% \text{ time in light zone} + \% \text{ entries in the light zone}) / 2]$ . The intensity of the lighting was 390 lux in the light compartment, and 4 lux in the dark compartment.<sup>(50)</sup>

### Tissue collection

All animals were euthanized 30 min after the last behavioral test (PND60). The sacrifice was performed by decapitation and trunk blood was collected. Blood samples were centrifuged at 1.000x g for 10 min, temperature set on 17 °C. Plasma was then separated and stored at -80 °C until the analysis. Immediately after decapitation, the mPFC was free-hand dissected with tweezers and scalpel. Following dissection, samples were frozen in dry ice. All samples were stored at -80 °C until the day of molecular analysis. For plasma CORT assay and transcript mRNA levels analyses we used samples from 6 animals per group for both cohorts (baseline and anxiety).

### Plasma Corticosterone Assay

For corticosterone assay, plasma samples were thawed and the Corticosterone Enzyme Immunoassay (Arbor Assays) ELISA kit was used with 5  $\mu$ L of each sample in accordance with the manufacturer's guidelines. The optical density was

determined at a wavelength of 450 nm in the ELISA plate reader. We subsequently transformed the value into pg/mL concentrations using standard curve parameters.

### Transcript mRNA levels

Total RNA was extracted using QIAzol (Qiagen) according to the manufacturer's protocol and reconstituted in 15  $\mu$ l of RNase-free water. RNA concentration was measured in the NanoDrop (Thermo Fisher) spectrophotometer. A total of 1  $\mu$ g of RNA from each sample was reverse transcribed into cDNA using the miScript II RT Kit (Qiagen). The following primers (IDT) were designed, tested and used: *Nr3c1* Forward (GGACCACCTCCCAAACCTCTG), *Nr3c1* Reverse (ATTGTGCTGTCCTTCCACTG), *Nr3c2* Forward (AGG TACTGGGGCAATCCATC), *Nr3c2* Reverse (AGTGC CACTGTCTTGCTTATG), *Pgk* Forward (TGCACGCTT CAAAAGCGCACG), *Pgk* Reverse (AAGTCCACCC TCATCAGACCC). Real-Time qPCR was performed using Rotor Gene (Qiagen) and each SYBR Green PCR reaction was run in duplicate. The fold change relative expression was calculated using the  $\Delta\Delta$  Ct method with the Basal AFR group as a reference and *Pgk* as endogenous controls. To verify primer specificities, melting curve analyses and agarose gels were performed.

### Composite Z-score analyses

A composite Z-score was calculated using the three main parameters used for measurement of anxiety-like behavior response in each task. We choose the time spent in center of OF, time spent in the open arms of EPM, and time spent in light compartment of LD. The calculation of Z-score for each parameter was based on, first, subtraction the mean of all animal's score from each individual score and, then, dividing each result by the standard deviation of all scores. After, we calculated the average of the sum of each Z-score parameter above-mentioned. The results were considered the 'Anxiety-like Phenotype' Z-score. In addition, we also performed a Z-score for RA phenotype. We included in the composite 'Risk Assessment Phenotype' Z-score the total number of rearing and stretch behaviors from OF, RA and gathering information index derived from EPM, and total number of rearing and RA behavior from LD.

### Statistical Analyses

Data are expressed as mean  $\pm$  standard error of the mean (SEM). Normal distribution of data was tested for all dependent variables using the Kolmogorov-Smirnov test. All variables showed normality and parametric analyses were assumed. Independent Student *t*-test was performed for all comparative group analyses. Two-way ANOVA was performed with group and cohort as fixed factors and biomolecular outcomes as dependent parameters (CORT, GR mRNA

and MR mRNA). Pearson's correlation analyses were used to examine possible associations between composite Z-scores for behavioral parameters and biomolecular variables. All analyzes were conducted considering the value of  $\alpha = 0.05$  using the statistical analysis software, SPSS version 21.0. Graphs were developed using GraphPad Prism 7 (GraphPad Software Inc., La Jolla, CA, USA).

## Results

### Weight control

No differences in body weight between MS and AFR animals were identified at weaning (PND21:  $t = 1.37$ ,  $df = 20$ ,  $p = 0.18$ ) and before behavioral tasking (PND57:  $t = 0.7$ ,  $df = 20$ ,  $p = 0.43$ ).

### Anxiety-like Behavioral Tasks

#### Open Field Test

Regarding locomotor activity measurements, no differences between groups were detected [AFR  $\times$  MS (meters):  $37.53 \pm 2.70 \times 38.80 \pm 2.98$ ,  $t = -0.31$ ,  $df = 20$ ,  $p = 0.75$ ] (Figure 3a). However, significant differences were found regarding the total time spent in the central zone [AFR  $\times$  MS (sec):  $207.42 \pm 12.14 \times 149.67 \pm 23.43$ ,  $t = 2.18$ ,  $df = 20$ ,  $p < 0.05$ ] (Figure 3b), and the number of total rearing behaviors in the central zone [AFR  $\times$  MS ( $n$ ):  $28.00 \pm 4.05 \times 8.81 \pm 2.29$ ,  $t = 4.11$ ,  $df = 20$ ,  $p < 0.001$ ] (Figure 3c). Animals exposed to MS spent less time in the central zone and performed fewer rearing behaviors in this zone. No differences were found on stretching behaviors in the central zone ( $t = 0.58$ ,  $df = 20$ ,  $p = 0.56$ ) and on number of fecal boluses produced ( $t = -1.61$ ,  $df = 20$ ,  $p = 0.12$ ) (data not shown).

#### Elevated Plus Maze

No differences between groups were observed in the EPM test (Figure 4), with the exception of the quantity of fecal

bolus, in which animals exposed to MS showed a higher quantity of fecal boluses produced when compared to AFR group [AFR  $\times$  MS ( $n$ ):  $5.72 \pm 0.85 \times 10.09 \pm 0.54$ ,  $t = -4.30$ ,  $df = 20$ ,  $p < 0.001$ ] (Figure 4c). The anxiety related behavior measurements were assessed through total time spent in the open arms ( $t = 0.40$ ,  $df = 20$ ,  $p = 0.96$ ) (Fig. 4a), number of entries in the open arms [ $t = -2.08$ ,  $df = 20$ ,  $p = 0.051$ ] (Figure 4b) and avoidance index [ $t = 0.37$ ,  $df = 20$ ,  $p = 0.71$ ].

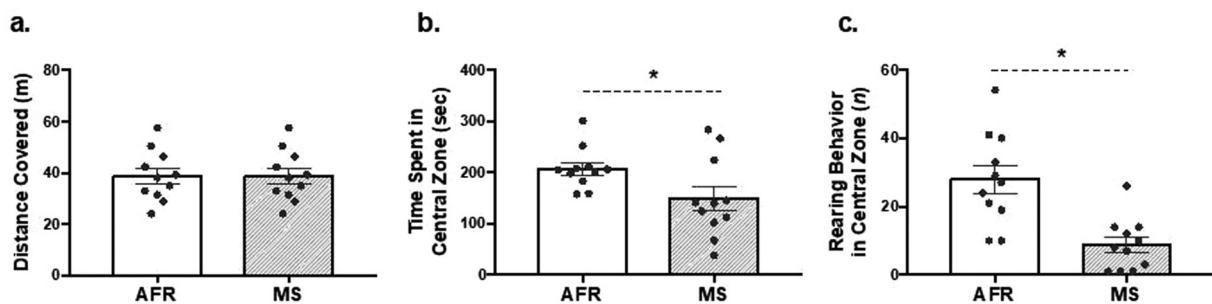
Regarding the ethological parameter related to RA behaviors in the open arms, we found significant differences between AFR and MS groups in both indexes. MS animals showed decreased RA behavior [AFR  $\times$  MS (index):  $1.10 \pm 0.24 \times 0.51 \pm 0.04$ ,  $t = 2.91$ ,  $df = 10.91$ ,  $p < 0.05$ ] (Figure 5a), as well as a decrease in exploration/information gathering [AFR  $\times$  MS (index):  $2.78 \pm 0.83 \times 0.91 \pm 0.14$ ,  $t = 2.32$ ,  $df = 14.32$ ,  $p < 0.05$ ] (Figure 5b).

#### Light Dark Test

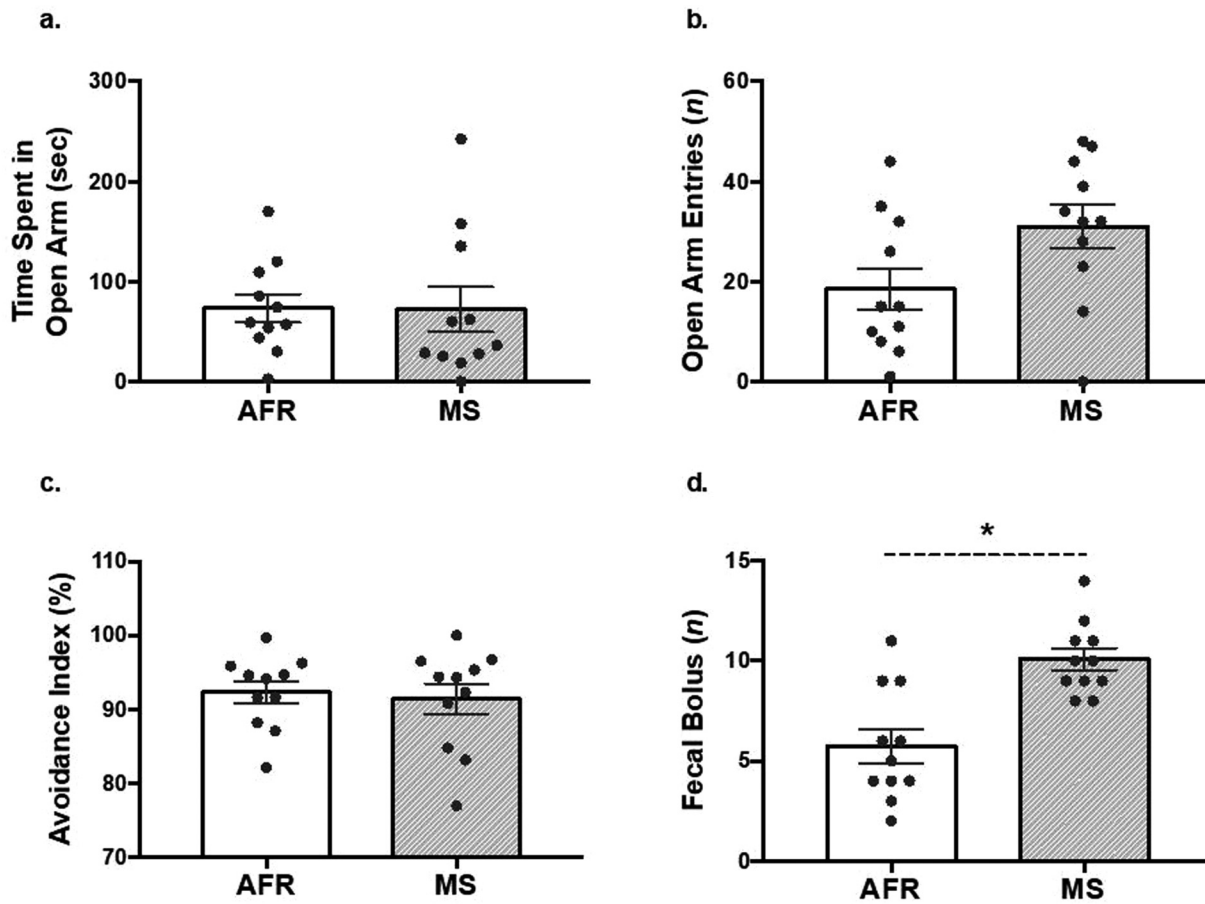
In the LD test we observed that the MS group spent significantly less time exploring the light compartment [AFR  $\times$  MS (sec):  $380.40 \pm 27.64 \times 268.71 \pm 18.10$ ,  $t = 3.38$ ,  $df = 20$ ,  $p < 0.05$ ] (Figure 6a). The MS group also had decreased number of rearing behaviors in the light compartment [AFR  $\times$  MS ( $n$ ):  $33.63 \pm 4.25 \times 19.70 \pm 2.76$ ,  $t = 2.74$ ,  $df = 20$ ,  $p < 0.05$ ] (Figure 6b), and increased avoidance index in the light compartment [AFR  $\times$  MS (%):  $43.77 \pm 2.31 \times 53.84 \pm 1.44$ ,  $t = -2.68$ ,  $df = 20$ ,  $p < 0.05$ ] (Figure 6c). Other parameters such as total number of entrances in light and dark compartments did not show statistical differences, respectively ( $t = -0.74$ ,  $df = 20$ ,  $p = 0.46$  and  $t = -0.85$ ,  $df = 20$ ,  $p = 0.40$ ), as well as the total number of bolus fecal produced ( $t = -1.40$ ,  $df = 20$ ,  $p = 0.17$ ) (data not shown).

#### Anxiety and Risk Assessment Phenotype Scores

We found an increase in the composite anxiety score in the MS group [AFR  $\times$  MS (score):  $-0.34 \pm 0.16 \times 0.34 \pm$



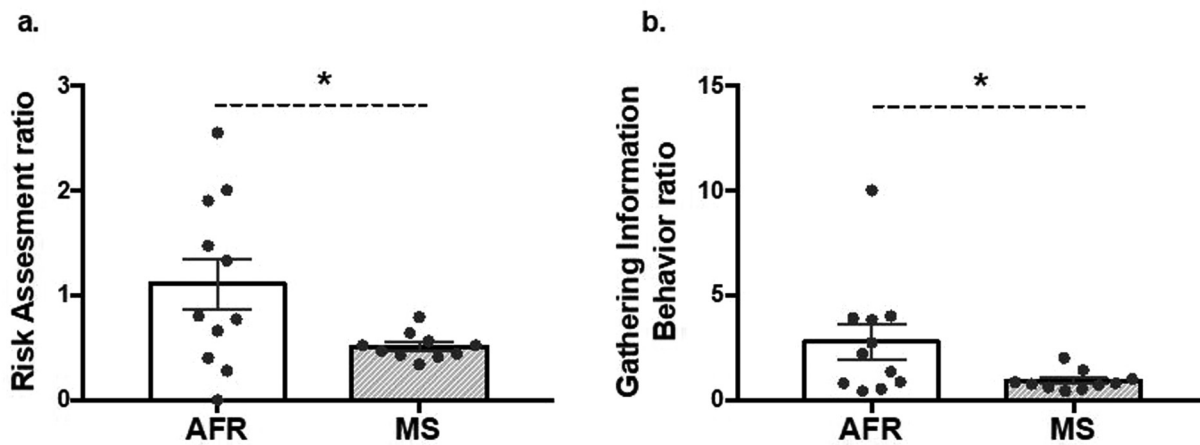
**Figure 3.** MS effects on anxiety-like behavior on OF. (a) Total distance covered in meters; (b) Total time spent in central zone; (c) Total number of rearing behaviors in central zone. \* $p < 0.05$  for MS  $\times$  AFR group differences in  $t$ -test. Number of animals per group: AFR,  $n = 11$ ; MS,  $n = 11$ . Results are expressed as the mean  $\pm$  SEM.



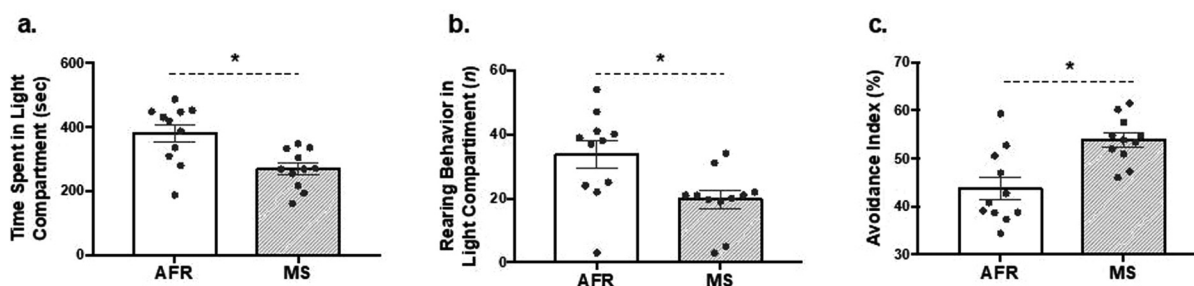
**Figure 4.** MS effects on anxiety-like behavior on EPM. (a) Total time spent in open arm; (b) Number of open arm entries; (c) Avoidance index; (d) Total number of fecal boluses produced during the task. \* $p < 0.05$  for MS  $\times$  AFR group differences in  $t$ -test. Number of animals per group: AFR,  $n = 11$ ; MS,  $n = 11$ . Results are expressed as the mean  $\pm$  SEM.

0.15,  $t = 3.00$ ,  $df = 20$ ,  $p < 0.01$ ], indicating that they presented higher avoidance and anxiety response in relation to the anxiogenic zone of the three apparatus (Fig. 7a).

Additionally, MS animals had lower composite RA score compared to AFR group [AFR  $\times$  SM (score):  $0.29 \pm 0.14$   $\times$   $-0.27 \pm 0.08$ ,  $t = 3.15$ ,  $df = 18$ ,  $p < 0.01$ ].



**Figure 5.** MS effects on risk assessment (RA) behaviors on EPM. (a) RA ratio; (b) Gathering information ratio. \* $p < 0.05$  for MS  $\times$  AFR group differences in  $t$ -test. Number of animals per group: AFR,  $n = 11$ ; MS,  $n = 10$ . Results are expressed as the mean  $\pm$  SEM.



**Figure 6.** MS effects on anxiety-like behavior on LD. (a) Total time spent in light compartment; (b) Total number of rearing behaviors in light compartment; (c) Avoidance index. \* $p < 0.05$  for MS  $\times$  AFR group differences in t-test. Number of animals per group: AFR,  $n = 11$ ; MS,  $n = 11$ . Results are expressed as the mean  $\pm$  SEM.

### Corticosterone

We observed that MS animals had higher levels of plasma CORT compared to AFR condition independent of cohort (Baseline: AFR  $\times$  MS [pg/ml]:  $13.17 \pm 2.44 \times 99.87 \pm 19.80$  and Anxiety: AFR  $\times$  MS [pg/ml]:  $93.80 \pm 54.59 \times 175.03 \pm 26.17$ ,  $F = 32.60$ ,  $dl = 1,21$ ,  $p < 0.001$ ). Moreover, animals that performed the behavioral tests had an overall increase in plasma CORT levels compared to baseline animals (Baseline  $\times$  Anxiety [pg/ml]:  $56.52 \pm 15.05 \times 130.72 \pm 17.98$ ,  $F = 28.05$ ,  $dl = 1,21$ ,  $p < 0.001$ ). No significant interaction effect was observed between group and cohort ( $F = 0.03$ ,  $dl = 1,21$ ,  $p = 0.85$ ) (Figure 8).

### GR and MR gene expression

GR expression analyses revealed no significant differences between groups ( $p > 0.05$ ) (Figure 9a). MR mRNA expression was decreased in MS animals compared to AFR independent of cohort (Baseline: AFR  $\times$  MS:  $1.03 \pm 0.30 \times 0.46 \pm 0.21$  and Anxiety: AFR  $\times$  MS:  $1.26 \pm 0.84 \times 0.77 \pm 0.39$ ,  $F = 5.74$ ,  $dl = 1,22$ ,  $p < 0.05$ ) (Figure 9b). No interaction effect was observed for both GR ( $F = 1.09$ ,  $dl = 1,22$ ,

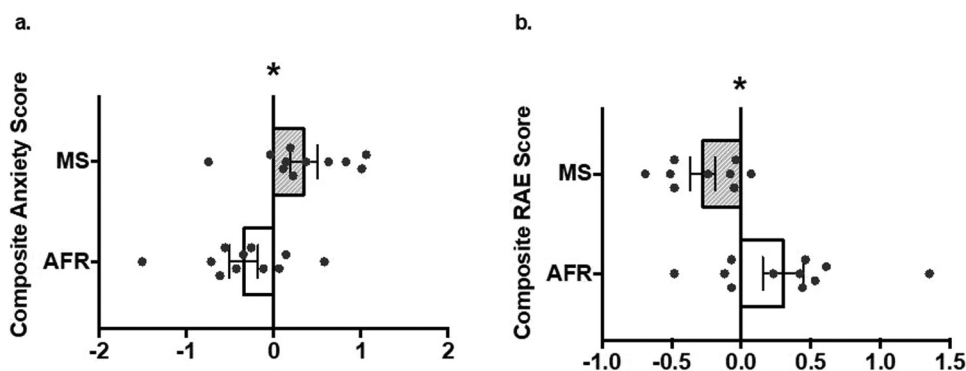
$p = 0.30$ ) and MR ( $F = 0.02$ ,  $dl = 1,22$ ,  $p = 0.87$ ) expression.

### Correlational Analyses

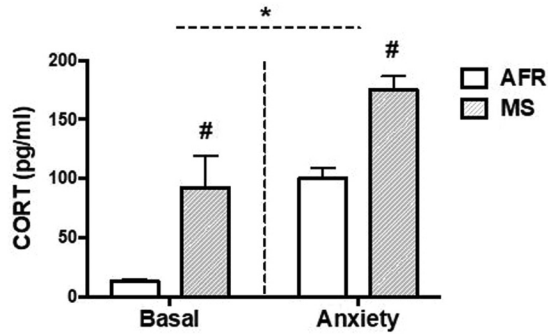
Finally, Pearson's correlation analysis evaluated the relationship between the composite Z-score for anxiety and RA, plasma CORT levels, and GR and MR gene expression. All correlations are presented in Table 1. Our results demonstrated a significant negative correlation between both composite Z-scores ( $r = -0.49$ ,  $p < 0.05$ ) and a significant positive correlation between GR and MR gene expression levels ( $r = 0.71$ ,  $p < 0.05$ ). Additionally, we found a significant positive correlation between composite Z-score for anxiety phenotype and CORT levels ( $r = 0.75$ ,  $p < 0.05$ ) and a significant negative correlation between CORT and GR levels ( $r = -0.88$ ,  $p < 0.05$ ) (Table 1).

### Discussion

In this study we aimed to investigate the effects of MS over anxiety-like behavior in BALB/c mice, as well as plasmatic



**Figure 7.** MS effects on composite anxiety score and composite risk assessment and exploration (RAE) score. (a) Composite Anxiety score = (time spent in center of OF + time spent in the open arms of EPM + time spent in light compartment of LD); (b) Composite RAE score = (total number of rearing and stretch behaviors from OF + RA and gathering information index derived from EPM + total number of rearing and RA behavior from LD). \* $p < 0.05$  for MS  $\times$  AFR group differences in t-test. Number of animals per group: AFR,  $n = 11$ ; MS,  $n = 9$ . Results are expressed as the mean  $\pm$  SEM.



**Figure 8.** MS effects on plasma CORT levels on baseline and anxiety cohorts. \*  $p < 0.05$  for cohort basal  $\times$  anxiety effects. #  $p < 0.05$  for group MS  $\times$  AFR effect. Number of animals per group: Basal cohort: AFR,  $n = 5$   $\times$  MS,  $n = 5$ ; Anxiety cohort: AFR,  $n = 6$   $\times$  MS,  $n = 5$ . Results are expressed as the mean  $\pm$  SEM.

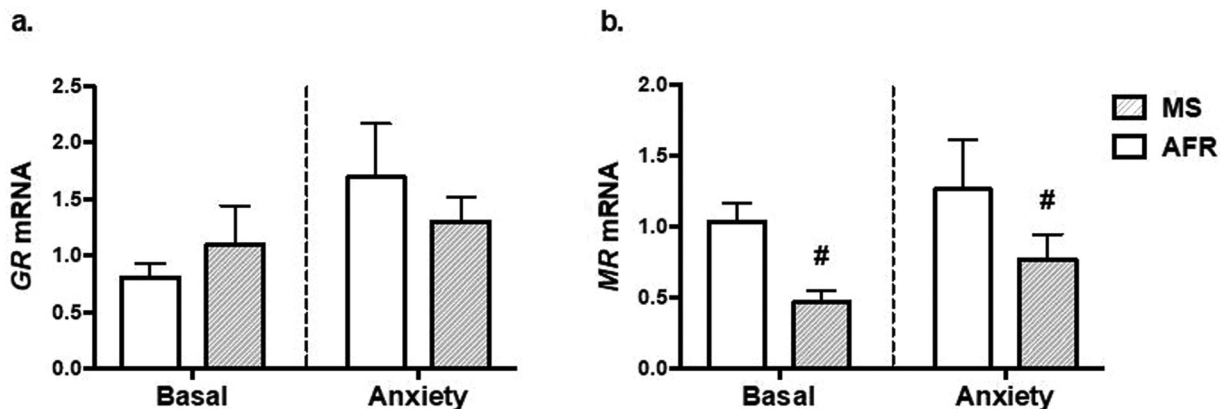
levels of CORT and GR/MR gene expression in the mPFC. The main findings of this study are: (1) MS induced anxiety-like behaviors in male Balb/c mice especially in OF and LD tasks, which is also demonstrated by the Z-score data. (2) MS led to decreased RA behavior in the EPM, as well as lower composite Z-score for RAE. (3) MS exposure increased levels of CORT and decreased expression of MR. (4) Correlation analyzes showed a positive association between plasma CORT levels and anxiety-like behavior. Taken together, those results strengthen the deleterious effects of MS on behavioral and neurobiological outcomes later in life.

The results of OF and LD tests indicated that the MS groups spent significantly less time exploring the anxiogenic and threatening zones of both tasks. Both tests have been showing consistent results in the literature and appear to be reliable paradigms for anxiety-like behavior investigations.<sup>(51–55)</sup> Our lab, for example, have previously found MS effects over anxiety-like phenotype implicated in both OF and LD tests in BALB/c mice. This strain has been

widely used for the investigation of anxiety and stress responsiveness among rodents. Especially in ELS paradigms, BALB/c is reported to be more responsive to the long-term stress effects compared to other strains, such as C57BL/6 mice.<sup>(12,56)</sup> In addition, facing anxiogenic environments BALB/c tend to reduce the locomotor activity and increase risk assessment behaviors for explore and gather information, which could provide complementary parameters for the evaluation of anxiety phenotype<sup>(41,57)</sup> reinforcing the validity of MS effects in BALB/c mice.<sup>(52)</sup>

Regarding the results from EPM test, there is an increasing number of studies failing to replicate ELS effects analyzing classical parameters of anxiety, such as time spent in open and closed arms and number of entries in each arm, as reported by our study.<sup>(33,37,58,59)</sup> The EPM test is described as anxiety sensitive, capable of comprehending anxiogenic and anxiolytic-like behaviors,<sup>(60)</sup> and is commonly used to investigate anxiety-like state induced or inhibited by anxiogenic or anxiolytic drugs.<sup>(61–64)</sup> In non-pharmacological experiments, such as the case of ELS studies evaluating long-term behavioral effects, EPM could induce to an extreme avoidance response between the animals and the results analyzes are susceptible to a floor effect, especially in the most classical parameters of evaluation.<sup>(63,64)</sup> This is why EPM is more commonly used for detecting anxiolytic effects. However, it has been suggested that some complementary parameters involving other ethological aspects, such exploration and gathering information, as the case of RA behavior could emerge as useful tool to better understand the animal's behavior in response to the anxiogenic stimuli of the apparatus.<sup>(27,61,64,65)</sup>

Our ethological investigation suggested that the MS group engaged less in assessing and gathering important environmental information compared to AFR mice. The decrease in RA behavior has been described as an impulsivity tendency, in which stressed mice engage in risk-taking behavior more often due to the lack of RA.<sup>(37)</sup> These behaviors have



**Figure 9.** MS effects on mRNA GR and MR expression on baseline and anxiety cohorts. (a) GR mRNA expression on basal and anxiety cohorts; (b) MR mRNA expression on basal and anxiety cohorts. #  $p < 0.05$  for group MS  $\times$  AFR effect. Number of animals per group: GR mRNA, Basal Cohort: AFR,  $n = 6$   $\times$  MS,  $n = 6$ ; Anxiety Cohort: AFR,  $n = 6$   $\times$  MS,  $n = 5$ . MR mRNA, Basal Cohort: AFR,  $n = 5$   $\times$  MS,  $n = 6$ ; Anxiety Cohort: AFR,  $n = 6$   $\times$  MS,  $n = 5$ . Results are expressed as the mean  $\pm$  SEM.



**Table 1.** Pearson's Correlation index Between Composite Z-Score Parameters, CORT and GR/MR mRNA Levels.

	Anxiety Phenotype		RA phenotype		mRNA GR		mRNA MR		CORT	
	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>
<b>Anxiety phenotype</b>										
<b>RA phenotype</b>	<b>-0.49</b>	<b>0.028*</b>								
<b>mRNA GR</b>	-0.35	0.28	0.15	0.69						
<b>mRNA MR</b>	-0.50	0.11	-0.13	0.72	<b>0.71</b>	<b>0.013*</b>				
<b>CORT</b>	<b>0.75</b>	<b>0.011*</b>	-0.53	0.10	<b>-0.88</b>	<b>0.019*</b>	-0.75	0.08		

been reported as a reliable way to observe the manifestation of anxiety-like behavior, since ethological assessment provides information about the emotional response to the environment.<sup>(66)</sup> RA behavior comes from the potential existence of threat in the environment, and the qualitative actions are based on the capability of the animal to relate to its surroundings.<sup>(67)</sup> This alteration in emotional reactivity facing an approach/avoidance conflict during the task can be interpreted as an anxiogenic-like state.<sup>(68)</sup>

Anxiety, risk-taking, and impulsivity-like behaviors are related to the adequate function of specific brain regions. One of these regions is the mPFC, which is capable of modulating such behavioral responses.<sup>(69)</sup> Furthermore, the mPFC is sensitive to HPA axis activation, indicating that the exposure to stressful events elicits functional alterations in this region in order to adapt to the environmental demands.<sup>(6)</sup> Our findings show that MR expression is reduced in stressed mice compared to controls regardless of cohort, which suggests that changes in MR expression are provoked by the MS protocol and not induced by the behavioral battery. Evidence suggest that MR deficient mice in forebrain regions had less exploratory activity following stress exposure in the OF, higher freezing in a fear conditioning paradigm, and higher levels of plasma CORT.<sup>(70)</sup> Furthermore, mice overexpressing MR in the forebrain had less anxiety in the OF. Therefore, absence or reduced levels of cortical MR is indeed associated with elevated anxiety and altered HPA axis activity in response to stress. Furthermore, our data on increased plasma CORT levels corroborates with the idea of higher stress reactivity in MS animals.<sup>(12,36,37,71)</sup>

Glucocorticoids and mineralocorticoids are endocrine hormones supporting the regulation and maintenance of several physiological functions.<sup>(72,73)</sup> In the brain, the action of these hormones is mediated through GR and MR receptors, which are mainly associated with CORT and with aldosterone expression, respectively.<sup>(72)</sup> While GR are spread over several different brain areas, MR appear to be more restricted to the limbic areas of the brain.<sup>(74)</sup> GR and MR receptors demonstrate both complementary and opposing actions, especially regarding psychopathologies. It was found that decreased MR activity is associated with the development of psychiatric disorders, while an overactivity of GR is associated with the later development of mood disorders.<sup>(75)</sup> Even though both receptors share the same target

genes and mechanisms of action, they are reported to have distinct effects on the brain.<sup>(75)</sup> Since they are tightly regulated by the HPA axis, both receptors play a key role in the stress response system.<sup>(72,75)</sup> Changes in GR and MR levels after exposure to stress have been reported by previous studies, and these alterations commonly imply the later development of psychiatric disorders.<sup>(76,77)</sup>

It is well documented that mPFC and amygdala connections work to adjust behavioral responses, such as fear expression and anxiety.<sup>(78)</sup> In normal conditions, mPFC inhibits amygdala activation in a top-down manner to prevent exacerbated emotion expression.<sup>(79,80)</sup> Impairment in the mPFC control over amygdala is also well documented in chronic stress,<sup>(81)</sup> suggesting a possible path to the development of pathological conditions. Recently, Rainekei et al. found a more prevalent effect of chronic mild stress in GR specifically in mPFC when compared to amygdala.<sup>(82)</sup> In our study we focused specifically in mPFC to explore the association between GR and MR gene expression related to RA parameters, considering that the mPFC is a key region for the regulation of risk assessment and decision-making behaviors.<sup>(83)</sup> Risk assessment is a behavioral parameter capable of revealing the animal's ability to assess the environment before taking action, thus possibly being able to reflect the mPFC-amygdala top-down control.<sup>(37,83,84)</sup>

Our findings must be carefully interpreted. In this research we focused exclusively in male mice, abdicating of sex differences investigations and limiting data generalization. ELS effects can lead to different behavioral responses depending on sex. However, regardless of restricting the investigations to males, our findings contribute to the validation of MS effects over anxiety phenotype in Balb/c mice. A second limitation of our study is the usage of only one brain region for gene expression analyses of MR and GR, it would be interesting for future studies to focus on the investigation of these targets in other key brain regions underlying HPA functioning. Besides that, despite our findings indicated reduced levels of MR gene expression, it is important to note that our analysis is limited to gene expression layer. Thus, we were not able to extend these results to the protein level or receptor function. Another limitation of the extension of our findings is related to the ending-point (PND60, early adulthood). The MS-induced effects seem to be persistent at this stage, but it still unclear whether these

effects would persist from early to middle and late adulthood. So, it could be interesting for future studies address such issue using different cohorts of animals at different ending-points along the adulthood.

In summary, exposure to MS leads to an increase in anxiety-like phenotype in Balb/c mice, triggering a disruption of the HPA axis functioning. Balb/c mice MS increased CORT response and led to persistent changes in MR levels in the mPFC. Suggesting that despite studies focusing on GR gene expression, the expression of MR seems to play an important role on stress reactivity. This research contributes to the discussion and understanding of ELS effects over anxiety phenotype in a preclinical model with translational potential.

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### Author Contributions

**Erika Kerstering-Ferreira:** Investigation, Methodology, Writing – original draft. **Saulo G. Tractenberg:** Conceptualization, Investigation, Formal analysis, Writing – original draft, Writing – review & editing. **Francisco S. Lumertz, Rodrigo Orso and Kerstin Creutzberg:** Writing – review & editing. **Luis Eduardo Wearick-Silve and Thiago W. Viola:** Conceptualization, Investigation, Methodology, Writing – review & editing. **Rodrigo Grassi-Oliveira:** Conceptualization, Project administration, Resources, Writing – review & editing, Supervision.

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The authors declare that there is no conflict of interest.

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### Ethical Approval

Ethics Committee on Animal Use (CEUA) of PUCRS - Registration number #14/00421.

### Informed Consent

Not applicable, because this article does not contain any studies with human or animal subjects.

### Trial Registration

Not applicable, because this article does not contain any clinical trials.

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