Received: 5 December 2019 | Accepted: 22 December 2019

DOI: 10.1002/jdn.10004

RESEARCH ARTICLE

INTERNATIONAL JOURNAL OF DEVELOPMENTAL NEUROSCIENCE

WILEY

Vulnerable and resilient cognitive performance related to early life stress: The potential mediating role of dopaminergic receptors in the medial prefrontal cortex of adult mice

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Funding information
This study was supported by grants [442776/2018-7] and [307130/2018-5] of RGO and [141182/2016-4] of SGT from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and [88881.189914/2018-01] of SGT from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES)

Abstract

Rationale: Exposure to early life stress (ELS) is known to have pronounced effects on the prefrontal cortex (PFC). However, not all individuals exposed to ELS manifest the same neurobiological and cognitive phenotypes when adults. Dopamine signaling could be a key factor in understanding the effects of stress on PFC-related cognitive function.

Objectives: We aimed to investigate the differential effects of ELS on cognitive performance of adult mice and the dopaminergic receptors expression in the PFC.

Methods: BALB/c males were exposed to the maternal separation (MS) procedure and their cognitive performance on the eight-arm radial maze (8-RAM) were assessed during adulthood. For molecular-level assessments, we performed mRNA expression analyses for dopamine receptors—*DRD1*, *DRD2*, *DRD3*—and *Hers1* expression in the medial PFC.

Results: While MS produced an overall impairment on 8-RAM, the stressed animals could be divided in two groups based on their performance: those with impaired cognitive performance (vulnerable to maternal separation, V-MS) and those without any impairment (resilient to maternal separation, R-MS). V-MS animals showed increased *DRD1* and *DRD2* expression in comparison with other groups. Errors on 8-RAM were also positively correlated with *DRD1* and *DRD2* mRNA expression.

Conclusions: Our findings suggest a potential role of the dopaminergic system in the programming mechanisms of cognitive vulnerability and resilience related to ELS.

KEYWORDS

dopamine, early life stress, maternal separation, resilience, stress, working memory

1 | INTRODUCTION

Adverse early life experiences, such as childhood maltreatment and other stressor events, are known to be risk factors for the development of neuropsychiatric disorders later in life (Lupien, McEwen, Gunnar, & Heim, 2009; Teicher & Samson, 2013; Teicher, Samson, Anderson, & Ohashi, 2016). One of the core aspects of this high-risk neuropsychiatric profile is related to the adverse effects of early life stress (ELS) on neurodevelopment and the persistent structural

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and functional changes in central brain regions such as the prefrontal cortex (PFC) and the hippocampus (Hipp). These regions were commonly found to be significantly impaired in both human (De Bellis, Hooper, Spratt, & Woolley, 2009; Mothes et al., 2015; Navalta, Polcari, Webster, Boghossian, & Teicher, 2006; Nikulina & Widom, 2013; Oswald et al., 2014) and animal (Aisa et al., 2009; Chocyk et al., 2013; de Azeredo et al., 2017; Grassi-Oliveira, Honeycutt, Holland, Ganguly, & Brenhouse, 2016; Reincke & Hanganu-Opatz, 2017) studies, addressing the effects of ELS later in life.

As the PFC is one of the brain regions that are most sensitive to the effects of ELS and pronounced cognitive deficits are observed in PFC-dependent tasks (Arnsten, 2009; Grassi-Oliveira et al., 2016; Viola et al., 2019), it is crucial to identify the potential mechanisms that can explain the adverse effects of stress on PFC functionality. One interesting hypothesis about PFC regulation is related to the role of dopamine signaling in specific networks and their output pathways, which affect distinct cognitive functions that are dependent on this brain region (e.g., working memory, attention, planning and solving problems, cognitive flexibility, decision making and goal-directed behaviors) (Holroyd & Umemoto, 2016; Jenni, Larkin, & Floresco, 2017; Kehagia, Murray, & Robbins, 2010; Puig, Antzoulatos, & Miller, 2014; Puig & Miller, 2015; Winstanley & Floresco, 2016). During cognitive tasks that require PFC recruitment, a significant release of endogenous dopamine (DA) as a consequence of DA neuron stimulation has been identified (Murphy, Arnsten, Goldman-Rakic, & Roth, 1996; Ott & Nieder, 2019; Vijayraghavan, Wang, Birnbaum, Williams, & Arnsten, 2007). DA acts by binding to two classes of receptors, D1- and D2-like receptors, and their respective subdivisions (D1- and D5-receptors—*DRD1* and *DRD5*—for the first class and D2-, D3- and D4-receptors—*DRD2*, *DRD3* and *DRD4*—for the second class). *DRD1* and *DRD2* are considered the most abundant receptors expressed in the brain, and DA action on both of these receptors is important to facilitate PFC engagement (Araki, Sims, & Bhide, 2007). Excessive stimulation of DA receptors in PFC, however, has been associated with deleterious effects on working memory functioning and other cognitive processes in an inverted-U shape relation (Murphy et al., 1996; Vijayraghavan et al., 2007; Williams & Castner, 2006).

A recent review and genome-wide studies (GWAS) (Knowles, Carless, et al., 2014; Knowles, Mathias, et al., 2014) compiled the potential target genes associated with PFC functionality, especially in relation to working memory performance. In their findings, it has been supported a mediating role of neuronal excitability through ion channel activity and/or dopaminergic signaling, highlighting the direct and indirect effects of DA expression in the PFC. Alterations in the excitability or efficiency of dopaminergic signaling were suggested to be involved in working memory dysfunction. Furthermore,

Highlights

- Maternal separation impacted working memory performance in a subsample of animals.
- V-MS animals showed significant deficits on working memory performance.
- V-MS cognitive deficits were associated with DA receptor expression changes.
- DA receptors in PFC could mediate the cognitive dysfunctions associated with ELS.

Knowles, Carless, et al. (2014) found an interesting candidate gene, the basic helix-loop-helix (bHLH) transcriptional factor *Hesr1* gene (hairy and enhancer of split-related 1, also called *HEY1*), which has been shown to be involved in neurodevelopmental processes (e.g., neurogenesis and differentiation), neuropsychiatric disorders and behavioral traits characterized by dysregulation of dopaminergic signaling. The *Hesr1* gene binds to 3′ non-coding region of the dopamine transporter gene (*DAT1*), repressing the expression of *DAT1* and indirectly affects the expression of dopamine-related genes (*D1, D2, D4, D5*) (Fuke et al., 2006). In *Hesr1* knockout mice, it was found an upregulation of *DAT1*, suggesting also a compensatory increase on dopamine receptor genes expression, which may be involved on working memory deficits and altered behavioral phenotype in classical behavioral tasks, such as open field and elevated plus maze (Fuke et al., 2006; Knowles, Mathias, et al., 2014).

Despite the significant data suggesting that ELS experiences lead to behavioral and cognitive dysfunctions in consequence of the abnormal neurodevelopment trajectory, it is difficult to infer a deterministic prediction. Inconsistent data among preclinical studies (Bonapersona, Joels, & Sarabdjitsingh, 2018; Molet, Maras, Avishai-Eliner, & Baram, 2014; Nylander & Roman, 2013; Tractenberg et al., 2016), and the lack of knowledge regarding why some stressed individuals are susceptible to the negative consequences of early life experiences, while others seem to be "unaffected" or resilient seem to contribute to these mixed findings (Miller, Chen, & Parker, 2011; Silk et al., 2007; Yehuda, Flory, Southwick, & Charney, 2006). Some efforts have been made in this direction, particularly when distinct expression patterns of specific target genes related to cognitive dysfunctions seen among subgroups of stressed animals were investigated. Studies addressing stress-reactive and non-stress reactive groups, or vulnerable and resilient groups, have advanced our knowledge of how distinctive ELS effects emerge and operate at the interface between gene–environmental factors (Calabrese et al., 2017; Clinton, Watson, & Akil, 2014; Rana et al., 2016; Spencer-Segal & Akil, 2019; Srinivasan, Shariff, & Bartlett, 2013).

Here, we aimed to investigate the effects of ELS on working memory and memory function and on the expression target genes related to dopaminergic signaling (*Hers1*, *DRD1*, *DRD2,* and *DRD3*) in the medial prefrontal cortex (mPFC). In addition, to improve our potential findings, we explored how animals subjected to ELS would differentially respond to stress exposure using an experimental design that separated the stressed animals that showed a cognitive response to ELS from those that were resilient.

2 | MATERIALS AND METHODS

2.1 | Animals

All animals were purchased from the animal facility of the Center for Experimental Biological Models (CeMBE) at Pontifical Catholic University of Rio Grande do Sul (PUCRS), Brazil. Male BALB/c mice (total, $n = 38$) were used for this experiment. All of them were housed in standard plastic mouse cages (22 cm × 16 cm × 14 cm), 2–3 animals per cage and kept under constant housing and handling conditions (light/dark cycle: 12 hr, lights on at 6:00 hr; room temperature: 21 ± 1°C; humidity: 55% ± 5%; controlled air circulation; and, food and water regime ad libitum).

Breeding procedures involved housing two female mice with one male mice per cage during a mating period of 2 weeks. At the end of this period, the male was removed and the females were individually housed and inspected daily at the same time until pregnancy confirmation. The day when pups were found was defined as postnatal day 1 (PND1). A cross-fostering procedure was run with half of the litter, followed by litter control from 5 to 8 pups per litter, when necessary. Subsequently, the animals were randomly assigned to undergo prolonged maternal separation (MS group) or animal facility rearing (AFR group). For this experiment, we used a total of 18 different litters, of which 11 were designated for the MS group and 7 were for the AFR group. We used a total of 25 MS animals (1–4 animals per litter) and 13 AFR (1–3 animals per litter). Surplus females were assigned to additional ongoing projects in the laboratory. Pups were not sexed until weaning day (PND21), preventing additional manipulations. At PND21, the males were weighed and group housed. Cage cleaning was performed once a week by the animal facility staff throughout the experimental period. The cognitive experimental procedures were assessed at early adulthood, from PND58 to PND67.

All procedures were approved by the Ethics Committee on Animal Use (CEUA) of PUCRS, Brazil under the registration #14/00421. We conducted the experiments in accordance with National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) and International Council for Laboratory Animal Science (ICLAS).

2.2 | Maternal Separation (MS) procedures

MS procedures were performed following the methodological recommendations proposed in our extended MS systematic review (for more details, see Tractenberg et al., 2016) and well established by prior studies (Bailoo, Jordan, Garza, & Tyler, 2014; Wang, Jiao, & Dulawa, 2011). From PND2 to PND15, the pups of the MS group were separated daily from their dams for a period of 180 min, during the end period of the light cycle (16:00–19:00 hr). The procedure involved first removing the dams from the home cage and reallocating them to a new cage containing standard clean bedding. Dams were also transferred to another room to avoid ultrasonic vocalization between dams and the pups. Pups were then removed from the home cage and individually distributed in a small plastic box containing a little amount of bedding. The temperature of the pups was controlled by a digital-regulated heating pad (around 32 ± 3°C) in order to prevent stress by hypothermia. After the 180-min period, the pups were returned to their home cage followed by the dam. The animals in the AFR group was left undisturbed in their home cages throughout the postnatal period.

2.3 | Eight-arm radial maze test (8-RAM)

To evaluate cognitive parameters related to working memory, visuospatial memory and learning, we conducted an adapted version of the 8-RAM protocol (Ammassari-Teule, Hoffmann, & Rossi-Arnaud, 1993; Umemori et al., 2013; Valentim, Olsson, & Antunes, 2013; Valladolid-Acebes et al., 2011; Yadav et al., 2013), with four-arm baited, as first described by Jarrard (1983). The apparatus consisted of eight arms extending radially from a central octagonal platform (5 cm wide × 30 cm long, extending from a central platform 25 cm^2). Each of the eight arms had internal fixed visual clues put on the floor below the apparatus. The clues were prepared as described in Ammassari-Teule's study (1993) by using different visual patterns (e.g., black, black squares, white, longitudinal stripes). Each arm, numbered from 1 to 8, had a specific visual pattern clue that was permanent throughout the experimental period. Additionally, four external clues were attached to the walls around the maze. The stimuli were shapes cut from black cardboard (e.g., a rectangle, a circle, a triangle and a half moon; Valladolid-Acebes et al., 2011). The overhead lights were on. We ran a 14-day session broken into three phases: training (days 1–4),

testing (days 5–13) and retention (day 14) explained in detail below. During all phases, the maze was kept in a constant orientation. The training phase occurred twice a day in the morning (9:00 hr) and in the afternoon (13:00 hr). As our protocol depends on goal-directed behavior, we submitted the animals to a 20-hr hydric restriction condition, as previously suggested by Rowland (2007). Thus, every day at the end of the procedures, the animals were placed in an ad libitum water condition and all bottles of water were removed 4 hr later, at 17:00 hr. The hydric restriction condition started 1 week before the testing phase to acclimate the animals with this regimen and minimize stress-induced effects. We used as reward a diluted solution of 10% condensed milk, using 20 μl of this solution as bait.

2.3.1 | Training phase

On day 1, all animals of the same home cage were placed together on the center platform and allowed to explore the entire maze for 20 min in the morning time. Later, during the afternoon, the animals were placed individually and were allowed to explore all eight arms for 10 min. At this time, all the arms were baited at the beginning and the ending; on day 2, the day 1 afternoon procedure was repeated as described above; on day 3, animals were placed individually on the central platform and allowed to explore all arms for 10 min or until all rewards were collected. All the arms were baited just at the ending (on the collecting area). The procedure was performed in the same form in the morning and in afternoon; on day 4, animals were placed individually and were free to explore all arms for 10 min; however, at this time, just four of the eight arms were baited. If the animals retrieved all four rewards before the time ran out, the trial was finished and the animal was removed from the maze. This procedure was performed twice on this day, in the morning and in the afternoon. The four baited arms were randomly assigned for each animal (e.g., 1–3–5–7 or 2–4–6–8) and were maintained throughout the testing phase.

2.3.2 | Testing phase

During days 5–13, the procedure was run in the same way throughout the testing phase. This phase consisted of two consecutive trials per animal performed once a day (13:00 hr). The inter-trial interval was about 2 min. For each trial, all animals were placed individually on the center platform inside a transparent cylinder; the trial started when the cylinder was removed (5-s delay) and the animal was free to explore all arms for 10 min or until all rewards were retrieved. In order to eliminate or reduce olfactory cues, before each animal and between trials, the maze was wiped with a 70% ethanol solution.

2.3.3 | Retention phase

At day 14, all animals were placed at the maze to run a single 10-min trial or until they collected all rewards.

The parameters evaluated for each trial were as follows: the time spent to collect all rewards; the number of arm entrances; the number of working memory errors—when the animal reentered a baited arm that had already been visited in that trial; the number of reference memory errors—number of entries in an arm that had never been baited; the number of perseverative errors—when the animal reentered an unbaited arm that had already been visited in that trial. An arm entry was defined when the animal was entirely (four paws and tail) inside the arm. A correct entry was defined as a first entry/reward consumed into a baited arm (Avdesh, Hoe, Martins, & Martin-Iverson, 2013; Nozari, Mansouri, Shabani, Nozari, & Atapour, 2015). All measures were registered by an observer while the experiment was running. We also calculated the sum of both trials of mentioned errors for each day. Following the data analyses of behavioral parameters, we used the sum of all 8-RAM errors to divide the MS group into two subgroups (those with significant deficits were considered vulnerable, V-MS group and those that performed similar than controls, were considered resilient, R-MS group).

2.4 | Euthanasia and brain tissue collection

All animals were euthanized by decapitation 30 min after the last trial of the testing phase. Trunk blood was immediately collected from the site where the animal is decapitated and stored in 1.5-mL Eppendorf tubes containing ≅ 100 μl of ethylenediaminetetraacetic acid (EDTA) solution at room temperature until all euthanasia procedures were finished. Next, we centrifuged the samples at 1.000 × *g* for 10 min at a temperature of 17°C. The plasma was collected and stored at −80°C until use. At the same time, the brains were removed and the mPFC region was dissected manually using the free-hand dissection method with tweezers and scalpel in the following bregma coordinates: anteroposterior from 1.34 to 1.98 mm, mediolateral ±0.5 mm and dorsoventral from −1.0 to −3.0 mm. Following dissection, samples were immediately frozen in dry ice. All samples were stored at −80°C until the day of molecular analysis.

2.4.1 | Plasma corticosterone (CORT) assay

Plasma was defreezed and the Corticosterone Enzyme Immunoassay (Arbor Assays) ELISA kit was used with 5 μl of the sample in accordance with the manufacturer's guidelines. From the total number of animals ($n = 38$) analyzed for obtaining behavioral data, 36 samples were used in CORT

analysis, while 2 samples were lost during plasma extraction. From the data analyses, we excluded five outlier values considering the SPSS cutoff point (1.5XIQR, Interquartile range) (AFR: $n = 3$ and V-MS: $n = 2$). 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.

2.4.2 | Transcript mRNA levels

A total of 18 samples were used for mRNA analysis. $N = 6$ samples from each group (V-MS, R-MS and AFR) were randomly selected and total RNA was isolated using QIAzol (Qiagen; Hilden, Germany) and chloroform standard protocols. The RNA concentration was measured using the NanoDrop spectrophotometer. Next, 1 μg of RNA from each sample was reverse-transcribed using the miScript II RT Kit (Qiagen). The following primers (IDT) were used: *Drd1* (For: ATGGCTCCTAACACTTCTACCA, Rev: GGGTATTCCCTAAGAGAGTGGAC), *Drd2* (For: ACCTGTCCTGGTACGATGATG, Rev: GCATGGCATA GTAGTTGTAGTGG), *Drd3* (For: CCTCTGAGCCAG ATAAGCAGC, Rev: AGACCGTTGCCAAAGATGATG) and *Hers1* (For: GAAGCGCCGACGAGACCGAATCAA, Rev: CAGGGCGTGCGCGTCAAAATAACC). The SYBR Green PCR was performed in duplicate for each sample using a Rotor Gene Real-Time PCR machine (Qiagen). Fold change relative expression was calculated through the ΔΔCt method with the AFR group as a reference. Probable phosphoglycerate kinase (Pgk): (For: TGCACGCTTCAAAAGCGCACG, Rev: AAGTCCACCCTCATCACGACCCAFR) values were used as the endogenous control for mRNA analysis. To verify primer specificities, melting curve analyses and agarose gel assessments were performed.

2.5 | Statistical analyses

Normal distribution and homogeneity of variances were tested using the Kolmogorov–Smirnov test. All variables showed normality, and parametric analyses were assumed. The analyses were performed using Student's independent *t* test to analyze the "total number of errors" between the MS and AFR groups; one-way analysis of variance (ANOVA) for dichotomization of groups and to compare the AFR, vulnerable to MS (V-MS) and resilient to MS (R-MS) groups; and repeated-measures ANOVA with the between-subjects factor "dichotomized groups" and the within-subject factor "days-block" for all evaluated 8-RAM parameters. The "days-block" variable was created using the mean score of the sum of 8-RAM parameters through three different intervals [block-1: Σ(days 1–3)/3; block-2: Σ(days 4–6)/3 and block-3: Σ(days 7–9)/3]. Post hoc analyses were adjusted with Turkey HSD correction. Pearson's correlation analyses were also used to examine possible associations between behavioral and biological parameters. *P*-values less than .05 were considered significant in our analyses. Values in graphs are expressed as the mean ± *SEM*. All results were explored and analyzed by SPSS 21.0 for Windows (SPSS Inc., Chicago, IL, USA). Graphs were generated using GraphPad Prism 7 (GraphPad Software Inc., La Jolla, CA, USA).

3 | RESULTS

3.1 | Group dichotomization

We created a variable based on the sum of all 8-RAM errors (working memory error, perseverative errors, and reference errors). The average number of total errors in the control group (AFR) ($n = 13$) was 93.61 ± 12.29, while that in the MS group ($n = 25$) was 146.76 ± 37.71, which was statistically different ($t = -6.42$, $df = 32.12$, $p < .0001$) (Figure 1a). Based on the cutoff point of three standard deviations from the mean (AFR group = 93.61 ± 12.29), our cutoff point was set as 131. MS animals with > 131 total errors were considered as V-MS and those with < 131 total errors were considered as R-MS. Significant differences between groups were identified considering the number of total errors ($F = 63.77$, $df = 2, 35$, $p < .0001$). Post hoc test comparisons revealed significant differences between the V-MS and R-MS groups ($p < .0001$) and V-MS and AFR groups ($p < .0001$). No differences were found between the R-MS and AFR groups ($p = .1681$) (Figure 1b). The percentage of animals who met the criteria within each group is shown in Figure 1c. $N = 15$ animals met the criterion for the V-MS group, while $n = 10$ met the criterion for the R-MS group.

3.2 | V-MS versus R-MS behavioral analyses

ANOVA showed significant intergroup differences for different parameters of the 8-RAM in both testing and retention phases (Figure 2). The total number of working memory errors (WMEs) was significantly different ($F = 38.80$, $df = 2, 35$, $p < .0001$). Post hoc analysis revealed significant differences between the V-MS and both R-MS and AFR groups (*V-MS* vs. *R-MS*, $p < .0001$ and *V-MS* vs. *AFR*, $p < .0001$). No difference was found between the R-MS and AFR groups ($p = .3997$) (Figure 2a). The total number of perseverative errors (PSEs) was also significantly different ($F = 44.09$, $df = 2, 35$, $p < .0001$). A post hoc test showed differences between V-MS and both R-MS and AFR groups (*V-MS* vs. *R*, $p < .0001$ and *V-MS* vs. *AFR*, $p < .0001$). No difference

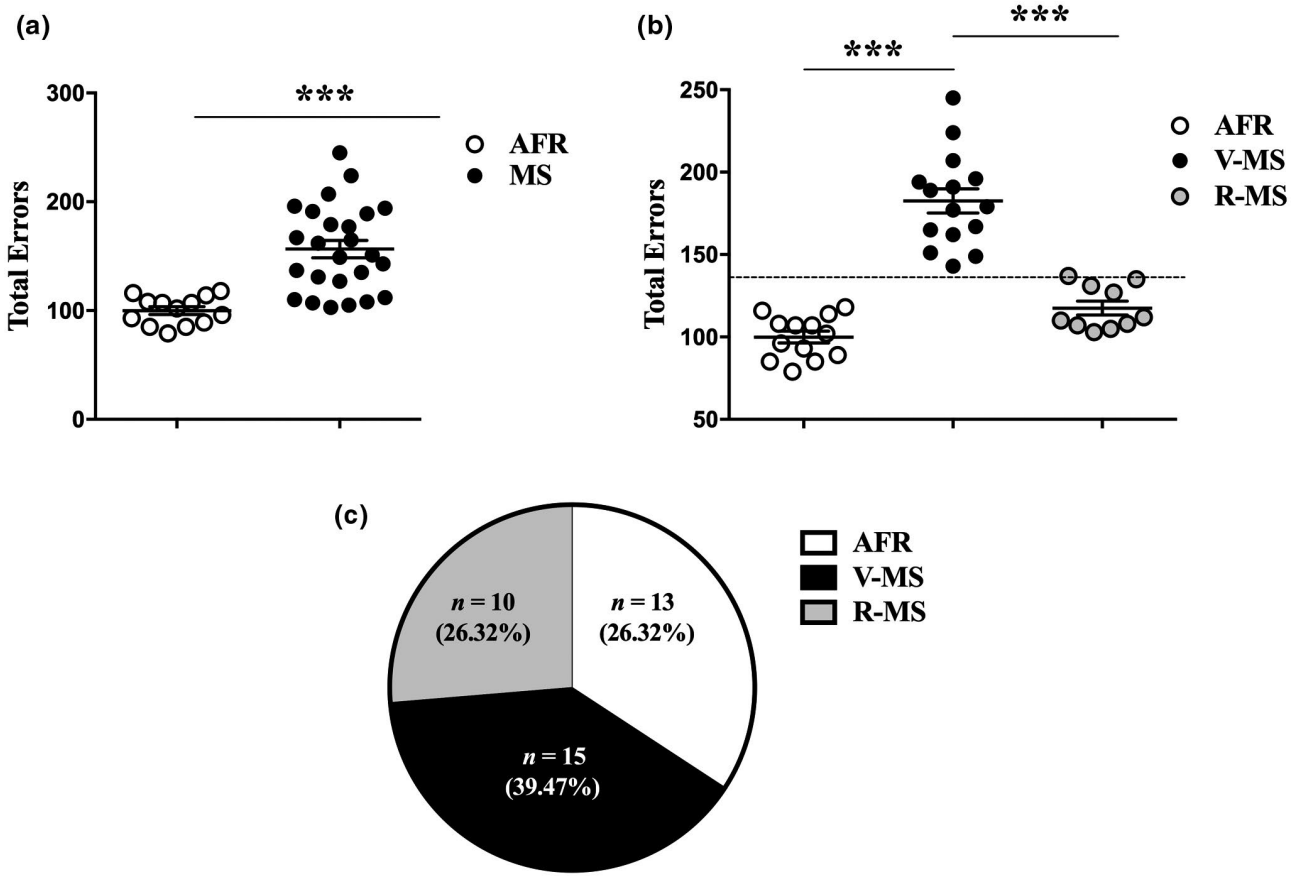

FIGURE 1 Maternal Separation (MS) effects on Eight-arm Radial Maze (8-RAM) and group's dichotomization based on total number of errors. (a) Number of total errors on 8-RAM along the testing days; (b) Number of total errors on 8-RAM based on group's dichotomization; (c) Percentage of animals who met criteria based on group's dichotomization for each experimental group. AFR = Animal Facility Rearing, R-MS = Resilient Maternal Separation, V-MS = Vulnerable Maternal Separation; ***$p < .00$ (*t*-test). Results are expressed as the mean ± *SEM*

was found between the R-MS and AFR groups ($p = .1803$) (Figure 2b). The total number of reference errors (REs) was significantly different ($F = 14.85$, $df = 2, 35$, $p < .0001$). Post hoc analysis revealed differences between the V-MS group and both R-MS and AFR groups (*V-MS* vs. *R-MS*, $p < .0022$ and *V-MS* vs. *AFR*, $p < .0001$). No difference was found between the R-MS and AFR groups ($p = .5127$) (Figure 2c). The number of retention working memory errors (r-WMEs) was significantly different ($F = 5.69$, $df = 2, 35$, $p < .0073$). Post hoc analysis revealed differences between the V-MS group and both R-MS and AFR groups (*V-MS* vs. *R-MS*, $p < .0427$ and *V-MS* vs. *AFR* =, $p < .0109$). No difference was found between the R-MS and AFR groups ($p = .7337$) (Figure 2d). Moreover, the number of retention perseverative errors (r-PSEs) was also significantly different ($F = 4.07$, $df = 2, 35$, $p < .0256$). Post hoc analysis revealed differences between the V-MS and R-MS groups (*V-MS* vs. *R-MS*, $p < .0301$). No difference was found between the AFR group and both V-MS ($p = .1115$) and R-MS ($p = .7482$) groups (Figure 2e). Lastly, the number of retention reference errors (r-REs) failed to reveal significant differences among the three groups ($p = .0831$) (Figure 2f).

3.3 | Days-block analyses

Block analysis was conducted in order to explore how the animals performed over three different phases of the task and to evaluate the transition from goal-directed behavior to habit-directed behavior. Analyses of the time spent to complete the task (TIME), WMEs, PSEs and REs during the testing phase at block-1, block-2 and block-3 using repeated-measures one-way ANOVA revealed a significant block effect (TIME: [$F = 19.63$, $df = 2, 70$, $p < .0001$, post hoc]; WME: [$F = 8.95$, $df = 2, 70$, $p < .0001$]; PSE: [$F = 22.8$, $df = 2, 70$, $p < .0001$]; RE [$F = 9.48$, $df = 2, 70$, $p < .0001$]) and group effect (TIME: [$F = 13.16$, $df = 2, 70$, $p < .0001$]; WME: [$F = 38.8$, $df = 2, 35$, $p < .0001$]; PSE: [$F = 36.2$, $df = 2, 35$, $p < .0001$]; RE [$F = 14.85$, $df = 2, 35$, $p < .0001$]). No interaction of block X group was found for all parameters (Figure 3). Post hoc analysis for block effect showed significant differences in TIME (block-1 > block-2, $p < .0301$ and block −3, $p < .0001$ and block-2 > block-3, $p < .0009$), revealing that animals were able to reduce the time spent to complete the task across the trial-blocks, and significant differences in WME (block-1 > block-3, $p < .0045$); PSE

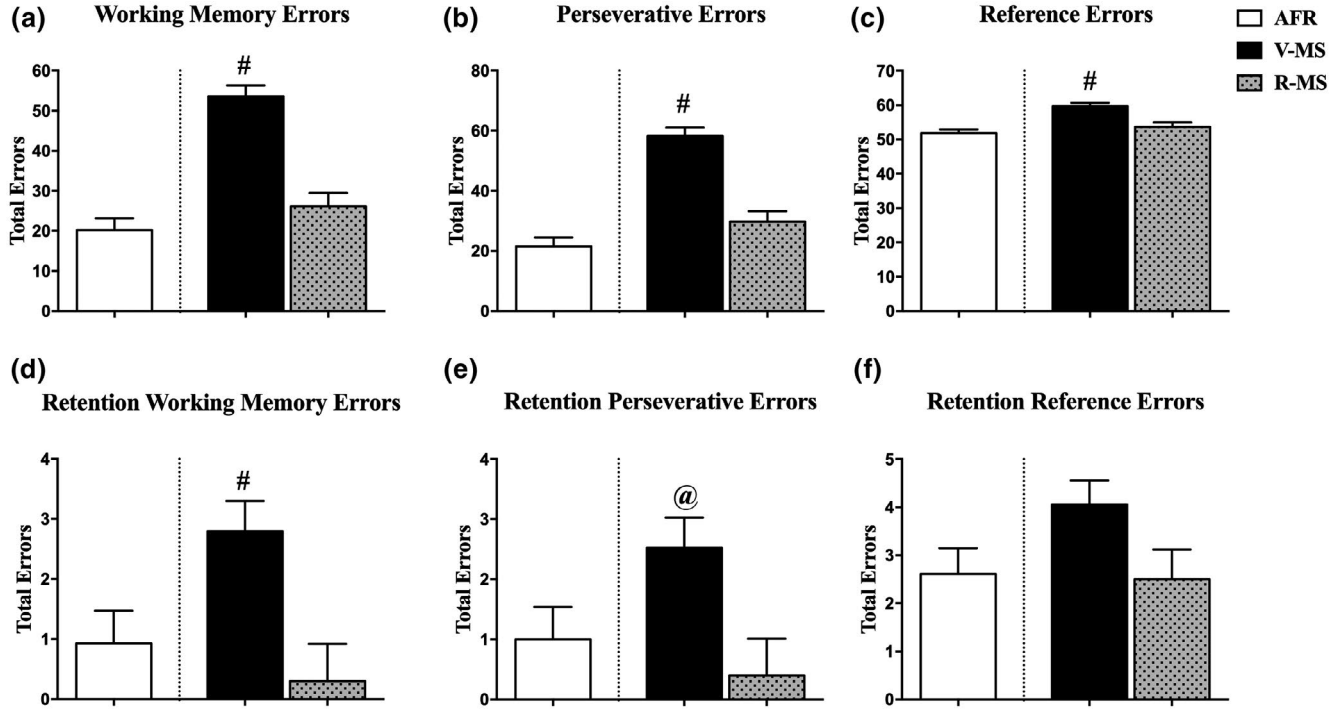

FIGURE 2 V-MS × R-MS analysis on Eight-arm Radial Maze parameters. (a) Number of total working memory errors; (b) Number of total perseverative errors; (c) Number of total reference errors; (d) Number of total retention working memory errors; (e) Number of total retention perseverative errors; (f) Number of total retention reference errors; #$p < .00$, Post hoc analysis (Tukey post-hoc test) revealed significant differences between V-MS and both R-MS and AFR groups; @$p < .05$, Post hoc analysis (Tukey post-hoc test) revealed significant differences between V-MS and R-MS. AFR = Animal Facility Rearing, V-MS = Vulnerable Maternal Separation, R-MS = Resilient Maternal Separation. Results are expressed as the mean ± *SEM*

(block-1 > block-2, $p < .0033$ and block-3, $p < .0001$ and block-2 > block-3, $p < .0134$) and RE (block-1 > block-2, $p < .0466$ and block-3, $p < .0004$), indicating that animals were able to reduce the number of errors from the block-1 to block-3. Post hoc analysis for group effect showed significant differences in TIME (V-MS > R-MS, $p < .0002$ and V-MS > AFR, $p < .0013$), revealing that V-MS group spent more time to complete the task compared to R-MS and AFR groups, and significant differences in WME (V-MS > R-MS $p < .0001$ and V-MS > AFR, $p < .0001$); PSE (V-MS > R-MS $p < .0001$ and V-MS > AFR, $p < .0001$) and RE (V-MS > R-MS $p < .0071$ and V-MS > AFR, $p < .0001$), indicating that V-MS group had higher number of errors than R-MS and AFR groups.

3.4 | V-MS × R-MS molecular analyses

Next, we investigated the plasma levels of corticosterone (CORT) as a proxy for hypothalamic pituitary adrenal (HPA) activity. Our comparative analysis revealed a significant difference between groups ($F = 7.83$, $df = 2, 32$, $p < .0002$). Post hoc analysis showed that CORT levels were significantly increased in the R-MS group, when compared to the AFR group (*R-MS* vs. *AFR*, $p < .0013$) No statistical differences were observed between the V-MS and AFR ($p = .0915$) or R-MS ($p = .1128$) groups (Figure 4). Our transcriptional analyses found significant differences between groups with respect to the mRNA levels of *DRD1* ($F = 5.09$, $df = 2, 17$, $p < .0218$) and *DRD2* ($F = 11.51$, $df = 2, 17$, $p < .0012$). Post hoc analysis revealed a significant increase in *DRD1* mRNA levels in the V-MS group, in comparison with the R-MS group (*V-MS* vs. *R-MS*, $p < .0221$), and in *DRD2* mRNA expression, in comparison with both R-MS and AFR groups (*V-MS* vs. *R-MS*, $p < .0038$ and *V-MS* vs. *AFR*, $p < .0016$). No differences among the experimental groups were found for *DRD3* ($p = .640$) and *Hers1* ($p = .6688$) mRNA levels (Figure 5).

3.5 | Correlational analyses

Finally, we performed Pearson correlation analyses with all 8-RAM parameters (WMEs, PSEs and REs) in both testing and retention phases, plasma CORT levels and the mRNA levels for *DRD1*, *DRD2*, *DRD3* and *Hers1*. All correlations are presented in Table 1. We found a significant positive correlation between both mRNA *DRD1* and *DRD2* for WME and PSE errors (Figure 6). mRNA *DRD1* was

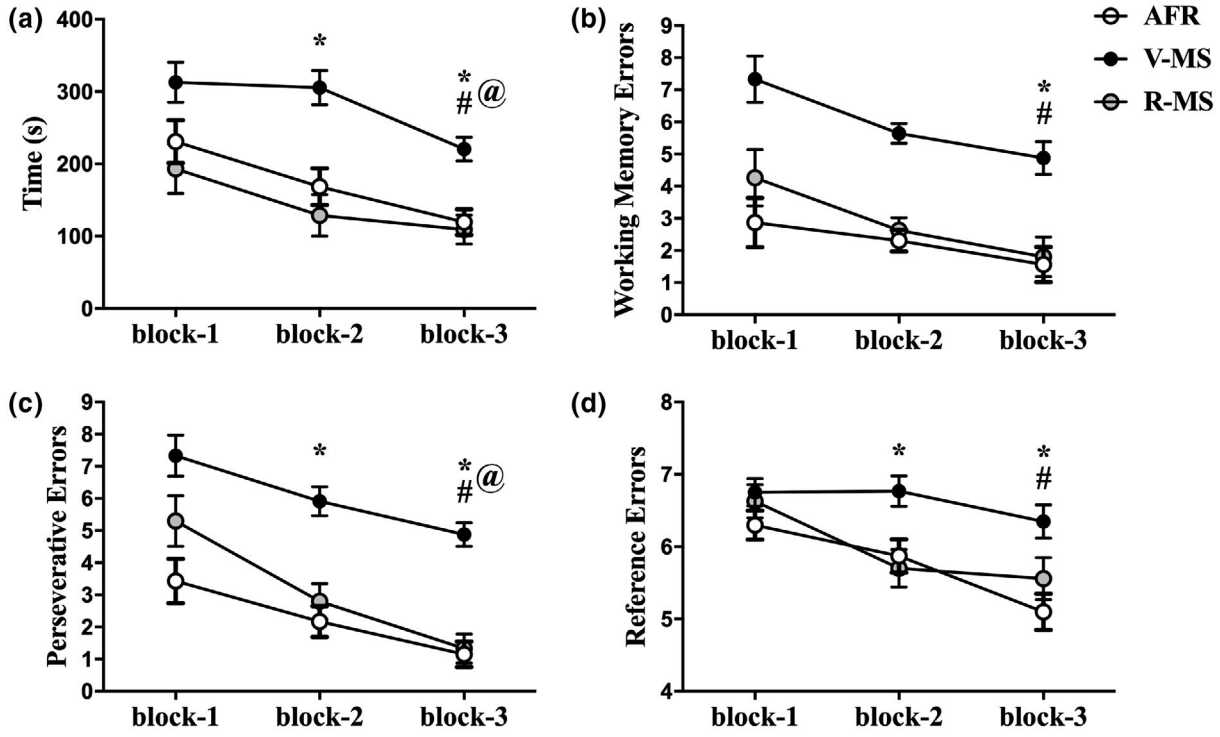

FIGURE 3 Days-block analyses on Eight-arm Radial Maze parameters. (a) Time spent to collect all the rewards; (b) Number of total working memory errors in each block; (c) Number of total perseverative errors in each block; and (d) Number of total reference errors in each block; Repeated measure one-way ANOVA showed significant block effect (TIME: $p < .00$, WME: $p < .00$, PSE: $p < .00$, RE: $p < .00$) and group effect (TIME: $p < .00$, WME: $p < .00$, PSE: $p < .00$, RE: $p < .00$]. AFR = Animal Facility Rearing, R-MS = Resilient Maternal Separation, V-MS = Vulnerable Maternal Separation. Results are expressed as the mean ± *SEM*

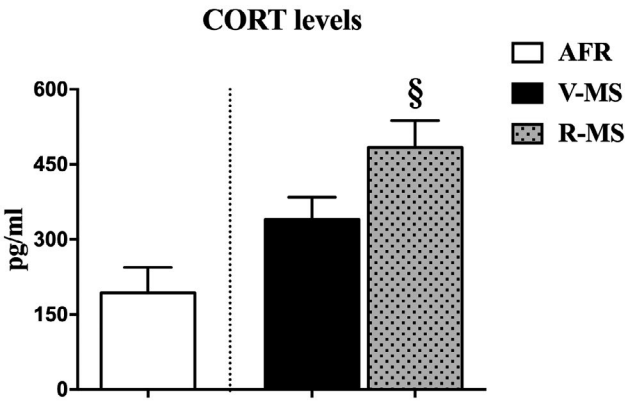

FIGURE 4 V-MS × R-MS analysis on plasma corticosterone (CORT) levels. §$p < .00$, significant groups effect, pos hoc analysis (Tukey post-hoc test) revealed significant differences on CORT levels between R-MS and AFR groups. AFR = Animal Facility Rearing, R-MS = Resilient Maternal Separation, V-MS = Vulnerable Maternal Separation. Results are expressed as the mean ± *SEM*

positively correlated with WME errors ($r = .53$, $p < .0216$) and PSE errors ($r = .53$, $p < .0234$) (Figure 6a) and mRNA *DRD2* was also positively correlated with WME errors ($r = .51$, $p < .0291$) and PSE errors ($r = .61$, $p < .0067$) (Figure 6b).

4 | DISCUSSION

This study investigated the effects of MS on working memory functioning and on DA receptors (*DRD1*, *DRD2* and *DRD3*) and *Hers1* mRNA expression in the mPFC, assessing the potential differences among maternally separated animals, as we were interested in determining how these animals could be differentially affected by ELS. Our findings revealed that, as expected, MS led to impaired working memory and reference memory in 8-RAM considering the number of total errors committed along all testing days. This result is consistent with prior studies showing that different ELS models can disrupt working memory and memory processes (i.e., consolidation and retrieval processes), through functional alterations of specific brain regions (Cattaneo et al., 2019; Chocyk et al., 2013; de Azeredo et al., 2017; Grassi-Oliveira et al., 2016; Jin et al., 2013; van der Kooij, Grosse, Zanoletti, Papilloud, & Sandi, 2015; Liu, Atrooz, Salvi, & Salim, 2017; Mehta & Schmauss, 2011). However, not all MS animals presented the same pattern of cognitive performance. In fact, there was a specific subgroup of MS animals that showed similar performance as controls, while another subgroup showed worse performance on the task.

Differences in stress responses between subjects exposed to stress paradigms have already been demonstrated by some

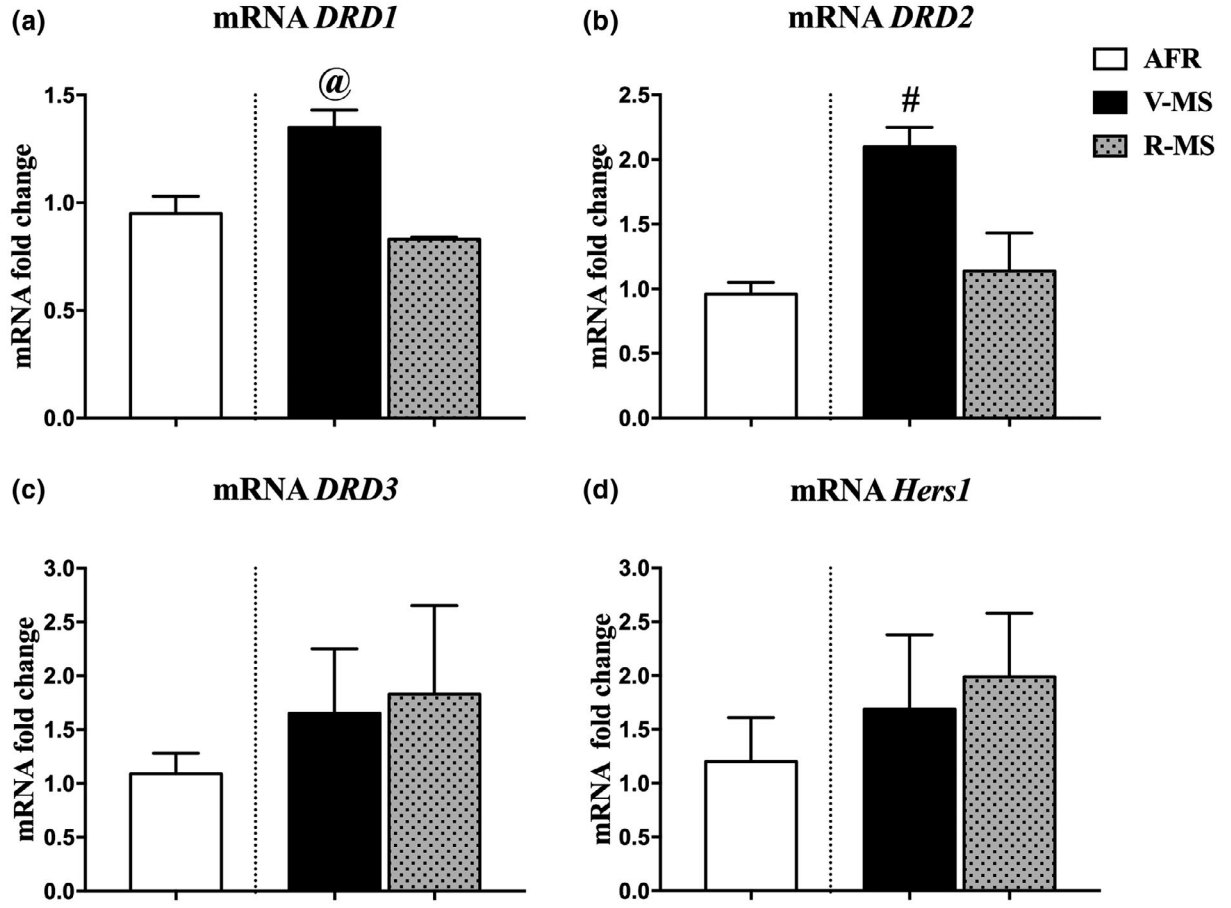

FIGURE 5 V-MS × R-MS analysis on mRNA dopamine receptor and Hers1 expression. (a) Drd1 mRNA levels; (b) Drd2 mRNA levels; (c) Drd3 mRNA levels; (d) Hers1 mRNA levels; @$p < .05$, Post hoc analysis (Tukey post-hoc test) revealed significant differences between V-MS and R-MS; #$p < .01$, Post hoc analysis (Tukey post-hoc test) revealed significant differences between V-MS and both R-MS and AFR groups. AFR = Animal Facility Rearing, R-MS = Resilient Maternal Separation, V-MS = Vulnerable Maternal Separation. Results are expressed as the mean ± *SEM*

TABLE 1 Pearson's correlation index between errors in 8-RAM and gene expression (*DRD1*, *DRD2*, *DRD3* and *Hers1*) in medial prefrontal cortex (mPFC)

	WME		PSE		REF	
	r	*p*	*r*	*p*	*r*	*p*
DRD1	**.53**	**.022***	**.53**	**.023***	.27	.27
DRD2	**.51**	**.029***	**.61**	**.007***	.23	.34
DRD3	−.09	.70	.13	.59	−.09	.71
Hers1	−.02	.93	.33	.18	.40	.10
Plasma CORT	.12	.47	.09	.59	.04	.80

Note: *r* = Pearson's correlation index; *p* = significance level; *statistically significant.

studies (Aydin, Frohmader, & Akil, 2015; Calabrese et al., 2017; Clinton et al., 2014; Rana et al., 2016; Spencer-Segal & Akil, 2019) and considering our data for distinct patterns of cognitive performance within the MS group, we focused on comparisons between the responsive and non-responsive MS animals (V-MS × R-MS) for all parameters of 8-RAM. Our data revealed that for all parameters of 8-RAM (WMEs, PSEs and REs), the V-MS group had a higher number of errors compared to the R-MS and control groups. The same results were found for the retention test (24 hr after), except for retention reference errors. Although V-MS animals were able to learn and reduce the number of errors and time spent to collect the rewards over the testing period, showing an improvement of their performance along the trials, the V-MS animals performed worse than resilient and control animals. This indicated that V-MS generally still showed a significant impairment on working memory processes compared to other groups. Such deficits probably narrow the ability to store essential information for learning and to move from goal-directed behavior to habit-directed behavior (Puig et al., 2014; Wickens, Horvitz, Costa, & Killcross, 2007), especially because they had higher number of perseverative errors. Thus, collectively, our findings indicate that not all maternally separated animals were equally affected by the stressor early in life, suggesting a discriminative cognitive

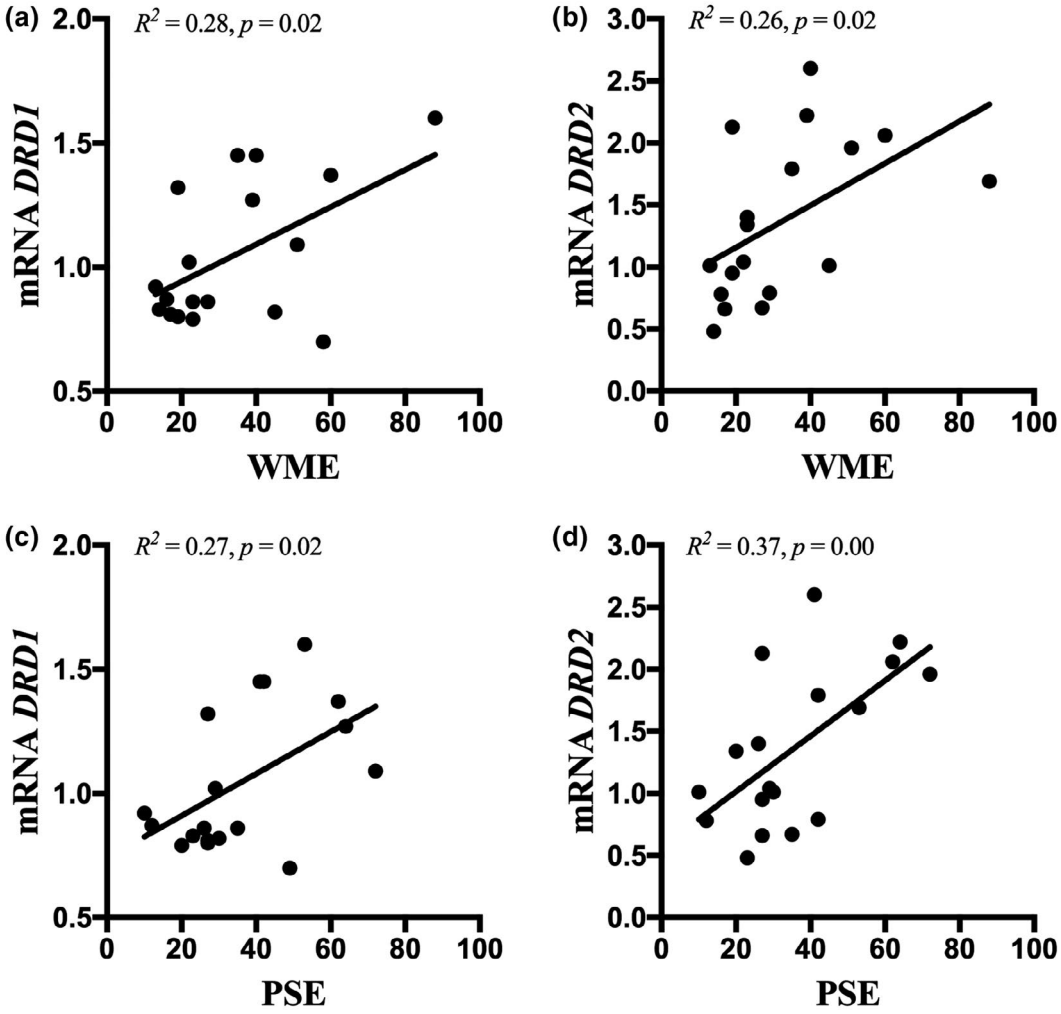

FIGURE 6 Correlational analyses between Eight-arm Radial Maze parameters and mRNA DRD1 and DRD2 expression. (a) Positive correlation between number of total working memory errors and DRD1 mRNA expression; (b) Positive correlation between number of total working memory errors and DRD2 mRNA expression; (c) Positive correlation between number of total perseverative errors and DRD1 mRNA expression; (d) Positive correlation between number of total perseverative errors and DRD2 mRNA expression

phenotype between vulnerable and resilient subgroups for ELS effects.

Because the biomolecular targets underlying the distinct patterns of response to early stress exposure are not fully elucidated, we decided to investigate the potential mechanisms that may contribute to the differential cognitive outcomes observed in V-MS and R-MS mice. We investigated the mRNA levels of DA receptors (*DRD1*, *DRD2* and *DRD3*) and *Hers1*. Our findings revealed alterations in DA receptors mRNA expression, especially in *DRD1* and *DRD2*. *DRD1* and *DRD2* mRNA expression were found higher in V-MS groups compared to the R-MS or AFR groups. Our correlational analysis revealed associations between different types of task errors (WMEs and PSEs) and *DRD1* and *DRD2* mRNA expression, corroborating the idea that DA receptors expression could regulate cognitive performance (Knowles, Mathias, et al., 2014; Murphy et al., 1996). Both *DRD1* and *DRD2* have been suggested as important contributors to several cognitive functions, including working memory, decision making, cognitive flexibility and learning processes under certain circumstances (Puig et al., 2014). Regarding their action on memory function, for example, it was demonstrated that both are essential to initial phases of learning processes and for transition from goal-directed behavior to habit-directed behavior during tasks with multiple trials (Mohebi et al., 2019; Puig et al., 2014; Vijayraghavan et al., 2007).

The overexpression of DA receptors in PFC and PFC-related networks has been suggested as a potential contributor for the inverted-U shape relationship between dopaminergic transmission and cognitive efficiency (Vijayraghavan et al., 2007). An excessive increase of activity in dopaminergic system was suggested as able to negatively impact PFC-dependent cognitive functioning (Azadmarzabadi, Haghighatfard, & Mohammadi, 2018; Murphy et al., 1996; Vijayraghavan et al., 2007). When *DRD1* agonists was administered in animals during working memory task, for example, it was observed

suppressive effects on dopaminergic neuronal firing in PFC in association with performance deficits (Vijayraghavan et al., 2007). Altered behavior phenotype was also found in a recent study from our lab (Wearick-Silva et al., 2019) in association with upregulation of *DRD1* in animals exposed to ELS through Limited Bedding protocol. It suggests that stress negative effects could be shape the brain development through dopamine system contributing for behavioral dysfunctional outcomes later in life. Thus, the remarkable deficits on working memory, cognitive flexibility and memory retention found in V-MS animals on 8-RAM could be related to *DRD1* and *DRD2* mRNA overexpression in response to ELS exposure. All these evidences of DA receptors expression changes and its relationship with behavioral and cognitive deficits could highlight the effects of potential genetic variants and/or epigenetic modifications in DA pathways, supporting potentials biomolecular markers of vulnerability characterized by DA expression (Vijayraghavan et al., 2007).

In addition, animal data (Fuke et al., 2006; Jenni et al., 2017; Puig & Miller, 2015; Vijayraghavan et al., 2007) have been found depletion on *Hers1* gene expression associated with increased *DAT* and DA receptors gene expression. This upregulation response as consequence of *Hers1* changes could influence the sensitivity of DA transmission, affecting the animal's behavior on cognitive tasks (Fuke et al., 2006; Knowles, Mathias, et al., 2014). In our study, however, we did not find any differences between groups to substantiate the prior findings regarding the role of *Hers1* in DA receptor expression imbalance. Only *DRD1* and *DRD2* mRNA expression were found upregulated. Decreased DA concentrations in synaptic transmission, for example, are able to disrupt signaling transmission, provoking a regulatory response at DA receptor level, which could explain an upregulation of DA receptors gene expression (Fuke et al., 2006; Vijayraghavan et al., 2007).

The overactivation of DA receptors in the PFC combined with activation of stress-induced hormonal response, such as an increase in CORT levels following a stress exposure, also have been associated with working memory impairments (Garrido et al., 2013; Park et al., 2006; Williams & Castner, 2006). In our study, unexpectedly, CORT levels were significantly increased in the R-MS group and no correlation was found between CORT levels and DA receptor mRNA levels. Altered HPA response and glucocorticoid levels were identified among ELS studies, supporting the negative effects of stress factors during the early periods of neurodevelopment (Anacker, O'Donnell, & Meaney, 2014; Cohen et al., 2006; Frodl & O'Keane, 2013; Yehuda et al., 2010). Acute and chronic exposure to glucocorticoids produces distinct effects on the organisms. A short-term increase in glucocorticoid levels has already been demonstrated as a neurobiological positive adaptation associated with reduction of dysfunctional anxiety-like behavior response, promoting resilience (Spencer-Segal & Akil, 2019). In addition, CORT administration in rats before stress-induced tasks was associated with attenuation of anxiety and defensive response (Zohar et al., 2011), whereas a blunted CORT response has been related to an inadequate behavioral response (Cohen et al., 2006; Danan, Matar, Kaplan, Zohar, & Cohen, 2018). This could be the case of our R-MS animals that showed an elevation of CORT levels following the cognitive task, considering the fact that our analyses were carried out 30 min after the last day of testing, elevations in CORT levels in R-MS animals might promote certain cognitive performances, although the control animals showed lower CORT levels. As our V-MS responded differently, it could represent an abnormal acute stress-induced response and, consequently, be deleterious for cognitive adaptation to task demand. Because our study did not access basal CORT levels before the test beginning, it is difficult to establish how both maternally separated animals would chronically respond to stress, narrowing the extent of our conclusions.

Finally, it is important to note that our findings should be interpreted with some limitations. We based our cognitive evaluation in only 8-RAM at early adulthood and could not extend the conclusions to other cognitive domains and/or developmental time-point. However, the 8-RAM is considered a complex cognitive task that has been used in studies with rodents to evaluate multiple parameters (e.g., working memory, cognitive flexibility, spatial reference memory and learning; Churchwell & Kesner, 2011; Dudchenko, 2004), most of them recruiting more or less the activity of both mPFC and hippocampus. It is difficult to assume that this task is specific for evaluation of either PFC or hippocampus functionality, but some authors have been proposing a prominent role of one or another brain region depending on the type of error committed by the animal (Bilkey & Liu, 2000; Yoon, Okada, Jung, & Kim, 2008). For example, when the animals are trained (thought spatial stimulus) to make a choice response to get the reward and, after, have to keep this information over the time, a working memory demand are imposed. PFC play a critical role for attend this demand, while hippocampus is suggested to be more implicated in the learning and retrieval processes along the testing days (Yoon et al., 2008). It seems that both brain regions influence the performance but in different temporal domains, then damage to either structure are able to produce impairments. Studies in this line have been providing conclusions that instead a structural specificity, a functional interaction between both is required to perform this task (Matzel & Kolata, 2010; Reincke & Hanganu-Opatz, 2017). Further, we just investigate molecular targets looking at the gene expression layer. Although it represents an interesting exploratory screening, investigations using other techniques, (e.g., Western blotting and microdialysis) may be useful to address the proteins at cell-type level and the levels of dopamine while mice are performing the task, respectively, to better determine the function of the studied

brain areas. To improve the cause–effect relationship studies should also explore specific receptor antagonists or agonists effects on dopaminergic function. In the same line, using a free-hand technique for brain dissection, we cannot be precise at subregion analysis level, which should be considered when generalizing the molecular results.

5 | CONCLUSION

Our results showed that V-MS had poor performance on 8-RAM and that such cognitive deficits were associated with significant changes in the DA receptor expression. We suggest that dopaminergic signaling in PFC could be a key player in the cognitive dysfunctions associated with this brain region in response to ELS exposure. Future studies should focus on improving our understanding of the role of dopamine signaling in cognitive and behavioral response to ELS, addressing potential biomolecular markers that could better elucidate how ELS affects dopaminergic signaling functionality during development and represent a susceptibility factor to neuropsychiatric diseases. In addition, such methodological approach could provide new insights into individual potential neurobiological distinctions than can bias the subjects toward vulnerability and resilience. Here, as an example, we highlighted the role of dopaminergic receptor expression changes in animals that revealed a vulnerable cognitive phenotype on a PFC-dependent task.

ACKNOWLEDGEMENTS

We would like to thank Emerson S. Hoffmann who helped in initiating the experimental procedures during his period at the Developmental Cognitive Neuroscience Lab.

CONFLICT OF INTEREST

The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS

S.G. Tractenberg, L.M.C. Malcon and F.S. Lumertz conducted stress experimental procedures. S.G. Tractenberg, R. Orso and K.C. Creutzberg conducted 8-RAM protocol. K.C. Creutzberg, L.E. Wearick-Silva and T.W. Viola ran and interpreted biomolecular analyses. S.G. Tractenberg, R. Orso and R. Grassi-Oliveira were the major contributors in writing and T.W. Viola, M.A. Riva and R. Grassi-Oliveira revised the final version of the manuscript. All authors declared that they read and approve manuscript final version.

ETHICS APPROVAL STATEMENT

This research was approved by the Ethics Committee on Animal Use (CEUA) of PUCRS, Brazil under the registration #14/00421.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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How to cite this article: Tractenberg SG, Orso R, Creutzberg KC, et al. Vulnerable and resilient cognitive performance related to early life stress: The potential mediating role of dopaminergic receptors in the medial prefrontal cortex of adult mice. *Int J Dev Neurosci*. 2020;80:13–27. https://doi.org/10.1002/jdn.10004