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Environmental enrichment improves lifelong persistent behavioral and epigenetic changes induced by early-life stress

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Abstract

This study aimed to evaluate the effects of environmental enrichment (EE) in Wistar rats subjected to maternal deprivation (MD). MD was performed in the first **post-natal days (PND) ten** for 3 h/day. The groups were: control; deprived without EE; and deprived with EE. The EE was applied for 3 h/day. Forced swimming test (FST) and open field test were performed, and histone deacetylase (HDAC) and DNA methyltransferase (DNMT) activities in the prefrontal cortex (PFC) and hippocampus were evaluated on 31, 41, and 61 PND. MD altered spontaneous locomotor activity and immobility time in FST, but the effects were sex- and developmental period dependent. In deprived females at **PND 31, 41, and 61,** HDAC and DNMT increased in the PFC and hippocampus. In females exposed to EE for 20 days, there was a decrease of HDAC in the hippocampus and DNMT in the PFC and hippocampus. Exposure of females to EE for 40 days can reverse HDAC and DNMT increase in all brain areas. In deprived **males** at **PND 31, 41, and 61,** HDAC and DNMT increased in the hippocampus, and in the group exposed to EE for 40 days,

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Author contributions

All authors participated in the design and interpretation of the studies, analyzed the data and review of the manuscript; ABM performed maternal deprivation protocol. LAB, MEMB, ABM, and JPB performed EE protocol, behavioral tests and the sample collecting. AH, LHK, and SSV performed the epigenetic analysis. SSV, GZR, and COA performed statistical analysis. LMM, LDRB, LAB, and GZR wrote the manuscript. JQ reviewed the manuscript. GZR did the design of experiment and reviewed the manuscript.

Authors statement

None.

Declaration of competing interest

None.

there was a decrease in hippocampal activity. In PFC of **male** deprived rats at PND 61 and EE for 40 days, there was a reduction of HDAC and DNMT. MD induced lifelong persistent behavioral and epigenetic changes, and such effects were more evident in female than male rats. EE can be considered **an essential** non-pharmacological strategy to treat long-term trauma-induced early life changes.

Keywords

Epigenetics; Maternal deprivation; Environmental enrichment; Sex difference; Major depressive disorder

1. Introduction

Major depressive disorder (MDD) is a highly debilitating mental disorder whose etiology can be attributed to a combination of genetic predisposition and life events, mainly exposure to stress. This mood disorder has a global reach, affecting people of all ages, races, and economic conditions. Several clinical symptoms may be present, including emotional, neurovegetative, and cognitive symptoms (Malhi and Mann, 2018).

The pathophysiology of this mood disorder is complex. **It can** involve several factors, including a decrease in monoamine (serotonin, dopamine, and norepinephrine) levels, decrease in neurotrophic factors, deregulation in the hypothalamic-pituitary-adrenal (HPA) axis, increase in glutamate (the **primary** excitatory neurotransmitter in the central nervous system), increase in neuroinflammation and oxidative stress (Beurel et al., 2020; Czarny et al., 2018; Levy et al., 2018; Malhi and Mann, 2018; Reus et al., 2017). There is also a growing interest in the role of epigenetics in MDD (Nestler, 2014; Peña and Nestler, 2018; Talarowska, 2020; Torres-Berrío et al., 2019).

Epigenetics refers to the study of mechanisms that control gene expression in a potentially heritable way. Epigenetic modifications can be grouped into three main categories: DNA methylation, histone modifications, and nucleosome positioning (Portela and Esteller, 2010). Here, it is worth mentioning the first two categories. DNA methylation occurs in a cytosine residue almost exclusively in the context of the CpG islands. CpG-island methylation is mediated by the DNA methyltransferase (DNMT) family of enzymes that catalyze **a methyl group's transfer** from S-adenosyl methionine to DNA**, and** this methylation is associated with gene silencing (Kacem and Feil, 2009; Portela and Esteller, 2010). Several post-transcriptional modifications occur in histone, including acetylation. Acetylation of lysine residues is highly dynamic and regulated by the opposing action of two families of enzymes, histone acetyltransferases and histone deacetylases (HDACs), that add and remove acetyl groups, respectively. Noteworthy, HDACs are predominantly transcriptional repressors (Bannister and Kouzarides, 2011). Interestingly, a growing body of evidence has suggested that epigenetic information may be transferred across generations (Chong et al., 2007; Gertz et al., 2011).

A growing body of evidence has suggested that epigenetic changes may be one of the mechanisms by which stressors interact with the genome, leading to **DNA structure**

changes, gene expression, and behavior (Park et al., 2019; Turecki and Meaney, 2016). Literature has **focused on** early life stress, including child maltreatment, parent neglect, undernutrition, or sexual abuse. Early life stress increases depressive disorders in later life and, at least in part, epigenetic mechanisms may be involved in the consequences of this early stress (Heim and Binder, 2012; Li et al., 2020; Torres-Berrío et al., 2019). Interestingly, researchers suggest that epigenetic mechanisms involved in early life stress can contribute **to** the development of new therapeutic interventions (Torres-Berrío et al., 2019). Moreover, there is also a growing interest in the sex differences in the **brain's epigenetic** (Gilbert et al., 2019; Hodes, 2013).

Studies evidenced that early maternal separation or maternal deprivation (MD) in rodents is a traumatic event that induces depressive-like behavior in adulthood (El Khoury et al., 2006; Lee et al., 2007; Réus et al., 2011; Vetulani, 2013). On the other hand, **an enriched environment** (EE) reduced depressive-like behaviors in stressed rodents (Seong et al., 2018; Shilpa et al., 2017). A growing body of evidence has suggested that EE can cause numerous benefits to the brain, including effect directed to the hippocampus, stimulates adult hippocampal neurogenesis, promotes the survival of adult-born neurons in the dentate gyrus, **improving** learning and memory, reduces anxiety, **influencing neurotransmitters systems**, such as serotonin and acetylcholine, increased neurotrophic factors, and others (Kempermann, 2019; Simpson and Kelly, 2011; van Praag et al., 2000). **Moreover, EE is a crucial experimental paradigm to understand the relationship between genes and the environment and how this interaction changes the brain's structure and function across an animal's lifespan** (Kempermann, 2019).

Thus, **this study aimed** to evaluate the activity of the HDAC and DNMT in the prefrontal cortex (PFC) and hippocampus at different **development stages** of male and female rats discovering MD and exposed to EE.

2. Methods

2.1. Animals

Female Wistar rats with **three** months of age and weighing 250–280 g were obtained from the breeding colony of Universidade do Extremo Sul Catarinense (UNESC, Criciúma, SC, Brazil) and were housed for one week in the presence of males for mating purposes. At the end of 7 days, the pregnant rats were housed individually with *ad libitum* access to food and water. The pregnant rats were housed individually for the birth of the pups and their identification. All mothers and pups were kept on a 12-h light/dark cycle (06:00 a.m. to 06:00 p.m.) at a temperature of 23 ± 1 °C. **One day after birthing occurred, the maternal deprivation protocol was applied to a percental of male and female pups from days 1–10 after birth (deprived); other males and females were used as controls (non-deprived).**

The choice of animals from birth to compose the control or deprived group was random based on the sample n for each group required until the end of each experiment. That is, **three** groups that would make up the control groups in each phase, 31, 41, and 61 days (n = 12–15 per phase) a total of approximately 72 animals (controls: males and females) and

six groups that would make up the deprived groups in each phase, 31, 41, and 61 days (n = 12–15 per phase), these **six** groups being divided as follows: 1) deprived without enrichment (3 groups) and 2) deprived plus enrichment (3 groups), adding up to approximately 144 animals (deprived: males and females). The average number of births is **eight** pups per litter, half male and half female. On the third day after birth, sexes were identified, and the distribution was organized according to the necessary sample at the end of the experiment.

All experimental procedures involving animals were performed according to the NIH Guide for the Care and Usage of Laboratory Animals within the Brazilian Society for Neuroscience and Behavior recommendations for animal care. The ethics committee approved the experimental protocol from UNESC under protocol number: 070/2018–1.

2.2. Maternal deprivation (MD)

The deprivation protocol consisted of removing the mother from the residence box and taking her to another room. Removing the mother from the original box consisted of placing her in a new box with new wood shavings **and** some of the wood shavings from the original box. Carefully by a trained researcher and with a minimum of noise, the box with the mother was taken to a nearby room with controlled temperature and humidity, as appropriate. The pups were maintained in their home cage (grouped in the nest in the presence of maternal **odor**). The pups were deprived of the mother for 3 h per day during the first **ten** days (Fig. 1). We prefer this protocol because it does not require **manipulating** the pups (Ignácio et al., 2017; Kosten et al., 2007; Mello et al., 2009; Réus et al., 2017). At the end of each daily deprivation session, the mothers were returned to their home boxes; this procedure was carried out during the light part of the cycle, between 8:00 a.m. and 12:00 p.m. The control rats (non-deprived) remained in their resident boxes together with their mothers throughout the experiment. **After MD protocol,** pups remained with their mothers for 21 days when the pups were weaned. Then the animals were again divided into new experimental groups: 1) non-deprived (control); 2) deprived + without EE, and 3) deprived + EE. Individual groups of rats (male and female) were evaluated at different periods of development after **post-natal** days (PND) 31, 41, and 61 (n = 4–5 animals/group for each stage of development: n = 12 = 15 for males and n = 12–15 for female). In the different **development stages** and the different experimental groups, behavioral (n = 12 = 15 for males and n = 12–15 for **females**) and epigenetic (n = 4 = 5 for males and n = 4–5 for **females**) tests were performed as described in the methods section.

2.3. Environmental enrichment (EE) and experimental groups

EE began after weaning (Barichello et al., 2014; Pereira et al., 2007; Rojas et al., 2013). Before starting exposure to EE, all animals in each box were identified with marking on the tails using non-toxic colored pens. During exposure to EE, **ten** animals were placed each time in the apparatus. Animals that were not exposed to EE were in the original boxes but in the same behavioral room in which the EE procedure was performed. For the EE procedure, **the deprived** + EE group was exposed to the EE for 3 h daily, for different periods. One group of animals was exposed to EE for 40 days, another group for 20 days**, and** a third for **ten** days. Each of these groups had experimental groups 1 (control) and 2 (deprived) for each **development stage**. More details can be seen in Fig. 1. EE consisted of a large cage

(40 × 60 × 90 cm) with three floors, ramps, running wheels, and several objects of different shapes and textures. **Minor** changes were made once a week by adding new objects and withdrawing others (Ohlsson and Johansson, 1995; Pereira et al., 2007). The running wheels and stairs enhanced voluntary exercise, a seesaw provided somatosensory stimulation, and large tubes, a set of tunnels, LEGOH blocks, wood pieces, and hanging items provided cognitive stimulation (Nithianantharajah and Hannan, 2006).

2.4. Open field test

The rats were exposed to the open field apparatus **to** assess the possible effects of maternal deprivation and/or EE on spontaneous locomotor activity. This apparatus consisted of a brown plywood arena 45 × 60 cm surrounded by wooden walls 50 cm high and containing a frontal glass wall. **The open field floor is divided by black lines into nine rectangles** (15 × 20 cm each). The animals were gently placed in the left rear quadrant and left to explore the arena for 5 min (training session). Any crossing (frequency with which the mice crossed one of the grid lines with all four paws) and rearing (frequency with which the mice stood on their hind legs in the maze) performed in both sessions was counted (Brown et al., 1999).

2.5. Forced swimming test (FST)

According to previous reports, the FST was conducted (Porsolt et al., 1977; Réus et al., 2015). In this study, the FST was carried to evaluate depressive-like behavior associated with MD and antidepressant-like effects of EE. Rats from all groups were individually placed in a cylinder containing water for 15 min (pre-test session) at a temperature around 25 °C. Twenty-four hours after the pre-test session, the rats were subjected to the FST for a 5-min session (test session) to evaluate the immobility time, which is reported in second.

2.6. Histone deacetylase and DNA methyltransferase activities analysis

The PFC and hippocampus were rapidly frozen and stored at −80 °C until nuclear proteins were extracted. The tissue samples from PFC and hippocampus were frozen at −80 °C **and subjected** to a nuclear extraction protocol from a Nuclear Extraction kit (Sigma, St Louis, USA). Briefly, samples were homogenized in cytoplasmic lysis buffer containing dithiothreitol (DTT) and protease inhibitors. The suspension was kept on ice for 15 min and then centrifuged at 250×*g* for 5 min at 4 °C. The supernatant containing the cytosolic fraction was discarded, and the pellet was resuspended in two volumes of cold cytoplasmic lysis buffer. The suspension was homogenized using a syringe with a small gauge needle and centrifuged at 8000×*g* for 20 min at 4 °C. The pellet was resuspended in an extraction buffer containing DTT and protease inhibitors. The resulting sample was kept in slow agitation for 30–60 min in an orbital shaker at 4 °C. After, the nuclear suspension was centrifuged at 16,000×*g* for 5 min at 4 °C **, and** the supernatant containing the nuclear extract was transferred to another tube and stored at −80 °C until further analysis. Nuclear extracts from the PFC and hippocampus were subjected to the HDAC and DNMT enzymatic activity assay (n = 4–5 for each brain region and enzyme) **using HDAC DNMT Assay kits** (Colorimetric Detection) according to the manufacturer's instructions. Briefly, nuclear extract samples were mixed with HDAC (EPIGENTEK; Base catalog #P-4034) or DNMT (EPIGENTEK; Base catalog #P-3009) assay buffer, more assay substrate of the same enzymes, in a 96 well

plate and incubated at 30 °C for 45 min. Concomitantly, a standard curve was performed with serial dilutions of HDAC or DNMT substrates, as also positive and negative controls were added to the plate. **Afterward**, the developer solution was added to the wells, and the plate was incubated at room temperature for 15 min. A colorimetric reading was performed on a fluorescence plate reader with 450 nm for HDAC and DNMT activities. Figures are expressed as OD/h/mg. OD is the reading value; h is related to incubation time, and mg is **enzyme amount. Each unit of analysis is a brain area of each rat.**

2.7. Protein dosage

Proteins were measured according to the method of Lowry et al. (1951). Bovine serum albumin was used as a standard.

2.8. Statistical analysis

The data are presented as mean ± standard error of **the mean** (SEM). Differences among experimental groups in the assessment of behavior and epigenetic were determined by one-way ANOVA, followed by Tukey post-hoc test when ANOVA was significant. **Two-way ANOVA determined differences between sex and groups interaction.** P values < 0.05 were considered to be statistically significant.

3. Results

3.1. Locomotor activity of MD and EE in the open field test

The spontaneous locomotor activity is illustrated in Fig. 2. At **PND 31** it was observed a reduction in the crossings number in deprived, compared to control and deprived + EE by **ten** days group, compared to control and deprived females ($F_{2–45} = 9.884$; $p < 0.0001$; Fig. 2A) and an increase in the crossings number in deprived, compared to control and deprived + EE by **ten** days group, compared to control and deprived in males ($F_{2–42} = 19.564$; $p < 0.0001$; Fig. 2B). Rearings number was not change in female rats at **PND 31** ($F_{2–45} = 2.500$; $p = 0.094$; Fig. 2A) and male rats ($F_{2–42} = 1.027$; $p = 0.367$; Fig. 2B). Two-way ANOVA revealed differences for sex and groups interaction in the crossings number at **PND 31** ($F_{2–67} = 5.806$; $p = 0.004$). **Effects were observed for sex in the control groups and in the rearings number** ($F_{2–67} = 3.444$; $p = 0.037$). Effects were observed for sex in the control groups, maternal deprivation, and EE exposure.

At **PND 41** there was a decrease in the crossings number in females exposed by EE by 20 days ($F_{2–36} = 6.610$; $p = 0.004$; Fig. 2C). Rearings number was not change in females at **PND 41** ($F_{2–36} = 0.539$; $p = 0.588$; Fig. 2C). In male at **PND 41** spontaneous locomotor activity was not change, crossings ($F_{2–37} = 0.580$; $p = 0.565$; Fig. 2D) and rearings ($F_{2–37} = 1.587$; $p = 0.219$; Fig. 2C). Two-way ANOVA did not revealed differences for sex and groups interaction in the crossings ($F_{2–69} = 2.067$; $p = 0.135$) and rearings numbers ($F_{2–72} = 0.337$; $p = 0.714$) at **PND 41**.

In female at **PND 61** there was an increase in the crossings number of deprived groups ($F_{2–40} = 5.853$; $p = 0.006$; Fig. 2D) and in the rearings number of deprived + EE by 40 days ($F_{2–40} = 5.465$; $p = 0.008$; Fig. 2D). In males at **PND 61** spontaneous locomotor activity

was not change in any group, crossings (F 2–39 = 0.395; p = 0.677 Fig. 2E) and rearings (F 2–39 = 0.902; p = 0.414; Fig. 2E). Two-way ANOVA did not revealed differences for sex and groups interaction in the crossings (F 2–75 = 0.805; p = 0.450) and rearings numbers (F 2–72 = 2.113; p = 0.128) at **PND 61**.

3.2. Behavior effects of MD and EE in the forced swim test (FST)

The immobility time in the FST at PND 31 of deprived rats exposed to EE for **ten** days **is** present **in** Fig. 3A and Fig. 3B. No significant alterations were found in male and female rats when evaluated the immobility time. Female (F = 2–35 0.419; p = 0.661). Male (F 2–42 = 1.296; p = 0.287) (Fig. 3B). Two-way ANOVA did not revealed differences for sex and groups interaction on the immobility time at **PND 31** (F 2–57 = 0.535; p = 0.588).

At PND 41, in deprived female rats, an increase in the immobility time (F 2–40 = 10.608; p < 0.0001) **was found. In contrast, after the EE exposition by ten days, a decrease in female rats' immobility time was observed compared to the deprived female rats.** The present data imply the antidepressant EE effects in female animals (Fig. 3C). No alterations were identified between immobility (F 2–43 = 1.287; p = 0.258) from male rats groups (Fig. 3D). Two-way ANOVA did not revealed differences for sex and groups interaction on the immobility time at **PND 41** (F 2–76 = 0.535; p = 0.588).

At PND 61, in female deprived + EE group, a reduction in immobility time was observed compared to MD and control groups (F 2–34 = 10.078; p < 0.0001) (Fig. 3E). The male deprived rats showed an increase in the immobility time (F 2–26 = 6.494; p = 0.006) related to the control group. On the other hand, the EE exposition exhibited a reduction in this parameter comparing to the MD animal group (Fig. 3F). Two-way ANOVA revealed differences for sex and group interaction on immobility time in the at **PND 61** (F 2–56 = 3.984; p = 0.024). Effects were observed for sex in the control groups.

3.3. Effects of EE by ten days under the HDAC and DNMT activities in the PFC and hippocampus in female and male rats submitted to maternal deprivation

Fig. 4 shows the results of HDAC and DNMT activities in the PFC and hippocampus of female **rats submitted to** maternal deprivation early in life and exposed to EE for **ten** days. In females at **PND 31**, who were exposed to EE for **ten** days, an increase in HDAC activity was found in the PFC (F 1–12 = 958.226; p < 0.0001; Fig. 4A) and hippocampus of the group that was deprived, and remained elevated in the deprived group exposed to EE for **ten** days (F 1–12 = 1282.467; p < 0.0001; Fig. 4A). In the PFC of males at **PND 31** no changes were found in HDAC activity (F 1–12 = 675.435; p < 0.0001; Fig. 4A). However, in the hippocampus, HDAC activity increased in the deprived group and remained in the group exposed to EE for **ten** days (F 1–12 = 1715.520; p < 0.0001; Fig. 4A), compared with the control group. Two-way ANOVA revealed differences for sex and groups interaction on HDAC activity in the PFC at **PND 31** (F 2–23 = 16.169; p <0.0001). Effects were observed for sex in the maternal deprivation and EE exposure. **However, two-way ANOVA did not reveal differences for sex and group interaction on the HDAC activity at PND 31 (F 2–24 = 2.322; p = 0.119) in the hippocampus.**

In female rats at PND 31, an increase in DNMT activity was found when submitted to maternal deprivation**,** and in those exposed to EE for **ten** days, DNMT activity remained high, both in the PFC (F 1–12 = 1164.260; $p < 0.0001$; Fig. 4B) and in the hippocampus (F 1–12 = 1014.953; $p < 0.0001$; Fig. 4B). DNMT activity did not change in the PFC of male rats at **PND 31**. In the hippocampus, there was an increase in DNMT activity in animals exposed to maternal deprivation, and the EE for **ten** days was not able to reverse this change, keeping the DNMT activity high ($p < 0.05$; Fig. 4B). Two-way ANOVA revealed differences for sex and groups interaction on DNMT activity in the PFC at **PND 31** (F 2–23 = 9.073; $p = 0.001$). Effects were observed for sex in the maternal deprivation and EE exposure. **However, in the hippocampus, two-way ANOVA did not reveal differences for sex and group interaction on the DNMT activity at PND 31** (F 2–24 = 0.149; $p = 0.862$).

3.4. Effects of EE by 20 days under the HDAC and DNMT activities in the PFC and hippocampus in female and male rats submitted to maternal deprivation

In females at **PND 41**, HDAC activity increased in the PFC and hippocampus in the deprived group ($p < 0.05$; Fig. 5A), compared to the control group. However, there was a reduction in hippocampal HDAC activity in the group exposed to EE for 20 days in the hippocampus (F 1–12 = 809.736; $p < 0.0001$; Fig. 5A). In the PFC of males at **PND 41**, no changes were found in HDAC activity (F 1–12 = 493.229; $p < 0.0001$; Fig. 5A). However, in the hippocampus, HDAC activity increased in the deprived group and remained in the group exposed to EE for 20 days (F 1–12 = 887.170; $p < 0.0001$; Fig. 5A), compared with the control group. Two-way ANOVA revealed differences for sex and groups interaction on HDAC activity in the PFC (F 2–24 = 10.804; $p < 0.0001$) and hippocampus at **PND 41** (F 2–24 = 27.402; $p < 0.0001$). Effects were observed for sex in the maternal deprivation for PFC and EE exposure for **the hippocampus**.

In deprived females at **PND 41**, DNMT activity increased in the PFC and hippocampus. However, in groups exposed to EE for 20 days, DNMT activity was decreased in both brain structures (PFC: F 1–12 = 217.632; $p < 0.0001$; hippocampus: F 1–12 = 696.591; $p < 0.001$; Fig. 5B). DNMT activity did not change in the PFC of male rats at **PND 41**. In the hippocampus, there was an increase in DNMT activity in animals exposed to maternal deprivation, and the EE for 20 days was not able to reverse this change, keeping the DNMT activity high (F 1–12 = 414.9\07; $p < 0.0001$; Fig. 5B). Two-way ANOVA revealed differences for sex and groups interaction on DNMT activity in the PFC at **PND 41** (F 2–24 = 10.214; $p < 0.0001$). Effects were observed for sex in the maternal deprivation. In the hippocampus also were found differences for sex and **group** interaction on DNMT activity at **PND 41** (F 2–24 = 20.840; $p < 0.0001$). Effects were observed for sex in the EE exposure.

3.5. Effects of EE by 40 days under the HDAC and DNMT activities in the PFC and hippocampus in female and male rats submitted to maternal deprivation

Both in the PFC and in the hippocampus of deprived female rats at **PND 61**, an increase in HDAC activity was found, compared with the control group (F 1–12 = 748.697; $p < 0.0001$; F 1–12 = 396.995; $p < 0.0001$; Fig. 6A, respectively). On the other hand, EE for 40 days **could** reverse the increase in HDAC activity compared to the deprived group ($p < 0.05$; Fig. 6A). In male rats at PND 61, a decrease in HDAC activity was found in the

PFC in the deprived group exposed to EE for 40 days ($p < 0.05$; Fig. 6A), compared to the control group. In the hippocampus of male rats at **PND 61,** an increase in HDAC was found, whereas, in the group exposed to EE for 40 days, HDAC activity was reduced ($F_{1–12} = 336.699$; $p < 0.0001$; Fig. 6A). Two-way ANOVA revealed differences for sex and groups interaction on HDAC activity in the PFC at **PND 61** ($F_{2–24} = 19.794$; $p < 0.0001$). Effects were observed for sex in the controls, maternal deprivation**,** and EE exposure. **However, in the hippocampus, two-way ANOVA did not reveal differences for sex and group interaction on the HDAC activity at PND 61** ($F_{2–24} = 0.708$; $p = 0.502$).

In deprived females at PND 61, DNMT activity increased in the PFC and hippocampus. However, in groups exposed to EE for 40 days, DNMT activity was decreased in both brain structures (PFC: $F_{1–12} = 217.632$; $p < 0.0001$; hippocampus: $F_{1–12} = 696.591$; $p < 0.001$; Fig. 6B). In deprived male rats at **PND 61** and exposed to EE, there was a reduction in DNMT in the PFC, compared to the control group ($F_{1–12} = 382.105$; $p < 0.0001$; Fig. 6B). In the hippocampus of male rats at PND 61 and submitted to maternal deprivation, there was an increase in DNMT activity; however, in animals that were exposed to EE for 40 days, DNMT activity was reduced in the hippocampus ($F_{1–12} = 600.173$; $p < 0.0001$; Fig. 6B). Two-way ANOVA revealed differences for sex and groups interaction on DNMT activity in the PFC ($F_{2–24} = 4.246$; $p = 0.026$) and hippocampus ($F_{2–24} = 4.700$; $p = 0.018$) at **PND 61**. Effects were observed for sex in the maternal deprivation.

4. Discussion

In the present study, we showed that rats exposed to MD have behavior and epigenetic changes that were influenced according to sex and stage of life and that an EE can reverse most of these changes.

MD induced depressive-like behavior in female rats at PND 41 (adolescence stage) and male rats at PND 61 (adulthood). These results suggest that stressful events experienced during the postnatal period may have altered the **ordinary** course of neurodevelopment, resulting in depressive-like behavior later, especially in males. **A previous study by our research group, using the same MD protocol, also showed that this protocol was able to induce depressive-like behavior in males at PND 60 but not at PND 40** (Réus et al., 2017). In another study with MD (but with a different protocol from this study), it was observed that **male rats subjected** to early stress developed depressive-like behavior at PND 45–50 but not at PND 21–28 (Shepard et al., 2018). In another MD protocol, both male e female rats showed a depressive-like behavior at PND 52–60, with a more significant effect when the MD protocol was applied at PND 3 **than** PND 11 (Miragaia et al., 2018). Based on our data and literature findings, it is observed that early life stress can generate depressive-like behavior. **However**, these effects are influenced by the age and sex of the animals. Although the depressive-like behavior occurred at different stages of development between males and females, the statistical analyzes did not differ between the sexes, except in the control females at PND 61 who had a longer immobility time than males.

EE exerted an antidepressant-like effect on males and females at PND 41 and PND 61. Literature data evidenced that EE can ameliorate depressive behavior, memory impairment

and reduced anxiety behavior in rats exposed to chronic stress (Seong et al., 2018; Shilpa et al., 2017). In another study, 45 days of EE induced antidepressant-like behavior in controls and male rats exposed to MD (Gonzalez-Pardo et al., 2019´). There is also evidence that EE during the peripubertal period reverses the effects of maternal separation on both HPA axis and behavioral responses to stress (Francis et al., 2002).

It is worth mentioning that any manipulations that can affect general activity levels can potentially alter immobility, leading to false conclusions in the FST. It is essential to verify the FST results with isolated behavioral tests that measure general activity, as the spontaneous motor activity in the open field test (Can et al., 2011). In the present study, MD protocol altered spontaneous locomotor activity. The open-field test is one of the most used tests to assess locomotor activity, and several factors can influence the results, including gender (ambulation of female rats is usually higher than that of males), stress, among others (Sestakova et al., 2013). **It is worth mentioning that none of the open field test changes impact the results obtained in the FST.**

Our study contributes to the literature by demonstrating that early life stress causes epigenetic changes at different **development stages**, especially in female rats. A recent review evidenced that DNA methylation might be the essential link between the environment and gene expression in MDD and that DNMTs are involved in the pathogenesis of MDD, although there are still contradictory observations (Duan and Lu, 2020). Histone acetylation also seems to be involved with the pathophysiology of depression (Abel and Zukin, 2008; Sun et al., 2013).

It is **essential** to consider that MDD is more common in women than men, and it has been suggested that epigenetic changes may be responsible, at least in part, for these differences between the sexes (Hodes et al., 2017). Analyzing the differences between the sexes, a study with rodents that were submitted to prenatal stress, it was evidenced that the number of genes that **changed** gene expression was more in females than in males, and this difference occurred both in the hippocampus and frontal cortex (Van den Hove et al., 2013). Interestingly, exposure to subchronic variable stress increased mRNA levels for DNMT3a in the nucleus accumbens of both male and female mice. However, female mice had overall higher DNMT3a levels **than** male mice (Hodes et al., 2015). **On the other hand, a study only with males submitted to maternal separation (3 h for 13 days) observed an increase in DNMT3a expression, DNMT3a1 protein, and DNMT activities in the PFC at PND 15** (Urb et al., 2019). In contrast, male rats subjected to MD had increased DNMT activity in the hippocampus, but nor in the PFC at PND 63 (Ignácio et al., 2017). It is worth mentioning that DNMT expression differs during development in females and males, leading to feminization and masculinization of brain structures (McCarthy et al., 2009; Nugent et al., 2015).

A study with maternal separation in male mice was observed many modifications in neocortical HDAC mRNA expression, but not in the hippocampus (Levine et al., 2012), a different result from this study. However, it is worth noting that our study was evaluated the activity and not the expression of HDAC. On the other hand, chronic stress in male rats induced an increase in HDAC5 expression in the hippocampus (Liu et al., 2014). **Moreover,**

in male rats, MD increased HDAC and DNMT activities in the hippocampus but not in the PFC in the PND 63 (Ignácio et al., 2017). Still, as far as we are aware, this was the first study that investigated gender differences in HDAC in an animal model of depression. It is worth mentioning that HDAC activity during the early postnatal period is essential for the **brain's masculinization** (Matsuda et al., 2011) and that HDACs modulate and respond to sex steroid hormones (Zhao et al., 2010).

Comparing genders, females exposed to MD and **ten** days of EE have **more significant** activity of HDAC and DNMT in the PFC. Also, HDAC activity in PFC is **more remarkable** in females exposed to MD and 40 days of EE. In the hippocampus, females exposed to MD and 20 days of EE have less HDAC and DNMT activity. **It is suggested** that EE, especially in the long term (40 days), can prevent epigenetic changes caused by early life stress in female and male rats. **Also, in females, 20 days of EE is enough to prevent MD's epigenetic effects in the hippocampus.** Mice exposed for four weeks to EE during adolescence altered some emotionality-related behavior and glucocorticoid receptor mRNA expression in a sex-specific manner (Lin et al., 2011). In line with this, another study with mice also demonstrated that the effect of EE in the BDNF mRNA was different in males and females (Chourbaji et al., 2012).

Regarding the effects of EE in DNMT or HDAC, there are few studies. **In male rats exposed to 15 days of EE, an increase in DNMT1 expression in the hippocampus was observed** (Cao et al., 2017). **That** may be why in the present study, neither 10 nor 20 days of EE in male rats was able to reverse the increase in DNMT and HDAC that occurred in the hippocampus. On the other hand, in a transgenic murine model of Alzheimer diseases, adult female mice exposed to EE for **eight** weeks (56 days) had an increase in the mRNA expression of DNMT3a, DNMT3b, and HDAC1 in the hippocampus, but EE had no change the mRNA expression of DNMT1 and HDAC2 (Griñán-Ferré et al., 2018). This controversial result in our study may have occurred due to the model used (model of depression versus model of Alzheimer diseases), due to the moment of exposure to EE (postnatal versus adult), or even because the present study evaluated the general activity of DNMT and HDAC and not the expression of specific subtypes of these enzymes. **As far as we are aware, our study was the first to compare these enzymes' activity in both sexes of animals exposed to EE.**

Our findings can show that early life stress caused by MD induces depressive-like behavior and epigenetic changes in the development of male and female rats. Depressive-like behavior was evidenced in females at PND 41 and males at PND 61 exposed to MD protocol, while EE reversed these changes. **Offspring deprived of maternal care showed an increase in HDAC and DNMT activities**. However, such effects were dependent on sex and developmental period. Noteworthy females were more susceptible to epigenetic changes induced by MD in PFC. On the other hand, the offspring exposed to MD, but which was subjected to EE, mainly due to prolonged exposure, could reverse the increase in these enzymes. Besides, EE for 20 days in females caused more epigenetic effects in the hippocampus when compared to males. Thus, EE can be considered an essential non-pharmacological strategy to prevent or reverse long-term trauma-induced early life changes.

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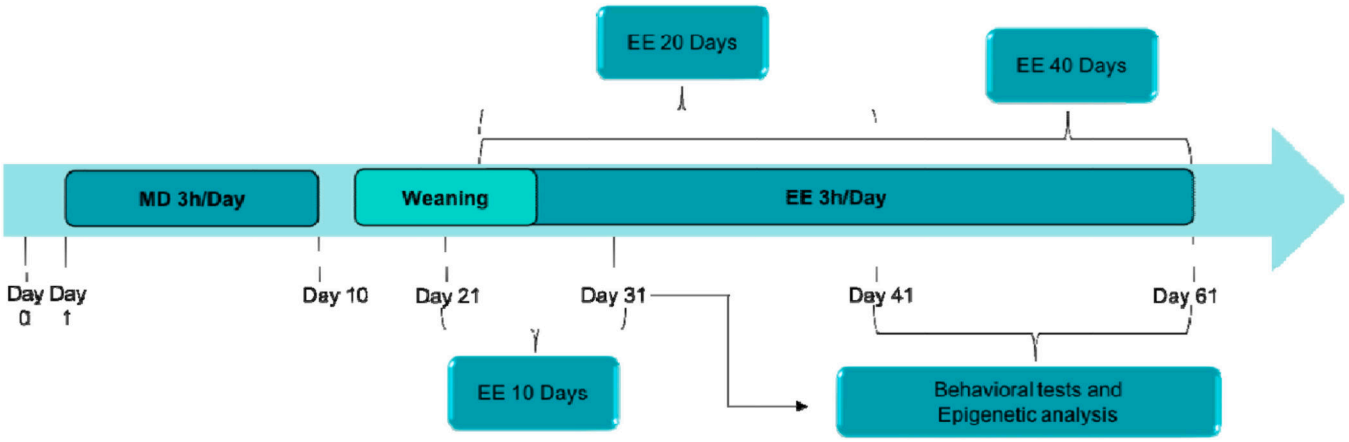

Fig. 1.

Maternal deprivation (MD) was performed during the first **ten** days of life (3 h a day). Weaning occurred on the 21st day after birth. Different groups of male and female rats were evaluated on days 31, 41**, and** 61 after birth. Environmental enrichment (EE) was carried out for 3 h/day. The group evaluated on day 31 was submitted to EE for **ten** days, the group evaluated on day 41 was submitted to EE for 20 days, **and** the group evaluated on day 61 was submitted to EE for 40 days. In each of the developmental stages, different animals (male and female) were submitted to the open field and forced swimming tests, then they were killed, and **the prefrontal** cortex and hippocampus were removed for analysis of epigenetic parameters.

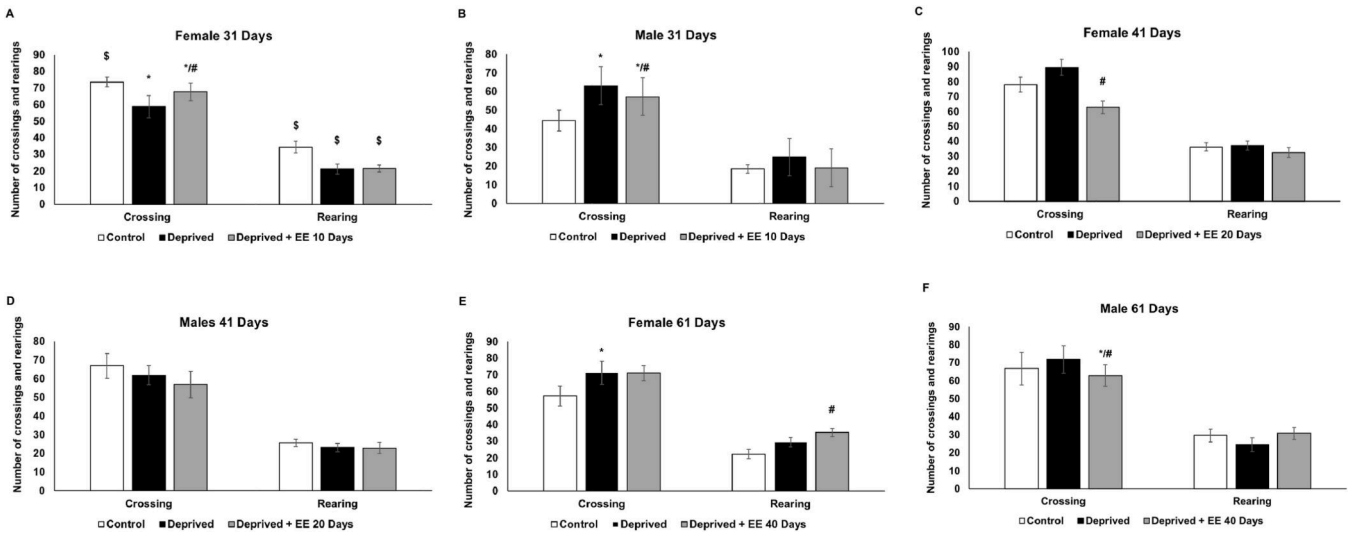

Fig. 2.

Spontaneous locomotor activity effects of maternal deprivation (MD) and/or environmental enrichment (EE) in the open field test. EE effects for **ten** days in rats subjected to MD in female (A) and male (B) rats. EE effects for 20 days in rats subjected to MD in female (C) and male (D) rats. EE effects for 40 days in rats subjected to MD in female (E) and male (F) rats. *$p < 0.05$ compared to the control group; $^{\#}p < 0.05$ compared to the deprived group. \$ $p < 0.05$ vs. sex and groups interaction according to two-way ANOVA.

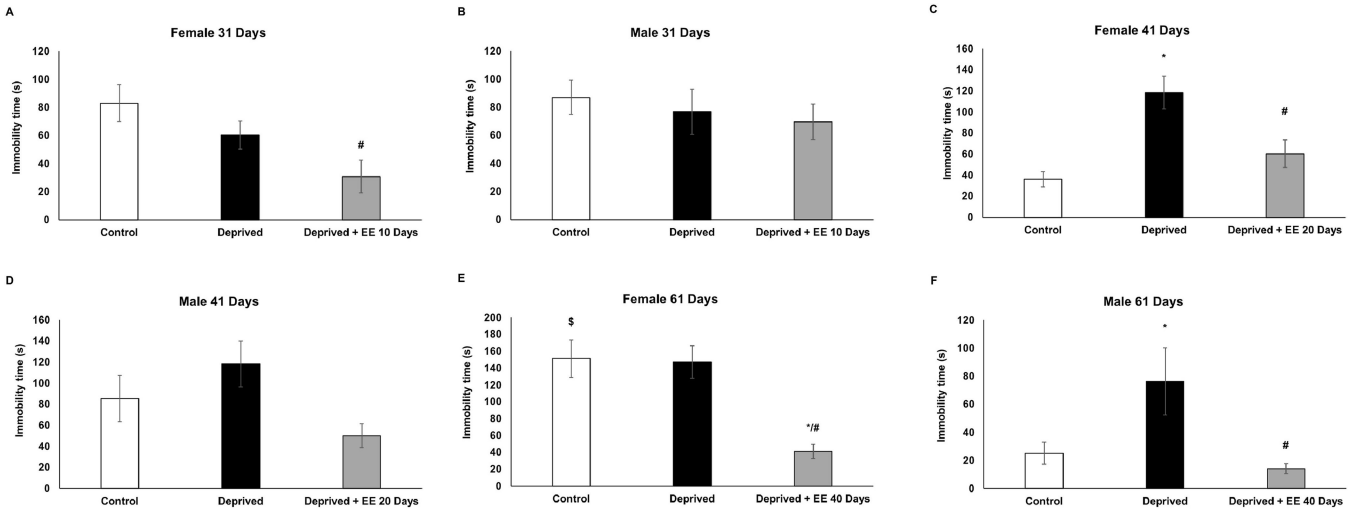

Fig. 3.

Immobility time effects of maternal deprivation (MD) and/or environmental enrichment (EE) in the forced swim test (FST). EE effects for **ten** days in rats subjected to MD in female (A) and male (B) rats. EE effects for 20 days in rats subjected to MD in female (C) and male (D) rats. EE effects for 40 days in rats subjected to MD in female (E) and male (F) rats. *$p < 0.05$ compared to the control group; $^{\#}p < 0.05$ compared to the deprived group. $ $p < 0.05$ vs. sex and groups interaction according to two-way ANOVA.

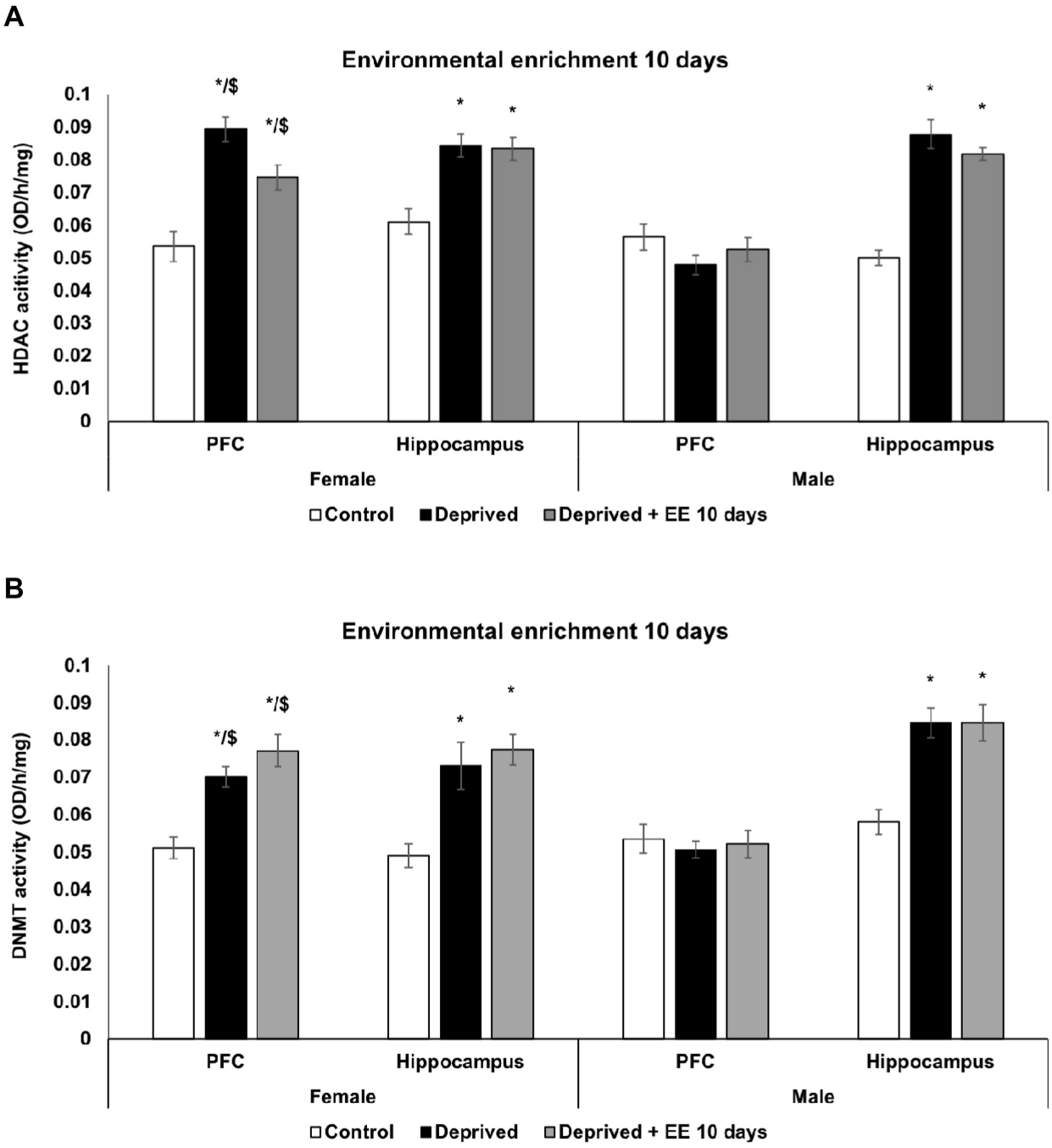

Fig. 4.

Effects of EE for **ten** days under HDAC (A) and DNMT (B) activities in the PFC and hippocampus of rats subjected to maternal deprivation. *p < 0.05 compared to the control group. $ p < 0.05 vs. sex and groups interaction according to two-way ANOVA.

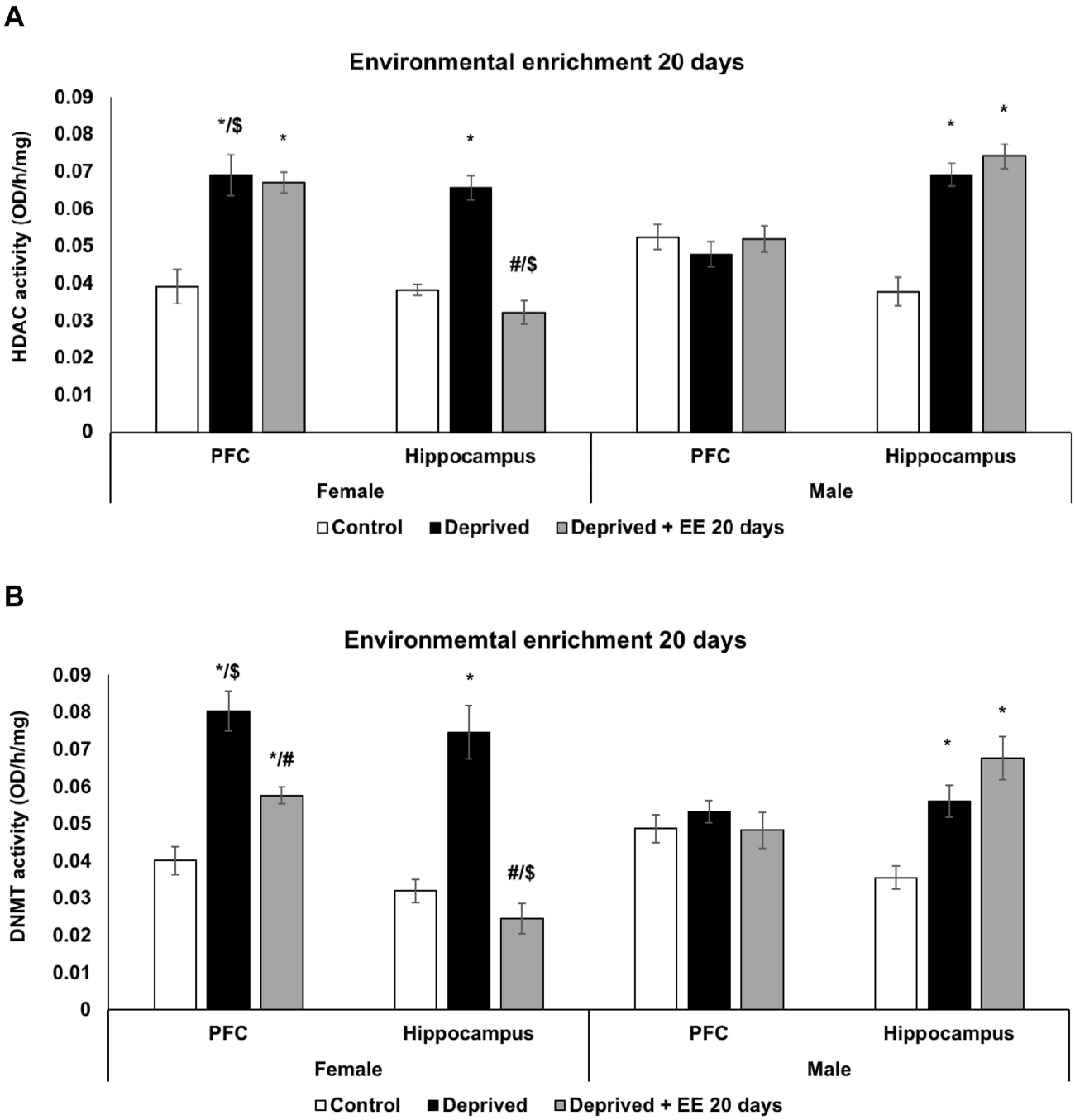

Fig. 5.

Effects of EE for 20 days under HDAC (A) and DNMT (B) activities in the PFC and hippocampus of rats subjected to maternal deprivation. *$p < 0.05$ compared to the control group; $^{\#}p < 0.05$ compared to the deprived group. $ $p < 0.05$ vs. sex and groups interaction according to two-way ANOVA.

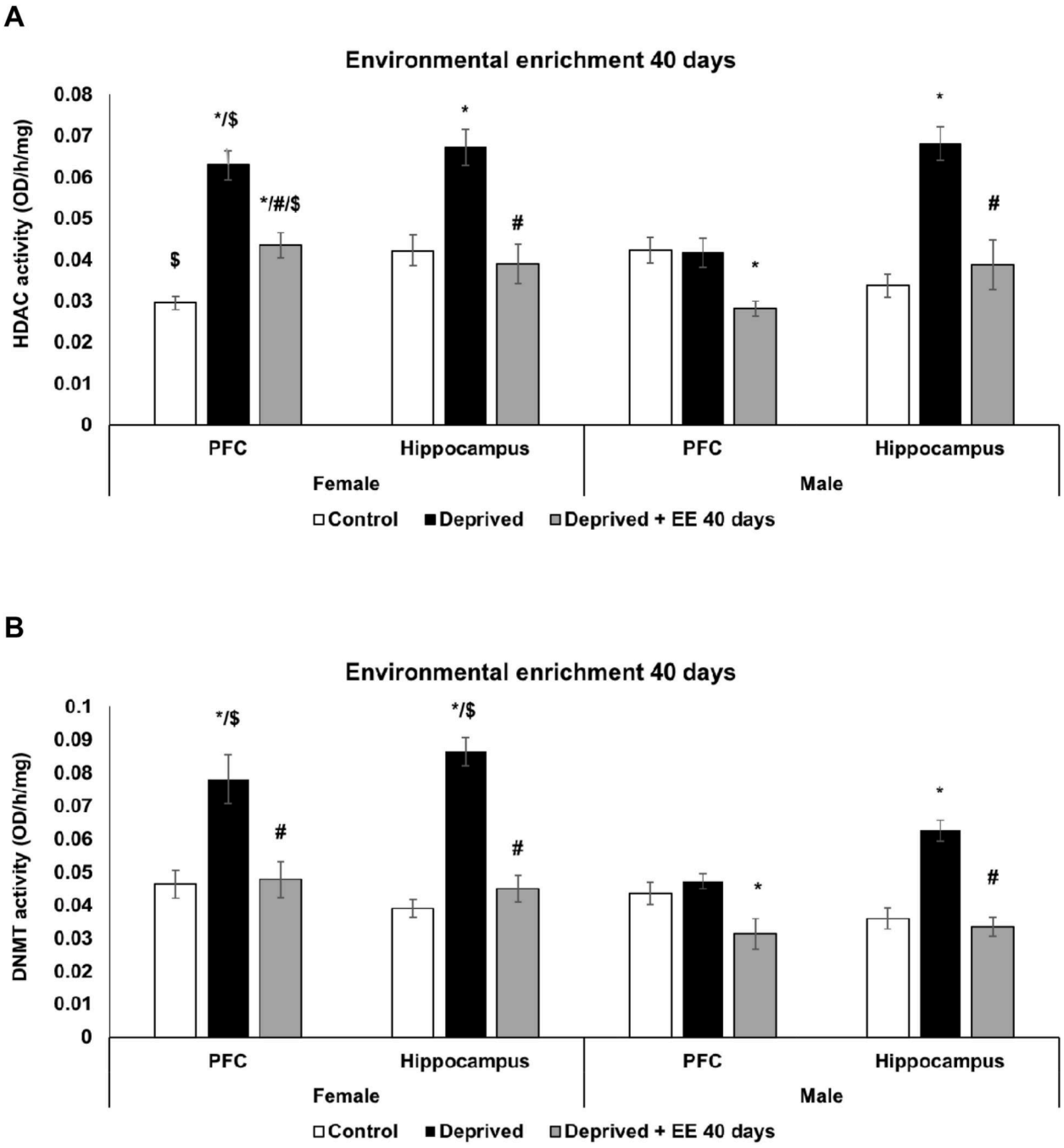

Fig. 6.

Effects of EE for 40 days under HDAC (A) and DNMT (B) activities in the PFC and hippocampus of rats subjected to maternal deprivation. *$p < 0.05$ compared to the control group; $^{\#}p < 0.05$ compared to the deprived group. \$ $p < 0.05$ vs. sex and groups interaction according to two-way ANOVA.