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Research report

Neurobiological effects of neonatal maternal separation and post-weaning environmental enrichment

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HIGHLIGHTS

- We examined whether environmental enrichment (EE) could compensate the effects of early maternal separation (MS).
- Maternal separation impaired inhibitory avoidance but had no effect in object recognition.
- Grooming behavior in the open field was significantly lower in maternally separated animals.
- EE reversed the effects of MS in the inhibitory avoidance task and in grooming behavior.
- c-Fos expression as well as glucocorticoid receptor in the hippocampus were increased by enrichment.

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ABSTRACT

Throughout the lifespan, the brain has a considerable degree of plasticity and can be strongly influenced by sensory input from the outside environment. Given the importance of the environment in the regulation of the brain structure, behavior and physiology, the aim of the present work was to analyze the effects of different environmental qualities during two critical ontogenic periods (early life and peripuberty) on behavior and hippocampal physiology.

Male Wistar rats were separated from their mothers for 4.5 h daily during the first 3 weeks of life. They were weaned on day 21 and housed under either standard or enriched conditions. At 60 d of age, all animals were then housed in same-treatment groups, two per cage, until testing began on day 74. Emotional and cognitive responses were tested using the open field, novel object recognition test and step-down inhibitory avoidance learning. In the dorsal hippocampus, glucocorticoid receptor expression and neuronal activity were examined by immunoreactivity.

Grooming behavior in the open field was found to be significantly lower in maternally separated animals, but post-weaning environmental enrichment completely reversed this tendency. Inhibitory avoidance but not object recognition memory was impaired in maternally separated animals, suggesting that early maternal separation alters learning and memory in a task-specific manner. Again, environmental enrichment reversed the effects of maternal separation on the inhibitory avoidance task. Even though maternal separation did not significantly affect Fos and glucocorticoid receptor (GR) expression, environmental enrichment increased both Fos expression in the total hippocampal area and also the overall number of GR positive cells per hippocampal area, mainly due to the changes in CA1.

These findings suggest that differential rearing is a useful procedure to study behavioral and physiological plasticity in response to early experience and that, although the effects of adverse experience early in life such as maternal separation can persist until adulthood, some of them can be compensated by early favorable environments, possibly through nervous system plasticity.

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1. Introduction

Over the past decade, the importance of environmental influences in regulating brain activity, behavior and physiology has been widely recognized [1–7]. There is compelling evidence that stressful experiences early in life affect neuroendocrine, cognitive and behavioral development and lead to greater susceptibility to psychopathology in adulthood [3,8]. A normal interaction between

Abbreviations: MS, maternally separated; EE, environmental enrichment; PND, postnatal day; AFR, animal facility rearing; NORT, novel object recognition test; GR, glucocorticoid receptor.

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mother and pups during early postnatal life is essential for proper growth and development in most mammals [6,3,9]. Stress affects early neurogenesis, synaptic production and retraction and myelination, during critical periods of postnatal development. Separation from dams represents one of the most potent stressors for pups. Both in rodents and primates, exposure to prolonged periods of maternal separation during early life increases the magnitude of neuroendocrine responses to stress and, consequently, vulnerability to stress-related psychopathology [10,11,6]. Maternal separation during the early postnatal period, when the rat brain is undergoing neural development, has been reported to produce structural disruptions in the brain and may also cause changes in learning and memory that persist into adulthood [12,7]. However, the results are inconclusive and the neural mechanisms underlying the long-term effects of early experiences still need to be fully investigated. In our laboratory, the model of early environmental manipulation of mother–infant relationships consists of 4.5 h daily separations performed from birth until weaning on postnatal day 21. As adults, compared with controls (non-maternally separated animals) this group has particular behavioral and endocrine changes (ACTH, corticosterone and catecholamines), both basal and in response to stress [13–15].

The brain also undergoes maturation and rearrangement of neurotransmitter pathways during adolescence, providing a critical window open to plastic changes that makes this period susceptible to both adverse and supportive shaping environmental forces. The neurobiological processes occurring in the brain during this developmental period have so far been poorly investigated [16].

Environmental enrichment (EE) in laboratory rodents increases brain plasticity at the structural level, producing functional neurophysiological and memory enhancement, increased thickness of the cerebral cortex, as well of dendritic arborization, neurogenesis, synaptogenesis and long-term potentiation [2,17]. This paradigm is a complex combination of social stimuli, intellectual and physical activity, that provides better opportunities to interact and explore the environment in what has been described as a continuous improvement of cognitive and sensory-physical activity [18,19]. The molecular substrate of the effects of EE on brain plasticity is multifactorial. In recent years, environmental enrichment has been used as a procedure that might prevent some of the deleterious effects of stress [18,20,21] and, moreover, EE could reverse most of the effects of juvenile stress at the behavioral and biochemical levels [22]. Bredy et al. showed that peripubertal environmental enrichment reversed the effects of reduced maternal care [23,24]. However, to our knowledge, very few studies have investigated the behavioral and neurochemical effects of environmental enrichment in the early maternal separation model [25]. In these studies Francis et al. demonstrate reversibility at the functional level, EE reversed effects of postnatal maternal separation on both endocrine and behavioral responses to stress. Nevertheless the underlying neural mechanism remains to be fully addressed but the hippocampus and the prefrontal cortex emerge as potentially interesting sites for consideration.

The hippocampal formation is one of the most extensively studied brain regions, showing a remarkable degree of structural and functional plasticity, and is particularly sensitive to stress. The hippocampus is a key limbic structure that inhibits the activity of the hypothalamic-pituitary-adrenal axis and plays a key role in the integration of learning and memory processes. This structure continues to develop after birth and may be the brain region that is most vulnerable to the effects of chronic stress. It is also one of the targets of glucocorticoids in the brain, which have a bimodal effect on it. While basal levels of these hormones are essential for neuronal maintenance, exposure to elevated levels of glucocorticoids (stress levels) causes morphological changes that are accompanied by cognitive deficits [26–30].

Given the above evidence highlighting the importance of the interaction of environment and genetic background in shaping the neural system and behavior, the aim of the present work was to study whether environmental enrichment during adolescence could reverse or compensate the effects of early maternal separation on learning and memory processes and hippocampal neuronal activity.

2. Materials and methods

2.1. Animals

Mothers were Wistar-derived rats bred in our colony and maintained on 12:12 h light–dark schedule (with lights on at 07:00 h), room temperature at 20 °C, with free access to water and food. The day of birth was designated as postnatal day (PND) 0. On PND 1 litters were culled to 10 pups with a 1:1 sex rate as far as possible.

All experimental procedures were performed according to International Guidelines on Care and Use of Laboratory Animals with protocols approved by the National University of Córdoba.

2.2. Maternal separation paradigm

Litters were randomly assigned to undergo maternal separation or to be reared under animal facility rearing (AFR) conditions. Maternal separation was carried out as previously described [14]. For MS, litters were separated from dams for 4.5 h per day, starting at 09:00 h and ending at 13:30 h, from PND 1 to 21 inclusive. Each separation consisted of removing dams from the home cage and placing them in an adjacent cage while the pups were kept together in the rearing site. After the separation period, the dam was returned to the home cage. Control litters were reared under standard (AFR) conditions, disturbed only by animal facility practices, twice a week, until weaning.

2.3. Post-weaning housing conditions

On PND 21, male offspring from both maternal separation and AFR conditions were randomly assigned to either environmental enrichment (EE) or standard housing conditions with food and water available ad libitum, giving rise to four treatments: animal facility-reared rats in standard housing conditions (AFR-NE); maternally separated (MS) rats housed in standard conditions (MS-NE); animal facility-reared rats housed in environmental enrichment (AFR-EE) and maternally separated rats in enrichment housing (MS-EE).

2.4. Environmental enrichment

Rats from the environmentally enriched group were housed in groups of 7–10 individuals in a complex enriched environment from PND 21 to PND 60. The enriched cage consisted of a large box (90 cm × 60 cm × 75 cm) containing a variety of toys, wooden blocks, climbing platforms, running wheels and plastic tubes as described previously [18,31–33]. Internal structure and toys were rearranged and renewed twice a week; feeding boxes and water bottles were also moved to different places to favor exploratory behaviors. Non-enriched (NE) animals were housed in pairs in standard laboratory cages (45 cm × 30 cm × 20 cm).

On postnatal day 60, enriched animals were transferred to two per cage for one week in order to favor equal conditions among groups, to allow habituation and to optimize experimental manipulations until testing began.

2.5. Behavioral testing

On PND 67–74 the animals' behavior was tested. All behavioral tests were conducted sequentially with the same animals. Animals were handled before the beginning of behavioral testing period to avoid a stress reaction to subsequent manipulation.

2.5.1. Open field test

The open field test was performed at PND 67 and carried out in a wooden box (50 cm × 60 cm × 60 cm) divided in 16 regular square areas. The animals were transported to the experimental room 2 h before experimental testing began. Each rat was gently placed in the center of the arena at the start of the session and was allowed to freely explore the novel environment for 15 min. The first 3 min of the open-field activities were recorded and subsequently analyzed. Locomotion (number of squares crossed with the four paws), number of rearings (posture sustained with hind paws on the floor), time of grooming (including licking the paws, washing movements over the head, fur-licking and genital cleaning) were the variables assessed as well as time spent in the center of the arena in order to typify exploratory behavior and habituation. The total number of fecal boluses left in the apparatus (defecation) was also noted. The arena was wiped with 70% ethanol between individuals.

2.5.2. Novel object recognition task

The novel object recognition test (NORT) was performed in the same chamber used in the open field test. Animals were habituated to the arena by allowing them to explore it for 15 min on two consecutive days (PND 67 and 68) before the experimental sessions, in the absence of stimulus objects. The stimulus objects varied in shape and color and were made of glass or plastic. All the rats were tested with the same objects. The training session (PND 69) consisted of placing the individuals in the apparatus containing two identical objects (two glass bottles of 12 cm high) designated A and A′. The discrimination session was performed 1 h later; in which one familiar (used in the training session) and one novel object were presented (A and B respectively; B: plastic rectangle). The objects were placed equidistant from two corners and were secured to the arena floor so that the subject could not displace them during exploration. The relative position of the two stimulus objects (familiar or novel) were randomly permuted for each experimental animal. At both sessions, individuals were allowed to freely explore the stimuli until they had accumulated 30 s of object exploration [34–36]. Exploration was defined as directing the nose toward the object at a distance of no more than 2 cm. Objects and arena were carefully wiped with 70% ethanol to prevent olfactory cues affecting the behavior of subsequently tested rats. Sessions were registered with a video camera placed over the field, and subsequently the percentage of time spent exploring the novel and familiar object and the total time spent in the arena were assessed.

2.5.3. Step-Down inhibitory avoidance

Twenty four hours later the same animals were submitted to the inhibitory avoidance task which was assessed in an acrylic box (50 cm × 25 cm × 30 cm). A grid floor made of parallel bronze rods (0.5 cm diameter) 1 cm apart was connected to a shock generator. An escape platform (20 cm × 25 cm) 2 cm above the steel floor was located at the left extreme of the grid. At the beginning of the training session, the individuals were placed on the platform and a transparent acrylic board was placed for 120 s, preventing the individual from descending from it and allowing contextual exploration (hippocampal dependent version). Following this delay, the board was removed and the latency to step down onto the floor with the four paws was recorded. Immediately after stepping down with all four paws, the animals received a 0.35 mA foot-shock for 2 s. In addition, animals were further exposed to the context for 30 s before being removed from the apparatus and placed back into their home cage. The retention test session was performed 1 h after training (short-term memory). No footshock was given in the retention session; step-down latencies (300 s ceiling) and freezing duration (in seconds) were taken as a measure of retention. Only individuals that visibly responded to the aversive stimulus were used for the analysis.

Freezing was defined as the lack of any movement except that required for respiration. Then, freezing time relative to the total time spent in the platform was calculated.

2.5.4. c-Fos and GR immunohistochemistry and immunocounting

c-Fos and GR expression in the hippocampus were measured by immunohistochemistry. Individuals were deeply anesthetized with chloral hydrate (0.54 g/kg i.p.) after the last session of the step-down inhibitory avoidance task and perfused transcardially with saline 0.9% followed by 4% paraformaldehyde in 0.1 M phosphate buffer solution. The brains were removed, post-fixed in 4% paraformaldehyde for 24 h at 4 °C and then transferred to 20% sucrose. Coronal sections of 40 μm through the hippocampus (bregma 2.8–3.3 mm) were collected using a freezing microtome. Immunohistochemistry was carried out by the free-floating method. After endogenous peroxidase blocking (10% methanol and H_2O_2) and incubation with albumin 5%, sections were incubated overnight with the respective primary antibody (anti Fos, Ab-5, Oncogen Science, 1: 1000 or anti GR, E-20 sc-1003 Santa Cruz Biotechnology, 1:500). Subsequently, the sections were incubated with the respective biotinylated secondary antibody for 2 h. Immuno signals were amplified with Avidin–Biotin–Peroxidase complex (ABC Elite kit, Vector Laboratories) using 3,3′-diaminobenzidine (DAB-Sigma) as chromogen. Sections were placed on glass slides using Albrecht gelatine (1.5% gelatine/80% alcohol), dried, dehydrated in xylene and coverslipped using DPX mounting medium (Fluka, Buchs, Switzerland). Sections were examined under a light microscope (Olympus) and hippocampal images were captured using a high-resolution digital camera.

Quantification of positive cells for immunohistochemistry on hippocampal CA1, dentate gyrus and CA3 areas was assessed using Image-J software. At least five sections per individual were analyzed. In every section, representative samples for each hippocampal area were taken. Positive cells in sample areas were manually quantified. Results were shown as positive cells/area (density of immuno-positive cells).

2.6. Statistical analysis

For behavioral tests, comparisons between experimental groups were made by using a two-way analysis of variance (ANOVA), considering rearing protocols (AFR × MS) and enrichment (NE × EE) as independent variables. When variables did not meet the requirements of normality and homogeneity of variance, data was transformed to ranks. Student's *t*-test was used to compare values of novel vs. familiar object exploration on NOR test.

For c-Fos and GR immunocounting, statistical analysis was carried out using a linear mixed effect model, which is a modified analysis of variance/covariance (ANOVA/ANCOVA) that allows for the analysis of dependencies in the data. Rearing protocol, environment and hippocampal area were modeled as factors and hippocampal sections were randomized. In all comparisons, p values of less than 0.05 were considered to indicate statistical significance.

Data were presented as mean ± standard error (SE). DGC post hoc multiple comparison analysis was performed for further examination of group differences, in case of significance for factors or interactions between factors of ANOVA.

3. Results

3.1. Open field task

Maternally separated animals housed in standard conditions displayed a markedly decreased grooming behavior in OF than the other groups. Rats raised in the enriched environment elicited high grooming behavior on this task (Fig. 1). A significant main effect of environmental enrichment $F(1,40) = 20.51$, $p < 0.001$ and

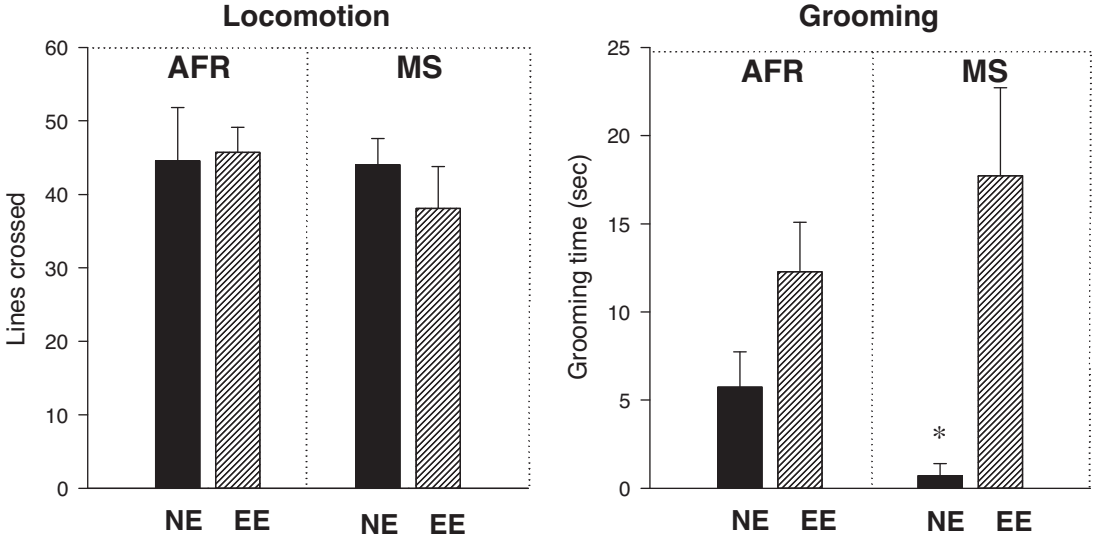

Fig. 1. Effect of maternal separation and environmental enrichment over locomotion and grooming time in the open field. Grooming behavior was significantly lower in maternally separated animals while postweaning environmental enrichment reversed this tendency in this group. Basal locomotion was not affected by any treatment. Values expressed as means ± SE. *$p < 0.05$ vs MS EE and AFR-groups. N size: AFR NE = 10, AFR EE = 14, MS NE = 10 and MS EE = 11.

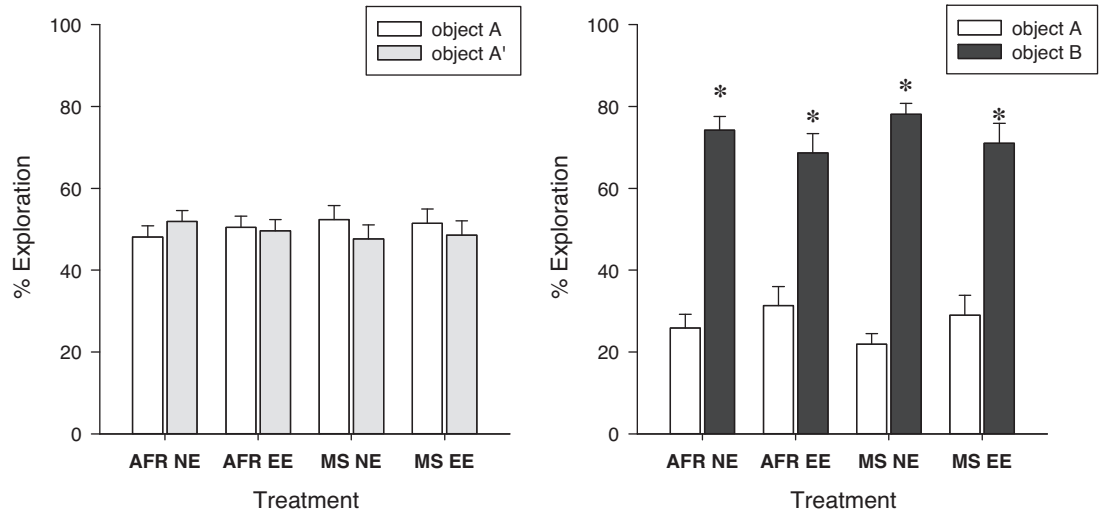

Fig. 2. NOR test results. Training session (left panel) and discrimination session (right panel). During discrimination session all groups were able to discriminate the new object (B) from the previously presented one (A). Values expressed as percentage of exploration means ± SE. *Significantly different from object A. N size: AFR NE = 11, AFR EE = 13, MS NE = 7 and MS EE = 10.

interaction among factors $F(1,40) = 3.85$, $p = 0.05$ was found in time of grooming behavior in the open field.

No significant difference was observed between the groups when comparing total locomotion $F(1,40) = 0.82$, $p = 0.54$ (Fig. 1), time spend in central area $F(1,40) = 0.92$, $p = 0.48$ or defecation $F(1,40) = 1.83$, $p = 0.12$ and vertical activity $F(1,40) = 0.75$, $p = 0.59$.

3.2. Novel object recognition task

Performance in the novel object recognition test during the training and discrimination sessions is shown in Fig. 2. All groups showed good memory performance in the object recognition task. During the test session, all groups were able to discriminate the new object (B) from the previously presented one (A) (AFR NE $t(11) = -10.88$, $p < 0.0001$; AFR EE $t(13) = -5.69$, $p < 0.0001$; MS NE $t(7) = -15.23$, $p < 0.0001$; MS EE $t(10) = -6.13$, $p < 0.0001$). Even though no significant differences were observed between groups, a main effect of environmental enrichment over total open field time was found. Enriched individuals presented larger latencies to accumulate 30 s object exploration, described as a tendency $F(1,37) = 2.70$, $p = 0.10$ at training sessions and a significant effect $F(1,37) = 5.24$, $p < 0.05$ at the discrimination session. Maternal separation also generated a slight tendency toward the reduction of exploration latency time in both cases, training $F(1,37) = 3.33$, $p = 0.07$ and discrimination $F(1,37) = 2.57$, $p = 0.11$. AFR-MS group had lower exploration latency means on both sessions (Fig. 3).

3.3. Step-down inhibitory avoidance task

3.3.1. Latency time to step-down

Latencies to step-down during training session were not significantly different across the groups (data are not shown because the latency to step-down in this session was basically non-existent).

The results show that the rearing protocol was a significant factor influencing step-down latency time during the retention test. Maternally separated animals displayed significantly shorter latencies to step-down when compared to AFR animals, which clearly indicates a worse performance in this aversive task suggesting

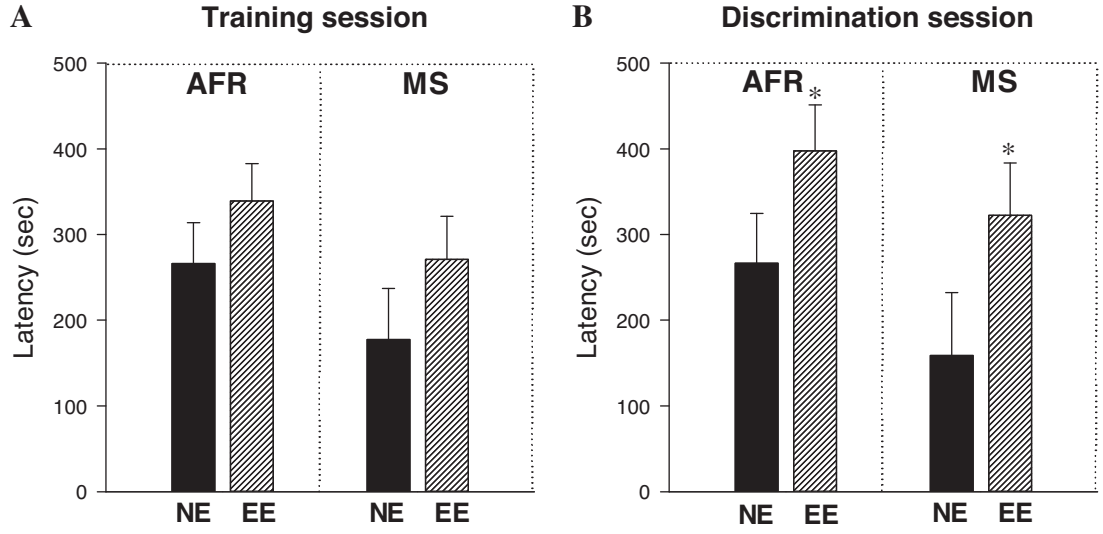

Fig. 3. Time latencies on open field to accumulate 30 s of object exploration during the NORT. In training session (panel A) and discrimination session (panel B). Enriched individuals presented larger latencies than not enriched ones. *Significantly different from non enriched. N size: AFR NE = 11, AFR EE = 13, MS NE = 7 and MS EE = 10.

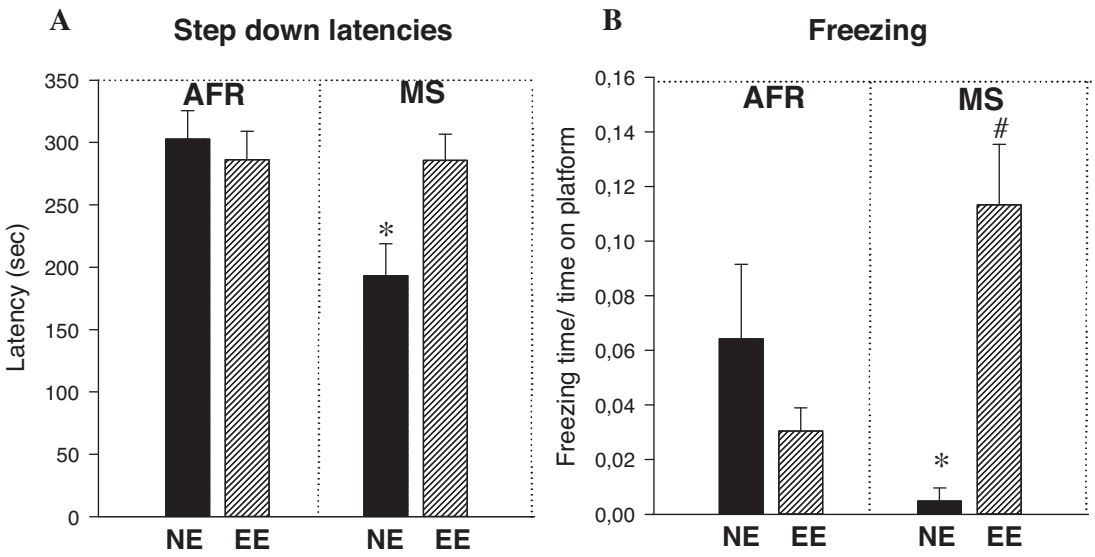

Fig. 4. Panel A: Step-down latencies on retention session in contextual version of the step down inhibitory avoidance test. AFR groups both NE and EE presented almost ceiling latency time whereas MS-NE animals showed decreased step down latencies indicating impaired memory retention skills. Subsequent enrichment generated responses similar to AFR groups. Values expressed as means ± SE. *Significantly different from AFR, $p < 0.01$. Panel B: Freezing response on step-down inhibitory avoidance task 1 h after shock delivery expressed as freezing time relative to total time on step down platform. MS NE animals presented the lowest levels of freezing behavior while MS EE ones displayed the highest. *Significantly different from AFR NE $p < 0.0001$; # significantly different from AFR EE $p < 0.0001$. N size: AFR NE = 9, AFR EE = 9, MS NE = 7 and MS EE = 10.

some type of deficit in memory acquisition, consolidation or retrieval $F(1,31) = 4.20$, $p < 0.05$. A significant interaction between rearing protocol and post-weaning environment was also found $F(1,31) = 8.01$, $p < 0.01$, and thus, MS-NE group's latencies were lower than those of AFR, while MS-EE individuals performed in the same way as the AFR groups (Fig. 4, panel A).

3.3.2. Freezing response on step-down inhibitory avoidance task

The statistical analysis revealed a significant effect of environmental enrichment $F(1,31) = 10.747$, $p < 0.005$, as well as a significant interaction between the rearing protocol and post-weaning environment $F(1,31) = 30.369$, $p < 0.0001$. Maternally separated rats reared in a non-enriched environment (MS-NE) spent significantly less time freezing than AFR animals. Environmental enrichment induced a marked increase in freezing, a measure of conditioned fear behavior (Fig. 4, panel B).

3.4. Immunohistochemistry

3.4.1. FOS expression

Analysis across subfields of the hippocampus (CA1, CA3 and dentate gyrus) revealed a significantly different expression of Fos-like immunoreactivity among hippocampal areas $F(1,21) = 5.08$, $p < 0.01$. CA1 showed significantly greater density of Fos-positive cells.

Maternal separation did not alter Fos immunoreactivity in the hippocampus. However, environmental enrichment increased Fos expression on total hippocampal area $F(1,21) = 6.22$, $p < 0.05$. The interaction of maternal separation and hippocampal area was also significant $F(1,21) = 3.56$, $p < 0.05$. Analysis revealed that MS-EE animals presented the highest density of Fos-positive cells in comparison with other groups, and this difference was restricted to the CA1 area (Figs. 5 and 6).

3.4.2. GR expression

Statistical analysis elucidated effects generated by several factors influencing GR expression. First of all, the number of GR-positive cells was not homogeneous among hippocampal areas. CA3 presented the lowest number of immunostained cells in comparison with CA1 and DG $F(1,22) = 257.62$, $p < 0.0001$.

Regarding environmental effects enrichment acted as a factor that affects the number of GR positive cells. Taking the hippocampus as a hole entity and avoiding its partitioning into areas, EE increased GR positive cells $F(1,22) = 6.48$, $p = 0.01$. Further study revealed that this effect was different between areas, since significant interaction was found among enrichment and the studied hippocampal areas $F(1,22) = 36.65$, $p < 0.0001$. The impact of environmental enrichment was higher in the CA1 and DG and lower in the CA3 area. In fact, EE did not affect the number of cells expressing GR in the CA3.

Triple interaction between the rearing protocol, enrichment and the hippocampal area was also significant $F(1,22) = 5.39$, $p < 0.01$,

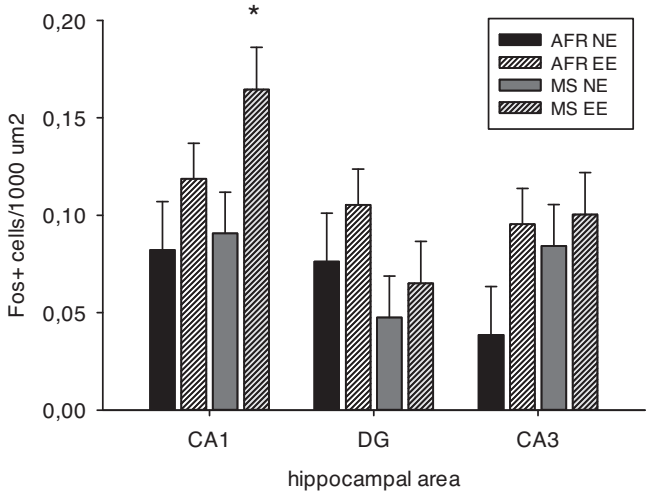

Fig. 5. Density of positive cells for Fos immunohistochemistry on hippocampus representative areas (CA1, CA3, and dentate gyrus). Statistical analysis showed that enrichment increased Fos expression on total hippocampal area. Even though a slight tendency is conserved among areas, this effect was more eminent on CA1 *significantly different from all other groups. Values expressed as means ± SE. N size: AFR NE = 5, AFR EE = 4, MS NE = 6 and MS EE = 4 subjects.

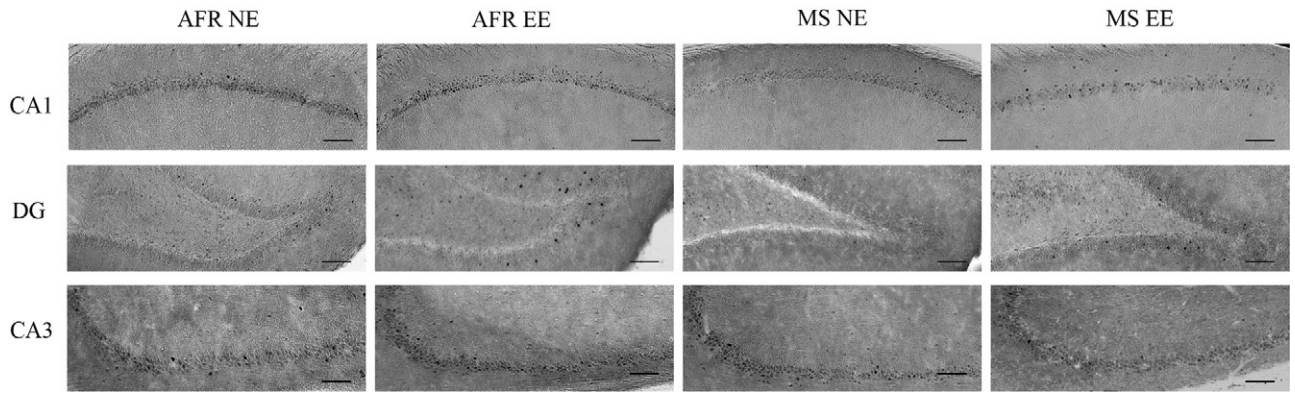

Fig. 6. Representative photomicrographs showing Fos immunoreactivity in the CA1, CA3 and DG subfields of the dorsal hippocampus from different experimental groups. Scale bar 100 μm.

and the post hoc analysis showed that AFR-EE individuals had the most increased level of GR-positive cells in the CA1 and DG areas. Maternally separated individuals, both enriched and not, presented low GR expression in these areas, as well as AFR-NE ones. Finally, regardless of treatment, the CA3 hippocampal area presented the lowest levels of GR-positive cells (Figs. 7 and 8).

4. Discussion

Postnatal maternal care as well post-weaning physical and social interactions are external environment factors that can model phenotypic features in adulthood [37]. Overall, our results indicate that environmental conditions, such as early maternal separation and environmental enrichment, have both independent and interactional effects, and demonstrate how environmental variations at two distinct developmental stages can modulate adult behavior and protein expression profiles in the hippocampus.

Stress effects on adult rats are generally transient, while early life stress, such as maternal separation, alters physiological functions expressed in adolescence and also in adulthood [38]. Our data

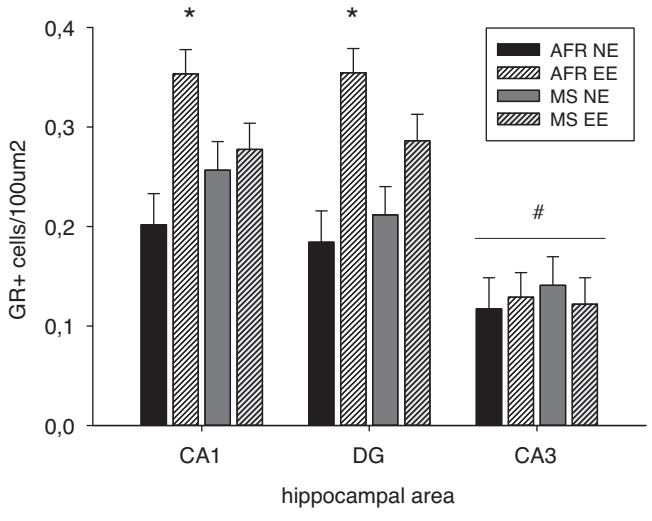

Fig. 7. Positive cells for glucocorticoid receptors immunohistochemistry on hippocampus representative areas (CA1, CA3, dentate gyrus). Both CA1 and DG present similar expression profiles generated by treatments, on which AFR EE individuals have increased levels of GR-positive cells per area. CA3 area presented the lowest GR density and remained unaffected by enrichment or separation treatments. *Significantly different from other groups; # significantly different from CA1 and DG. N size: AFR NE = 5, AFR EE = 4, MS NE = 6 and MS EE = 4 subjects.

show that, in the open field task, maternally separated rats exhibit decreased grooming behavior, and that environmental enrichment reversed this response. Open-field exposure allows an approximation to responses toward novel stimuli, such as exploratory and habituation behavior. Self-grooming is an ancient innate behavior seen across most animal species and is a particularly important part of rodent behavioral repertoires [39]. Grooming behavior is often displayed as a reaction to unexpected stimuli, conflict situations or frustration [40]. Even so, it may be involved in the habituation process. Previous evidence has shown that environmental enrichment increases grooming behavior [16,41–43], and it has been suggested that whole grooming or at least some of its structural and sequential components may be involved in the habituation process. Our results fit this interpretation and support the idea of grooming as a habituation index. The fact that maternal separation decreased levels of grooming adds new data to this perspective and suggests inappropriate habituation to novelty in this group, which can be reversed by environmental enrichment.

Controversial data about the MS group's performance on the NORT is found in the literature. Adult cognitive function in MS rats as measured by novel object recognition was shown to be either unaffected [44] or impaired [45]. Our data support the idea that maternal separation does not affect recognition memory in rodents. However, the absence of group differences at relatively short intervals (1 h) should not be interpreted as evidence that manipulations do not impair memory, since increasing the delay increases the demand on memory [46]. Furthermore, discrepancies in results among studies can be generated by different maternal separation protocols or testing techniques. Our results differ from Asia's in maternal separation protocol, habituation and testing time extension and analysis procedure (fixed recognition time versus fixed OF time). Fixed recognition time seems to be more suitable than fixed field time on NORT analysis, particularly in cases in which treatment generates uneven exploratory motivation. This procedure assures that all animals accumulate the same amount of time exploring the objects. A comparison has been made of the outputs of both analysis variants, demonstrating that in some tasks hippocampal lesions generated apparent recognition memory deficit in fixed session time, but this result is due to less object exploration during the sample phase, while fixing 30 s of object exploration time makes these differences disappear [47].

Environmental enrichment generated differences in exploratory behavior during NORT. The total time it took for enriched animals to accrue 30 s of object exploration was significantly higher. Although this could be interpreted as reduced motivation to explore, it is also possible that these animals exhibited a greater habituation to novel stimuli. Enriched rats seems to habituate to novel

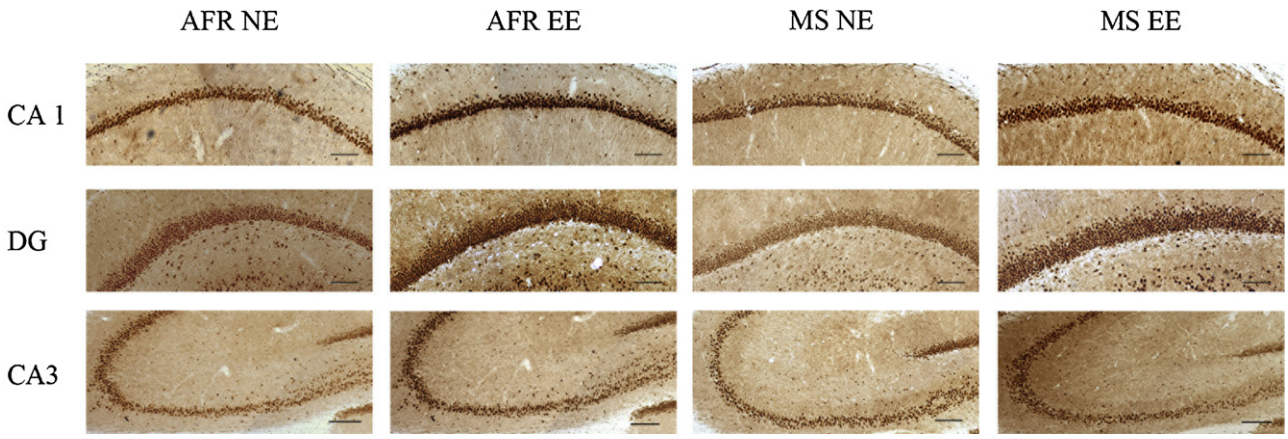

Fig. 8. Effect of different environmental manipulations on GR expression in the hippocampus. Representative photomicrographs of coronal sections at the CA1, CA3 and DG subfields from different experimental groups. Scale bar 100 μm.

stimuli more rapidly suggesting that the relative incentive value of the novel object may be reduced in enriched rats. It was important to use an exportation time-fixed NORT protocol, which is the best way to reflect the treatment's impact on cognitive function.

Regarding the step-down inhibitory avoidance task, we found that latency results agree with previous work that demonstrates lower latency time to step-down in separated animals [48], even when the maternal separation protocols differ. Our results help to validate maternal separation effects and also to identify general tendencies in maternal separation effects beyond variations in the separation protocol, providing further evidence of the importance of maternal care in the development of hippocampal-dependent learning and memory. The amount of freezing behavior in the step-down task in the test session, 1 h after the aversive stimulus, was different between groups. Maternally separated individuals that presented lower step-down latencies also have reduced levels of freezing when tested. Together, these results indicate diminished memory retention in MS animals 1 h after an aversive stimulus (electric footshock) was applied.

Step-down and Nobel Object Recognition tasks were both used to assess short-term memory and retention events, but showed different behavioral outcomes. Interestingly, in our animal model, inhibitory avoidance but not object recognition memory was impaired in maternally separated animals. These data suggest that early maternal separation alters learning and memory in a task-specific manner that may reflect alterations in emotional reactivity. Although these two tasks are commonly used in order to analyze short term memory deficits, they differ significantly in contextual aversiveness and the emotional component of the task. Therefore, the differential responses observed among groups may be mainly elicited by an emotional or highly unpleasant context. Higher levels of aversiveness may elicit maternal separation responses that could be masked in normal or non-aversive contexts.

Interestingly, environmental enrichment appears to generate a lower reactivity to novel stimuli in general, as can be seen both in the open field (as higher levels of grooming) and in NORT (as increased latency to accumulate the 30 s of object exploration). Animals in enriched environments are exposed to novel objects repeatedly, that may contribute to their relatively faster habituation when facing novelty. An opposite response was observed in maternally separated and non-enriched individuals.

Experiences during critical periods of development can affect the formation of neuronal circuits and exert long-lasting influences on neural function. Previous studies showed that environmental enrichment, exerts significant effects on animals' emotionality and that this behavioral difference is accompanied by differential c-Fos activation in the amygdala, whereas the hippocampus was notably not labeled by c-Fos antibody [49]. Although maternal separation did not have any effect in the number of Fos-immunoreactive cells, EE individuals showed raised levels of Fos immunoreactivity, especially in the CA1 hippocampal area. Fos expression patterns in the hippocampus show activation and therefore possible linkage of these areas with environmental experiences. This also indicates that not all the hippocampal areas respond in the same way to environmental challenges suggesting that neuronal activity in the hippocampus may be affected in region- and temporal specific manners by environmental experiences.

GR immunohistochemical expression profiles generated by treatments were homogeneous in the CA1 and DG areas, but not in CA3, where GR expression was the lowest and the treatments seem not to have a significant impact. In CA1 and DG, non-maternally separated and enriched individuals presented the greatest number of GR-positive cells. Previous work indicates that EE induces GR gene expression in specific hippocampal subfields (CA1 and CA2), comparing enriched individuals with isolated individuals [50]. Even though this comparison is not entirely appropriate, considering that isolation produces selective changes in stress profiles [51], our results also show increased GR labeling in the CA1 area in enriched animals.

Increased GR expression in specific hippocampal subfields of EE rats may relate to changes in neuronal anatomy and function, attenuating adrenocortical stress responses and thus allowing more efficient hippocampal neuronal function, as many studies have demonstrated [52,53].

Results show consistency within behavioral assessments and demonstrate how EE treatment can generate changes in previously maternally separated animals. Maternal separation effects persist during adulthood, but can be compensated by early favorable environments through nervous system plasticity. On the other hand, maternal separation effects on hippocampal physiology were less conspicuous, but altered to some extent by enrichment effects. For example, increased GR immunolabeling was found only in EE animals that had not been through maternal separation. Thus, maternal separation to some extent conditioned EE responses.

5. Conclusion

To conclude, early and peripubertal environment can alter, either independently or in conjunction, some important phenotypic profiles with high adaptive significance on adulthood, such

as habituation, defensive behaviors and emotional reactivity to a novel or avoidant context. In a general way, this study demonstrates the importance of environment as a generator of plastic changes in behavioral systems. Early plasticity can represent an important and interesting tool to aid in understanding phenotypic expression patterns in adulthood.

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