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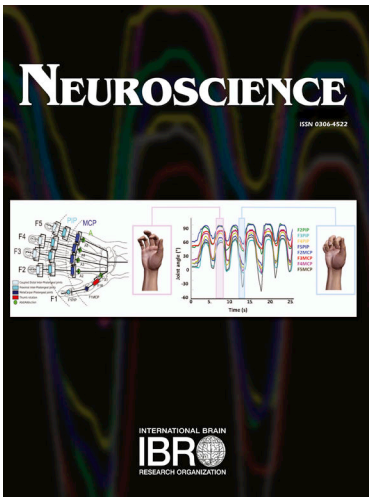

Postweaning enriched environment enhances cognitive function and brain-derived neurotrophic factor signaling in the hippocampus in maternally separated rats

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PII: S0306-4522(20)30647-3
DOI: https://doi.org/10.1016/j.neuroscience.2020.09.058
Reference: NSC 19914

To appear in: *Neuroscience*

Received Date: 16 April 2020
Revised Date: 24 September 2020
Accepted Date: 28 September 2020

Please cite this article as: J. Maximiliano Cordier, J. Paola Aguggia, V. Danelon, F. Rafael Mir, M. Angélica Rivarola, D. Mascó, Postweaning enriched environment enhances cognitive function and brain-derived neurotrophic factor signaling in the hippocampus in maternally separated rats, *Neuroscience* (2020), doi: https://doi.org/10.1016/j.neuroscience.2020.09.058

TITLE

Postweaning enriched environment enhances cognitive function and brain-derived neurotrophic factor signaling in the hippocampus in maternally separated rats

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Declarations of interest: none

ABSTRACT

Adverse environments during early life may lead to different neurophysiological and behavioral consequences, including depression and learning and memory deficits that persist into adulthood. Previously, we demonstrated that exposure to an enriched environment during adolescence mitigates the cognitive impairment observed after maternal separation in a task-specific manner. However, underlying neural mechanisms are still not fully understood. The current study examines the effects of neonatal maternal separation (MS) and postweaning environmental enrichment (EE) on spatial learning and memory performance in a short version of the Barnes Maze, active and passive behaviors in the forced swim test, and on TrkB/BDNF receptor expression in the hippocampus. Our results revealed that MS impaired acquisition learning and that enriched rats performed better than non-enriched rats in acquisition trials, regardless of early conditions. During the probe, enriched-housed rats demonstrated better performance than those reared in standard conditions. No significant differences between groups were found in the forced swim test. Both MS and EE increase full-length TrkB expression, and the combination of MS and EE treatment caused the highest levels of this protein expression. Similarly, truncated TrkB expression was higher in the MS/EE group. Animal facility rearing (AFR) non-enriched groups present the lowest activation of phosphorylated Erk, a canonical downstream kinase of TrkB signaling. Taken together, our results demonstrate the importance of enriched environment as an intervention to ameliorate the effects of maternal separation on spatial learning and memory. TrkB/BDNF signaling could mediate neuroplastic changes related to learning and memory during exposure to enriched environment.

KEYWORDS

Enriched Environment - Maternal Separation-Brain derived neurotrophic factor receptors-Behavior

INTRODUCTION

The mother-infant relationship is unquestionably one of the most important in early life, playing a pivotal role in emotional and cognitive development. Even subtle modifications of this early relationship may result in long-term changes in later patterns of physiological, neuroendocrine and behavioral responses. The maternal separation (MS) paradigm in rodents is one of the most widely used animal models to study the effects of early adverse experience on physiology and behavior. Basically, it consists of removing pups from their mother during the early postnatal period (3-6 hours of separation from the mother daily). Despite the vast number of reports on the effects of maternal separation, general conclusions are difficult, and many times contradictory results emerge, which may be attributable to differences in experimental design between laboratories (Lehmann and Feldon, 2000; Murthy and Gould, 2018). Nevertheless, it is well established that maternal separation is a traumatic event that alters the programming of neuroendocrine and behavioral systems (Pryce and Feldon, 2003; Veenema and Neumann, 2009; Schmidt et al., 2011; Vetulani, 2013).

In addition to the perinatal period, adolescence is a critical sensitive period of brain development, susceptible to environmental stimuli (Dumontheil, 2016), and therefore environmental enrichment (EE) during this period has been recognized as a possible behavioral neuroprotective strategy. Although EE varies between laboratories, it usually involves increasing the complexity of the environment, using a combination of social interaction, physical exercise, and exposure to novel inanimate objects. This paradigm results in positive effects such as improvement in learning and memory on spatial tasks, and anatomical and physiological effects in the brain such as increased hippocampus neurogenesis and increased dendritic branching, (for review please see Nithianantharajah and Hannan, 2006; Baroncelli et al., 2010; Pang and Hannan, 2013; Voss et al., 2013; Eckert and Abraham, 2014; Fischer, 2016; Ohline and Abraham, 2019).

Previous data from our laboratory and others demonstrate that some of the effects of early adverse experience can be compensated by more favorable environments than those of the standard home cage environment, possibly through nervous system plasticity (Hui et al., 2011; Vivinetto et al., 2013; Doreste-Mendez et al., 2019; Gonzalez-Pardo et al., 2019). However, the underlying mechanisms are not fully understood. Neurotrophins are good candidates for mediating brain plasticity and some of the effects of environmental conditions on brain function (Bekinschtein et al., 2011). Among these, brain-derived neurotrophic factor (BDNF), with its high affinity receptor TrkB, is the most widely distributed neurotrophin in the brain and plays a significant role in many central nervous system (CNS) functions, such as neuronal differentiation, synaptogenesis and synaptic plasticity (Karpova, 2014), as well as having a neuroprotective role under adverse conditions. The expression of BDNF protein and its

receptors changes over the course of development and in response to environmental conditions. During development, the expression of BDNF and its two primary receptors (TrkB and p75NTR) are closely controlled to guide the survival, differentiation, and plasticity of cells of the CNS (Labelle and Leclerc, 2000; Kuma et al., 2004). Different studies have shown that manipulations of the early environment can affect the expression of neurotrophins both during development and in adulthood (Cirulli et al., 2003; Roceri et al., 2004; Sale et al., 2004; Branchi et al., 2006; Mosaferi et al., 2015). Changes in the expression of neurotrophins as a result of early adverse experiences affect short- and long-term neurobehavioral plasticity, leading to important changes in stress response, social skills and the development of psychiatric disorders in adulthood, including an increased risk for major depression (Roceri et al., 2004; Nithianantharajah and Hannan, 2006; Sullivan et al., 2011; Bohlen and Bohlen, 2018).

Although several studies reported that maternal separation affects BDNF expression in the hippocampus (Daskalakis et al., 2015; Wang et al., 2015; Ohta et al., 2017), few studies have reported changes in BDNF receptors or BDNF-signaling pathways following both maternal separation and enriched environment.

The aim of the present work was to determine whether exposure to environmental enrichment during adolescence can revert or compensate the effects of early maternal separation on hippocampal-dependent memory, and whether the effects of environmental enrichment on cognitive function are associated with changes in the expression of BDNF receptors.

EXPERIMENTAL PROCEDURES

Animals

Dams were Wistar outbred rats (n=12) purchased from the breeding colony of the animal facility of the Mercedes y Martin Ferreyra Medical Investigation Institute (INIMEC-CONICET-UNC) Córdoba. They were housed in a temperature-controlled animal room (20–22°C) on a 12 h light/dark cycle (lights on from 7:00 to 19:00) with *ad libitum* access to commercial pelleted rodent food and tap water in drinking bottles. Pregnancy was induced by mating each female with a sexually experienced male. Pregnant females were checked twice-daily by visual observation to establish the exact date of birth of the offspring, designated as postnatal day (PND) 0. Each litter was randomly assigned to a different neonatal treatment - maternal separation (MS) or non-maternal separation to be reared under standard animal facility rearing (AFR) conditions - and all animals within a litter received the same experimental manipulations.

All experimental procedures were performed according to the International Guidelines on Care and Use of Laboratory Animals with protocols approved by the Institutional Ethical Committee for Animal Welfare at the National University of Córdoba.

Maternal Separation Protocol

Maternal separation was performed as previously described (Aguggia et al., 2013)(Vivinetto et al., 2013). Briefly, on PND 1, each litter was sexed and culled to 10 pups (aiming at 5:5 males-females whenever possible). For maternal separation, litters were separated from the mother daily for 4.5 h between 09:00 and 13:30, from PND 1 to 21 inclusive. Dams were removed from the rearing cage and placed in a new cage containing clean bedding material in the same animal room while the pups were kept together in the rearing cage. After the separation period, the dam was returned to the home cage. AFR litters were left undisturbed, except for the change of bedding twice a week. Weaning took place at PND 21 and male offspring from both maternal separation (n=32) and AFR conditions (n=28) were pseudo randomly assigned to either environmental enrichment (EE) or standard non-enriched (NE) housing conditions with food and water available *ad libitum*. To avoid litter effect only 2-3 pups from each litter were assigned to the same postweaning housing condition. There were thus four treatments: animal facility-reared rats in standard housing conditions (AFR/NE, n=14); maternally separated (MS) rats in standard conditions (MS/NE, n=16); animal facility-reared rats housed with environmental enrichment (AFR/EE, n=14) and maternally separated rats in enriched housing (MS/EE, n=16).

Enriched and Non-Enriched Environments

Enriched environment exposure was carried out from PND 21 to PND 62. Each AFR/EE (n=14) and MS/EE (n=16) animal group were further divided into two groups and housed in EE cages. The enriched environment consisted of housing groups of 8-10 (completed with individuals of same preweaning rearing condition) in specially designed cages (90 cm × 60 cm × 75 cm) containing a variety of toys of different sizes, textures and colors (wood, metal, and plastics), climbing platforms, running wheels and plastic tubes, as described previously (Vivinetto et al., 2013). Animals were exposed to novelty stimulation by rearranging the internal structure of the cage and renewing toys twice a week. Food and water were provided *ad libitum* but, in order to favor exploratory behavior, feeding boxes and water bottles were rearranged twice a week. The non-enriched condition was defined as two animals housed in open-top standard laboratory plastic cages (20 cm x 45 cm x 30 cm) with no other elements than wood chip bedding.

Behavioral Testing

Each behavioral test was performed with different animal cohorts in which all treatments were represented. Once finished, the animals were returned to their corresponding enriched environment or standard laboratory cages.

Barnes Maze

To assess variations in learning and memory performance in the experimental groups, we conducted an adapted version of the Barnes maze. Most of the Barnes test protocols involve multiple training sessions. The protocol used in the present work is a short and spaced adaptation of a training paradigm previously described by other authors, used to assess subtle variations in cognitive performance (Attar et al., 2013).

Apparatus: The maze consisted of a round open platform, 122 cm in diameter, mounted 60 cm above the ground. Along its perimeter were 18 equally spaced holes, each 10 cm in diameter, located 2 cm from the platform edge. The maze also had one removable black goal box (23 × 17 × 10.2 cm). All holes appear dark and looked identical from the maze surface to minimize intra maze visual cues. A removable opaque open cylinder (20 cm in diameter, 30 cm high) was used to position animals in the center of the maze for the start of each trial. The maze was placed in the center of an illuminated room. Two high contrast signals (one large square and one large triangle) were located around the maze as visual cues. All sessions were recorded with a high definition video camera mounted from the ceiling above the center of the maze. The experimenter stood in a fixed position in one corner of the room approx. 1.5 m away from the maze. The maze and goal box were wiped with 70% ethanol between individuals to dissipate odor cues and provide a standard olfactory context for each trial.

Testing Procedures:

Testing began on PND 61. Animals were habituated to the testing room 1 hour before testing. The acquisition phase consisted of two training days with two trials on the first day (15 min intertrial) and one trial on the next day. At the beginning of each trial, rats were placed in the start cylinder at the center of the Barnes maze. After 30 sec, the cylinder was removed, and they were allowed to freely explore the maze for 3 min. to find the escape box. If the animal did not find the target hole before the time ran out, it was gently guided into it. Once the animal was in the escape box, it was allowed to stay in there for 1 min and then was returned to its holding cage for a 15 min inter-trial interval. During acquisition training, the following measures of learning performance were recorded: (1) the latency (sec) to initiate search, defined as the first investigation of any hole; (2) the latency to locate/enter the escape hole, defined as the time to identify the target hole the first time; (3) number of errors, head-dips into non-target hole.

24 h after the last training session, on the probe day (PND 63), spatial memory retrieval was tested. For this purpose, the escape box was removed, the rat was placed inside the cylinder

in the center of the maze for 15 sec, and then was allowed to explore the maze for 90 sec, at the end of which it was returned to its holding cage. During the probe phase, memory was assessed by recording (1) the time spent per quadrant (the maze was divided into quadrants consisting of 3 holes with the target hole in the center of the target quadrant); (2) number of errors (number of incorrect holes visited); (3) distance (total path length); (4) velocity (average speed); (5) latency to escape (time to find the hole where the escape box used to be); (6) time in the quadrant opposite to the escape hole. The rats' search strategies were also analyzed and classified into one of three categories: Direct: when going directly into the escape hole or the two adjacent holes; Serial: when systematic search along the maze perimeter was in a clockwise or counterclockwise direction or 80% of investigated holes were adjacent until the escape hole was located; Random: 60% of investigated holes were non-adjacent and animals made more than two crossings through the center of the maze to check different holes.

Forced Swimming test

The forced swimming test was performed on two consecutive days, as previously described (Aguggia et al., 2013), in a 40cm high, 20cm diameter acrylic cylinder. It was filled with tap water at 25±1°C to an approximate height of 30 cm, so that the animal could not touch the bottom with its hind legs. On PND 61, a pre-test session was conducted for 12 min in order to minimize the acute stress of water and to acclimatize them to testing situation. Twenty-four hours later, each rat was tested for 5 min in the same cylinder and same conditions as the previous day. After each session, the animal was removed from the cylinder, gently dried with a towel and placed in a transient drying cage with a heat lamp above it until its fur was dried. Test sessions were registered by a video camera positioned in front of the cylinder and analyzed later. Water was changed after every animal session. During the 5-minute test, the following behaviors were recorded: (1) immobility time (absence of movements except for those necessary to keep the nose above water); (2) swimming time (energetic movements of forelimbs or hind limbs); and (3) climbing time (energetic vertical movements with the forelimbs against the cylinder).

Tissue Collection and Western Blot Analysis

Immediately after the Barnes test, the animals were euthanized by decapitation using a guillotine for small rodents, and brains were removed immediately. Hippocampi were dissected on ice and immediately lysed and homogenized with radioimmunoprecipitation assay buffer containing Tris-HCl pH7.6 25mM; 150mM NaCl; 1% NP-40; 1% sodium deoxycholate; 0.1% sodium dodecyl sulfate; phosphatase and protease inhibitors for 3 min at 4°C. The samples were then centrifuged at 15000 g at 4ºC. The supernatant was transferred to Eppendorf tubes and stored at -80ºC until use.

The protein concentration of each sample was determined using the DC Protein Assay Kit (Bio-Rad Laboratory, Hercules, CA, USA), and equal protein amounts (25µg) were denatured for 5 min at 95ºC, electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gel and then transferred to nitrocellulose membranes (Bio-Rad Laboratory, Hercules, CA, USA). Membranes were blocked with 1% skimmed milk in 10 mM Tris-HCl at pH 7.4, and then incubated in 150 mM NaCl, 0.1% Tween 20 (TBST) overnight at 4ºC with their primary antibodies. The antibodies used were: anti-Erk (1:2000, Sigma-Aldrich, St. Louis, MO, USA), anti-pErk (1:1000, Sigma-Aldrich, St. Louis, MO, USA), anti TrkB (1:1000, Abcam, Cambridge, UK), anti-α-tubulin (1:3000, DM1A; Sigma-Aldrich, St. Louis, MO, USA). Membranes were then washed three times in TBST for 10 min each followed by incubation with a horseradish peroxidase-conjugated secondary antibody (1:2000; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 1 h at room temperature. After two washes with TBST, bands were visualized using a chemiluminescence detection kit (ECL; Amersham Life Science, Buckinghamshire, England). The plates were digitized in a scanner and then quantified by densitometry using the Fiji-ImageJ analysis software (NIH, Bethesda, MD, USA).

Statistical Analysis

All statistical tests were performed with SPSS and InfoStat software. Homogeneity of variances and normal distribution of behavioral and biochemical data were tested by Levene's test and Shapiro-Wilk test respectively ($p>0.05$). Data from the Barnes test assessing learning capacity (acquisition phase) were analyzed by a linear mixed model test for repeated measures, with maternal separation x environmental enrichment as factors. When the interaction between factors was significant, to further characterize the behavioral response, single effects were analyzed by one-way ANOVA. All other analyses were conducted with 2x2 factorial ANOVA (maternal separation x environmental enrichment as factors). Post hoc tests were completed with the Bonferroni or Fisher LSD test. The level of significance was set at $p<0.05$. Categorical data from search strategies behavior were analyzed using Chi-Square. Results are expressed as mean ± standard error of the mean (SEM).

RESULTS

Barnes Maze

Acquisition training: Latency to find the goal

Analysis with repeated measures of the data revealed a significant interaction effect of maternal separation, enriched environment and trial in the latency time to find the goal $F_{2,30}= 5.377$ ($p= 0.008$).

All the animals showed a decreased latency to locate the escape hole compared with the first trial, $F_{2,30}=37.032$ ($p=0.001$), except for the MS/NE group which showed a virtually unchanged

latency to reach the escape hole, indicating that they did not learn the spatial memory task (Fig. 1-A).

Pairwise comparisons showed that, during the last training, both groups that were reared in an enriched environment took a significantly shorter time than the groups without environmental enrichment ($p = 0.0019$) and MS/EE animals took less time to find the target hole than the AFR/EE ($p= 0.0001$).

Acquisition training: Number of incorrect holes

There was a triple interaction between maternal separation, environmental enrichment and trial, $F_{2,30}=13.517$, ($p<0.001$). The number of incorrect holes explored decreased over time except for the maternally separated animals reared in standard environment (MS/NE group) in which the statistical analysis showed a significant increase in the exploration of non-goal holes as the trial progressed ($p<0.05$). In animals with EE, there was a decrease in the number of incorrect hole investigations during the last trial regardless of the early treatment ($p<0.001$) (Fig 1-B).

Acquisition training: Latency to initiate search

Considering the latency to initiate search (i.e. total time between start cylinder removal and first hole investigation), the results showed that there were no significant differences between the groups $F_{2,30}=1.380$, ($p=0.261$). That is, all groups of animals showed similar performance in all trials, which indicates that in no case did the treatments interfere with the motor abilities or the level of motivation to explore the maze (Fig. 1-C).

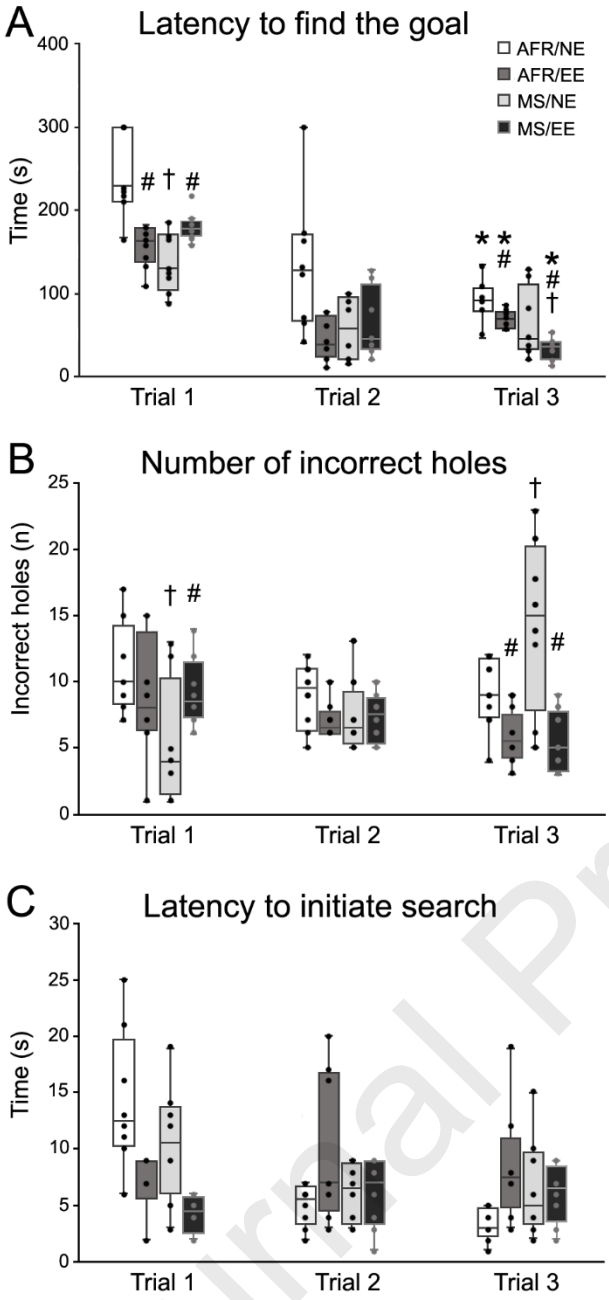

Figure 1: Performance in the Barnes maze during training days (A)-Latency time to find the escape hole over the training trials. (B)-Number of errors locating the escape box. (C)-Latency to initiate search over training trials. Box plot shows 25th to 75th percentiles, inner line represents the median, whiskers extend to the 5th and 95th percentile, (n=7-8 animals for each group). Visits to incorrect holes decreased over time in environmentally enriched groups, indicating that the animals were learning the location of the box.

* p≤ 0.05 when compared to the trial 1

p ≤ 0.05 when compared to NE

† p ≤ 0.05 when compared to AFR

Testing- Probe day

A single retention trial was conducted 24 h after acquisition training. Statistical analysis revealed a double interaction between maternal separation and environmental enrichment $F_{3,29}$=13.409, (p=0.001) in the number of incorrect holes visited. Post hoc analysis showed that the number of errors was significantly higher in the MS/NE group than in the AFR/NE (p=0.008). However, MS rats in the EE group had a lower number of visits to incorrect holes and performed at the same cognitive level as the non-maternally separated groups (p=0.001) (Fig. 2-A).

There was also a significant maternal separation x environmental enrichment interaction on the time spent in the target quadrant $F_{3,29}$= 25.256, p=0.001. Post hoc analysis revealed that this was significantly higher in enriched animals regardless of rearing condition (p<0.005), indicating that the ability to remember the location of the target hole is favored by environmental enrichment. Surprisingly, animals with maternal separation and MS/EE showed a higher percentage of time in the target quadrant than animals raised in standard conditions (p=0.001) (Fig. 2-B).

Statistical analysis revealed a significant interaction between maternal separation x environmental enrichment in the time to find the hole where the escape cage was located $F_{3,29}$= 35.296, (p=0.001). Enriched animals regardless of early stress condition showed significantly lower latency time to find the target hole (p<0.001). Maternally separated animals took less time to find the target hole than their respective AFR controls (p<0.001) (Fig 2-C).

No significant differences were found between groups in the time spent in the opposite quadrant to where the escape hole was located in acquisition training $F_{3,29}$= 2.090, nor in average speed $F_{3,29}$= 0.440 or in total path length $F_{3,29}$= 2.445 (Figs 2-D-E-F, respectively).

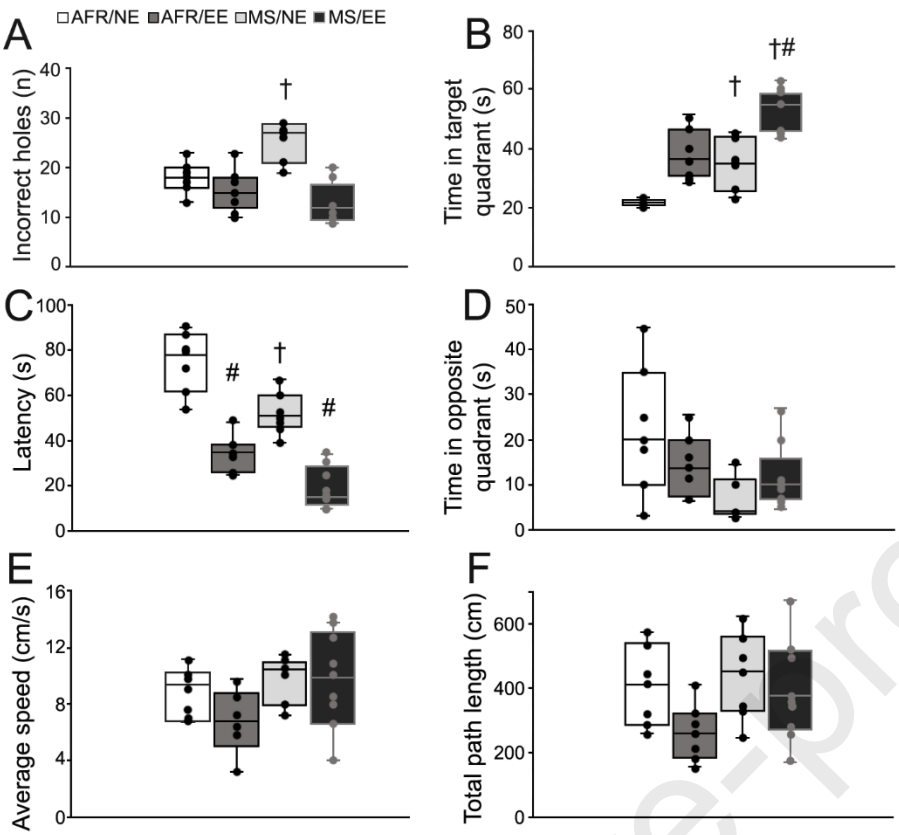

Figure 2: Probe day. Memory retention assessed 24h after last training exposition. (A)-Number of incorrect holes visited, (B)-Time spent in the target quadrant, (C)- Latency time, (D)-Time spent in the opposite quadrant, (E)-In average speed, (F)-Total path length. Box plot shows 25th to 75th percentiles, inner line represents the median, whiskers extend to the 5th and 95th percentile, (n=7-8 animals for each group).

p≤ 0.05 when compared to NE

† p≤ 0.05 when compared to AFR

Search strategy use

Animals exposed to the enriched environment, regardless of early life stress condition, showed around 80% use of strategies that involve visual cues (direct and serial) to find the escape hole during the probe, and less than 20% in random strategy (Chi Square=29.88, p<0.001), suggesting improved navigational abilities. Groups from the standard non-enriched environment used mostly random search (almost 60% of the total) (Fig 3).

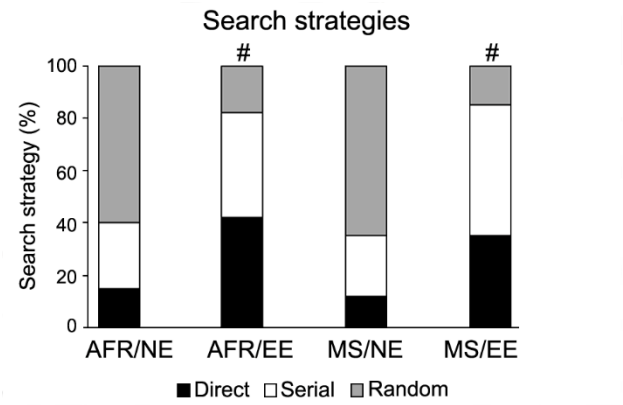

Figure 3: Stacked bar graph indicating the percentage of time engaged in specific search strategy usage during probe day.

#: p<0.001 Chi Square

Forced swimming test

No significant differences were found among experimental groups either in active behaviors, swimming time $F_{3,29}$= 1.178 (p=0.341) or climbing $F_{3,29}$= 0.761 (p=0.528); nor in passive behaviors, immobility time $F_{3,29}$= 0.260 (p= 0.854) during the forced swimming test (Fig 4 A-B-C).

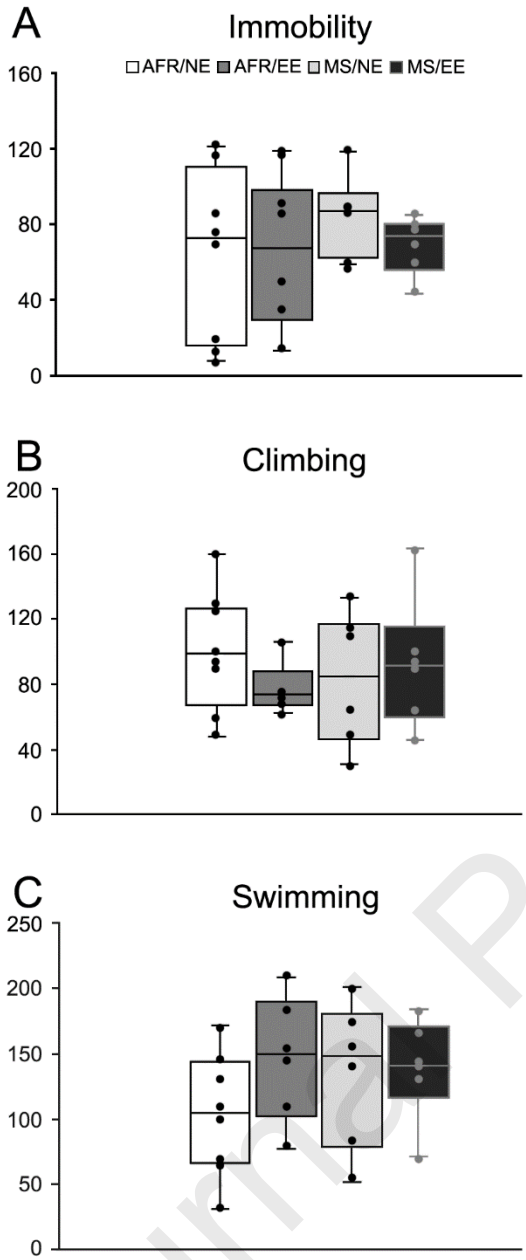

Figure 4: Forced Swimming Test. (A)-Immobility, (B)-Climbing and (C)-Swimming time (seconds). Box plot shows 25th to 75th percentiles, inner line represents the median, whiskers extend to the 5th and 95th percentile (n = 8 animal for each group).

Hippocampal BDNF-receptors and pERK/ERK expression

The effects of early adverse maternal separation and housing environment during adolescence on TrkB and pErk/ Erk expression in the hippocampus were analyzed by means of Western blot. Two-way ANOVA demonstrated that environmental enrichment increased the expression levels of full-length TrkB (FL TrkB) protein expression, regardless of early maternal

condition. $F_{3,22}$= 14.97, p= 0.02 (Fig 5-A). The expression of truncated TrkB was significantly higher in the MS/EE compared to the other groups $F_{3,22}$= 14.80, p= 0.021 (Fig 5-B).

Finally, phosphorylated Erk levels (a downstream TrkB receptor signaling kinase) were significantly lower in the control group (i.e. AFR/NE) $F_{3,22}$= 15.81, p= 0.01. There was no difference in phosphorylated Erk levels between the rest of the groups (Fig 5-C).

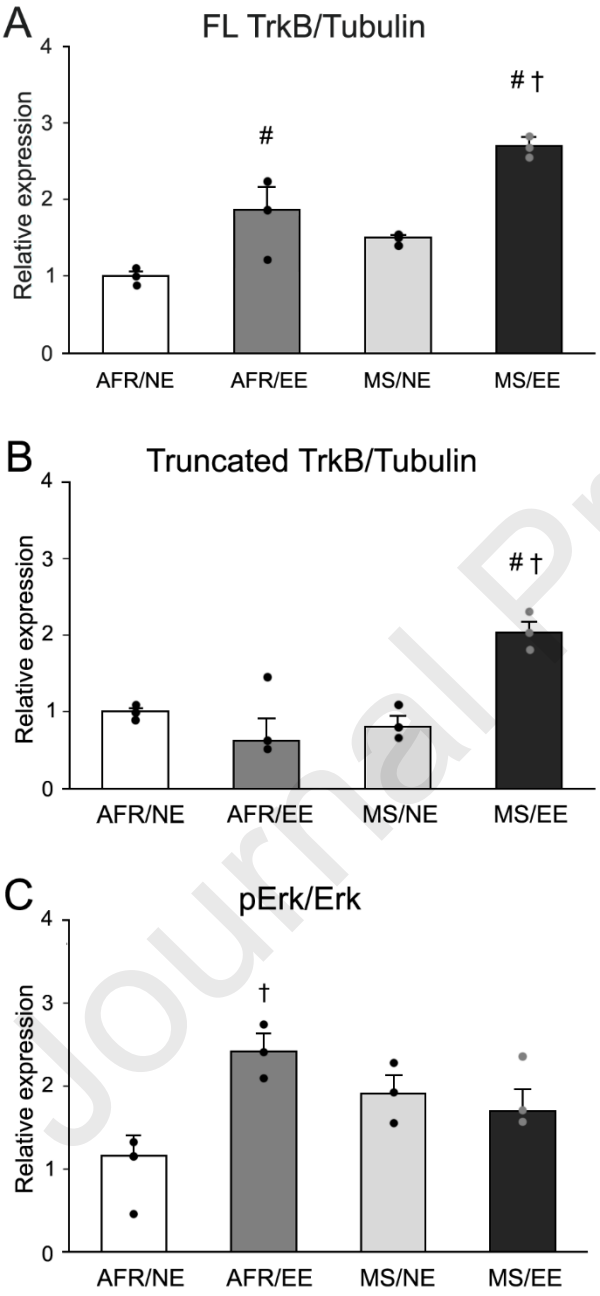

Figure 5: BDNF-receptor expression in the hippocampus. (A)-Full length TrkB protein expression. (B)- Truncated TrkB protein expression. (C)- Phosphorylated Erk/Erk protein levels. Data are presented as median ± SE.

p≤ 0.05 when compared to NE
† p≤ 0.05 when compared to AFR

DISCUSSION

This work studied how early maternal separation and subsequent environmental enrichment influence cognition, emotional behavior and hippocampal BDNF-receptor and signaling proteins expression, which play key roles in neuronal plasticity. The data indicate that early maternal separation impaired spatial learning abilities in the short version of the Barnes test.

During acquisition, the escape latency time decreased in all experimental groups as training progressed, except in MS/NE. During this phase also, as the animal learns where the target hole is, the number of errors (exploration of incorrect holes) should decrease over time. In this experiment, there was a significant difference between the subjects (groups). All groups could be expected to have similar latency time to reach the target hole and a similar number of errors during the first trial, and the animals that learned to locate the escape hole during the training sessions would then be expected to have fewer errors and shorter latencies to find the escape hole again. This was the case for AFR/NE, AFR/EE and MS/EE, which showed improved performance during the trials, indicating that they learned to solve the maze correctly. The MS/NE group, however, did not show this progression. It is interesting to note that, in the MS/NE group, both parameters were significantly lower during the first training and then, as the training continued, the latency was unchanged and the number of errors increased. This might mean that the MS/NE animals learn to solve the maze quickly and are then able to maintain this performance during the rest of the training. In that case, it could be said that the early life adversity of MS did not necessarily result in learning deficit, but rather in a different coping strategy in the challenging situation of the Barnes maze. However, the number of errors also increased in this group. Studies have concluded that the number of errors is the more sensitive measure for detecting memory impairment during acquisition trials (O'Leary and Brown, 2013; Gawel et al., 2019), which would indicate that MS/NE animals indeed have a cognitive deficiency in learning to solve the maze. Exposure to environmental enrichment decreases the number of errors as training progresses regardless of early life stress. Comparing the starting and ending latencies, the EE groups showed the lowest escape latency at the end of the acquisition trial indicating that learning velocity was higher in enriched animals.

Taken together, the data from the training stage indicate that all the experimental groups, except those separated from the mother raised in a standard non-enriched environment, fully acquired the spatial task through the training in the abbreviated version of the Barnes maze.

In the literature there are methodological differences in running the Barnes maze (Gawel et al., 2019). In the acquisition phase, although most protocols include several training sessions per day usually during 4-5 days, abbreviated versions of the Barnes test training protocol have been previously reported (Vargas-lópez et al., 2011; Attar et al., 2013; Morel et al., 2016), and indicate that this version is sufficient to induce learning in rats and that it has the advantage of simplifying behavioral assessment. Results from the protocol presented here indicate that, in this short version of the acquisition phase, AFR animals can efficiently acquire and retain the location of the escape box, while MS animals showed a deficiency or inability to properly acquire learning. Environmental enrichment during adolescence reverses the deficit in animals exposed to maternal separation and improves cognitive performance in AFR groups.

Considering the latency to explore the first hole (latency to initiate search), the results showed no significant differences between the groups nor between different training sessions, suggesting that the treatments did not alter motor skills or the level of motivation to explore the maze and that the motivation to explore the maze and find the escape hole is maintained during the training. During the retention test, enriched animals showed a significant preference for the target quadrant, indicating that they not only learn the test faster but also retain their enhanced performance more efficiently, showing preference in exploring the quadrant where the escape hole is located.

An unexpected finding was that maternally separated animals spent more time in the target quadrant and had a lower latency in reaching the target hole. Nevertheless, the frequency of exploration of the incorrect holes was significantly higher in MS/NE animals, while in the group exposed to environmental enrichment the number of errors decreased to levels comparable with animals not separated from their mother. Regardless of early life condition, enriched animals used spatial strategies (Serial + Direct) to find the target hole, while a random strategy predominated in non-enriched animals, indicating that environmental enrichment increases the use of spatial strategies to solve the test.

There is no general agreement about the effects of early manipulations, like maternal separation, on behavioral aspects related to memory and learning. Such discrepancies may be associated with differences in maternal separation protocols, such as the time of separation or its duration in the postnatal period. Our results match other reports that indicate that prolonged maternal separation impaired cognitive performance on spatial tasks in rodents (Aisa et al., 2007; Wang et al., 2011; Holubová et al., 2018; Maghami et al., 2018). However, it has been suggested that early manipulation alters learning in a task-specific manner and

that the duration and timing of early manipulation are critical factors determining performance in spatial memory in rats (Kosten et al., 2012).

Studies have shown that early adverse experiences may predispose to affective disorders in adulthood, both in human and animal models (Juruena, 2014; Chen and Baram, 2015; Syed and Nemeroff, 2017). While maternal separation in an animal model has been previously reported to induce depression-like behaviors in adulthood (Vetulani, 2013), in our study, unexpectedly, early maternal separation did not generate immobility in adulthood in the forced swimming test (considered to reflect a measure of behavioral despair) compared to normally reared animals, nor did the enriched environment affect behavior. Other authors also reported that maternal separation impaired adult cognitive performance but did not affect emotional behavior in the forced swimming test (Wang et al., 2011), or reported no differences for total time immobile in a model of maternal separation and postweaning enrichment (Doreste-Mendez et al., 2019). Likewise, the EE did not produce significant changes in the behaviors evaluated during the FST.

Probably the maternal separation protocol used in this study, in which the mother is removed from the home cage, while leaving the offspring in the familiar environment and in the company of their siblings, is less stressful for the pups and failed to induce immobility or passive coping behaviors by itself, but it is possible that it increases the susceptibility to develop behavioral despair when the animals are exposed to later stress situations. According to the “two hit” model, maternal separation generates an internal vulnerability that alone is not enough to express behaviorally but behavioral changes emerge when animals are exposed to subsequent stress (Murthy and Gould, 2018) (Marais et al., 2008).

Finally, it is important to consider that, while immobility in the FST is widely interpreted as depressive-like behavior in different animal models, some studies consider that it actually reflects a coping strategy in the face of a situation of acute stress.(Molendijk and Kloet, 2015). Additional behavioral tests to assess depressive behavior should be addressed in future experiments.

The hippocampus has been extensively studied, due to the importance of this region in memory and learning processes, besides being a key structure in the neuroendocrine circuit of the stress response. It has been previously reported that maternal separation results in long-term changes in BDNF expression (Sullivan et al., 2011; Bath et al., 2013; Wang et al., 2015) but contrasting findings have been reported. In addition, few studies have addressed the impact of early environmental manipulations on BDNF receptors and signaling pathways. BDNF binds the TrkB receptor with high affinity and activates different signaling pathways that mediate neurotrophin functions, such as cell survival, synaptogenesis, synaptic remodeling, and growth of axon and dendrites, and this could at least partly explain the effects of enrichment conditions in the brain and in behavior.

In the analysis of BDNF receptor expression in the hippocampus, FL TrkB levels showed a direct relation with performance during the probe phase of Barnes maze, where the MS/EE group showed the highest levels of this receptor, coinciding with the increase in time in the target quadrant and the lower number of errors observed in this group. Although maternal separation does not significantly reduce the expression of FL TrkB, EE increases it significantly in both groups separated from the mother and raised in standard conditions.

Truncated TrkB levels also showed the highest level of expression in the MS/EE group. The truncated TrkB is normally expressed in astrocytes and it is plausible that the negative experience of maternal separation may first induce an increase in these levels as a neuroprotective mechanism, and then sensitize the system against a new exposure to mild stress, such as the enriched environment. Vulnerable individuals, such as those maternally separated, could experience EE as mild stress given the presence of social competitors, physical exercise, novel objects, rearrangement of space, while resilient individuals in the same environment may develop or display an adaptive neurophysiological response.

The pErk/Erk ratio showed that the AFR/NE group had a significantly lower activation of this TrkB downstream kinase than the rest of the groups. Data from Ohta et al. reveal that prolonged maternal separation reduced BDNF expression and BDNF-Erk activation signaling in a period-specific way (postnatal day 7), corresponding to synaptogenesis in the hippocampus. Disruption of synaptogenesis during early development could explain deficits in hippocampal-dependent learning and memory. However, BDNF expression as well as BDNF-Erk activation increased in the weaning period (Ohta et al., 2017). The increase we observed in our work in the pErk/Erk ratio could reflect a compensatory effect by repeated MS, considering that BDNF has a neuroprotective and repair function.

Taken together, our behavioral results show that spatial memory is impaired in maternally separated animals, and that environmental enrichment during adolescence reverses these alterations. The maternal separation model does not affect the expression of immobility in the forced swimming test. This suggests that certain systems are more vulnerable, and that early MS stress affects them differently when cognitive function is impaired but that the strategies for coping with situations of acute stress assessed in the FST are not affected. While BDNF receptors may be involved as possible underlying mechanisms, new studies are necessary to further elucidate their role.

ACKNOWLEDGMENTS

This work was supported by National University of Cordoba-SECyT grant [Res Nº.411-18].MAR and DM conceived the study, participated in the design and coordination of

experiments and drafted the manuscript. FRM participated in the design and interpretation of data and helped to draft the manuscript. JMC, VD, JPA participated in the design of the study, completed data acquisition and performed statistical analysis and data interpretation. All authors discussed the results and contributed to the final manuscript.

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HIGHLIGHTS

EE reverts the effects of maternal separation on learning and memory in a short Barnes maze protocol.

Either MS or EE paradigm did not produce significant effects on the immobility in the forced swim test in adulthood.

The short Barnes maze training protocol evidences changes in learning ability and memory.

Effects of combined early MS and EE during adolescence on cognition are related to BDNF signaling in the hippocampus.