Early life stress due to repeated maternal separation alters the working memory acquisition brain functional network

Journal:	*Stress*
Manuscript ID	EDS-2631.R2
Manuscript Type:	Original Research Report
Date Submitted by the Author:	n/a
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Keywords:	Early life stress, Maternal separation, Working Memory, Cytochrome c oxidase, Brain energy metabolism, Mammillary bodies

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Stress. The International Journal on the Biology of Stress
Editorial Office

April, 19th, 2020

Dear Editor in chief Prof. James P. Herman:

Please find attached the original manuscript entitled "Early life stress due to repeated maternal separation alters the working memory acquisition brain network" by Banqueri et al. in order to be re-submitted for review in Stress. The International Journal on the Biology of Stress. The strength of our article is that we demonstrate that early stress delays the acquisition of a working memory task but not completely alter this memory. We also measured oxidative metabolism, using cytochrome c oxidase histochemistry, in the brain of control and maternally separated subjects, discovering a greater energy expenditure for solving the task in stressed subjects in prefrontal cortices, hippocampi and mammillary bodies: to our knowledge for the first time.

All authors declare that there are no actual or potential conflicts of interest including any financial, personal or other relationships with other people or organizations that could inappropriately influence this work. The work described has not been submitted for publication, in whole or in part, elsewhere and all the authors listed have approved the manuscript that is enclosed.

Sincerely,

María Banqueri

Early life stress due to repeated maternal separation alters the working memory acquisition brain functional network

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Running title: Early life stress alters working memory

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Abstract

Unfortunately, adverse environments in early life are frequently found in most human populations. Early life stress leads to diverse cognitive impairments, some of them related to learning and memory and executive functions such as working memory (WM). We employ an animal model of early stress using repeated maternal separation (MS) for four hours a day on 21 consecutive days, pre-weaning. In adulthood, we tested their spatial WM using the Morris water maze. MS subjects showed a marked delay in the acquisition of the task. In addition, we explored brain energy oxidative metabolism and found an increase in cytochrome c oxidase (CCO) activity in the cingulate cortex, anterior thalamus, and supramammillary areas, indicating an intense effort to successfully solve the WM task. However, decreased CCO activity was found in the medial-medial mammillary nucleus in MS animals, which would partially explain the delayed acquisition of the WM task. Further studies are needed to explore the long-term alterations produced by early stress.

Key Words: Early life stress; Maternal separation; Working Memory; Cytochrome c oxidase; Brain energy metabolism; Mammillary bodies

Lay summary: A stressful environment caused by separation of baby rats from the mother for several hours a day in the first stages of postnatal life can be devastating to brain cells, making them look for alternative sources of energy, among other changes. These alterations in brain functional networks would lead to cognitive impairments such as delayed acquisition of new learning and strategies.

Introduction

Adverse experiences in early life are frequent in human populations. In fact, 63.9% of children experience at least one adverse event in their infant years (Philip et al., 2013). Children exposed to early stress obtain worse scores in different cognitive domains, mainly related to executive functioning, cognitive flexibility, inhibitory control, or sustained attention (Bos, 2009; Feifel et al., 2017), as well as their use of Theory of Mind Abilities (Simon et al., 2019). Early deprivation in humans could lead to circuit miswiring in the immature brain (Bos, 2009), suggesting a relationship between an adverse environment and aberrant neurodevelopment.

The first socioeconomic and emotional environment is relevant in cognitive development. In fact, childhood poverty seems to be inversely related to adult working memory (WM) in a dose dependent manner (Evans and Schamberg, 2009). In addition, this variable also influences the self-regulatory capacity, and so these children have greater difficulty ignoring distractors (Evans and Fuller-Rowell, 2013). Therefore, early life stress is associated with persistent WM impairment in humans (Fuge et al., 2014) because WM is one of the most sensitive neurocognitive systems to early stress (Evans and Fuller-Rowell, 2013). In fact, alterations in WM after early stress are a good marker of major depression in this population (Fuge et al., 2014).

Whereas reference memory is long-term stable memory, WM involves the manipulation and retrieval of information to carry out prospective actions (Barha et al., 2007). Hence, WM is a temporary storage mechanism that makes it possible to hold active information and manipulate it (Evans and Schamberg, 2009). According to the multi-component model of WM, it has different components involving control, manipulation, and protection from being distracted by irrelevant information (Baddeley and Hitch, 1974). Among these characteristics, the hippocampus (HC) is particularly relevant for the

maintenance of memory (Fuge et al., 2014), and it is known that early stress produces long-term prefrontal cortex (PFC) and HC alterations (Mizoguchi et al., 2000). Spatial WM is PFC-HC dependent (Zhang and Cai, 2008), which means that WM alterations would probably be found in adulthood in early stressed subjects.

In humans and other animals such as rodents, maternal care is important for neurodevelopment. In fact, subjects raised by attentive mothers (dams that show high rates of licking and grooming) have shown better outcomes on different cognitive tasks, such as spatial memory and WM (Barha et al., 2007). Hence, dam-litter relationship disturbances can act as a model for early life stress. One of the most widely used early stress animal models is maternal separation (MS) (Banqueri et al., 2017a; Plotsky and Meaney, 1993).

In this study, our aim was to explore early stress effects on adult performance on a PFC-dependent task, and the possible differences in the main brain-related functional networks. Specifically, we wanted to analyze whether a 21-day MS model would alter WM, and how metabolic brain activity would be affected, to our knowledge for the first time. In order to achieve this goal, we performed a WM test and then explored brain energy oxidative metabolism and the possible functional networks, using cytochrome c oxidase (CCO) histochemistry (Gonzalez-Lima and Cada, 1994).

Methods

Animals

Ten adult Wistar rats (*Rattus norvegicus*) (seven females (dams) and three males) were purchased from the vivarium at Oviedo University. The subjects used for this experiment were their litters (247-398.7g at the end of the experiment). All the animals received *ad libitum* food and tap water and were maintained at a constant room

temperature (22 +/-2 ºC), with a relative humidity of 65-75% and a 12h artificial light-dark cycle (08:00-20:00/20:00-08:00). The procedures and manipulation of the animals used in this study were carried out according to the Directive (2010/63/EU), Royal Decree 53/2013 of the Ministry of the Presidency related to the protection of animals used for experimentation and other scientific purposes, and they were approved by the Principality of Asturias committee for animal studies.

Maternal separation

The maternal separation procedure was carried out according to a previously published standardized protocol (Banqueri et al., 2017b; González-Pardo et al., 2019). Briefly, litters were randomly assigned to the MS or AFR condition. Litters with more than 10 animals were culled to 10, with approximately the same number of males and females in each. For MS, litters were separated from the dams for 4 hours per day, starting at 10:00 hours and ending at 14:00 hours. The MS group was separated from PND 1 to PND 21 (Figure 1A). Each separation consisted of removing the dams from the home cage and placing them in an adjacent cage (to keep them from witnessing the procedure) while the pups were kept together in a new cage. Litters remained together during the separation time in an incubator (30 ºC, 55-65% relative humidity). After the separation period, the dam and the litter were returned to the home cage (placing the litter in the home cage first). Control litters were reared under standard animal facility rearing (AFR) conditions, disturbed only by animal facility husbandry practices once a week until weaning. On PND 21, all the animals were weaned and segregated by sex, and only males were selected for the study. Therefore, two groups of male animals were included in the experiment, the control group (AFR, n = 12) and experimental group (MS, n = 12).

Morris Water Maze

On PND 100 (Figure 1A), the animals' behavior was tested in the Morris Water Maze (MWM) (Morris, 1984), as previously described (Méndez-López et al., 2009c). The apparatus consisted of a black cylindrical fiberglass tank measuring 150 cm in diameter by 75 cm in height, placed 35 cm above the floor. The water level was 30 cm, with a temperature of 22±2 °C. The escape platform used was a black cylinder measuring 10 cm in diameter and 28 cm in height placed 2 cm below the surface of the water, and it was not visible to the animals. The MWM was located in the center of a 16 m2 lit room (two lamps of 4000 lx oriented towards the walls), surrounded by black panels (30 cm from the maze) on which the spatial cues were placed (horizontal line, vertical line, and a square rotated 45°, all yellow or black and yellow). The pool was divided into four imaginary quadrants (A, B, C and D) to locate the start positions and platforms. The animal's behavior was recorded, and its path was analyzed using a computerized video-tracking system (Ethovision Pro, Noldus Information Technologies, Wageningen, The Netherlands).

Working memory task

In the learning protocol (Méndez-López et al., 2009a), the first day was devoted to the animals' habituation to the task. The animals received a three-trial habituation session with different starting and platform positions in a small square water tank (47×75×38 cm) one day prior to the initial test. The spatial memory task consisted of a paired sample task. Each daily session (6 days of training) was composed of two trials (sample and retention). The sample consisted of releasing the animal from one of the four starting points in the pool and letting it swim until it reached the hidden platform (maximum trial length, 60 s; time on platform, 15 s). To begin each trial, we placed the rats in the water, facing the maze wall in one of four quadrants, and the daily order of entry into these quadrants was pseudo-randomized (Figure 1B for examples). During

the inter-trial interval, we placed the animals in a black bucket for 30 s. After this time, we proceeded with the retention trial, which was identical to sample one every day. Latencies were our main dependent variable. The learning criterion was a lower escape latency on retention trials compared to sample trials.

Cytochrome oxidase histochemistry

Ninety minutes after the behavioral task in the MWM ended, the animals were decapitated. Brains were removed, frozen rapidly in N-methyl butane (Sigma-Aldrich, Madrid, Spain), and stored at -40 ºC until processing with quantitative CCO histochemistry, as described by González-Lima and Cada (Gonzalez-Lima and Cada, 1994). Coronal sections (30 µm) of the brain were cut at -22 ºC in a cryostat (Leica CM1900, Germany) and mounted on non-gelatinized slides. To quantify enzymatic activity and control staining variability across different baths, sets of tissue homogenate standards from the Wistar rats' brains (12 brains were used to create tissue homogenate, and they were treated in the same way as experimental brains) (Poremba, Jones, & Gonzalez-Lima, 1998) were cut at different thicknesses (10, 30, 50 and 70 µm). These tissues were included with each bath of slides to generate a single regression equation between CCO activity and the optical density of the sections for the subsequent comparison of all the tissues in the present experiment. The sections and standards were incubated for 5 minutes in 0.1 phosphate buffer with 10% (w/v) sucrose and 0.5 (v/v) glutaraldehyde, pH 7.6. Next, baths of 0.1M phosphate buffer with 10% (w/v) sucrose were given for 5 minutes each. Subsequently, 0.05M Tris buffer, pH7.6, with 275 mg/l cobalt chloride, 10% w/v sucrose, and 0.5 (v/v) dimethyl-sulfoxide was applied for 10 min. Then, sections and standards were incubated in a solution of 0.0075% cytochrome c (w/v), 0.002% catalase (w/v), 5% sucrose (w/v), 0.25% dimethylsulfoxide (v/v), and 0.05% diaminobenzidine tetrahydrochloride (w/v) (Sigma-Aldrich, Madrid, Spain) in

800ml of 0.1M phosphate buffer at 37ºC for 1h, in agitation to provide a homogenous oxygen distribution. The reaction was stopped by fixing the tissue in buffered formalin for 30 minutes at room temperature with 10% (w/v) sucrose and 4% (v/v) formalin. Finally, the slides were dehydrated, cleared with xylene, and cover-slipped with Entellan (Merck, Germany).

CCO optical density quantification

The CCO histochemical staining intensity was quantified by means of densitometric analysis, using a computer-assisted image analysis workstation (MCID, Interfocus Imaging Ltd., Linton, England) composed of a high precision illuminator, a digital camera, and a computer with specific image analysis software. The mean optical density (OD) of each region was measured on bilateral structures using three consecutive sections in each subject. In each section, four non-overlapping readings were taken, using a square-shaped sampling window adjusted for each region size (See Figure 3C). A total of twelve measurements were taken per region by an investigator who was blind to the groups. These measurements were averaged to obtain one mean per region for each animal. OD values were then converted to CCO activity units, determined by the enzymatic activity of the standards measured spectrophotometrically (Gonzalez-Lima & Cada, 1994). The regions of interest were anatomically defined according to Paxinos and Watson's atlas (Paxinos & Watson, 2005). The regions of interest and the distance in mm of the regions counted from bregma were: +3.20mm for the infralimbic (IL), prelimbic (PL), and cingulate (CG) cortices; -1.20 mm for the CA1, CA3, and dentate gyrus (DG) subfields of the dorsal HC; -2.04 for anterodorsal, anteroventral, and anteromedial (AD, AV, AM) thalamic nucleus; and -4.56 mm for the supramammillary (SuM), medial medial mammillary (MMM), and medial lateral mammillary (MML).

Statistical Analysis

The data recorded were analyzed using the SigmaStat software version 3.2 (Systat, Richmond, USA). In all cases, significance was accepted when $p<0.050$. Mauchly's test was used to test the sphericity assumption in repeated-measures analysis. Because the data met the sphericity assumption, uncorrected F tests were presented.

Behavioral data

We analyzed escape latencies for each day (sample and retention) separately for each group, using a paired T-test. If the data did not meet the normality assumption, we used Wilcoxon's Signed Rank test. In addition, we performed two further t-test analyses with total means for sample and retention in each group.

CCO activity

Group differences in CCO activity measured in each brain region were evaluated by one-way ANOVAs. A Kruskal–Wallis one-way analysis of variance of Ranks (H) was performed when equal variance failed. When statistical significance was found, Tukey's test was applied as a post-hoc test when ANOVA was used, and Dunn's method when Kruskal–Wallis was used.

Correlations

We performed the analysis of interregional correlations by calculating Pearson product-moment correlations. In order to avoid errors due to an excessive number of significant correlations in small sample sizes, we used a 'jackknife' procedure (McIntosh and Gonzalez-Lima, 1994), based on the calculation of all the possible pairwise correlations resulting from removing one subject each time, and taking into consideration only those correlations that remained significant ($p < 0.05$) across all possible combinations.

Results

Working memory task

We measured escape latencies during WM training in the MWM. We compared sample trial escape latency times with retention trial escape latencies between groups on each day of training. The animal facility reared (AFR) group (n=12) showed lower retention escape latencies than sample latencies. They learned the task on day 4, which means that they reached the learning criterion (lower escape latency on retention trials compared to sample trials). (D4 t $_{(11)}$: 3,042, p = 0.011), and this learning was maintained until *day 6* (D6 $t_{(11)}$: 2.648, p = 0.023). They did not reach the learning criterion on the first three days (D1 $t_{(11)}$: 2.024, p = 0.068; D2 Z: -1.752, p = 0.078; D3 $t_{(11)}$: 1.434, p = 0.179) (Figure 2A).

The MS group did not show learning on any training day (D1 $t_{(5)}$: 1.813, p = 0.097; D2 $t_{(11)}$: 0.219, p = 0.831; D3 $t_{(11)}$: 0.967, p = 0.454; D4 $t_{(11)}$: -1.017, p = 0.331; D5 $t_{(11)}$: 1.505, p = 0.161), except the last one (D6 $t_{(11)}$: 2.646, p = 0.023) (Figure 2B).

CCO activity

We measured CCO activity as a regional marker of brain energy metabolism. We used CCO histochemistry and optic densitometric analysis over 12 brain areas. First, we checked differences between groups using a one-way ANOVA, and then we explored possible functional networks using Pearson correlations between areas. Regarding energy metabolism, we found increased metabolic activity in the MS groups in the CG ($H_{(1)}$ = 5.143, p= 0.023), AD ($H_{(1)}$ = 4.710, p= 0.03), AM ($F_{(1, 21)}$ = *9.347*, p= 0.006), and SuM ($F_{(1, 21)}$ = *5.207*, p= 0.034). However, brain metabolism of MMM in MS subjects decreased ($H_{(1)}$ = 13.183, p< 0.001). No differences between groups were found in the rest of the probed areas: IL ($H_{(1)}$ = 1.286, p= 0.257), PL ($H_{(1)}$ = 1.587, p= 0.208), AV ($F_{(1, 21)}$ = 3.544, p= 0.074), CA1 ($F_{(1, 22)}$ = 3.578, p= 0.072), CA3 ($F_{(1, 22)}$ =

0.121, p= 0.731), DG ($F_{(1, 22)}$ = 1.675, p= 0.210), and MML ($H_{(1)}$ = 0.397, p= 0.529). See figure 3.B and table 1&2 for Pearson correlation results.

Discussion

MS leads to WM delayed acquisition in males when stress is present in early stages of neurodevelopment (from PND 1 to 21). Previous literature indicates that acute stress facilitates WM (Barha et al., 2007); however, when stress becomes chronic, as in early life stress, studies have shown that it leads to long-term WM impairments when early restraint stress plus adult stress are present (two-hit approach) (Jin et al., 2013) or adult chronic stress (Mizoguchi et al., 2000). It has been consistently reported that early perturbations can lead to long-term behavioral alterations (Bouet et al., 2011; Zhang and Cai, 2008) that can be noticed as early as the adolescent period in WM (Viola et al., 2019). Here we propose that male rats submitted to a long period of MS (4h per day during the entire pre-weaning phase) will display delayed acquisition of a WM task, which means that the WM skill, and probably its related functional networks, develop differently in these subjects.

Some authors have claimed that MS affects WM in adulthood in stress-susceptible mice strains (Feifel et al., 2017). However, although some genotypes (polymorphisms of corticotropin releasing factor receptor 1) are more sensitive to early stress than others, severe early stress produces WM disturbances independently of the previous genotype (Fuge et al., 2014). Nonetheless, other authors found WM deficits in mice submitted to post-weaning isolation procedures, but not after MS (Bouet et al., 2011). The rationale behind this apparent controversy lies, in our opinion, in the MS model used. These authors used maternal *deprivation* (24h PND 9) and not prolonged MS (for 21 days), which represents sustained and repeated episodes of early stress. In our view, this repeated model could emulate the human scenario better.

Other memory types, such as reference memory, are not impaired after chronic stress exposure, as studies have shown (Banqueri et al., 2017b; Mizoguchi et al., 2000). This could mean that WM impairments after MS might be specific. The model of MS that we use could be specifically affecting PFC-related skills such as cognitive flexibility (Banqueri et al., 2018) and, as we propose here, WM. However, MS would not lead to a general memory impairment, with navigational and spatial memory skills remaining intact.

CCO has repeatedly been used as a neural marker of learning and memory neural substrate changes related to different behavioral performance. This marker shows the differences between regional oxidative metabolism, and these changes denote increases and decreases in the activity and function of brain areas (Fidalgo et al., 2014; Méndez-López et al., 2009c; Poremba et al., 1998; Rojas et al., 2012; Sampedro-Piquero et al., 2013). We observed several brain areas that could have been functionally impaired in these subjects.

First, CG that belong to the mPFC, which has been repeatedly related to WM (Hanson et al., 2012; Yuen et al., 2009), may be altered. In this prefrontal cortex, MS subjects displayed greater metabolic activity, probably indicating, finally, the acquisition of the task. After the acquisition of a given task, the energy needed to complete it decreases (Conejo et al., 2010; Méndez-López et al., 2009a). The AFR animals, which learned the task 3 days earlier, showed brain activity levels that were already normalized. MS subjects only showed success on a spatial WM memory task on day 6 of training (Figure 2B), whereas AFR rats showed learning from day 3. Therefore, MS can lead to WM deficits in adulthood, perhaps mediated by alterations in the PFC. Remarkably, the opposite scenario to parental neglect, animal models of early stimulation such as neonatal tactile stimulation, produce long-term PFC potentiation along with improved

spatial WM (Zhang and Cai, 2008). One explanation for WM impairment related to CG could be a decrease in interneurons in this brain region. Particular functions of this population of cells in the PFC have been related to proper cognitive function (do Prado et al., 2016). Indeed, accumulative stress has been shown to produce impairments in spatial WM, along with smaller PFC volumes, in humans (Hanson et al., 2012). Both cellular and chemical disturbances have been proposed to explain these PFC changes after early life stress. One neurochemical mechanism related to cognitive impairments is PFC dopaminergic dysfunction because the mesocortical dopamine path is vulnerable to stress. Chronic stress produces an increase in D1 receptor density in this area, and antidepressants with dopamine-related actions improve WM in depressed subjects (Mizoguchi et al., 2000).

In addition to CG, the anterior thalamus areas, AD and AM, showed an increase in oxidative metabolism. The WM task in the MWM has a strong spatial navigation aspect, and the anterior thalamus, as a key node in Papez's circuit, is a key region for all spatial navigation tasks (Aggleton et al., 2010; Jankowski et al., 2013). Using scopolamine, which impairs acetylcholinergic neurotransmission, known for having a pivotal role in learning and memory, decreased activity in the anterior thalamus was found. This decreased metabolism correlated with WM impairment (Méndez-López et al., 2011), showing that correct functioning of the anterior thalamus is necessary for WM task acquisition.

Interestingly, no changes in HC were found. It is known that the PFC influences adult HC neurogenesis; in fact, PFC activity after a WM task can decrease HC proliferation rates (Schaefers, 2015). Hence, we also measured CCO units in the HC, which is also frequently associated with spatial WM. An intact PFC is essential for WM, and the HC represents another crucial node in the WM function network (Evans and Schamberg,

2009). However, we observed no changes in this area, and we could hypothesize that altered HC functioning could be helping the delayed acquisition. Early stress challenges and hippocampal impairment have been consistently found in both humans and rodent models (Blankenship et al., 2019; Nouri et al., 2020).

Finally, we also explored MB. These hypothalamic nuclei are consistently related to spatial memory tasks (Méndez et al., 2008; Vann, 2010; Vann and Aggleton, 2004). Using an analysis of early gene activity as an approach, some groups have found a relationship between SuM and WM (Santín et al., 2003). In SuM, we found the same pattern of increased activity in the MS group. However, in MMM, we saw the opposite pattern; MS showed less activity than AFR. MMM lesions have been shown to lead to WM impairments (Méndez-López et al., 2009b) because this area is essential to spatial WM in rodents, which could explain why this region is still very active even though AFR animals learned the task on earlier trials. In general, MS subjects that just learned the task showed an increase in all the areas needed to solve it, except the MMM. This delayed MMM recruitment could be one of the keys to the late acquisition of the task in MS animals.

MB, often neglected in WM studies, have been found to play a key role in spatial memory tasks that do not involve intense stressful components. They also provide the essential head direction information and help with the allocentric navigation (Kinnavane et al., 2018) needed for WM tested in the MWM. Hence, we propose that the differential energy expenditure found in MMM and SuM in MS subjects may be preventing task acquisition. This would make MB alterations a pivotal area in cognitive delays due to early life stress.

When we focus on CCO unit correlations between areas, which allow us to infer joint functional brain metabolic activity, AFR did not show any particular pattern of shared

activation between areas. We think this could be a sign of a well-acquired task that is not currently requiring too many brain resources. However, the MS functional network is still complex (Figure 3B), requiring the collaboration of the PFC, thalamus and MB, the areas usually required to successfully perform spatial WM tasks (Méndez-lópez et al., 2010; Méndez-López et al., 2009b), except for the HC, which is another explanation for their acquisition delay.

Early life stress compromises typical neurodevelopment. Nevertheless, this *miswiring* does not seem to be homogeneous, with the PFC-related cognitive skills being more damaged, such as cognitive flexibility (Banqueri et al., 2018) or WM. One possible limitation of our research could be the lack of a basal control group that we could use to measure the CCO activity prior to learning experience, to detect more precisely which CCO changes are due to the learning experience and which are related to the stress itself. We aimed to explore the neural substrates of stressed subjects while performing a WM task, and we found an overall hypermetabolism in classical related areas. Further studies are needed to more thoroughly explore metabolic and cellular changes after early stress in this network.

Acknowledgements

This research was supported by Projects Grants of the MINECO (Ministerio de Economía y Competitividad del Gobierno de España): PSI2017-90806-REDT, PSI2017-83893-R, PSI2017-83038-P, PSI 2015-73111-EXP, BES-2014- 070562 to M.B.

Disclosure of interest

The authors report no conflicts of interest.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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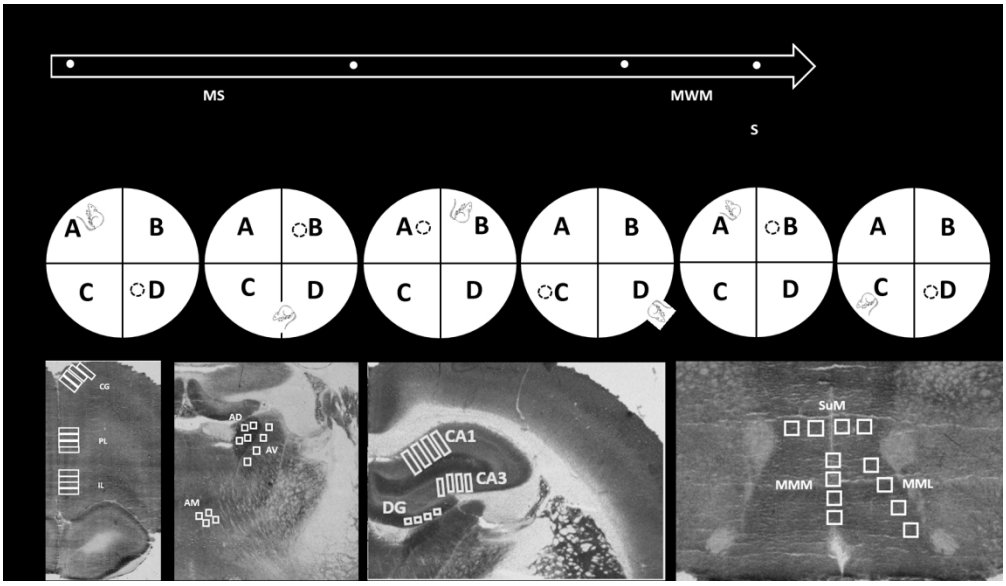
Figure captions

Fig. 1: A) Experimental timeline. PND: Postnatal day, MS: maternal separation, MWM: Morris Water Maze, S: Sacrifice. B) Representation of the different training days in a working memory task. C) Square shaped sampling frames of CCO histochemistry in the regions of interest. Each sampling frame was adjusted to the neuroanatomical shape of the given region of interest. Infralimbic (IL), prelimbic (PL), and cingulate (CG) cortices, CA1: *Cornu Ammonis* 1; CA3: *Cornu Ammonis* 3; dentate gyrus (DG, anterodorsal, anteroventral and anteromedial (AD, AV, AM) thalamic nucleus, supramammillary (SuM), medial medial mammillary nucleus (MMM), medial lateral mammillary nucleus (MML).

Fig. 2: Behavioral results. Escape latencies in MWM over days A) AFR group. This group acquired the task on day 4 of training. B) MS group escape latencies. This group learned the task only on the last day. *$p<0.05$. AFR: Animal facility rearing. MS maternal separation.

Fig. 3: CCO results. A) CCO units in the areas of interest. B) Functional network diagram of statistically significant Pearson correlations between areas in MS group. *$p<0.05$. AFR: Animal facility rearing. MS maternal separation. Infralimbic (IL), prelimbic (PL), and cingulate (CG) cortices, CA1: *Cornu Ammonis* 1; CA3: *Cornu Ammonis* 3; dentate gyrus (DG, anterodorsal, anteroventral and anteromedial (AD, AV, AM) thalamic nucleus, supramammillary (SuM), medial medial mammillary nucleus (MMM), medial lateral mammillary nucleus (MML).



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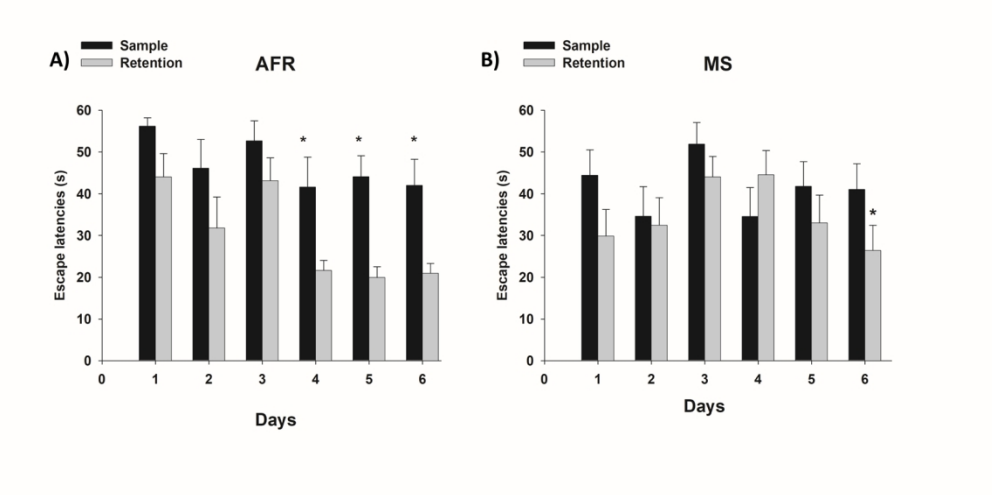

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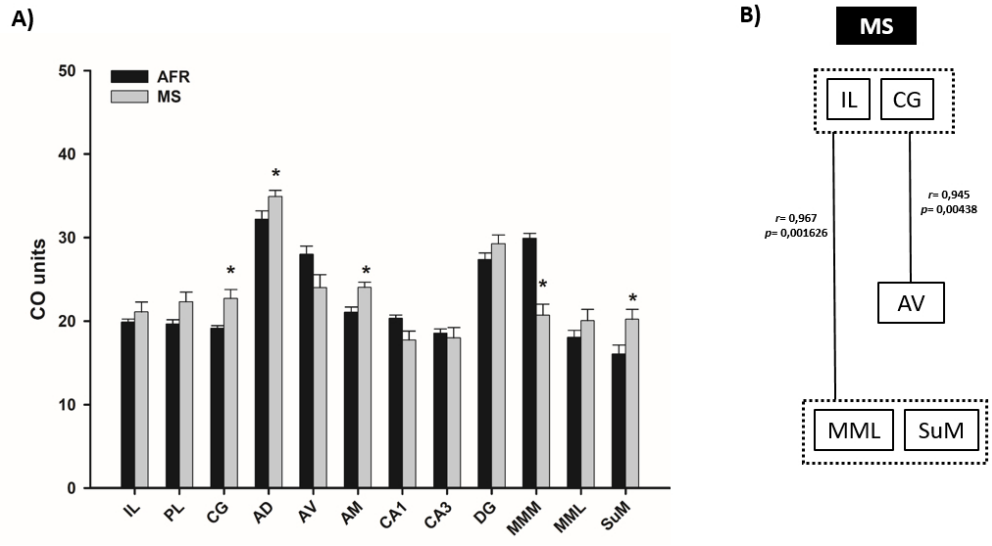
594x297mm (72 x 72 DPI)



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359x200mm (72 x 72 DPI)

	PL	CG	AD	AV	AM	CA1	CA3	DG	MMM	MML	SuM
IL	0,797	**0,885**	-0,237	0,872	-0,278	0,785	0,846	0,2	0,916	**0,967**	0,889
	0,0579	**0,0189**	0,651	0,0235	0,594	0,0642	0,0337	0,705	0,0103	**0,00162**	0,0178
PL		0,907	-0,243	0,762	-0,0545	0,297	0,443	0,05	0,765	0,801	0,812
		0,0127	0,642	0,0784	0,918	0,568	0,38	0,925	0,0761	0,0553	0,0499
CG			0,0423	**0,945**	-0,205	0,568	0,691	-0,0791	0,763	0,797	0,712
			0,937	**0,00438**	0,697	0,239	0,128	0,882	0,0773	0,0576	0,112
AD				0,174	-0,381	0,111	0,118	-0,402	-0,302	-0,458	-0,555
				0,742	0,457	0,834	0,824	0,43	0,561	0,361	0,253
AV					-0,447	0,711	0,841	0,063	0,784	0,763	0,604
					0,374	0,113	0,0358	0,906	0,065	0,0778	0,204
AM						-0,567	-0,649	-0,654	-0,512	-0,228	-0,113
						0,24	0,163	0,158	0,299	0,663	0,831
CA1							0,967	0,252	0,714	0,682	0,551
							0,0016	0,63	0,111	0,136	0,257
CA3								0,322	0,81	0,751	0,593
								0,534	0,0507	0,0853	0,215
DG									0,542	0,354	0,345
									0,266	0,491	0,502
MMM										0,943	0,898
										0,00479	0,0151
MML											**0,954**
											0,00315

Table 1: Shows the Pearson correlations between brain areas in the AFR group for all the structures studied. Significant correlations in bold. Each table cell shows the calculated Pearson's correlation r value and the P level for the calculated correlation coefficient. Infralimbic cortex= IL, Prelimbic cortex=PL, Cingulate cortex= CG, Dentate Gyrus= DG, Anterodorsal Thalamus= AD, Anteroventral thalamus= AV, Anteromedial Thalamus= AM, Supramammilar=SuM, Medial Medial Mammillary= MMM, Medial lateral Mammillary= MML $p<0.050$

362x162mm (72 x 72 DPI)

	PL	CG	AD	AV	AM	CA1	CA3	DG	MMM	MML	SuM
IL	0,0341	0,227	-0,291	-0,459	0,165	0,401	0,287	-0,0954	-0,0732	0,0726	0,236
	0,931	0,557	0,447	0,214	0,671	0,284	0,454	0,807	0,851	0,853	0,574
PL		-0,309	-0,197	0,0401	0,0247	0,111	-0,495	-0,462	-0,0424	0,284	-0,399
		0,418	0,611	0,918	0,95	0,775	0,176	0,211	0,914	0,458	0,327
CG			0,225	-0,115	0,219	0,0712	0,177	0,000209	0,193	0,237	0,518
			0,561	0,769	0,572	0,855	0,649	1	0,619	0,54	0,188
AD				0,878	0,761	-0,14	0,288	0,5	0,403	-0,0029	-0,208
				0,00186	0,0173	0,719	0,452	0,171	0,282	0,994	0,621
AV					0,679	-0,171	0,194	0,314	0,449	0,183	-0,276
					0,0444	0,661	0,617	0,411	0,226	0,638	0,508
AM						0,0719	0,397	0,348	0,605	0,364	-0,0335
						0,854	0,29	0,358	0,084	0,336	0,937
CA1							0,686	0,499	0,582	-0,0231	0,506
							0,0414	0,171	0,0999	0,953	0,201
CA3								0,739	0,7	0,0551	0,635
								0,0228	0,0359	0,888	0,0904
DG									0,54	-0,422	0,141
									0,133	0,258	0,739
MMM										0,397	0,545
										0,29	0,162
MML											0,189
											0,654

Table 2: Shows the Pearson correlations between brain areas in the AFR group for all the structures studied. No significant correlations were found. Each table cell shows the calculated Pearson's correlation r value and the P level for the calculated correlation coefficient. Infralimbic cortex= IL, Prelimbic cortex=PL, Cingulate cortex= CG, Dentate Gyrus= DG, Anterodorsal Thalamus= AD, Anteroventral thalamus= AV, Anteromedial Thalamus= AM, Supramammilar=SuM, Medial Medial Mammillary= MMM, Medial lateral Mammillary= MML

339x161mm (72 x 72 DPI)