Spatial memory-related brain activity in normally reared and different maternal separation models in rats

María Banqueri[a,b,][*], Marta Méndez[a,b], Jorge L. Arias[a,b]

[a] Laboratory of Neuroscience, Department of Psychology. University of Oviedo, Plaza Feijoo, s/n, E-33003, Oviedo, Spain
[b] Instituto de Neurociencias del Principado de Asturias (INEUROPA), Spain

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A B S T R A C T

Early life stress comprises a wide range of adverse events that can occur in the subject's early developmental stages: from child abuse to rodent repeated maternal separation models. This study used young adult male Wistar rats that were maternally raised (AFR), maternally separated from post-natal day (PND) 1 to PND10 (MS10), or maternally separated from PND1 to PND21 (MS21), to assess the effects of maternal separation on spatial learning and memory using the Morris Water Maze (MWM). We also performed quantitative cytochrome oxidase (COx) histochemistry on some selected brain areas in order to find out whether maternal separation affects brain energy metabolism. We obtained a similar spatial learning pattern in maternally raised and maternally separated subjects, with MS10 showing faster acquisition; however, different brain networks with different energy consumptions were used by each group. MS10 spent more energy to get the same behavioral outputs, whereas MS21 resembled AFR more in term of energy demands.

1. Introduction

Neglectful parenting is a severe worldwide problem [1,2] due to the emotional and cognitive alterations found in subjects who experience it [3–5]. In an attempt to explore the biological basis of these alterations and try to reduce them in the future, several models of early stress have been developed. One of the most widely used is maternal separation (MS) [6–8].

MS produces a wide range of changes in behavior, and one of the most studied is spatial learning. Alterations in spatial memory and learning have been tested in early stress models [9–13]; however, some groups claim that spatial memory is not specifically affected. Some authors suggest that prefrontal-related tasks are altered after early stress (shifting and reversal learning, for instance), whereas classic reference memory tested with the MWM is preserved [14]. However, other authors propose that spatial memory is preserved, pointing to social behavior as the most affected dimension [15]. This controversy could be due to differences in the exact protocol used by each research group, or MS animals might be successful on memory tasks if they consume more metabolic energy than controls. Thus, our main goal was to explore whether MS and control brains work in similar ways to solve the task.

In this study, we aimed to explore the effects of two MS models, from postnatal day (PND) 1 to PND 10 and from PND 1 to PND21, on

spatial memory on a reference memory task. We chose 10 first days because some interesting developmental processes start just after this period: the granular hippocampal cells migration occurs between postnatal days 10 to 25 in rodents [16] and allopregnelonone levels reach its peak in PND10. Neonatal allopregnelonone levels in the second week of life seem to be important for the maturation of dopaminergic systems and GABAergic thalamo-cortical connections [17]. Our second goal was to explore whether the MS and control rats were using the same networks to solve the task. In order to explore the contribution of different brain areas during the spatial task, we used quantitative cytochrome oxidase (COx) histochemistry as a reliable marker of brain energetic metabolic activity [18,19]. COx activity increases with sustained energy demands [20]. This technique has been used to explore brain regions involved in spatial memory tasks [21–23].

Spatial learning and memory have been related to broad brain areas with diverse functions [24]. To solve a spatial memory task, subjects need to recall the information from the cognitive map stored in memory (prefrontal cortex) [25] and be sensitive to reinforcement related to the accumbens core, which codifies the platform location, also known as relief memory [26]. In addition, proper navigation and consolidation are supported by classic spatial-related areas, such as the hippocampus (HC), related cortices, and mammillary nuclei [27,28]. We explored all these areas as well as anxiety and fear-related areas, such as the bed nucleus of stria terminals (BNST) or the amygdala [29], because MS

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animals have been shown to be anxious [30]. Different networks could be used in stressed brains to solve the task, and it is also possible that even when two subjects are using the exact same regions, they could be using different metabolic expenditures.

Therefore, our first aim was to explore the differences between the two MS length models and their performance on a spatial learning task, and the second aim was to verify whether stressed and non-stressed animals were using the same neural networks with the same metabolic expenditure in them.

2. Materials and methods

2.1. Animals

A total of 30 newborn male Wistar rats were taken from the animalarium at Oviedo University. All the animals had ad libitum food and tap water, and they were maintained at a constant room temperature (22 ± 2 °C), with a relative humidity of 65–75% and an artificial light-dark cycle of 12 h (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.

2.2. Maternal separation

8 Litters were randomly assigned to maternal separation or to being reared under animal facility rearing (AFR) conditions. Litters with > 10 animals were culled to 10, we maintained the previous female/male ratio after culling process. For MS, litters were separated from dams for 4 h per day, starting at 10:00 h and ending at 14:00 h as other authors [31,32]. The MS group was separated from PND 1 to PND 21, whereas the MS10 group was separated from PND 1 to PND 10. See Fig. 1. Each separation consisted of removing the dams from the home cage and placing them in an adjacent cage while the pups were kept together in a new cage, with some sawdust: from the shared cage. Litters remained together during the separation time in an incubator (30 °C, 55–65% relative humidity), and also they were placed together in the shared cage. Use of an incubator is preferred in order to avoid hypothermia [33]. After the separation period, the dam and the litter were returned to the home cage (first, the litter). Control litters were reared under standard animal facility rearing (AFR) conditions, disturbed only by animal facility practices once a week until weaning. On PND 21, all the animals were weaned, placing 5 animals per cage, and segregated by sex, and only males were selected for the study. Therefore, 3 groups of male animals were included in the experiment, one control group or AFR (n = 10) and two experimental groups: MS10 (n = 10) and MS21 (n = 10).

2.3. Morris water maze task

On PND 85–90, the animals' behavior was tested in the Morris water maze (MWM), as previously described in [34]. 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. Water level was 30 cm, and its temperature was 22 ± 2 °C. The escape platform used was a cylinder, 10 cm in diameter and 28 cm high, placed 2 cm below the

surface of the water. The MWM was in the center of a 16 m^2 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 start positions and platform. 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).

In the learning protocol, the first day was devoted to the habituation of the animals to the task: Thus, the animals performed four trials with a visible platform that jutted out 4 cm from the water and was located in the center of the pool. On the following 4 days, the animals were required to locate a hidden platform located in the center of quadrant D in relation to the external visual cues on training days. Training was performed in blocks of six trials per day. To begin each trial, the rats were placed in the water, facing the maze wall in one of four quadrants, and the daily order of entry into these quadrants was pseudo-randomized. Each trial ended once the animal had found the hidden platform, or when 60 s had elapsed. If the animal had not reached the hidden platform after this time, it was placed on the platform for 15 s. During the inter-trial interval, the animals were placed in a black bucket for 30 s. After the first four trials, a probe test was applied in which the pool platform was removed and the rat was introduced into the pool for 25 s in the quadrant opposite to where the platform had been located in previous trials, in order to check whether the animal remembered the position of the platform. Immediately after the probe test, the animals were subjected to an additional trial with the hidden platform placed in its usual position to avoid any possible interference with the probe test. Latencies, the time that every animal needed to find the platform of the first four trials each day, were recorded during the acquisition, as well as the time of permanence in each quadrant during the probe test.

2.4. Cytochrome oxidase histochemistry

Ninety minutes after the behavioral task ended, the animals were decapitated. Brains were removed, frozen rapidly in *N*-methyl butane (Sigma-Aldrich, Madrid, Spain), and stored at − 40 °C until processing with quantitative CO histochemistry, described by González-Lima and Cada [35]. Coronal sections (30 μm) of the brain were cut at − 22 °C in a cryostat (Leica CM1900, Germany). The sections were 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 [36] were cut at different thicknesses (10, 30, 50 and 70 μm) and included with each bath of slides. The sections and standards were incubated for 5 min in 0.1 phosphate buffer with 10% (w/v) sucrose and 0.5 (v/v) glutaraldehyde, pH 7.6. Next, baths of 0.1 M phosphate buffer with 10% (w/v) sucrose were given for 5 min each. Subsequently, 0.05 M Tris buffer, pH 7.6, 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 0.06 g cytochrome *c*, 0.016 g catalase, 40 g sucrose, 2 ml dimethyl-sulfoxide, and 0.4 g diaminobenzidine tetra-hydrochloride (Sigma-Aldrich, Madrid, Spain) in 800 ml of .1 M phosphate buffer at 37 °C for 1 h. The reaction was stopped by fixing the tissue in buffered formalin for 30 min 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).

2.5. CO optical density quantification

The CO histochemical staining intensity was quantified by means of densitometric analysis, using a computer-assisted image analysis workstation (MCID, Interfocus ImagingLtd., and Linton, England) composed of a high precision illuminator, a digital camera, and a

Fig. 1. Timeline of the experiment. From PND 1 to PND 10, the MS10 animals were separated from their dams (10 MS10 males) From PND 1 to PND 21, the MS21 animals were separated from their dams (10 MS21 males). PND = Post-natal day, MS = Maternal separation, MWM = Morris Water Maze, S = Sacrifice.