

CELLULAR COUNT CHANGES IN DIFFERENT RAT BRAIN AREAS DUE TO EARLY MATERNAL SEPARATION

ALEJANDRO MUÑOZ VALENCIA¹, JONATAN VELÁSQUEZ QUIROGA²,
JAVIER SADDAM LÓPEZ ARIAS³, JHON CHAVARRO TIBACAN⁴, ZULMA DUEÑAS⁵

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Summary

Objective: to identify whether maternal separation during breastfeeding (MSDB) affects the cellular count in different rat brain areas. The continuous mother-child interaction, adjusts and modulates the offspring behavioral response to environmental stimuli and also affects their development and homeostasis. Morphological and physiological changes in the offspring brains have been observed, including cell count changes in different brain areas with differences between males and females. **Materials and methods:** this study compared albino Wistar rats in a protocol of MSDB with a control group. Brain tissue was fixed with paraformaldehyde, cut in cryostat and either stained with Hematoxylin-Eosin (H&E) or processed for immunohistochemistry against glial fibrillary acidic protein (GFAP). All sections were analyzed using a cell count protocol including statistical analysis with Students T test at a significance level of $P \leq 0.05$. **Results:** the MSDB group of male subjects presented higher GFAP-marked cell count in primary motor cortex and hippocampus; while female subjects, showed less GFAP-marked cell count in these same areas. **Conclusions:** MSDB produces sex-specific changes in the number of glial cells especially in the primary motor cortex, this finding may be considered as associated factor of alterations in motor responses to stress in these subjects, in addition to other known causes such as the Hypothalamic-Pituitary-Adrenal Axis dysfunction.

Key Words: breast feeding, motor cortex, hippocampus, child development.

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- 1 Medical Student. Universidad Nacional de Colombia. Bogotá. Colombia. ORCID: 0000-0001-6845-5130. Correo e.: alemunozvalunal.edu.co.
- 2 Urology Resident. Universidad Nacional de Colombia. Bogotá. Colombia. Correo e.: javelasquezq@unal.edu.co.
- 3 Medical Doctor. Universidad Nacional de Colombia. Bogotá. Colombia. Correo e.: jaslopezar@unal.edu.co.
- 4 Pharmacy Student. Universidad Nacional de Colombia. Bogotá. Colombia. Correo e.: jachavarrot@unal.edu.co.
- 5 Professor. Facultad de Medicina. Departamento de Ciencias Fisiológicas. Universidad Nacional de Colombia. Bogotá. Colombia. ORCID: 0000-0001-6068-4174. Correo e.: zjduenasg@unal.edu.co. Corresponding author.

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Cambios en el recuento celular en diferentes áreas del cerebro de rata debido a la separación materna temprana

Resumen

Objetivo: *identificar si la separación materna durante la lactancia (MSDB) afecta el conteo celular en cortezas motoras y otras áreas del cerebro de la rata. La interacción continua entre la madre y su hijo, ajusta y modula la respuesta comportamental de las crías hacia estímulos ambientales, afectando además su desarrollo y homeostasis. Cambios morfológicos y fisiológicos han sido descritos en la descendencia, incluyendo diferencias en conteos celulares de varias áreas de la corteza cerebral.* **Materiales y métodos:** *este estudio se compararon ratas Albino Wistar bajo un protocolo de MSDB contra un grupo control. El tejido cerebral fue fijado con paraformaldehído, cortado en criostato, y tratado con tinción Hematoxilina-Eosina (H&E) o procesado con inmunohistoquímica contra proteína ácida glial fibrilar (GFAP). Todas las secciones fueron analizadas usando un protocolo de conteo que incluyó análisis estadístico con el test de T de Student con significancia a nivel de $P \leq 0.05$.* **Resultados:** *el grupo de machos que tuvo MSDB presentó mayor conteo de células marcadas contra GFAP en las cortezas motoras primarias y el hipocampo; mientras que las hembras con MSDB, mostraron menor conteo de células marcadas contra GFAP en estas mismas áreas.* **Conclusión:** *la MSDB produce cambios específicos de acuerdo al sexo del sujeto, en el número de células gliales especialmente en las cortezas motoras primarias, este hallazgo puede ser considerado como causante parcial de las alteraciones en las respuestas motoras a estrés en estos sujetos, además de otras causas conocidas como la disfunción del eje Hipotálamo-Pituitario-Adrenal (HPA).*

Palabras clave: *lactancia materna, corteza motora, hipocampo, desarrollo infantil.*

Introduction

Epidemiological evidence in humans associates early exposure to adverse events with greater vulnerability to develop mental diseases such as anxiety attacks, depression, substance abuse and behavioral changes, among others, including subsequent social misbehavior [1-4]. Such evidence has allowed to postulate that the

acquired mental dysfunctions may depend on alterations of the neuronal circuits and failure to accomplish different developmental mechanisms. With this background, it is important to take into account that maternal bond is fundamental to establish synaptic networks and also plays a crucial role in the postnatal development of individuals [5-9]. Observational studies

that can be done with human subjects are not able to show the underlying mechanisms that influence the offspring in terms of stress response and neuro-histological correlation. Thus, experimental methods with other mammal species as animal models allow different approaches to this issue. Most researches have been done with rodents, especially rats, for which the widest and most detailed knowledge exists [10-13].

The maternal separation during breastfeeding (MSDB) animal protocol in rats has revealed numerous anatomical and physiological changes in the hypothalamus-pituitary-adrenal axis (HPA), amygdala, hippocampus, prefrontal and medial cortex, and peri hypothalamic areas such as the medial preoptic area [14-16]. The GABAergic system, closely related to the regulation of anxiety, is one of those most affected by MSDB, especially the expression of GABA_A receptors in the hippocampus [17] and medial prefrontal cortex [18]. However, there are few references regarding cellular changes in the amygdala, hypothalamus and peri hypothalamic area [18], which are important in the regulation of responses to fear and anxiety [19].

Furthermore, the response to stress and anxiety is strongly mediated by factors related to the sex of the animals [16]. Few studies using MSDB that have explored the neurobiological and behavioral effects, had grouped or analyzed according to each sex group [16-18]. Previous studies have reported that exposure to a protocol of MSDB causes differences in anxiety-like behaviors, which seem to depend on subject's sex and also can be associated with the alteration of expression by GABA_A receptors. Other authors support evidence regarding the number of astrocytes in certain brain areas of separated rats compared with those that were not separated [18, 20, 21].

The maternal separation has also been linked to postnatal maturation of neural cells, specifically of microglial cells in various areas of the central nervous system [22]. Other associations were observed between the maternal

separation and lower gene expression of GFAP in glial cells at the prefrontal cortex at different postnatal stages, including adult rats up to 10 weeks old [23]. Morphometric abnormalities of the ventricles seen in rodent models have been associated with different pathologies including substance abuse disorders and Alzheimer's disease [24,25].

Taking in to account all these antecedents, the objective of the present study was to identify the possible changes induced by MSDB in the total cell counting of GFAP and non-GFAP cells, along different areas including motor cortices of the rat brain and to describe whether these changes were different between male and female groups, comparing all data with control groups.

Materials and methods

Maternal Separation During Breastfeeding

Female and male Wistar rats were obtained from the in-house animal facility of the Universidad Nacional de Colombia and housed at the veterinary school. Animals were housed in standard rat cages (40 x 31 x 22 cm) and kept under standard laboratory conditions with reversed light/dark cycle (lights off at 07:00 h), 22 ± 2°C temperature, 55 ± 10% humidity and food and water *ad libitum*. Animals were mated during 1 week; and housed alone during the last 2 weeks of gestation. Litters were randomly assigned to separation or animal facility-reared (control) groups to equally distribute each gender. All experimental protocols were approved by the Ethics Committee of the Facultad de Medicina de la Universidad Nacional de Colombia and according to Colombian law (Resolución N°. 8430 de 1993) and international regulations regarding research using animal subjects i.e. National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications) and in accordance with the rules and procedures of the APA.

Ethics Committee. Four dams were used. Litters were undisturbed at birth: postnatal day zero (P0) and were homogenized to 10. Four groups were set, two with only males and two with only females, setting one control and one experimental group for each sex, respectively. The MSDB protocol was the same as reported in previous studies [18]. From day 0 to day 22, the protocol for experimental groups comprised 180 minutes of separation from the mother during the morning and 180 minutes during the afternoon. Then, on day 22 all subjects were separated from the mother, and four groups were set by subject's sex and previous exposure to MSDB protocol or control, setting up each group with 10 subjects procuring equal male: female ratios. Conditions of illumination, temperature, food, and drink were equal for all subjects. The rats continued their growing and development with habituation to minimal human handling, including the change of bedding and food supply until maturation. On day 60, the subjects were anesthetized, then, transcardial perfusion was carried out with saline solution 0.9% and then 4% paraformaldehyde (PFA). The brains were preserved in PFA for further histological analysis.

Histological processing for cellular identification

The brains were cryoprotected in a saturated sucrose solution four days before sectioning in cryostat. The brains were later processed by carrying out a series of coronal sections of 20 μ m thick. Half of the slices were stained with H&E to identify the total number of cells in each of the structures and the remaining half was stained for immunohistochemistry against GFAP.

Histological processing for immunohistochemistry

The procedure was performed as previously reported [20]. The 20 μ m slices were incubated with blocking serum [bovine serum

albumin, 0.3% Triton X-100, normal goat serum] for 1 h. Then, incubated with primary antibody anti-GFAP (Sigma-Aldrich Cat# G4546, RRID:AB_1840895) for 12h at room temperature before undergo incubation with biotinylated anti-rabbit secondary antibody (Vector Laboratories Cat# PK-6100, RRID:AB_2336819) at a dilution of 1:500 in 0.3 % PBS Triton for 90 min, and then with antibody (AB) complex for 90 min. Finally, the slices were treated with Diaminobenzidine (Vector Laboratories Cat# SK-4100, RRID:AB_2336382). Four rinses with 0.3% PBS Triton were carried out for 15 minutes between each stage. After clearing the DAB and adding 0.9% NaCl solution, the slices were mounted on slides with a fine paintbrush for later analyses.

Cell count and statistical analysis

Photographic records were taken of each sections using the 2X and 4X objectives of a CARL ZEISS inverted microscope linked to a Cannon® G10 digital camera, connected to a computer where the images were stored. Three photos of each brain area of interest were taken from each subject; the photos were sorted by region, staining and sex.

Analyzed areas were identified according to the stereotaxic coordinates described by Paxinos & Watson [26] as follows: Area 1 including M1 – primary motor cortex, LO – lateral orbital cortex, and VO – ventral orbital cortex at Interaural 14.20 mm and Bregma 4.20mm; Area 2 including AOP – posterior part of anterior olfactory nucleus, AOM – medial part of anterior olfactory nucleus, and AOV – ventral part of anterior olfactory nucleus at Interaural 12.70mm and Bregma 2.70mm; Area 3 including Cg1 – area 1 of cingulate cortex and Cg2 – area 2 of cingulate cortex at Interaural 11.20mm and Bregma 1.70mm; Area 4 including S1J – jaw region of primary somatosensory cortex, GI – granular insular cortex, and AID – dorsal part of agranular insular cortex at Interaural 10.6mm and Bregma 1.00mm; Area 5 including RsGb – retrosplenial granular b cortex and RSA –

retrosplenial agranular cortex at Interaural 9.70mm and Bregma 0.20mm; Area 6 including M1 – primary motor cortex and M2 – secondary motor cortex at Interaural 8.70mm and Bregma 0.80mm; Area 7 including S1Tr – trunk region of primary somatosensory cortex and S1DZ – dysgranular region of primary somatosensory cortex at Interaural 8.08mm and Bregma -1.80mm; Area 8 including CeC – capsular part of central amygdaloid nucleus, CeL – lateral division of central amygdaloid nucleus, and CeM – medial division of central amygdaloid nucleus at Interaural 6.88mm and Bregma 3.30mm; Area 9 including CA1 – field CA1 of hippocampus, CA2 – field CA2 of hippocampus, and CA3 – field CA3 of hippocampus at Interaural 5.70mm and Bregma 4.30mm.

Cell counting was performed using Image J software (ImageJ, RRID:SCR_003070) with the blue filter to count the sections stained with H&E, and the green filter to count those stained with anti-GFAP.

The cell counting was performed using both stains. In order to obtain the non-GFAP count, we subtracted the anti-GFAP cell count from the absolute count observed in the H&E slides at the same analyzed area. All the collected data were analyzed using Student's t-test, performed for each group according to experimental group, subject's sex, brain area, and staining of the slide. Statistical analyses were carried out based on a population of 10 subjects per experimental and sex groups. We used an alpha level of .05 for all statistical tests.

Results

Regarding GFAP marked cell count in males shown in Figure 1, differences were found in Areas 2, 4, 6, 8 but only reaching strong correlation in the motor cortex located in Area 6 $t(1,83) = 3,92, p = <0,01$, and amygdala cortex located in Area 9 $t(1,83) = 2,18, p = 0,03$.

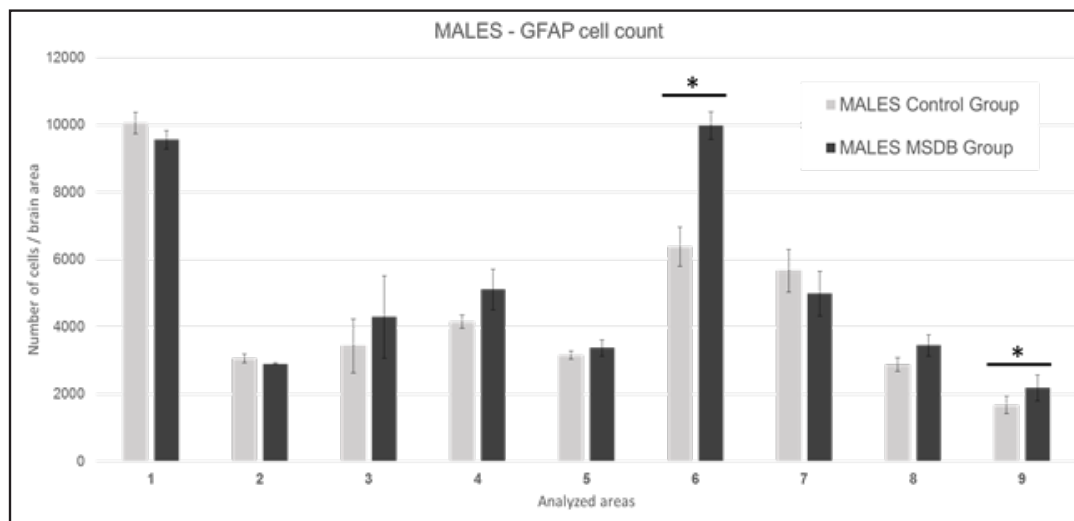


Figure 1. Males: Mean of GFAP marked cells in analyzed areas. Data plotted with 2 SD error bar. 1: M1 - primary motor cortex, LO - lateral orbital cortex, and VO - ventral orbital cortex. 2: AOP - posterior part of anterior olfactory nucleus, AOM - medial part of anterior olfactory nucleus, and AOV - ventral part of anterior olfactory nucleus. 3: Cg1 - area 1 of cingulate cortex, and Cg2 - area 2 of cingulate cortex. 4: S1J - jaw region of primary somatosensory cortex, GI - granular insular cortex, and AID - dorsal part of agranular insular cortex. 5: RsGb - retrosplenial granular b cortex,

and RSA - retrosplenial agranular cortex. 6: M1 - primary motor cortex, and M2 - secondary motor cortex. 7: S1Tr – trunk region of primary somatosensory cortex and S1DZ –dysgranular region of primary somatosensory cortex. 8: CeC – capsular part of central amygdaloid nucleus, CeL – lateral division of central amygdaloid nucleus, and CeM – medial division of central amygdaloid nucleus. 9: CA1 - field CA1 of hippocampus, CA2 - field CA2 of hippocampus, and CA3 - field CA3 of hippocampus. Source: authors.

GFAP marked cells count in female subjects is shown in Figure 2, strong statistically correlation was found in: Area 1 $t(1,83)= 5,40$, $p= <0,01$; Area 2 $t(1,83)= 3,91$, $p= <0,01$; Area 3 $t(1,83)= 2,21$, $p= 0,03$; Area 5 $t(1,83)= 6,01$, $p=$

$<0,01$; Area 6 $t(1,83)= 4,21$, $p= <0,01$; and Area 9 $t(1,83)= 3,64$, $p= <0,01$. These areas include orbital, olfactory, cingulate, retrosplenial, motor, and hippocampus cortices, respectively.

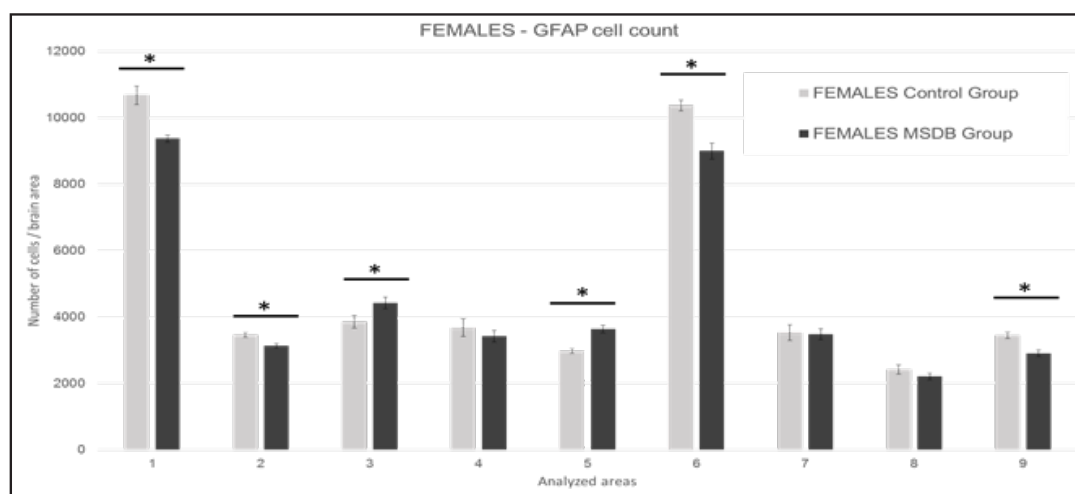


Figure 2. Females: Mean of GFAP marked cells in analyzed areas. Data is presented with 2 SD error bar. 1: M1 - primary motor cortex, LO - lateral orbital cortex, and VO - ventral orbital cortex. 2: AOP - posterior part of anterior olfactory nucleus, AOM - medial part of anterior olfactory nucleus, and AOV - ventral part of anterior olfactory nucleus. 3: Cg1 - area 1 of cingulate cortex, and Cg2 - area 2 of cingulate cortex. 4: S1J - jaw region of primary somatosensory cortex, GI - granular insular cortex, and AID - dorsal part of agranular insular cortex. 5: RsGb - retrosplenial granular b cortex, and RSA - retrosplenial agranular cortex. 6: M1 - primary motor cortex, and M2 - secondary motor cortex. 7: S1Tr – trunk region of primary somatosensory cortex and S1DZ –dysgranular region of primary somatosensory cortex. 8: CeC – capsular part of central amygdaloid nucleus, CeL – lateral division of central amygdaloid nucleus, and CeM – medial division of central amygdaloid nucleus. 9: CA1 - field CA1 of hippocampus, CA2 - field CA2 of hippocampus, and CA3 - field CA3 of hippocampus. Source: authors.

The cell count of non-GFAP cells was calculated as previously detailed, data of male subjects are shown in Figure 3. Differences were found in Areas 3, 4, 5, 6, 9, but taking into

consideration the previous GFAP and H&E cell counts with their respective statistical significances, we reached strong correlation only in Area 4. This area includes the insular cortex.

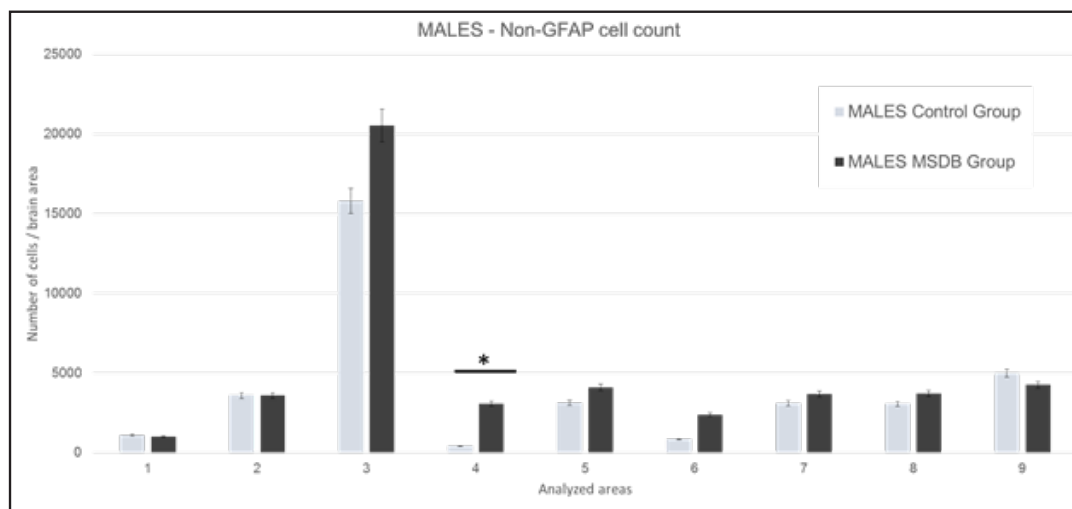


Figure 3. Males: Mean of non-GFAP cells in analyzed areas. Data is presented with 0.05 error bar. 1: M1 - primary motor cortex, LO - lateral orbital cortex, and VO - ventral orbital cortex. 2: AOP - posterior part of anterior olfactory nucleus, AOM - medial part of anterior olfactory nucleus, and AOV - ventral part of anterior olfactory nucleus. 3: Cg1 - area 1 of cingulate cortex, and Cg2 - area 2 of cingulate cortex. 4: S1J - jaw region of primary somatosensory cortex, GI - granular insular cortex, and AID - dorsal part of agranular insular cortex. 5: RsGb - retrosplenial granular b cortex, and RSA - retrosplenial agranular cortex. 6: M1 - primary motor cortex, and M2 - secondary motor cortex. 7: S1Tr - trunk region of primary somatosensory cortex and S1DZ - dysgranular region of primary somatosensory cortex. 8: CeC - capsular part of central amygdaloid nucleus, CeL - lateral division of central amygdaloid nucleus, and CeM - medial division of central amygdaloid nucleus. 9: CA1 - field CA1 of hippocampus, CA2 - field CA2 of hippocampus, and CA3 - field CA3 of hippocampus. Source: authors.

Regarding female subjects, data for non-GFAP cells is shown in Figure 4. Differences were found in Areas 1, 2, 4, 5, 7, 9, but taking into consideration the statistics values for significance in previous anti-GFAP and H&E cell counts, strong correlation was only found in Areas 1, 2, and 9. These areas include the orbital, olfactory, and hippocampus cortices, respectively.

Discussion

The neurobiological consequences of maternal separation during breastfeeding have been approached using animal models. In fact, alterations of the axis that regulates the response to stress have been reported using MSDB model

in rats [8]. Our research group has contributed studies regarding behavioral changes of the separated subjects and differences associated with subject's sex [18, 20].

In this study, statistical significance was obtained at various cortices for both sexes. In GFAP-marked cells count of male subjects, changes were noticed at the motor and hippocampus cortices, Areas 6 and 9, respectively; meanwhile, in non-GFAP cells count, changes were found at the insular and motor cortices, Areas 4 and 6, respectively. However, in GFAP-marked cells count of female subjects, variation was observed at the orbital, olfactory, cingulate, retrosplenial, motor, and hippocampus cortices, Areas 1, 2, 3, 5, 6, 9, respectively; while in non-

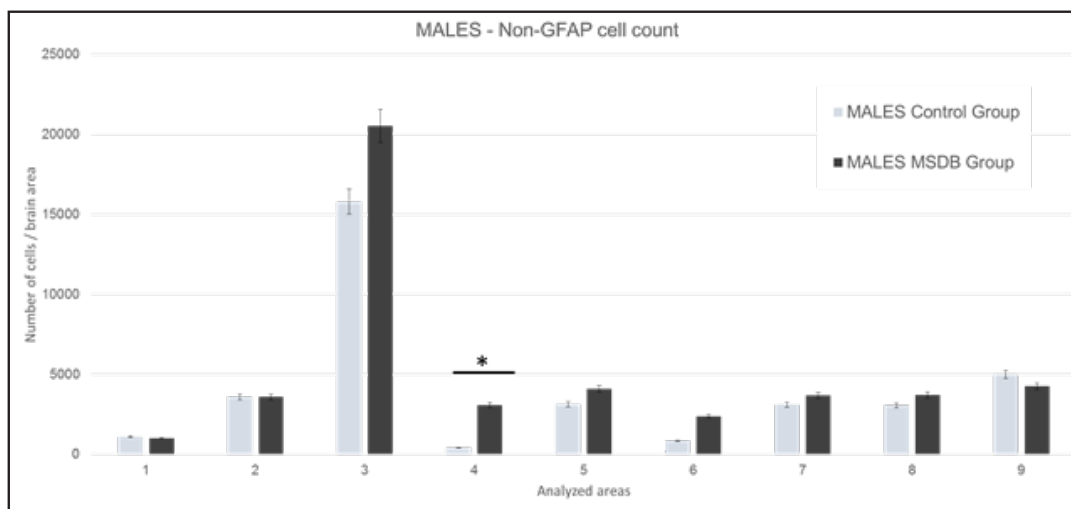


Figure 4. Females: Mean of non-GFAP cells in analyzed areas. Data is presented with 0.05 error bar. 1: M1 - primary motor cortex, LO - lateral orbital cortex, and VO - ventral orbital cortex. 2: AOP - posterior part of anterior olfactory nucleus, AOM - medial part of anterior olfactory nucleus, and AOV - ventral part of anterior olfactory nucleus. 3: Cg1 - area 1 of cingulate cortex, and Cg2 - area 2 of cingulate cortex. 4: S1J - jaw region of primary somatosensory cortex, GI - granular insular cortex, and AID - dorsal part of agranular insular cortex. 5: RsGb - retrosplenial granular b cortex, and RSA - retrosplenial agranular cortex. 6: M1 - primary motor cortex, and M2 - secondary motor cortex. 7: S1Tr - trunk region of primary somatosensory cortex and S1DZ - dysgranular region of primary somatosensory cortex. 8: CeC - capsular part of central amygdaloid nucleus, CeL - lateral division of central amygdaloid nucleus, and CeM - medial division of central amygdaloid nucleus. 9: CA1 - field CA1 of hippocampus, CA2 - field CA2 of hippocampus, and CA3 - field CA3 of hippocampus. Source: authors.

GFAP cells count, differences were detected at the orbital, olfactory, and hippocampus cortices, Areas 1, 2, and 9, respectively.

The most evident differences were found in counts of cells marked with anti-GFAP in both sexes along the primary motor and hippocampus cortices. The hippocampus is recognized as one of the principal areas in control of the response to stress, learning, and memory [27]. Early studies with other maternal separation protocols, indicated the presence of changes in the architecture of the hippocampus, specifically in CA1, CA3 and the dentate gyrus [12, 28, 29] as a consequence of chronic stress, as well as reduction in the abilities of learning and memory during adult life [27]; moreover,

a predisposition to stress-related disorders characterized by disruption in glucocorticoid secretion [30]. The results at the hippocampal area, indicate a higher cell count for marked and non-marked GFAP cells in male subjects, on the other hand, female subjects showed less cell count for marked and non-marked cells; this discrepancy between sex groups has been largely found in previous studies from our research group and other groups. Previous articles from our research group have explained other details addressing the changes in the hippocampus cell count [18, 20].

Indeed, no other previous studies have carried out a specific cell count for GFAP-marked cells along the primary motor cortex. Discrepan-

cy was observed regarding the direction of the changes between sex groups. For male subjects, there was an increase in GFAP-marked cells in primary motor cortices, M1 and M2; in contrast, for female subjects, there was a GFAP-marked cells count decrease in these same areas. Some articles have detailed the morphometrical changes in the gross anatomical thickness of these cortices [31], but using a protocol of maternal deprivation that is not fully related to the MSDB protocol used in this study. Many authors have shown the association between changes in the cell counting and other alterations regarding cellular structure and metabolism [32], the regulation of nervous impulses [27], and behavioral changes related with anxiety [17]. Recent studies described specific changes in interneuron maturation triggered by MSDB protocol, also contributing to decrease the total cell count in analyzed cortices [33], leading to the possibility of a sequence of events started by MSDB and ending in cortical dysfunction of this areas.

The findings of this study are probably associated with changes in the HPA axis response related to alterations in corticosterone levels, as well as motor responses to anxiety [6, 8, 17, 34]; subsequently, these changes could also involve modifications in the mechanisms of response to anxiety. In addition, several articles have related connectivity between the hippocampus and the amygdala, including their association with anxiety disorders [35]. Previous findings by other authors [8, 19, 36], suggested that changes in glial cells and neurons in areas connected with the amygdala are a possible adaptive mechanism to stress with a later increase of outputs towards the amygdala nuclei. It is important to note that these increases in GFAP-marked cell count were not observed in female subjects, indicating the presence of other factors affecting the GFAP-positive cells

population, leading to a more complex needing for understanding the role of those cells in this MSDB protocol scenario, according to the subject's sex group.

Moreover, changes in immunoreactivity to GFAP imply modifications in the neuroglia that can be linked directly to the astrocytes and its maturation probably affected by the MSDB protocol; on the other hand, as part of the limitations of this study, this changes in GFAP-marked cell may be partially caused by brain repair activity due to the fundamental role of glial cells to keep the ionic equilibrium, nutrition, and stability of synaptic communication in the brain, as part of the process known as reactive gliosis [37].

The findings of this study should be reinforced by other studies with larger sample size, and specific staining to differentiate the cell types summarized here as non-GFAP, allowing also a greater number of sections to be analyzed. It is important to conduct further studies that correlate the motor cortex alterations found in this study and the motor response to stress, carrying out behavioral studies that aim to unveil the possible role of these cell count changes in the alteration of the motor neural circuits and subsequently transforming the motor response to stress, altered motor response largely seen in rodent subjects exposed to MSDB protocols.

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