Maternal separation during breastfeeding induces changes in the number of cells immunolabeled to GFAP

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Abstract

The mother–child relationship is fundamental to the establishment and maintenance of synaptic networks and physiological and emotional development. Animal models including maternal separation have been used to study changes at behavioral and neurobiochemical levels. Due to the relevance of glial cells during development, our aim was to determine if short periods of maternal separation during breastfeeding induce permanent changes in a number of astrocytes labeled with the glial fibrillary acidic protein in different brain areas. Wistar rats were housed under standard laboratory conditions with reversed light/dark cycle; food and water *ad libitum*. Pups were separated from their mothers for 6 h daily during breastfeeding period. On day 22, pups were separately housed according to gender and treatment. At day 60, subjects were evaluated in the elevated plus maze and, after processing for immunohistochemistry, 20-μm sections were made. Prefrontal cortex, paraventricular nucleus, preoptic area, hippocampus and amygdala were localized. Labeled cells were quantified using Image-J program. Results showed that separated females had more entries into open arms and spend more time as compared with the control groups. In the prefrontal cortex we identified a decrease in staining cells in separated females, whereas there was an increase in staining cells in separated males. In the hippocampus and preoptic area, we observed a decrease only in separated males. We did not find any differences in the paraventricular nucleus or amygdala. Our results indicate that maternal separation during breastfeeding induces permanent changes in the number of astrocytes in different brain areas of both males and females.
Keywords: maternal separation; elevated plus maze; glia, GFAP; immunohistochemistry; astrocytes; brain plasticity; stress.

Introduction

Stressful or traumatic experiences that occur in the first years of life affect brain development and behavior of individuals (Neigh, Gillespie, & Nemeroff, 2009). Clinical studies that evaluate the consequences of early adverse experiences such as child abuse, maternal neglect and psychosocial stress suggest that a history of adverse occurrences could promote the development of various psychopathologies (Cicchetti & Manly, 2001; Salzberg et al., 2007; Lai & Huang, 2011; Mesa-Gresa & Moya-Albiol, 2011). To study how negative early experiences affect the development of the individual, various animal model studies have been implemented including those investigating alterations in mother–pup interactions (Moriceau, Roth, & Sullivan, 2010; Lesch, 2011; Schmidt, Wang, & Meijer, 2011). One of these models is early maternal separation, which has been widely used as a model of the anxiety, depression and stress caused by neglect (Duque et al., 2011).

As maternal separation is considered a stress model (Litvin et al., 2010), it is possible to inquire about the neurobiological mechanisms involved in individual responses because of the activation of the hypothalamus–pituitary–adrenal axis (HPA) (Lajud, Roque, Cajero, Gutiérrez-Ospina, & Torner, 2011), which shows the physiological mechanisms that enable an organism to respond adaptively to threatening stimuli. This response occurs, for example, by increasing glucocorticoids and adrenalin from the adrenal glands (Wilber & Wellman, 2009). Stress hormones also play an important role at the core level because action facilitates the consolidation of memories, which are mediated by the action of glucocorticoids in the amygdala (AM) and the hippocampus (HP) (Starkman, Giordani, Gebarski, & Schteingart, 2003). However, high exposure to glucocorticoids during chronic stress causes alterations in the function of the axis and behavioral alterations such as depression and anxiety disorders (Ladd, Huot, Thrivikraman, Nemeroff, & Plotsky, 2003). In addition, continuous exposure to glucocorticoids induces disordered activity of neurotransmitters such as glutamate and GABA monoamines (Reagan et al., 2004). In fact, it has been shown that chronic stress affects the electrophysiological properties of glutamate ionotropic receptors in the HP and prefrontal cortex

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(PFC), which are associated with atrophy of pyramidal neurons in these areas (Kole, Swan, & Fuchs, 2002).

Not only are stress responses mediated by the reaction of the HPA axis and by the action of neurotransmitters but also by interneuronal communication and glia. It is known that astrocytes play an important role in brain function during development by interacting with neurons, helping migration during embryonic stages, secreting neurotrophins, and contributing to synaptogenesis and to processes of brain plasticity, among others (Newman, 2003). In this way, it has been shown that synaptic transmission can be modulated by changes in astrocytes that surround these synapses, producing morphological changes that affect synaptic transmission (Newman, 2003).

In previous studies we found that short periods of maternal separation during breastfeeding (MSDB) induced a decrease in anxious behavior as evaluated by the elevated-plus maze (EPM) in adult females but not in males, and those results were associated with a decrease in the immunoreactivity in alpha subunits of GABA-A receptors in the HP, paraventricular nucleus (PVN) and preoptic area (POA) (Moreno, Lamprea, & Dueñas, 2009; León, Riveros-Barrera, & Dueñas, 2012).

Taking into account the existing literature and our previous results, in addition to the known importance of astrocytes in different processes including stress response, learning and memory, the purpose of this work was to assess whether subjects undergoing MSDB presented differences during adulthood in the number of astrocytes immunostained with glial fibrillary acidic protein (GFAP) in the PFC, PVN, POA, AM and HP compared to the same areas in a nonseparated control group.

Methods

Subjects

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; each was housed alone during the last 2 weeks of gestation. Litters were randomly assigned to separation or animal facility-reared (AFR) 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 conformed to the 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.

Maternal separation procedure

Litters were undisturbed at birth: postnatal day zero (P0). The mothers were first removed from their home cages in the main colony room and placed into a fresh cage at P1. Pups were subsequently sexed and randomly assigned to undergo maternal separation or AFR procedures. Both male and female pups were used. Handling was kept to a minimum throughout the procedure to avoid ''handling effects'' on the pups. The experimental groups (MSDB) were placed together in a separate small cage (P1–P21) and then were moved to a different room to prevent communication with their mothers by means of ultrasonic vocalizations (Hofer et al., 1994). The separated pups were placed on a heating pad at 25–28°C for 6 h (3 in the morning and 3 in the afternoon). Pups were returned to their original home cages in the main colony room after the 3-h separation immediately followed by the return of the mother. Control litters remained undisturbed except for routine cage cleaning, which was performed once a week. For each pup, the separation procedure was carried out at the same time every day (7:00–10:00 and 13:00–16:00). At day 22, litters were sexed and separated by gender and treatment until day 60 and kept under standard conditions with free access to water and food. In total, three littermates were used (12 males and 18 females).

Apparatus: elevated plus maze (EPM) test

The EPM was made from dark black acrylic material and consisted of two open arms (50 × 10 cm) crossed at right angles with two opposed arms of the same size. Two of the opposed arms were enclosed by 40-cm-high walls except for the central part where the arms crossed. The whole apparatus was raised 50 cm above the floor. A Plexiglas rim (1-cm high) surrounded the perimeter of the open arms to prevent the rats from falling off. The experimental sessions were recorded by video camera interfaced with a monitor and a DVD in an adjacent room. A rat was placed in the central area of the maze facing one of the open arms at the beginning of each session and was allowed free exploration for 5 min. Female rats were fist-tested to identify the estrus cycle by examining vaginal smears; all were evaluated during the diestrus phase. Results were expressed as the percentage of entries and time spent in the open arms. Free, specially designed software for recording behavior (X-PloRat) was used for behavioral analysis.

Procedure: histological procedure

After behavioral testing, subjects were perfused with paraformaldehyde (PFA 4% in phosphate buffer: PBS) after anesthesia with chloral hydrate intraperitoneally (400 mg/kg). We then extracted the brains and preserved them in PFA. Three days prior to brain processing, it was cryoprotected with 30% sucrose in PBS. Areas of interest were placed based on the coordinates of Rat Brain Atlas of Paxinos (Paxinos, 2004). We used a cryostat (Leica CM1850) at -21°C and 20-μm-thick cuts were made.

Immunohistochemistry

Brain sections from both AFR and experimental rats were selected according to anatomical landmarks corresponding to the selected areas using a Paxinos (2004) rat brain atlas. The sections were simultaneously processed in a free-floating state for GFAP detection. Brain sections were treated for 1 h with 3% v/v normal goat serum in PBS (0.1 M) containing 1.5% BSA and Triton X-100 to block nonspecific binding sites. Afterwards, sections were incubated for 12 h with anti-GFAP (Dako Cytomation) and diluted 1:500 in PBS-BSA. After five rinses in PBS (15 min each), sections were incubated for 1 h at room temperature with biotinylated secondary antibodies diluted 1:500 (Kit Vectastain, Vector Laboratories, Burlingame, CA, USA). Subsequent to further washing in PBS, sections were incubated for 1 h with streptavidin-peroxidase complex diluted 1:500. Sections were then washed five times in PBS and twice in 0.9% NaCl buffer, and peroxidase activity was carried out with diaminobenzidine hydrochloride (DAB) included in the same kit. Sections were washed three times with saline solution at the end of the enzymatic reaction step. The sections were then mounted on gelatin-coated slides, air dried and coverslipped using cytoresin for observation with a light microscope.

Quantification of immunolabeled cells

For each subject, four sections were made and four pictures from each area were taken. All images were captured with a light microscope (Carl Zeiss-AxioVert 40 CFL) and a digital camera (Cannon Power Shot 640). In total, 250 μm^2 per area labeled cells were counted manually using ImageJ software. Investigators were blind to the groups while taking the photomicrographs and performing the image analysis. All images used in the analysis were taken on the same microscope at the same optical settings.

Statistics

The evaluated variables were considered in terms of mean; to estimate data dispersion between groups average error was taken into account. For inferential analysis, SIGMA STAT 3.11 program was used. To compare means when normality criteria apply we use parametric statistics (t-test), otherwise Mann–Whitney U test was used. For statistical analysis of labeled cells, the R program was used and, taking into account the normality, a t-test or Wilcoxon analysis was applied; p value < 0.05 was considered statistically significant. Data are presented as mean ± standard error of the mean (SEM).

Results

In the present study we first analyzed the behavioral response of separated and nonseparated subjects in the EPM in order to confirm our previous results. Second, we compared the number of astrocytes immunolabeled for GFAP in the PFC, PVN, POA, AM and HP. Our sample consisted of adult rats, both males (12: 7 control, 5 separated) and females (18: 8 control, 10 separated) who had been separated from their mothers during breastfeeding compared with a group of nonseparated controls.

Figure 1 shows the females' behavior in the EPM. As we reported previously, separated females had more entries and spent more time in open arms compared with nonseparated females.
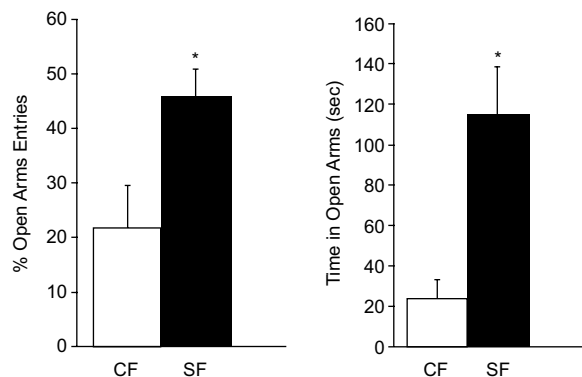

Figure 1. Plots of percentage of entries and time spent in the open arms of the elevated-plus maze in separated *(n = 10)* and nonseparated *(n = 8)* females. Bars represent the mean ± standard error of the mean (SEM) of controls and separated subjects. *p = 0.017. (t-test, t = -2.7) and for time *p = 0.005 (Mann–Whitney U = 44.000).

In Figure 2 we show the lack of differences in separated and nonseparated males in the same parameters evaluated with females in the EPM. Both results corroborate our previous findings.
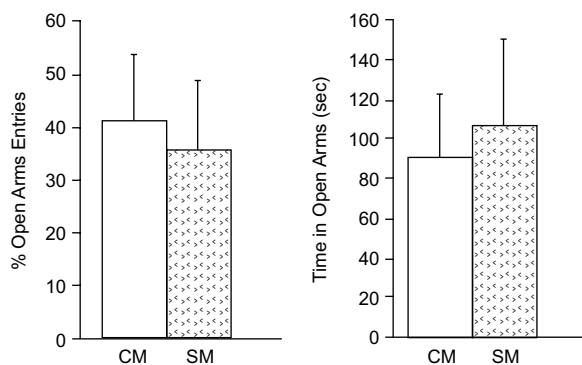

Figure 2. Behavioral responses of non-separated (*n*=7) and separated (*n*=5) males in the elevated plus maze. Bars represent the means ± s.e.m. of controls and separated subjects. % Open arms * p = 0.781 T-Test t = 0.286, Time in Open Arms * p = 0.766, t= 306.

Figure 3 shows representative photographs of immunostainning cells against GFAP in PFC, HP and POA, areas in which we found statistically significant differences.
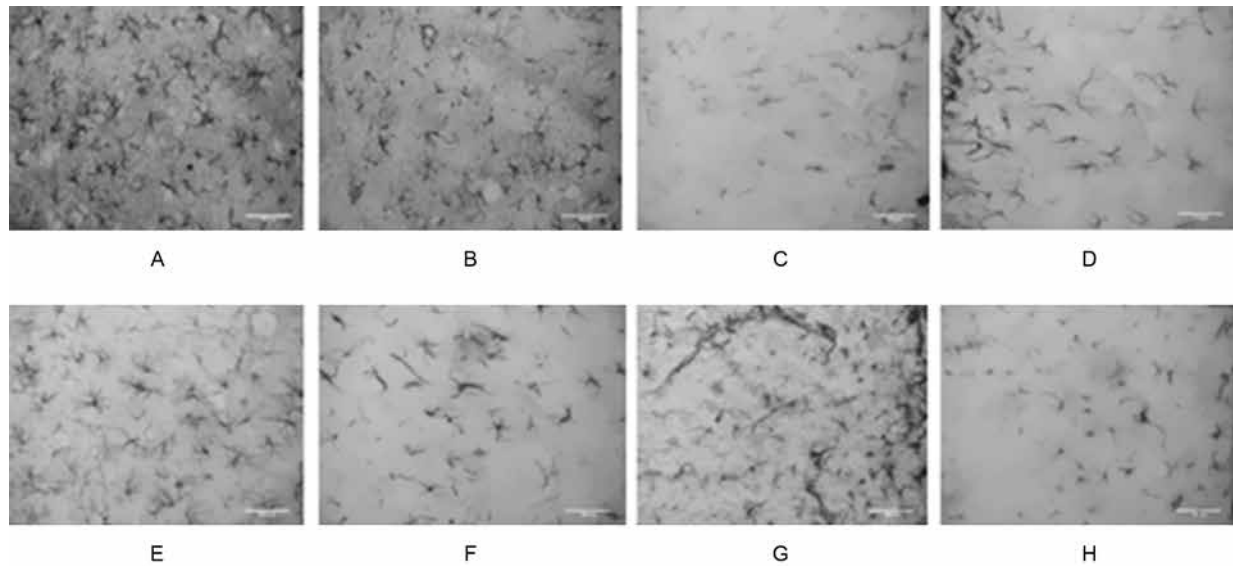
Figure 3. Representative photos of glial cells immunolabeled with GFAP antibodies. (A) PFC control female. (B) PFC separated female. (C) PFC control male. (D) PFC separated male. (E) HP control male. (F) HP separated male. (G) POA control male. (H) POA separated male.

Astrocyte count is shown in the area equivalent to the PFC. Statistically significant differences were found in the number of cells immunostained with GFAP in separated rats in both females and males ($p < 0.05$). These differences correspond to reduced numbers of astrocytes in separated females compared with nonseparated females and an increase of the immunostained cells in separated males compared with nonseparated control group males (Figure 4).
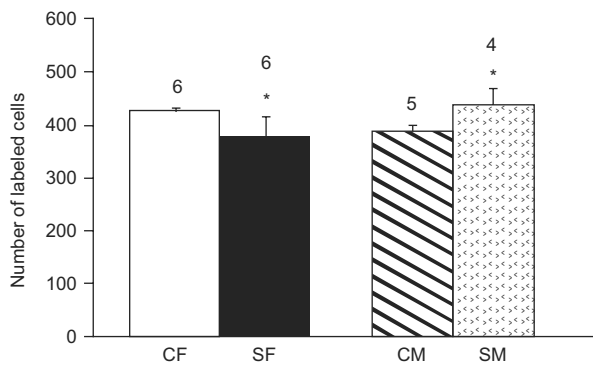

Figure 5. Number of immunoreactive cells in the hippocampus. Control females (CF, $n = 5$) and separated females (SF, $n = 5$) vs. control males (CM, $n = 5$) and separated males (SM, $n = 5$). Bars represent the mean ± SEM of controls and separated subjects. *$p < 0.05$.

Figure 4. Number of GFAP-immunoreactive cells in the prefrontal cortex. CF, control females; SF, separated females; CM, control males; SM, separated males. Bars represent the mean ± SEM of controls and separated subjects. *$p < 0.05$. Numbers at the top of the bars indicate the number of brains analyzed.

With respect to the count and analysis of cells in the HP and POA, the only clear difference is a decrease in the labeling of astrocytes in separated males only (Figures 5 and 6).

No significant differences were apparent in the astrocyte count immunostained with GFAP in the PVN or AM for female or male subjects, for either those nonseparated or separated.
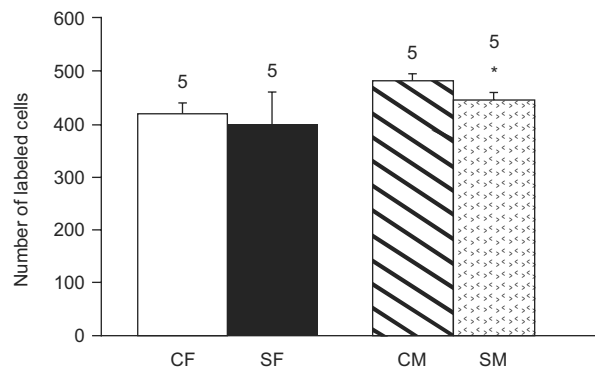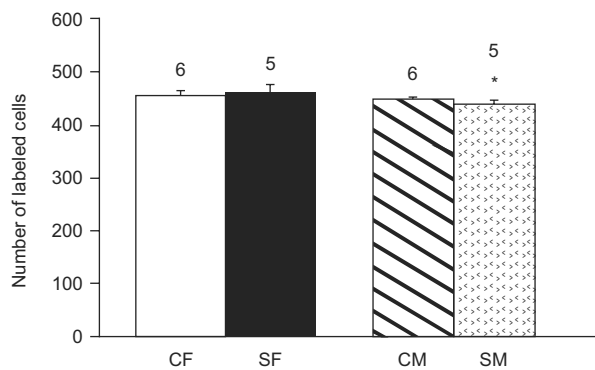

Figure 6. Number of immunostaining cells in preoptic area. Control females (CF, $n = 6$) and separated females (SF, $n = 5$). Control males (CM, $n = 6$) and separated males (SM, $n = 6$). Bars represent the mean ± SEM of controls and separated subjects. *$p < 0.05$.

Discussion

Consistent with our previous work, the present study revealed that moving pups away from their dams for two 180-min periods each day in the dark cycle during nursing had gender-specific consequences on anxiety-like behavior. We found different behavioral responses in separated females compared with nonseparated females, and we did not find differences between males (data not shown). Females generally exhibited less anxiety behavior than males, and this effect was increased in the MS pups. The nonanxiogenic profile of the MS females was reflected in increased time spent in the open arms (Figure 1). Because the effect of maternal separation during the dark cycle has not been reported in the literature, we cannot make a direct comparison. In fact, many authors point to the differences between protocols and strains. Toward the second goal of this study, we found that short periods of maternal separation during breastfeeding induce permanent changes in the number of astrocytes immunolabeled with GFAP in different brain areas in different ways according to gender. In this context, our results are novel because others have not performed this type of study in this separation model and have not analyzed the differences between males and females. Here we present an analysis of the possible implications of these differences based both on the literature and results generated by our research group.

Prefrontal cortex

In the PFC of separated males, we found an increase of astrocytes marked with GFAP. Knowing that astrocytes are regulators of synaptic transmission (Newman, 2003), changes in the PFC could be associated with increased cortical activity in males where they could be potentially involved in a differential mechanism of response to anxiety as previously observed. In fact, it has been reported that changes in connectivity between the AM and the cortex are associated with anxiety disorders (Etkin & Schatzberg, 2011). Here we suggest that because GFAP is essential in the maintenance of the morphology of the astrocytes, the decreased immunoreactivity of this protein may be due to a reordering of these cells in response to a stressful stimulus; this effect is called glial retraction (Salm, 2000). Mechanisms for the reordering of astrocytes include reorganization of the cytoskeleton, re-entry in the cell cycle and astrocyte death, which influences neuronal excitability and peptide release (Perea & Araque, 2007; Slezak and Pfrieger, 2003).

Regarding females who showed a decrease of anxious behavior in this protocol (Figure 1), we found a lower marking of astrocytes in the PFC. Based on the above analysis, we could suggest that synaptic regulation that exerts astrocytes could be associated with a decrease in cortical activity in females. Experimentally, this hypothesis could be checked using electrophysiological experiments.

Hippocampus

The HP is one of the main areas of focus in a study related to the regulation of response to stress, learning and memory (McClelland, Korosi, Cope, Ivy, & Baram, 2011). In general, studies on maternal separation as a cause of chronic stress show early changes in the cytoarchitecture of the HP, in particular in CA1, CA3 and in the dentate gyrus (Huot, Plotsky, Lenox, & McNamara, 2002; Jahanshahi, Sadeghi, Hosseini, Naghdi, & Marjani, 2008). In this study we found that the number of astrocytes in this area was reduced only in males who had been separated from their mothers during breastfeeding. Brunson et al. (2005) showed that stress early in life was associated with a progressive decrease in learning and memory in adult life. On the other hand, loss of glia in the HP has been suggested as a determining factor in brain disorders related to stress, characterized by a poor secretory regulation of glucocorticoids (Hamidi, Drevets, & Price, 2004). In addition to the importance of the HP during learning processes, it has been shown that maternal separation induces a decrease in the labeling of cells that colocalize pCREB and GFAP in hippocampal CA1. CREB is a transcription factor that can be involved in memory and learning processes and, as a conveyor of lactate, this neuron–astrocyte is required for the formation of spatial memory (Yin, Del, Zhou, & Tully, 1995; Gibbs, Hutchinson, & Hertz, 2008).

Preoptic area

The POA was the first area described as sexually dimorphic and continues to be one of the clearest examples of dimorphism both in laboratory animals and in humans. If the POA is removed, male rats do not express sexual behavior in the presence of a receptive female and, in addition, the POA is one of the structures that mediate the regulation of the HPA axis (Tsukahara, 2009).

Previous results reported by our research group demonstrate that the amount of cells immunolabeled by IHC against the α1 of the GABA-A receptor subunits decreases significantly in both males and females. However, with respect to the staining of GFAP that was evaluated in this work, we found a significant decrease only in separated males. There are still some studies related to the glia in this area. Taking into account that POA is a dimorphic region, we could suggest that this is one of the most susceptible areas to change caused by chronic stress and, in the future, could be evaluated separately in females and males by comparing maternal behavior in females or sexual behavior in males.

Paraventricular nucleus

The PVN receives important inhibitory signals from different structures of the limbic system including hypothalamic nuclei, POA, the bed of the striatum nucleus, nucleus of the solitary tract and others. Additionally, the PVN also has local inhibitory activity mediated by GABA-A receptors, which makes modulation of the PVN very complex (Jankord & Herman, 2008).

Previous results from our laboratory demonstrated a reduction in the expression of GABA-A receptors in separated females; therefore, we expected alterations in glia. However, the analysis showed no differences in the marking of GFAP in any of the separated groups. Although there have been reports in the literature on the effects of maternal separation on the PVN, these have mainly focused on the effect of lung function; therefore, this result, while novel, requires further studies to be explained comprehensively.

Amygdala

The AM is one of the regulatory centers for both stress and emotional responses. Although previous results showed that separated males have a significant reduction in the immunoreactivity from the AM to the GABA-A receptor α1 subunit, we found no differences in the quantification of astrocytes immunolabeled with GFAP, which could be interpreted as an alteration in communication but not in morphology. It is known that stressful events activate the AM and a network of associated brain areas, including the PFC. In fact, it has been reported that strong connectivity between the AM and the PFC improves response behavior to anxiety so that a lower activity in the AM results in increased activity in the cortex (Kim et al., 2011). However, not having found any differences in the labeled astrocytes does not necessarily mean that other cells in the glial tissue did not change; it may be a result of the alteration of receptors or transporters expressed in these cells.

In summary, a possible explanation for the decrease in the immunolabeled GFAPs in the different areas studied could be that stress right after birth induced by separation may alter the transition process between the expression of vimentin by astrocytes and the subsequent expression of GFAP. Because astrocytes play an important role in response to brain damage, an increase in the marking of these cells with GFAP could be explained by the reactivation of astrocytes during the process of gliosis in response to central nervous system damage with the subsequent formation of a glial scar.

The results reported here allow us to suggest that short periods of maternal separation during breastfeeding permanently alter the number of labeled astrocytes in some brain areas, which are different for males and females. These changes are reflected in the modulation of different types of stress responses or alterations in processes of learning and memory.

Reports in the literature together with our results support the hypothesis that maternal separation permanently alters the growth of astrocytes, which could influence synaptic communication.

Acknowledgments

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