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Effects of perinatal undernutrition on the basilar dendritic arbor of the anterior cingulate pyramidal neurons in lactating dams

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In altricial species, early pre- and neonatal undernutrition interferes with the neuronal organization of several brain structures that have critical time windows for synaptic organization, including the prefrontal cortex. In Golgi-Cox stained tissue the basilar dendritic arbor of pyramidal neurons in the anterior cingulate cortex of early underfed adult lactating dams was evaluated. The anterior cingulate of the rat plays a major role in the execution of sexual, maternal and visual attentional control and other cognitive responses. The effects of neonatal undernutrition on the basilar dendritic tree and perikaryon measurements in layer II/III pyramidal neurons of the anterior cingulate were examined in lactating dams at postpartum days 8 and 12. In the underfed dams the distal portions of the basilar dendrites had fewer branches and a lower dendritic density of dendrites, and neurons had perikarya with reduced perimeter and cross-sectional area. Thus, the neuronal alterations may interfere the plastic synaptic activity and with maternal cognitive performance of rats subjected to early underfeeding. These anatomical alterations of the anterior cingulate may help to understand the disruption of long-term cognitive processes associated with perinatal food restriction.

Key words: Early undernutrition, anterior cingulate, dendritic arbors, maternal response

INTRODUCTION

The remarkable sensorial, motor and homeostatic immaturity of the altricial newborn species requires a number of sequential, genetic, hormonal, and basic brain and systemic anatomical and functional changes during gestation in the female culminating with the maternal response repertoire, which is essential for the survival of progeny and their social and cognitive development (Rosenblatt et al. 1988, Numan 2007, Kim et al. 2010, Kinsley and Amory-Mayer 2011). The care and the protection of the pups is regulated by a large number of closely interconnected brain structures (the maternal circuitry) including the medial prefrontal cortex, the medial preoptic area, the lateral habenula complex, the nucleus accumbens shell, the olfactory system, the medial and central amydala, the hippocampus and the periaqueductal gray (Beach 1937, Slotnick and Nigrosh 1975, Numan et al. 1977, Corodimas et al. 1993, Lonstein et al. 1998, Li and Fleming 2003). Among these neuronal substrates of the lactating dams, the medial and lateral prefrontal cortex play a fundamental role in the integration of the sensorial, motivational, attentional control, and motor information that are essential for successful maternal care of the pups (Groenewegen and Uylings 2000, Pawluski et al. 2006, Dalley et al. 2004, Afonso et al. 2007, Febo et al. 2010).

Early food restriction during gestation and/or sucking and the associated sensory deprivation in the newborn rat results in delayed sensory maturation and in permanent anatomical and functional abnormalities of the central nervous system (CNS) (Galler and Propert 1981, Kehoe et al. 2001, Salas et al. 2002, Felix et al. 2014). These alterations are associated with a reduced number of neurons, loss of spines, decreases in the number of dendrites and

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synaptic contacts, and atrophy of the perikarya. These changes are well-known in CNS structures undergoing relevant modifications in neurogenesis, migration, and synaptic connectivity that correlate with long-lasting deficiencies in the mother litter-bonds, social learning, and the performance of maternal behavior (Frankova 1974, Smart 1976, Galler and Propert 1981, Salas et al. 1984, Salas et al. 2002). Although previous studies of acute cytotoxic lesions of different percentages and regions medial and lateral prefrontal, or cingulate cortex have demonstrated deficiencies in nest building, relocation of pups in response to nest destruction, or picking up and dropping pups with aimlessly into the nest (Beach 1937, Stamm 1955, Slotnick and Nigrosh 1975, Afonso et al. 2007). However, little is known about the diffused brain lesions resulting from perinatal food restriction and the plastic reorganization of the basilar dendritic arbor of pyramidal neurons in the anterior cingulate (AC) of the dams, which is a key for pup´s retrieval. Furthermore, morphofunctional studies on brain areas involved in cognitive processes are relevant to understand possible mechanisms related with early food restriction or other stressful conditions that may correlated with the long-term human brain disorders or abnormal environmental adaptive conditions (Murmu et al. 2006, Glatz and Lewis 2000).

The present study anticipates that the AC neuronal alterations elicited by pre- and neonatal food restriction may reflect a disrupted maternal circuit that alters maternal responsiveness including the pup retrieval deficiencies shown by the lactating dams that had been underfed during the perinatal period.

METHODS

Subjects and experimental design

Subjects were twenty female Wistar rats obtained from progenitors from Harlan Industries, Sprague-Dawley, (IN, USA). The animals were housed in polycarbonate cages (50×40×20 cm^3) in a room maintained at 24±2˚C and at 50% humidity on a 12-h:12-h light-dark cycle (lights on at 7:00 AM), with water and food (Purina chow) *ad libitum*. For mating, groups of four, 90-day-old virgin female rats were housed with two males of similar age. Sperm-positive females were placed one week before delivery in individual plastic maternity cages (35×27×17 cm^3) with grill tops and wood shavings as nesting material. Births were verified daily at 8:00 AM and 18:00 PM. Newborns found at that time were considered to be 0 days of age. The day after birth, pups were weighed and sexed, and the litter (average at birth 10 pups) was adjusted to 8 pups per mother (four males and four females). During this time most of the pups of the UG maintained the distribution in the litter but when one of them die it was replaced by a pup of the same age, sex, and condition to minimize a possible influence on the maternal behavior and brain morphology. By contrast CG subjects were maintained healthy throughout the study. The redistribution was intended to minimize possible genetic and nutritional differences that may influence the experimental data. The presence of bilateral thoracic and abdominal lines of nipples and the shorter anogenital distance in the females were used as criteria for sex recognition (Vanderbergh 2003). Animal care and protocols were approved by local Animal Committees and

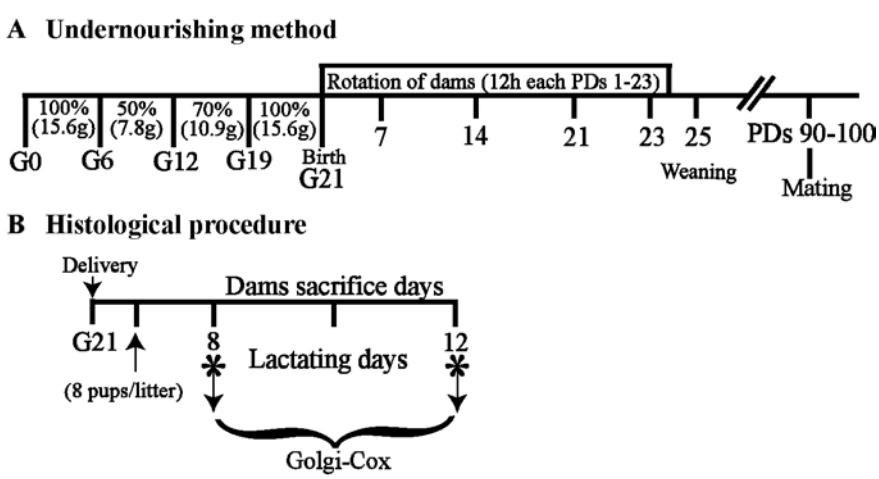

Fig. 1. Experimental design for (A) dietary treatments and (B) histological procedure.

adhered to the National Research Council guide for the use and care of mammals (NRC 2003).

Nutritional paradigms

The control group (CG) subjects (*n*=10) were female mothers obtained from four litters normally nourished by well-fed mothers with free access to food and water during the gestation and lactation periods. After birth, pups were fed and handled by interchanging a pair of normally lactating mothers every 12 h for 24 days as described elsewhere (Rubio et al. 2004). The female rats obtained by this method were mated and maternally tested as adults.

The underfed group (UG) of female rats (*n*=10) came from at least four different litters. The normal diet requirement was calculated by measuring the food intake of a group of four pregnant rats (200–250 g) every week during a 24-day period. The resulting average food intake for each week was the basal level used to calculate the food-intake percentage of the UG females. Dams were fed from gestational day 6 (G6) to G12 with 50% (7.8 g) of the balanced diet (Purina chow), from G13 to G19 with 70% (10.9 g), and then with 100% (15.6 g) of the same diet until parturition to avoid resorption or cannibalism of newborns (Fig. 1A). The current protocol was chosen because neurogenesis of the cortical and subcortical maternal circuit and afferent connectivity occur between days G12-G21 of gestation (Altman and Bayer 1995). At birth, prenatally underfed female newborns were nursed by two, gestationally underfed dams, in one the main galactophorous ducts was tied subcutaneously. The two lactating mothers were interchanged every 12 h between litters from post-natal days 1 to 24. The CG and UG groups were weaned at postnatal day 25, after which the rats were allowed free access to water and food (Purina chow). The females were maintained in groups of 4–6 until reaching 90–100 days of age, when they were mated and tested for maternal behavior with their own litter. This cross-fostering procedure ameliorates the effects on the pups of maternal sensory deprivation (Lynch 1976). Approximately 80% of the total UG dams were undernourished during the light phase of cycle. The current study evaluates the effects of a pre- and neonatal underfeeding paradigms on body and brain weights, the dendritic orders and density, the cross-sectional area and the perimeter of the perikarya of layer, II/III AC pyramidal neurons. These neuronal parameters may correlate with the long-term maternal cognitive performance during the early and late lactating periods when the maternal responsiveness is maximal (pup retrieval, nest building, and the kyphosis posture for pups'sucking) (Salas et al. 1984).

Histology

A total of 20 lactating dams were subjected to two dietary treatments (*n*=10, CG, and *n*=10, UG). Body weight of dams and pups on the PDs 1, 4, 8, and 12, and brain weight of the dams before sacrifice at postpartum days (PDs) 8 and 12 during the lactating period were recorded. The aim of these measurements was to detect the long-term effects of perinatal undernourishment on the physical growth of dams, and the effects of possible maternal care deficiencies upon the pups´ development. On PDs 8 and 12 of the lactation period, the dams were deeply anesthetized with ether, and perfused transcardially first with saline and then with buffered 4% paraformaldehyde (JT Baker, Co), pH 7.4. Thereafter, dams were decapitated; the brains were removed, weighed wet, cut into three coronal blocks, and immersed in the Golgi-Cox solution for impregnation (Fig. 1B). Briefly, the blocks were immersed in a mixed solution A (mercuric chloride and potassium dichromate), and solution B (potassium chromate and potassium tungstate dilute in distillate water). Three weeks later, the blocks were dehydrated for 24 h in

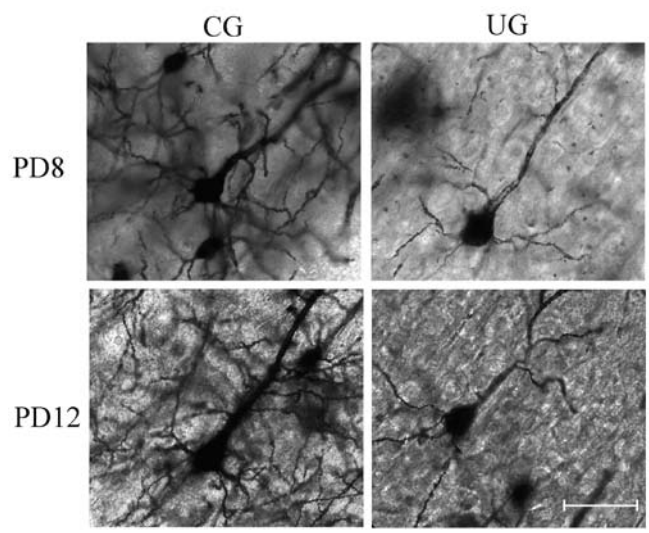

Fig. 2. Representative photomicrographs of layer II/III AC pyramidal neurons from CG and UG lactating dams at PDs 8, and 12. Bar, 50 μm.

alcohol/acetone, followed by an ether/alcohol solution and embedded in a gradually pro-celloidine moistened with 35% isopropyl alcohol (Fluka) (15 days). Thereafter, to drain alcohol from the blocks they were expose to chloroform in a glass container (15 min). Subsequently, they were cut in coronal sections of 120–150 μm; the staining process following (distilled water, Microdol-X liquid developer (Kodak), distilled water and gradually alcohol solution, until absolute alcohol. Finally were put in propanol and tolueno solutions, and mounted serially with Permount and exposed to air dry before microscope observation (Narayanan et al. 2014). The slides were coded to ensure blind evaluation with respect to age and dietary treatment of the dams. During the digitizing of the neuronal images the experimenter had access only to the code numbers and not to the ages and dietary treatments. Identification and location of the AC cortex were based on Paxinos and Watson's atlas (1986). Anterior-posterior coordinates for the location of the AC corresponded to values ranging from Bregma 4.70 to 1.70 mm. For each experimental group a total of 60 AC pyramidal neurons was analyzed.

Morphometric evaluations

A total of 120 well-impregnated, layer II/III AC pyramidal neurons whose basilar dendritic arbors were confined to one section was evaluated for each experimental condition, age group, and neuronal parameter (Fig. 2). Basilar dendritic order measurements were obtained by counting the number of 1st, 2nd, 3rd, 4th, and 5th dendritic

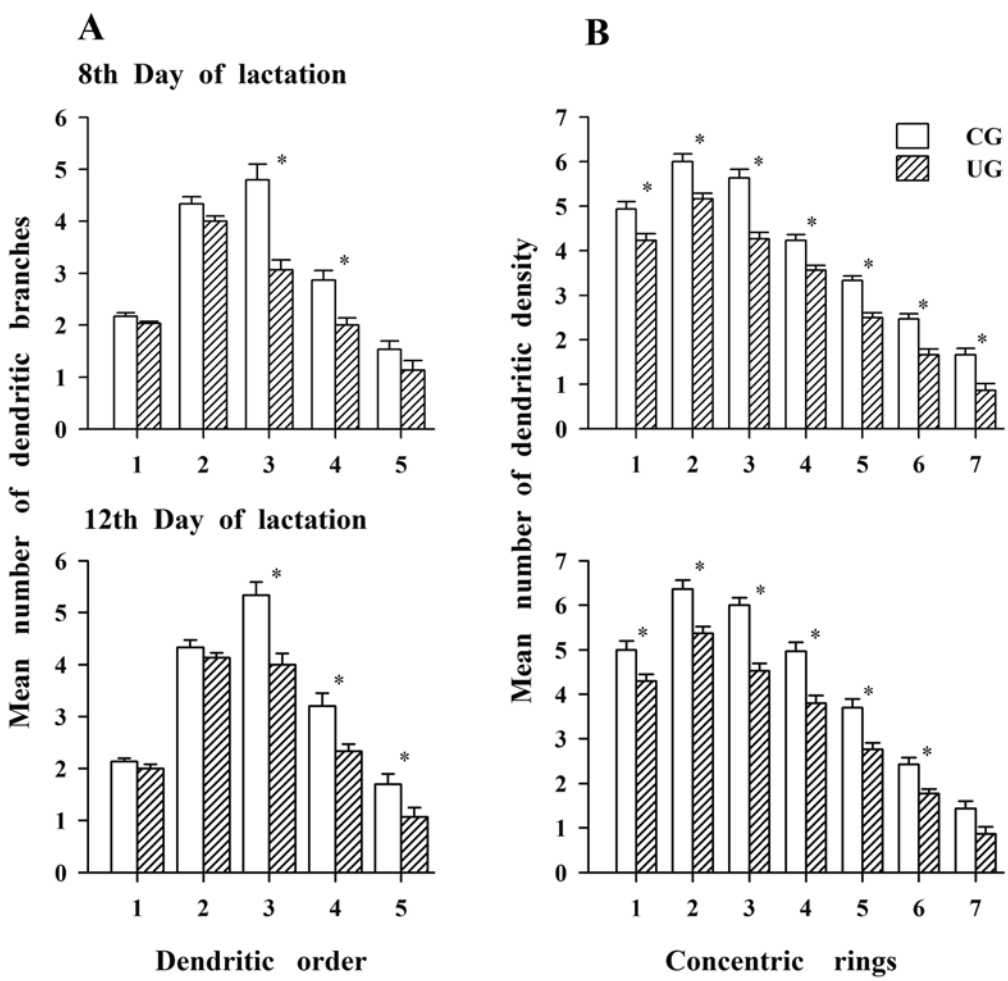

Fig. 3. Mean values ±SEM for the (A) number of dendritic branches of different orders and (B) number of dendritic crossings of pyramidal neurons (n=30/age group) at PDs 8 and 12 and under different experimental conditions. Note, the reduced number of branches and crossings in the UG at most ages. **Post hoc* statistical differences between groups, $P<0.05$.

Table I

Mean values ±SEM of dams and pups weights (g) in CG and UG rats during the lactation period

Age (days)	Body weigh mothers		Body weight pups	
	CG	UG	CG	UG
Prep	419.33±4.65	316.41±14.79*		
1	340.08±7.83	281.75± 7.23*	7.10±0.05	6.93±0.09
4	343.00±7.80	287.75± 9.16*	9.72±0.09	9.75±0.17*
8	356.83±6.68	299.08± 6.72*	15.94±0.87	14.93±0.19*
12	361.00±6.26	308.00± 9.66*	21.21±0.21	19.25±0.31*

Factors	df	F	p<	df	F	p<
Nutr (A)	1,66	30.57	0.0001	1,198	8.63	0.003
Age (B)	3,66	20.54	0.0001	3,594	585.24	0.0001
A*B	3,66	0.25	NS	3,594	3.45	0.016

P<0.05. Fisher LSD test.

orders. Dendritic branches leaving the cell body were defined as first order, while those which branched from the former were considered second order, and so on. The dendritic density was measured by placing the cell body and primary dendrites at the center of the first of a series of seven concentric rings (spaced at 40-μm intervals) and counting all dendritic intersections with larger individual rings (Sholl 1956). The cross-sectional area and the perimeter of the pyramidal neuron perikarya were also measured. In all cases neuronal measurements were obtained at a magnification of 40× using an image digitizing system (Perception Analysis System by Human-Computer Interface, Cambridge, UK). No attempt was made to correct for compression of the three-dimensional dendritic arbor to a two-dimensional sketch, since the relative differences between neurons remain constant when transformed from three to two dimensions (Spinelli et al. 1980). Furthermore, because the dendritic arbor is confined to the tissue section no stereological method was used. Additionally, for the soma parameters the image analyzer did some calculations similar to those previously described.

Statistics

All measurements were analyzed using the following ANOVA comparisons.

1) Scores for body weight of dams and pups were compared with a two-way ANOVA, 2 (dietary conditions) X 4 (ages) each;

2) the effects of dams wet brain weights were compared with a two-way ANOVA 2 (dietary conditions) X 2 (ages);

3) the effects of undernutrition on the dendritic order and dendritic density of basilar branches of pyramidal neurons during the lactating period were analyzed using a three-way ANOVA, 2 (dietary conditions) X 2 (ages) X 5 (dendritic orders) or 7 (concentric rings);

4) the cross-sectional area and the perimeter of perikaryon measurements were compared with a two-way ANOVA, 2 (dietary conditions) X 2 (ages).

The *post hoc* statistical comparisons between experimental groups were made using the Fisher LSD *post hoc* test. The significant threshold for all comparisons was set at $P<0.05$.

RESULTS

Effects on body and brain weight

The ANOVA comparisons of the body weight scores between the CG and the UG dams after giving birth indicated that the UG dams weighed significantly less than CG mothers, ($F_{1,66}$=30.57, P<0.0001), with a significant effect of age, ($F_{3,66}$=20.54, P<0.0001), and without interaction between factors. *Post hoc* comparisons showed significant body weight reductions (P<0.05) in the UG dams at all ages tested, with slight increases in both experimental groups (Table I). Comparisons in brain wet brain of dams showed significantly reduced values by the diet, ($F_{1,20}$=1176, P<0.0001); and age, ($F_{1,20}$=6.66, P<0.01), without interactions between factors. The ANOVA analysis of pups´ body weight comparisons between CG and the UG on the same lactating days indicated significant effects of diet, ($F_{1,198}$=8.63, P<0.003); and age, ($F_{3,594}$=585.24, P<0.0001), and a significant interaction of diet by age, ($F_{3,594}$=3.45, P<0.016). *Post hoc* comparisons between groups at different ages indicated significantly reduced weights (P<0.05) of the UG pups at PDs 4, 8, and 12 (Table I).

Effects on the dendritic arbor

ANOVA comparisons of the dendritic order measurements indicated significant reductions associated with the diet, ($F_{1,116}$=4.807, P<0.03) and age, ($F_{1,116}$=39.983, P<0.0001), with no interaction between factors. *Post hoc* comparisons indicated that UG pyramidal neurons showed significant reductions (P<0.05) in the 3rd and 4th dendritic orders on day 8 of lactation, as well as in the 3rd, 4th and the 5th dendritic orders on day 12 of lactation (Fig. 3A).

The density of the dendritic branches of AC pyramidal neurons, measured as the number of crossings of dendrites with 7 circles, showed significant reductions associated with the diet, ($F_{1,116}$=97.682, P<0.0001) and age, ($F_{1,116}$=4.785, P<0.03), with no significant interaction between factors. *Post hoc* comparisons of dendritic crossings in the pyramidal neurons indicated that in the UG they were consistently reduced at all ages during lactation (P<0.05) (Fig. 3B).

Effects on perikarya

The ANOVA comparisons of the perikaryon cross-sectional area measurements indicated significant reductions in the pyramidal neurons of the UG subjects, ($F_{1,116}$=67.79, P<0.0001), with not significant effects of the age, and with no interaction between factors. *Post hoc* comparisons showed significant cross-sectional area reductions (P<0.05) in the UG subjects at PDs 8 and 12 (Fig. 4A). ANOVA comparisons of the soma perimeter measurements indicated significant reductions associated with the diet, ($F_{1,116}$=87.43, P<0.0001) but no significant interaction between factors. *Post hoc* comparison showed significant reductions (P<0.05*)* in the soma perimeter of the UG at PDs 8 and 12 (Fig. 4B).

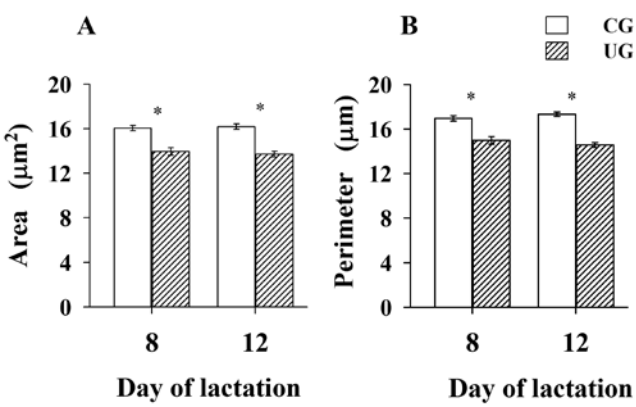

Fig. 4. Mean values ±SEM of cross-sectional area (A) and parikaryon perimeter (B) of pyramidal neurons of CG and UG subjects at PDs 8 and 12 of lactation. Note, the reduced values in the two perikaryon measures in the UG groups. *Significant differences between experimental groups, P<0.05. Fisher LSD test.

DISCUSSION

The present results indicated that pre- and neonatal food restriction results in delayed physical development of the dams, as evidenced by their significantly lower body and brain weights during the lactation period. In this regard, the findings agree with previous data showing that low brain and body weights are associated with reduced size and abnormal structure and function of the placenta affecting, the nutrients supply to the fetus, with delayed physical and sensory development, and ear- and eye-opening, altered locomotor patterns during infancy, and poor maternal responsiveness (Altman et al. 1970, Salas et al. 2002, Wu et al. 2004, Belkacemi et al. 2010). Additionally, the body weight reduction of pups may be reflecting

the inadequate maternal behavior of the early UG dams and the brief experimental separation of pups from their mothers when dams were rotated between litters during the underfeeding procedure that may disrupt their bonding interactions (Bousalham et al. 2013). Additionally, the perinatal body weight reduction of pups delays their physical activity, body movements, and tactile stimulation under the maternal ventrum, thereby affecting the pups´ demands for maternal care, altered ultrasound vocalizations and interactions within the litter (Smart 1976, Evoniuk et al. 1979, Salas et al. 1984, Salas et al. 2002, Schanberg et al. 2003, Tonkiss et al. 2003, Febo et al. 2008, Richards et al. 2012).

Our findings also indicated that pre- and neonatal food restriction resulted in significantly reduced dendritic orders and density of the basilar cingulate dendritic branches, both of which interfere with the excitability dynamics, and the elaboration of spatiotemporal patterns of discharge by the AC pyramidal neuron population to activate the maternal circuitry and its functions. The neuronal hypoplasia associated with the undernourishment procedure interferes with the postsynaptic neuronal geometry and affects the AC neuronal excitability, the threshold required to trigger electrical activity, and possibly, the dynamics of the afferent synaptic contacts from the anterior thalamic nuclei, and limbic areas of a number of sensory, motor, and visceral brain sources (Williams and Stuart 2000, Febo et al. 2008). These synaptic level distortions of the AC neurons may affect the stream of spatiotemporal information codes from different anatomical sources that are modulated by the AC neuronal substrate, to coordinate complex physiological phenomena such as attentive responses, visual discrimination, and the cognitive maternal processes for the development and protection of the progeny (Williams and Stuart 2000, Froemke et al. 2010).

Previously a number of studies examined the effects of ablation of medial and lateral prefrontal cortex, and of electrolytic lesions using a constant, 1-ma anodal constant current for 3–8 sec. Thus, rats with large cortical or small AC ablations showed variations that correlated with the extent of cortical damage in nest-building ability, relocation of pups in response to nest destruction, or duration of picking up and dropping pups aimlessly into the nest (Beach 1937, Slotnick 1967, Slotnick and Nigrosh 1975, Afonso et al. 2007). In other cases the lesions were elicited by the local administration in the AC locus of tetrodotoxin (TTX), a cytotoxic chemical that inactivates the medial prefrontal cortex (mPFC) or by the GABA-mediated inhibition during tests for retrieval-behavior latencies, with impaired or reduced maternal retrieval and no effects on locomotor or other maternal activity respectively (Febo et al. 2010). Many of these studies have provided valuable information about the brain circuitry that regulates the maternal behavioral efficiency, however, complete knowledge about the integration of signals from different sources, and their interactions with the forebrain structures is still under investigation. Previous studies on the effects of pre- and neonatal underfeeding paradigms provided very consistent evidence of a reduction in the number of neurons and poor dendritic arbor and spines, with different levels of chronic damage in both the motor and sensory neuronal relays (Pascual and Zamora-Leon 2007, Breton et al. 2009, Florian and Nunez 2010, Salas et al. 2012). The morphological alterations of the AC neurons identified here may suggest that the neuronal disruption may be occurring at the level of synaptic connectivity, with effects on the plastic properties of complex underlying cognitive processes like the visual attention and the maternal care (Broersen and Uylings 1999, Afonso et al. 2007, Febo et al. 2010). The characteristics of the underfeeding model used here to provoke forebrain brain damage may mimick a maternal response deficiency observed in human mothers that suffer early malnutrition or complete food restriction in the poor or underdeveloped countries. However, little is known about the diffuse brain lesions elicited by perinatal food restriction and the plastic reorganization of basilar dendritic arbor profiles of forebrain neurons like those in the AC, which are key for newborn maternal retrieval.

CONCLUSION

The current study showed that adult lactating rats subjected to pre- and neonatal food restriction, showed significant long-term reductions in the number and density of the dendritic branches, reduced cross-sectional area, and reduced perikaryon perimeter of AC pyramidal neurons. The findings suggest that in these dams the AC neuronal integration of different afferent patterns of coding information, and the appropriate efferent electrical discharges to other neuronal targets may be disrupted, impairing the cognitive performance of dams subject to early food restriction. The AC anatomical alterations

characterized here may help to understand the disruption of long-term cognitive processes, such has the abnormal maternal behavior and mental disorders associated with perinatal food restriction.

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