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The effect of chronic stress on prenatal development of the central nervous system

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Abstract

The survival of developing embryos depends on the control and maintenance of homeostasis. Stress caused by chronic immobilization during pregnancy in rats may alter the normal development of the nervous system and increase susceptibility to psychiatric disorders. We investigated the effects of chronic stress on cell proliferation in the forebrains of embryos at 12 days of gestation, and in the hippocampus, dentate gyrus and cortex in embryos at 17 and 21 days of gestation. We examined serial sections of the embryonic brains of control and stressed rats at days 12, 17 and 21 of gestation. Brain sections were immunolabeled with anti-PCNA and stereological analysis was performed on 540 images. The results showed no statistical differences on days 12 and 17 of gestation in the proliferation area of the structures studied, whereas on day 21 of gestation, proliferation decreased in the cortex and dentate gyrus of embryos of the stressed group. These changes were related to decreased prolactin and increased corticosterone concentrations in the plasma.

Key words: central nervous system, gestation, PCNA, prenatal stress, proliferation, rats

The development of the central nervous system (CNS) requires complex interactions of genetic and environmental factors (Wadhwa et al. 2001). If the environmental factor is detrimental, it may cause short- or long-term deleterious consequences for the health. Stress is produced by external or internal stimuli that alter homeostasis (Armario et al. 2012). Prenatal stress is an epigenetic factor that can cause changes in brain structure and function (Lemaire et al. 2000, Weinstock 2001).

The hypothalamus-pituitary-adrenal (HPA) axis is altered under stressful conditions. The paraventricular nucleus (PVN) neurons of the hypothalamus synthesize and release corticotrophin releasing hormone (CRH). The CRH stimulates adrenocorticotrophin-releasing hormone (ACTH) secretion by the pituitary. In turn, ACTH stimulates the glucocorticoids (GC) secretion by the adrenal cortex (Weinstock 2001, Smith et al. 2006). The fetal sympathetic-medullary adrenal axis releases adrenaline (A) and noradrenaline (NA) to restore the homeostasis (Mayer et al. 2011). During pregnancy, CRH is produced also by the placenta and it causes activation of the fetal HPA axis (Mulder et al. 2002). The GC in turn impacts the fetal programming of nervous tissues, which are especially sensitive (Sandman et al. 1997). It has been demonstrated that GC also can cause a decrease of cell proliferation in the dentate gyrus (Lemaire et al. 2000).

It has been demonstrated that under stress conditions in rats, morphological changes in the hippocampus, amygdala and prefrontal cortex, which are associated with learning, memory and

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emotional responses, were produced (Vyas et al. 2002). The neurobiological consequences of the stress during early stages of the development may promote behavioral and emotional problems, which may increase the risk of psychopathologies in adulthood (Gressens et al. 1992, Weinstock 2001). The prenatal stress exposition increases stress hormone production by the mother, such as prolactin (PRL), which are released in addition to GC. PRL regulates HPA axis activity by exerting an inhibitory effect (Torner et al. 2002). Because stress plays an important role in the anatomical and physiological development of the CNS during the prenatal period, we investigated the effects of prenatal stress on cell proliferation area (CPA) in fetal rat brains at 12, 17 and 21 days of gestation.

Material and methods

Animals

The *Conclusions and Recommendation on the Reduction, Refinement and Replacement of Laboratory Animals Procedure of the Declaration of Bologna* were followed for animal experimentation. All experiments were conducted according to the principles and procedures of the *NIH Guide for the Care and Use of Laboratory Animals* (NIH publication no. 85-23, revised 1985). Thirty 90 − 120-day-old 200-300 g primiparous female Wistar rats were used. Animals were maintained under controlled laboratory conditions at 20 ± 2° C and a 12/12 h light/dark cycle, and food and water were provided *ad libitum*. Rats were cycled using colpocytograms and were mated during pro-estrous with a male of the same strain. Pregnancy day 0 was defined by the presence of spermatozoa in the vaginal fluid. Pregnant rats were separated into control rats (CR) and stressed rats (SR) groups.

Experimental treatment

SR were subjected to sessions of immobilization stress in a tubular clamp made of perforated plastic. The clamp was anchored to a wood base and padded for animal comfort. Each immobilization session lasted 45 min. Rats were subjected to these sessions every other day beginning on the fourth day of gestation to prevent embryo resorption before sacrifice. The method was adapted from the National University of Rio Cuarto biotherius under ethical rules for manipulation of animals for experimentation.

The stress procedure affects the mother directly and the embryo indirectly. Chronic stress was evaluated by measuring the plasma corticosterone (CORT) levels. CR and SR were sacrificed by decapitation on days 12, 17 and 21 of gestation. Five fetuses at each gestation age were obtained randomly for each group (n = 15), the minimum number of animals required to determine the statistical significance of the sample according to data supplied by the Research Ethics Committee of the UNRC). No anesthetics were applied, because ether, ammonium compounds, barbiturates and other anesthetics can act as stressors (MacLusky et al. 1979). After sacrifice, the adrenal glands of each group at each gestational stage were obtained and weighed using a microanalytical balance.

The fetuses extracted from each pregnant rat at each gestational age were fixed in 10% buffered formaldehyde for 24 − 48 h, dehydrated and embedded in paraffin. Sections 7 μm thick of the heads of the embryos were obtained using a Reichert-Young 2065 microtome. Twelve alternating sagittal sections were obtained from each head. Sections were mounted with Vectabond (Vector Laboratories, Inc., Burlingame, CA).

PCNA immunocytochemistry

An indirect immunocytochemical method was used to detect the immunolocalization of PCNA. Three microscope slides per animal, each with six sections of the same head (90 total) were used. Histological sections were de-waxed and hydrated in phosphate buffered saline, pH 7.4 (PBS), and a 20% solution of hydrogen peroxide was used to block the endogenous peroxidases. Nonspecific antibodies were blocked using blotto (5 g powdered skim milk dissolved in 100 ml PBS + 1 ml Triton). The samples incubated with primary anti-PCNA antibody, a mouse monoclonal antibody, diluted 1:50 (Enzo Life Science, Miami, FL) overnight at 4° C, followed by incubation with a biotinylated secondary antibody and avidin-biotin-peroxidase complex (Vectastain ABC Elite Kit 6200; Vector) for 60 min at room temperature. The reaction was visualized using diaminobenzidine 3-3´ tetrahydrochloride (DAB) (Vector) for 3 min for the 12 day gestation embryos and for 10 − 15 min for the 17 and 21 days of gestation embryos. Finally, the histological sections were dehydrated, cleared and mounted. The negative control was the reaction applied to a head sections without the primary antibody; the positive control was the reaction on intestinal tissues.

Stereology analysis

Two fields (100×) for each section at 12 days of gestation and four fields (25×) for each section at 17 and 21 days of gestation were scanned to build a raw image data base for CR and SR. A Zeiss Axiophot microscope with a built-in AxioVision Zeiss digital camera was used (Carl Zeiss MicroImaging GmbH, Jena, Germany). The images were analyzed using Fiji Image J software (Rasband 2011; National Institute of Health-NIH; Java). Quantitative analysis of the proliferating structures was performed in relation to the total forebrain area. The total forebrain area was measured in fetuses at 12 days of gestation by measuring the forebrain rostrally and ventrally at the level of the nasal turbinates, caudally at the level of the median trabecula and dorsally surrounding the forebrain vesicle at the beginning of the mesencephalon. In fetuses at 17 and 21 days of gestation, the total forebrain area was measured rostrally and ventrally at the level of the nasal turbinates to the pituitary, caudally crossing the midline and dorsally surrounding the cortex The total forebrain areas were measured in the same way for the CR and SR. Data are expressed as CPA, which was obtained as the ratio of the cell proliferation area for each structure/total area ×100.

Statistical analysis

For comparison of CR and SR at day 12 of gestation, a one-way ANOVA was applied. For comparison of CR and SR at days 17 and 21 of gestation, a nonparametric test was performed (Kruskal Wallis test). In each case, a value for $p \leq 0.05$ was considered statistically significant. Statistical analyses were performed using Infostat software.

Results

Qualitative analysis

In both groups, on days 12 and 17 of gestation, the neurons showed intense and homogeneous PCNA immunostaining (Fig. 1A − D). On day 21 of gestation, the PCNA immunostaining intensity of the neurons in the hippocampus, cortex and dentate gyrus was less than that in the embryos at 17 days of gestation (Fig. 1E–F).

Quantitative analysis of CPA

No significant differences in CPA between CR and SR were observed in embryos at 12 days of gestation. In embryos at 17 days of gestation, we observed no significant differences in CPA between the CR and SR in the cortex, hippocampus or dentate gyrus (Table 1). In embryos at 21 days of gestation, we observed a significant decrease in CPA in the cortex ($p < 0.0001$) and dentate gyrus ($p = 0.01$) in SR compared to CR. No significant differences between groups were observed in the hippocampus (Table 1).

Analysis of CPA at 17 and 21 days of gestation

For both groups, we found a significant increase in CPA at 21 days of gestation compared to day 17 for the cortex ($p < 0.0001$), hippocampus ($p < 0.0001$) and dentate gyrus ($p < 0.0001$).

Adrenal weights of pregnant rats

The adrenal weights of SR showed a significant increase compared to CR on days 17 ($p = 0.02$) and 21 ($p = 0.03$) of gestation. No significant difference was observed on day 12 of gestation (Table 2).

Discussion

We demonstrated earlier (Mugnaini et al. 2006, Bozzo et al. 2011, 2013) the impact of chronic stress by immobilization on pregnant rats; however, the effects of chronic stress on neurogenesis during embryonic development is unknown. The plasma levels of CORT at the end of gestation were significantly increased under stress conditions (Romanini et al. 1999). We observed that adrenal weight at days 17 and 21 of gestation also was significantly increased in SR compared to CR.

Our immunohistochemical study showed that CPA increased gradually from day 17 to day 21 in CR; the immunostaining pattern was similar for SR. The increased CPA by day 21 of gestation may be due to increased expression of trophic factors, such as brain neurotrophin, which may increase neuron survival or decrease apoptosis, processes by which embryonic neural tissue is remodeled (Holcomb et al. 1995).

We observed decreased CPA in the dentate gyrus and cortex of SR embryos at day 21 of gestation. This decrease was closely related to decreased plasma levels of PRL (Soñez et al. 1996) and increased plasma levels of CORT in chronically stressed rats at the end of the gestation. In normal pregnancies, PRL promotes cell proliferation and inhibits cell death through Bcl-2 anti-apoptotic protein expression; however, lower plasma levels of PRL under stress

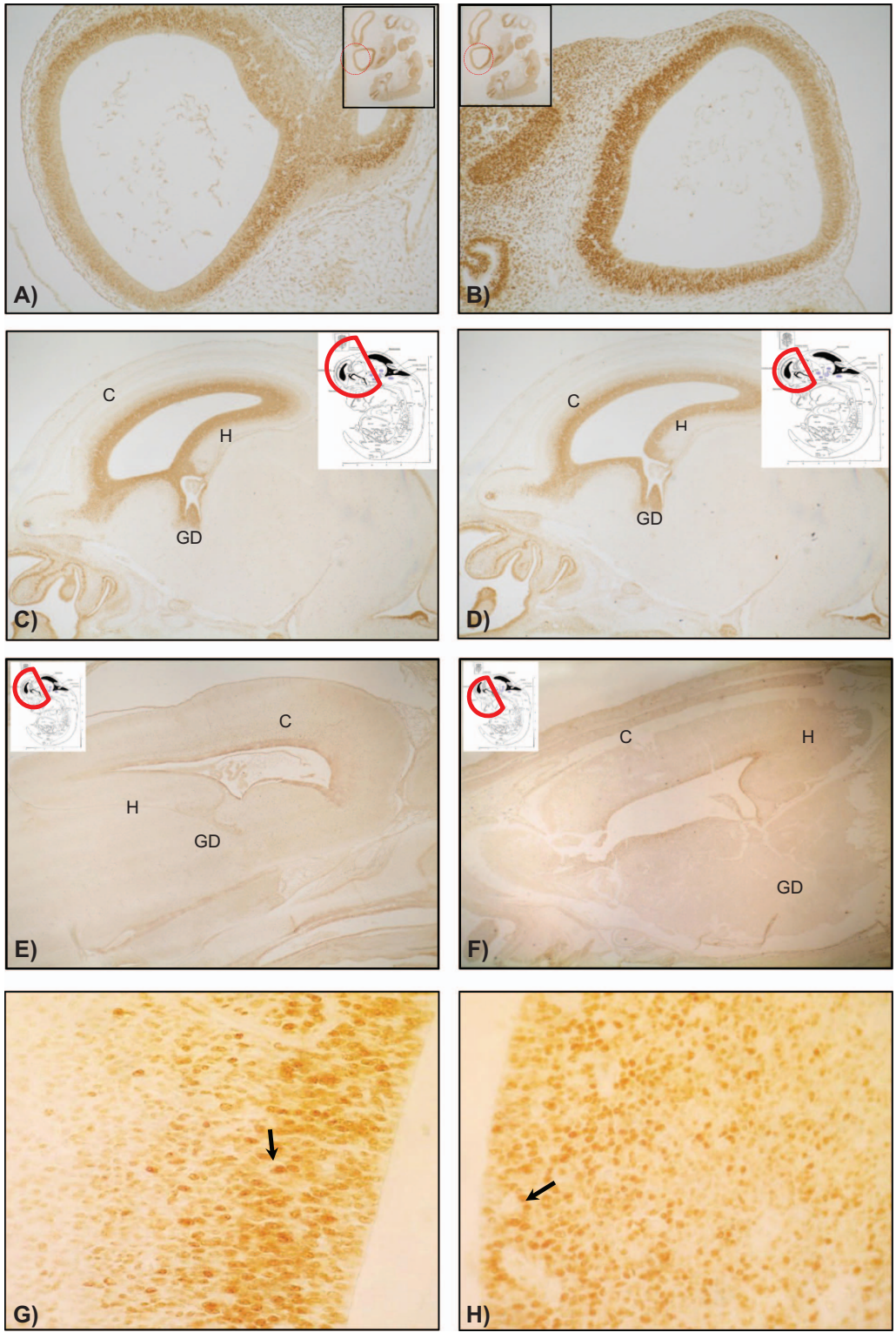

Fig. 1. A) Forebrain of a CR group embryo head at 12 days of gestation. 100 ×. B) Forebrain of an SR embryo head at 12 days of gestation. 100 ×. C) Forebrain of a CR embryo head at 17 days of gestation. 25 ×. D) Forebrain of an SR embryo head at 17 days of gestation. 25 ×. E) Forebrain of a CR embryo head at 21 days of gestation. 25 ×. F) Forebrain of an SR embryo head at 21 days of gestation. 25 ×. C, cortex; H, hippocampus; GD, dentate gyrus. Micrographs obtained with a higher magnification to indicate proliferation nuclei: G) Cortex of an embryo head of the SR of 17 days of gestation (400 ×) and H) Dentate gyrus of an embryo head of the SR of 17 days of gestation (400 ×). The arrow indicates immunolabeled nuclei with PCNA.

Table 1. CPA in the three areas of the forebrain of embryo heads at 17 and 21 days of gestation

	17 Days		21 Days	
	CR	SR	CR	SR
Cortex	6.65 ± 1.48	7.33 ± 1.48	47.77 ± 6.46	21.1 ± 5.21*
Hippocampus	2.91 ± 0.85	3.89 ± 2.01	14.77 ± 1.77	15 ± 2.96
Dentate gyrus	0.50 ± 0.58	0.37 ± 0.19	19.92 ± 7.27	11.18 ± 2.30*

Data are ± means SD.
*$p < 0.05$.

conditions could cause decreased cell proliferation (Weimann et al. 1999).

GC are required for normal embryonic development; however, overexposure could have deleterious effects depending on the time of exposure (Welberg et al. 2001). The brain is particularly sensitive to deleterious effects; the NA and GC alter neuron activity differentially in areas that play important roles in attention, vigilance, determination of appropriate behavioral strategies and long-term memory, which may cause adult behavioral disorders (Joe¨ls and Baram 2009). These behavioral alterations appear to be related to gradual changes in the morphology, electrical potential and proliferative capacity of neurons (Joe¨ls et al. 2007). The high CORT levels observed may cause decreased CPA in the nervous system by blocking gene expression, such as c-fos and c-jun, which are involved in progression of the cell cycle (Rubenstein and Beachy 1998).

Earlier reports demonstrated that the morphology of the amygdala and cortex were altered in chronically stressed rats (Wellman 2001, Vyas et al. 2002, Radley et al. 2004); this observation is consistent with our observation of decreased CPA in the embryonic cortex of stressed rats on day 21 of gestation. Van Den Hove et al. (2006) demonstrated that prenatal stress produced a 58% decrease in cell proliferation in hippocampal, olfactory bulb, cerebellum and subventricular zone tissues during early neonatal development. Chronic stress inhibits the proliferation and survival of new neurons in the hippocampus of rats (Duman 2004, Gould et al. 1997, Heine et al. 2004). We found no significant differences in the hippocampus of SR compared to controls on the gestation days that we studied, however.

Table 2. Adrenal weight in pregnant rats

Days	CR	SR
12	51.99 ± 8.86	51.52 ± 8.72
17	60.10 ± 6.80	74.03 ± 9.12*
21	65.25 ± 7.27	77.92 ± 7.64*

Data are ± means SD.
*$p < 0.05$.

Changes in the nervous system produced by stress are related to the stage of development of this system at which the stress is applied (Aizawa et al. 2004). During the prenatal and early postnatal periods, maternal environmental factors affect the ultimate development of the nervous system (Soto-Moyano et al. 1999). Changes in the environment may affect the interaction of neurons with their synaptic targets, which would become evident by altered development of the embryonic nervous system (Jolicoeur and Pirlot 1988).

Chronic stress applied during gestation has important effects on neuronal development. Mechanisms that may be involved in altering neurogenesis include the interaction of hormones and neurotransmitters released by both the mother and the embryo in response to stressful stimuli. Stress conditions could cause psychopathological behavior, affective disorders and hyperactivity (Aizawa et al. 2004). It is important to preserve a normal maternal environment during gestation to prevent psychiatric disorders in the progeny.

In the future, it would be useful to evaluate whether decreased cell proliferation in specific areas of the nervous system is related to changes in behavior and learning in adult rats that had been stressed prenatally.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

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