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Prenatal Protein Malnutrition Affects the Density of GABAergic Interneurons During Hippocampus Development in Rats

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ABSTRACT

Background: Prenatal protein malnutrition disrupts the pattern of maturation and development of the hippocampus and its neuroanatomy and increases inhibition of the granular cell layer of the *fascia dentata*. If local gamma-aminobutyric acid interneurons are partly responsible for inhibition of the hippocampus, it is reasonable to assume that there may be an increase in the gamma-aminobutyric acid cell population of prenatal protein malnutrition rats. **Objective**: This experimental study was conducted to ascertain the effects of prenatal protein malnutrition on the density of GABAergic interneurons at the *cornus ammonis* and *fascia dentata* in rats. **Methods**: Animals were investigated under two nutritional conditions: (i) prenatal protein malnutrition group fed 6% protein, and (ii) well-nourished control group fed 25% protein. Using an antibody for gamma-aminobutyric acid, immunoreactive cells (GABAergic) were assessed in the rostral-caudal direction of the dorsal hippocampus at four levels. **Results**: (i) In 30-day-old rats with prenatal malnutrition, the *fascia dentata* had an average of 27% more GABAergic cells than the control group; this higher amount was not detectable at 90 days. (ii) There was a significant 18% increase in GABAergic neurons at level 1 of the *cornus ammonis* at 90 days of age. **Conclusions**: There was an increase in the population of interneurons in the *fascia dentata* and *cornus ammonis* in prenatal protein malnutrition rats. We conclude that prenatal hypoprotein malnutrition produces changes at 30 days in the *fascia dentata*. Results suggest that prenatal malnutrition also produces a delay in the programmed chronology of gamma-aminobutyric acid interneurons. Finally, in *cornus ammonis*, at 90 days of age, prenatal protein malnutrition showed an increase only at level 1; this effect may be evidenced in the long term, despite postnatal rehabilitation. (REV INVES CLIN. 2015;67:296-303)

Key words: Cornus ammonis. Fascia dentata. Hippocampus. Prenatal protein malnutrition. GABAergic neurons.

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INTRODUCTION

Malnutrition affects functions, such as learning and memory[1-4], because it alters the development of the nervous system by damaging brain cell structures included in the substrate that controls these functions, i.e., the hippocampus.

Physiological studies have shown that rats with prenatal protein malnutrition show a delay in the establishment and maintenance of long-term potentiation[5], most likely due to an increase in the inhibition of the granular cell layer of the *fascia dentata* (FD)[3,6-8]. Similarly, high gamma aminobutyric acid (GABA)-mediated levels of inhibition have been found by electroencephalogram in prenatally malnourished rats during slow-wave sleep and inactive awakening (non-theta frequency) stages[9]. Prenatal protein malnutrition animals show an increase in activity of the inhibitory system that modulates the function of granular cells, thereby altering the stimulation of the perforant pathway[10,11].

According to anatomical, pharmacological, and physiological studies, the substrate of the intrinsic hippocampal inhibitory system is located, at least partially, at the GABAergic interneurons in the *cornus ammonis* (CA1-CA3) and the FD[12]. Other structures involved in the inhibition of granular cell activity are the extra-hippocampal supplies of GABAergic cells at the *septum medium*[13,14], noradrenergic cells at the *locus coeruleus*[15], and serotonergic cells in the median raphe[16]. To date, extra-hippocampal serotonergic or noradrenergic anatomical alterations generated by protein malnutrition have been found at the dorsal raphe and *locus coeruleus* cells[6,17].

Prenatal protein malnutrition causes an increase in inhibition of the granular cell layer of the FD. If local GABA interneurons are partly responsible for the inhibition of the hippocampus, it is reasonable to assume that there may be an increase in the GABA population of prenatal protein malnutrition rats. The present study was conducted to ascertain the effects of prenatal protein malnutrition on the density of GABAergic interneurons at the CA and FD.

MATERIALS AND METHODS

Experimental design

Wistar rats of reproductive age (80-90 days of age) were used and maintained under identical light-darkness (12:12 hours), temperature (22-24°C), and humidity (49-50%) conditions, with free access to food and water. Male rats used for mating were maintained under the same conditions.

The rats were housed in acrylic cages with five animals per cage until being treated with the appropriate experimental condition. Rats were treated according to the Guide for the Care and Use of Experimental Animals[18].

The hypoprotein malnutrition state was achieved according to the method previously described by Morgane, et al.[3] using two isocaloric diets (Teklad-Harlan, Madison, WI, USA). The sources of protein included two different amounts of casein: 25% casein in the control group, and 6% casein in the prenatal protein malnutrition group.

Prenatal protein malnutrition and postnatal rehabilitation nutrition

Fifteen female rats were fed the 6% protein diet for five weeks prior to mating to create malnourished mothers, and another 15 female rats received the 25% protein diet to form the control and nursing mother group. After the five-week nutritional diet period, female rats from both groups were mated with normally fed (conventional rodent feed) male rats. Day one of gestation was recorded, and the presence of spermatozoids was taken with a vaginal smear each morning. Based on the registered data, female rats with positive smears were separated and placed in plastic boxes (48 × 27 × 16 cm) according to their corresponding diets. During the gestation period, animals were maintained under the previously described conditions. At delivery, litters born on the same day and fed the same diet were weighed and their sex determined. Male rats from each litter were intermixed to obtain genetically heterogeneous litters. Each litter was adjusted to groups of eight pups. Rats from malnourished mothers were placed together with well-nourished nursing mothers to obtain animals with prenatal malnutrition and postnatal rehabilitation (6/25%). Animals born from well-nourished female rats (25%) were maintained under the same conditions to obtain a set of control animals (25/25%). Pups were weaned at 21 days, and rats from both groups were fed a 25% protein diet.

The effects of prenatal protein malnutrition were studied in six male rats from each group at 30 and 90 days of age and were randomly assigned to one of two groups: prenatal malnutrition or control. Each animal was

anaesthetized with sodium pentobarbital (55 mg/kg of body weight) and intracardially perfused with a cold phosphate buffer saline (PBS) 0.1 M, pH 7.4, followed by a mixture of 4% paraformaldehyde and 0.5% glutaraldehyde in the same buffer. Vibratome® serial slices (30 μm) were cut to include all the dorsal hippocampus (rostral-dorsal direction), a total of 1,320 μm.

Immunocytochemistry to detect gamma aminobutyric acid-positive interneurons

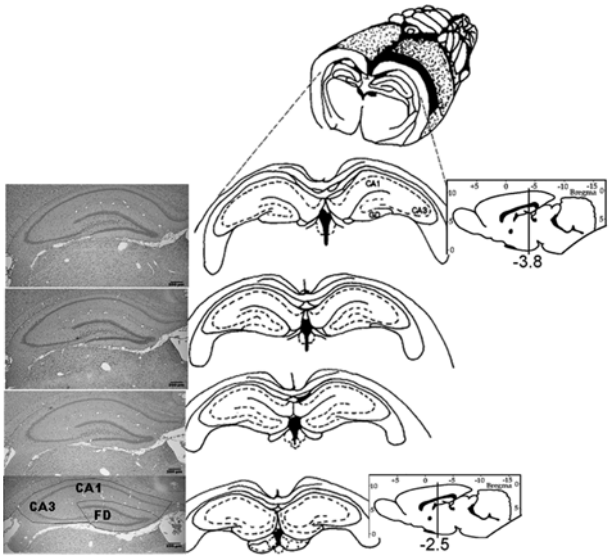
Immunocytochemistry was performed to detect the GABA-positive interneuron which coexists with other neurotransmitters and a large number of neuromodulators such as substance P, parvalbumin, somatostatin, neurotensin, and enkephalin, among others. Use of an antibody against GABA ensured the detection of the total population of GABAergic interneurons in the hippocampus.

The immunocytochemistry sections[19] matched with cut levels of bregma: 2.5-3.8 ± 0.15 mm (−2.5, 2.8, 3.3, 3.8. Fig. 1). Sections were incubated by free flotation for two hours in 10% normal goat serum (Vector Laboratories, Inc., Orton Southgate, UK), followed by incubation with a primary antibody against GABA (Incstar 1:1000) in 0.1 M PBS containing 3% normal goat serum for 48 hours at 4°C. Subsequently, the avidin-biotin-peroxidase method (ABC kit, Vector Laboratories, Inc., Orton Southgate, UK) was used, and the reaction was revealed with nickel ammonium sulphate, which intensified the chromogen 3'3'-diaminobenzidine (Sigma Aldrich, St. Louis, USA). The slices were dehydrated and flat embedded in Epoxy resin (Epon). As a control in immunochemical experiments, the primary antibody was omitted.

Quantitative analysis

Quantitative analysis was done in eight sections per animal, two sections at each level, with 50 μm between each section, to assure that the GABA-positive cell count was made in sections containing different cells; and 220 μm between sections at levels of bregma −2.5, 2.8, 3.3, 3.8 ± 0.10 mm (Fig. 1). Because, during hippocampal formation, cell populations originate at different times, and GABAergic cell distribution varies within the structure[20], cells were separately identified by dividing the structure into two regions, CA and FD.

Figure 1. Diagram showing slices of bregma −2.5 to −3.8 of the rat brain where the density of GABA-positive neurons was estimated (Paxinos and Watson, 1998). On the left are micrographs of the hippocampal slices at the same level (5×). CA: cornus ammonis.

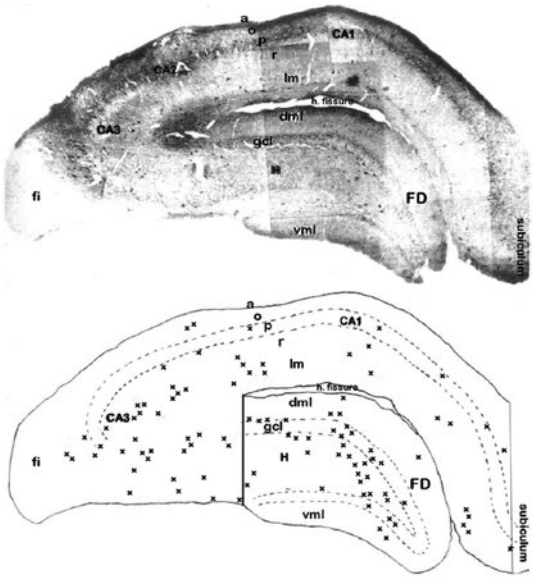


Definition of anatomical areas

The regional borders used for quantitative analysis are illustrated in figure 2. The limits of CA are: top, alveus (thin white layer that covers the CA); lateral, fimbria (fi) and subiculum; lower, hippocampal fissure (h. fissure). Cell counts were performed in the all layers of CA1, CA2 and CA3 in: stratum oriens, stratum pyramidale, stratum radiatum, and stratum lacunosum-moleculare. In FD, a structure limited by hippocampal fissure, cell counts included all layers: granular cellular layer, dorsal molecular layer, ventral molecular layer, and hilus. The hilus of FD includes the entire polymorphic cellular layer situated between the two blades of the FD, but excludes the densely packed CA3 cells that often extend into the hilus. The lateral boundary of the FD was defined by tracing a line connecting its upper and lower lips, perpendicular to the stratum pyramidale of CA3 (Fig. 2).

Quantification of GABA neurons was performed using the AxioVision 4.8.2 Rel. Program (Carl Zeiss) on a computer connected to an Axio Scope 2 Plus microscope and a digital camera (Axio Cam MRc, Zeiss). Micrographs were taken of hippocampal formation at 5× magnification; the borders of the FD and CA1-3 were outlined on the micrographs on the monitor and their areas were automatically measured. The

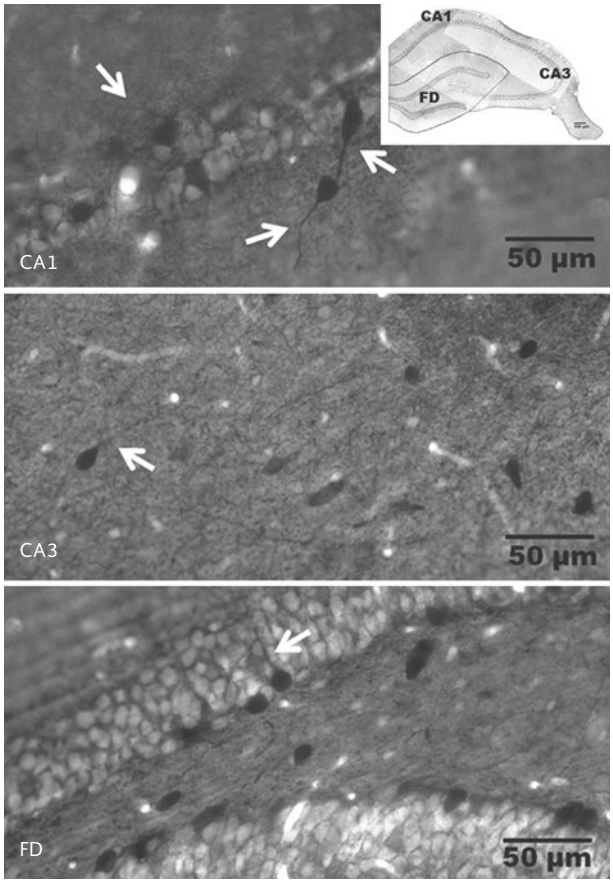
Figure 2. Hippocampal formation; shown are the regional borders used for quantitative analysis of GABA interneurons. A. Microphotographs reconstruction; B, drawing made on the photography. Limits of *cornus ammonis*: upper, alveus; lateral, fimbria and subiculum; lower, hippocampal fissure. Limits of *fascia dentate*: upper, hippocampal fissure, lateral boundary is a line connecting its upper and lower lips, perpendicular to the *stratum pyramidale* of CA3. Each × denotes a positive GABA neuron in the different layers of *cornus ammonis* and *fascia dentate*. Interrupted lines outline the layers of neurons, pyramidal in *cornus ammonis,* and granular in *fascia dentate*. CA: *cornus ammonis;* FD: *fascia dentate;* a: alveus; fi: fimbria; h fissure: hippocampal fissure; o: *stratum oriens*; p: *stratum piramidale*; r: *stratum radiatum*; lm: *stratum lacunosum-moleculare*; gcl: granular cellular layer; dml: dorsal molecular layer; vml: ventral molecular layer; H: hilus.



A

B

Figure 3. Representative microphotographs of the hippocampus showing GABA-positive interneurons in the *cornus ammonis* (CA1, CA3) and *fascia dentata*. 40×. Inset, reconstruction of the hippocampus at low magnification (10×) of different regions.



GABA-positive neurons were marked by a click of the mouse on their micrographs on the monitor at a magnification of 20×, and automatically counted.

The total number of labeled GABA neurons in both hemispheres was assessed, and the density of each area, CA and FD, was estimated. We defined the density as the total number of GABA-positive cells in an area in μm^2. The assessments were performed in a blind fashion by a well-trained person who assigned a numerical code to each.

Statistical analysis

Statistical analysis was done using Excel (Microsoft) and SPSS (V. 19). Effects of the treatments with both diets and at both ages were analyzed using a two-way analysis of variance (ANOVA). Significant differences for a diet or age, or for the interaction of the two, were determined using a two-way ANOVA followed by the Tukey *post hoc* test (program).

RESULTS

In both the control and prenatal protein malnutrition groups, the reaction to GABA was identified as a brown precipitate at the soma, the proximal dendrites, and the axon. GABAergic neurons were observed in both the CA and FD (Fig. 3). No apparent changes in morphology were found in these cells in the prenatal protein malnutrition animals or in the control group. Slices incubated without the primary antibody were immune-negative.

Effects of diet and age on the population of GABAergic interneurons

Fascia dentata

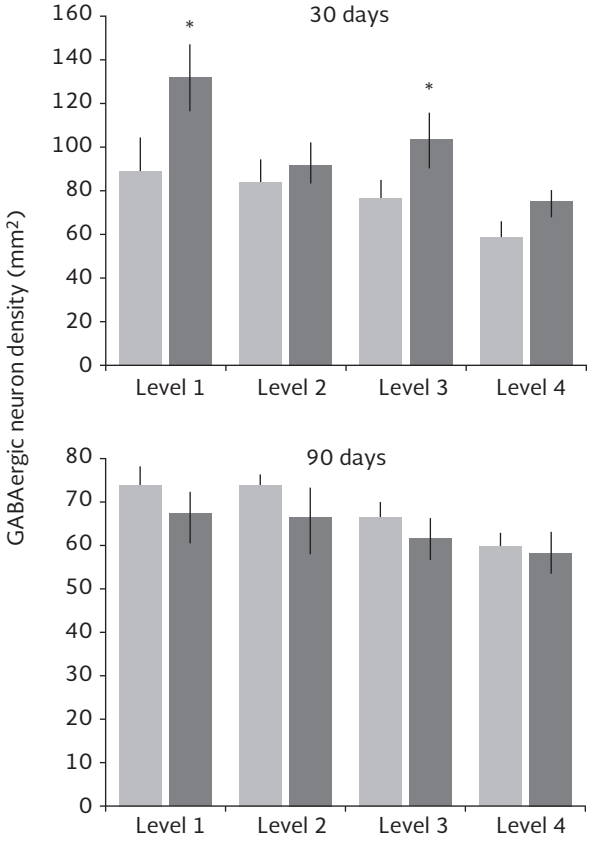
The two-way ANOVA showed a significant effect due to the difference in diets ($F = 22.47$; $p < 0.001$), a non-significant effect due to age ($F = 4.58$), and a significant effect due to interaction effects ($F = 12.35$; $p < 0.001$).

The ANOVA and the Tukey test for each hippocampal level showed that FD slices from the 30-day prenatal protein malnutrition group had a significant increase in cells in levels 1 and 3 of 47 and 35%, respectively. These increases were not found at 90 days (Fig. 4).

When the density of GABAergic interneurons in the prenatal protein malnutrition group at 30 and 90 days was compared, two significant decreases were found: 50% at level 1 (132 ± 43 to 66 ± 6 neurons) and 58% at level 3 (104 ± 31 to 60 ± 2 neurons).

In the 30-day rats with prenatal protein malnutrition, the FD had an average of 27% more GABAergic neurons (99 ± 10) than in the control group (78 ± 5). However, this higher number was not found at 90 days, although there was a specific distribution pattern of GABAergic cells along the hippocampal formation's rostral-caudal axis, particularly at levels 1 and 3.

Figure 4. The number of GABAergic interneurons (density) in rostral-dorsal levels 1-4 of *fascia dentata* (bregma –2.5, 2.8, 3.3, 3.8 mm according to Paxinos and Watson, 1998) of control (light gray bar) and prenatal protein malnutrition (dark gray bar) rats at 30 and 90 days of age (mean ± SE). Statistically significant ($p < 0.001$) differences (*) compared with control.



Cornus ammonis

The ANOVA revealed non-significant differences due to diet ($F = 1.61$) and age ($F = 0.53$), but found a significant difference due to interaction effects ($F = 5.76$; $p < 0.01$).

The prenatal protein malnutrition group did not show a significant difference at 30 days; however, a significant 18% increase in GABAergic interneurons was found at level 1 at 90 days of age (Fig. 5). The 30- and 90-day control groups showed a significant 19% decrease of GABAergic neurons at level 1.

GABAergic interneurons at different rostral-caudal fascia dentata and cornus ammonis levels

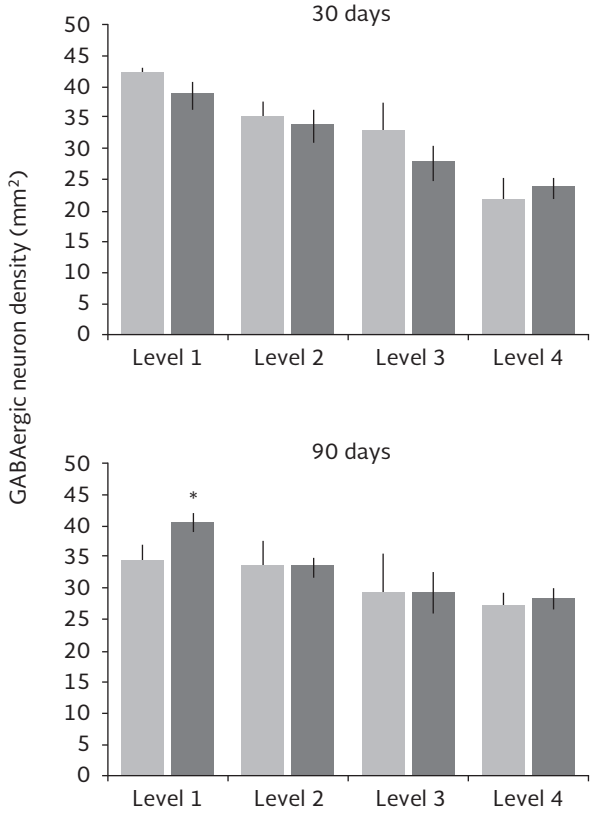
No significant difference was found between FD levels at any age (30 or 90 days) in the control group, while in the prenatal protein malnutrition group there were changes at 30 days of age in levels 1 and 3. These changes, however, were reversed at 90 days.

A different intrinsic CA population distribution of GABAergic interneurons between levels was found at 30 days, but the distribution was expressed as a non-significant decrease in cell density along the rostral-caudal axis. This distribution pattern was not modified by malnutrition and disappeared at 90 days of age. Finally, at 90 days, the prenatal protein malnutrition group showed a single increase in level 1.

DISCUSSION

Our results showed a similar process of false gain in cell population with respect to the synapse-neuron

Figure 5. The number of GABAergic interneurons (density) in rostral-dorsal levels 1-4 in CA1-CA3 (bregma –2.5, 2.8, 3.3, 3.8 mm according to Paxinos and Watson, 1998) of control (light gray bar) and prenatal protein malnutrition (dark gray bar) rats at 30 and 90 days of age (mean ± SE). Statistically significant ($p < 0.001$) differences (*) compared with control. CA: *cornus ammonis*.



proportion in prenatal protein malnutrition rats, which was previously described by Thomas, et al.[21] This event is referred to as "catch up". However, the similarity in GABA neuronal density at 90 days generates the possibility that rehabilitation at birth is important for these cells. These data contrast with those found in other studies in prenatal malnutrition rats, where different hippocampal neuronal populations of granular cells at FD and pyramidal cells in CA1 and CA3 have been studied after 90 days[1,10,22,23].

Studies have been conducted to evaluate subpopulations of GABA neurons in the hippocampus in prenatal malnutrition rats. In this context, our results differ from those reported[15], where they demonstrated that prenatal malnutrition at 90 days of age did not affect the number of parvalbumin-immunoreactive (PV-IR) GABAergic interneurons, in any region or layer of the hippocampal formation. The PV-IR is a subpopulation of total GABA neurons; they are predominantly chandelier and basket cells, constitute approximately half of the interneurons localized in the pyramidal layer, and regulate output from the pyramidal cells. There are some possible reasons for the discrepancy. First, we characterized the overall population of GABA neurons in the FD and CA, including fusiform neurons found at the edges and between granular layer cells, and polymorphic interneurons of the hilar region; these are local circuits of FD. Other researchers have used population-specific markers to quantify varieties of interneurons, including only a portion of GABA interneurons, and it should be noted that no studies have assessed the total population of GABA interneurons in the hippocampal formation. Second, the interneurons were assessed at two postpartum ages during development, and the most important change was found on postpartum day 30. Third, the quantitative analysis we used; we assessed the density of GABA immunoreactive interneurons in FD and CA, the opposite to estimating the total neuron number. This complicates direct comparison with the current findings.

Investigations using paired-pulse stimulation of the perforant pathway, a technique designed to elucidate the effects of net modulatory influences on the level of granule cell excitability, revealed significantly higher levels of inhibitory control of granule cell activity during the theta behaviors of active waking and REM sleep[24]. In whole-cell patch clamp recordings of spontaneous and of miniature inhibitory postsynaptic currents (mIPSC) generated by CA1 pyramidal cells, performed *in vitro* in hippocampal slices from control and prenatally protein malnourished adult male rats, investigators found that the frequency of mIPSCs, was significantly increased in CA1 pyramidal cells in slices prepared from prenatally malnourished compared with control rats[24]. This shows that the increase in inhibition affects the different regions of the hippocampal formation. The argument proposed to explain the increase in inhibition in CA1 is an altered ratio of PV-IR interneurons to total neuron numbers[25].

The GABA neuronal networks in the hippocampal formation are especially capable of ordering, controlling, coordinating, and synchronizing the activity of hippocampal principal cells, including FD granule cells[6]. We propose that an increase in the density of GABAergic cells may explain in part the increase in

inhibition mediated interneuronally on electrophysiological responses of granule cell in FD of prenatal protein malnutrition rats.

The similar percentage of GABAergic cells found in the FD and CA at both ages in the control group suggests that the development of GABA cells may be completed. This was not observed in the case of the prenatal protein malnutrition group, where an increase in GABAergic cell density was found in FD at 30 days, which was most likely due to a delay in the development and apoptosis of GABAergic cells. Another possible explanation is the delay in brain growth, because the measurements of the CA and FD areas are within the previously described interval[26]. We conclude that neither age or diet induced significant changes in the measurement of the area, except for a transitory and not statistically significant reduction found at the first rostral-caudal level of FD at 30 days, a reduction that recovers at 90 days. This suggests that prenatal protein malnutrition may have caused a delay in the events related to the growth of the brain and that these events were subsequently reactivated[3,6].

The affectation of brain growth only at level 1 of the FD, in addition to the specific distribution pattern of GABAergic cells along the CA's rostral-caudal axis at 30 days, are perhaps related to the gradient being determined for the rostral-caudal maturation of pyramidal cells and for the GABA-mediated synaptic inhibition[27,28]. A topographic gradient has also been found for granular and GABAergic cells[28]. Furthermore, an increase found at level 1 of the CA at 90 days may contribute to an increase in inhibition in malnourished rats. It is particularly interesting to study the vulnerability of the hippocampal function after malnutrition with regard to hippocampal function and its relationship with learning and memory. Anatomical changes correlating to memory deficiency have been found after combining pre- and postnatal malnutrition[5,29,30].

It is well-known that prenatal protein malnutrition generates differential changes in the hippocampal structure, i.e., reduction of the segment number, increased mean length of terminal segments of the granular cells, and morphological changes in their synapses[31,32]. After a review of several aspects of the way cell proliferation, migration, differentiation, and death are affected by malnutrition[6], it is important to verify whether a delay in the establishment of the GABAergic cell population also has implications on the integrity of synaptic connectivity in granular cells.

Changes in synaptic connectivity due to prenatal protein malnutrition may provide information about the way that brain development is affected by malnutrition, as it can generate attention, learning, and memory disorders. In addition, experimentally malnourished animals may serve as a brain dysfunction model for malnourished humans in that brain dysfunction associated with the abnormal development of hippocampal neurons may generate hyperactivity and learning disabilities[22,31,33]. The hyperactivity syndrome involves an increase in inhibition, thus suggesting attention problems and poor discrimination in learning performance.

From these results, we conclude that prenatal protein malnutrition produced changes in the FD at 30 days. The results suggest that prenatal protein malnutrition also produced a delay in the programmed chronology for GABA-interneurons. Finally, in the CA at 90 days of age, prenatal protein malnutrition showed a single increase at level 1, a result that may be affected in the long term despite postnatal rehabilitation.

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