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Effects of perinatal protein malnutrition and fenfluramine action on food intake and neuronal activation in the hypothalamus and raphe nuclei of neonate rats

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HIGHLIGHTS

- Protein undernutrition and serotonin-system action in neonates in P10 and P18.
- c-Fos in response to fenfluramine activation of the PVN, MnR and DR is lower.
- Undernutrition immunoreactive c-Fos increase in the hypothalamus and raphe.
- Serotonin action in neonates delayed by early protein undernutrition.

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ABSTRACT

In neonatal rats, hunger and satiety responses occur particularly via dehydration and gastric distention, respectively. The control of food intake in newborns is yet to be fully consolidated, particularly with respect to the participation of the hypothalamic nuclei and their relationship with the serotonergic pathway. Moreover, it is unclear how the environmental stressors in early life, like undernutrition, interfere in these events. Therefore, this study examined the serotonin-system's impact on food intake in rat neonates at postnatal day (P) 10 and P18 and the manner in which protein undernutrition during pregnancy and lactation interferes in this behavior. To accomplish this, Wistar rats were used, nutritionally manipulated by a diet having two protein levels, (8% and 17%) during pregnancy and lactation, to form the Control (n = 10) and Low protein groups (n = 10). At 10 and 18 postnatal days pups received an acute dose of fenfluramine (3 mg/kg) or saline (0.9% NaCl) and subjected to milk consumption testing and then perfused to obtain the brains for the analysis of cell activation of the immunoreactive c-Fos in the hypothalamic and raphe nuclei. At 10 days a reduction in weight gain was observed in both groups. On comparison of the neuronal activation for the paraventricular nucleus, an increased activation in response to fenfluramine was observed. At 18 days, the weight gain percentage differed between the groups according to the nutritional manipulation, in which the control animals had no significant change while the undernourished presented increased weight gain with the use of fenfluramine. The marking of c-Fos in response to fenfluramine in the hypothalamic and raphe nuclei revealed, an especially lower activation of the PVN, MnR and DR compared intra-group. However when evaluating the effect of undernutrition, marking activation was observed to increase in all the nuclei analyzed, in the hypothalamus and raphe. Data from this study indicate that the action of serotonin via food intake in the neonates may have been delayed by early protein undernutrition.

1. Introduction

In neonatal rats, after six days of life, the satiety response is via gastric distention and the most important food stimulus is dehydration [1,

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2]. The control mechanisms of food intake are considered rudimentary because, in the neonate, the response of this mechanism occurs by hydration status and not by the energy state [3]. On the first day of life, they are able to discriminate aversive reinforcements to food intake [4]. The perception of macronutrients is also triggered at different ages during the neonatal period. Glucose perception occurs at 12 days of life [5]; that of lipids between 15 and 18 days [6] and proteins between 13 and 18 days, post-natal [7].

The hypothalamus plays a key role in regulating the food intake and body weight, with a neural circuit formed by the projections arising from the arcuate nucleus (ARH) to the other hypothalamic nuclei like the paraventricular (PVN), dorsomedial (DMH) and ventromedial (VMH) [8,9]. The projections originating in the arcuate nucleus are developed in the postnatal period, following a temporal domain. [8,9]. The ARH projections for the dorsomedial and the ventromedial develop rapidly and are consolidated at P6, while the paraventricular have a standard initial innervation between P8 and P10 and the lateral hypothalamus (LHA) to the late mature P12 [8,9]. The adult pattern is set to P30 [10]. Milk intake gradually decreases to P18 and disappears completely in the fourth week of life [11], consistent with the maturation of the hypothalamic tract. The first serotonergic neurons in the mouse are observed between 10 and 14 days of gestation [12]. These neurons together form the raphe nuclei, positioned along the brainstem [13,14]. This region sends out projections to the different brain parts enabling the serotonergic system to participate in different behaviors [15], among them is the control of food intake, through the action of serotonin on the 5HT1B and 5HT2C receptors in the hypothalamus via stimulating satiety [16].

The first serotonergic projections to the hypothalamus arise during E16 [17]. Serotonin administered during the neonatal period appears to stimulate the suckling behavior up to the 9th day of lactation [18]. After this period the serotonin stimulates satiety favoring the transition between feeding and eating solid food [19]. Full transition from breastfeeding occurs at 30 days of postnatal life, related to the neurochemical changes, and serotonin seems to be directly involved in this event [18].

Malnutrition in the perinatal period can program the mechanisms that control the eating behavior. Collaboration of our research group recently observed an increase in the expression of the orexigenic hypothalamic peptides and reduction of the anorexigenic hypothalamic peptides, associated with high food consumption in malnourished rats [20,21]. Therefore, we observed that perinatal malnutrition causes a reduction in the hypophagia serotonin action acting on the 5-HT 1B receptors, although it does not alter the effect promoted on the 5-HT2C receptors [22]. Therefore, we attempted to evaluate the action of the serotonergic system on food intake in rat neonates in P10 and P18 and the mechanism by which the protein malnutrition during pregnancy and lactation interferes in this behavior. We hypothesized that the protein malnutrition promotes hyporesponsivity of the action of the serotonergic system on food intake in neonatal rats, thus interfering with the maturation of this system.

2. Methods

2.1. Animals

All the experiments were approved by the National Council of Animal Experimentation Control (Concea) N° process 23076.026104/2012-16. Wistar Albino rats (200–250 g body weight) were reared in the Nutrition Department of the Federal University of Pernambuco. The rats diagnosed as pregnant were transferred to individual cages and during pregnancy and lactation received isocaloric diets with different protein concentrations, forming the groups: Control (normal protein diet - 17% protein), n = 08 or Low Protein (Low protein diet - 8% protein), n = 08. The litters were culled after birth to 8 pups per mother in the ratio of 4:4 (male: female). Throughout the experiment, the animals were maintained under the standard *vivarium* conditions of temperature of 22 ± 10 °C under light/12 h dark inverted, light on at 18 h), receiving food and water ad libitum.

2.2. Experimental procedures

2.2.1. Food intake test

At 10 and 18 days the pups were separated from their mothers for a 4-h period and placed in an incubator at 33 °C. Then, voiding was performed through a soft object of friction over the genital region of the pups to promote the excretion of urine and feces. Subsequently, the animals were weighed. Shortly thereafter, the food intake procedure was done for a 30-min test [1,23]. In this test, the pups were placed in an incubator (33 °C) on a milk-soaked surface (infant formula - NESTLÉ®). The milk consumption was calculated as the difference between the weight before and after the test according to the formula [(final weight − initial weight) × 100] − 100. To test the effect of the fenfluramine on food consumption by the animals of the Low protein and Control groups, this drug was administered 30 min before testing.

2.2.2. Neuronal activity in response to the consumption of milk or fenfluramine

The stimuli for the analysis of neuronal activation were performed at 10 and 18 days of age. Sixty minutes prior to perfusion, the animals were submitted to the following conditions: injection of saline solution (0.9% NaCl, sc) and acute exposure to milk or fenfluramine injection (3 mg/kg bw) and milk. Initially, the animals received fenfluramine acute application or saline 30 min before the milk was supplied in the surface, soaked for 30 min, and then targeted for the infusion. Thus, the experimental groups were formed: Control-Saline (CS), Control-Fenfluramine (CF), Low protein-Saline (LPS), Low protein-Fenfluramine (LPF). Each group included five animals from different litters.

2.2.3. Transcardial perfusion in neonatal rats in 10 and 18 days of life

The animals were perfused after being deeply anesthetized with ketamine (Dopalen-Ceva®, Paulinia-São Paulo, Brasil, 1 ml/kg de p.c.) and xylazine (Anasedan – Ceva® Paulínia-São Paulo, Brasil, 0.10 ml/kg de p.c.), by intramuscular injection. For perfusion, the chest cavity was opened to gain access to the heart [24]. Then, a cannula was attached to a peristaltic pump (Milan® BP-600, Colombo, Paraná-PR, Brasil) and inserted into the left heart ventricle. A peristaltic pump infusion was connected at a speed compatible with that required to maintain the integrity of the blood vessels. Initially, the rats were infused with 150 ml of saline (NaCl, 0.9%) at room temperature to remove the blood inside the vessels. The saline infusion was then infused with a fixative solution (4% paraformaldehyde at pH 7.4, at 4 °C).

At the end of the passage of the fixative solution, the brains were removed from the skulls and post-fixed in the same fixative solution plus sucrose (20%) for 4 h. Next, they were stored in cryoprotectant solution (sodium phosphate buffer - PBS - over 20% sucrose) for 6–24 h.

2.2.4. The c-Fos immunohistochemistry

The cuts in the series containing the raphe and hypothalamic nuclei were processed for immunohistochemistry against the c-Fos by the DAB-peroxidase method. To achieve this, after successive washing with the sodium phosphate buffer, the sections were incubated with the primary antibody (anti-c-Fos (4) SC-52, rabbit polyclonal IgG, Santa Cruz Biotechnology) in a dilution of 1:200 for 48 h in the cold room at 4 °C. Subsequently, they were incubated in biotinylated secondary antibody (B-8895 Anti-Rabbit IgG-Biotin antibody produced in goat Sigma-Aldrich®, São Paulo-SP, Brazil) diluted in the ratio of 1:800 for 90 min in the refrigerated chamber at 4 °C. After amplification of the signal with the Avidin-Biotin Complex (Vectastain Kit P-4000, Vector Laboratories®, California, USA) with incubation for 90 min, the revelation was performed using a solution of 3,3-diaminobenzidine (Sigma-Aldrich® DAB-, São Paulo-SP, Brazil) diluted in H_2O with ammonium