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UNDERNUTRITION DURING EARLY LIFE ALTERS NEUROPEPTIDE Y DISTRIBUTION ALONG THE ARCUATE/PARAVENTRICULAR PATHWAY

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Abstract—Perinatal nutrient restriction exerts profound influences on brain development. Animals that suffer undernutrition during lactation also display impaired weight gain. Feeding behavior is mainly modulated by neural and hormonal inputs to the hypothalamus. The arcuate–paraventricular neuropeptidergic Y pathway has a prominent role in appetite regulation. The aim of this work was to study the effects of protein undernutrition during lactation on this hypothalamic pathway. We used rats from 5 to 60 postnatal (P) days whose dams were fed a 0% protein diet (PFG) or a normoprotein diet (CG) from P1 to P10. To reproduce the same amount of calorie ingested by the PFG we used an underfed group (UFG). Immunohistochemistry was performed to assess neuropeptide Y (NPY) distribution in the arcuate, periventricular and paraventricular nuclei. Our results showed a NPY immunostaining peak at P10 in all nuclei in CG animals. In UFG animals this peak was observed by P15, while, in the PFG animals only by P20. Our results suggest that the neuropeptidergic arcuate–paraventricular pathway suffered a delay in NPY distribution in undernourished animals, particularly those fed a 0% protein diet, reflecting an effect on this pathway maturation that could explain previously reported alterations on feeding behavior in these animals.

Key words: undernutrition, hypothalamus, feeding behavior, leptin.

INTRODUCTION

Fetal and neonatal growth regulation is a complex interactive process controlled by genetic, nutritional and environmental factors. The most influential factor during fetal and neonatal life limiting growth potential is probably substrate supply (Patel et al., 2000). Animals that suffer nutritional changes during lactation display an altered pattern of weight gain, which is increased in overnourished animals and decreased in undernourished ones (Vadlamudi et al., 1995). According to the nutritional programing hypothesis (Barker et al., 1989; Lucas et al., 1999), perinatal nutritional status also has profound and persistent influences on neural development and cognitive function (Morgane et al., 1993).

Weight gain and feeding are mainly modulated by neural and hormonal inputs to the hypothalamus, a structure that is subdivided into interconnecting nuclei including the arcuate (ARC), paraventricular (PVN), ventromedial (VMN) and dorsomedial nuclei (DMN) and the lateral hypothalamic area (LH). Hypothalamic pathways between these nuclei are complex and involve numerous neurotransmitter and neuropeptide systems, many of which function in parallel to either increased or decreased energy availability (Schwartz et al., 2000).

The ARC is a key nucleus in the regulation of appetite. It comprises two major neuronal subpopulations: neuropeptide Y (NPY) and Agouti-related peptide (NPY/AGRP) neurons and pro-opiomelanocortin and cocaine/amphetamine-regulated transcript (POMC/CART) neurons. Both neuronal populations make connections within the PVN by afferents that pass through the periventricular nucleus (Pe). Activation of NPY/AGRP neurons induces ingestion whereas activation of POMC/CART neurons signals satiety. Leptin has opposite effects on these groups of neurons: inhibiting NPY/AGRP and activating POMC/CART ones (Cowley et al., 2001). These two subpopulations of ARC neurons have efferent projections to numerous hypothalamic regions such as the paraventricular (PVH), dorsomedial (DMH) and lateral (LH) ones, and the development and maturation of these projections occur during the first postnatal weeks in rodents (Bouret and Simerly, 2004).

NPY is a potent orexigenic agent (Flood and Morley, 1991; Kask et al., 1998) and inhibition of its effects causes a decrease in food intake (Walter et al., 1994; Schaffhauser et al., 1997). Leptin receptors coexist with NPY mRNA in the ARC (Hakansson et al., 1996; Wang et al., 1997) and leptin binding downregulates NPY

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Abbreviations: 3V, third ventricle; AGRP, Agouti-related peptide; ANOVA, analyses of variance; ARC, arcuate nucleus; CART, cocaine/amphetamine-regulated transcript; CG, control group; DMH, dorsomedial hypothalamic region; ELISA, enzyme-linked immunosorbent assay; NADPH, nicotinamide adenine dinucleotide phosphate; NO, nitric oxide; NPY, neuropeptide Y; OpD, optical density; P, postnatal day; PBS, phosphate-buffered saline; Pe, periventricular nucleus; PFG, protein-free group; POMC, pro-opiomelanocortin; PVN, paraventricular nucleus; rANOVA, repeated measures analyses of variance; ROI, region of interest; UFG, underfed group; VAT, visceral adipose tissue.

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(Schwartz et al., 1996). NPY can also carry out its orexigenic effect by stimulating nitric oxide (NO) in PVN neurons (Morley et al., 1999, 2011; Sadler and Wilding, 2004). It has been reported that NO can have an important role in the regulation of food intake (Morley et al., 1995; Ueta et al., 1995).

Although most studies have been carried out during the prenatal period (Koski et al., 1986; Kozak et al., 2000), studies performed during the postnatal period have also shown that nutritional status causes impairments in the structure of organs whose critical developmental windows occur during weaning (Hausman et al., 1991; Lanoue and Koski, 1994). Previous studies using a protein undernutrition protocol (0% protein diet) during the first half of the lactation period in rats demonstrated that the offspring presented an altered feeding pattern, consisting in a dampened appetite, which reflected a metabolic imprinting effect on feeding behavior (Moura et al., 2002). These animals also showed a delay in NOS presence/distribution in the PVN and VMH, which may have affected the development of the hypothalamic circuitry, leading to metabolic imprinting (Marcelino et al., 2004).

Taking into account that foodstuffs with high protein content are the most expensive ones and that, as a consequence, protein is a nutritional factor that is commonly reduced or nearly absent in a poor family's diet, it would be interesting to assess the effects on the offspring of a severe protein restriction in the diet of the nursing dams. This work is the first one to study the ARC–PVN NPY system during hypothalamic nuclei development using this postnatal undernourishment protocol. Impairment in the maturation of this system may contribute to changes in the feeding behavior previously observed in this model.

EXPERIMENTAL PROCEDURES

Animals

All studies were conducted in accordance with the principles and procedures approved by the university's Animal Care Committee (CEA/055/2009-UERJ), and National Institutes of Health Guide for the Care and Use of Laboratory Animals. Pregnant dams (Wistar rats) were housed in individual cages at 23 °C on a 12-h light/dark cycle. Filtered water was always available in the housing cages throughout the experiment. The pregnant dams were fed *ad libitum* with a normoprotein diet during gestation. The normoprotein diet consisted of 72.6% carbohydrate, 22.0% protein and 5.4% fat (% total kcals) and contained 4.21 kcal/g. After delivery, all litters were culled to six male pups so as to maximize lactation performance (Fischbeck and Rasmussen, 1987). Litters with less than six male pups were not used. The anogenital distance was used to differentiate females from males and all experimental animals were accurately sexed.

Animals were distributed into three groups: control group (CG), underfed group (UFG) and protein-free group (PFG). The diet protocols were implemented 5 h after parturition. PFG dams received the 0% protein diet while CG dams received the 22% protein diet. The protein-free diet was prepared in our laboratory and consisted of 88.9% carbohydrate, 0% protein, and 11.1% fat (% total kcals) and contained 4.05 kcal/g. The UFG was given the 22% protein diet limited to the same caloric amount as the PFG dams. Diet composition is depicted in Table 1. The amount of chow provided to UFG animals was selected based on a previous study using this animal model (Marcelino et al., 2004) and in the quantification of calories ingested by PFG animals. After 11:00 a.m. of the 10th lactation day, all dams received the normoprotein diet. At weaning (P21), pups were separated from their progenitors and continued to be fed a normoprotein diet. All animals were fed *ad libitum* and the diets were supplemented with vitamins and minerals following the recommendations of the American Institute of Rodent Nutrition Diet (Reeves et al., 1993).

Table 1. Composition of control and protein-free diets

	Control diet[a]	Protein-free diet[b]
Ingredients (g/kg)		
Soybean + wheat	220	0
Corn starch	744	1006
Soybean oil	24.5	50
Vitamin mixture[c]	4	4
Mineral mixture[c]	40	40
Macronutrient composition (g/kg)		
Protein	22	0
Carbohydrate	72.6	89
Fat	5.4	11
Total energy (KJ/kg)	17680	17000

[a] Standard diet for rodents.
[b] The protein-free diet was prepared using the control diet and replacing the protein by corn starch (Moura et al., 1997).
[c] Vitamin and mineral mixtures were formulated according to the American Institute of Nutrition ATN-93G recommendation for rodent diets (Reeves et al., 1993).

Body mass, visceral adipose tissue (VAT) mass and food consumption

Dams from all groups were weighed just after delivery (P0) and at the last day of the diet (P10). Mothers' food consumption was also calculated by weighing pellet chows every day. Animals were weighed every other day during the first 10 days of lactation beginning at P0, and then at P15, P20, P30, P45, P60 and P90. From P0 to P8, litter averages (4 per group) at each day were used as data points. VAT mass was removed from rats of all three groups, ranging from P45 to P90, under sodium pentobarbital (50 mg/kg) anesthesia. Fat composition was measured by weighing retroperitoneal, mesenteric and epididymal fat.

Immunohistochemistry

Offspring immunohistochemistry was carried out at the following postnatal (P) days: P5, P10, P15, P20, P30, P45 and P60. At least 6 animals per age per group were used. Only a limited number of animals (n = 1–3)