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Hypothalamus integrity and appetite regulation in low birth weight rats reared artificially on a high-protein milk formula☆

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Abstract

High-protein (HP) milk formulas are routinely used in infants born with a low birth weight (LBW) to enhance growth and ensure a better verbal IQ development. Indirect evidence points to a link between an HP intake during early life and the prevalence of obesity in later life. We hypothesized that HP milk supplementation to LBW pups during early postnatal life would impact hypothalamic appetite neuronal pathways development with consequences, at adulthood, on energy homeostasis regulation. Rat pups born with a LBW were equipped with gastrostomy tubes on the fifth day of life. They received a milk formula with either normal protein (NP, 8.7 g protein/dl) or high protein content (HP; 13.0 g protein/dl) and were subsequently weaned to a standard, solid diet at postnatal day 21. Rats that had been fed HP content milk gained more weight at adulthood associated with an increase of plasma insulin, leptin and triglycerides concentrations compared to NP rats. Screening performed on hypothalamus in development from the two groups of rats identified higher gene expression for cell proliferation and neurotrophin markers in HP rats. Despite these molecular differences, appetite neuronal projections emanating from the arcuate nucleus did not differ between the groups. Concerning feeding behavior at adulthood, rats that had been fed HP or NP milk exhibited differences in the satiety period, resting postprandial duration and nocturnal meal pattern. The consequences of HP milk supplementation after LBW will be discussed in regard to neural development and metabolic anomalies.

Keywords: Hypothalamus; Feeding behavior; Artificial rearing; Low birth weight; Protein; Intrauterine growth restriction

1. Introduction

Approximately 10% of human infants are born with a low birth weight (LBW) due to intrauterine growth restriction (IUGR), which usually results from placental insufficiency through a down-regulation of maternal–fetal amino acid transport [1]. Infants born with IUGR are routinely fed enriched formulas in which protein content reaches 160% to 190% of the normal protein (NP) content in human milk (e.g., 2.0–2.3 vs. 1.0–1.2 g/100 ml in human milk) so as to ensure catch-up growth. This practice ensures a better verbal IQ development at 7 1/2 to 8 years of children born preterm [2], but recent studies suggest that an accelerated growth velocity in early life may increase the risk of developing metabolic disorders like type 2 diabetes [3], cardiovascular disease [4] and obesity [5] in adult life. This is reported as the thrifty phenotype hypothesis defined as an adverse effect of poor fetal nutrition and infant growth on adult health [6]. An adverse fetal environment may "program" the fetus to select an appropriate trajectory of growth in response to environmental cues. This pivotal observation led to the concept of fetal metabolic programming. In this paradigm, the supply of specific nutrients (or lack of them) during a short, early "time window" either before or after birth may permanently alter the function or structure of tissues or metabolic pathways.

Energy homeostasis is centrally regulated by hypothalamus, which develops during pre- and postnatal period in rodents [7]. Hypothalamus nuclei are mainly organized during embryonic days through cell proliferation, migration and cell adhesion steps, whereas neural projections and synaptogenesis occur during postnatal periods [7,8]. The establishment of the neuronal pathways involved in appetite regulation and energy homeostasis takes place during postnatal period [9] and is, in part, under the control of neurotrophic factors such as leptin, insulin, Bdnf (brain-derived neurotrophic factor) or growth factors (insulin-like growth factor 1). Leptin is

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thought to be a key neurotrophic factor during the postnatal period [10] as it facilitates the growth of arcuate nucleus (ARC) neurons projections to paraventricular nucleus (PVN), dorsomedian nucleus (DMD) and lateral hypothalamic area (LHA) [10]. Malnutrition leads to malformation of hypothalamus nuclei [11], reduction of the postnatal leptin surge, and reduction of α-melanocyte-stimulating hormone [α-MSH, anorexigenic peptide derived from proopiomelanocortin (POMC) cleavage] fiber density in PVN at weaning such as we and others demonstrate [12,13]. A causal link with food behavior disorders at adulthood is therefore suspected [14–16].

In addition to the effect on brain development, a large body of literature presents alteration of development of peripheral organs implicated in energy regulation such as the endocrine pancreas [17], adipose tissue [18], liver or gut [19].

Since we previously demonstrated alterations in feeding behavior observed in adult rats born with IUGR [16], it remained to establish what part of the effect was due to restricted nutrient supply in utero or to the excess nutrient supply administered in early life. We therefore hypothesized that, in a rodent model of IUGR, a high level of protein present in milk could have harmful consequences on the development of hypothalamus and impact feeding behavior and energy homeostasis. Artificial rearing of rat neonates through a gastrostomy was previously used by Patel et al. [20–22] and des Robert et al. [23] to test the effects of various milk nutrient content on rat pups born with a normal birth weight. Yet, we adapted this technique to IUGR rats since the potential effect of high-protein (HP) feeding in early life would be particularly relevant in infants born with a LBW. We first examined metabolic parameters at adulthood and then focused our description study on brain development, cerebral response to leptin and feeding behavior. We compared, 12 days after birth, the effect of the amount of proteins provided in milk on the hypothalamic expression of 45 genes involved in brain development. We then estimated by immunodetection the orexigenic and anorexigenic ARC projections to the PVN, dorsomedial and ventromedial hypothalamus and LHA implicated in the control of food intake and the ability of ARC neurons to respond to neurotrophic factors such as leptin during the postnatal period. We also tested, by fine meal pattern and satiety sequence analysis, the possible consequences on the feeding behavior of adult rats.

2. Materials and methods

2.1. Animals

Sprague–Dawley rats (Janvier, Le Genest-Saint-Isle, France), 8 weeks old, were maintained under controlled conditions (22°C, 12:12-h dark/light cycle) with free access to food (A04, SAFE, Augy, France) and water. After 10 days of habituation, virgin female rats (10 weeks old and weighted between 250 and 300 g) were housed overnight with a male. After confirmation of mating by visualization of spermatozoa in vaginal smears, dams were placed individually and were fed a low-protein diet (8% of protein, $n=15$) purchased from Arie Block (Woerden, the Netherlands) (Table 1), which leads to the delivery of LBW pups as described previously [16,19]. At delivery, litter size was adjusted to eight male pups per dam. Pups were pooled from the 15 litters and randomly attributed to foster mothers fed a low-protein diet. At postnatal day 5 (PND5), rat pups were anesthetized with isoflurane and an intragastric cannula was implanted percutaneously by a nonsurgical technique to create an artificial nutrition model, "the pup in the cup" model (Fig. 1A), which was previously described in our laboratory [24]. Briefly, after cannula implantation, pups were randomly assigned to two groups: a NP group (NP milk intake, $n=35$) and HP group (HP milk intake, $n=34$). The daily amount of milk formula to be infused was determined according to the pups' age and weight (from 1.9 ml/day at PND5 to 11.2 ml/day at PND20) (Fig. 1B). Pups were stimulated five times per day for 1 to 2 min with a wet cotton swab on the anogenital region to cause urination and defecation and gentle stroke on the head and the body with a warm wet paintbrush to reduce behavioral and brain development alterations [25,26]. On PND21, rat pups were weaned to standard chow (A04, SAFE, Augy, France, 290 kcal/100 g with 16.1 g of proteins/100 g of diet, 3.1 g of lipids/100 g of diet and 60 g of carbohydrates/100 g of diet) and individually housed (Fig. 1A). All experiments were performed in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC), regarding the care and use of animals for experimental procedures.

Table 1
Energy and nutrient composition of experimental diets

	8% protein	P14
Energy (kcal/100 g)	367.40	356.10
Protein and amino acid[a]		
Casein	9.00	–
Methionine	0.08	–
Total milk protein	–	14.00
Digestible carbohydrate[a]		
Dextrose	68.17	–
Cornstarch	8.00	62.24
Sucrose	–	10.03
Fat[a]		
Soya oil	4.30	4.00
Fiber[a]		
Cellulose	5.00	5.00
Vitamin and mineral mix[a]	5.05	4.50
Choline[a]	0.40	0.23

[a] Values are in grams per 100 g of diet.

2.2. Milk formulas preparation

The rat milk substitute with NP contained 8.7 g/dl protein (casein/whey protein ratio: 37/63), 11.9 g/dl fat and 3.3 g lactose/dl and was prepared from powdered rat milk replacer (Wombaroo Food Products, Glen Osmond, Australia). In HP milk, protein content was increased by 50% (13.1 g/dl), with a preserved casein/whey protein ratio (37/63) and nonprotein energy not significantly higher (fat, 12.1 g/dl; lactose, 3.5 g/dl). The supplementation was done with calcium caseinate (Protilight IP3®, Armor Protéines, Saint-Brice-en-Coglès, France), whey protein (Protarmor 800Li®, Armor Protéines) and five free amino acids (L-arginine, glycine, L-histidine, L-phenylalanine, L-serine) to reach a 50% increase of all amino acids (Table 2).

2.3. Gene expression by TaqMan low-density array

RNA was isolated from snap-frozen hypothalamus, reverse transcribed and amplified by real-time polymerase chain reaction (PCR) using a TLDA (TaqMan low-density array) array as recently described [13]. The accession number of each gene was summarized in Supplemental Table 1.

The TLDA uses TaqMan real-time PCR-based technology with eight assays run in parallel for 48 genes and requires a low volume for each assay. Forty-four genes set and

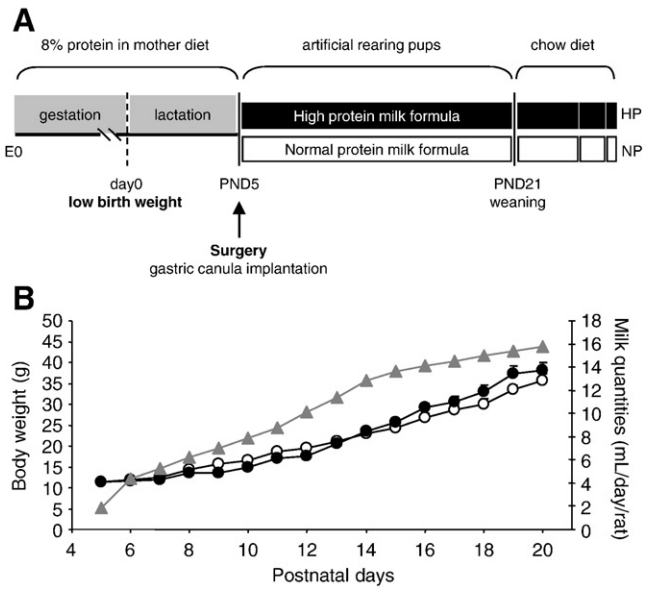

Fig. 1. Experimental model of artificial rearing pups after IUGR (A). Low birth weight pups were obtained by maternal protein restriction during gestation. At PND5, pups were gastrostomized and were nourished by enteral route with high or NP milk formula until weaning to create two groups of rats: HP rats and NP rats, respectively. Effects of HP (black circles) milk vs. NP (white circles) milk feeding during neonatal period on body weight from PND5–20 (B). Milk quantity was determined according to the pups' age and weight (grey triangles).

Table 2
Milk composition

	NP	HP
Energy (kcal/100 ml)	155.00	172.60
Protein[a] ratio (casein/whey protein)=1.7	8.70 (3.20/5.50)	13.10[b] (4.82/8.28)
Fat[a]	11.90	12.10
Lactose[a]	3.30	3.50

[a] Values are in grams per 100 ml of milk.
[b] Supplementation with calcium caseinate, whey protein and five free amino acids (L-arginine, glycine, L-histidine, L-phenylalanine, L-serine).

four housekeeping genes were studied (18S, Gapdh, Polr2a and Ppia). After extraction and quantification as described above, cDNA was synthesized from 1 μg of total RNA using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA). Real-time PCR was carried out using Applied Biosystems TaqMan reagents and run on ABI Prism 7900HT Sequence Detection System (Applied Biosytems). Data were collected with Applied Biosystems SDS 2.1 software and analyzed with RQ Manager 1.2 software (Applied Biosystems). Relative expression of each gene was determined using the comparative $2^{-\Delta\Delta Ct}$ method [27]. Each sample was normalized to 18S and Polr2a since their expressions were not influenced either by age or by pup growth status. The mean of 18S and Polr2a expression was used as calibrator.

2.4. Tissue and blood collection

Rats were rapidly euthanized between 9:00 and 11:00 a.m. by CO_2 inhalation. Blood was collected in heparinized tubes (Laboratoires Léo, Saint-Quentin-en-Yvelines, France) and centrifuged at 2500 g for 15 min at 4°C. The hypothalamus was dissected out (according to Paxino's atlas coordinates: −1.0 to −4.5 mm from Bregma and 3 mm in depth [28]) on ice tray, snapped frozen in liquid nitrogen and stored at −80°C.

2.5. Immunohistochemistry

pSTAT3-immunoreactivity (IR) was performed on hypothalamus dissected from 12-day-old male rats after a single intraperitoneal (ip) injection of recombinant rat leptin (10 mg/kg body weight), n=5 (PreproTech by tebu-bio, Le Perray-en-Yvelines, France) or saline solution (NaCl 0.9%), n=5. After injection, pups were replaced in their cup and were deeply anesthetized 90 min later by isoflurane inhalation. Transcardiac perfusions were performed with first NaCl 0.9% followed by PFA (paraformaldehyde) 4%. Brains were removed, postfixed and frozen. Twenty-micrometer coronal sections were realized between −0.60 and −4.08 mm of Bregma coordinates, according to Paxino's atlas [28], with a HM560 cryotome (MM, Francheville, France), collected in six series and stored at −20°C under dry atmosphere until further use. pSTAT3-IR was performed as described previously [29] using the rabbit anti-pSTAT3 antibody from Cell Signaling (Ozyme, Saint-Quentin-en-Yvelines, France) and a biotinylated goat anti-rabbit secondary from Jackson (AbCys, Paris, France). The peroxidase reaction product was revealed by the avidin–biotin peroxidase complex and the glucose oxidase–DAB(3,3'-diaminobenzidine)–nickel method [30].

AgRP and α-MSH-IR were performed at PND70 (after 12 h of fast) on sections using rabbit anti-AgRP (1/1000; Phoenix Pharmaceuticals, Karlsruhe, Germany) and sheep anti-α-MSH (1/2000; Chemicon, Millipore, France) antibodies, which were incubated 48 h at 4°C. AgRP and α-MSH-IR were revealed, respectively, by incubation with a goat anti-rabbit Alexa 568 (1/500) and a donkey anti-sheep Alexa 488 antibody (1/500) (for both: Molecular Probes, Invitrogen, Cergy-Pontoise). Sections were coverslipped with glycerol/PB (phosphate buffer) 0.1 M (v/v) and antifade kit (Fluoprobes, Interchim, Montluçon, France).

2.6. Cell count and fibers estimation

pSTAT3-IR was quantified on five animals per group and per treatment after observation under a Nikon eclipse E400 microscope. The images captured by a Nikon digital camera DXM1200F were then quantified using the Nikon Imaging Software element 2.3. All pSTAT3-positive cells were counted in ARC between Bregma −1.72 and −3.60 mm coordinates according to Paxino's atlas [28]. A mean value was obtained for each animal from at least three representative sections of ARC.

AgRP and α-MSH fiber density was estimated on PVN, DMD and perifornical LHA of five animals per group using a Zeiss Axiovert 200 microscope with Apotome module (Carl Zeiss, Le Pecq, France) equipped with a ×20 objective, which took seven virtual optical sections in the three dimensions throughout each hypothalamic structure. Image analysis was performed using the free ImageJ software. Briefly, a stack of images was binarized to isolate labeled fibers from background. A threshold was defined to recover labeled fibers and was maintained between all images from the two experimental groups. The value obtained per rat corresponded to the mean area of labeled fibers estimated in two or three representative sections of each structure.

2.7. Mesenteric fat

Mesenteric fat pads were collected with great care at sacrifice (PND160), fixed in 4% phosphate-buffered formalin and paraffin embedded. Sections (5 μm) were stained with hematoxylin–eosin and examined at ×4 magnification using an Eclipse E400 NIKON light microscope equipped with a video camera (digital camera DXM 1200F). Adipocyte number was normalized to a mean section area of 1 mm^2. Cells (n=1500–2000 cells/section) were counted from a section in entirety for each mesenteric fat tissue per rat. Results were expressed as number of cells/mm^2. The surface area of adipocytes (n=1100–1400) was measured from one to three randomly selected fields in sections examined at ×10 magnification using Image J free software (1.40 g, NIH [National Institutes of Health]) and expressed in μm^2.

2.8. Biochemical analysis

Plasma leptin and insulin concentrations were determined with specific ELISA kits following the manufacturer's instructions for insulin (Rat/Mouse Insulin Elisa kit; LINCO Research, St. Charles, MO, USA) and leptin (Rat/Mouse Leptin Elisa kit, LINCO Research). Glucose and triglycerides were measured by using colorimetric enzymatic reactions with specific kits (glucose and triglycerides PAP 150 kits; BioMérieux, Marcy-l'Etoile, France).

2.9. Behavioral satiety sequence

The analysis of the behavioral satiety sequence (BSS) was performed as described by Halford et al. [31] in animal home cage where habituation trial had been performed. To promote feeding in young rats (PND50), we removed food for 3.5 h before the presentation of regular chow. The observation period lasted 50 min. To analyze feeding and other types of behavior, two trained observers who were unaware of the nutritional status of the animals videotaped animals and scored films. Quantified activities were feeding, grooming and resting. The test period was divided into ten 5-min time bins, which allowed to determine meal duration, feeding rate, grooming and resting time, as well as the time of occurrence of satiety.

2.10. Meal pattern analysis

At PND50, seven NP and seven HP rats were housed individually under temperature-controlled conditions (22±2°C). They were maintained under an artificial 12:12-h light/dark cycle (lights on at 5:00 a.m.) and had free access to food, a moistened (powered diet: water, 1:1) P14 diet (AIN93M-modified diet, see reference and Table 1 for details [32]) for a preliminary habituation period of 10 days. After the habituation period, the rats were then housed for 2 days in cylindrical Plexiglas cages equipped to monitor meal patterns by means of continuous weighing of the food cups. Meal pattern recording started the second day (after 1 day of acclimatization to the cage) at 5:00 p.m. and ended at 4:00 p.m. on the subsequent day and was recorded each 5 s during 23 h. Food containers were refilled daily at 9:00 a.m. with fresh food. The cumulative ingested quantity (g) was converted to the metabolizable energy ingested (kJ) during the 23 h of food presentation using the conversion factor (14.6 kJ/g). The exact feeding pattern was defined as follows: a meal needed to be larger than 0.1 g and longer than 10 s. Two distinct meals needed to be separated by an interval >10 min [33]. With these filters, parameters characterizing feeding pattern (meal size, kJ; meal duration, min; and ingestion rate, kJ/min) were obtained. The meal number was also determined as well as cumulative energy intake (kJ) and average intermeal interval (IMI). All of these parameters were studied during the 23 h of food presentation and were analyzed separately during the dark and the light phases.

2.11. Statistical analyses

Analyses were performed using Statview 5.0 (SAS Institute, Cary, NY). Differences among groups were determined by Mann–Whitney test and presented as mean±S.E.M. In all tests, $P \leq .05$ was considered significant.

3. Results

3.1. Body weight, growth and metabolic parameters

The body weight of NP- and HP-fed male pups did not differ at PND12, PND18 (Fig. 1B) and PND70, but an increase of HP rats body weight was observed at PND160 (Table 3). A significant increase of plasma leptin, triglycerides and insulin levels without change in plasma glucose concentration were measured in 160-day-old HP rats (Table 4).

Table 3
Body weight (g) of males NP and HP pups

	PND12	PND18	PND70	PND160
NP	17.8±0.5	28.4±1.0	325.0±4.6	534.8±13.8
HP	18.7±0.4	30.4±1.0	325.3±10.2	575.8±15.1 *

Values are means±S.E.M., n=7 to 14 per group.
* P<.05.

3.2. Adipose tissue

At PND160, mesenteric fat mass was increased in HP group, whether expressed as absolute weight or as a fraction of body weight correlated with plasma leptin (data not shown). Comparison of the adipocyte surface distribution confirmed a significant difference between the two groups (χ^2=57.90, P<.0001), with a significantly larger number of large adipocytes in HP group (Supplemental Fig. 1).

3.3. Differential expression of neurodevelopmental factors in the hypothalamus

At PND12, the expression of four genes out of the 42 detectable genes on the TLDA was significantly different between NP and HP rats and represented proteins implicated in cell cycle proliferation (Cdkn1b), neurogenesis (Pcna) and neurotrophins (Bdnf and Igf1). These four genes were overexpressed in HP rats compared to NP and increased by 30% for Bdnf (P<.05), Cdkn1b (P<.05) and Pcna (P<.05) and by 100% for Igf1 (P<.01) (Fig. 2).

3.4. Immunoreactivity of AgRP and α-MSH containing fibers in the PVN, DMD and LHA

AgRP and α-MSH-IR detection were performed at PND70 in the two groups of rats and were analyzed in PVN (Fig. 3), DMD and perifornical LHA (Supplemental Fig. 2). No difference of intensity of IR was found between the two groups.

3.5. Hormone levels during postnatal development

Plasma leptin levels were measured at PND12 and 18 (Fig. 4A). A significant increase of plasma leptin concentration was observed between PND12 (1.59±0.40 ng/ml) and PND18 (3.86±0.79 ng/ml, P<.05) for rats of the NP group but not for HP rats (PND12, 3.34 ±1.31 ng/ml, vs. PND18, 3.72±0.81 ng/ml, NS [non significant]).

No significant rise in plasma insulin levels was measured at PND12 and 18 in either group even though a trend toward higher insulin was noticed in HP group (P=.06) (Fig. 4B). No difference was observed between NP and HP rats at PND12 (NP, 0.50±0.04 ng/ml, vs. HP, 0.75±0.17 ng/ml, NS). At PND18, plasma insulin levels was higher in HP rats (1.18±0.15 ng/ml) when compared to NP rats (0.83±0.26 ng/ml, P<.05).

Table 4
Metabolic parameters of males NP and HP pups at PND160

	NP	HP
Leptin (ng/ml)	5.55±1.18	9.68±1.04 *
Triglycerides (g/L)	0.73±0.09	0.95±0.08 *
Insulin (pM)	54.63±5.59	121.93±13.85 *
Glucose (mmol/L)	9.77±1.15	9.72±0.96

Values are means±S.E.M., n=7 per group.
* P<.05.

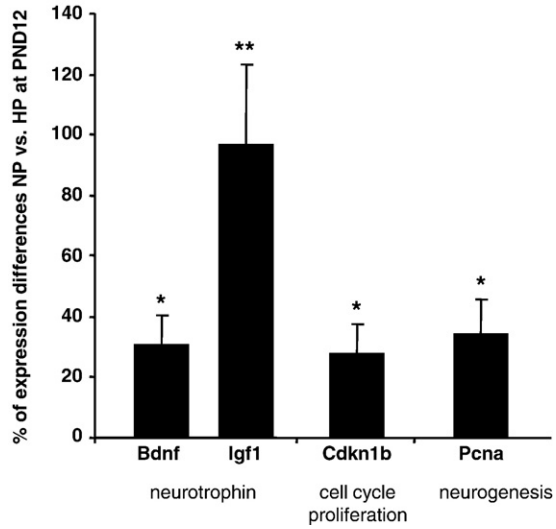

Fig. 2. Percentage of expression difference for each significant gene at PND12 for HP vs. NP rats. Values are means, n=5 per group. *P<.05, **P<.01: HP vs. NP rats.

3.6. Hypothalamic pSTAT3-IR after leptin challenge

The number of pSTAT3-positive cells detected in ARC of PND12 NP and HP rats was significantly increased in response to an ip injection

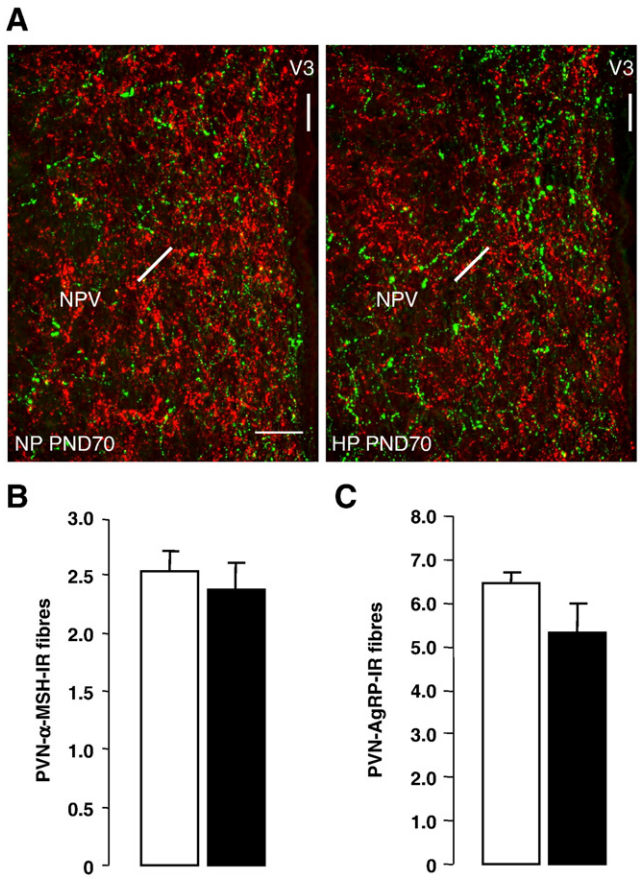

Fig. 3. Photomicrographs of α-MSH-IR (green) and AgRP-IR (red) fibers innervating PVN at PND70. (A) Fibers were shown at PND70 in PVN. Scale bar=100 μm. Fibers density of the two groups: NP (open bars) and HP (closed bars) were evaluated in the PVN for (B) α-MSH and (C) AgRP. Values are means±S.E.M., n=5 per group.

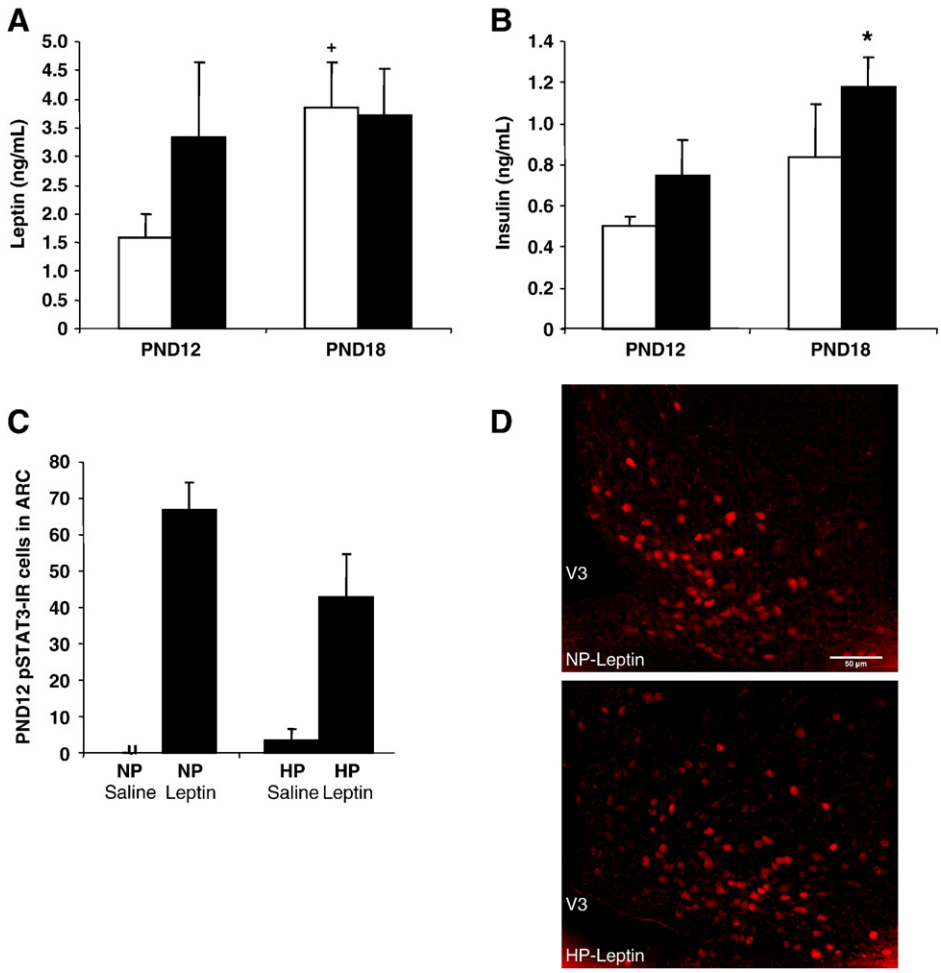

Fig. 4. Plasma (A) leptin and (B) insulin concentrations in NP and HP at PND12 and 18. (C) pSTAT3-IR-positive cells evaluated 90 min after ip saline or leptin (10 mg/kg) injections at PND12 in ARC. (D) pSTAT3-IR-positive cells evaluated 90 min after ip saline or leptin (10 mg/kg) were represented at PND12 in ARC. Scale bar=50 µm. Normal protein group (open bars) and HP group (solid bars). Values are means±S.E.M., n=5/6 per group. *P<.05, NP vs. HP. ^{+}P<.05, PND12 vs. PND18. u: undetectable.

of leptin when compared to saline treatment. However, no differences were observed between NP and HP rats (Fig. 4C, D).

3.7. Behavioral satiety sequence

BSS monitored over a period of 50 min with rats aged 50 days. As shown in Fig. 5, only HP group exhibited a typical BSS characterized by an initial phase of eating followed by an active phase of grooming, and ending with a phase of resting. Even though feeding time did not differ between NP (14.7±3.2 min) and HP (13.1±2.3 min) rats, HP rats consumed during the first five 5-min period, whereas NP rats ate until the end of the experiment. Additionally, the resting time was largely decreased in NP rats (NP, 1.6±0.6, vs. HP, 11.0±0.4 min, P<.05). A clear satiety state occurred around the sixth period for HP rats but was difficult to delineate for the NP group. Meal size was not significantly different between the two groups of rats (NP, 5.4±0.4 g, vs. HP, 9±1.6 g, P=.08), nor the speed of ingestion (NP, 0.5±0.1 g/min, vs. HP, 0.8±0.2 g/min, NS).

3.8. Meal pattern analysis

Cumulative energy intake, parameters of the feeding pattern during the 23 h of food presentation and their evolution between the light and dark phases are presented in Fig. 6. The 23-h energy intake (NP, 614.51±46.54 kJ, vs. HP, 671.34±54.07, kJ) did not differ between NP and HP rats and was also similar during the light and dark phases (Fig. 6A). Intermeal interval did not differ between the two groups (NP, 120.53±15.92 min, vs. HP, 104.16±14.07 min) (Fig. 6D). The contrast between the light and dark period was more pronounced in HP than in NP rats, however. In NP rats for instance, IMI was not significantly lower during the dark period than during the light one (day, 161.07±34.24 min, vs. night, 101.37±15.71 min, NS), and meal number was not significantly higher (NP day, 5.14±0.99, vs. NP night, 6.57±0.75). In contrast, in HP rats, IMI was significantly larger during the light phase than during the dark phase, and meal number was larger at night. Despite these differences in the day/night contrast between HP and NP rats, meal number, meal size, meal duration and ingestion rate were not significantly different between NP and HP rats at any time (Fig. 6).

4. Discussion

The current study, which is unique so far, deals with a topic of considerable interest on infant nutrition, which is the potential beneficial or detrimental effects derived from the use of HP milk formulas on LBW infants. The results of our experimental study, on an animal model of LBW rats, link high neonatal protein intake and the later occurrence of alterations in weight gain and metabolic disorders in adulthood. To our surprise, although no difference in hypothalamic appetite neuronal pathways development was revealed

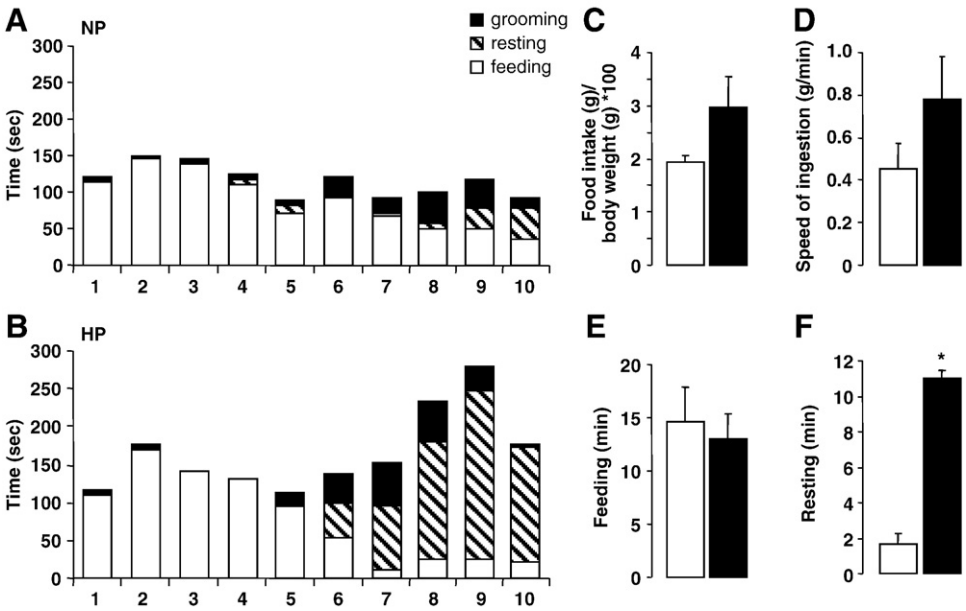

Fig. 5. Time course of the BSS for (A) NP and (B) HP groups at PND50. Each type of behavior (feeding, resting, and grooming) is expressed as the time spent (seconds) during a 5-min period, over 90 min of monitoring. (C) Relative food intake ((g)/body weight (g)*100), (D) speed of ingestion (g/min), (E) feeding (min) and (G) resting (min) during the refeeding period. Normal protein group (open bars) and HP group (closed bars). Values are means±S.E.M., $n=7$ per group. *$P<.05$, NP vs. HP.

between rats, our study highlighted the fact that high neonatal protein intake had a positive impact on expression pattern of genes known to play a role in neurodevelopment. It also demonstrated positive effect of high neonatal protein intake on meal sequence and satiety appearance. In such situation, beneficial and detrimental effect of high neonatal protein intake need to be further discussed and mechanisms proposed.

Artificial rearing of newborn rats through gastrostomy is unique as it allows for a manipulation of specific nutrient intake to individual pups during the suckling period. This approach was used to specifically determine whether increasing the protein content of a milk formula in the preweaning period would have a direct impact on neuronal organization and latter consequence on feeding behavior.

During the preweaning period, feeding an HP formula failed to enhance growth. Therefore, the main finding of the current study is the delayed onset of differences in weight gain and metabolic parameters and feeding behavior measured in the two groups of adult rats depending on the composition of the milk substitute received earlier in neonatal life.

Rats that had received HP milk during preweaning period were indeed 13% heavier as adults. A concomitant increase in plasma triglycerides, leptin and insulin level was observed in adult animals that had received HP milk in neonatal life. The high level of leptin was associated with a greater mass of adipose tissue and adipocytes of larger size. We speculate that neonatal HP feeding may enhance triglyceride synthesis and fat deposition and, consequently, leptin secretion by adipose tissue. Additionally, given that glycaemia is not different between groups, it is possible that increased insulin concentrations represent normal compensation to increased insulin resistance in the HP group. In our study, the development and functions of fetal pancreas and adipose tissue were probably already altered due to gestational dietary restriction. The current study nevertheless is first to demonstrate that just changing one nutrient, protein intake had an additional long-term effect on organs of LBW pups. To our knowledge, a single experimental study (des Robert et al. [23]) directly addressed the effect of feeding gastrostomized normal weight neonatal pups with formulas supplying graded amounts of protein. They described that higher protein intake led to faster weight gain and insulin resistance and altered the expression of glucose transporters in the short term but did not last at adulthood. A number of differences in the protocol (IUGR vs. normal weight pups, normal litter vs. very large litters for the fist days, 16 days of protein intake vs. 8 days) make the comparisons of the results hazardous.

On the brain, we then demonstrated that high neonatal protein intake after IUGR slightly modified the expression pattern of genes known to play a role in the neurodevelopment of the hypothalamus when compared to pups reared under NP intake. From a behavioral point of view, IUGR rats fed with HP milk diet demonstrated a clear distinction between night and day meal pattern and a clear satiety state after an ingested meal in contrast to the other group. Regarding neurodevelopmental factors expression in the hypothalamus, we observed an up-regulation of four genes involved in cell proliferation and tissue modeling in the HP pups. Indeed, expression of neurotrophins such as Bdnf and Igf1 and marker of neurogenesis as Pcna and Cdkn1b, a cell cycle proliferation gene, are in favor of a high proliferation rate taking place at PND12 in the hypothalamus of HP rats. What will be the consequence of this finding is difficult to extrapolate since higher expression of neurotrophins could favor the development of this cerebral structure, but greater numerical density of Nissl-stained neurons in VMH (ventromedial hypothalamus) and PVN [11] during postnatal period is also associated with alterations of appetite pathway development [12] and lead to metabolic disorders during adulthood [15]. In contrast, in a recent study, we demonstrated that higher amount of nutriments provided during the preweaning period reduces abnormal organization of hypothalamic pathway involved in energy homeostasis and implies the same neurodevelopment factors [13].

Concerning ARC AgRP and POMC projections establishment to PVN, DMD and LHA areas, no differences were observed between HP and NP rats (see additional data). Moreover, neonatal plasma leptin, insulin levels and pSTAT3-IR in ARC did not differ between HP and NP rats. The ontogeny of orexigenic and anorexigenic pathway is dependent on neonatal leptin levels and ARC activation by leptin [10]. Since neonatal leptin levels of the two groups of artificially reared pups were lower than concentrations measured in plasma of mother-fed pups [13], the extension of ARC AgRP and POMC

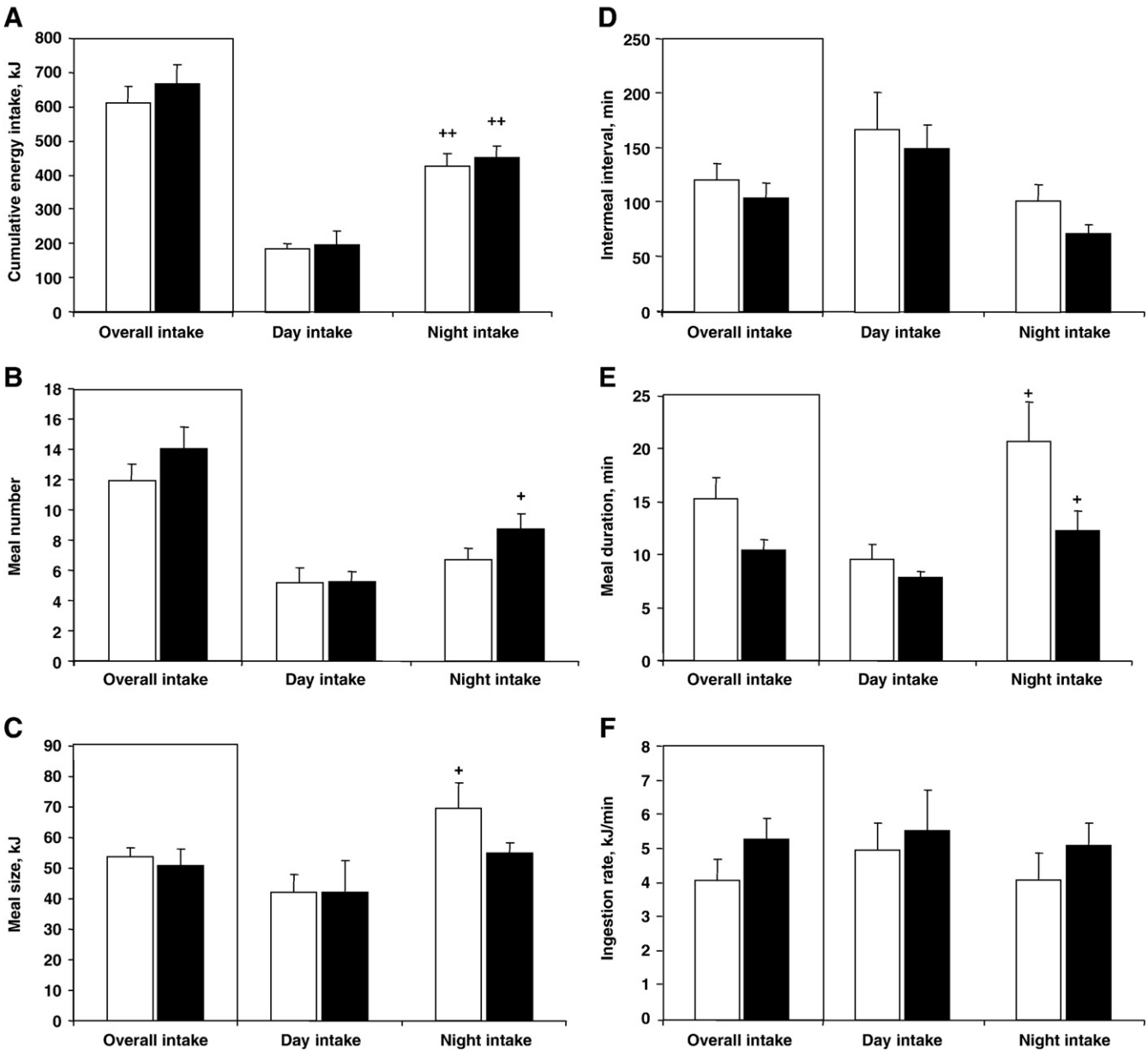

Fig. 6. Meal pattern recording during 23 h at PND70. (A) Cumulative energy intake (kJ), (B) meal number, (C) meal size (kJ), (D) IMI (min), (E) meal duration (min), (F) ingestion rate (kJ/min). Normal protein group (open bars) and HP group (closed bars). Values are means±S.E.M., $n=7$ per group. $^{+}P<.05$, $^{++}P<.01$, day vs. night intake.

projections of NP and HP rats was reduced compared to control rats nourished by lactating mother (data not shown). The increase of proliferation factors found at PND12 in HP rats could lead to a beneficial effect on brain development although not visible on the brain structure under investigation. Such an effect needs to be analyzed on other hypothalamic populations of neurons or on hippocampus, cortical layers and, globally, on second-order structures related to feeding behavior [34,35].

At adulthood, some feeding behavior differences were observed between HP and NP groups. Indeed, after 1 h of refeeding, HP rats exhibited a normal BSS with the usual succession of behaviors: feeding, grooming and resting. On the contrary, NP rats displayed a very short resting period with signs of excessive motor activity in their home cage during the test period. As a consequence, we were not able to delineate when the satiety period occurred since the rats were still eating at the end of the test period. In addition to the locomotor activity measurement of the NP rats, we also propose to further study the functionality of the gastrointestinal tract of these two groups of rats since no results are available yet on this parameter. In particular, the capacity of the stomach and the upper intestine to sense nutriments and send back satiety signals as cholecystokinin and peptide YY [36] in NP rats will be interesting to evaluate.

A fine analysis of the meal patterns recorded for 23 h also revealed some subtle differences between the two groups of rats: No differences were observed on cumulative energy intake, meal number, meal size, meal duration and IMI during the day as well as the night period. However, the contrast during the high food intake at night and the reduced food intake during the day that lead normally to differences in food intake, meal number, IMI and sometimes meal size between night and day was not observed in the NP rats of this study. This suggests an alteration of the circadian rhythm of feeding, as previously described by Orozco-Sólis et al. [37] for IUGR rats. This suggests a beneficial role of HP milk content on circadian rhythm

establishment and could be related to the effect of high protein intake on regulation of clock genes or genes under the regulation of these promoters [38–40].

In conclusion, we demonstrate, for the first time, a number of new and important findings. First, in preweaned IUGR rats, the development of hypothalamic pathways important for regulation of appetite is not affected by early low or HP milk intake. Second, in the young adult, meal pattern and feeding behavior demonstrate irregularities according to previous feeding status of the pups. Third, a well-established feeding behavior observed in HP rats is not sufficient to protect them from the detrimental effect of high neonatal protein intake as metabolic alterations occurred around 160 days of life. The impact of proteins on peripheral organs as pancreatic cells, adipocytes and even intestinal tract requires further investigation to unravel the cellular and molecular mechanism involved.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jnutbio.2010.08.007.

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