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Maternal Perinatal Undernutrition Drastically Reduces Postnatal Leptin Surge and Affects the Development of Arcuate Nucleus Proopiomelanocortin Neurons in Neonatal Male Rat Pups

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A growing body of evidence suggests that maternal undernutrition sensitizes the offspring to the development of energy balance metabolic disorders such as type 2 diabetes, dyslipidemia, and obesity. The present study aimed at examining the impact of maternal undernutrition on leptin plasma levels in newborn male rats and on the arcuate nucleus proopiomelanocortin (POMC) and neuropeptide Y (NPY) neurons that are major leptin targets. Using a model of perinatal maternal 50% food-restricted diet (FR50) in the rat, we evaluated leptin plasma levels and hypothalamic POMC and NPY gene expression from postnatal day (PND) 4 to PND30 in both control and FR50 offspring. In control rats, a postnatal peak of plasma leptin was observed between PND4 and PND14 that reached a maximal value at PND10 (5.17 ± 0.53 ng/ml), whereas it was dramatically reduced in FR50 pups with the higher concentration at PND7 (0.93 ± 0.23 ng/ml). In FR50 animals, using semiquantitative RT-PCR and *in situ* hybridization, we showed that the hypothalamic POMC mRNA level was decreased from PND14 until PND30, whereas NPY gene expression was not significantly modified. In PND21 FR50 animals, we observed strikingly reduced immunoreactive β-endorphin nerve fibers projecting to the hypothalamic paraventricular nucleus without affecting NPY projections. Our data showed that maternal undernutrition drastically reduces the postnatal surge of plasma leptin, disturbing particularly the hypothalamic wiring as well as the gene expression of the anorexigenic POMC neurons in male rat pups. These alterations might contribute to the adult metabolic disorders resulting from perinatal growth retardation. (*Endocrinology* 149: 470–475, 2008)

IT IS NOW WELL recognized that neurodevelopmental alterations such as maternal undernutrition have long-lasting consequences and sensitize to the development of cognitive and metabolic disorders in the offspring (1–5). Evidence indicates that offspring from undernourished mothers are predisposed to become obese when placed into a high-fat diet (6, 7). Although it has been shown that overexposure of the fetus to maternal glucocorticoids could participate to the so-called fetal programming of adult diseases (4, 8), little is known about the factors involved in the developmental programming of energy balance. However, it is clearly established that the regulation of food intake and energy expenditure takes place in specific hypothalamic brain areas (9). In the hypothalamus, the arcuate nucleus (Arc), which is located in the mediobasal part, is considered to be outside the blood-brain barrier and is therefore accessible to circulating factors. In adults, peripheral hormones such as leptin and insulin control the nutritional status and energy storage level and act on feeding centers by modulating the expression and release of hypothalamic orexigenic and anorexigenic peptides such as neuropeptide Y (NPY) and α-MSH, a neuropeptide derived from proopiomelanocortin (POMC) processing in the hypothalamus, respectively (10). It has been shown in mice that these peripheral hormones display a surge of their plasma levels between birth and the third week of life (11). Studies using the leptin-deficient ob/ob mice have indicated that leptin plays a crucial neurotrophic role in the development of hypothalamic circuits regulating food intake and adiposity (12, 13). In these genetically deficient mice, it has been demonstrated that the neurodevelopmental action of leptin is restricted to a critical neonatal period that coincides with its postnatal surge (12, 13). In rat pups, it has been reported that neonatal leptin regulates the gene expression of POMC and NPY without affecting food intake (14). Recently, it has been shown that injections of exogenous leptin from postnatal day (PND) 3–13 to female rat pups from prenatally undernourished mothers prevent the occurrence of several metabolic disorders usually observed in adult animals. In particular, this postnatal treatment normalized the caloric intake, locomotor activity, body weight, and fat mass as well as insulin and leptin plasma levels in adult offspring (15).

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Abbreviations: AgRP, Agouti-related peptide; Arc, arcuate nucleus; FR50, 50% food-restricted; NPY neuropeptide Y; PND, postnatal day; POMC, proopiomelanocortin; PVN, paraventricular nucleus.

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In view of these data, the present study examined the impact of a maternal perinatal undernutrition on leptin plasma levels and on the development of the hypothalamic POMC and NPY systems regulating energy balance in the male rat pups. It shows that, as already observed in mice (11), a plasma surge of leptin is present in control rats between PND 4 and 14. Importantly, maternal perinatal undernutrition drastically reduced the postnatal leptin plasma levels and decreased the hypothalamic gene expression of POMC as well as the nerve fiber projections from the Arc-POMC neurons to the paraventricular nucleus (PVN). These observations shed new lights on how maternal undernutrition may contribute to the programming of the feeding behavior in the offspring (1, 3, 5, 16).

Materials and Methods

Subjects

All experiments were conducted in accordance with the European Communities Council Directive of 1986 (86/609/EEC). Animal use accreditation by the French Ministry of Agriculture (No. 04860) has been granted to our laboratory for experimentation with rats. Adult Wistar rats were purchased from Charles Rivers Laboratories (L'Arbresle, France) and housed five per cage. Animal rooms were maintained on a 12-h light, 12-h dark schedule, and animals were permitted free access to food (regular rat chow No. 113; Usine d'Alimentation Rationelle, Villemoisson sur-orge, France) and tap water.

Maternal perinatal undernutrition

Two groups of pregnant rats were studied. In the control group (n = 15), dams were fed *ad libitum* during gestation, from embryonic d 1 (E1) to E21 for fetuses, and lactation, from PND1 to PND21 for pups. In the 50% food-restricted (FR50) group, females (n = 16) received 50% of the daily food intake of control mothers from E14 until the end of lactation as already published (17). Dams delivered spontaneously during the night between E21 and E22, and litter size was adjusted to eight pups per litter in both groups. Each litter usually contained between seven and 13 fetuses. Experiments were conducted only on male pups. For each measurement, only a limited number of animals (n = 1–3) was used from each litter to obviate a putative litter effect. Pups were killed at several postnatal stages from PND4 to weaning (PND21). After weaning, all rats were placed into individual cages, fed *ad libitum*, and then killed at PND30.

Plasma and tissue collections

At each stage (PND4, -7, -10, -14, -17, -21, and -30), pups were rapidly weighed and decapitated between 0800 and 1100 h. Trunk blood samples were collected in tubes prerinsed with 5% EDTA and centrifuged. Plasma samples were stored at −80 C until determination of circulating leptin. Hypothalami were frozen in liquid N_2 and stored at −80 C until semiquantitative RT-PCR experiments. For *in situ* hybridization, whole brains were frozen on dry ice and stored at −80 C until sectioning. For immunohistochemistry, brains were postfixed for 24 h in 4% paraformaldehyde in PBS and cryoprotected by incubation for 24 h in 0.05 M PBS containing 20% sucrose. The hypothalami were cut into serial 12-μm sections, mounted on gelatin-coated slides, and directly stored at −80 C for immunohistochemistry or dried at 63 C for 1 min before performing *in situ* hybridization.

Leptin measurements

Leptin plasma levels were measured with a conventional two-site ELISA (Active murine ELISA; Diagnostic Systems Laboratories, Cergy-Pontoise, France) according to the manufacturer's protocol. At each stage, at least seven pups were used both in the control and in FR50 groups. Each point has been measured in duplicate using 100 μl plasma. The assay sensitivity was 0.04 ng/ml, and the intra- and interassay coefficients of variation were 5.4 and 7.3%, respectively.

NPY and POMC gene expression analysis

Hypothalamic NPY and POMC gene expression was determined in male rat pups using semiquantitative RT-PCR as described and validated previously (18). RNA was extracted and purified from hypothalami of each postnatal stage (n = 5 rats per group) using the TRIzol reagent (Life Technologies, Inc., Strasbourg, France). The quality of total RNA was assessed by determining the 260/280 absorbance ratio and by agarose gel electrophoresis. Three micrograms of total RNA were reverse transcribed into cDNA using 3 μg random hexamers and 200 U Moloney murine leukemia virus reverse transcriptase (Life Technologies). One thirtieth of the first-strand synthesis reaction was amplified using 1 U *Taq* DNA polymerase (Qbiogen, Illkirch, France) and 2 μM of each forward and reverse primer. The cycling parameters were 94 C for 1 min 30 sec, 60 C for 1 min 30 sec, and 72 C for 2 min. Negative control RT-PCR were performed by omitting RT from the reaction mixture. The position of the primers as well as the predicted size of amplification products are summarized in Table 1. Cyclophilin B was used as an internal standard. Each experiment was performed in triplicate and gave similar results. After amplification, the samples were separated on a 2% agarose gel, visualized by ethidium bromide, and quantified by the Multi-Analyst (Bio-Rad Laboratories, Gif-sur-Yvette, France) software.

In situ hybridization studies were also performed to investigate particularly the POMC and NPY gene expression in the Arc, among other nuclei. Coronal sections (12 μm) of brains (n = 5 PND21 animals per group) through the hypothalamic Arc [ranging from −2.12 to −3.30 mm posterior to bregma, according to the atlas of Paxinos and Watson (19)] were realized and mounted on gelatin-coated slides, dried, and kept at −80 C. *In situ* hybridization was carried out as previously described (20). Both POMC and NPY RT-PCR fragments (Table 1) were subcloned into pGEM-T easy, linearized, and used as riboprobes after labeling using [^{35}S]dUTP (1300 Ci/mmol; Amersham Biosciences, Freiburg, Germany) with the Sp6/T7 Transcription Kit (Roche Diagnostics, Mannheim, Germany). Hybridization with the sense probes was used as control. For each probe, all the slides were exposed together on one x-ray film (Biomax-MR; Kodak, Le Pontet, France). Autoradiograms were digitized during the same session. Hybridization signals were quantified on the autoradiogram films as previously described (20). The OD of the hybridized signals were measured using a GS-700 densitometer coupled with a computer-assisted image analysis using Multi-Analyst software (Bio-Rad) and were expressed as OD per square millimeter. No measurable OD was observed using the sense probes.

NPY and β-endorphin (β-End) immunohistochemistry

Sections from PND21 FR50 and control neonate brains (n = 5 rats per group) were incubated overnight at room temperature in the primary antibodies at the appropriate dilutions in PBS containing 0.3% Triton

TABLE 1. Primers used for semiquantitative RT-PCR analysis

Sequence (mRNA)	Accession no.	PCR product (bp)	Primer position		No. of cycles
			Forward	Reverse	
Cyclophilin B	AF071225	456	155–179	586–610	23
POMC	AF510391	379	79–103	434–458	25
NPY	M20373	354	63–87	393–417	30

Accession numbers, primer positions, PCR products, and number of RT-PCR cycles are indicated. Accession numbers correspond to the mRNA sequences.

X-100 and 10% lactoproteins. The rabbit NPY antiserum was generously provided by Dr. H. Vaudry (University of Rouen, France) and was used at a dilution of 1:500 (21). The rabbit antiserum to β-End was prepared in our laboratory and used at a dilution of 1:200 (21, 22). Labeling was revealed with secondary antibodies conjugated to Cy3TM (1:400; Jackson ImmunoResearch Laboratories, Inc., Interchim) or Alexa Fluor (1:400; Molecular Probes, Interchim) for 2 h at room temperature. Observations were made on a fluorescence microscope (Olympus, Rungis, France) using an image analysis software (analySIS 3.0 Soft Imaging System; Olympus).

Statistical analysis

All data are presented as mean ± SEM. Statistical analysis was performed using two-way and one-way ANOVA and *post hoc* comparison by Dunnett's test. $P < 0.05$ was considered significant. Analyses were performed using SigmaStat software (Systat Software, Port Richmond, CA).

Results

Maternal undernutrition reduces body weight in neonates

As already published (17), when compared with controls, FR50 neonates showed a significant reduction in body growth increase. This decreased body weight was observed as early as PND4 until PND30 ($P < 0.001$, for each stage studied). For example, FR50 rats' body weight was 9.1 ± 0.27 *vs.* 11.21 g ± 0.46 g in controls, 18.34 ± 0.63 *vs.* 33.43 ± 1.27 g in controls, and 46.29 ± 2.57 *vs.* 85.01 ± 1.38 g in controls at PND4, -14, and -30, respectively.

Maternal undernutrition diminishes the postnatal surge of plasma leptin in neonates

Figure 1 illustrates the changes in plasma leptin both in control and in FR50 male rats during the postnatal period. Plasma leptin was significantly modulated during the early developmental stages studied (age effect: $F_{6,96} = 11.88$; $P < 0.001$), and an interaction between group and age was observed (group × age: $F_{6,96} = 9.64$; $P < 0.001$). In controls, plasma leptin increased between PND4 and -14 and reached a peak level of 5.17 ± 0.53 ng/ml at PND10. Although a plasma leptin peak was still present in FR50 neonates, its maximal value observed in PND7 rats was dramatically reduced when compared with controls (0.93 ± 0.23 *vs.* 5.10 ± 0.47 ng/ml in controls). At each stage examined (from PND4–21), maternal undernutrition drastically decreased leptin plasma levels, particularly in PND10 animals in which a 55-fold reduction was observed in FR50 rats (0.09 ± 0.03 ng/ml in FR50 *vs.* 5.17 ± 0.53 ng/ml in controls, $P < 0.001$). However, at PND30, leptin levels were comparable between control and FR50 rats.

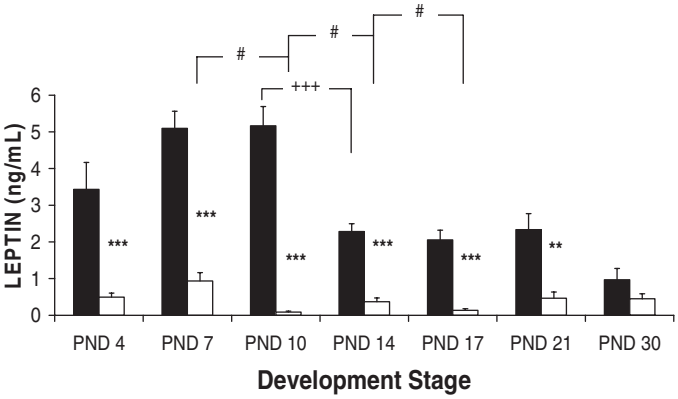

FIG. 1. Maternal undernutrition drastically reduces plasma leptin levels in male rat neonates. Postnatal ontogeny of plasma leptin concentration in pups whose mothers were fed a control diet (*black bars*) or a 50% restriction diet (FR50, *white bars*) during the last week of gestation and lactation. Values represent mean ± SEM (7–10 animals per group per age). **, $P < 0.01$; and ***, $P < 0.001$, FR50 *vs.* control animals; #, $P < 0.05$ between FR50 groups; +++, $P < 0.001$ between control groups.

Maternal undernutrition decreases hypothalamic POMC gene expression in neonates

Using semiquantitative RT-PCR, hypothalamic POMC mRNA expression was modulated during the postnatal period (age effect: $F_{6,33} = 59.61$; $P < 0.001$), and an interaction between group and age was observed (group × age: $F_{6,33} = 8.96$, $P < 0.001$). In controls, POMC mRNA exhibited a low expression level from PND4–10, reached a peak level at PND14, and decreased at PND17 before reaching similar values to those measured at PND14, -21, and -30 (Fig. 2A). Although a similar pattern of gene expression was observed between FR50 and control animals, FR50 neonates displayed significantly reduced POMC mRNA levels from PND14 until PND30 when compared with controls. The maximal effect of maternal undernutrition was observed on PND21, at which a 2-fold reduction ($P < 0.05$) of POMC mRNA level was evidenced in FR50 rats (Fig. 2A). For hypothalamic NPY mRNA content, the expression level was affected during development (age effect: $F_{6,36} = 17.68$; $P < 0.001$), but no difference was observed between experimental groups (Fig. 2B).

To more precisely quantify POMC and NPY gene expression in the hypothalamic Arc nucleus, *in situ* hybridization experiments were performed in both FR50 and control rats at PND21. According to RT-PCR analysis, maternal undernutrition significantly ($P < 0.01$) reduced POMC gene expression in the hypothalamic Arc (Fig. 2, C and D), whereas it did not affect NPY mRNA level (data not shown).

Maternal undernutrition affects POMC neurons nerve fibers projections in neonates

Both NPY and β-End antisera labeled cell bodies with the expected pattern in the Arc of control animals, with POMC-containing neurons being more ventrally located than the NPY-containing perikarya. Labeled axons were observed in many brain regions, with a particularly intense innervation of the PVN of the hypothalamus with both antisera. In FR50 neonates, intense labeling in cell bodies in the Arc was still observed with both antisera (data not shown). No obvious difference was observed in the innervation of the PVN by NPY-containing projections. However, a marked decrease in the fluorescence intensity and number of β-End-labeled axons was apparent in the PVN of all FR50 animals (Fig. 3).

Discussion

The principal finding of this study is that maternal perinatal undernutrition drastically reduced the surge of plasma leptin and is accompanied by marked alterations of the Arc-

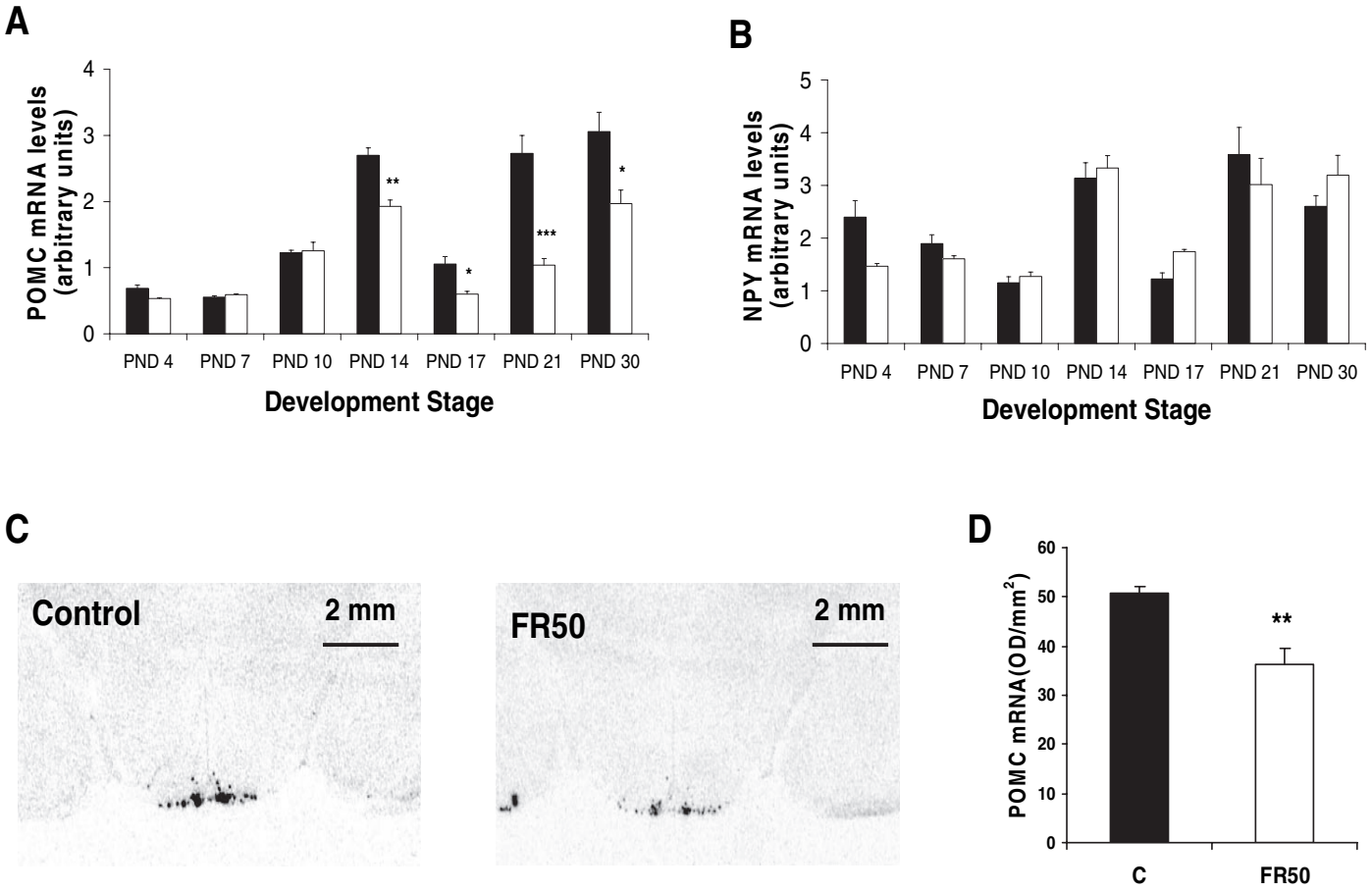

FIG. 2. Maternal undernutrition decreases hypothalamic POMC gene expression in neonates. Semiquantitative RT-PCR analysis of POMC (A) and NPY (B) mRNA level in control (*black bars*) and FR50 (*white bars*) hypothalami (n = 5 animals per group per age). Note that the maximal difference between control and FR50 neonates was observed at PND21. C, Photomicrograph of brain coronal section showing specific *in situ* hybridization signal for POMC mRNA in the Arc of PND21 control and FR50 rats (n = 5 animals per group per age). Labeling, visible in dark, is confined to the Arc (stereotaxic coordinates: −3.14 mm posterior to bregma). D, Semiquantification of the Arc-POMC mRNA level in PND21 animals using *in situ* hybridization. C, Control. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, significant differences between groups.

POMC system in male rat neonates. We also showed that, as earlier reported in mice (11), male rat neonates exhibit an early postnatal peak of plasma leptin, reinforcing the idea that leptin could play a key role during the development of hypothalamic nuclei controlling food intake and energy expenditure in rodents (13). Indeed, using the ob/ob mutant mice (12), it has been demonstrated that neonatal leptin acts as a neurotrophic factor promoting the development of projections from the Arc of the hypothalamus (12). Interestingly, in neonates, addition of exogenous leptin prevents the developmental hypothalamic alterations observed in adult ob/ob mice, whereas leptin injections to these adult mutant mice do not seem to counteract these defects (12). These data indicate the existence of critical windows during early developmental stages in mice and are in line with several reports that have shown that the manipulation of maternal nutrition during the perinatal period has both short- and long-term consequences on the programming of feeding behavior in the rat offspring (1, 3, 5, 16, 23).

The origin of the postnatal surge of plasma leptin is still controversial. It has been suggested that maternal milk could constitute one of the main sources of circulating leptin in neonatal rats because leptin concentrations rapidly decline to undetectable levels in pups separated from their mothers for 24 h (24). The importance of lactation has been strengthened by the observation that rat neonates reared in small litters exhibit a disorganization and malprogramming of the hypothalamic NPY system and are hyperphagic and overweight throughout life (23), indicating thus that maternal overnutrition during lactation sensitizes to the development of obesity and metabolic abnormalities. In contrast, adult rats underfed during lactation due to nurturing in large litters never developed obesity (23). It has also been suggested that an alternative source of the leptin peak could result from endogenous production by brown and white adipose tissues (25). Among several tissues analyzed, we detected leptin mRNA solely in brown and white adipose tissues in PND10 control neonates (data not shown). However, if fat is responsible for the surge of leptin, it remains to be determined why the plasma peak observed in neonates is transient. Using nursing mothers during lactation, it has been reported that offspring from prenatally undernourished female rats may also develop hyperphagia and obesity, particularly when they are placed into a hypercaloric regimen after weaning (6).

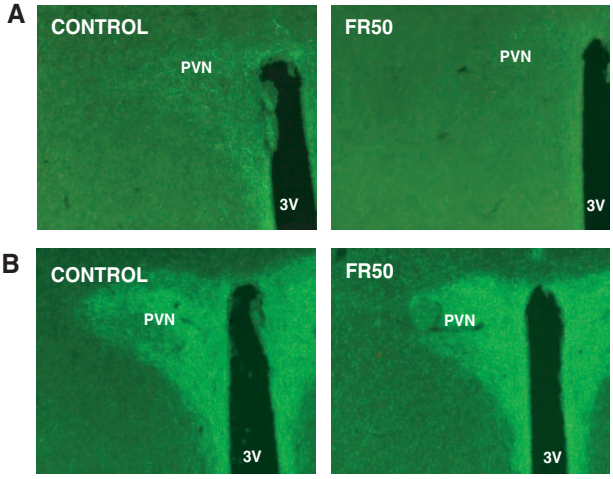

FIG. 3. Maternal undernutrition affects POMC neuron nerve fiber projections in neonates. Immunohistochemical detection of POMC (A, using an anti-β-End antiserum) and NPY (B) in the PVN of control or FR50 PND21 neonates. Although fibers immunostained for NPY are abundant in the PVN of both control and FR50 pups, only rare fibers labeled for β-End are detected in the same nucleus of FR50 rats compared with controls. Magnification, ×130. 3V, Third ventricle.

In addition, the same group has recently reported that exogenous leptin injections during the neonatal period prevent the development of metabolic alterations usually observed in adult rats (15). These latter data indicate that the milk production of leptin from nursing mothers is insufficient to deprogram the effects of prenatal undernutrition, suggesting that a complex interaction between pups and mother is required for the full efficiency of postnatal leptin. However, it is difficult to draw conclusions because leptin plasma levels in rat neonates from prenatally undernourished mothers have not been reported in this study.

The present study showed, for the first time in a nongenetically modified rodent model, that the marked decrease of plasma leptin levels in neonates induced by maternal undernutrition precedes the diminution of the hypothalamic Arc-POMC mRNA expression of these developing rats. This result is in line with previous observations indicating that, in PND10 rats, exogenous leptin is already able to modify Arc-POMC and NPY gene expression (14). In the ob/ob mutant mice, the absence of endogenous leptin markedly affects both agouti-related peptide (AgRP)/NPY and α-MSH pathways (12). In contrast, we did not observe any detectable modification of the AgRP (data not shown) and NPY mRNA levels, suggesting that the very low levels of leptin in FR50 rat neonates are sufficient to maintain a normal AgRP/NPY gene expression. This assumption is corroborated by immunohistochemistry studies that showed no gross abnormalities in the projection of the NPY nerve fibers from Arc to PVN. In contrast, β-End immunolabeling of nerve fibers projecting to the PVN is drastically reduced, indicating that maternal perinatal undernutrition seems to preferentially affect the POMC anorexigenic pathway in rats. Interestingly, rats overfed during the lactation period showed an increased number of NPYergic neurons in the hypothalamus at weaning, indicating that perinatal under- and overnutrition lead to distinct hypothalamic disturbances (25). Indeed, nutritional surplus and deficiency during the perinatal period seem to converge toward a common phenotype responsible for an increased propensity to develop obesity and engender metabolic disturbances (26). Although the programming of obesity is a multifactorial process, the diversity of animal models with a common endpoint might suggest some shared pathways. Several factors such as the adipoinsular axis and glucocorticoid signaling are undoubtedly important, but the plasticity of the hypothalamus in late pregnancy and early postnatal life appears to be a crucial determinant to the programming of appetite and metabolism toward establishing a modified body weight set point, which may or may not be adjustable over time and upon subsequent environmental conditions.

It is noteworthy that adult FR50 male rats fed under a standard diet from weaning are not overweight, suggesting that the drastic decrease of postnatal leptin is not sufficient to promote severe alterations in body weight regulation. As already observed in the rat offspring from prenatally undernourished mothers, a hypercaloric regimen might be required to unmask the metabolic alterations programmed in these animals (6, 15). Interestingly, a growing body of evidence indicates that neonatal leptin could play, in addition to the regulation of energy homeostasis, important roles in the control of various neuroendocrine functions, hematopoiesis, lymphopoiesis, and maturation of small intestine (27, 28). In particular, it has been reported earlier that neonatal leptin influences the activity of both gonadotroph and hypothalamo-pituitary-adrenal axes (29, 30). Because we have previously reported that FR50 animals of both sexes present a delayed puberty (31) and an altered hypothalamo-pituitary-adrenal axis activity throughout life (4), it remains to be determined to what extent the dramatic decrease of plasma leptin observed in rat neonates from undernourished mothers could contribute to these physiological defects.

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