Early Metabolic Programming of Puberty Onset: Impact of Changes in Postnatal Feeding and Rearing Conditions on the Timing of Puberty and Development of the Hypothalamic Kisspeptin System

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Kiss1 neurons have recently emerged as a putative conduit for the metabolic gating of reproduction, with leptin being a regulator of hypothalamic *Kiss1* expression. Early perturbations of the nutritional status are known to predispose to different metabolic disorders later in life and to alter the timing of puberty; however, the potential underlying mechanisms remain poorly defined. Here we report how changes in the pattern of postnatal feeding affect the onset of puberty and evaluate key hormonal and neuropeptide [*Kiss1*/kisspeptin (Kp)] alterations linked to these early nutritional manipulations. Female rats were raised in litters of different sizes: small (four pups per dam: overfeeding), normal (12 pups per dam), and large litters (20 pups per litter: underfeeding). Postnatal overfeeding resulted in persistently increased body weight and earlier age of vaginal opening, as an external sign of puberty, together with higher levels of leptin and hypothalamic *Kiss1* mRNA. Conversely, postnatal underfeeding caused a persistent reduction in body weight, lower ovarian and uterus weights, and delayed vaginal opening, changes that were paralleled by a decrease in leptin and *Kiss1* mRNA levels. Kisspeptin-52 immunoreactivity (Kp-IR) in the hypothalamus displayed similar patterns, with lower numbers of Kp-IR neurons in the arcuate nucleus of postnatally underfed animals, and a trend for increased Kp-positive fibers in the periventricular area of early overfed rats. Yet, gonadotropin responses to Kp at puberty were similar in all groups, except for enhanced responsiveness to low doses of Kp-10 in postnatally underfed rats. In conclusion, our data document that the timing of puberty is sensitive to both overfeeding and subnutrition during early (postnatal) periods and suggest that alterations in hypothalamic expression of *Kiss1*/kisspeptin may underlie at least part of such programming phenomenon. **(*Endocrinology* 152: 3396–3408, 2011)**

In mammals, many neuroendocrine networks responsible for the homeostatic control of essential body functions become organized during the intrauterine and perinatal stages of development (1). This organizational process is essential for the generation of proper adaptative responses throughout the lifespan (2–4). Because the developing organism uses early external cues to anticipate future environmental settings (5, 6), exposure to extreme

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Abbreviations: ARC, Arcuate nucleus; AVPV, anteroventral periventricular nucleus; BW, body weight; IHC, immunohistochemistry; icv, intracerebroventricular; IR, immunoreactive/immunoreactivy; LL, large litter; NL, normal litter; semi-Q, semiquantitative; SL, small litter; VO, vaginal opening; VO50, 50% VO.

conditions during this period results in perturbed maturation of key neuroendocrine networks and, hence, may compromise the capacity to generate homeostatic responses later in life (7–9). As a paradigmatic example, data from different species, including humans, have documented that severe alterations of the nutritional status or hormonal milieu during the gestational and early postnatal life predispose to later development of metabolic diseases, such as type 2 diabetes and obesity (2, 3, 10–14). Accordingly, early nutritional malprogramming of different metabolic and hypothalamic pathways is considered as a potential contributing factor for the rising incidence of obesity and its related sequelae (12, 15).

Although the impact of metabolic programming on other physiological functions tightly coupled to energy homeostasis is yet to be fully characterized, compelling evidence has mounted recently, suggesting that puberty onset and reproductive capacity are also sensitive to nutritional influences during early stages of development (16–23). In this context, human studies evidenced that puberty onset is accelerated in girls with low birth weight that subsequently develop obesity in childhood (24, 25). In rats, different manipulations of gestational and postnatal feeding have been shown to variably impact puberty onset, although the nature of such changes depends on the type and timing of the nutritional challenge. Thus, in females rats, prenatal overfeeding has been reported to cause early puberty onset (19), whereas delayed puberty has been observed in rats submitted to intrauterine growth retardation or early postnatal underfeeding (16, 20–22). However, caloric restriction during pregnancy and lactation has also been reported to advance puberty in the female offspring (19). Most of the above studies were descriptive, and little is known regarding the neuroendocrine mechanisms involved (21). However, the pathophysiological relevance of the above observations is stressed by the plethora of adult reproductive defects described in animal and human models of early nutritional challenge (23, 24, 26, 27), a phenomenon the translational interest of which is reinforced by the rising incidence of disorders of body weight, especially obesity, during gestation and childhood.

Puberty is triggered by the heightening of the secretory activity of GnRH neurons, which results in the full activation of the gonadotropic axis and attainment of reproductive capacity (28, 29). The mechanisms of puberty onset have been the subject of active investigation, especially after recent reports of possible changes in the timing of puberty in humans (30, 31), and the potentially associated health problems (32–36). A major breakthrough in our understanding of the neuroendocrine basis of puberty came from the identification of Kp, products of the *Kiss1* gene, and their receptor, GPR54 (also termed Kiss1R), as major regulators of GnRH secretion and, hence, reproductive maturation and function (37, 38). Indeed, compelling evidence indicates that the hypothalamic Kiss1 system, which in rodents comprises two major neuronal populations, located in the arcuate (ARC) and anteroventral periventricular (AVPV) nuclei (37, 38), undergoes a complex pattern of activation during puberty that appears essential for its proper timing (39, 40). This maturational process involves not only an increase in the hypothalamic Kp tone (41, 42) but also an elevation of the number of Kp neurons and their projections to GnRH neurons (43, 44). These plastic changes are sensitive to the organizing and activational effects of sex steroids during postnatal and prepubertal development (40, 44). Whether pubertal maturation of the hypothalamic Kiss1 system is influenced by additional modifiers has not been explored in detail to date.

Hypothalamic Kiss1 neurons are highly sensitive to body energy status and metabolic cues, as evidenced by suppressed *Kiss1*/kisspeptin expression in conditions of negative energy balance, which are also linked to inhibition of the reproductive axis (45, 46). Likewise, deregulated hypothalamic *Kiss1*/kisspeptin levels have been reported in mouse models of obesity (47). The metabolic signals that regulate the Kiss1 system are likely multiple, but the adipokine, leptin, has been proposed as a key modulator, and Kiss1 neurons have been suggested to participate in mediating leptin regulation of GnRH secretion, therefore defining a pathway for the permissive actions of leptin on puberty onset (45, 46). It must be stressed, however, that the experimental data supporting a role of leptin in the control of hypothalamic Kiss1 system come from studies in adult models of metabolic stress and may not be fully representative of physiological conditions at puberty. In addition, it is noted that recent functional genomic studies in mice lacking leptin receptors in Kiss1 neurons suggest that the effects of leptin might be indirect and/or dispensable, at least in conditions of congenital absence of leptin signaling in this neuronal population (48).

In the present study, we evaluated the impact of nutritional challenges during the critical postnatal period on the onset of puberty, and the putative neurohormonal mechanisms involved. To this end, a rat model of manipulation of litter size, to induce conditions of underfeeding (large litters) or overnutrition (small litters) during lactation, was used (49). This model has been proposed to mimic nutritional challenges during the last trimester of human gestation (50). As neuroendocrine parameters, leptin levels and hypothalamic expression of *Kiss1* mRNA, as well as Kp immunoreactivity (IR) and gonadotropin responses to exogenous Kp-10, were selected for analysis (37, 45). Of note, whereas the effects of acute metabolic

stress on Kiss1 expression and gonadotropin responsiveness to Kp had been previously explored in pubertal and adult animals (45, 46), the impact of early (postnatal) metabolic challenges on the subsequent expression and function of the hypothalamic Kiss1 system later during puberty remains largely unexplored to date.

Materials and Methods

Animals and drugs

Wistar female rats bred in the vivarium of the University of Córdoba were used. Pregnant dams were obtained by mating with adult Wistar male rats and were kept under constant conditions of light (14 h of light, from 0700 h) and temperature (22 C), with free access to standard laboratory chow and tap water. On d 1 postpartum, female pups were selected, cross-fostered, and grouped into three different litter sizes: small litters (SL; four pups per litter), normal litters (NL; 12 pups per litter), and large litters (LL; 20 pups per litter), as described elsewhere (16, 18, 49, 51). This procedure has been previously used to induce over- or underfeeding during early postnatal maturation (*i.e.* lactation). The animals were weaned on d 21 postpartum, when the pups of each litter size were housed in groups of four rats per cage, with free access to pelleted food and tap water. The experimental procedures were approved by the Córdoba University Ethical Committee for animal experimentation and conducted in accordance with the European Union guidelines for use of experimental animals. Mouse Kiss1 (110–119)-NH_2, also termed Kp-10, was obtained from Phoenix Pharmaceuticals (Belmont, CA). For immunohistochemical (IHC) analyses, a mouse Kp-52 antiserum (code JLV-1), which recognizes the longer form of rodent Kp (Kp-52) without any detectable cross-reactivity with Arg-Phe-related peptide, was used in accordance with previous literature (52–54).

Experimental design

In experiment 1, the effects of changes in postnatal feeding on the timing of puberty were evaluated in female rats derived from different litter sizes (SL, NL, and LL). Body weight (BW) gain was evaluated in the different groups (n = 36/group) at weekly intervals during the first month of postnatal life. Thereafter, BW and vaginal opening (VO; as external sign of puberty) were monitored daily between d 30 and d 37 postpartum. Detailed inspection was conducted in each animal to determine the age of complete canalization of the vagina (55). In addition, in a subset of animals from each group, ovarian and uterus weights were recorded, and blood samples were collected for hormonal determinations, upon decapitation of the animals at d 31 or d 36 postpartum (n = 12/age-point).

In experiment 2, analyses of hypothalamic expression of *Kiss1* mRNA and Kp-IR were conducted in 36-d-old female rats, raised in SL, NL, and LL. For RNA analyses, hypothalamic samples (n = 6/group) were excised immediately after decapitation of the animals, by dissection with a horizontal cut of approximately 2 mm depth, using the following limits: 1 mm anteriorly from the optic chiasm, the posterior border of the mammillary bodies, and the hypothalamic fissures. Brain samples for IHC analyses (n = 5/group) were obtained after *in vivo* perfusion and fixation of the animals (see below).

In experiment 3, gonadotropin responsiveness to Kp was studied in our models of early nutritional manipulation by assessing the effects of acute central injection of Kp-10 on LH and FSH secretion in 31-d-old females. The procedure of intracerebroventricular (icv) injection of Kp-10 was as previously published (55); icv cannulae were implanted to a depth of 3 mm beneath the surface of the skull, with an insert point 1 mm posterior and 1.2 mm lateral to bregma (55). Two doses of Kp-10 were tested: 1 nmol (maximal stimulation) and 10 pmol (submaximal stimulation), to estimate potential changes in the sensitivity to Kp. Blood samples were obtained 15 min after Kp-10 injection. Animals injected with vehicle served as controls.

Hormone measurements

LH and FSH levels were measured using RIA kits supplied by the National Institutes of Health (National Hormone and Peptide Program, Torrance, CA). Rat LH-I-10 and FSH-I-9 were labeled with ^{125}I using Iodo-gen tubes (Pierce Chemical Co., Rockford, IL). Hormone concentrations were expressed using reference preparations LH-RP-3 and FSH-RP-2 as standards. Intra- and interassay coefficients of variation (CV) were less than 8 and less than 10%, respectively. Leptin levels were determined using a commercial RIA kit from MP Biomedicals (Costa Mesa, CA). The sensitivity of the assay was 0.05 ng/tube, and the intraassay CV was less than 5%.

RNA analysis by semi-Q (semiquantitative) RT-PCR

Relative *Kiss1* mRNA levels were assayed in hypothalamic preparations by RT-PCR, optimized for semi-Q detection, using previously defined primer pairs and conditions (41, 56). As internal control, amplification of a 240-bp fragment of S11 ribosomal protein mRNA was carried out in parallel in each sample. In keeping with previous optimization tests, 32 and 24 PCR cycles were chosen for semi-Q analysis of the specific target (*Kiss1*) and *RP-S11* internal control, respectively (41, 56). Specificity of PCR products was confirmed by direct sequencing. Quantification of intensity of RT-PCR signals was carried out by densitometric scanning, and values of the specific target were normalized to those of internal control to express arbitrary units of relative expression. Liquid controls and reactions without reverse transcription resulted in negative amplification.

Immunohistochemistry

IHC assays were conducted in hypothalamic sections of brain samples from 36-d-old female rats. The animals were perfused through the ascending aorta, under thiobarbital anesthesia, with 250 ml fixative solution (4% paraformaldehyde) (57, 58). Brains were collected, immersed in fixative overnight, and dehydrated in 30% sucrose for 2–4 d. Coronal sections (40 μm) were cut in parallel series of four on a freezing microtome, and one series of sections were processed for Kp-IR. The sections were incubated in 1% H_2O_2-PBS to block endogenous peroxidase activity and in 0.01 M PBS with 0.3% Triton X-100, 5% swine serum, and 1% BSA to block nonspecific binding sites. The sections were then incubated at 4 C for 24 h with a purified rabbit antiserum against mouse Kp-52 (JLV-1), diluted 1:200. Kp-IR was detected by the avidin-biotin method using diaminobenzidine as chromagen. The sections were incubated for 60 min in biotinylated secondary anti-rabbit IgG (The Jackson Laboratory, Bar Harbor, ME) diluted 1:1000, washed, and transferred to the avidin-biotin complex (Vector Laboratories, Burlingame, CA) diluted 1:250. Thereafter, the

sections were incubated in 0.1% diaminobenzidine (Sigma Chemical Co., St. Louis, MO). The sections were mounted on gelatinized glass slides, dried, and coverslipped in Pertex.

In keeping with our previous protocols (54), IHC analyses involved counting of the number of immunoreactive (IR) neuronal cell bodies in the ARC and AVPV. Nevertheless, the numbers of identifiable Kp-positive cell bodies in the AVPV were very low in all groups; thus, quantitative analyses were only conducted in the ARC. In addition, Kp fibers were stereotaxically quantified in the anterior periventricular area. To this end, horizontal lines with a spacing of 43 μm were placed randomly across a region covering the area between the anterior commissure and the optic chiasm, with a maximum limit of 200 μm into the parenchyma from the ventricle, including the AVPV. The number of fiber crossings of these arbitrary lines was counted, using the microscope software AxioVision (Zeiss MicroImaging, Thornwood, NY). As complement of this stereotactic approach, Kp-IR fibers were also quantified by measuring OD in the same anterior periventricular region (maximum limit of 200 μm into the parenchyma from the ventricle), using the software Image-J (National Institutes of Health, Bethesda, MD). Background was measured in a region with no immunoreactivity (IR) outside the region of interest, and this value was subtracted from the specific OD of the anterior periventricular region and expressed as arbitrary units. Because of high density, fiber numbers could not be reliably estimated in ARC.

Presentation of data and statistics

Hormonal determinations were conducted in duplicate, with a minimum of 10 samples per group. Semi-Q RT-PCR analyses were carried out in duplicate from at least five independent RNA samples per group. IHC analyses were carried out in five brain samples of each group. The numbers of Kp-positive (IR) cells and fibers were counted using a light microscope (Nikon Biophot-20 × magnification; Nikon, Inc., Melville, NY) by an observer blind to treatment regimens. Kp-IR cells and fibers, located within the ARC and periventricular regions, were counted in one of the series of brain sections. Data are presented as the means ± SEM. Results were analyzed for statistical differences, using unpaired Student's *t* test or ANOVA followed by Student-Newman-Keuls multiple-range tests (SigmaStat 8.0; Jandel, San Rafael, CA). $P \leq 0.05$ was considered significant.

Results

Impact of changes in postnatal feeding on the timing of puberty and related parameters

The effects of changes in early postnatal feeding on BW gain and puberty onset were monitored in female rats bred in litters of different size (SL, NL, and LL), in order to induce conditions of early overfeeding or undernutrition (49). BW curves progressively diverged between the three groups, starting from d 7 (Fig. 1A), so that at weaning, SL rats were 33% heavier than NL controls, whereas LL rats were 38% leaner that NL animals. Such differences in BW persisted after weaning, even though the animals were allowed to eat *ad libitum*. Indeed, BW curves from the three groups displayed parallel slopes, with SL females being

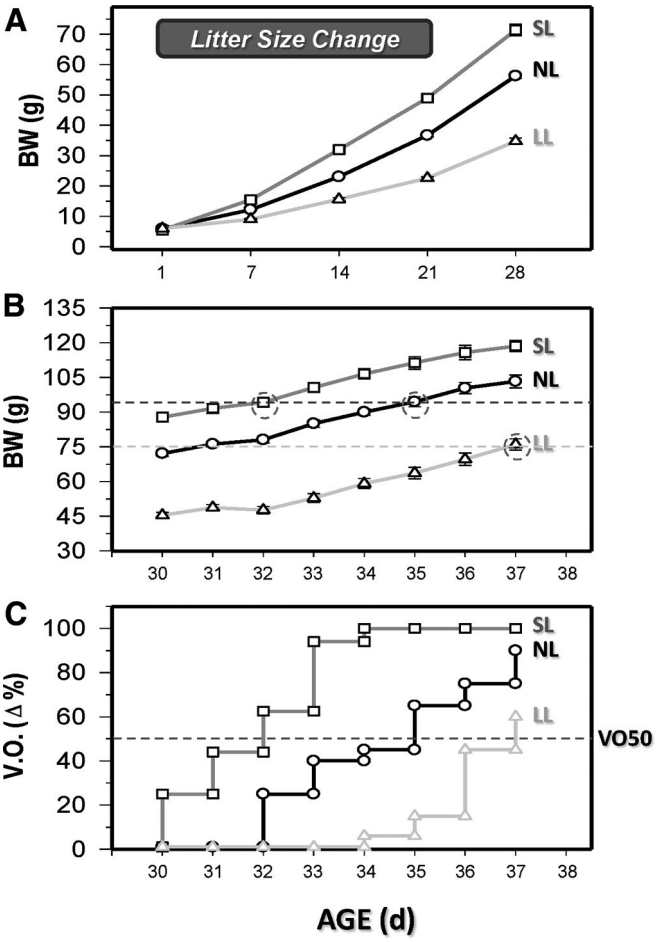

FIG. 1. Impact of changes in early postnatal feeding on BW gain and puberty onset, assessed by VO. Female rats were raised in litters of different size: four pups per dam (SL), 12 pups per dam (NL), and 20 pups per dam (LL), as a mean to induce overfeeding or undernutrition during early postnatal stages of development. The period of litter size manipulation (between d 1 and d 21 postpartum) is denoted by the *gray box* in panel A. BW was monitored in the groups on a weekly basis up to d 30 postpartum (panel A). Thereafter, BW was daily monitored up to d 37 postpartum (panel B). In addition, between d 30 and d 37 the animals were inspected daily for canalization of vagina; the day of complete canalization being considered as VO. For presentation of data, cumulative percentage data of VO are presented for the three groups in panel C. For each group, the age when 50% of the animals displayed VO was considered as VO50, indicated in panel C as a *gray dotted line*. In addition, the values of BW when each group achieved VO50 are *circled* and represented by *dotted lines* in panel B.

20% heavier than control NL rats throughout pubertal maturation and LL animals showing approximately 30% lower BW than controls during this period (Fig. 1B). These changes in BW were paralleled by similar trends in terms of VO (Fig. 1C). Thus, SL females displayed earlier VO, with all animals showing complete canalization of vagina by d 34 postpartum and a mean age of VO of approximately 32 d. In contrast, by d 37, 10% and 40% of females from NL and LL had not yet displayed VO, which prevented calculation of mean ages of VO. Alternatively, the mean age for completion of 50% VO (VO50) was estimated, with values of 32 d, 35 d, and 37 d for SL, NL, and

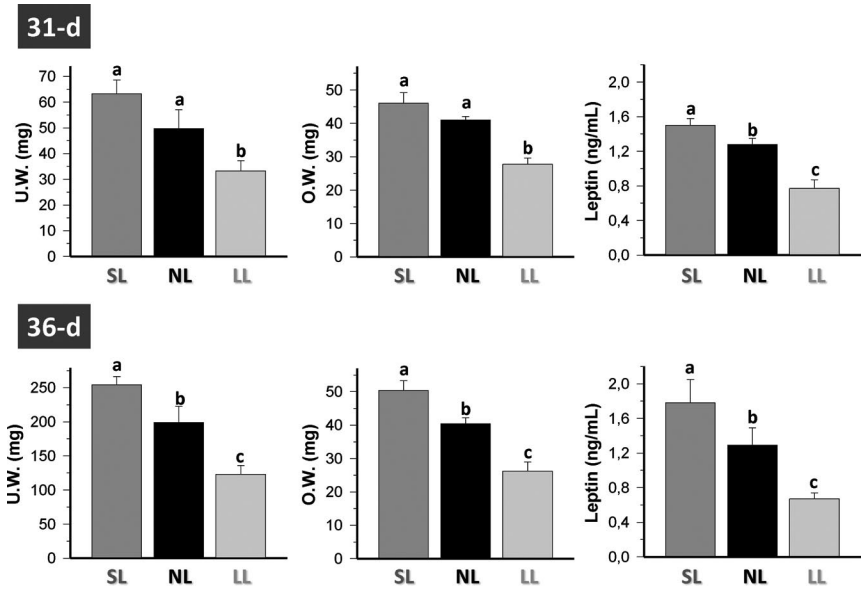

FIG. 2. Impact of changes in early postnatal feeding on key phenotypic and neurohormonal parameters of puberty. Uterus and ovarian weights (U.W. and O.W., respectively), as well as serum leptin levels (ng/ml), were recorded in female rats from three groups: SL (overnutrition), NL (normal nutrition), and LL (subnutrition). Determinations were conducted at two age points: d 31 and d 36 postpartum (*upper* and *lower panels*, respectively). Values are given as the mean ± SEM of at least 10 independent determinations per group. Groups with *different superscript letters* are statistically different. ($P < 0.05$; ANOVA followed by Student-Newman-Keuls multiple range test).

LL, respectively (*dotted line* in Fig. 1C). BW at VO50 was similar between SL and NL groups (~95 g BW); in contrast, VO50 was achieved at a much lower BW in LL females (~75 g BW) (see *dotted lines* in Fig. 1B).

As additional indices of pubertal maturation and activation of the reproductive axis, uterus and ovarian weights were also monitored in the different groups, at d 31 and d 36 postpartum (*i.e.* during the pubertal transition). Postnatally overfed rats had higher ovarian and uterus weights than control NL animals on d 36 postpartum (Fig. 2); similar trends were detected on d 31, but these differences did not reach statistical significance. In clear contrast, underfed females had severely reduced uterus and ovarian weights at the two age points, which were 37% and 50% lower than in control (NL) and overfed (SL) animals, respectively. Serum leptin levels on d 31 and d 36 were higher in SL animals than in NL rats. In contrast, leptin concentrations were significantly lower in underfed LL rats at both age points. Indeed, serum leptin levels in LL females on d 36 (close to VO50) were lower than in SL and NL rats on d 31, *i.e.* before VO50 was achieved (0.67 ± 0.07 ng/ml in LL rats on d 36 *vs.* 1.28 ± 0.07 ng/ml in NL and 1.51 ± 0.07 ng/ml in SL on d 31; $P < 0.01$). In addition, basal serum LH levels in SL females on d 31 were higher than in NL females (3.55 ± 0.5 *vs.* 2.3 ± 0.29 ng/ml; $P < 0.01$), whereas LH concentrations in LL females tended to be lower (1.78 ± 0.25 ng/ml), but this trend was not statistically significant.

Kiss1 expression and Kp-IR in pubertal female rats after early nutritional challenges

Based on the above data, changes in the hypothalamic expression of *Kiss1* mRNA and the patterns of Kp-IR were evaluated in our models. Pubertal levels of *Kiss1* mRNA in whole hypothalamic fragments were significantly higher in SL females than in NL animals (Fig. 3). Conversely, *Kiss1* mRNA expression in LL females was significantly decreased *vs.* corresponding control values on d 36, with its relative levels being approximately half of those detected in SL animals at the same age.

RNA expression analyses were complemented by IHC studies. As shown in Fig. 4, SL and NL rats displayed similar number of Kp-IR neurons in the ARC at puberty; the slightly higher mean number of Kp cells in the ARC of SL rats (20% over NL values) was not statistically significant. In contrast, the number of Kp-positive cells in the ARC of LL females was significantly decreased, with approximately 70–75% reduction in the mean cell number *vs.* NL and SL rats. In addition, Kp-positive fibers in the AVPV region were evaluated by a combination of stereotactic and densitometric approaches; the former provides an estimate of fiber numbers whereas the latter gives an integral account of fiber peptide content (including fiber and terminal densities). The maximum area of parenchyma for analysis was set at 200 μm from the third ventricle. Nevertheless, to provide a more thorough estimation of regional changes in fibers and density, additional quantitative analyses were selectively conducted at the limit of the first 100 μm from the ventricle. As a whole, these data indicate that postnatally overfed animals tend to have higher numbers and density of Kp fibers in the AVPV area, although substantial variations were detected across this region (as indicated by comparison of results at 100- and 200-μm width areas) and the high in-group variability prevented these differences from reaching statistical significance. In any event, our analyses indicated that pubertal SL females had a mean 2-fold elevation in the number of Kp fibers in the 200-μm periventricular region compared with control NL rats ($P = 0.058$; Student's *t* test), whereas no difference was detected between NL and LL groups (Fig. 5). Like-

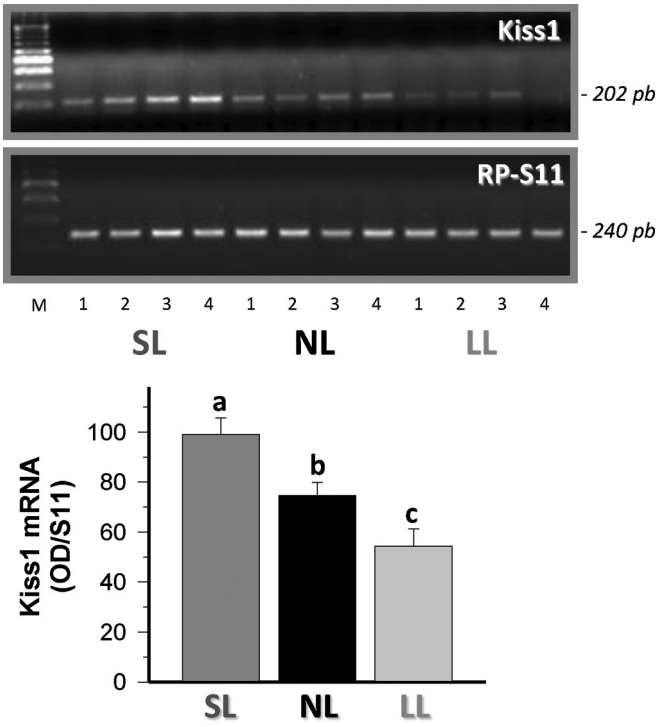

FIG. 3. Profiles of hypothalamic expression of *Kiss1* mRNA in the three experimental groups of female rats: SL (overnutrition), NL (normal nutrition), and LL (subnutrition). Hypothalamic samples were obtained at d 36 postpartum, as described in *Materials and Methods*. In the *upper panel*, a representative RT-PCR assay is shown of expression levels of *Kiss1* mRNAs in hypothalamic samples from the three experimental groups under analysis. Four independent samples per group are presented. Parallel amplification of S-11 ribosomal protein mRNA served as an internal control. In the *lower panel*, semi-Q relative levels of *Kiss1* mRNA are presented as the mean ± SEM of at least five independent determinations. Groups with *different superscript letters* are statistically different. ($P < 0.05$ by ANOVA followed by Student-Newman-Keuls multiple range test).

wise, the mean Kp fiber density within the first 100-μm width periventricular area was 2-fold higher in SL than in NL and LL rats, although this difference was not statistically significant. Admittedly, no detectable change in mean fiber density was observed among the groups when the total 200-μm width periventricular region was considered for quantitative analysis (Fig. 5).

Gonadotropin responses to Kp-10 in pubertal female rats after early nutritional challenges

Acute gonadotropic responses to central administration of Kp-10 were also explored in peripubertal (31-d-old) female rats raised in SL, NL, and LL, as indirect index of changes in endogenous Kp tone and proxy measurement of altered sensitivity to Kp (59, 60). icv injection of 1 nmol Kp-10 evoked very robust LH and FSH responses in control (NL) animals at 15 min after injection (maximal stimulation). In contrast, the submaximal dose of 0.01 nmol induced a 3-fold lower LH secretory response, with no effect on FSH secretion (Fig. 6). Similar patterns were detected in SL rats, with potent LH and FSH responses to 1 nmol Kp-10 and modest LH secretion after icv injection of 0.01 nmol Kp-10; the absolute magnitude of these responses was similar to that of NL rats. In contrast, pubertal LL females displayed enhanced gonadotropic responses to Kp-10 *in vivo*, as evidenced by 1) higher net LH responses to 1 nmol Kp-10 ($P < 0.05$ *vs.* SL rats); 2) enhanced LH responses to 0.01 nmol Kp-10 ($P < 0.05$ *vs.* NL rats); and 3) significant FSH secretory responses to 0.01 Kp-10, which were not detected for this dose in SL or NL females (Fig. 6).

Discussion

We provide herein an integral characterization of the impact on female puberty of different conditions of nutritional distress during the early postnatal period. Of note, rat models of nutritional challenge during early postnatal life have been proposed to mimic conditions of metabolic perturbation during late gestation in humans (50). Indeed, a substantial component of neuroendocrine maturation, which in primates occurs in late intrauterine life, takes place during the early postnatal period in rodents (50, 61). Accordingly, rat models of nutritional manipulation during these postnatal stages are likely more appropriate to analyze conditions of neurohormonal malprogramming of eventual translational interest (50). To gain a mechanistic insight into the pubertal phenotypes observed, we focused our studies on the hypothalamic Kiss1 system, as essential element for the timing of puberty, sensitive to the activational regulatory effects of metabolic signals (37, 45, 46).

Although the impact of early nutritional or metabolic cues on the later development and functioning of the hypothalamic Kiss1 system has received limited attention to date, a very recent report suggested that intrauterine undernutrition impairs the rise of *Kiss1* mRNA levels at the hypothalamus during the pubertal transition (21). However, that study was focused on the effect of underfeeding during the gestation, a model that probably mimics metabolic insults during human midgestation (16), *i.e.* before completion of the organization of multiple neuroendocrine systems. Our present results complement and extend that previous study and unveil also interesting differences between these two models of perinatal subnutrition, which are discussed in detail below.

Postnatal subnutrition and puberty onset

Most of the rodent studies addressing the impact of early nutritional programming of puberty have focused on the consequences of undernutrition during pregnancy or

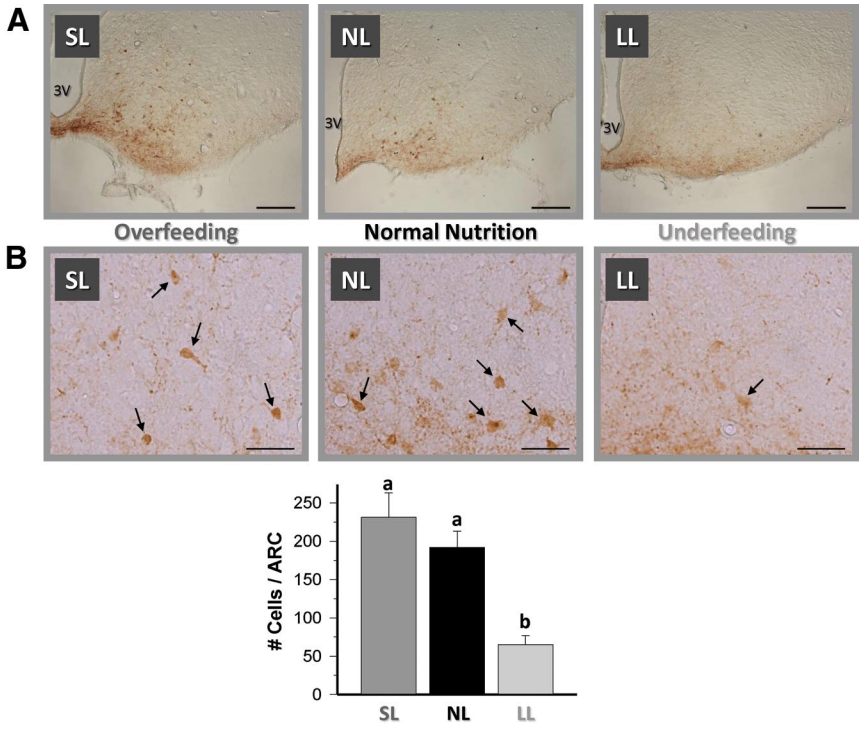

FIG. 4. IHC analysis of the number of Kp neurons (detected as cell bodies with discernible Kp-IR) in the ARC in the three experimental groups under analysis: SL (overnutrition), NL (normal nutrition), and LL (subnutrition). In the *upper panels*, representative photomicrographs of Kp-IR cell bodies in the ARC from SL, NL, and LL female rats at puberty are shown, at two different magnifications: Panels A (lower magnification) and B (higher magnification). In the latter, Kp-IR cell bodies are indicated with *arrows*. In addition, in the *lower panel*, quantification of the number of Kp-positive cells in the ARC is shown for each experimental group. Cell numbers are represented as the mean ± SEM. Groups with *different superscript letters* are statistically different ($P < 0.05$ by ANOVA followed by Student-Newman-Keuls multiple range test). *Scale bars*, 100 μm (*panels A*, lower magnification) or 50 μm (*panels B*, higher magnification). 3V, Third ventricle.

lactation (16, 18, 20–22). However, these studies remain scarce and have yielded contradictory findings. In most cases, gestational subnutrition or postnatal underfeeding have been reported to induce a variable delay in puberty, as monitored by the age of VO and/or first estrous cycle, as well as other indices of ovarian maturation (16, 20–22). However, overtly delayed VO has not been detected in all studies involving postnatal malnutrition due to large litter size (16, 18). Our results in LL rats are in keeping with the predominant literature and document an unambiguous delay of the age of VO, together with decreased uterus and ovarian weights at puberty, in females subjected to postnatal subnutrition. Interestingly, a recent study reported a slight advancement in the age of VO in the offspring of mothers submitted to 50% calorie restriction during lactation; yet, in that study, indirect evidence for some degree of postpubertal ovarian failure was also observed (19). It is possible that the impact of nutritional manipulations on the developmental programming of puberty might depend not only on the magnitude and timing of energy restriction, but also on the type/features of such challenge, because food deprivation of the lactating mother or the pups may associate with different covariables (50) with different impact on the onset of puberty.

The mechanism behind the above alterations is likely to involve disturbed development and/or expression of the hypothalamic Kiss1 system. Thus, relative *Kiss1* mRNA levels at the hypothalamus were notably decreased in peripubertal female rats submitted to postnatal undernutrition. Although our data did not allow us to discriminate the hypothalamic site (ARC *vs.* AVPV) where mRNA levels are actually decreased, they provide indirect evidence for the disruption by postnatal malnutrition of the increase in *Kiss1* expression reported to occur during normal pubertal maturation in the female rat. This phenomenon was analogous to that described recently in a model of intrauterine undernutrition (21), suggesting that both gestational and early postnatal conditions of energy deficit have a durable inhibitory impact on the expression levels of *Kiss1* mRNA in the hypothalamus.

As complement to RNA expression data, our anatomical IHC analyses demonstrated a marked suppression of the number of Kp-IR neurons in the ARC of LL rats at puberty, without changes in the number of Kp fibers at the periventricular area. The putative function of ARC Kiss1 neurons in the control of male and female puberty remains somewhat contentious. Nonetheless, *in situ* hybridization and quantitative RT-PCR analyses of specific hypothalamic regions have demonstrated a rise of *Kiss1* mRNA levels in the ARC during the pubertal transition in the rat (62, 63). Moreover, Kp-IR has been shown to increase in the ARC of male and female rats during puberty (53, 62). Importantly, *Kiss1* mRNA expression at the ARC is also sensitive to metabolic cues, as documented by its partial suppression in conditions of transient subnutrition at puberty (64). Thus, the decrease in Kp-positive neurons in the ARC of LL females may contribute to the delay in puberty onset in this model.

Previous studies demonstrated a marked increase in the number of Kp-IR neurons in the AVPV of female mice during postnatal maturation, especially between d 15 and d 30 postpartum (43, 44). Our IHC analyses, using the validated JLV-1 antiserum against rodent Kp-52 (52–54), intended to

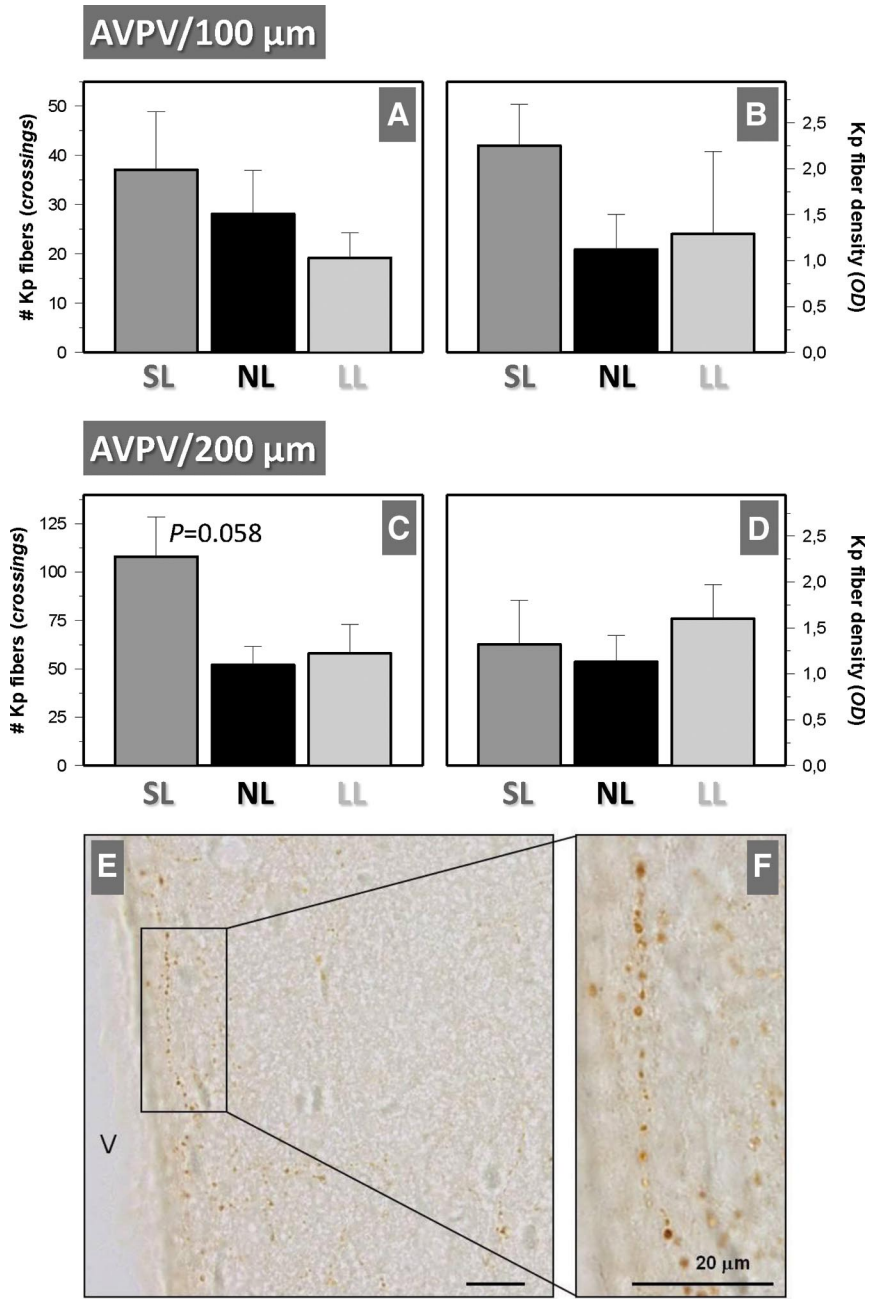

FIG. 5. In the *upper panels*, quantitative data from immunohistochemical analyses of the number and density of Kp-positive fibers in the hypothalamic periventricular region. The area of interest encompassed a section of up to 200 μm apart from the third ventricle. However, to provide a more thorough analysis of potential regional variations, additional quantitative analyses were specifically conducted in a subsection of the above area, spanning only the first 100 μm from the third ventricle. Two complementary quantitative analyses were conducted: 1) by counting the number of IR-fiber crossings (panels A and C); and 2) by measuring OD of the Kp-IR fibers (panels B and D), in the above hypothalamic areas of the three experimental groups: SL (overnutrition), NL (normal nutrition), and LL (subnutrition). For further details, see *Materials and Methods*. In addition to quantitative data, in the *lower panels*, representative photomicrographs of Kp-IR fibers and terminals in the same coronal section, containing the anterior periventricular region at the level of the preoptic nucleus and the AVPV, are shown from a NL female rat. Two different magnifications are presented in panels E and F. Most Kp-positive fibers possessed many boutons, were predominantly located near the ependyma of the third ventricle (3V), and displayed a ventrodorsal orientation. In panel C, $P = 0.058$ *vs.* NL group, (Student's *t* test). *Scale bar*, 20 μm.

evaluate IR cells in the ARC and AVPV regions (37, 38). However, only the ARC data were quantitatively analyzed, because very few Kp-IR cell bodies were detected in the AVPV. Considering the proven expression of *Kiss1* mRNA at the AVPV in rats (65, 66), the lack of detection of Kp-IR neurons at this site is intriguing. Nonetheless, we consider that this is not due to technical limitations of our approach, because we obtained similar results with another (universally used) antibody targeting rodent Kp-10 (Caraty's antiserum, ref. AC 566; data not shown). Moreover, a recent study in adult female rats confirmed that, in contrast to mice, Kp-IR cell bodies are not easily visualized in the rat AVPV regardless of the use of JLV-1 or AC-566 antiserum (67), unless colchicine is used, an approach that is likely to have an impact on quantitative IHC results. Indeed, the differential labeling of Kiss1 neurons between the ARC and AVPV in the rat might reflect physiological differences in the processing and/or secretory dynamics of mature Kp that warrant specific investigation.

The potential changes in responsiveness and sensitivity to Kp were also analyzed in LL rats. Acute fasting at puberty has been previously shown to augment gonadotropin responses to Kp-10 (59), a phenomenon that was associated to the lowering of Kp tone caused by energy deficit, which could induce a compensatory state of enhanced responsiveness. Albeit modest, a similar trend for higher gonadotropin responsiveness to Kp-10 was detected in pubertal LL female rats. In the above context, and considering our current expression/IHC data, it is tempting to propose that this moderate elevation of Kp responsiveness may reflect a proportional decrease of its basal hypothalamic tone in pubertal female rats after postnatal underfeeding.

Postnatal overfeeding and puberty onset

There was so far limited information regarding the impact of overfeeding

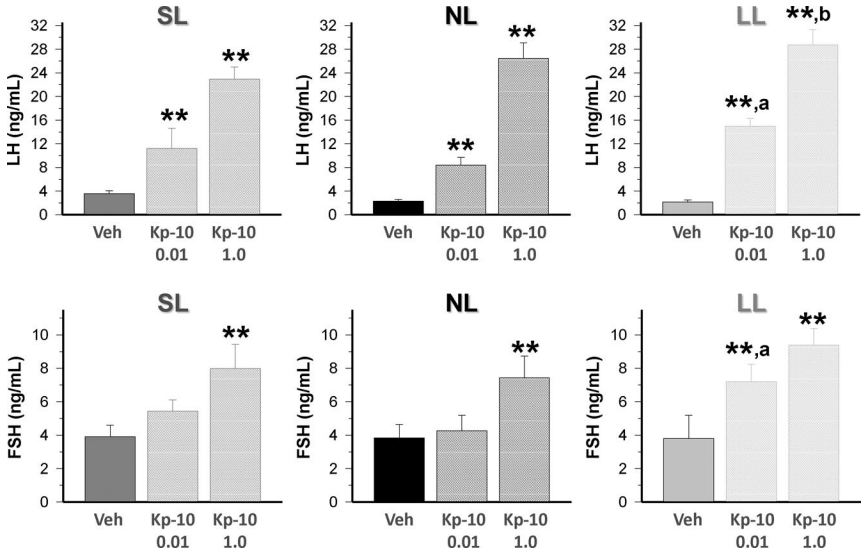

FIG. 6. Gonadotropin responses (LH, *upper panel*; FSH, *lower panel*) after central (icv) injection of a single bolus of Kp-10. Tests were conducted in 31-d-old female rats from one of the experimental groups: SL (overnutrition), NL (normal nutrition), and LL (subnutrition). Two doses of Kp-10 (0.01 and 1 nmol) were tested in icv tests. Hormonal data were assayed 15 min after icv injection of Kp-10; LH and FSH values are mean ± SEM of at least 10 independent determinations per group. **, $P < 0.01$ *vs.* corresponding control values (rats injected with vehicle); a, $P < 0.05$ *vs.* responses to a similar dose of Kp-10 in NL rats; b, $P < 0.05$ *vs.* responses to a similar dose of Kp-10 in SL rats (ANOVA followed by Student-Newman-Keuls multiple range test). Veh, Vehicle.

during early critical periods on the developmental programming of puberty. This, however, has become an issue of translational interest, given the rising incidence of obesity during human gestation and childhood. Of note, whereas our model of overfeeding during lactation had been extensively used to assess the effects of early overweight on the developmental programming of BW homeostasis and metabolism, to our knowledge this is the first report to characterize changes in the timing of puberty and the Kiss1/kisspeptin system in such conditions of early overfeeding. In keeping with a previous study involving maternal feeding on a high-fat diet during pregnancy and/or lactation (19), our model of postnatal overnutrition induced a persistent increase in BW gain and advanced the timing of puberty. These data demonstrate that persistent energy excess during early development might contribute to precocious pubertal activation, a phenomenon that, in face of the escalating incidence of child obesity, may contribute to the trends of earlier puberty onset reported recently, especially in girls (30, 31).

Our data in SL rats suggest that postnatal overfeeding is associated with elevated Kp tone in the hypothalamus at puberty, as evidenced by higher expression levels of *Kiss1* mRNA and the trend for a higher density of Kp-positive fibers in the anterior periventricular area of the hypothalamus. In addition, a nonsignificant 20% increase in the number of Kp-IR neurons in the ARC was detected in the overfed group. Considering that our IHC analysis of Kp-positive cell bodies does not allow quantification of discrete/continuous changes in Kp expression, but rather variations in the number of Kp-positive cells, it remains possible that the increase of Kp expression might have not been of sufficient magnitude as to result in a significant rise of IR cell bodies in the ARC. In addition, we detected some degree on interindividual variation (eventually linked to subtle changes in BW within the same group), which might have masked clearer changes between SL and NL groups. In the same vein, although a tendency for increased numbers of Kp fibers and density was observed in SL animals at this periventricular area, these changes displayed some regional and interindividual variability, which prevented them from reaching statistical significance. In any event, the functional relevance of such trends of increased Kp-IR is yet to be defined, in particular as to whether this leads to enhanced release of mature Kp, because changes in peptide content or even Kiss1 mRNA levels may not necessarily translate into equivalent fluctuations in neuropeptide release. Similarly, the origin, projections, and eventual neuronal targets of Kp fibers remain unknown and merits specific investigation. In this sense, solid evidence for the origin of the Kp fibers in the periventricular region is still missing, although it is most likely that they stem from the neurons in the vicinity. Notwithstanding these uncertainties, our expression data are compatible with an increase in the turnover of Kp synthesis and axonal transport in postnatally overfed animals. Such putative elevation of Kp tone is further supported by the observed increase of basal LH levels in SL females at puberty. However, such elevation does not appear to be associated with any detectable desensitization to further stimulation with Kp, in keeping with our previous data that pubertal female rats are less prone to desensitization than adult rats (68).

Mechanistic implications

A key issue emerging from our data are the underlying mechanisms for the observed changes in the timing of puberty. Previous studies on the metabolic control of puberty have mainly focused on the impact of acute nutritional stress, mainly subnutrition, or actual BW; analyses that have supported the contention that threshold energy (fat) stores are permissive for puberty (46). Nonetheless, limited attention has been paid to the eventual neurohor-

monal mechanisms for early developmental programming of puberty. Noteworthy, leptin has been shown to be a relevant neurotrophic factor during early postnatal life in rodents, promoting the development of hypothalamic neural pathways essential for BW homeostasis (69, 70). Such actions appear to be conducted by the postnatal surge of leptin, the timing of which is affected by perinatal malnutrition (71–73). Our current data make it tempting to propose that postnatal development of the Kiss1 system is also affected by metabolic cues, eventually including leptin, a phenomenon that may impact on the timing of puberty and its modulation by later challenges. Importantly, the different models of early nutritional challenge used to evaluate puberty induce also persistent modifications in BW gain after weaning, with the potential confounding influence of changes in fat stores and leptin levels at the time of puberty. However, detailed analysis of our results unveils potential interactions between developmental (early) influences and later changes in the modulation of puberty onset by BW and (putatively) leptin levels. With regard to the latter, however, it is stressed that a recent report failed to demonstrate a substantial role of Kiss1 signaling in mediating leptin effects on puberty in mice (48), although the use of models of congenital lack of leptin receptors in Kiss1 neurons does not allow us to exclude the possibility of indirect actions of leptin on Kp networks and/or the occurrence of compensatory phenomena during development.

In SL rats, early onset of puberty takes place at a BW and, presumably, leptin levels equivalent to those of control NL animals. This is illustrated by analogous values of BW at VO50 between these two groups. This would suggest that within the nutritional range from normal nutrition to overfeeding the timing of puberty may be the consequence of achieving threshold levels of BW and fat stores, in keeping with the original Frisch hypothesis (74); early developmental influences would operate mainly as durable modifiers of postnatal BW gain. In this sense, maternal obesity has been described to amplify the neonatal surge of leptin in the offspring, with deferred influences in terms of leptin resistance and perturbation of key hypothalamic networks, such as AgRP, which may lead to hyperphagia and obesity (72). Whether these early alterations of the postnatal surge of leptin also affect (directly or indirectly) the developmental maturation of the hypothalamic Kiss1 system merits further investigation.

In clear contrast, BW values at VO50 in animals subjected to postnatal undernutrition were substantially lower (>20%) than those in the control NL group. Similarly, leptin levels in LL rats were persistently decreased throughout the pubertal transition, with values in 36-d-old LL females being approximately half of those in control NL rats on d 31. In the same vein, pubertal maturation of the hypothalamic Kiss1 system, as estimated by *Kiss1* mRNA levels and number of ARC Kp-IR cells, was severely impaired by postnatal subnutrition. Yet, approximately 60% of LL females displayed VO on d 37 postpartum. The above data would suggest that early malnutrition resets the permissive threshold levels of BW (and eventually leptin), allowing pubertal maturation even in the face of suboptimal metabolic conditions. Indeed, puberty took place in LL rats earlier than it would be predicted on the basis of their BW/leptin levels. This adaptative response is reminiscent of other catch-up manifestations of developmental malprogramming associated with early subnutrition (19). It is tempting to propose, although yet to be proven, that the mechanism for pubertal changes in the expression of Kiss1 system in postnatally underfed rats may involve perturbations in the shape and trophic actions of the postnatal surge of leptin.

As a final note, early nutritional manipulations in rodents have been shown to disturb the development of several hypothalamic pathways involved in energy homeostasis that might have an impact of the Kiss1 system as well. For instance, perinatal under- and overfeeding has been shown to persistently alter hypothalamic neuropeptide Y expression (49), neuropeptide Y being a putative regulator of *Kiss1* expression (75). Similarly, altered metabolic responses to melanin-concentrating hormone and melanocortins (α-MSH) have been described in postnatally overfed rats; melanin-concentrating hormone and α-MSH have been shown to affect Kp responsiveness and *Kiss1* mRNA expression in mice and sheep, respectively (76, 77). In addition, peripheral hormones other than leptin, such as ghrelin, with potential effects on *Kiss1* expression and puberty (78, 79), might also be altered in our models of early nutritional challenge and, thus, could contribute to the observed effects in terms of pubertal timing reported herein. Finally, it is noted that manipulations of litter size may have an impact on additional hormonal signals (*e.g.* sex steroids) and neuroendocrine systems (*e.g.* the adrenal/stress axis), that might have contributed to part of the changes in the maturation of the hypothalamic Kiss1 system and the timing of puberty reported herein (80, 81).

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