Blockage of the neonatal leptin surge affects the gene expression of growth factors, glial proteins and neuropeptides involved in the control of metabolism and reproduction in peri-pubertal male and female rats

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Leptin is important in the development of neuroendocrine circuits involved in metabolic control. As both leptin and metabolism influence pubertal development, we hypothesized that early changes in leptin signaling could also modulate hypothalamic systems involved in reproduction. We previously demonstrated that a single injection of a leptin antagonist on postnatal day (PND) 9, coincident with the neonatal leptin peak, induced sexually dimorphic modifications in trophic factors and markers of cell turnover and neuronal maturation in the hypothalamus on PND13. Here our aim was to investigate if the alterations induced by leptin antagonism persist into puberty. Accordingly, male and female rats were treated with a pegylated super leptin antagonist from PND5 to 9 and killed just before the normal appearance of external signs of puberty (PND 33 in females and PND 43 in males). There was no effect on body weight, but in males food intake increased, subcutaneous adipose tissue decreased and hypothalamic NPY and AgRP mRNA levels were reduced, with no effect in females. In both sexes, the antagonist increased hypothalamic mRNA levels of the kisspeptin receptor, Gpr54. Expression of the leptin receptor, trophic factors and glial markers were differently affected in the hypothalamus of peri-pubertal males and females. Leptin production in adipose tissue was decreased in antagonist treated rats of both sexes, with production of other cytokines being differentially regulated between sexes. In conclusion, in addition to the long-term effects on metabolism, changes in neonatal leptin levels modifies factors involved in reproduction that could possibly affect sexual maturation.

The 16-kDa protein leptin, produced mainly by adipocytes, is a pleiotropic hormone involved in many physiological processes (1–3). Not only is it important in central regulation of body weight and food intake (4–6), but leptin also has important developmental effects. In fact, there is a postnatal leptin surge in rodents, beginning around postnatal day (PND) 5 in males and peaking between PND9 and 10 (7), that has been implicated in hypothalamic development by modifying neuronal outgrowth and synaptic connectivity, as well as neurogenesis

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Abbreviations:

and neuronal and glial survival (8). Numerous studies have analyzed the importance of leptin in metabolism (9, 10), the immune response (11, 12) and reproduction (13, 14), especially in adulthood, but there are few studies concerning the effects of early changes in leptin levels on peri-pubertal development.

It has recently become apparent that leptin has important effects on the kisspeptin system (15). Kisspeptins are a family of structurally related peptides encoded by the KISS1/Kiss1 gene that act through binding and subsequent activation of the G protein-coupled receptor, GPR54, also termed Kiss1R (16). This system is mainly expressed in the hypothalamus and is essential in the control of puberty and reproduction (17). In fact, changes in leptin levels are known to affect Kiss1/kisspeptin expression in the hypothalamus, suggesting that at least part of leptin's effects on the central reproductive axis occur through this neuronal network, either directly or indirectly (15); however, whether there are effects of the neonatal leptin surge on this system remains unclear. In addition, other neuropeptide systems are suggested to interact with kisspeptins in the central control of puberty and reproduction. Among these, considerable interest has recently focused on the putative roles of the gonadotropin-inhibitory hormone, RFRP, which has been proposed to oppose the stimulatory effects of kisspeptins in the central control of the HPG axis (18). Yet, the effect of leptin, and specifically the neonatal leptin surge, on the maturation and function of this system remains completely unexplored. We have previously shown that disrupting the leptin surge in Wistar rats with a pegylated leptin antagonist on PND9 results in multiple changes in hypothalamic levels of trophic factors, neuropeptide expression, receptor levels, cell turnover and cell specific markers up to at least PND13 (19). We hypothesized that this early modification in leptin signaling has diverse effects on hypothalamic neuronal circuits throughout postnatal life and could affect the normal physiological changes in neuroendocrine events during the peri-pubertal period. Thus, in the present study we analyzed how blockage of the postnatal leptin surge affects the expression of neuronal trophic factors, metabolic function, and hypothalamic expression of anorexic and orexigenic neuropeptides and the kisspeptin system in the peri-pubertal period.

Materials and Methods

Animals

Adult Wistar rats were purchased from Harlan Interfauna Ibérica S.A. (Barcelona, Spain) and allowed to acclimate for 2 weeks before mating. One male was placed in a cage with two females for 10 days. On the day of birth (PND0), litters were culled to eight pups per dam (4 males and 4 females). No cross-fostering was employed. In all experimental groups at least three different litters were used to reduce the litter effect, with a total of 12 rats in each experimental group.

Rats were maintained at a constant temperature (22 ± 1°C) and humidity (50 ± 2%) in a reversed 12-hour light-dark cycle (red light on at 08:00 and white light on at 20:00). Pregnant rats were given free access to rat chow (commercial diet for rodents; A03, Safe, Augy, France) and water. As our aim was to study the pubertal transition, right before the appearance of external phenotypic signs of puberty, and because the timing of puberty differs between the sexes, females were killed on PND33 and males on PND43. In our animal facility the normal age of vaginal opening (VO) is between 32–35 days of age. In males balano preputial separation (BPS) normally occurs between days 42–44. Thus, all animals were monitored daily to assure that they did not display these external signs of puberty. This suggests that the control animals were approaching VO or BPS and that treatment with the leptin antagonist did not advance this process, at least not significantly by this external sign. However, when an individual approaches puberty, one would also expect an increase in gonadal size and an increase in the pulsatile secretion of gonadotropin and sex steroids. Whether these processes are delayed by blocking leptin signaling during early development cannot be determined from this experimental paradigm.

These studies were approved by the local ethics committee and complied with the Royal Decree 1201/2005 (BOE n° 252) pertaining to the protection of experimental animals and with the European Communities Council Directive (86/609/EEC).

Leptin antagonist treatment

From PND5 until PND9 rats were injected *sc* with 5 mg/kg bodyweight (bw) of rat pegylated super leptin antagonist (mutant D23L/L39A/D40A/F41A). The material was a gift of Protein Laboratories (Rehovot, Israel) and was prepared as described in Jamroz-Winiewska et al (20). The animals received one injection per day at 9:00. Control rats were injected with the same volume of distilled water as vehicle (2.5 ml/kg). After each injection the animals were immediately returned to their mothers.

Weight, food and pubertal control

All rats were left undisturbed until weaning at PND22 at which time they were separated into 4 rats of the same sex/cage. Body weight was monitored weekly until 5 days before sacrifice. Food intake was measured until 5 days before sacrifice by placing a known amount of food in each cage and measuring the remaining amount at the same time the following week. Puberty was monitored by checking daily for VO or BPS. No animal used in the study had external phenotypic signs of puberty. The experimental design is shown in Figure 1. Each experimental group consisted of 12 rats.

Tissue collection

On PND33 for females and PND43 for males, rats were killed after a 12h fast by rapid decapitation. The brain was rapidly removed and the hypothalamus (HT), hippocampus (HC) and pituitary were dissected. The hypothalamus, defined rostrally by the optic chiasm and caudally by the anterior margin of the mam-

millary bodies, was dissected out with a depth of approximately 2 mm. Each hypothalamic block was immediately divided in the coronal plane using the optic chiasm as the boundary, as previously described (21). The anterior dissection (rostral hypothalamus; HTr) contains the rostral periventricular area of the third ventricle (RP3V), while the posterior dissection (medial-basal hypothalamus; MBH) includes the entire ARC. As a control for the amount of tissue dissected, hypothalamic tissue blocks were weighed. No significant differences in hypothalamic tissue block weight were noted between comparable experimental groups. In addition, the gonads and subcutaneous (inguinal), visceral (retroperitoneal) and brown adipose tissue were dissected and weighed. Tissue was then frozen in liquid nitrogen and stored at −80° until processed.

Estradiol-17β and testosterone ELISAs

Plasma estradiol levels were determined by using an ELISA from Enzo (Farmingdale, NY, USA). This assay has a sensitivity of 14.0 pg/ml and interand intra-assay coefficients of variation of 8.3% and 2.1%, respectively.

Plasma testosterone levels were determined by using an ELISA from Cusabio (Wuhan, P.R. China). This assay has a sensitivity of 0.06 ng/ml and interand intra-assay coefficients of variation of $< 15\%$.

All samples were run in duplicate and within the same assay for all analyses. For each assay absorbance in each well was measured by using a Tecan Infinite M200 (Grodig, Austria) and serum concentrations were calculated from the standard curve.

Corticosterone radioimmunoassay (RIA)

Corticosterone was measured using a solid phase I125 RIA (Immunochem Double Antibody Cort; MP Biomedicals, Illkirch, France). Detection limit was 7.7 ng/ml and the interand intra-assay coefficients of variation were $< 10\%$.

Multiplexed magnetic bead immunoassay

Leptin, insulin and interleukin 6 (IL6) were measured in duplicate by a multiplexed magnetic bead immunoassay kit (Millipore Corporation) as previously described (22). Briefly, beads conjugated to the appropriate antibodies and serum samples (25 μl each) were incubated over night at 4°C with shaking. Wells were washed three times using a wash buffer and antibody conjugated to biotin (50 μl) was added. After incubation for 30 minutes at room temperature with shaking, beads were incubated during 30 minutes with 50 μl streptavidin conjugated to phycoerythrin. Beads were analyzed in the Bio-Plex suspension array system 200. Raw data (mean fluorescence intensity) were analyzed using the Bio-Plex Manager Software 4.1 (Bio-Rad Laboratories).

Quantitative real-time PCR

Total RNA from 50–100 mg of hypothalamus or hippocampus tissue was isolated using TRIsure Reagent (Bioline, London). High Capacity cDNA Reverse Transcription kits (BioRad Laboratories, Hercules, CA) were used according to the manufacturer's protocol on a iCycler iQ PCR Thermal Cycler (BioRad) to transcribe 0.5 μg total RNA isolated from each tissue.

Aliquots of the resulting cDNA samples were amplified by PCR with specific oligonucleotide primer pairs designed to span intron/exon borders. The sequence of the primer pairs used to study the Kiss1 system in the hypothalamus were: 1) rat Kiss1, forward 5′ GCT GCT GCT TCT CCT CTG TG 3′ and reverse 5′GCA TAC CGC GGG CCC TTT T 3′; 2) rat Gpr54, forward 5′GCC ACA GAT GTC ACT TTC CTT C 3′ and reverse 5′GCC ACA GAT GTC ACT TTC CTT C 3′; 3) rat RFRP, forward 5′ AAT CCC TGC ACT CCC TGG CCT 3′ and reverse 5′AAG GAC TGG CTG GAG GTT TCC 3′; and 4) rat NPFFR1, forward 5′ AAC CGG CAC ATG CGC ACT GTC 3′and reverse 5′ GAC ATG CCC TGC ACC AAG CCG 3′. Amplification of S11, forward 5′ CAT TCA GAC GGA GCG TGC TTA C 3′ and reverse 5′ TGC ATC TTC ATC TTC GTC AC 3′ served as control for these RT-PCR reactions. Amplification of the cDNA template was performed using 1x iQ Supermix and SYBR green for each detected gene (Promega, Wisconsin, USA). According to manufacturer's guidelines, the $\Delta\Delta$CT method was used for relative quantification. Statistics were performed using ΔCT values.

For the remaining RNA studies a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) was used according to the manufacturer's protocol on a Peltier thermal Cycler Tetrad2 (BioRad) for RT-PCR was used to transcribe 2 μg total RNA isolated from each individual tissue. Amplification of the cDNA template was performed with an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) using TaqMan Universal PCR Master Mix (Applied Biosystems) and TaqMan Gene Expression Assay Mix kits for each detected gene (Applied Biosystems). The commercial reference for each predesigned gene expression assay is as follows: In the hypothalamus we analyzed the metabolic neuropeptides neuropeptide Y (NPY, Rn01410145_m1), proopiomelanocortin (POMC, Rn00595020_m1), Agouti-related peptide (AgRP; Rn014311703_g1), cocaine-and amphetamine-regulated transcript (CART; Rn00567382_m1) and orexin (Rn00565995_m1). To determine if the sensitivity to leptin was affected, expression of the leptin receptor (LepR; Rn01433250_m1) and suppressor of cytokine signaling 3 (SOCS3; Rn00585674_s1) were analyzed.

As glial cells are involved in pubertal onset (23–25), and their development seems to be modulated by leptin (26, 27), the glial cell markers vimentin (RN00579738_m1) and glial fibrillary

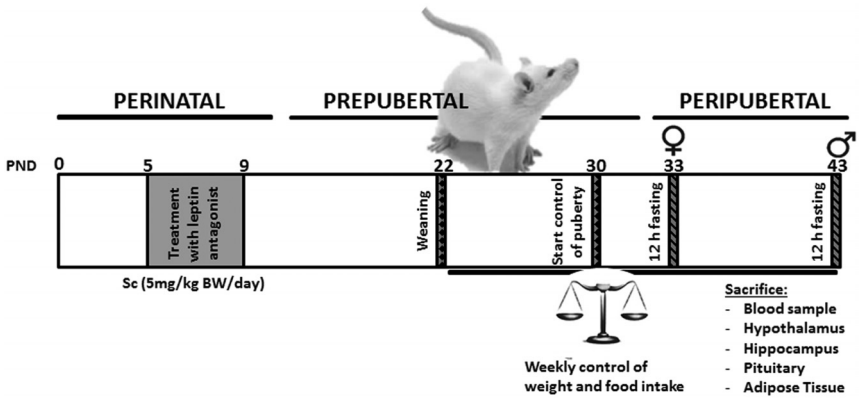

Figure 1. Diagram of the experimental design.

acidic protein (GFAP; Rn00566603), were measured. The trophic factors brain-derived neurotrophic factor (BDNF, Rn01484924_m1) and insulin-like growth factor 1 (IGF1, Rn99999087_m1) and its - receptor (IGF1R, Rn01477918_m1) were also analyzed in the hypothalamus.

In the pituitary the gonadotropins follicle-stimulating hormone (FSH) (βFSH, Rn01484594_m1) and luteinizing hormone (LH) (βLH, Rn00563443_g1) were determined.

We studied whether the production of leptin (Lep, Rn00565158_m1) or its receptor is altered in subcutaneous white adipose tissue (WAT). To determine if other cytokines were affected in WAT the proinflammatory cytokine tumor necrosis factor (TNF)-alpha (TNF-α, Rn01525859_g1) and anti-inflammatory cytokine interleukin-10 (IL10, Rn00563409_m1) were chosen for analysis. To determine if thermoregulation was affected mitochondrial uncoupling proteins 1 (Rn00562126_m1) and 2 (Rn00571166_m1) were analyzed in brown adipose tissue (BAT).

Various housekeeping genes were analyzed in each tissue and the gene that showed no variation between groups was chosen to normalize the data in that tissue. Results were normalized to GAPDH (Rn99999916_s1) in the hypothalamus, actin (Rn00667869_m1) in pituitary and 18S (Rn01428915_g1) in adipose tissue. According to manufacturer's guidelines, the $\Delta\Delta$CT method was used for relative quantification. Statistics were performed using ΔCT values.

Data analysis

Data were analyzed by using SPSS for Windows, version 15.0. Normality was checked by Shapiro-Wilks's test ($P > .05$). Data were analyzed by Student's *T* test. As males and females were of a different chronological age due to the differential timing of the pubertal process, data were not directly compared between sexes.

Results

Body weight gain, accumulated food intake and fat mass

In males, antagonist treatment had no effect on body weight gain (Figure 2A), but increased accumulated food intake ($P < .05$; Figure 2B) and decreased subcutaneous WAT ($P < .05$; Figure 2C). The amount of visceral WAT (Vh 0.62 ± 0.06 g, Antag 0.60 ± 0.04 g) and BAT in males (Vh 0.39 ± 0.03 g, Antag 0.37 ± 0.01 g) was unaffected by the antagonist treatment. Neonatal leptin antagonist treatment resulted in an increase in mean testicular weight ($P < .05$; Figure 2D).

There was no effect on body weight, food intake or subcutaneous WAT in peri-pubertal females (Figure 2 E-G). Likewise, agonist treatment had no effect on the amount of visceral WAT (Vh 0.44 ± 0.02 g, Antag 0.38 ± 0.04 g) or BAT (Vh 0.23 ± 0.02 g, Antag 0.23 ± 0.01 g). Although ovarian weight tended to increase, this change was not significant (Figure 2H).

Serum hormone levels

Serum insulin, leptin, IL6, and corticosterone levels were not modulated by neonatal leptin antagonist treatment in peripubertal males or females (Table 1).

Leptin antagonist treatment increased serum estradiol levels in females ($P < .05$). Testosterone levels were unaffected in males (Table 1).

Hypothalamic mRNA levels

Genes related to cell turnover

In males the mRNA levels of vimentin, which is expressed in tanycytes and proliferating and activated astrocytes, tended to increase in the HTr (Figure 3A), but this was not statistically significant due to the high variability in the data. There was no effect in the MBH. The mRNA levels of the astrocytic intermediate filament GFAP increased in the hypothalamus of male rats (Figures 3C), with this increase being significant in the HTr ($P < .05$).

Vimentin mRNA levels were increased in the MBH of females ($P <$

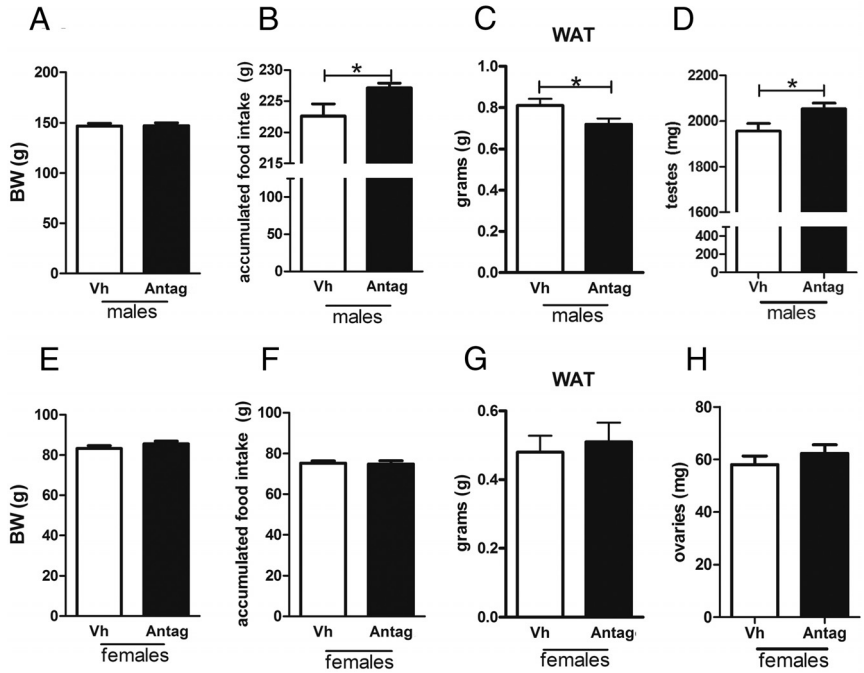

Figure 2. A, Mean body weight (BW), (B) accumulated food intake per rat from the time of weaning until 5 days before, (C) subcutaneous white adipose tissue (WAT) (D) and testicular weight at sacrifice in male rats treated with a leptin antagonist (Antag) or vehicle (Vh) from postnatal day (PND) 5 until PND9 (n = 12). E, Mean BW, (F) food intake, (G) WAT and (H) ovarian weight in female rats treated with a leptin antagonist. Student's t: * $P < .05$.

Table 1. Circulating hormone levels

	Vh males	Antag males	Vh females	Antag females
IL6 (pg/ml)	77.4 ± 13.6	72.3 ± 14.5	103.3 ± 37.5	123.2 ± 38.4
Insulin (pg/ml)	209.5 ± 94.2	488.8 ± 191.3	350.7 ± 131.3	612.3 ± 326.1
Leptin (ng/ml)	1.2 ± 0.2	1.2 ± 0.2	0.9 ± 0.1	0.9 ± 0.1
Corticosterone (ng/ml)	594.5 ± 59.3	505.2 ± 45.3	677.1 ± 143.2	587.8 ± 45.7
Estradiol-17β (pg/ml)			36.5 ± 12.8	145.2 ± 41.6*
Testosterone (ng/ml)	2.1 ± 0.4	2.3 ± 0.5		

Data are expressed as mean ± SEM from rats treated with vehicle (Vh) or with leptin antagonist treatment (Antag) from postnatal day (PND) 5 to PND9. $n = 8$ per experimental group. Student's t: *$P < 0.05$

.03; Figure 3B), with the rise in the HTr not being statistically significant. In females GFAP mRNA levels were unaffected (Figures 3D).

Neonatal treatment with the leptin antagonist modified the expression of important hypothalamic trophic factors during the peri-pubertal period. In males the mRNA levels of BDNF (Figure 3E) were higher in the HTr ($P < .05$) and lower in the MBH ($P < .03$) of antagonist treated rats compared to controls. The mRNA levels of IGF1 (Figures 3G) and IGF1R (Figures 3I) were unaffected.

In females BDNF levels were unaffected (Figures 3F). IGF1 mRNA levels were increased in the MBH ($P < .02$), while the increase in the HTr did not reach statistical significance (Figure 3H). In contrast, mRNA levels of the IGF1R were increased in the HTr in antagonist treated females (Figure 3J; $P < .01$).

Genes related to metabolic control

Regarding neuropeptides involved in food intake, in the MBH the mRNA levels of NPY ($P < .02$) and AgRP ($P < .05$) were decreased in males, with no effect on POMC, CART or orexin (Table 2). In males, the inhibitor of leptin signaling SOCS3 was unaffected (Vh 100 ± 7.2, Antag 116.7 ± 20.9% Vh) but there was an anatomically specific effect on LepR mRNA levels. LepR mRNA increased in the HTr (Figure 4A; $P < .04$) and decreased in the MBH (Figure 4B; $P < .04$).

In females, we found no effect on NPY, AgRP, POMC, CART, orexin (Table 2) or SOCS3 (Vh 100 ± 9.6%, Antag 137.5 ± 18.6% Vh) mRNA levels. LepR mRNA levels increased in the HTr (Figure 4C; $P < .002$), but were unaffected in the MBH (Figure 4D).

Puberty related genes

In males there was no significant effect on Kiss1 mRNA levels in the HTr or MBH (Table 2). In contrast, neonatal treatment with the leptin antagonist increased the mRNA levels of the Kiss1 receptor Gpr54 (Figure 4E, F and I) in HTr ($P < .02$), MBH ($P < .003$) and hippocampus ($P < .04$). The mRNA levels of the inhibitor of the gonadal axis Rfrp (Figure 4J) and of Npff1r (Figures 4 M and N) were unaffected.

In females there was no effect on Kiss1 (Table 2) or Npff1r (Figures 4O and P) mRNA levels. However, the mRNA levels of the Kiss1 receptor Gpr54 were increased by the antagonist in the HTr (Figure 4G; $P < .004$), MBH (Figure 4H; $P < .03$) and hippocampus (Figure 4K; $P < .03$). The mRNA levels of Rfrp were increased by the antagonist in the MBH (Figure 4L; $P < .05$).

Pituitary gonadotropins

In males, pituitary mRNA levels of βFSH (Vh 100 ± 29, Antag 110.9 ± 24.4% Vh) and βLH (Vh 100 ± 12.7, Antag 93.8 ± 19.3% Vh) were unaffected.

In females βFSH mRNA levels were increased by leptin antagonist treatment (Vh 100 ± 10.9, Antag 338.5 ± 74.3% Vh), with no effect on βLH mRNA levels (Vh 100 ± 27.3, Antag 96 ± 16.7% Vh).

Adipose tissue

In peri-pubertal males neonatal treatment with leptin antagonist reduced the mRNA levels of leptin in subcutaneous WAT (Figure 5A; $P < .03$), but had no effect on LepR (Figure 5C), IL10 (Figure 5E) or TNFα (Figure 5G) mRNA levels.

In peri-pubertal females, leptin antagonist treatment reduced the mRNA levels of IL10 (Figure 5F. $P < .02$) and TNFα (Figure 5H. $P < .05$) in subcutaneous WAT of females rats, with the decreases in Lep (Figure 5B) and LepR (Figure 5D) not reaching statistical significance.

In BAT there was no effect on the mRNA levels of UCP1 in males (Vh 100 ± 11.2, Antag 85.7 ± 13.4% Vh) or females (Vh 100 ± 19.7, Antag 129.2 ± 11.6% Vh) or UCP2 in males (100 ± 7.5, Antag 98.3 ± 7.7% Vh) or females (Vh 100 ± 9.8, Antag 135.7 ± 13.5% Vh).

Discussion

Leptin is a key factor in the development of metabolic circuits (4), and modification of the neonatal leptin surge

has been shown to have long-term effects on metabolism (28, 29). Here we show that blockage of the neonatal leptin surge not only alters metabolic neuropeptide expression during the peri-pubertal period, but also affects the expression of elements of the kisspeptin system and related signals (namely, RFRP), as well as neurotrophic factors and glial cell markers in the hypothalamus. Thus, it is conceivable that neonatal leptin levels could also be involved in the programming of reproductive neuronal circuits and possibly pubertal onset. Indeed, a rise in circulating leptin levels is thought to be involved in the advancement of pubertal onset observed in rats with increased prepubertal adiposity (29, 31). However, in this study we were unable to determine the effect on pubertal onset as rats were killed close to, but before external signals of puberty were detected in order to limit the effect of increased gonadal steroid secretion. Moreover, this resulted in males and females of different ages being used in these studies, as the temporal development of puberty differs between the sexes, and limiting the direct comparison between sexes.

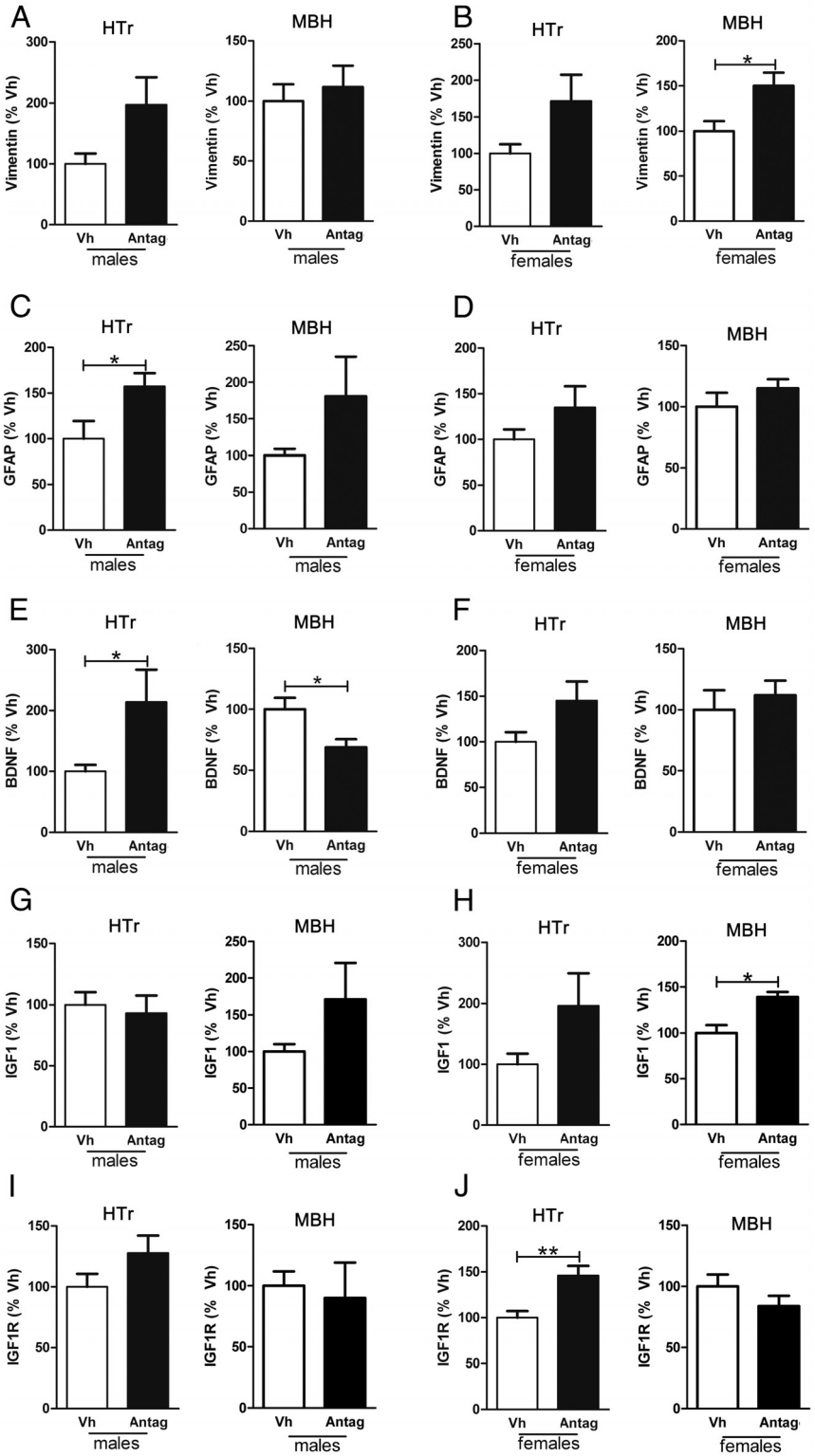

Figure 3. Levels in the rostral (HTr) and medial-basal (MBH) hypothalamus of the mRNAs encoding (A & B) vimentin, (C & D) glial fibrillary acidic protein (GFAP), (E & F) brain derived neurotrophic factor (BDNF), (G & H) insulin-like growth factor (IGF)1 and (I & J) IGF1 receptor (IGF1R) in male (left panel) and female (right panel) rats treated with a leptin antagonist (Antag) or vehicle (Vh) from postnatal day (PND) 5 until PND9 (n = 5–6). Student's t: $*P < .05$.

We have previously shown that blockage of the leptin peak at PND9 modifies hypothalamic expression of neurotrophic factors and markers of cell turnover up to 4 days later in a sexually dimorphic manner, with females being more affected than males (19). Here we show that neurotrophic factor levels continue to be affected even during the peri-pubertal period of both sexes. At PND13 BDNF mRNA levels were increased in the MBH of females and unaffected in males (21). However, in males approaching pubertal onset this neurotrophic factor was increased in the rostral hypothalamus and decreased in the MBH after neonatal antagonism of leptin signaling, with no significant effect on IGF1 or its receptor. In peri-pubertal females early leptin antagonism increased the mRNA levels of IGF1 in the MBH and of the IGF1 receptor in the

Table 2. Neuropeptide mRNA levels in the medial-basal (MBH) and rostral (HTr) hypothalamus.

Neuropeptide	Vh males	Antag males	Vh females	Antag females
NPY (MBH)	100 ± 5.9	70.5 ± 7.5**	100 ± 20.8	108.4 ± 11.2
AgRP (MBH)	100 ± 13.6	56.9 ± 12.6*	100 ± 14.3	92.7 ± 9.7
POMC (MBH)	100 ± 17.3	73.9 ± 15.9	100 ± 23.1	126.9 ± 13.7
CART (MBH)	100 ± 8.5	71.4 ± 11.6	100 ± 15.6	129.4 ± 16.1
Orexin (MBH)	100 ± 9.6	86.7 ± 18.9	100 ± 20.1	147.8 ± 20.9
Kiss1 (MBH)	100 ± 8.3	112.8 ± 14.6	100 ± 8.7	114.9 ± 7.6
Kiss1 (HTr)	100 ± 8.3	198.9 ± 49.7	100 ± 14.1	110.0 ± 13.7

Data are expressed as mean ± SEM from rats treated with vehicle (Vh) or with leptin antagonist treatment (Antag) from postnatal day (PND) 5 to PND9. Data are normalized to Vh treated rats for each sex. $n = 5–6$ per experimental group. Student's t: $*P < 0.05$, $**P < 0.01$.

rostral hypothalamus. At PND13 IGF was unaffected in either sex (21). Thus, there appears to be a time-dependent, anatomical and sex specific effect of neonatal leptin blockage on these two neurotrophic factors and this could have an important impact on puberty associated neurogenesis (30). Although there was a clear sexually dimorphic effect at PND13 (21), here the differences observed between peri-pubertal males and females could be due to the fact that they were killed at different ages.

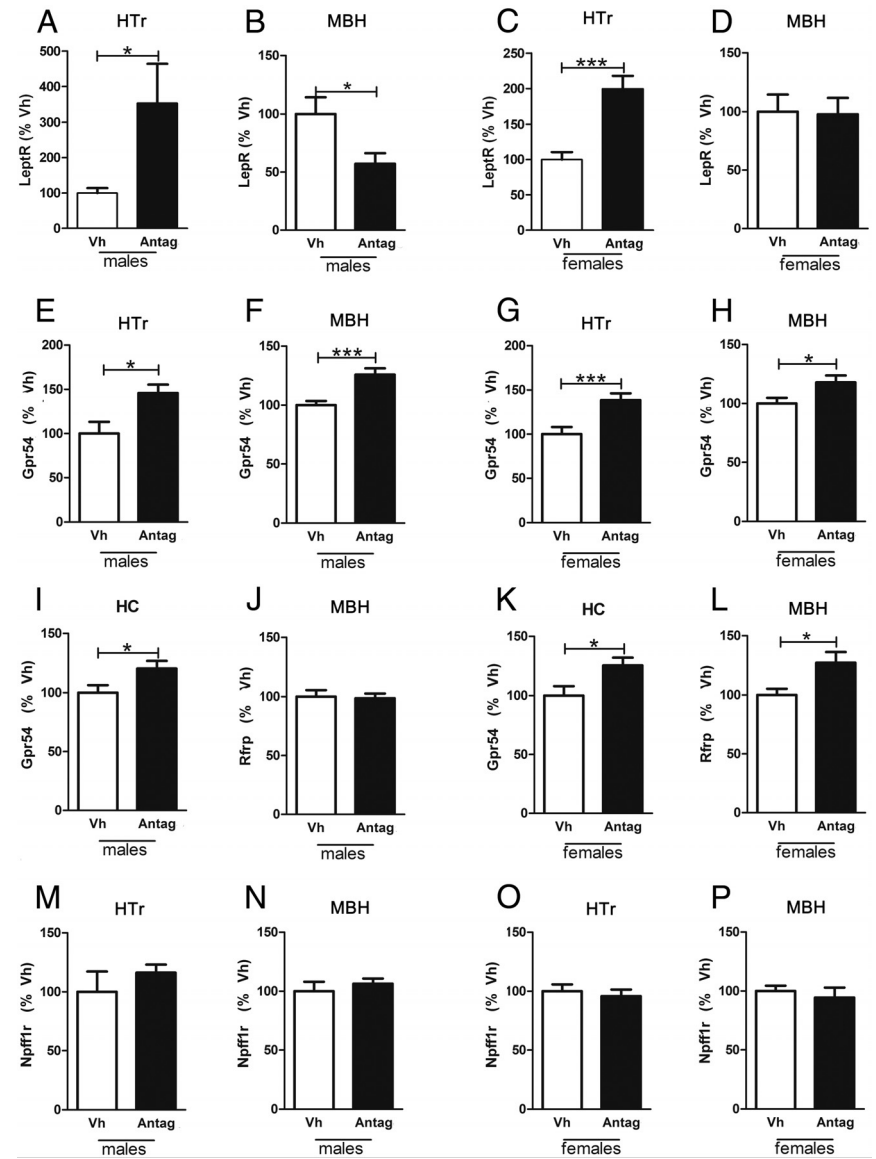

Figure 4. Levels of the mRNAs encoding (A-D) leptin receptor (LepR), (E-I, K) Kiss1 receptor (*Gpr54*), (J & L) RFamide-related peptide (*Rfrp*) and (M-P) neuropeptide FF receptor 1 (*Npff1r*) in the rostral (HTr) and medial-basal (MBH) hypothalamus and hippocampus (HC) of male (left panel) and female (right panel) rats treated with a leptin antagonist (Antag) or vehicle (Vh) from postnatal day (PND) 5 until PND9 (n = 5–12). Student's t: $*P < .05$, $***P < .005$.

One potential caveat that must be taken into consideration is that the males and females used in this study were of different chronological ages as pubertal onset occurs earlier in females than in males. Indeed, it is impossible to directly compare puberty and its associated processes between the sexes. Not only is pubertal onset sex dependant, but the reproductive axis, including at the hypothalamic level, is innately sexually dimorphic. Thus, even comparisons between males and females of the same age must be interpreted with caution. This does not negate the fact that blockage of neonatal leptin signaling affects the expression of important neurotrophic factors during this critical period close to pubertal onset in both sexes. Interestingly, glial cell markers were also modulated at this time-point by neonatal leptin blockage. As glial cells play an important role in puberty (31) and metabolic control (32), some of the long-term neuroendocrine effects due to changes in neonatal nutrition and

leptin levels, including the timing of pubertal onset (33), could be mediated through glial cells. This possibility deserves further investigation. Indeed, analysis of the anatomical specificity of the changes in glial cells, as well as neurotrophic factors, is of great interest and necessary in order to associate these changes with specific neuroendocrine functions.

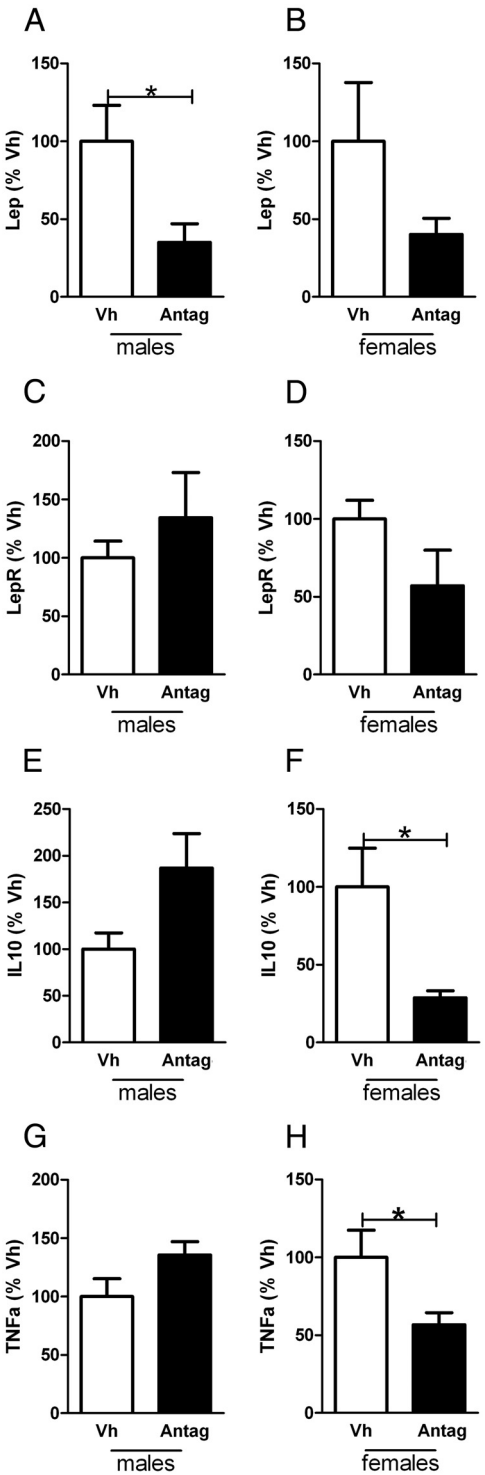

Figure 5. Levels of the mRNAs encoding (A & B) leptin (Lep), (C & D) leptin receptor (LepR), (E & F) interleukin 10 (IL10), and (G & H) TNFα in subcutaneous white adipose tissue in male (left panel) and female (right panel) rats treated with a leptin antagonist (Antag) or vehicle (Vh) from postnatal day (PND) 5 until PND9 (n = 5–6). Student's t: $*P < .05$; $**P < .01$.

Modifications in early nutritional status can disrupt the normal development of central reproductive circuits and alter pubertal timing (33–37) and the results reported here suggest that some of these effects are possibly due to changes in neonatal leptin levels. The Kiss1/Gpr54 system is essential for stimulating pubertal onset and reproductive function (15), while the more recently described Rprp/Npff1r system may participate in these functions as a gonadotropin inhibitor (16). Blockage of the neonatal leptin surge increased the peri-pubertal levels of Gpr54 mRNA in both sexes with no detectable effect on Kiss1 mRNA levels. Leptin can stimulate the Kiss1/GPR54 system in neuroblasts in vitro (38) suggesting that early leptin exposure could have an organizational effect on the expression of this receptor, and possibly modulate the responsiveness of the system to kisspeptins. Gpr54 was also found to be increased in the hippocampus of both sexes, where it is highly expressed in granule cells of the hippocampal dentate gyrus and is correlated with plasticity and could possibly influence cognition (39). As mentioned above, it remains to be determined if blockage of leptin signaling at PND9 actually affects pubertal timing since this study was terminated immediately before completion of pubertal maturation as our objective was to study molecular events at the time preceding/leading to puberty. However, although ovarian weight was not significantly affected, a trend for increased ovarian weight was detected and there was a rise in serum estradiol levels and βFSH mRNA levels in the pituitary of antagonist treated females, which might be compatible with a possible advancement in pubertal onset in this sex. This possibility, however, is yet to be experimentally demonstrated. No change in βLH mRNA levels was found and the α-subunit, which is identical for both gonadotrophins, was not measured. The α-subunit is reported to be produced in excess, with the expression of LH and FSH being dependent on the transcription of their specific β-subunits (40).

In males, testicular weight was significantly increased in the antagonist treated rats, although circulating testosterone levels or gonadotropin mRNA levels were not modified. Increased testicular weight could indicate an advancement of pubertal development even though testosterone levels are not yet elevated (41). Again, further studies are necessary to determine how pubertal transition is affected when leptin signaling is blocked during early neonatal life.

Pegylated leptin antagonists have been previously shown to block leptin-induced food intake and weight

gain when administered to adult female mice (42). By contrast, in nursing pups no effect was found on weight gain (19), supporting the concept that during this period of development leptin's main role is as a neurotrophic factor and not an anorexic signal (43). Moreover, the timing of leptin exposure during critical periods of development is of great importance. For example, while pharmacological antagonism of leptin during the first 13 days of postnatal life resulted in a delayed increase in weight gain (44), blockage of the leptin surge by a single injection of leptin antagonist on PND9 decreased weight gain, with this also becoming apparent with advancing age (28). Similar to previous studies (19, 28, 44) we found no effect of the super leptin antagonist used here on body weight during neonatal and prepubertal life. However, there was a slight, but significant, increase in accumulated food intake in males and this was associated with a decrease in white adipose tissue. As these rats showed no difference in total body weight, one would suspect an increase in lean body mass. As lean mass has a higher resting energy expenditure than fat mass (45), this might also suggest higher energy expenditure in these animals, which could explain the increase in food intake in leptin antagonist treated males while maintaining normal weight gain. However, no changes in UCP1 or UCP2 levels were found in BAT.

The decrease in subcutaneous WAT in males treated with the leptin antagonist could be due to perturbation of direct actions of leptin on adipocyte development (46–49). Adipose distribution and function differs between males and females and this is partially due to their differential genetic make-up (50). Moreover, leptin has differential effects on male and female adipocytes (47); thus, it might be expected that they differ in response to the blockage of this hormone's actions during development, resulting in different effects on long-term adipose mass acquisition.

Neonatal leptin antagonism reduced leptin mRNA levels in subcutaneous WAT in both peri-pubertal males and females. Early nutritional status has been shown to impact on adipogenesis and adipokine expression in the adult animal (51), thus early leptin levels could be involved in programming of later leptin production. Although leptin mRNA levels were reduced in subcutaneous WAT, circulating leptin levels were not affected. This could be due to a change in leptin turnover or in its production by other fat depots. Unfortunately, we were unable to analyze leptin production in visceral adipose of these animals due to technical difficulties. Early nutritional status also affects later inflammatory responses and the propensity to develop metabolic disease (52). We show that cytokine production in WAT is affected by neonatal leptin inhibition, as there was a decrease in IL10 and TNFα mRNA levels in females. Although we found no significant changes in males, there was a tendency towards an increase in both cytokines. Together, these results indicate that early metabolic programming by neonatal leptin signaling not only involves affectation of neuroendocrine circuits, but also long-term effects on adipose tissue function.

Leptin activates thermogenesis and energy expenditure; one potential mechanism for this to occur is through the UCP system in BAT (53). We found no significant change on either UCP1 or 2 in BAT, suggesting that the leptin blockade does not affect BAT thermogenesis, at least in this time-point. However, as this treatment induces delayed changes in body weight, while food intake is not modified (28), it is possible that there is a delayed modification in resting energy expenditure due to perturbation of early leptin effects. Of note, mean resting energy expenditure is normally reduced with aging (54–56); thus, our data are compatible with the possibility that early developmental events could have an impact on key ageing processes.

In conclusion, these studies further support the concept that disruption of early postnatal leptin signaling produces long-term effects on diverse neuroendocrine systems. The observation that levels of growth factors, glial structural proteins, and neuropeptides and/or their receptors are modified by neonatal treatment with a potent leptin antagonist during the peri-pubertal phase suggests that this important organizational period in the hypothalamus is altered. This indicates that not only puberty may be modified, but that the response of the hypothalamus to the numerous physiological changes that can occur during adolescence, as well as other environmental challenges, is modified. This could in turn further exacerbate the long-term effects induced by early nutritional/leptin modification. One should take into consideration that only mRNA levels were measured in the hypothalamus and adipose tissue. A change in static mRNA levels is generally accepted to indicate a modification in protein production; suggesting, for example, that the increase in Gpr54 mRNA levels reflects an increase in receptor protein levels. However, the lack of a detectable change in the mRNA level of a protein does not necessarily mean that protein production or processing is unaffected. Indeed, GFAP mRNA levels were only significantly increased in the HTr of male rats, but tended to increase in the MBH, as well as in females and these changes could possibly be detected if protein levels were measured. It is also possible that the pattern of release of a hormone or neuropeptide is modified and this cannot be determined by static measurement of either mRNA or protein levels. This is especially important for hormones secreted in a pulsatile manner, such as FSH and LH where the physiological outcome is mod-

ified by the hormonal secretory pattern. Moreover, receptor signaling or sensitivity could also be modified. Thus, the results reported here should be interpreted with these caveats in mind. Indeed, further studies are necessary in order to better understand the long-term effects of leptin signaling during development on the reproductive axis.

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