Effects of Acute Changes in Neonatal Leptin Levels on Food Intake and Long-Term Metabolic Profiles in Rats

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In rodents there is a rise in serum leptin levels between postnatal days (PND) 5 and 14, with this neonatal leptin surge reported to modulate the maturation of hypothalamic circuits involved in appetite regulation. We hypothesized that acute changes in neonatal leptin levels have different long-term metabolic effects depending on how and when this surge is modified. To advance the timing of the normal leptin peak, male Wistar rats were injected with leptin (sc, 3 μg/g) on PND 2. To ablate the leptin peak on PND 10, a pegylated leptin antagonist (sc, 9 μg/g) was injected. Controls received vehicle. All rats were allowed to eat *ad libitum* until PND 150. Increased leptin on PND 2 reduced food intake ($P < 0.01$) after 3 months of age with no effect on body weight. Levels of total ghrelin were reduced ($P < 0.001$) and acylated ghrelin increased ($P < 0.05$), with no other modifications in metabolic hormones. In contrast, treatment with the leptin antagonist on PND 9 did not affect food intake but reduced body weight beginning around PND 60 ($P < 0.02$). This was associated with a reduction in fat mass, insulin ($P < 0.01$), and leptin ($P < 0.007$) levels and an increase in testosterone levels ($P < 0.01$). Hypothalamic neuropeptide Y ($P < 0.05$) and leptin receptor ($P < 0.005$) mRNA levels were reduced, whereas mRNA levels for uncoupling protein 2 ($P < 0.005$) were increased in visceral fat, which may indicate an increase in energy expenditure. In conclusion, acute changes in neonatal leptin levels induce different metabolic profiles depending on how and when leptin levels are modified. **(*Endocrinology* 152: 4116–4126, 2011)**

Leptin, a pleiotrophic hormone primarily produced by adipocytes, exerts an anorexic effect at the level of the hypothalamus by modulating the synthesis and secretion of specific neuropeptides to control appetite and metabolism (1–3). This adipokine is also implicated in hypothalamic development and most specifically in development of neuronal circuits that control metabolic function (4–6). Animals genetically lacking leptin have modifications in the number of projections and synaptic inputs to hypothalamic areas and neuronal populations that control metabolism (5, 6). Leptin is not only important for neuronal outgrowth (4), but also for neuronal survival and development of other brain cells such as astrocytes (7).

In rodents there is a postnatal leptin surge that in males begins after postnatal day (PND) 5 and peaks between PND 8 and 10 (8, 9). This early increase in leptin is important for normal physiological development of metabolic systems because pharmacological ablation of leptin during the first 13 d of life disrupts leptin responsiveness, increases weight gain, and induces hyperleptinemia in response to a high-fat diet in adulthood (10). Increasing leptin levels in the postnatal period also augments diet-

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Abbreviations: AgRP, Agouti-related protein; AUC, area under the curve; BDNF, brain-derived neural growth factor; BW, body weight; CART, cocaine amphetamine-related transcript; FAS, fatty acid synthetase; GLUT-4, glucose transporter 4; HDL, high-density lipoprotein; HSL, hormone-sensitive lipase; Ins-R, insulin receptor; LDL, low-density lipoprotein; LPL, lipoprotein lipase; NPY, neuropeptide Y; Ob-R, leptin receptor; OGTT, oral glucose tolerance test; PND, postnatal day; POMC, proopiomelanocortin; PPAR, peroxisome proliferator-activator receptor; UCP, uncoupling protein.

induced weight gain (11) and induces leptin resistance in adult males (12). Indeed, the long-term effects induced by changes in prenatal nutrition may be mediated, at least in part, through modifications in neonatal leptin levels (8, 9).

Precisely when during development the leptin surge is modified may determine the long-term outcomes. We recently reported that separation of rat pups from their mothers for 24 h beginning on PND 9 drastically reduced leptin levels during the separation, with leptin levels being significantly lower at PND 75 (13, 14) and bodyweight decreasing compared with controls as aging advances (personal unpublished observation V.M., M.-P.V., J.A.C.). This is in contrast to what occurs when leptin is blocked during the first 13 d of life (10). The neonatal decrease in leptin induced by maternal separation is coincident with modifications in hypothalamic levels of IGF-I and brain-derived neural growth factor (BDNF), trophic factors important for brain development, and markers of cell turnover and maturation (13). Adult serum testosterone and IGF-I levels were also affected (14), suggesting that other neuroendocrine systems are also modulated.

Much attention has been paid to the effects of maternal or neonatal nutrition on later metabolic function, but other early environmental factors also have important influences. Stress, illness, or infections can acutely affect both maternal and neonatal metabolism, as well as leptin levels, with these situations having long-term effects on metabolic control (15–18). Thus, whereas maternal or neonatal nutritional changes are generally chronic, other environmental influences may be more acute with their long-term effects also being mediated, at least partially, through changes in neonatal leptin levels. Moreover, it is conceivable that modifications in leptin levels at specific developmental time points could selectively affect development of central and peripheral systems that are undergoing modifications at this moment, resulting in differential metabolic and endocrine outcomes.

Our aim was to compare the metabolic outcomes in adult rats in response to acute modifications in neonatal leptin levels at different time points. To determine whether the metabolic changes induced by maternal deprivation on PND 9–10 can be attributed to the decline in leptin levels (13) a pegylated leptin antagonist (LeptAntag) was administered at PND 9. Addition of a polyethylene glycol molecule increases the half-life of this LeptAntag by 14.4-fold, with the antileptin effects being mediated, at least in part, by inhibiting leptin's transport across the blood-brain barrier, thus inhibiting its central activity (19). This time point is of physiological importance because leptin levels are elevated at this moment (8, 9), and specific connections in hypothalamic metabolic circuits are being formed (5). To determine whether the timing of the onset of the leptin surge is critical, neonatal rats were treated with a single injection of leptin on PND 2 to advance this phenomenon.

Materials and Methods

Animals

All experiments were designed according to the European Union laws for animal care, and the study was approved by the local institutional ethical committee.

Male Wistar rats were used for these studies (Harlan interfauna Ibérica S.A., Barcelona, Spain). After mating and pregnancy were confirmed, dams were housed individually and fed *ad libitum*. On the day of birth litters were adjusted to eight pups per mother. In all experiments rats were kept under constant conditions of temperature (20–22 C) and light/dark cycles (lights on from 0730 h to 1930 h).

Experiment 1: acute neonatal leptin treatment

To advance the timing of the leptin surge, on PND 2 male offspring (n = 12) were injected sc with 3 μg/g body weight (BW) of rat leptin (National Hormone & Pituitary Program, Torrance, CA). This dose induces leptin signaling and modulates hypothalamic neuropeptide expression and fat mass in neonatal animals (20). Control rats (n = 12) were injected with the same volume of vehicle (saline + 0.1% BSA, 10 ml/kg). This time point was chosen as leptin levels have not yet begun to rise under normal physiological conditions (8, 9).

To determine the levels of circulating leptin achieved with this treatment, PND 2 males were injected sc with 3 μg/g BW of leptin or vehicle and killed 0, 1, 3, 6, or 24 h later (n = 6–7).

After weaning rats were housed three to four per cage with free access to rat chow (Panlab, Barcelona, Spain) and water. BW and food consumption were assessed weekly until decapitation on PND 150. Food intake was determined by placing a precise amount of rat chow in the cage and measuring the remaining amount the following day. The amount of chow ingested per animal was calculated by dividing by the number of rats per cage, with each cage representing an n of 1.

After decapitation, blood was collected in tubes containing EDTA to obtain plasma after 20 min of centrifugation at 3000 rpm. Plasma samples were stored at −80 C until hormone levels were measured. The brains, visceral and sc fat pads, and gastrocnemius were removed, weighed, and stored at −80 C until processed.

Experiment 2: LeptAntag treatment

To blunt the leptin peak on PND 9–10 male rats (n = 10) were injected sc (1900 h) with 9 μg/g BW of rat pegylated LeptAntag (PLR, Rehovot, Israel). This time point was chosen because leptin levels are elevated at this moment (8, 9), and specific connections in hypothalamic metabolic circuits are being formed (5). Control rats (n = 11) were injected with the same volume of vehicle (saline + 0.1% BSA, 10 ml/kg).

After weaning rats were housed three to four per cage with free access to food and water. BW and food consumption were assessed weekly as described above from weaning until decapi-

tation on PND 150. Plasma, brains, visceral and sc fat pads, and gastrocnemius muscle were obtained and stored as described above.

Oral glucose tolerance test (OGTT)

Rats were subjected to an OGTT between PND 125 and 130. They were fasted overnight (from 1900 h to 0900 h) and at 0900 h basal glycemia was measured (Optium Plus, Abbot Diabetes Care, Witney Oxon, UK). Rats were then orally administered a bolus of 3 mg/g-BW of glucose. Glycemia was measured at 30, 60 and 120 min by venous tail puncture. The total area under the curve (AUC) for the glucose response was calculated with the following formula: AUC = 0.25 × (fasting value) + 0.5 × (30 min value) + 0.75x (1-h value) + 0.5x (2-h value) (21, 22).

Hormone measurements

Plasma levels of leptin, insulin, and adiponectin were measured by ELISA following the manufacturer's instructions (Linco Research, St. Charles, MO). Absorbance in each well was measured with a Tecan Infinite M200 (Grödig, Austria). The sensitivity of the method for leptin, insulin, and adiponectin was 0.04, 0.2, and 0.16 ng/ml, respectively. The intraassay variation was 2.2% for leptin, 1.9% for insulin, and 1.3% for adiponectin.

Total and acylated ghrelin levels were measured in plasma by RIA following the manufacturer's instructions (Linco Research). The sensitivity of the method was 93 pg/ml, and the intra- and interassay variations were 6.4 and 16.3% for total ghrelin and 7.4 and 13.4% for acylated ghrelin, respectively. Because freeze-thaw cycles can degrade acylated ghrelin, an aliquot of plasma from each animal was rapidly frozen and only used for this determination.

IGF-I concentrations were measured by a double-antibody RIA. Serum IGF-I binding proteins were removed by acid-ethanol extraction. The IGF-I antiserum (UB2-495) was a gift from Dr. Underwood and Dr. Van Wyk and is distributed by the Hormone Distribution Program of NIDDK through the National Hormone and Pituitary Program (Gropep Ltd., Adelaide, Australia). The intraassay coefficient of variation was 8%, and the assay sensitivity was 6 ng/ml.

Plasma testosterone levels were measured using an enzyme immunoassay kit by Assay Designs, Inc. (Ann Arbor, MI). The sensitivity of the method was 5.67 pg/ml, and the intraassay variation was between 7.8% and 10.8%.

For all measurements samples were run in duplicate and within the same assay.

Analysis of glycerol and lipid levels

Plasma glycerol levels were determined by using a free glycerol determination kit (Sigma-Aldrich, St Louis, MO). This method measures endogenous circulating glycerol by coupled enzyme reactions with the concentration being directly proportional to absorbance at 540 nm.

Total lipids, triglycerides, cholesterol, low-density lipoprotein (LDL), and high-density lipoprotein (HDL) were measured in plasma by using commercial kits purchased from SpinReact (Sant Esteve de Bas, Gerona, Spain). Plasma samples were diluted 1:2 with saline, and the assays were performed following the manufacturer's instructions.

RNA preparation and quantitative real-time PCR

Hypothalami were isolated on ice using the following boundaries: an anterior cut was made at the level of the optic chiasm, a posterior coronal section anterior to the mammillary bodies, two sagittal cuts parallel to the lateral ventricles, and a dorsal horizontal cut at the level of the anterior commissure.

Total RNA was extracted from the hypothalamus, sc and epididymal adipose tissue, and gastrocnemius muscle according to the Tri-Reagent protocol (23). cDNA was synthesized from 1 μg of total RNA by using a high-capacity cDNA RT kit (Applied Biosystems, Foster City, CA).

Agouti-related protein (AgRP), proopiomelanocortin (POMC), neuropeptide Y (NPY), cocaine amphetamine-related transcript (CART), insulin receptor (Ins-R), leptin receptor (Ob-R), IGF-I, and BDNF, were assessed in hypothalamic samples by quantitative real-time PCR. In epididymal and sc adipose tissue Ins-R, Ob-R, fatty acid synthetase (FAS), hormone-sensitive lipase (HSL), glucose transporter 4 (GLUT-4), peroxisome proliferator-activator receptor γ (PPARγ), lipoprotein lipase (LPL) and uncoupling protein 2 (UCP-2) gene expression was measured. In the gastrocnemius Ins-R, GLUT4, and UCP-2 were measured.

Quantitative real-time PCR was performed by using assay-on-demand kits (Applied Biosystems) for each gene (Table 1). TaqMan Universal PCR Master Mix (Applied Biosystems) was used for amplification according to the manufacturer's protocol in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Values were normalized to the housekeeping genes actin or GAPDH. According to manufacturer's guidelines, the $\Delta\Delta C_T$ method was used to determine relative expression levels. Statistics were performed using $\Delta\Delta C_T$ values (24).

Statistical analysis

All data are presented as mean ± SEM. Unpaired two-tailed Student's *t* tests were used to compare the two experimental groups. Repeated measures were used for analysis of glycemia changes in the glucose tolerance test and changes in weight or circulating leptin levels over time. The values were considered significantly different when the $P < 0.05$.

TABLE 1. Reference of primers employed for real-time PCR (Applied Biosystems)

Primer	Reference
AgRP	Rn01431703
NPY	Rn01410145
POMC	Rn00595020
CART	Rn00567382
Ob-R	Rn01433205
FAS	Rn00569117
HSL	Rn00563444
PPAR-γ	Rn00440945
LPL	Rn00561482
UCP-2	Rn00571166
GLUT-4	Rn00563565
Ins-R	Rn00567670
BDNF	Rn01484924
IGF-I	Rn99999087

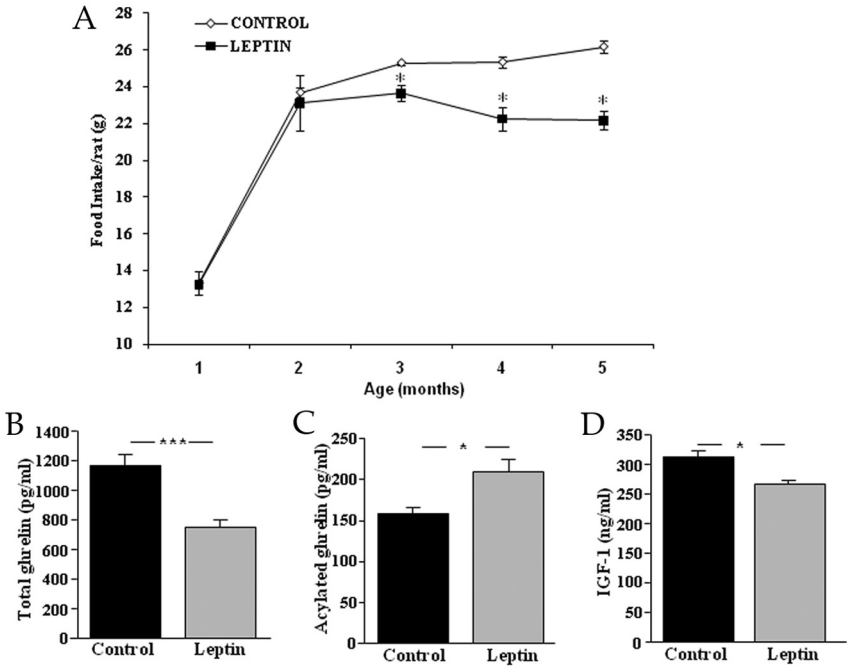

FIG. 1. A, Mean daily food intake from weaning until 150 d of age in male rats treated on PND 2 with leptin (3 μg/g BW). Circulating levels of total ghrelin (B), acylated ghrelin (C), and IGF-I (D) on PND 150. *, $P < 0.05$; ***, $P < 0.001$.

Results

Experiment 1: acute neonatal leptin treatment on PND 2

Circulating leptin levels in response to acute neonatal leptin treatment

Leptin levels were elevated 1 h after the injection, remained elevated at 3 h, began to decline by 6 h, and were normalized 24 h after the injection (control 0, 1.1 ± 0.4; control 1 h, 2.7 ± 0.4; Lept 1 h, 1671.3 ± 173.3; control 3 h, 1.3 ± 0.6; Lept 3 h, 1827.6 ± 90.3; control 6 h, 4.5 ± 0.2; Lept 6 h, 580.5 ± 59.1; control 24 h, 3.0 ± 0.3; Lept 24 h, 3.7 ± 0.5 ng/ml; $P < 0.0001$).

Food intake, BW, and adipose tissue, muscle, and brain weight

Until approximately 3 months of age there was no difference in food intake. In later adulthood rats treated with leptin began to eat less than the controls such that the mean daily intake from approximately 3 months of age on was significantly lower than that of controls (Fig. 1A; $P < 0.001$).

At no time during the study was there an effect of neonatal leptin treatment on BW, including the final weight at PND 150 (control, 453.8 ± 4.8 g; Lept, 443.7 ± 6.1 g). Visceral fat pad weight was reduced (control, 9982.1 ± 812.1 mg; Lept, 7986 ± 310 mg; $P < 0.05$), but this difference was not significant when normalized for BW (control, 2.2 ± 0.2 mg/100 g BW; Lept, 1.8 ± 0.1 mg/100 g BW). There was no difference in the absolute (control, 2128.3 ± 138.0 mg; Lept, 2143.6 ± 100.9 mg) or normalized (control, 0.47 ± 0.03 mg/100 g BW; Lept, 0.48 ± 0.02 mg/100 g BW) weight of sc fat pads. Although the weight of the gastrocnemius muscle was not affected (control, 2109.2 ± 50.7 mg; Lept, 2203.6 ± 41.3 mg), when normalized for BW it was increased by leptin treatment (control, 0.465 ± 0.11 mg/100 g BW; Lept, 0.497 ± 0.08 mg/100 g BW; $P < 0.05$).

Glycemia and OGTT

There was no significant difference in fasting glucose levels or food intake (control, 84.5 ± 2.0 mg/dl; Lept, 74.0 ± 5.1 mg/dl). In the OGTT there was a change in glucose levels over time ($P < 0.0001$) in both groups, with no difference found at any time point or in the AUC for glucose (AUC: control, 240 ± 10; Lept, 217 ± 10). Glycemia at time of decapitation was not different (control, 95.1 ± 3.2 mg/dl; Lept, 96.6 ± 2.8 mg/dl).

Plasma hormone levels

There was no effect of neonatal leptin treatment on adult levels of insulin (control, 2.0 ± 0.4; Lept, 1.2 ± 0.2 ng/ml), leptin (control, 16.4 ± 1.4; Lept, 13.7 ± 0.8 ng/ml), adiponectin (control, 35.1 ± 2.7; Lept, 38.6 ± 2.6 ng/ml), or testosterone (control, 1.23 ± 0.22; Lept, 0.76 ± 0.16 ng/ml). Total ghrelin levels declined (Fig. 1B, $P < 0.001$), and acylated ghrelin levels increased (Fig. 1C, $P < 0.05$) in the leptin-treated group. This resulted in the percent acylation of ghrelin being twice as much after leptin treatment (control, 14.1 ± 1.4%; Lept, 28.4 ± 1.9%; $P < 0.0001$).

There was a decrease in circulating IGF-1 levels in adult rats treated with leptin (Fig. 1D; $P < 0.005$).

Plasma total lipids, triglycerides, glycerol, total protein, cholesterol, LDL, and HDL levels

Neonatal leptin treatment reduced total cholesterol (control, 76.4 ± 2.7; Lept, 51.7 ± 2.9 mg/dl; $P < 0.0001$), HDL (30.7 ± 2.4, Lept, 20.9 ± 1.5 mg/dl; $P < 0.001$), and LDL levels (26.3 ± 1.5; Lept, 17.6 ± 0.7 mg/dl; $P < 0.0005$), with no significant changes in total lipids, triglycerides, free glycerol, or total protein levels (data not shown).

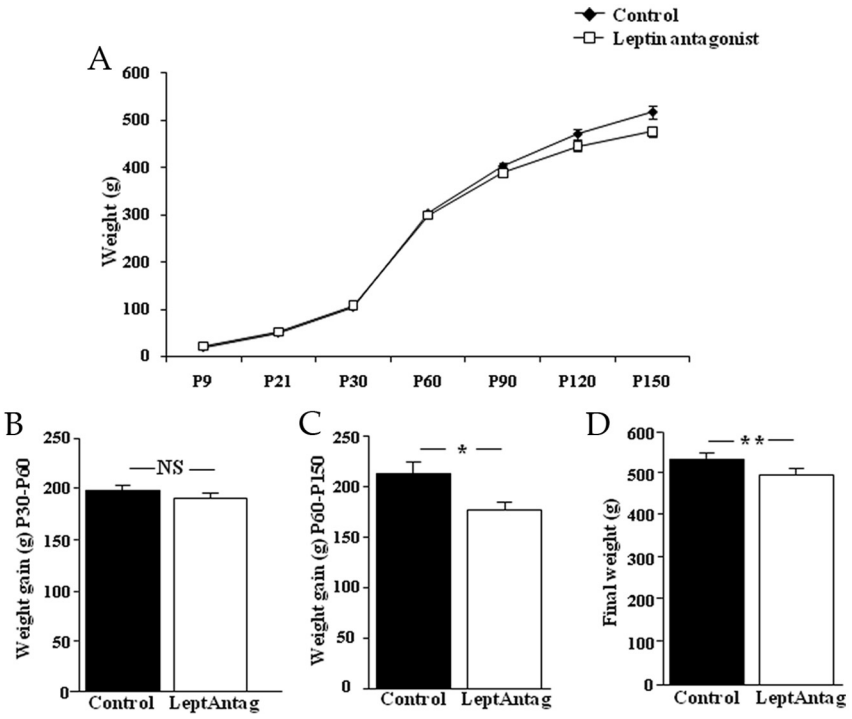

FIG. 2. A, BW gain in male rats from the time of injection with a pegylated LeptAntag on the afternoon of PND 9 until decapitation on PND 150. Weight gain between PND 30 and PND 60 (panel B) and PND 60 and PND 150 (panel C) in control rats and rats treated with the LeptAntag. D, Weight at the time of decapitation on PND 150. *, $P < 0.02$; **, $P < 0.03$. P, Postnatal day.

FAS, HSL, PPAR-γ, GLUT-4, LPL, UCP-2, Ob-R, and Ins-R mRNA levels in visceral and sc adipose tissue

In sc adipose tissue there was no effect of neonatal leptin treatment on the expression of any of the genes studied (data not shown). In visceral adipose, rats treated neonatally with leptin had increased mRNA levels for the Ob-R (control, 100 ± 37.8; Lept, 2075.7 ± 340.0% control; $P < 0.0001$) and Ins-R (control, 100 ± 8.9; Lept, 711.4 ± 177.4% control; $P < 0.001$), with no change in any of the other genes analyzed.

Hypothalamic trophic factors and neuropeptides

Neonatal leptin treatment increased CART mRNA levels in the adult hypothalamus (control, 100 ± 1.0; Lept, 129.9 ± 7.6; $P < 0.0001$), but had no effect on the hypothalamic mRNA levels of any of the other neuropeptides measured or for the Ob-R (data not shown). The mRNA levels for IGF-I or BDNF were not modified in the adult hypothalamus (data not shown).

Experiment 2: neonatal LeptAntag treatment on PND 9

Food intake, bodyweight, and adipose tissue, muscle, and brain weight

At no time during the study was there a difference between the groups in food intake (mean daily intake from weaning until decapitation: control, 25.0 ± 0.4 g; and LeptAntag, 25.6 ± 0.8 g).

During the first 3 months of life weight gain in the antagonist treated rats was indistinguishable from the control group (Fig. 2A). During the pubertal/early adult period with the most rapid growth velocity (PND30-PND60), there was no difference between the groups in total weight gain (Fig. 2B). However, after PND60 the antagonist-treated rats began to gain weight at a slower rate than controls, such that their weight gain during the last 3 months of the study was significantly less (Fig. 2C; $P < 0.02$), resulting in BW at PND150 being lower (Fig. 2D; $P < 0.03$).

The lower BW in the LeptAntag-treated rats was coincident with a reduction in the absolute weight of visceral fat pads (Fig. 3A; $P < 0.004$), as well as visceral fat weight adjusted for BW (Fig. 3B; $P < 0.02$). There was also a reduction in the absolute weight (Fig. 3C; $P < 0.005$) and weight adjusted for BW (Fig. 3D; $P < 0.02$) of sc fat. Neither the absolute weight of the gastrocnemius muscle (Fig. 3E) nor its weight normalized for BW (Fig. 3F) differed between the two groups. Brain weight was reduced in rats treated with the LeptAntag (2.13 ± 0.03 g) compared with controls (2.24 ± 0.02 g; $P < 0.01$) but when adjusted for BW this difference was not significant (control: 0.44 ± 0.1/100 g BW; LeptAntag, 0.46 ± 0.1/100 g BW).

Glycemia and OGTT

Glucose levels were not different at the onset of the OGTT (control, 80.4 ± 2.2 mg/dl; LeptAntag, 81.3 ± 2.7 mg/dl), and there was no difference in BW at this time (control, 463.5 ± 10.5 g; LeptAntag, 439.4 ± 9.9 g). In both groups glucose levels changed throughout time ($P < 0.0001$), with no significant difference between the groups at any time point. However, the rise in glycemia tended to be reduced at each time point in the antagonist-treated rats (Fig. 4A), such that the overall AUC was reduced (control, 247.0 ± 8.6; LeptAntag, 225.2 ± 2.9; $P < 0.05$). At time of decapitation blood glucose levels were lower in rats treated with the LeptAntag than in controls (control, 106.1 ± 3.1; LeptAntag, 94.6 ± 3.0 mg/dl).

Plasma hormone levels

Circulating insulin (Fig. 4B; $P < 0.01$) and leptin (Fig. 4C; $P < 0.007$) levels were reduced in LeptAntag-treated

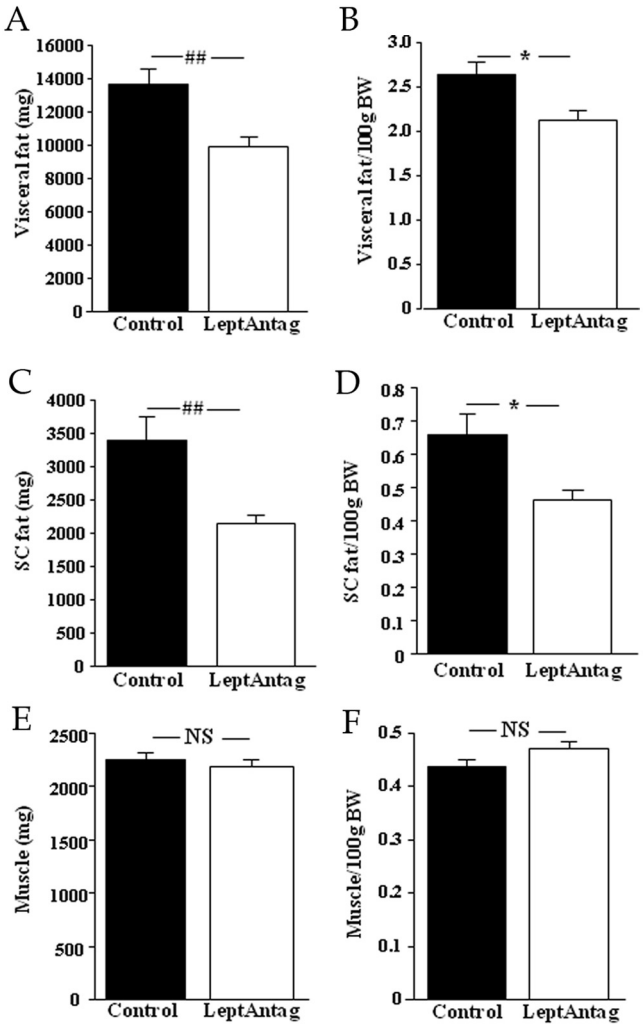

FIG. 3. Effect of treatment with a pegylated LeptAntag on PND 9 on the weight of visceral fat (A), visceral fat normalized for BW (B), sc fat (C), sc fat normalized for BW (D), gastrocnemious muscle (E), and gastrocnemious muscle normalized for BW (F) in male rats at 150 d of age. *, $P < 0.02$; ##, $P < 0.005$; NS, Nonsignificant $P > 0.05$.

rats, with no differences in adiponectin levels (control, 29.8 ± 1.7; LeptAntag, 27.9 ± 2.7 ng/ml). There was no change in total ghrelin (control, 975.5 ± 68.6; LeptAntag, 1156.6 ± 101.4 pg/ml) or acylated-ghrelin levels (control, 198.9 ± 25.8; LeptAntag, 165.3 ± 26.1 pg/ml), or in the percentage of ghrelin acylation (control, 22.0 ± 3.7%; LeptAntag, 15.0 ± 2.7%).

Plasma testosterone levels were increased in rats treated with the LeptAntag (Fig. 4D; $P < 0.01$), as were circulating IGF-I levels (Fig. 4D, $P < 0.05$).

Total lipids, triglycerides, cholesterol, LDL, and HDL levels

Plasma total lipids, total cholesterol, LDL, and glycerol levels were significantly decreased in LeptAntag-treated rats, whereas plasma triglycerides and HDL levels were not modified (Table 2).

FAS, HSL, PPAR-γ, GLUT-4, LPL, UCP-2, Ob-R, and Ins-R mRNA levels in visceral and sc adipose tissue

LeptAntag treatment up-regulated UCP-2 gene expression in visceral fat (control, 100 ± 15.5; antagonist, 163.4 ± 16.3% control; $P < 0.005$), whereas FAS, HSL, PPAR-γ, GLUT-4, LPL, Ob-R, and Ins-R mRNA levels were not modified in this tissue (data not shown).

In sc fat Ob-R gene expression was up-regulated (control, 100 ± 40; antagonist, 587.1 ± 197.1% control; $P < 0.05$), with no change in the mRNA levels of FAS, HSL, PPAR-γ, GLUT-4, LPL, UCP-2, or Ins-R (data not shown).

Gastrocnemius muscle

There was no effect of the antagonist treatment on the mRNA levels of Ins-R (control, 100 ± 6.5; antagonist, 97.1 ± 10.5% control), GLUT-4 (control, 100 ± 8.1; antagonist, 92.8 ± 10.6% control), or UCP-2 (control, 100 ± 8.2; antagonist, 110.3 ± 10.8% control) in muscle tissue.

Hypothalamic neuropeptides and trophic factors

Hypothalamic mRNA levels of NPY (Fig. 5A) and CART (Fig. 5D) were reduced ($P < 0.05$ for both), with no significant change in AgRP (Fig. 5B) or POMC (Fig. 5C). Ob-R mRNA levels were also reduced ($P < 0.005$) in the hypothalamus of antagonist-treated rats (Fig. 5E).

Treatment with the LeptAntag increased hypothalamic IGF-I mRNA levels (Fig. 5F; $P < 0.005$), but not BDNF mRNA levels (control, 100 ± 10.0; LeptAntag, 88.8 ± 10.3% control).

Discussion

Inducing a premature rise in leptin levels on PND 2 decreased food intake in the adult rat, with no modification in BW. In contrast, blunting of leptin's actions at PND 9 decreased BW and fat mass in adult males without changing food intake. In both experimental paradigms the metabolic effects were delayed with significant changes not being observed until adulthood, with the differences becoming more apparent as aging advanced. Thus, early environmental effects on metabolism may be overlooked because they can be subtle and occur gradually, possibly not being observed until later adulthood or earlier only if the animals are subjected to a metabolic challenge that accentuates these metabolic differences (11).

The age of onset, duration, and amplitude of the neonatal leptin surge are all reported to affect metabolic equilibrium in the adult animal (25–27). These characteristics of the leptin surge can be influenced by the maternal nu-

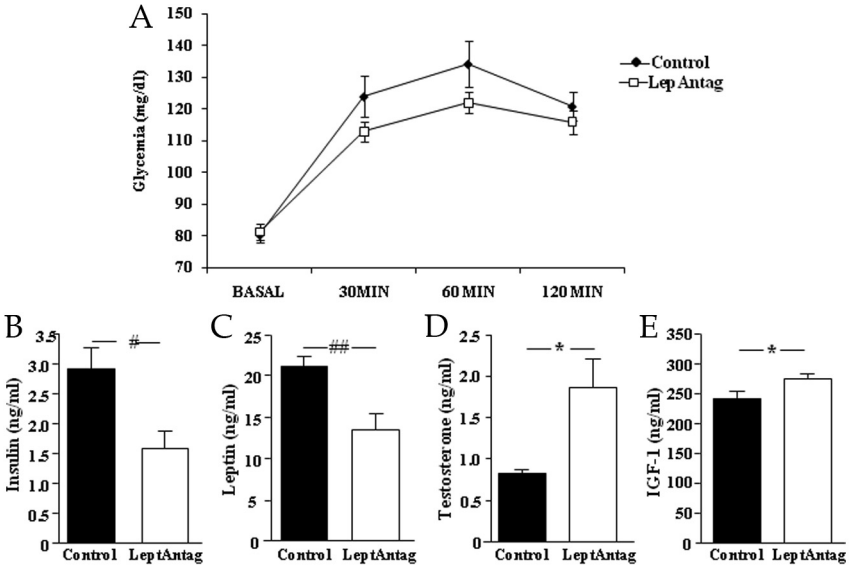

FIG. 4. A, Blood glucose levels during an OGTT in male rats treated with a pegylated LeptAntag or vehicle on the afternoon of PND 9. The test was performed between PND 125 and PND 130. Serum levels of insulin (B), leptin (C), adiponectin (D), total ghrelin (E), acylated ghrelin (F), and testosterone (G) in male rats treated with a pegylated LeptAntag on the afternoon of PND 9 at the time of decapitation on PND 150. *, $P < 0.02$; #, $P < 0.01$; ##, $P < 0.005$.

tritional status during pregnancy and lactation and neonatal food availability (11, 28), as well as maternal deprivation (13, 14). Physiological modifications of leptin levels during this time period are suggested to relay information to the fetus or neonate regarding the perinatal environment, such that development of structures controlling metabolism is attuned to the surrounding nutritional environment (25). Thus, an individual born into an adverse environment, such as reduced food availability, would adapt to this situation by developing a more efficient metabolism (29). Other pathophysiological processes such as stress, illness, or infections can acutely affect both maternal and neonatal metabolism, as well as leptin levels, with these situations also having long-term effects on metabolic control (15–18), suggesting that environmental changes may not have to be chronic to have long-term consequences.

A single injection of leptin on PND 2, which is before its physiological neonatal rise (30), affected food intake but not bodyweight. Although the dose used here resulted in pharmacological levels of circulating leptin, it is lower than that used in other studies analyzing the effects of neonatal leptin (4). This large increase in circulating leptin levels, which returned to control levels by 24 h, had a delayed effect on food intake. Adult rats decreased their food consumption, while maintaining a normal BW and percentage body fat. These rats also had decreased circulating IGF-I levels at 150 d of age. Whether this decline in IGF-I is also delayed or occurs throughout development, as well as the possible effect on longitudinal growth, remains to be determined.

The decrease in food intake in leptin-treated rats was coincident with increased circulating acylated ghrelin levels. Because acylated ghrelin stimulates food intake (31, 32), this rise could be a compensatory signal for the animal to increase food intake. Acylated ghrelin also stimulates energy storage into adipose tissue (33, 34), which could increase metabolic efficiency and might explain, at least in part, the fact that weight gain and fat mass were not affected with decreased food intake. In addition, although insulin and glucose levels were not modified, the rise in Ins-R gene expression in adipose tissue could indicate increased sensitivity to insulin. Higher Ob-R expression in adipose tissue suggests that modulation of neonatal leptin levels may affect the response of this tissue to leptin in adulthood. Total lipids and total, HDL, and LDL cholesterol levels were also decreased in these rats, which could be related to the reduction in food intake and hence total fat intake.

Leptin treatment on PND 2 had no effect on hypothalamic mRNA levels for any of the neuropeptides, receptors, or growth factors analyzed, except for CART. The mRNA levels for this neuropeptide were increased, which is opposite to that observed in LeptAntag-treated rats. Because CART has anorexigenic effects (35, 36), the increased levels of this neuropeptide could mediate the decreased food intake of leptin-treated rats. However,

TABLE 2. Circulating levels of lipids and total protein in adult (150 d of age) control rats, and rats treated with a LeptAntag on the afternoon of PND 9

	Total lipids (mg/dl)	Total cholesterol (mg/dl)	HDL (mg/dl)	LDL (mg/dl)	Triglycerides (mg/dl)	Free glycerol (μM)	Total protein (g/dl)
Control	716.3 ± 39.7	82.0 ± 4.1	48.9 ± 4.0	33.6 ± 1.5	178.4 ± 12.6	810.4 ± 59.3	7.5 ± 0.3
LeptAntag	606.3 ± 15.7[a]	65.5 ± 3.3[b]	34.6 ± 5.3	27.7 ± 1.5[b]	172.1 ± 23.9	588.5 ± 64.3[a]	6.3 ± 0.2[a]

[a] $P < 0.05$; [b] $P < 0.01$.

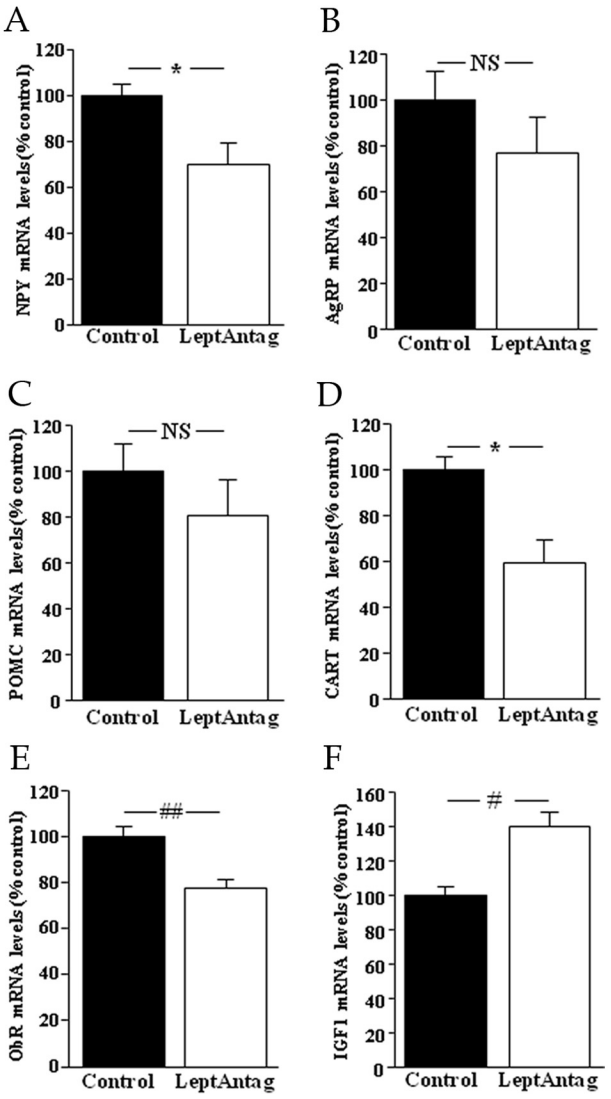

FIG. 5. Hypothalamic mRNA levels for NPY (A), AgRP (B), POMC (C), CART (D), Ob-R (E), and IGF-I (F) in male rats treated with a pegylated LeptAntag on the afternoon of PND 9 at the time of decapitation on PND 150. *, $P < 0.05$; ##, $P < 0.005$; NS, nonsignificant $P > 0.05$.

because CART is involved in other physiological functions, such as stress or reproduction (37), these changes may not necessarily be related to metabolism.

The pegylated LeptAntag employed in these studies has been previously shown to block leptin's actions both centrally and systemically (19). Treatment of neonatal rats with this antagonist modifies markers of cell turnover and maturation in the hypothalamus (unpublished observations V.M., M.-P.V., J.A.C.), suggesting that hypothalamic development is affected, and this could at least partially underlie the long-term effects on metabolism. These modifications in hypothalamic development could also affect other neuroendocrine systems, possibly explaining the rise in circulating testosterone and IGF-I levels observed here.

In contrast to the effects of hyperleptinemia on PND 2, antagonizing leptin's actions on PND 9 decreased BW, visceral and sc fat mass, and circulating leptin, insulin, and total and LDL cholesterol levels at 5 months of age. These modifications were not associated with changes in food intake, suggesting a change in metabolic efficiency. These rats were also more glucose tolerant, as indicated by a decrease in the AUC during the OGTT. The higher IGF-I levels could be involved in this increased tolerance because this growth factor has hypoglycemic effects even with concomitant suppression of insulin secretion (38).

The decreased weight gain in LeptAntag-treated rats was associated with a decline in both total and relative fat mass, which could be due to decreased lipogenesis and/or increased lipolysis. Unchanged adipose FAS and LPL mRNA levels suggest no modification in lipogenesis, whereas no change in adipose HSL expression and a decrease in plasma glycerol levels indicate that lipolysis is not increased. However, higher UCP2 expression in visceral fat of LeptAntag-treated rats indicates that their basal metabolic rate may be increased, which could decrease fat accumulation (39).

Diverse factors could be involved in the decreased fat mass as disruption of the neonatal leptin surge modified circulating levels of various hormones in the adult animal. Insulin is an important regulator of fat accumulation, increasing both lipogenesis and adipogenesis and decreasing lipolyisis (40–42), and levels of this hormone were decreased in LeptAntag-treated rats. Circulating IGF-I levels were increased and a negative relationship between this growth factor and visceral fat mass accumulation is reported in humans (43). In addition, because testosterone inhibits lipogenesis and adipogenesis and stimulates lipolysis (44–48), the higher testosterone levels in the antagonist-treated rats are also compatible with decreased adipose accrual. This increase in testosterone could possibly be due to a direct effect of the LeptAntag on the testes because leptin is involved in both testicular development and function, including testosterone production (49, 50), and blockage of the postnatal leptin surge alters gonadal development (51).

The effect on fat mass could also be due to altered adipogenesis as leptin is reported to have effects on adipocyte development (52–55). Because the genesis and major expansion of adipose tissue occur during gestation and early postnatal life (56), blunting the effects of leptin during this period could modulate adipose tissue development, as well the expression of its receptor on adipocytes. Indeed, Ob-R mRNA levels in sc fat were dramatically increased in antagonist-treated animals. The up-regulation of the Ob-R in sc adipose tissue could also be secondary to the reduced levels of this hormone.

The decrease in the mRNA levels of NPY and CART in total hypothalamic lysates of LeptAntag-treated rats

could indicate a decrease in the synthesis of these neuropeptides or a decreased number of NPY and CART neurons. Because NPY is orexigenic (57–59) a decline in its actions would increase energy expenditure, which could help to explain the decreased weight gain with no change in food intake. The decline in CART mRNA could be in response to the decreased weight gain because this neuropeptides is anorexigenic (35, 36), and a decrease in its actions could increase food intake and decrease energy expenditure. The effect of this experimental paradigm on the development of metabolic circuits, including the number of specific neuronal populations and their projections, as well as changes in specific synaptic inputs, deserves further investigation.

We previously reported that maternal deprivation on PND 9–10 dramatically reduces circulating leptin levels, and this is associated with modifications in the hypothalamic levels of IGF-I and BDNF, in conjunction with changes in markers of cell turnover and maturation (13), with these factors normalizing in the adult animal (14). Here we found that the mRNA levels of IGF-I were increased in the hypothalamus of antagonist-treated rats, with no change in BDNF levels. Modifications in IGF-I levels, both circulating and at the hypothalamic level, in the adult animal could be involved in the direct control of endocrine systems, including food intake and BW.

Treatment with the LeptAntag appears to improve metabolism as aging advances. These rats have decreased weight gain and fat accumulation, decreased cholesterol levels, increased glucose tolerance, and increased IGF-I and testosterone levels. Because circulating IGF-I and testosterone levels decrease with aging (60), it remains to be demonstrated whether this treatment is causing an actual increase in these parameters or is preventing the age-induced decline in these hormones. Likewise, whether this improved metabolism has implications in longevity is an important possibility to be analyzed.

In addition to the specific day of leptin administration/blockade, other factors such as the duration of treatment, the animal's sex, and subsequent alimentation may also have an impact on the adult metabolic response. Vickers *et al.* (28) reported that injection of leptin from PND 3 to PND 13 to female rats had no effect on weight gain or food intake at PND 170 or in the response to a high-fat diet. However, the same treatment increased weight gain in response to a high-fat diet in male rats from *ad libitum* fed dams but not in offspring from unnourished dams (11), indicating that the response depends on the maternal nutritional status during pregnancy. Increased maternal weight has been shown to augment and prolong the leptin surge, resulting in increased weight gain in later life in both males and females (9). Maternal food restriction during gestation reduces birth weight, with subsequent rapid postnatal catch-up growth advancing the neonatal leptin surge, resulting in normal adult BW on a normal diet, but exaggerated weight gain on a high-fat diet (61). However, maternal undernutrition during both gestation and lactation dramatically reduces the leptin surge in male offspring, and this is associated with increased weight gain in adulthood (8), similar to that observed with pharmacological blockage of leptin between PND 2 and PND 13 (10). In contrast, decreased circulating leptin levels at PND 9 s due to maternal separation reduces BW and leptin levels in adulthood (14), similar to that found here by injection of a LeptAntag at this same time period. Hence, depending on when the leptin surge is reduced, opposite effects on weight gain can be observed.

In conclusion even short-term modifications in neonatal leptin levels can have specific long-term metabolic effects. These outcomes may occur gradually and may not become manifest until adulthood. These data further stress the importance of monitoring maternal and neonatal diets, as well as other pathophysiological situations, to combat the rising incidence of obesity.

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