Postnatal intracerebroventricular exposure to leptin causes an altered adult female phenotype

Amit Varma, Jing He, Bo-Chul Shin, Lisa A. Weissfeld, and Sherin U. Devaskar

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LEPTIN, THE TRANSLATED AND SECRETED PRODUCT OF THE ob gene (65), is primarily synthesized by adipose tissue (25), interacts with membrane-associated leptin receptors in various tissues (13, 24, 54), and regulates energy homeostasis by matching rates of energy expenditure with energy intake (25, 42, 45, 46, 61).

The biological actions of leptin are composed of direct peripheral (on target organs) and central (via hypothalamic and sympathetic nervous system) effects. Peripherally, leptin affects the metabolism of various tissues including the pancreatic β-islets (30, 66), adipocytes (38, 42), skeletal muscle (50, 62), and liver (41), thereby modulating insulin secretion and sensitivity (30, 41, 42, 50, 62, 66). Centrally, leptin traverses the blood-brain barrier via receptor-mediated transcytosis (ObRa) or, in the absence of a blood-brain barrier, directly accesses the intracellular signal-transducing long isoform of the leptin receptor (ObRb) expressed in the hypothalamus (2, 6, 8, 54). In the hypothalamus, leptin alters the synthesis and release of key orexigenic (neuropeptide Y, agouti-related peptide) and anorexigenic (proopiomelanocortin and cocaine and amphetamine-related transcript) peptides in the adult, thereby suppressing appetite resulting in anorexia, increasing sympathetic nervous output resulting in enhanced energy expenditure, and ultimately producing weight loss (16, 46, 61).

Although most of the studies involving leptin action have been reported in the adult, some information during the postnatal period exists. Circulating leptin concentrations peak in the suckling mouse at age 7–10 days and in the rat at age 14–18 days (4, 18). In addition, leptin is also made by the placental trophoblasts, thereby contributing toward the net circulating fetal leptin concentrations (5, 24). Although a wide distribution of leptin receptors has been observed in the murine fetus (24), the functional role of leptin during fetal and/or postnatal periods of development remains controversial. Investigations so far have only examined the effect of systemic leptin administration during the postnatal period (3, 10, 15, 28, 36, 63). Systemic leptin administration in the postnatal rat was observed to cause a disinhibition of nonshivering thermogenesis (10), an increase in energy expenditure in a diurnal manner (4, 51), a decline in the fat pad weight (36, 44, 63) and body fat content (36, 44), and a decrease in body weight (3, 28, 36, 63), attesting to the presence of leptin sensitivity during this period of development. Investigations centered on the hypothalamic action of leptin have yielded differing results, with some studies reporting no effect, lending support to the concept of hypothalamic leptin resistance during the postnatal period (3). Other studies have demonstrated leptin-induced changes in the neuronal connectivity (11) and expression of key neuropeptides that are known to regulate ingestive behavior in the adult (36).

Recent investigations have focused on postnatal systemic leptin administration having long-term effects mainly on adult male food intake and body weight (11, 15). Thus whether postnatal hypothalamic leptin resistance exists is controversial. Further, whether postnatal leptin resistance, if it exists, persists into the adult stage is unknown. Finally, whether leptin resistance is at the blood-brain barrier or in the absence or immaturity of a blood-brain barrier at the hypothalamus cannot be resolved by systemic leptin studies alone. We hypothesized that postnatal chronic intracerebroventricular (ICV) leptin administration will have a direct and immediate effect on key neuropeptides (neuropeptide Y and α-melanocyte-stimulating hormone).

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hormone), which in turn will have a permanent impact on the ingestive behavior of the animal, thereby altering the postnatal and adult phenotype, and will indirectly disprove the existence of postnatal leptin resistance. To test this hypothesis, we undertook the present investigation to determine the hypothalamic and metabolic/hormonal effects of ICV leptin administration on the postnatal and subsequent adult male and female phenotypes.

MATERIALS AND METHODS

Animals. Gestationally timed pregnant Sprague-Dawley rats (Taconic Farm, Germantown, NY) were housed in individual cages, exposed to 12:12-h light-dark cycles at 21–23°C, and allowed access to standard rat chow (Purina, St. Louis, MO) ad libitum. As approved by the Magee-Women’s Research Institute’s Animal Care and Use Committee at the University of Pittsburgh and the Animal Research Committee at the University of California Los Angeles, the guidelines of the National Institutes of Health were followed. The animals were allowed to deliver, and the number of newborn pups per litter was culled to 10 to minimize the effect of litter size on nutrition and body weight.

Postnatal studies. The litters of pups (n = 160 pups from 16 litters) were arbitrarily divided into two major groups. One received ICV murine leptin (1 μg·2.5 μl·dose−1; Peninsula Laboratories, Belmont, CA) daily between 2 and 7 neonatal days of age (n = 70 pups), and the second group received 2.5 μl of vehicle (n = 90 pups) under mild restraint. The dose of leptin given during the torpor-like state of energy conservation in the morning (51) translated into an age-dependent stepwise decrease with an initial dose of 0.16 μg·body wt (BW)·dose−1 on day 2, 0.14 μg·g BW·dose−1 on day 3, 0.12 μg·g BW·dose−1 on day 4, 0.10 μg·g BW·dose−1 on day 5, 0.09 μg·g BW·dose−1 on day 6, and 0.08 μg·g BW·dose−1 on day 7. In a subset of pups, bovine serum albumin (2.5 μg·2.5 μl·dose−1; n = 4) was injected daily between 2 and 7 days of age and compared with the vehicle group (n = 4) to ensure no effect due to nonspecific protein administration into the cerebral ventricles. Leptin doses and route of administration were chosen on the basis of our preliminary experiments, which revealed that daily (2–7 neonatal days of age) ICV doses ranging from 0.05 to 1 μg·g BW·dose−1 led to a dose-dependent diminutive effect on the postnatal body weight gain pattern. The study design accounted for interlitter and intralitter variations by administration of leptin and vehicle to either littermates or pups from different litters. The 2–7 neonatal days of age were chosen for ICV leptin administration because this postnatal period was optimally responsive to leptin, as determined previously (15). All ICV injections were performed with the use of conventional stereotaxic coordinates for the lateral ventricle, as previously described (31, 47, 53, 57). The coordinates were set at 0.4 mm lateral to the skull surface (57). Individual pups in each litter were weighed daily between 8:00 and 10:00 AM. On weaning of pups at 21 days, body weight was assessed once every 10 days until 120 days of age. Food intake was assessed in the morning between 8:00 and 10:00 AM by measuring over a 24-h period the food consumed by weighing the rat chow at the start and end of the 24-h period after accounting for spillage and evaporation.

Glucose tolerance test. At day 8 and days 60–150, awake animals were placed in a plastic container (day 8) or in rat restrainers (days 60–150) and subjected to a glucose tolerance test. The animals received 0.5 g/kg BW of glucose intraperitoneally (day 8) or via the tail vein (days 60–150), and blood was obtained subsequently at 0, 15, 30, 60, and 120 min from the jugular vein (day 8) or the tail vein (days 60–150) to measure glucose concentrations as previously described (55). Glucose tolerance tests were performed in pooled suckling pups and individual adult animals.

Plasma assays. Animals were euthanized with intraperitoneal pentobarbital sodium (50 mg/kg), blood was collected from the left ventricle, and the plasma was separated and aliquoted for measurement of glucose by the glucose oxidase method (Sigma Diagnostics, St. Louis, MO; sensitivity = 1.8 mg/dl). Insulin, leptin, and corticosterone were quantified by double-antibody RIAs using rat standards and anti-rat insulin, anti-rat leptin (Linco Research, St. Charles, MO), or anti-rat corticosterone (Amersham Life Science, Buckinghamshire, UK) antibodies (sensitivity: insulin = 0.1 ng/ml, leptin = 0.5 ng/ml, and corticosterone = 0.06 ng/ml). Leutinizing hormone was also assessed by RIA using rat specific standards and antibodies (sensitivity = 0.005 ng/ml) (57).

Hypothalamic tissue assays. The hypothalamus was obtained as a frontal slide by vertical cuts 1 mm anterior to the body of the optic chiasm and 1 mm posterior to the mammillary bodies, with lateral boundaries demarcated by the ventral aspects of the cerebral cortices, and a single horizontal cut that separated the hypothalamus from the thalamus (31). The tissue block was weighed and extracted in four volumes of 0.1 N HCl (wt/vol). The extract was sonicated for 10 s (sonic dismembrator Fisher Scientific, Pittsburgh, PA) using 10 W of output power. The sonicated acid extracts were centrifuged at 10,000 rpm for 10 min to remove the tissue debris. The supernatant was freeze dried. The freeze-dried extracts were reconstituted in 0.05 M Tris·HCl buffer containing 0.1% bovine serum albumin (pH 7.8) for neuropeptide Y (NPY) measurements by RIA. NPY was assessed by an RIA that employed a polyclonal rabbit anti-rat NPY antibody and rat NPY standards (Peninsula Laboratories, Belmont, CA). NPY was expressed as picograms per unit of hypothalamic protein and as nanograms per gram of hypothalamic wet weight (49, 57).

Immunohistochemical analysis. The day 8 (n = 5), day 21 (n = 5), and day 120 (n = 5) rats from the leptin (0.16 μg·g BW·dose−1 initially) and vehicle treatment groups were anesthetized with intraperitoneal pentobarbital sodium (50 mg/kg), and their brains were perfused and fixed as previously described (40, 57). Serial rostrocaudal brain sections were obtained (35 μm) and subjected to immunohistochemical analysis as previously reported (40, 48, 57). Rabbit anti-rat NPY (1:8,000; Peninsula Laboratories, Belmont, CA), lepton receptor (1:200; Research Diagnostics, Flanders, NJ), or α-melanocyte-stimulating hormone (α-MSH; 1:10,000) (22) IgGs served as primary antibodies. Preimmune serum and the peptide preabsorbed antibodies were used as appropriate controls. After incubation with a biotinylated secondary antibody (Vector Lab, Burlingame, CA), sections were treated with the avidin-biotin complex (Vector Lab) and incubated with diaminobenzidine tetrahydrochloride solution containing hydrogen peroxide subsequently to generate a color reaction. Sections containing the hypothalamic region were subjected to image analysis under ×40 magnification with the use of the Simple C-32 software program (C-Imaging Series SIMPLE 32; Complix Imaging Systems, Cranberry, PA). After the background was subtracted, a gray scale was developed on the basis of the intensity of the immunoreactivity. This gray scale provided relative intensity of the neuropeptide immunoreactivity. In addition, the area of the immunoreactivity was circumscribed as per the anatomic landmarks described in the rat brain atlas (31) and measured. The measured intensity multiplied by the area of NPY immunoreactivity equaled the total amount of NPY immunoreactivity observed and was expressed in arbitrary units per section (57). A total of five sections per brain were analyzed to obtain a mean value of n = 1.

Data analysis. Data are expressed as means ± SE; n = 1 in the postnatal stage represented pooled pups (usually 5) from a single litter, each n = 1 arising from a separate litter for all experimental assays. In the postweaned animals, n = 1 represented a single animal obtained from different litters. An ANOVA model that included group effect (leptin vs. vehicle), age effect, and group × age interaction terms was fit to all the outcome variables. An overall F value for the significance of the model was computed in addition to an F value for the group effect, age effect, and group × age interaction effect. In the
RESULTS

Body weight gain pattern. Figure 1 demonstrates the changes in body weight gain in response to ICV leptin (0.16 μg·g BW⁻¹·dose⁻¹ initially) administration. Males and females were not separated during the suckling phase. A 10% decline in body weight gain was observed during the early postnatal phase (2–7 days) of development (Fig. 1A). Administration of bovine serum albumin had no impact on the body weight gain and was statistically similar to the vehicle-treated group (hence not shown). After cessation of the leptin intervention during the late postnatal phase of the suckling period, which spanned 8–21 days, a persistent decline in body weight gain was observed in the leptin group (Fig. 1B). The leptin-induced decline in postnatal body weight gain persisted in the adult females from 35 to 120 days of age (Fig. 2A). In contrast, no change in the body weight gain pattern was noted in adult males (Fig. 2B).

Food intake. A decline in food intake was evident in the leptin group (0.16 μg·g BW⁻¹·dose⁻¹ initially), paralleling the changes in body weight gain (Fig. 2A) and reaching statistical significance from 90 to 120 days only in the females (Fig. 3A). No similar statistically significant difference in food intake was noted in the adult males, although the actual mean values were lower with the leptin treatment compared with age-matched controls (Fig. 3B). When food intake was standardized to the body weight of the animals, a 10% decline was still observed in the 60- to 120-day-old females (Fig. 3C), with no difference in the males (Fig. 3D).

Glucose tolerance tests. Glucose tolerance testing at 8 days in the leptin group revealed a relatively faster glucose clearance (P < 0.02 at 60 min) in the leptin treatment group.
compared with the vehicle-treated group (Fig. 4A). In contrast, glucose intolerance ($P < 0.0098$ at 15 and 30 min) was observed in the leptin treatment group as early as 60 days in females (Fig. 4B). The males did not demonstrate glucose intolerance as late as 150 days in age (Fig. 4C).

**Plasma glucose and hormone concentrations.** Postnatal ICV administration of leptin (0.16 μg·g BW$^{-1}$·dose$^{-1}$ initially) led to no changes in circulating glucose concentrations in the *day 8*, *day 21*, and *day 120* males. The *day 120* females demonstrated hyperglycemia (Table 1). A 30% increase in plasma insulin concentrations was present at 8 days, and a 50% increase persisted in the *day 120* females alone (Table 1). Furthermore, although no difference in circulating leptin concentrations was observed at 8 and 21 days, a significant decline was noted in both females and males at 120 days (Table 1). ICV leptin administration from *days 2* to *7* led to an 80% decrease in endogenous corticosterone concentrations at *day 8* but caused a 60–70% increase in the *day 120* females and males (Table 1). Whereas no change was observed at 8 days, an increase in circulating leutinizing hormone concentrations occurred in the *day 21* (~70%) and *day 120* males and females. The increase in leutinizing hormone was more pronounced in males (2-fold) compared with females (~66%; $P < 0.01$) (Table 1).

**Hypothalamic neuropeptide concentrations.** ICV leptin (0.16 μg·g BW$^{-1}$·dose$^{-1}$) administration led to a decline in the paraventricular NPY immunoreactivity at 8 days that persisted in the *day 21* and *day 120* female adults. No effect was noted on the arcuate nuclear NPY immunoreactivity in the *day 8*, *day 21*, and *day 120* males, but there was a decline in the *day 120* females (Fig. 5, A–L; Table 2). The leptin-induced alterations in the total hypothalamic NPY concentrations paralleled the changes observed in the paraventricular nucleus (Table 2). Similar to the NPY changes, a persistent decline in hypothalamic (paraventricular nuclear mainly) leptin receptor immunoreactivity was noted at *day 8* (60%) and *day 21* (25%) and in the *day 120* females (20%) (Fig. 6, A–L; Table 2). In contrast, postnatal ICV leptin treatment led to no statistically

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**Table 1. Plasma glucose and hormone concentrations**

<table>
<thead>
<tr>
<th>Age</th>
<th>Glucose, mM</th>
<th>Insulin, ng/ml</th>
<th>Leptin, ng/ml</th>
<th>Corticosterone, ng/ml</th>
<th>LH, ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>8 Days</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VEH (n = 6)</td>
<td>7.28±1.39</td>
<td>2.1±0.1</td>
<td>1.67±0.1</td>
<td>36.4±2.6</td>
<td>0.6±0.08</td>
</tr>
<tr>
<td>LEP (n = 6)</td>
<td>7.0±0.56</td>
<td>3.1±0.1*</td>
<td>1.2±0.1</td>
<td>7.2±1.4*</td>
<td>0.54±0.1</td>
</tr>
<tr>
<td>21 Days</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VEH (n = 6)</td>
<td>12.27±1.2</td>
<td>3.7±0.5</td>
<td>3.27±0.7</td>
<td>19.6±3.3</td>
<td>0.18±0.02</td>
</tr>
<tr>
<td>LEP (n = 6)</td>
<td>11.89±1.17</td>
<td>4.28±0.5</td>
<td>3.46±0.4</td>
<td>26.4±1.4</td>
<td>0.31±0.06*</td>
</tr>
<tr>
<td>120 Days</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VEH (n = 6)</td>
<td>11.78±0.22</td>
<td>7.8±1.7</td>
<td>9.12±0.7</td>
<td>40.6±3.2</td>
<td>0.5±0.1</td>
</tr>
<tr>
<td>LEP (n = 6)</td>
<td>14.72±0.44*</td>
<td>15.7±0.4*</td>
<td>1.56±0.1*</td>
<td>140±2.8*</td>
<td>0.83±0.1*</td>
</tr>
<tr>
<td>Female</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VEH (n = 6)</td>
<td>11.78±0.67</td>
<td>3.6±0.02</td>
<td>9.43±0.3</td>
<td>33±2.9</td>
<td>0.27±0.01</td>
</tr>
<tr>
<td>LEP (n = 6)</td>
<td>13.17±0.61</td>
<td>2.77±0.3</td>
<td>4.2±0.5*</td>
<td>77±2.6*</td>
<td>0.81±0.05*</td>
</tr>
</tbody>
</table>

*Values are means ± SE; n = no. of animals. VEH, vehicle treated; LEP, leptin treated; LH, leutinizing hormone. P = 0.001 (ANOVA) for group, age, and group × age effect for glucose, insulin, leptin, and LH in females and leptin and LH alone in males, with a group effect in females and males for corticosterone; however, the age effect and group × age effect were not significant, and the group effect for glucose and insulin in males was not significant. *P < 0.01 (post hoc testing) vs. age-matched VEH group.*
significant change at day 8 but caused an increase in the dorsomedial nuclear α-MSH immunoreactivity alone at day 21 (4-fold). In the day 120 females, a twofold increase in the dorsomedial and arcuate nuclear α-MSH immunoreactivity was noted (Fig. 7, A–L; Table 2). Unlike the dorsomedial nucleus, minimal amounts of α-MSH were observed in the paraventricular nucleus, and no major change in paraventricular nucleus α-MSH immunoreactivity due to leptin administration was observed at the ages examined (negative data not shown). No similar changes were noted in the day 120 male hypothalami for all three peptides, namely NPY, leptin receptor, and α-MSH (Table 2).

Table 2. Hypothalamic neuropeptide concentrations

<table>
<thead>
<tr>
<th>Age</th>
<th>NPY ir, AU</th>
<th>NPY RIA†</th>
<th>LR ir, AU</th>
<th>α-MSH ir, AU‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>8 Days</td>
<td></td>
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<td></td>
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<tr>
<td>VEH</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>PVN or DMN</td>
<td>93±2 (5)</td>
<td>16±1.4 (6)</td>
<td>132±1.2 (5)</td>
<td>71±2 (5)</td>
</tr>
<tr>
<td>ARC</td>
<td>92±1 (5)</td>
<td>4±0.2 (6)</td>
<td>122±0.8 (5)</td>
<td>84±1.1 (5)</td>
</tr>
<tr>
<td>LEP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PVN or DMN</td>
<td>31±0.8* (5)</td>
<td>7.5±0.6* (6)</td>
<td>51±1.1* (5)</td>
<td>67±1.2 (5)</td>
</tr>
<tr>
<td>ARC</td>
<td>85±2 (5)</td>
<td>0.4±0.09* (6)</td>
<td>113±0.9* (5)</td>
<td>93±0.8 (5)</td>
</tr>
<tr>
<td>21 Days</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VEH</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PVN or DMN</td>
<td>167±0.8 (5)</td>
<td>80±1.6 (5)</td>
<td>105±0.6 (5)</td>
<td>42±0.5 (5)</td>
</tr>
<tr>
<td>ARC</td>
<td>127±2 (5)</td>
<td>106±0.6 (5)</td>
<td>109±1 (5)</td>
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</tr>
<tr>
<td>LEP</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>PVN or DMN</td>
<td>67±1.5* (5)</td>
<td>65±0.6* (5)</td>
<td>125±2.4* (5)</td>
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<tr>
<td>ARC</td>
<td>113±1.3 (5)</td>
<td>101±1.6 (5)</td>
<td>100±1 (5)</td>
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</tr>
<tr>
<td>Female</td>
<td></td>
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<tr>
<td>120 Days</td>
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<tr>
<td>VEH</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>PVN or DMN</td>
<td>169±0.5 (5)</td>
<td>28±2.9 (6)</td>
<td>90±2 (5)</td>
<td>51±1.7 (5)</td>
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<tr>
<td>ARC</td>
<td>136±0.4 (5)</td>
<td>9.4±0.2 (6)</td>
<td>57±1.6 (5)</td>
<td>55±1.3 (5)</td>
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<td>LEP</td>
<td></td>
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<tr>
<td>PVN or DMN</td>
<td>38±0.8* (5)</td>
<td>12±0.6* (6)</td>
<td>51±1.3* (5)</td>
<td>110±3* (5)</td>
</tr>
<tr>
<td>ARC</td>
<td>51±0.8* (5)</td>
<td>2.8±0.2* (6)</td>
<td>53±2.2 (5)</td>
<td>102±1.5* (5)</td>
</tr>
<tr>
<td>Male</td>
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<tr>
<td>120 Days</td>
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<td>VEH</td>
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<tr>
<td>PVN or DMN</td>
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<td>16±2 (6)</td>
<td>89±1 (5)</td>
<td>72±3 (5)</td>
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<tr>
<td>ARC</td>
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<td>64±2 (5)</td>
<td>81±2.7 (5)</td>
</tr>
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<td>LEP</td>
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<td></td>
</tr>
<tr>
<td>PVN or DMN</td>
<td>112±3 (5)</td>
<td>16±0.6 (6)</td>
<td>84±4 (5)</td>
<td>63±0.9 (5)</td>
</tr>
<tr>
<td>ARC</td>
<td>98±2 (5)</td>
<td>7.5±0.3 (6)</td>
<td>60±3 (5)</td>
<td>76±2.5 (5)</td>
</tr>
</tbody>
</table>

Values are means ± SE, with n in parentheses. NPY, neuropeptide Y; LR, leptin receptor; α-MSH, α-melanocyte-stimulating hormone; ir, immunoreactivity; AU, arbitrary units; PVN, paraventricular nucleus; DMN, dorsomedial nucleus; ARC, arcuate nucleus. †Units for NPY RIA: PVN or DMN, pg/0.1 mg protein; ARC, ng/g brain weight. ‡Values for α-MSH are DMN and ARC; all other values are PVN and ARC, respectively. P = 0.008 (ANOVA) for group, age, and group × age effect for NPY RIA and NPY, α-MSH, and LR immunoreactivity in females, with only an age effect in males. Group effect and group × age effect in males were not significant. *P < 0.008 (post hoc testing) vs. age-matched VEH group.
DISCUSSION

Early postnatal body weight gain pattern. We have demonstrated a decrease in early postnatal body weight (2–7 days) due to ICV leptin administration in the animals that received six doses of a stepwise decreasing regimen of leptin dosing (0.16 μg·g BW\(^{-1}\)·dose\(^{-1}\) initially). Previous investigations have revealed that the leptin-induced decline in postnatal body weight reflects a diminution in the fat pad weight and body fat content in rats (36, 44, 63). The mechanism behind the leptin-induced decline in body fat pad weight and content may relate to adipocyte apoptosis (2, 22, 38, 63). The decrease in postnatal body weight is also due to increased energy expenditure (20, 64) and disinhibition of nonshivering thermogenesis (10, 42), caused by leptin’s stimulation of the sympathetic nervous system (10, 20, 42, 64). No postnatal role for a decrease in energy intake due to altered ingestive behavior has been assigned so far (36, 52, 64). Further, no changes in hypothalamic neuropeptide expression were found in response to systemic leptin (3). Thus there was the need for our present studies employing ICV leptin to distinguish between the previously described systemic leptin-induced effects (3) and direct hypothalamic effects. Our present postnatal time line (at leptin doses of 0.08–0.16 μg·g BW\(^{-1}\)·dose\(^{-1}\)) is in keeping with previous reports involving systemic administration of leptin at 0.08 μg·g BW\(^{-1}\)·dose\(^{-1}\) (15). In both cases, no effect on body weight was evident until 5 days of age, 72 h after initiation of leptin administration, supporting the fact that both systemic and ICV leptin work through a common mechanism toward reducing postnatal body weight. This reduction in early postnatal body weight suggests the presence of leptin sensitivity, since previous studies in fa/+/fa rats that carry a mutation in the leptin receptor and exhibit leptin resistance demonstrated no decline in postnatal body weight in response to systemic leptin administration (28). In contrast, the wild-type and fa/+ rats with varying degrees of leptin sensitivity demonstrated a decline in postnatal body weight (28). Thus the leptin-induced reduction in postnatal weight gain signifies the presence of leptin sensitivity.

Late postnatal and adult body weight gain pattern. Leptin (0.16 μg·g BW\(^{-1}\)·dose\(^{-1}\) initially) administered postnatally in our study led to a persistent decline in body weight during the later postnatal phase (8–21 days) and in the adult female. Our findings contrast previous studies in male Wistar rats that received subcutaneous leptin (8 μg/100 g) over the first 10 days.

Fig. 6. Immunohistochemical analysis: LEP receptor. Representative photomicrographs demonstrating high-power (scale bar = 0.008 mm; A–L) magnification of day 8 (top), day 21 (middle), and day 120 (bottom) female coronal floating microtome brain sections with LEP receptor immunoreactivity (antibody dilution = 1:200) noted in the ICV VEH (B, D, F, H, J, and L)- and LEP (A, C, E, G, I, and K)-treated groups within the PVN (shown by arrows, A–F) and ARC (shown by arrows, G–L) regions in the LEP (n = 5)- and VEH (n = 5)-treated day 8, day 21, and day 120 female rats.

Fig. 7. Immunohistochemical analysis: α-melanocyte-stimulating hormone (α-MSH). Representative photomicrographs demonstrating high-power (scale bar = 0.008 mm; A–L) magnification of day 8 (top), day 21 (middle), and day 120 (bottom) female coronal floating microtome brain sections with α-MSH immunoreactivity (antibody dilution = 1:10,000) noted in the ICV VEH (B, D, F, H, J, and L)- and LEP (A, C, E, G, I, and K)-treated groups within the dorsomedial nuclear (DMN; shown by arrows, A–F) and ARC (shown by arrows, G–L) regions.
days of life (15). Unlike this previous report (15), we did not observe an increase in the body weight of the adult male Sprague-Dawley rats within the 120-day age range. Further, the findings with leptin are dissimilar to those seen in the intrauterine growth-restricted (IUGR) offspring that exhibit postnatal growth restriction as well, but develop hyperphagia, and become obese and hyperleptinemic adults (58, 59). Instead it may relate to the degree of leptin sensitivity that is present despite exogenous chronic leptin administration. The postnatal administration of leptin (0.16 μg·g BW⁻¹·dose⁻¹ initially) is possibly associated with intact leptin sensitivity throughout. This is exaggerated in the leptin-deficient state encountered in ob/ob mice that exhibited a decrease in body weight at 32 days of age in response to postnatal leptin administration (11).

Postnatal hypothalamic changes. Assessment of the effect of postnatal ICV leptin on hypothalamic NPY (orexigenic peptide) and α-MSH (anorexigenic peptide) is important, since previous studies have demonstrated a leptin-induced increase in energy expenditure (20, 64), but its effect on hypothalamic neuropeptides remained controversial (3, 15, 36). In the present study, we observed a decline in paraventricular NPY at day 8, with no statistically significant effect on α-MSH. Our present observations are similar to previous rat investigations where chronic systemic leptin administration (3 μg/g) led to a decline in hypothalamic NPY mRNA that was more pronounced than the increase in proopiomelanocortin (POMC) mRNA (36). These observations are partly similar to those in the adult, where leptin decreases hypothalamic NPY and increases α-MSH (16, 45). These leptin-induced changes in NPY and α-MSH, downstream of the leptin receptor, signify leptin sensitivity and mediate the diminution of postnatal body weight gain.

Postnatal leptin administration induced a decline in ObRb mRNA only in the postnatal caudal part of the arcuate nucleus, with an increase in suppressor of cytokine signaling-3 mRNA, which inhibits leptin signaling. In addition, a disconnection between the leptin effect on ObRb mRNA (36) and leptin binding to its hypothalamic receptors was observed (44). Thus it was critical to assess the hypothalamic neuropeptide concentrations rather than mRNA concentrations in our current study. Assessment of the total leptin receptor amounts in the paraventricular nucleus demonstrated a decline in the postnatal leptin-treated group, with no difference in the arcuate nucleus. This suggests a leptin-induced downregulation of total leptin receptors only in the paraventricular nucleus and not the arcuate nucleus. This observation is in keeping with previous reports involving the effect of postnatal leptin on ObRb mRNA concentrations alone in the arcuate nucleus (40). However, the changes we observed with total ObR in the postnatal period did not impair leptin’s action on hypothalamic NPY initially and α-MSH subsequently.

Investigations that have shown no effect of postnatal systemic leptin on hypothalamic NPY, agouti-related peptide, or POMC have undertaken experiments involving either only male animals (3, 15) or administered leptin (2 μg/g dose) between 10 and 17 days, when circulating leptin concentrations peak (3), rather than between 2 and 7 days, when maximal leptin sensitivity exists (15); assessed total hypothalamic mRNA concentrations (3); or administered differing doses of leptin systemically (3, 15, 36). Thus valid interstudy comparisons have been difficult. As is evident in our current study, neuropeptide changes are specific to the paraventricular nucleus in certain cases (NPY) or to the dorsomedial nucleus in other situations (α-MSH). In addition, changes in peptide immunoreactivity may be a net reflection of various processes that include synthesis, transport to, and release at the nerve terminal; intracellular peptide degradation; and/or growth of specific nerve termini. Combination of our present immunohistochemical protein data with previous in situ hybridization mRNA data (36) may lend credence to a biological role for leptin in the postnatal hypothalamus. Additionally, because both systemically and ICV-administered leptin produced similar hypothalamic changes in NPY and POMC/α-MSH mRNA (36) and protein concentrations, further confirmation of leptin sensitivity in the postnatal hypothalamus is forthcoming.

Postnatal ingestive behavior and energy expenditure. To determine whether postnatal leptin sensitivity includes appetite control, assessment of ingestive behavior would have been revealing. However, accurate measurement of ingested milk during the suckling phase is difficult without introducing other variables such as separation from the mother that in itself alters hypothalamic neuropeptides and ingestive behavior (26). Despite previously detected changes in hypothalamic neuropeptides due to systemic leptin, no change in postnatal milk ingestion at 5–10 days of age was reported (36). However, milk ingestion assessments involved separation of pups from the mother and measuring pre- and postprandial pup weights after the pups ingested milk from blotting papers placed below them (36, 52). Thus it is difficult to know whether the milk ingested was accurately measured. This is a universal limitation to postnatal studies where pup weights are used as a surrogate for milk ingestion (36, 52). Even though postnatal leptin alters hypothalamic neuropeptides, these changes may predominantly perturb energy expenditure rather than energy intake between 10 and 15 days of age (20, 64). NPY, in addition to causing hyperphagia in the adult, also conserves energy (16, 45, 61). On the other hand, α-MSH, while suppressing appetite, enhances energy expenditure (15, 45, 61). Both of these biological functions are mediated via the hypothalamus. Previous studies have established that leptin affects the metabolic rate in the suckling rat more than energy intake, while predominately affecting energy intake during the postsuckling phase. In the rat, a postnatal leptin surge occurs at 15 days (40); hence, this phase may coincide with a minimal effect on milk ingestion. Our study assessed hypothalamic changes due to leptin administration in the immediate postnatal period of leptin sensitivity (15) before the circulating leptin surge and the potential emergence of postnatal “leptin resistance.” Whether these neuropeptide changes affect suckling behavior in addition to the metabolic rate during this postnatal period (2–7 days) remains unknown. Recently, systemic leptin treatment in the postnatal period restored permanently disrupted neural projection pathways from the arcuate nucleus to leptin-deficient mice, which translated into permanent postsuckling changes in food intake (11). These results strongly support our current investigations.

Post suckling food intake and hypothalamic neuropeptide concentrations. Postnatal ICV leptin administration (0.16 μg·g BW⁻¹·dose⁻¹ initially) led to a postsuckling decline in food intake that closely paralleled the decline in body weight, achieving statistical significance in the adult females alone. This suggests that the leptin-induced decline in food intake...
(energy intake) was regulated to meet the decreasing demands of a diminished body weight gain pattern. This observation is dissimilar to that with maternal starvation or bilateral uterine artery ligation leading to IUGR. In the former, the offspring exhibit hyperphagia with an energy intake that is higher than the rate of body weight gain (58). The latter IUGR offspring demonstrate an increase in hypothalamic NPY concentrations (40), whereas the adult female offspring in our study that received postnatal ICV leptin exhibited a decline in paraventricular and arcuate nuclear NPY concentrations. This change was associated with an increase in the dorsomedial and arcuate nuclear α-MSH concentrations. This permanent effect of postnatal leptin on the hypothalamic neuropeptides suggests the persistence of hypothalamic leptin sensitivity even after the cessation of leptin administration.

**Glucose tolerance states.** ICV leptin administration caused no change in circulating leptin concentrations, attesting to a sound ICV injection technique with no vascular leak. ICV leptin treatment led to hyperinsulinemia at 8 days that persisted in the day 120 female. At 8 days, a relatively improved glucose tolerance was observed. A similar observation was reported in adult mice and rats because of acutely enhanced muscle glucose utilization (41, 62). In contrast, at 60–150 days, glucose intolerance was observed. Hyperinsulinemia with glucose intolerance is a hallmark of insulin resistance. Thus, in the adult that received leptin postnatally, the female animals were hyperinsulinemic, hyperglycemic, and glucose intolerant. In addition, these animals were hypoleptinemic and hypercorticosteronemic because of a catabolic state. Body weight reduction with hypoleptinemia signifies a diminution of fat content (3, 23, 37, 39, 45, 64), which, along with insulin resistance, characterizes a state of lipoatrophic diabetes mellitus as previously described (1).

**Sex-specific perturbations.** Although no difference between the vehicle-treated day 120 male and female rat leptin concentrations was observed in our present investigation, a greater reduction of circulating leptin concentrations in the leptin-treated group was observed in the female, reflective of a change in fat mass. In addition, sex-specific effects in the adult hypothalamus have also been reported (14). Information in the adult rat revealed that this female-preferred leptin effect on food intake is upstream of the hypothalamic melanocortin-3 and -4 receptors and perhaps involves NPY (14). Estrogen suppresses NPY, whereas androgens augment NPY synthesis in the hypothalamus (9, 29, 43, 56, 57). Unlike the effect on NPY, leptin caused an increase in circulating leutinizing hormone in the day 21 (pubertal) and the day 120 (postpubertal) adult females and males, serving as a positive outcome measure for the postnatal leptin treatment. This change is aligned with prior observations of a prepubertal surge in leptin being responsible for the sexual maturation in rats (60). Furthermore, leptin is known to enhance hypothalamic gonadotropin-releasing hormone that in turn increases the pituitary synthesis of leutinizing hormone that is secreted into the circulation (33).

The hormonal changes we observed during or after the suckling phase in the adult as a consequence of postnatal leptin administration are distinct from what has been reported in adult rats subjected to ICV or systemic leptin administration. Previous studies in the adult have shown leptin to suppress adrenal glucocorticoid synthesis, resulting in low circulating glucocorticoid levels (34), and inhibit insulin secretion from isolated rat pancreatic β-islet cells (30, 66). On the other hand, both glucocorticoid and insulin augment adipocyte leptin synthesis and secretion (7, 12, 19, 27). In our present study, neonatal ICV leptin administration over 6 days caused no change in circulating leptin or glucose concentrations but led to a decline in corticosterone and an increase in insulin concentrations at day 8. The decrease in endogenous corticosterone by exogenous leptin administration at day 8 has previously been related to reduced glucocorticoid production (34) and/or an enhanced ability to inhibit the stress-enhanced suppressive effect on hypothalamic corticotropin-releasing factor mRNA levels and ACTH secretion (23, 35). This is partly due to increased glucocorticoid receptor expression in the hippocampus and hypothalamic paraventricular nucleus (35). In the day 120 adult that received leptin postnatally, hyperinsulinemia was associated with hyperglycemia and glucose intolerance in the female, whereas a decline in circulating leptin with an increase in corticosterone levels was seen in both the male and female. Hypercorticosteronemia is more prominent in the female compared with the male, in keeping with the concomitant hypoleptinemia. This lowering of circulating leptin concentrations may have led to a disinhibition of adrenal corticosterone (34, 35) and augmented pancreatic insulin synthesis/release (30, 66), the latter being more pronounced in the female due to the additional complication of insulin resistance.

Overall, postnatal intervention with ICV leptin caused a sex-specific permanent alteration in the adult phenotype partly due to persistent perturbations in the hypothalamic neuropeptides and the metabolic/hormonal milieu. We speculate that these changes may ultimately result in a lipoatrophic diabetes mellitus-like phenotype signifying the presence of postnatal hypothalamic leptin sensitivity. This is an example of a sex-specific “neuropeptide imprinting” effect secondary to postnatal leptin exposure constituting “hormonal imprinting.” The significance of our observations relates to the offspring of a diabetic mother exhibiting high levels of circulating leptin at birth (17, 32) and low concentrations of brain NPY just before birth (49). The level of hyperleptinemia at birth may result in either leptin sensitivity or resistance, thereby contributing toward heterogeneity in clinical presentation by predetermining the adult phenotype. Future studies targeted at the postnatal receptor-signaling pathway are warranted to decipher the mechanisms that predispose toward leptin resistance vs. retention of sensitivity in the adult.

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