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Leptin Regulates Appetite-Related Neuropeptides in the Hypothalamus of Developing Rats without Affecting Food Intake

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Leptin regulates food intake in adult mammals by stimulating hypothalamic anorexigenic pathways and inhibiting orexigenic ones. In developing rodents, fat stores are low, yet circulating leptin levels are high and do not appear to regulate food intake. We determined whether two appetite-related neuropeptides [neuropeptide Y (NPY) and proopiomelanocortin (POMC)] and food intake behavior are sensitive to leptin [3 mg/kg body weight (BW), ip] in neonates. We measured the effects of 1) acute leptin administration (3 mg/kg BW, ip, 3 h before testing) on food intake on postnatal day (PND) 5, 8, and 10; and 2) chronic leptin treatment (3 mg/kg BW, ip, daily PND3-PND10) on BW gain and fat pads weight on PND10. In addition to hypothalamic POMC and NPY expression, we determined the expression of suppressor of cytokine signaling-3, all subtypes of leptin receptors, and corticotropin-releasing factor receptor-2 mRNA in PND10 pups receiving either an acute (PND10) or a chronic (PND 3–10) leptin (3 mg/kg BW, ip) or vehicle treatment. Brains were removed 30 or 120 min after the last injection. Acute leptin administration did not affect food intake at any age tested. Chronic leptin treatment did not change BW but decreased fat pad weight significantly. In the arcuate nucleus (ARC), acute leptin increased SOCS-3 and POMC mRNA levels, but decreased NPY mRNA levels in the rostral part of ARC. Chronic leptin downregulated all subtypes of leptin receptors mRNA and decreased NPY mRNA levels in the caudal ARC but had no further effect on POMC expression. Chronic leptin increased corticotropin-releasing factor receptor-2 mRNA levels in the ventromedial hypothalamus. We conclude that despite adult-like effects of leptin on POMC, NPY, and CRFR-2 expression in neonates, leptin does not regulate food intake during early development. (*Endocrinology* 143: 4683–4692, 2002)

LEPTIN, A HORMONE SECRETED by the adipose tissue, is well known for its critical role in the regulation of ingestive behavior and thermogenesis in adult rodents and humans (1, 2). Furthermore, leptin is implicated in the control of several neuroendocrine functions, including the hypothalamo-pituitary-adrenal (HPA) axis (1, 3–6). Leptin signals nutritional status and energy storage levels to feeding centers, through its action on the expression and release of orexigenic and anorexigenic neuropeptides including neuropeptide Y (NPY) and proopiomelanocortin (POMC), respectively. In adults, leptin has been shown to inhibit the expression of NPY, a potent stimulator of food intake (7–9), whereas it stimulates the expression of neuropeptide inhibitory to feeding such as the biologically active α-MSH (by way of reducing expression of the precursor POMC) in the rostral arcuate nucleus (ARC) (10), and corticotropin-releasing factor (CRF) from the paraventricular portion of the hypothalamus (PVN) (8, 11). In the arcuate nucleus, NPY and POMC neurons express Ob-Rb (the long form of leptin receptor) that are functionally coupled to the Janus kinase-signal transducer and activator of transcription intracellular signaling cascade and produce an endogenous inhibitor [suppressor of cytokine signaling (SOCS)-3] upon activation (12). Reduced POMC mRNA expression has been reported in animal models of reduced (ob/ob mouse) or defective (fa/fa Zucker rat) leptin signaling (13).

In neonates, hypothalamic NPY levels are initially low, dramatically increase at postnatal d 16 (PND16), and subsequently decline to reach adult levels after weaning (14). Recently, Grove *et al*. (15) demonstrated that the ARC NPY/agouti-related peptide fibers do not start to significantly innervate the PVN until PND10–11 and that even by PND15–16, these fibers have still not reached adult levels in the rostral part of the PVN. However, whether NPY expression is regulated by leptin during development is still unclear. Studies have demonstrated that NPY expression is sensitive to feeding status in developing rats. For instance, the expression of prepro-NPY and NPY is increased by maternal deprivation as early as PND2 in lean Zucker rat pups (16).

In contrast to NPY mRNA expression, POMC mRNA expression is hardly detectable in 5-d-old mice but then increases to reach adult levels around weaning (17). There is indication, however, that POMC mRNA expression in the ARC is not sensitive to the effect of chronic leptin treatment in 10-d-old mice (17). In the rat, adult-like pattern of distribution of POMC perikarya and fibers are observed by late gestation although the peak of POMC immunoreactivity occurs between PND21–28 (18). At present, there is a paucity of information on the regulation of POMC neurons in the ARC during the early neonatal period in this species.

Abbreviations: ARC, Arcuate nucleus; BW, body weight; CRF, corticotropin-releasing factor; CRFR2, CRF receptor type 2 ; HPA, hypothalamo-pituitary-adrenal; L, leptin; NPY, neuropeptide Y; Ob-Rall, all subtypes of leptin receptors; Ob-Rb, long form of leptin receptor; PND, postnatal day; POMC, proopiomelanocortin; PVN, paraventricular portion of the hypothalamus; S, saline; SOCS, suppressor of cytokine signaling; TEA, trietholamine; V, vehicle; VMH, ventromedial hypothalamus.

Similarly to POMC, CRF has been demonstrated to induce anorexia in adult rats (19). CRF receptor type 2 (CRFR-2) in the ventromedial hypothalamus (VMH) is the receptor through which CRF is thought to exert its action on feeding (20). Decreased VMH CRFR-2 mRNA levels have been reported in obese Zucker (fa/fa) rats (21) and also in starved and adrenalectomized rats (22). Moreover, both acute and chronic systemic administration of leptin in rats stimulated the expression of CRFR-2 mRNA in the VMH (20). Eghbal-Ahmadi *et al*. (23) found that CRFR-2 mRNA is expressed in the VMH as early as E16 and seems to follow a gradual pattern of increase, with a peak between PND15–25. Maternal deprivation has been shown to reduce the expression of CRFR-2 mRNA in the VMH of developing rats, whereas artificial feeding increased the expression of CRFR-2 mRNA to an intermediate level between maternally deprived unfed pups and control animals (24). However, to our knowledge, no study has analyzed the effects of leptin on the expression of this receptor during development.

Several authors, including ourselves (6, 25), reported that leptin is already effective in reducing body weight and body fat during early development, although this effect might be due to increased thermogenesis rather than anorexigenic action (26–28). Although the effects of leptin on NPY, POMC, and CRFR-2 are well established in adults, little is known about the ontogeny and the timing of the functional activation of these neuropeptides by leptin in rat pups. Hence, in our study we tested the effect of acute leptin on food intake in the early postnatal period and determined whether acute or chronic leptin treatment during the first 10 d of life induces functional intracellular responses and modifies the expression of hypothalamic neuropeptides such as NPY and POMC. We also examined leptin-induced changes in CRFR-2 expression in the ventromedial hypothalamus. We found that although acute leptin administration did not modify food intake in pups, leptin signaling was functional in the ARC of 10-d-old pups. Chronic and/or acute leptin treatment reduced the expression of NPY and all forms of Ob-R in the ARC, whereas it increased the expression of POMC in the ARC and CRFR-2 in the VMH.

Materials and Methods

Animals

Pregnant Sprague Dawley females (Charles River, St-Constant, Canada) were received on d 17–18 of gestation and housed individually in plastic cages with food and water available *ad libitum*. All animals were housed under constant environmental conditions of temperature (22–25 C), humidity (70–80%) and light (12-h light, 12-h dark cycle with lights on at 0800 h). The day of birth was considered d 0, and litters were culled to 10 pups/mother on d 2 of life. Other than daily disturbances due to the administration of the treatment (5-min maximum), pups were kept undisturbed with their mother until tested on d 5, 8, and 10. Litter weight was recorded every morning before the injections. All protocols were approved by the Animal Care Committee at McGill University and followed ethical guidelines from the CCAC.

Leptin treatments

Murine leptin was obtained lyophilized from Preprotech Inc. (Rocky Hill, NJ) and reconstituted in 10 mM Tris buffer at a pH of 9.5. After dissolution, the pH was readjusted to 7.4 by addition of 1 N HCl. For the chronic treatment, each litter was subdivided into three groups receiving either no injection (uninjected, n = 2 pups) or daily ip injections of 50 μl of either leptin (3 mg/kg BW, L, n = 4 pups) or vehicle (10 mM Tris-HCl, pH 7.4; V, n = 4 pups) between d 3 and 9 of life. All injections were given in the morning between 0830 and 1000 h.

Tissue collection

At 0900 h on PND10, rat pups from each chronic treatment group (V and L) were weighed and separated into two experimental groups receiving an ip injection of either saline (saline 0.9%, S) or leptin (3 mg/kg BW, L). Injected pups were then returned to their mothers in a quiet room and euthanized 30 or 120 min after the injection. Control pups (uninjected) were euthanized without any injection. The time interval of 30 min was chosen to be optimal for the determination of SOCS-3 induction as shown previously in adults (29), and the 120-min interval was chosen to be more optimal for the detection of stable changes in neuropeptide mRNA expression. Trunk blood was collected in chilled microcentrifuge tubes containing 10 μl of EDTA (60 mg/ml) and plasma was kept frozen at –20 C until assayed for leptin and corticosterone concentrations. Brains were rapidly collected and post-fixed in a chilled solution of 4% paraformaldehyde in phosphate buffer (0.1 M, pH 7.4, 4 C) for 2 d followed by immersion in a solution of 10% sucrose in phosphate buffer (0.1 M, pH 7.4) for 2 d at 4 C. Brains were then frozen at −80 C until processed for *in situ* hybridization. Retroperitoneal fat pads and adrenal glands were dissected and weighed.

To determine the possible down-regulation of leptin receptors (Ob-R) following chronic daily injection, a subset of V and L injected pups were euthanized on PND10, 24 h after the last injection to determine Ob-R mRNA levels in the ARC.

Time course after exogenous leptin injection

To determine the peak and duration of leptin increases in the plasma after exogenous administration, 10-d-old pups from 8 different litters were injected ip with 50 μl of either vehicle (0.9% NaCl) or leptin (3 mg/kg BW). Pups were euthanized at 1, 3, 6, 13, 18, or 24 h after injection (n = 6/treatment and per time point), and trunk blood was collected on EDTA as described above. A control group was euthanized without injection at the beginning of the experiment (t = 0 h, n = 5). Plasma concentrations of leptin and corticosterone were determined by sensitive RIAs.

Determination of food intake in pups

On PND5, PND8, and PND10, pups received an acute injection of leptin (3 mg/kg BW, ip) or saline (NaCl 0.9%) between 0900 and 1000 h, and they were removed from the dam for 3 h thereafter. This interval between injection and testing was chosen because we determined that the peak plasma leptin occurred 3 h after exogenous administration (Fig. 1). Before testing, pups were stimulated to urinate and defecate by stroking the anogenital region with an artist's brush wetted with warm water, then pups were weighed. During the intake test, pups were placed individually into plastic containers lined with paper towels wetted with a commercial lukewarm half and half milk solution. Pups were kept on a warming pad at 32–35 C and allowed to consume the milk solution for 30 min during which the paper towels were rewet with warm solution as necessary. Pups were then removed from the containers, dried carefully, and reweighed. Because pups at this age do not readily urinate and defecate spontaneously, unless stimulated manually, the amount of weight gained (expressed as a percentage of the pup's predeprivation body weight) during the intake test was used as a reliable measure of milk intake (30). Using this method of determination of food intake in neonatal rats, others have demonstrated both stimulatory effects of 2-mercaptoacetate (30) and inhibitory effects of glucose gastric preloads (31)

In situ hybridization in neonatal brain tissues

Twenty-micrometer coronal brain sections were collected onto poly-L-lysine-coated slides, allowed to desiccate overnight under vacuum at 4 C and kept at −80 C until processed for hybridization. For the determination of SOCS-3 (32), CRFR-2 (33), and all subtypes of leptin receptors (Ob-Rall) (34), we used specific cDNA fragments as described previously (35). The cDNA fragment for SOCS-3 (760 bp, mouse SOCS-3)

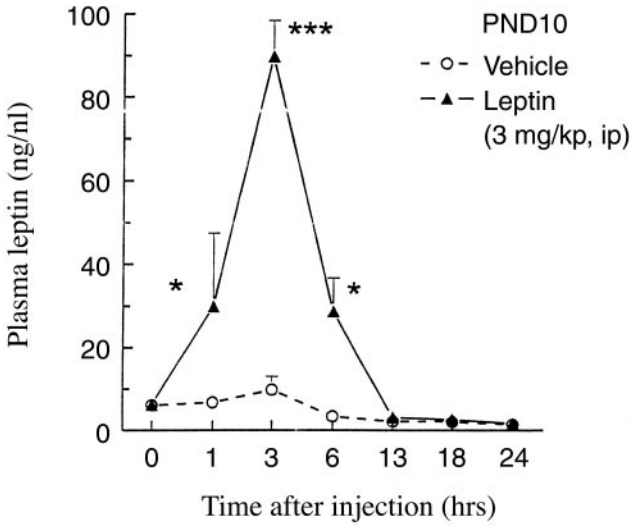

FIG. 1. Time course of changes in plasma concentrations of leptin after exogenous ip leptin administration (3 mg/kg BW, ip) on PND10. In the control group, pups were injected with saline (0.9%). Values represent the mean ± SEM of five to seven pups per group and per time point. *, $P < 0.05$ compared with the control group (Student-Newman-Keuls). ***, $P < 0.001$ compared with the control group (Student-Newman-Keuls).

was kindly provided by Dr. D. Hilton (the Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia) and inserted into a PCR-II vector. The antisense and sense probes were produced using *Xho*I and SP6, and *Bam*HI and T7, respectively. For the CRF-R2 hybridization, the 275-bp insert (rat insert, kindly provided by Dr. T. Lovenberg, Neurocrine Biosciences, San Diego, CA) was linked to the Bluescript vector. The antisense and sense probes were produced using *Eco*RI and T3, and BamHI and T7, respectively. The cDNA fragment used for hybridization of Ob-Rall (rat Ob-Rall, 500 bp) was kindly provided by Dr. C. Bjorbaek (Harvard Medical School, Boston, MA) and inserted into a Pgem11ZF vector. The antisense and sense probes were produced using Sac-I and SP6, and *Hin*dIII and T7, respectively. Briefly, sections were fixed for 20 min in paraformaldehyde (4%), digested for 30 min at 37 C with proteinase K (10 μg/ml in 100 mM Tris-HCl containing 50 mM EDTA, pH 8), acetylated 10 min with acetic anhydride [0.25% in 0.1 M triethanolamine (TEA), pH 8] and dehydrated through graded concentrations (50, 70, 95 and 100%) of alcohol. After vacuum drying for at least 2 h, 90 μl of hybridization solution mixture was spotted on each slide. Hybridization buffer contained 500 μl of formamide, 60 μl of 5 M NaCl, 10 μl of 1 M Tris (pH 8.0), 2 μl of 0.5 M EDTA (pH 8.0), 50 μl of 20 × Denhart's solution, 200 μl of 50% dextran sulfate, 50 μl of 10 mg/ml tRNA, 10 μl of 1 M dithiothreitol (118 μl diethylpyrocarbonate water/volume of probe used), and 1×10^7 cpm/ml of the ^{35}S-labeled cDNA SOCS-3, CRFR-2 or Ob-Rall probe. The slides were sealed with coverslips and incubated overnight at 60 C. The next day, slides were rinsed once for 20 min and 4 times for 5 min in 4× SSC (0.6 M NaCl; 60 mM sodium citrate buffer, pH 7), digested 30 min at 37 C with Ribonuclease-A (20 μg/ml in 10 mM Tris-500 mM NaCl containing 1 mM EDTA), washed in descending concentrations of SSC (2×, 10 min; 1×, 5 min; 0.5×, 5 min; 0.1×, 30 min at 60 C), and dehydrated through graded concentrations (50, 70, 95, 100%) of alcohol. After 2 h of vacuum drying, the slides were exposed to x-ray film (Eastman Kodak Co., Rochester, NY) for 5 d (CRFR-2), 3 d (SOCS-3), and 2 d (Ob-Rall). Radioactive standards prepared from brain paste with ^{14}C were exposed simultaneously. Hybridization signal on the autoradiograms was quantified from brain sections selected throughout the ARC for SOCS-3 and Ob-Rall and the VMH for CRFR-2 using a computerized densitometry by means of a microcomputer imaging device image analyzer system (Imaging Research, Inc., Ste-Catherine, Ontario, Canada). The data for the mRNA expression of SOCS-3, Ob-Rall and CRFR-2 were averaged throughout the ARC (SOCS-3 and Ob-Rall) and the VMH (CRFR-2), as there were no regional differences in the expression of each mRNA within the respective structures.

For each experimental group, two to six animals were analyzed with an average of three to six sections/animal. Once removed from the autoradiography cassettes, the slides were defatted in xylene and dipped in NTB2 nuclear emulsion (Eastman Kodak Co.) for 5 min. Exposure time to emulsion varied for each transcript. After developing, slides were rinsed in running distilled water for 1–2 h, counterstained with thionin (0.25%), and coverslipped.

For the determination of POMC and NPY mRNA, we used oligonucleotides probes with some variations in the hybridization protocol. Sequences were 5′-ATGAGATGTGGGGGGAAACTAGGAAAAGTC AGGAGAGCAAGTTTCATT-3′ and 5′-CTTGCCCACCGGCTTGC-CCCAGCG-3′ for NPY and POMC, respectively. The brain sections were postfixed for 10 min in paraformaldehyde (4%), washed 3 × 15 min in phosphate buffer 0.1 M, 1 × 1 h in 4× SSC (containing 1% of Denhardt's solution), and 5 min in a mixture of 1:1 (TEA 0.2 M and NaCl 18%), acetylated 15 min with acetic anhydride (0.25% in a mixture 1:1 of TEA 0.2 M and NaCl 18%), washed 3 × 5 min in 2× SSC, defatted through graded concentrations (70, 95, and 100%) of alcohol, then placed in chloroform 5 min and rehydrated through graded concentrations (100, 95, 70%) of alcohol. After air-drying for at least 2 h, 90 μl of hybridization solution mixture was spotted on each slide. Hybridization buffer contained 5% formamide, 600 mM NaCl, 10 mM Tris-HCl (pH 7.5), 1 M EDTA, 0.02% Denhardt 50×, 100 μg/ml of denatured salmon sperm DNA, 50 μg/ml of yeast tRNA, 10% dextran sulfate, 10 mM dithiothreitol, 1×10^7 cpm/ml of the ^{35}S-labeled POMC or NPY probe and volume was completed to 1 ml with sterile water. The slides were coverslip-sealed and incubated overnight at 40 C. The next day, the coverslips were removed and the slides were rinsed 10 min in 2× SSC, 5 min in 1× SSC, followed by a stringent wash for 60 min in 100 ml of 0.1× SSC with 1.5 ml of β-mercaptoethanol at 35 C under constant stirring. The sections were quickly rinsed in tap water and dehydrated through graded concentrations (50, 70, and 100%) of alcohol. After at least 2 h of air-drying, slides were exposed to β-max Hyperfilm (Amersham Pharmacia Biotech) together with radioactive ^{14}C standards for 6 d (POMC) and 12 d (NPY). Matching sections selected at four different levels of the ARC were analyzed. The whole ARC was subdivided into four levels (A through D) as previously described by Steiner and colleagues (36, 37) and adapted according to the atlas of Sherwood and Timiras (38) for 10-d-old pups. Level A corresponded to plate A 3.5 mm to A 3.2 mm (excluded), level B, plate A 3.2 mm to A 2.6 mm (excluded); level C, plate A 2.6 mm to A 2.0 mm (excluded); and level D, plate A 2.0 mm to the end of the ARC plate A 1.6 mm (included). For each experimental group, two to six animals were analyzed with an average of three to nine sections/animal.

Hormone assays

Plasma corticosterone concentrations were determined by RIA using a kit from ICN Biomedicals, Inc. (Costa Mesa, CA) with small modifications. The limit of detection was 0.2 μg/dl, interassay and intraassay variability was 12% and 3%, respectively (25). Plasma leptin levels were measured by specific RIA using a kit from Linco Research, Inc. (St. Charles, MO). The limit of detection was 0.5 ng/ml, and interassay variability was 9% (25).

Statistical analysis

All results were analyzed using the ANOVA followed by *post hoc* Student-Newman-Keuls or Tukey's highest significant difference tests where appropriate. Significant differences between two groups were determined by Student's *t* test. The level of significance was set as $P < 0.05$. All values are expressed as mean ± SEM.

Results

Effects of leptin treatment on plasma leptin levels, food intake, body and fat pads weight

In PND10 pups, plasma levels of leptin following exogenous ip leptin administration at the dose used in this study (3 mg/kg BW) were significantly increased already by 1 h after injection, peaked at 3 h, and returned to levels similar

to vehicle-treated pups by 13 h after injection (Fig. 1). At the time of the peak circulating leptin (*i.e.* 3 h after injection), food intake in leptin-treated pups was not modified over that of vehicle-treated pups on PND5, PND8, or PND10 of life (V *vs.* L: PND5, $P = 0.890$; PND8, $P = 0.438$; and PND10, $P = 0.353$, Table 1). When administered chronically between PND3–9, leptin slightly reduced body weight gain in PND10 pups ($P = 0.331$) but significantly reduced fat pads weight ($P < 0.01$) compared with vehicle-treated rats in agreement with previous data (6, 25).

Consistent with our previous studies (6) and the present time course study of plasma leptin, we found no significant difference in plasma leptin levels between pups that received a chronic leptin or vehicle treatment, as determined 24 h after the last injection (V/S = 23.58 ± 11.43 ng/ml; L/S = 16.4 ± 4.2, n = 6/group, $P = 0.466$). When the interval between injection and euthanasia was reduced to 30 min, plasma leptin levels were higher in pups receiving leptin compared with vehicle on PND10 (V/S = 23.58 ± 11.4 ng/ml; V/L = 82.56 ± 11.43, $P < 0.01$; L/S = 16.4 ± 4.2; L/L = 92.95 ± 4.47, $P < 0.01$; uninjected = 18.46 ± 3.24, n = 6/group). Circulating corticosterone levels at the time of euthanasia (30 or 120 min) were similar between groups at both time points (Table 1). However, plasma corticosterone was higher at 30 min compared with 120 min following treatment, most likely reflecting the stress of ip injection.

Effects of leptin on NPY, POMC, and SOCS-3 expression in the arcuate nucleus

Consistent with the function of SOCS-3 as an acute marker of leptin's intracellular response, changes in SOCS-3 mRNA expression were determined 30 min following acute leptin or vehicle treatment in PND10 pups. Thirty minutes after acute treatment, SOCS-3 mRNA expression was seen specifically in the epithelium lining the lateral ventricles, the dentate gyrus of the hippocampus (not shown), and the ARC (Fig. 2, *top*). In the ARC, SOCS-3 mRNA levels were significantly higher in pups injected with leptin compared with saline-injected pups ($P < 0.05$) (Fig. 2, *bottom*) and the increase in SOCS-3 expression was observed independently of the nature of chronic treatment (V/L *vs.* L/L pups, $P = 0.227$). In the dentate gyrus of the hippocampus, SOCS-3 mRNA levels were also significantly elevated after leptin treatment (V/S = 233.2 ± 17.2 nCi/g, V/L = 282.5 ± 11.5, $P < 0.05$). The short increase in corticosterone secretion is unlikely to have contributed significantly to SOCS-3 stimulation because previous reports have failed to document a direct effect of glucocorticoids on SOCS-3 expression throughout the adult rat brain (32).

Expression of NPY and POMC mRNA in PND10 pups was found throughout the rostro caudal extent of the arcuate nucleus (Fig. 3). Expression of POMC mRNA was restricted to the ARC, whereas NPY mRNA expression was found both in the ARC and the dorsomedial hypothalamus (Fig. 3C). No NPY mRNA expression was detected in the PVN of PND10 pups.

To determine the acute effects of leptin on neuropeptide expression, pups receiving vehicle (V) chronically were injected with either leptin (V/L) or saline (V/S) on PND10. Results are expressed as a percentage of levels found in V/S pups. Similarly, to investigate the chronic effects of leptin, pups receiving chronic leptin injections between PND3–9 were tested on PND10 with either leptin (L/L) or saline (L/S) and results are expressed as a percentage of levels found in L/S pups. As shown in Fig. 4, the effects of acute or chronic leptin on NPY mRNA were dependent upon the level of the ARC analyzed and the time point used after the injection. Whereas acute leptin reduced NPY mRNA levels 30 min after the injection in the more rostral portion of the ARC (level A, $P < 0.05$), it increased the expression of this peptide in a more caudal region (level C, $P < 0.05$) (Fig. 4, *top*). However, these effects were only transient because they were observed at 30 min, but not at 120 min after injection (level A, $P = 0.737$; and level C, $P = 0.180$ compared with V/S). The effects of an acute injection of leptin in pups chronically treated with leptin were also manifest on different levels of the ARC (Fig. 4,

TABLE 1. Effects of chronic (PND3–9) or acute (PND10) neonatal leptin treatment on body parameters, food intake, and corticosterone in 10-d-old pups

	Vehicle	Leptin (3 mg/kg BW)
Chronic treatment		
Body weight PND10 (g)	23.35 ± 0.53 (20)	22.53 ± 0.6 (19)
BW gain PND3–10 (g)	13.0 ± 0.83 (5)	11.94 ± 0.88 (5)
Fat pads weight (mg)	21.73 ± 1.94 (20)	15.74 ± 1.27 (19)[a]
Corticosterone (μg/dl)		
30 min	4.14 ± 0.43 (V/S) (6)	3.77 ± 0.33 (L/S) (6)
120 min	1.23 ± 0.17 (V/S) (4)	0.93 ± 0.15 (L/S) (4)
Acute treatment		
Food intake (g) PND5	0.15 ± 0.04 (10)	0.10 ± 0.03 (10)
PND8	0.24 ± 0.03 (11)	0.16 ± 0.04 (9)
PND10	0.34 ± 0.03 (20)	0.40 ± 0.04 (19)
Corticosterone (μg/dl)		
30 min	4.14 ± 0.43 (V/S) (6)	3.97 ± 0.35 (V/L) (6)
120 min	1.23 ± 0.17 (V/S) (4)	1.36 ± 0.09 (V/L) (4)

For determination of body weight gain, data from an entire treatment group (leptin or vehicle) within a litter (n = 5 litters) rather than individual pup weights were used because pups were weighed as a whole group (within a litter) between PND3 and 9. On PND10, pups were weighed individually. Food intake is expressed as changes in body weight within a period of 30 min of milk consumption. All parameters for weight and corticosterone secretion are from chronically treated pups, whereas data for food intake were determined after acute injection. For corticosterone secretion, the 30- and 120-min time points refer to the time after leptin injection. Values are mean ± SEM and the number of determinations is indicated in *parentheses*.

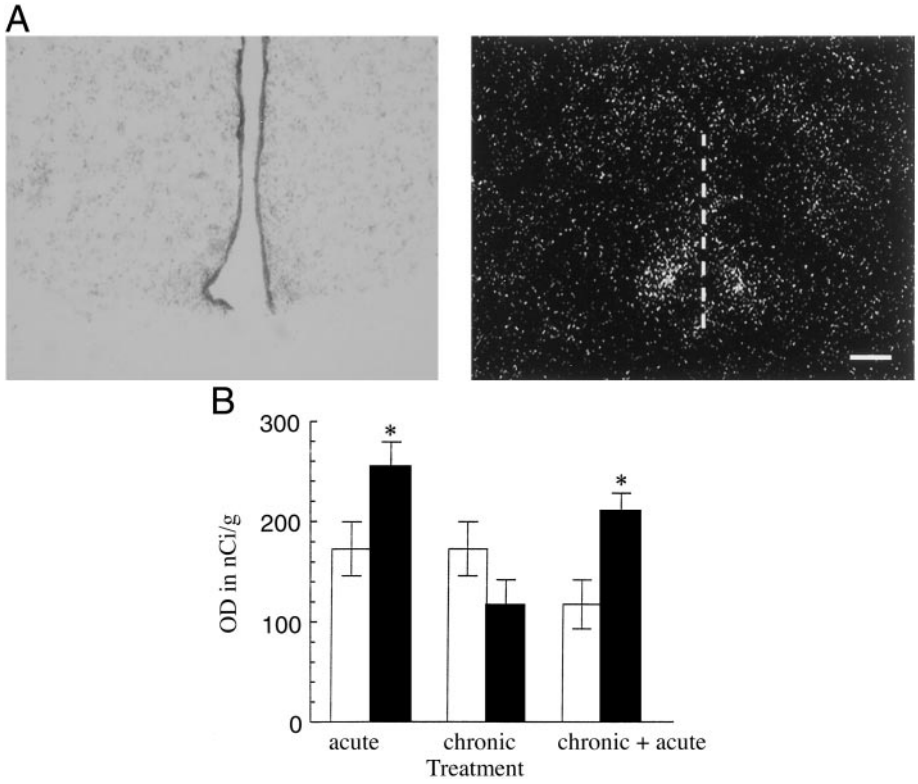

FIG. 2. Induction of SOCS-3 mRNA in the ARC of PND10 pups (*top, right*) 30 min after exogenous administration of leptin (3 mg/kg BW, ip). The *top left panel* depicts the region of the ARC that was analyzed. Changes in the expression of SOCS-3 mRNA levels in the ARC were semiquantified (*bottom*) in PND10 pups following acute (PND3–9 = V, PND10 = L), chronic (PND3–9 = L, PND10=S) and chronic + acute (PND3–10=L) leptin (*dark bars*) or saline (*open bars*) administration. *In situ* hybridization was performed as described in *Materials and Methods*. An average of three to five animals/group were used. Values represent the mean ± SEM of three to six determinations per animal. *Bar* represents 0.5 mm. *, $P < 0.05$ compared with the saline-injected group (Student-Newman-Keuls).

bottom). In this situation, cumulative leptin injection consistently reduced NPY mRNA expression 30 or 120 min after injection in all levels, but the more rostral portion of the ARC. The reduction in NPY mRNA expression after leptin treatment was consistent for both time points (30 and 120 min) only at the more caudal level of the ARC (level D, $P < 0.05$) (Fig. 5).

In agreement with adult studies (10), acute leptin treatment increased the expression of POMC mRNA only in the more rostral portion of the ARC (Figs. 5 and 6, *top*, level A). POMC mRNA levels increased both 30 and 120 min after an acute leptin injection in level A ($P < 0.05$), but were unchanged in other levels (30 min, level B, $P = 0.519$; level C, $P = 0.451$; level D, $P = 0.347$ compared with V/S and 120 min; level B, $P = 0.484$; level C, $P = 0.775$; level D, $P = 0.057$ compared with V/S). Acute leptin-induced stimulation of POMC expression was probably maximum because there was no further increase in POMC mRNA expression when an acute injection was superimposed on chronic leptin treatment (Fig. 6, *bottom*).

Effect of chronic leptin on leptin receptors (Ob-Rall) in the arcuate nucleus and CRFR-2 in the VMH

To determine whether chronic neonatal leptin treatment could down-regulate leptin receptors in the ARC, we determined changes in Ob-Rall mRNA expression in PND10 pups 24 h after the last injection of leptin. As expected, chronic leptin produced a down-regulation of all forms of leptin receptors (Ob-Rall) in the ARC, which was still apparent 24 h after the last injection (Fig. 7). In contrast, leptin increased CRFR-2 mRNA levels in the VMH of PND10 pups only when the interval between injection and euthanasia was 30 min ($P < 0.05$) and when the pups received chronic leptin treatment during PND3–9 (L/L *vs.* L/S) (Fig. 8). There was no significant effect of acute leptin on CRFR-2 expression in the VMH (V/S *vs.* V/L; 30 min, $P = 0.784$; 120 min, $P = 0.812$) and no residual effect seen 24 h after the last injection (V/S *vs.* L/S; 30 min, $P = 0.170$; and 120 min, $P = 0.490$). Consistent with the chronic effect of leptin on CRFR-2 expression demonstrated in adults (20), our results show that CRFR-2 expression is only transiently increased by leptin and that the acute effects of leptin on CRFR-2 expression are only revealed after chronic neonatal treatment with leptin.

Discussion

In these studies, we demonstrate that acute or chronic leptin administration during the first 9–10 d of life differentially modulate the expression of neuropeptides and receptors involved in the regulation of feeding behavior, including NPY, POMC, Ob-Rall, and CRFR-2. Regulation by leptin was observed in hypothalamic neurons despite the lack of effect of leptin on food intake in young neonates.

During the neonatal period, the elevated circulating levels of leptin (39) acting as a catabolic and anorexigenic hormone in adults might appear to be paradoxical at a time of rapid growth and development. Other studies and our present results however failed to demonstrate a decrease in food intake in pups treated with leptin (6, 26, 40, 41) suggesting either 1) that leptin signaling is not functional in the neonatal brain, or 2) that anorexigenic or orexigenic circuits are modulated differently compared with adults.

Using the production of SOCS-3 as a marker of functional activation of leptin receptor and intracellular signaling (29), we showed that an acute injection of leptin on PND10 in-

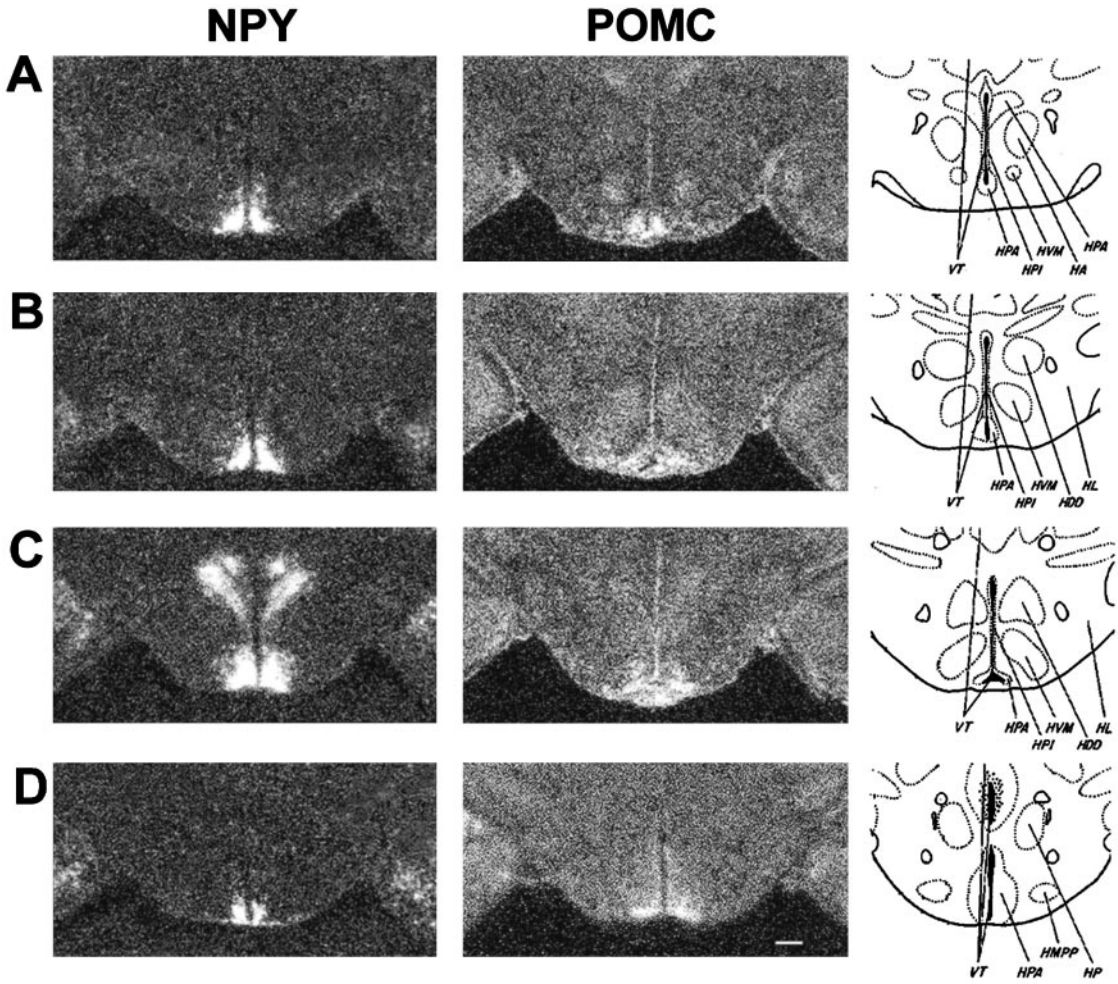

FIG. 3. *Left,* Representative sections depicting the rostro-caudal distribution of NPY (*left*) and POMC (*right*) mRNA expression in four subdivisions (A–D) of the ARC of PND10 vehicle-treated pups. *Right*, Schematic illustrations of the ARC levels analyzed for NPY and POMC mRNA expression taken from the atlas of Sherwood and Timiras for 10-d-old pups. These levels correspond to the first section used in the analysis of each level. Level A, Plate A 3.5 mm; level B; plate A 3.2 mm; level C, plate A 2.6 mm; level D, plate A 2.0 mm. Photographs were taken from the autoradiographic β-max films. VT, Ventriculus tertius; HPA, nucleus periventricularis arcuatus (hypothalami); HPI, nucleus periventricularis (hypothalami); HVM, nucleus ventromedialis (hypothalami); HA, nucleus anterior; HDD, nucleus dorsomedialis pars dorsalis (hypothalami); HL, nucleus lateralis (hypothalami); HMPP, nucleus premamillaris (hypothalami) (HMPP). *Bar* represents 1 mm.

creased the expression of SOCS-3 in the ARC. This effect disappeared 24 h after leptin administration in pups chronically injected with leptin between PND3–9. Interestingly, leptin retained its ability to activate intracellular signaling after chronic daily administration and despite the down-regulation of leptin receptors that we observed in the ARC. This result demonstrates that leptin receptors, and in particular Ob-Rb, can mediate the activation of SOCS-3 in the ARC and that leptin signal is functional in the hypothalamus of developing rats.

One of our salient observations is that POMC mRNA expression in PND10 pups was stimulated by acute leptin administration (V/L), although only in the rostral part of the ARC as demonstrated in adults (10). The acute effects of leptin on POMC mRNA were not observed in neonates treated daily with leptin, suggesting that POMC expression had reached a ceiling and could not be increased further in this group (L/L). The acute effects of leptin on POMC neurons could have been mediated directly through the binding of leptin to Ob-Rb expressed on these neurons or indirectly via the modulation of other neuropeptides such as NPY. Indeed, in addition to both being under the control of leptin, NPY, and POMC neurons regulate each other through reciprocal interactions (42) involving NPY Y1 receptors (43, 44) and/or GABA release (45). A recent study demonstrated that GABA and NPY are colocalized in axon terminals of the ARC that establish inhibitory synapses on POMC neurons. Leptin may directly depolarize POMC neurons and simultaneously hyperpolarize the somata of NPY/GABA neurons, thus removing the GABA inhibitory tone exerted on POMC neurons (45).

Because the product of POMC processing, α-MSH, is considered to be the predominant pathway regulating food intake in adults (42), it is surprising to observe leptin-induced increase in POMC expression, whereas we failed to observe any significant effect on food intake in neonates. This might point out to immaturity of POMC projections to target nuclei expressing melanocortin-4 receptors in the early postnatal period or suggest that POMC is not the

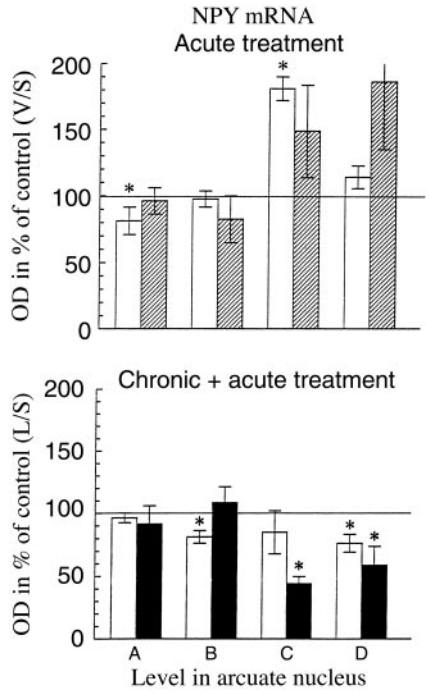

FIG. 4. Changes in the expression of NPY mRNA levels in the ARC 30 min (*open bars*) and 120 min (*dark or hatched bars*) following either acute (*top*) or chronic + acute (*bottom*) leptin administration (3 mg/kg BW, ip) in PND10 pups. *In situ* hybridization was performed as described in *Materials and Methods*, and the hybridized surface was expressed as a percentage of that in the respective control group (acute = V/S, chronic + acute = L/S). For each level illustrated in Fig. 3, an average of three to nine sections were analyzed per animal and three to six animals were used (except for level D in the 120-min chronic + acute group, n = 2). Values represent the mean ± SEM. *, $P < 0.05$ compared with the control group (V/S or L/S) (Student-Newman-Keuls).

primary regulator of food intake during development. Other systems like those stimulating food intake may rather be predominant at this time.

Similarly to POMC, NPY neurons appear to be already responsive to leptin treatment on PND10 as we found a decrease in NPY expression in various levels of the ARC after acute or chronic leptin injection. In the acute treatment, leptin reduced NPY expression in the more rostral part of the ARC but increased it in a more caudal level. These effects were transient because they were not present 120 min after acute leptin injection. If NPY is a primary regulator of food intake in neonates, the transient nature of the leptin effect on NPY expression might explain why we did not measure any significant difference in food intake between leptin and vehicle-injected groups 3 h after an acute treatment. The inhibition of NPY mRNA expression induced by an acute injection of leptin was more consistent and prolonged in animals chronically treated with leptin during the first 10 d of life. Again, it is remarkable that changes in NPY expression occurred despite leptin receptor down-regulation in the ARC. In contrast with the acute leptin injection, in the chronic condition all levels of the ARC, but the more rostral showed reduced NPY expression. It is possible that populations of NPY neurons along the ARC are differentially sensitive to acute and/or chronic leptin treatment due to the pattern of distribution of Ob-Rb or to the differential distribution of neuronal inputs to NPY neurons. An interesting parallel can be drawn between our results showing reduced NPY expression by increasing circulating leptin levels and other studies reporting increased NPY expression after maternal separation and food deprivation during neonatal development (16, 46).

Our results are in contrast with those reported by Ahima *et al.* (17) who failed to observe any difference in POMC or NPY mRNA content in the ARC of 17-d-old pups receiving a chronic leptin treatment. Methodological differences could account for the discrepancies because Ahima and Hileman (17) used RT-PCR to determine hypothalamic content of neuropeptides expression and we used *in situ* hybridization, which offers a better spatial resolution than RT-PCR. Moreover, we subdivided the ARC nucleus into four different levels allowing for a more refined level of analysis. Indeed, Smith and colleagues (46, 47) previously showed that failure to distinguish the different levels of the ARC does not permit the detection of region-restricted changes in NPY expression.

As an index of modifications in energy balance, we reported a tendency for leptin to lower body weight and a significant decrease in fat pads weight in pups receiving chronic leptin administration compared with vehicle-treated pups, even though we did not detect significant changes in food intake following an acute injection of leptin. Rather than being a consequence of reduced food intake, the effect of leptin on body and fat pads weight could be due to an increase in sympathetic activity as demonstrated in 2-wk-old neonates (40). In adult rodents, leptin activates sympathetic outflow via stimulation of hypothalamic POMC neurons and inhibition of the NPYergic system (8, 48). In our study, the observed changes in NPY and POMC expression induced by leptin might be more closely related to changes in sympathetic activation in neonates than to processes regulating food intake *per se*.

Consistent with adult experiments (49), chronic leptin administration in neonates down-regulated all types of leptin receptors, including probably the most abundant and functional form in the brain, Ob-Rb. Conversely, conditions reducing circulating leptin levels, such as fasting, have been reported to up-regulate Ob-Rb in the ARC and the VMH of adult rats (49). Although Ob-Rb are localized on NPY and POMC-producing neurons in the ARC of adults, the phenotype of neurons expressing leptin receptors in neonates is still unknown. Given the enhancement of leptin effect on NPY expression with chronic treatment and the lack of further effect of leptin on POMC in the same condition, it is tempting to speculate that leptin receptor down-regulation might have affected POMC neurons predominantly over those expressing NPY. Confirmation of this awaits further investigation.

Expression of CRF and its receptors, in particular CRFR-2, represents another important system regulating food intake (50, 51). Okamoto *et al.* (52) recently demonstrated that some of the anorexigenic effect of leptin are mediated through CRF, but not urocortin in adult rats. In neonates, acute leptin injection up-regulated the expression of CRFR-2 in the VMH, but only in pups chronically treated with leptin from PND3–9. This suggests that transient leptin's effects on CRFR-2 expression are only revealed when superimposed on chronic elevation of leptin levels and possibly reduced CRF release. When leptin levels

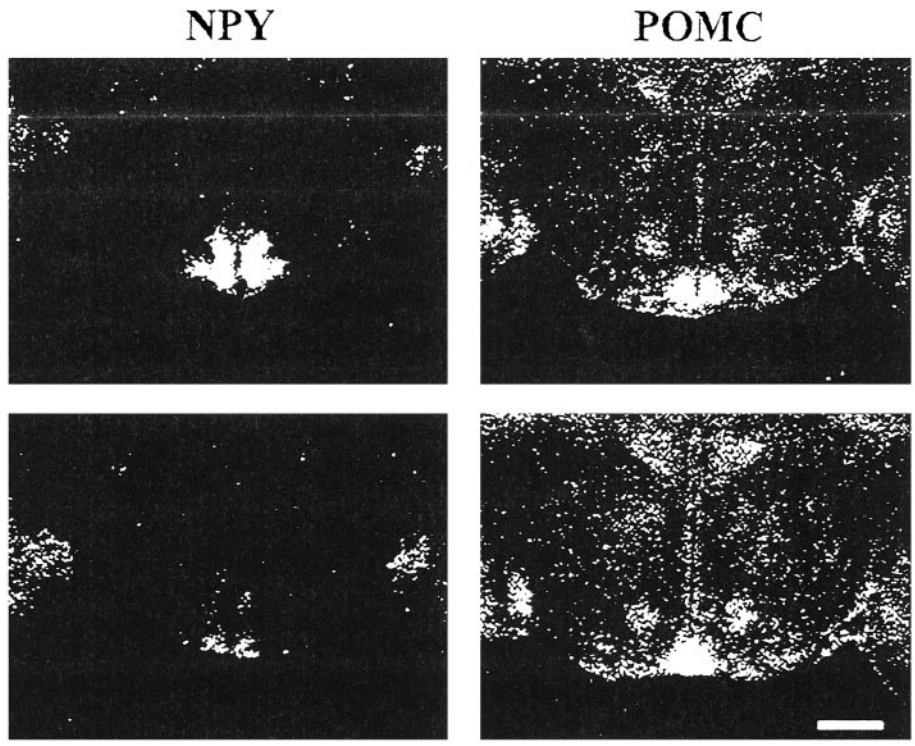

FIG. 5. *Left,* Representative sections at level D of the arcuate nucleus, illustrating the effects of chronic + acute leptin treatment (3 mg/kg BW) on NPY mRNA expression (*top*, vehicle-injected; *bottom*, leptin-injected). *Right,* Representative sections at level A of the arcuate nucleus, illustrating the effects of acute leptin administration (3 mg/kg BW) on POMC mRNA expression (*top*, vehicle-injected; *bottom*, leptin-injected). Photographs were taken from the autoradiographic β-max films. *Bar* represents 1 mm.

are dramatically reduced in neonates, such as after 24 h of maternal separation and food deprivation (Walker, C.-D., and M. Oates, unpublished), a strong down-regulation of CRFR-2 mRNA expression in the VMH was reported (24). What remains unclear is how leptin exerts its action on CRFR-2 expression in the VMH, whether it is directly via Ob-Rb present on VMH neurons harboring CRFR-2 (53) or indirectly through the regulation of CRF release (20). In the present study, we observed no SOCS-3 mRNA labeling in the VMH, suggesting rather an indirect action of leptin. Although some controversy remains as to the effects of leptin on CRF, it is generally accepted that leptin is inhibitory to CRF production in the PVN (54) and to HPA axis activity in neonates (25, 39). We previously showed that stimulated but not basal CRF mRNA levels were reduced by leptin in 10-d-old pups (25). Thus, the observed elevation in CRFR-2 mRNA expression could represent a compensatory mechanism induced after the reduction of CRF expression.

In summary, our results demonstrate that the expression of NPY, POMC, and Ob-Rall in the arcuate nucleus, and CRFR-2 in the VMH is differentially sensitive to acute and/or chronic leptin treatment in developing rats. Because leptin did not reduce food consumption in the same period, we conclude that the neuropeptides studied in these experiments are not predominant to determine food intake during early development. In particular, because adequate feeding is essential for survival in the early stages of life, neonatal hypothalamic regulation protects the organism against threat to this behavior by establishing several compensatory mechanisms aimed at maintaining energy homeostasis. Orexigenic circuits other than or in addition to the ones that we have studied here might have provided stronger signals to maintain food intake in leptin-treated pups. For example, agouti-related peptide, orexins, and ghrelin are all very po-

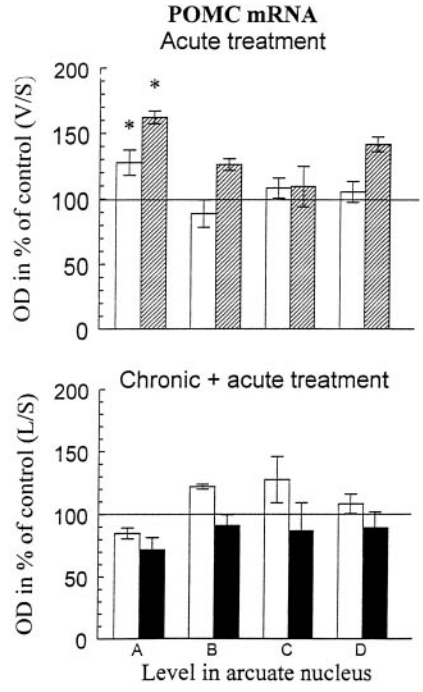

FIG. 6. Changes in the expression of POMC mRNA levels in the ARC 30 min (*open bars*) and 120 min (*dark or hatched bars*) following either acute (*top*) or chronic + acute (*bottom*) leptin administration (3 mg/kg BW, ip) in PND10 pups. *In situ* hybridization was performed as described in *Materials and Methods*, and the hybridized surface was expressed as a percentage of that in the respective control group (acute = V/S, chronic + acute = L/S). For each level illustrated in Fig. 3, an average of three to nine sections were analyzed per animal, and two to six animals were used. Values represent the mean ± SEM. *, $P < 0.05$ compared with the control group (V/S or L/S) (Student-Newman-Keuls).

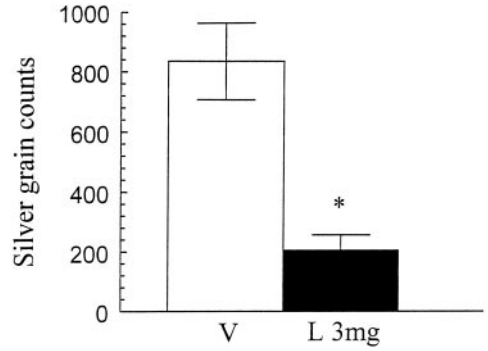

FIG. 7. Down-regulation of leptin receptors (Ob-Rall) mRNA levels in the ARC of PND10 pups following chronic vehicle or leptin (3 mg/kg BW, ip) administration between PND3 and 9. *In situ* hybridization was performed as described in *Materials and Methods* and expression was determined by counting silver grains on emulsion-dipped sections. An average of three to five sections per animal were analyzed, and two to six animals per group were averaged. Values represent the mean ± SEM. *, $P < 0.05$ compared with vehicle-injected pups (Student-Newman-Keuls).

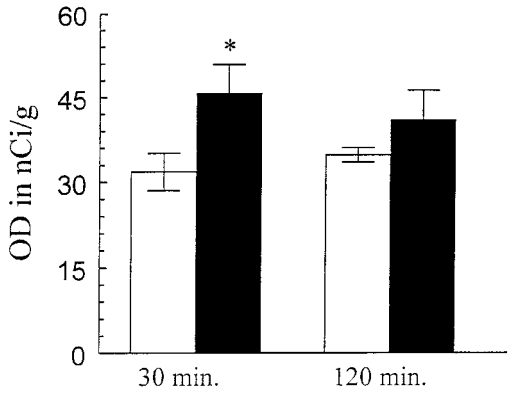

FIG. 8. Transient increase in CRFR-2 mRNA expression in the VMH of PND10 pups after chronic + acute (PND3–10) leptin administration (3 mg/kg BW, ip, L/L, *dark bars*) compared with vehicle-treated pups (L/S, *open bars*). Changes in the expression of CRFR-2 mRNA levels induced by leptin were seen at 30 min, but not 120 min, after injection on PND10. *In situ* hybridization was performed as described in *Materials and Methods*, and the hybridized surface was expressed as optical density measured on three to six sections per animal and four animals per group. Values represent the mean ± SEM. *, $P < 0.05$ compared with the control (L/S) group (Student-Newman-Keuls).

tent neuropeptides that have been shown to strongly stimulate appetite in adulthood. Future experiments are designed to investigate the regulation of these neuropeptides by leptin during development.

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