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Am J Physiol Regul Integr Comp Physiol 276:R1425-R1433, 1999.

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Marie-Pierre Ruffin, Tiziana Adage, Folkert Kuipers, Jan H. Strubbe, Anton J. W. Scheurink and Gertjan van Dijk

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Alfonso Abizaid, Diana Kyriazis and Barbara Woodside

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I. Swart, J. W. Jahng, J. M. Overton and T. A. Houpt

Am J Physiol Regul Integr Comp Physiol, November 1, 2002; 283 (5): R1020-R1026.

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Tiziana Adage, Anton J. W. Scheurink, Sietse F. de Boer, Koert de Vries, Jan Pieter Konsman, Folkert Kuipers, Roger A. H. Adan, Denis G. Baskin, Michael W. Schwartz and Gertjan van Dijk
J. Neurosci., May 15, 2001; 21 (10): 3639-3645.

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Am J Physiol Regul Integr Comp Physiol, October 1, 1999; 277 (4): R1020-R1024.

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Metabolic, gastrointestinal, and CNS neuropeptide effects of brain leptin administration in the rat

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Van Dijk, Gertjan, Randy J. Seeley, Todd E. Thiele, Mark I. Friedman, Hong Ji, Charles W. Wilkinson, Paul Burn, L. Arthur Campfield, Renata Tenenbaum, Denis G. Baskin, Stephen C. Woods, and Michael W. Schwartz. Metabolic, gastrointestinal, and CNS neuropeptide effects of brain leptin administration in the rat. *Am. J. Physiol.* 276 (*Regulatory Integrative Comp. Physiol.* 45): R1425–R1433, 1999.—To investigate whether brain leptin involves neuropeptidergic pathways influencing ingestion, metabolism, and gastrointestinal functioning, leptin (3.5 µg) was infused daily into the third cerebral ventricular of rats for 3 days. To distinguish between direct leptin effects and those secondary to leptin-induced anorexia, we studied vehicle-infused rats with food available ad libitum and those that were pair-fed to leptin-treated animals. Although body weight was comparably reduced (–8%) and plasma glycerol was comparably increased (142 and 17%, respectively) in leptin-treated and pair-fed animals relative to controls, increases in plasma fatty acids and ketones were only detected (132 and 234%, respectively) in pair-fed rats. Resting energy expenditure (–15%) and gastrointestinal fill (–50%) were reduced by pair-feeding relative to the ad libitum group, but they were not reduced by leptin treatment. Relative to controls, leptin increased hypothalamic mRNA for corticotropin-releasing hormone (CRH; 61%) and for proopiomelanocortin (POMC; 31%) but did not reduce mRNA for neuropeptide Y. These results suggest that CNS leptin prevents metabolic/gastrointestinal responses to caloric restriction by activating hypothalamic CRH- and POMC-containing pathways and raise the possibility that these peripheral responses to CNS leptin administration contribute to leptin's anorexigenic action.

OB protein; sympathetic nervous system; corticotropin-releasing hormone; proopiomelanocortin; food intake

EVIDENCE SUGGESTS that food intake and body adiposity are controlled, in part, by hormones that modulate neuropeptides within areas in the central nervous system (CNS) that are involved in the regulation of eating behavior. After its discovery by Zhang et. al. (60) in 1994, attention has focused on leptin (also named OB protein), the 167-amino acid protein product of the *ob*

gene that is mainly synthesized in adipose tissue and secreted in proportion to body adiposity (e.g., 11, 31, 44, 52). Leptin gains access to the CNS, apparently via a receptor-mediated transport system (2, 44), where it interacts with neuronal leptin receptors in brain areas that are involved in the control of ingestive behavior (e.g., 10, 45, 57). A central site of action is suggested by the observation that administration of relatively low doses of leptin into the CNS causes a reduction of food intake and body weight (e.g., 9, 13, 49) without producing incapacitation or malaise (55).

Body adiposity is affected not only by changes in food intake, but also by changes in energy metabolism. Accordingly, leptin also affects fuel metabolism, including metabolic rate, thermogenesis, cellular fat oxidation, and glucose turnover (16, 21, 25, 26, 32, 34, 42, 51, 61). Although direct effects of leptin on peripheral tissue have been reported (51, 61), alterations in metabolic processes may also result from leptin effects in the CNS (8). Hence, central leptin administration influences the synthesis and/or release of CNS peptides, such as neuropeptide Y (NPY) and corticotropin-releasing hormone (CRH) (4, 23, 24, 37, 43, 45), that regulate autonomic outflow. However, many studies investigating the effect of central leptin on CNS neuropeptides involved in regulation of autonomic outflow were either performed using 1) fasting animals to circumvent leptin's effect on food intake or 2) leptin-deficient rodents (e.g., *ob/ob* mice) that display supersensitivity to exogenous leptin. To investigate the role of central leptin in the control of peripheral metabolism and hypothalamic neuropeptides in normal lean (non-fasting and nonleptin deficient) rats, we injected leptin into the third cerebral ventricle (i3vt) over a 3-day period and then measured a variety of metabolic parameters [oxygen consumption ($\dot{V}O_2$), carbon dioxide production ($\dot{V}CO_2$)], circulating levels of fuels and hormones, hepatic glycogen content, body adiposity, gastrointestinal fill, and hypothalamic gene expression of several neuropeptides that respond to changes in energy balance and that regulate neuroendocrine outflow and metabolism [NPY, CRH, and proopiomelanocortin (POMC)]. To distinguish between the metabolic, neural and gastrointestinal effects of intracerebroventricular leptin and those that are secondary to the decrease in

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food intake resulting from leptin treatment, rats given central leptin were compared with vehicle-treated rats that had food available ad libitum and rats that were pair-fed to the leptin-treated animals. The results confirm other studies showing that leptin reduces circulating levels of free fatty acids and ketones, increases fat oxidation (Fat-ox), and prevents the fall in resting energy expenditure (EE) that normally occurs with reduced caloric intake. In addition, relative to pair-fed animals, leptin increased gastrointestinal fill to the level found in ad libitum-fed controls. Here we show that these effects can be mediated by leptin acting in the CNS and suggest that they may, in part, be explained by leptin-mediated increases in hypothalamic CRH and/or POMC gene expression.

MATERIAL AND METHODS

Animal preparation. Twenty-seven male Long-Evans rats obtained from the breeding colony maintained by the Department of Psychology of the University of Washington were housed individually in a temperature-controlled environment (22°C) in stainless steel hanging cages and maintained on a 12:12-h light-dark cycle. All procedures were performed in accordance with guidelines for animal use at the University of Washington. Pelleted chow and water were available ad libitum (except where noted). Under Equithesin anesthesia, the animals were implanted with 21-gauge stainless steel cannulas (Plastic One, Roanoke, VA) aimed at the third ventricle, according to techniques described elsewhere (57). The cannulas were fitted with removable obturators that extended 0.5 mm beyond the tip of the guide cannulas. After surgery, each rat was given 0.15 ml Chloromycetin (100 mg/ml sc) and Gentamicin (40 mg/ml ip) prophylactically. One week after surgery, cannula placements were confirmed by administration of 10 ng angiotensin II in 1 μ l of saline. Animals that did not drink 5 ml of water within 60 min were not used ($n = 3$). Rats were allowed to recover for at least an additional 2 wk, during which time they were handled twice daily for adaptation to the experimental procedures. All rats had returned to above presurgical weights by the time of testing.

Assessment of food consumption and body weight. On 3 consecutive days, freely feeding rats ($n = 8$) received an infusion (over 1 min) of 3.5 μ g human leptin dissolved in 3.5 μ l synthetic cerebrospinal fluid (sCSF) i3vt 2 h before the onset of the dark phase. Human leptin was chosen because this allowed us to identify unwanted leakage of i3vt-administered leptin to the periphery (by analysis of human and rat leptin levels in plasma with specific RIAs sensitive to either form of leptin). Hence, absence of human leptin in plasma (or at least nondetectable) argues against the possibility that the peripheral effects of i3vt leptin are mediated by a direct peripheral action of the hormone. Leptin (at least 90% pure) was harvested from an expression system in which recombinant DNA of the human gene was overexpressed in *Escherichia coli* (for details, see Ref. 8). Food intake and body weight of these and all other rats included in the present experiments were assessed daily starting 1 wk before infusions until the end of the 3-day treatment period. I3vt administration of the vehicle (sCSF alone) was given to rats that either had food ad libitum (ad libitum/sCSF; $n = 8$) or that were given the same amount of food as consumed by the leptin-treated rats on each day (pair-fed/sCSF; $n = 8$). The pair-feeding procedure consisted of matching (on the basis of identical body weights, within 2 g, at the start of i3vt treatments) of eight sCSF-treated animals to eight leptin-treated animals. On each sCSF treatment day, half the amount of food that was

consumed by a leptin-treated rat on that day was provided at the onset of the dark phase, whereas the other half was given after 4 h in the dark phase, except for the final treatment day. On that day, half the food was given after 3 h into the dark phase and the other half 3 h later.

Indirect calorimetry and behavioral assessment. Immediately after i3vt infusion on the last day of the 3-day treatment period, animals were placed in an indirect calorimeter chamber (Oxyeco, Columbus Instruments) until 3 h into the dark phase with access to water only. As soon as the lights went off, VO_2 and carbon dioxide production (VCO_2) were assessed. EE and carbohydrate oxidation (CHO-ox) and Fat-ox were determined from VO_2 and VCO_2 using the equations of Ferranini (18). During gas exchange measurements, behavior of the animals was recorded and later assessed by an investigator who was blind to the treatments. Each minute, the behavior of animals was assigned as "grooming" (whenever an animal groomed during that minute), "resting" (if an animal was lying motionless on the cage floor throughout the whole minute), or "alertness" (when assigned neither grooming nor resting). Assessment of behavior allowed distinction between the indirect calorimetry data under resting and nonresting conditions. For analyses of indirect calorimetry data, we assumed (on the basis of our unpublished observations of the relationship between behavior and VO_2) that animals approached a stable resting metabolic state when they displayed ongoing resting behavior for at least 15 min after grooming and for at least 5 min after alertness. Directly after the 3-h period in the calorimeter, animals were transported back to their home cage, where they were allowed to feed.

Blood and tissue collection and analyses. The following morning (1 h after lights on), animals were taken from their home cages and, within 2 min after opening their cages, were anesthetized by brief exposure to CO_2 and decapitated. Immediately thereafter, brains and liver samples were taken and frozen ($-80^\circ C$) and trunk blood was collected in cooled ($0^\circ C$) heparinized borosilicate tubes. Brains, liver tissue, and plasma (after centrifugation for 10 min at 1,500 g, $4^\circ C$, separation into different vials) were stored at $-80^\circ C$ until analyses. Carcasses were eviscerated and stored at $-20^\circ C$. Weights of whole livers, intestines (from stomach to the distal rectum), and retroperitoneal fat pads were assessed and also stored at $-20^\circ C$. Gastrointestinal fill was assessed from the weight difference of the intestines with and without its contents. Plasma glucose, triglycerides, and free fatty acids were assayed using commercial kits (Sigma 510-DA, Sigma 337-B, and Waco, respectively). Total plasma ketone bodies (acetoacetate plus hydroxy butyrate) and glycerol were measured using enzymatic procedures with fluorometric detection (38). Sensitive RIAs assessed the plasma level of human leptin (Linco; containing antibodies to human leptin without cross-reactivity to rodent leptin), rat leptin (Linco; containing antibodies to mouse and rat leptin without cross-reactivity to human leptin), insulin (47), and corticosterone. Release of glucose from liver homogenates incubated with amyloglucosidase was used to calculate liver glycogen. Carcasses and organs were dried, and fat was extracted according to the method of Leshner et al. (28). Percentage fat of carcasses and organs was determined from weight differences before and after the fat extraction procedure.

In situ hybridization. Coronal sections (14 μ m) of frozen rat brain were cut on a cryostat, mounted on RNase-free slides, and hybridized using antisense oligonucleotide probes based on cDNA sequences of rat CRH and NPY or with riboprobes complementary to mRNA for POMC or the mRNA encoding for the long-form of the leptin receptor (OB-Rb). The probes were labeled with [^{33}P]adenosine as described elsewhere (46),

and after hybridization, slides were rinsed under high-stringency conditions and apposed to X-ray film to generate autoradiographs, which were analyzed by computerized image analysis (53). Determination of NPY, POMC, and OB-Rb mRNA levels were made on sections from the midregion of the rostrocaudal extent of the arcuate hypothalamic nucleus (Arc) selected by an investigator blind to the study conditions. Measurements of CRH mRNA used a similar approach on sections obtained from the paraventricular hypothalamic nucleus (PVN). All densitometry data were collected by a technician blind to the conditions. The product of hybridization area and density was used as an index of relative mRNA levels.

Statistical analyses. ANOVA with repeated measures with three levels (ad libitum/sCSF vs. ad libitum/leptin vs. pair-fed/sCSF) and four factors (days 0–3) was used for analyses of body weight and food intake data. Further data analyses (except hypothalamic mRNA) included standard one-way ANOVA with three levels (ad libitum/sCSF vs. ad libitum/leptin vs. pair-fed/sCSF). Post hoc analyses were conducted only if the ANOVA was significant at the $P < 0.05$ (2 sided) level and used Tukey's highly significant difference test, also set at $P < 0.05$.

It was previously observed that food-deprivation reduces hypothalamic POMC mRNA (46), whereas it increases hypothalamic NPY mRNA (43–46). Because leptin treatment in the same dose used in the present study partially restores the expression of mRNA levels of POMC and NPY to levels found in rats under ad libitum feeding conditions (45, 46) and increases hypothalamic CRH mRNA (45), we ran planned comparisons (58) between mRNA levels of ad libitum/sCSF-treated and pair-fed/sCSF-treated animals (effect of negative energy balance) and between mRNA levels of leptin-treated and pair-fed/sCSF-treated animals (effect of leptin).

RESULTS

Food consumption and body weight. Figure 1 shows food intake (Fig. 1, top) and whole body weight (Fig. 1, bottom) of 3-day leptin-treated, ad libitum/sCSF-treated, and pair-fed/sCSF-treated animals. Food intake ($F_{6,63} = 37.51$; $P < 0.0001$; for time \times treatment interaction) decreased by $\sim 50\%$ over the 3-day treatment period in the leptin-treated and pair-fed animals compared with the ad libitum/sCSF-treated rats, and this led to an 8% reduction in body weight after 3 days in leptin-treated (and pair-fed/sCSF treated) animals relative to ad libitum/sCSF-treated animals ($F_{6,63} = 18.91$; $P < 0.0001$; for time \times treatment interaction). Whole body weights of pair-fed/sCSF-treated animals were not different from those of leptin-treated animals (respectively, 447.3 ± 5.5 and 447.9 ± 7.3 g on the final treatment day).

Behavior and indirect calorimetry. The relative time spent on grooming, resting, and alertness of leptin-treated, ad-libitum/sCSF-treated, and pair-fed/sCSF-treated animals is shown in Table 1. The different behaviors were not significantly different among groups. Table 2 shows indirect calorimetry data of leptin-treated, ad libitum/sCSF-treated, and pair-fed/sCSF-treated rats under resting metabolic conditions and over the entire (i.e., total) 3-h period. Throughout the 3-h period in the calorimeter, animals had between two and six phases in which they displayed ongoing resting behavior for at least 15 min after grooming behavior

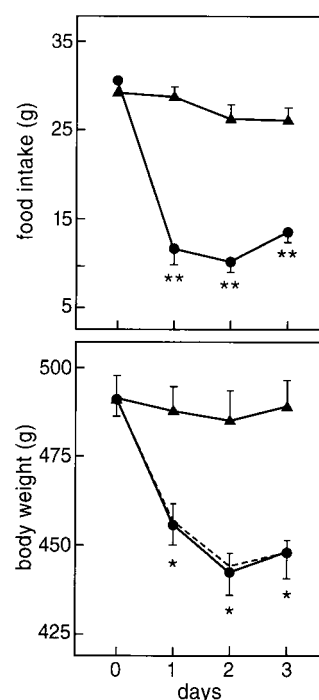


Fig. 1. Effect of daily intracerebroventricular infusions of synthetic cerebrospinal fluid (sCSF; ▲; $n = 8$) or $3.5 \mu\text{g}$ of leptin (●; $n = 8$) on food intake (top) and body weight (bottom) over a 3-day period. A third group of animals received intracerebroventricular infusions of sCSF, but animals in this group were pair-fed to leptin-treated animals (dashed line; $n = 8$). Statistical significance between ad libitum/sCSF group and leptin group * $P < 0.001$ and ** $P < 0.0001$.

and 5 min after alertness. Resting levels of VO_2 ($F_{2,23} = 5.40$; $P = 0.013$) and VCO_2 ($F_{2,23} = 8.59$; $P = 0.002$) were, respectively, 16 and 17% lower in pair-fed/sCSF-treated rats than in leptin-treated rats. VCO_2 was also lower in pair-fed/sCSF-treated rats (-13%) than in ad libitum/sCSF-treated rats. Over the entire 3-h period, VCO_2 ($F_{2,23} = 5.41$; $P = 0.013$) was lower in the pair-fed/sCSF-treated group relative to VCO_2 in leptin-treated (-12%) as well as in ad libitum/sCSF-treated rats (-11%). Respiratory quotient tended to be lower in both leptin-treated and pair-fed groups relative to ad libitum controls, but these differences did not reach statistical significance.

Blood and tissue analyses. Table 3 shows the plasma concentrations of hormones, fuels, hepatic glycogen content, percentage body fat (derived from carcass and

Table 1. Percentage of time spent on grooming, resting, and alertness displayed by experimental rats

Treatment	Ad Libitum/sCSF	Ad Libitum/Leptin	Pair-Fed/sCSF
Resting	60.8 ± 2.0	60.7 ± 4.3	55.5 ± 4.4
Grooming	11.3 ± 2.0	7.5 ± 1.5	11.9 ± 1.8
Alertness	27.9 ± 3.0	31.6 ± 4.1	32.6 ± 3.8

Values are means \pm SE in percentage of time (over 3-h period in indirect calorimeter chamber). Rats received daily intracerebroventricular infusion of synthetic cerebrospinal fluid (Ad Libitum/sCSF; $n = 8$) or $3.5 \mu\text{g}$ of leptin (Ad Libitum/Leptin; $n = 8$) or they received intracerebroventricular infusion of sCSF and were pair-fed to leptin-treated animals (Pair-Fed/sCSF; $n = 8$).

Table 2. VO_2 , VCO_2 , and RQ under resting metabolic conditions or over entire 3-h period of experiment of experimental rats

Treatment	Ad Libitum/ sCSF	Ad Libitum/ Leptin	Pair-Fed/ sCSF
Resting			
VO_2	752.5 ± 29.1	836.8 ± 34.4†	699.0 ± 25.5
VCO_2	669.8 ± 28.8	696.7 ± 16.2‡	576.5 ± 17.1*
RQ	0.89 ± 0.03	0.84 ± 0.03	0.82 ± 0.03
Total			
VO_2	848.5 ± 25.7	917.9 ± 35.5	830.1 ± 16.1
VCO_2	748.0 ± 31.3	760.0 ± 12.9†	667.0 ± 16.6*
RQ	0.88 ± 0.03	0.83 ± 0.03	0.80 ± 0.02

Values are means ± SE; $ml \cdot kg^{-1} \cdot h^{-1}$ for oxygen consumption (VO_2) and carbon dioxide production (VCO_2) and in VCO_2/VO_2 for respiratory quotient (RQ). Rats received daily intracerebroventricular infusion of sCSF ($n=8$) or 3.5 μg of leptin ($n=8$) or they received intracerebroventricular infusion of sCSF and were pair-fed to leptin-treated animals ($n=8$). *Statistical significance between ad libitum/sCSF-treated group and other groups ($P<0.05$); †‡ statistical significance between leptin and pair-fed/sCSF-group ($P<0.05$ and $P<0.01$, respectively).

organ fat extraction) of i3vt leptin-treated, ad libitum/sCSF-treated, and pair-fed/sCSF-treated rats. Relative to the ad libitum/sCSF group, the leptin-treated animals and pair-fed animals had lower levels of plasma leptin (measured with mouse RIA: $F_{2,23} = 16.12$; $P < 0.0001$; levels of leptin with a human-leptin RIA were nondetectable), hepatic glycogen content ($F_{2,23} = 8.84$; $P = 0.002$), and percentage body fat ($F_{2,23} = 9.54$; $P = 0.001$). The plasma triglyceride level was lower in leptin-treated (-40%) and pair-fed (-65%) rats relative to the level in ad libitum/sCSF-treated rats ($F_{2,23} = 6.71$; $P = 0.006$). There was a tendency of percentage body fat to be lower in leptin-treated rats than in pair-fed/sCSF-treated rats ($P = 0.08$). No treatment differences were observed in plasma levels of glucose, corticosterone, or insulin among groups. The plasma levels of corticosterone are relatively high compared with those in other studies (e.g., 48). Although repetitive i3vt administration of leptin and/or vehicle is not necessarily associated with negative side effects (see

Table 3. Plasma concentrations of fuel and hormones, hepatic glycogen, and percentage body fat of experimental rats

Treatment	Ad Libitum/ sCSF	Ad Libitum/ Leptin	Pair-Fed/ sCSF
Pl glucose, mM	8.32 ± 0.23	8.44 ± 0.20	8.51 ± 0.16
Pl triglycerides, mM	1.57 ± 0.23	0.93 ± 0.23*	0.55 ± 0.12†
Pl corticosterone, μg/dl	1.61 ± 0.64	2.63 ± 0.76	1.43 ± 0.45
Pl insulin, μU/ml	81.0 ± 11.3	73.7 ± 12.7	62.3 ± 7.4
Pl leptin, ng/ml	4.59 ± 0.17	2.00 ± 0.44‡	2.11 ± 0.42‡
Hepatic glycogen, mg/g	75.2 ± 6.4	38.3 ± 5.6†	45.2 ± 7.7†
%Body fat	16.1 ± 1.3	8.4 ± 0.9‡	11.5 ± 1.6*

Values are means ± SE. Rats received daily intracerebroventricular infusion of synthetic cerebrospinal fluid ($n=8$) or 3.5 μg leptin ($n=8$) or they received intracerebroventricular infusion of sCSF and were pair-fed to leptin-treated animals ($n=8$). Pl, plasma. *†‡Statistical significance between ad libitum/sCSF group and other groups ($P<0.05$, $P<0.01$, and $P<0.001$, respectively).

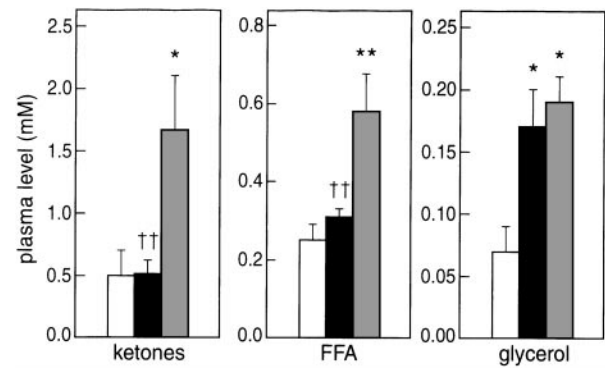


Fig. 2. Plasma concentrations of ketones, free fatty acids (FFA), and glycerol in ad libitum-feeding rats that received daily intracerebroventricular infusion of sCSF (open bars; $n=8$) or 3.5 μg leptin (filled bars; $n=8$) or rats that received intracerebroventricular infusion of sCSF and were pair-fed to leptin-treated animals (shaded bars; $n=8$). Statistical significance between ad libitum/sCSF-treated group and other groups: * $P < 0.05$ and ** $P < 0.01$. Statistical significance between leptin and pair-fed group: †† $P < 0.01$.

Ref. 55), it might be that this procedure has caused some conditioned (i.e., to the investigator) arousal in these animals, leading to an elevation of plasma levels of corticosterone at the time of death. Figure 2 shows the plasma concentrations of ketones, free fatty acids, and glycerol of i3vt leptin-treated, ad libitum/sCSF-treated, and pair-fed/sCSF-treated rats. Relative to ad libitum-fed controls, the leptin-treated (142%) and pair-fed animals (171%) had an increased level of plasma glycerol ($F_{2,23} = 9.44$; $P = 0.001$). In contrast, plasma levels of both free fatty acids ($F_{2,23} = 7.83$; $P = 0.003$) and ketones ($F_{2,23} = 6.30$; $P = 0.007$) were only higher in pair-fed animals (respectively, 132 and 234%), but not in the leptin-treated animals relative to the levels in ad libitum controls. Figure 3 shows gastrointestinal fill of i3vt leptin-treated, ad libitum/sCSF-treated, and pair-fed/sCSF-treated rats. Relative to pair-fed/sCSF-treated rats, leptin-treated rats had more than twice the amount of gastrointestinal fill ($F_{2,23} = 14.53$; $P = 0.0001$), which was, in fact, undistinguishable from the gastrointestinal fill in ad libitum/sCSF-treated animals.

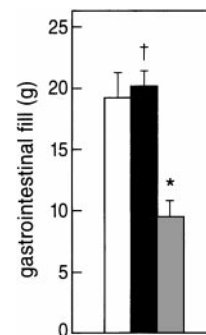


Fig. 3. Gastrointestinal fill of ad libitum-feeding rats that received daily intracerebroventricular infusion of sCSF (open bars; $n=8$) or 3.5 μg leptin (filled bars; $n=8$) or rats that received intracerebroventricular infusion of sCSF and were pair-fed to leptin-treated animals (shaded bars; $n=8$). Statistical significance between ad libitum/sCSF-treated group and other groups: * $P < 0.001$. Statistical significance between leptin and pair-fed group: † $P < 0.001$.

Table 4. CHO-ox and Fat-ox under resting metabolic conditions or over entire 3-h period of experiment of experimental rats

Treatment	Ad Libitum/ sCSF	Ad Libitum/ Leptin	Pair-Fed/ sCSF
Resting			
CHO-ox	705 ± 94	495 ± 84	389 ± 75*
Fat-ox	121 ± 28	260 ± 47*	245 ± 39
Total			
CHO-ox	764 ± 120	524 ± 91	391 ± 73*
Fat-ox	200 ± 44	293 ± 51	317 ± 33

Values are means ± SE in mg·kg lean mass⁻¹·h⁻¹. Rats received daily intracerebroventricular infusion of synthetic cerebrospinal fluid (*n* = 8) or 3.5 µg of leptin (*n* = 8), or they received intracerebroventricular infusion of sCSF and were pair-fed to leptin-treated animals (*n* = 8). CHO-ox, carbohydrate oxidation; Fat-ox, fat oxidation. *Statistical significance between Ad Libitum/sCSF group and other groups (*P* < 0.05).

EE. Because stored triglycerides in fat tissue is a metabolically inactive compartment, resting and total CHO-ox and Fat-ox and resting and total EE of animals were calculated for lean body masses of animals (by correcting for the amount of body fat from Table 3). The relative CHO-ox and Fat-ox of leptin-treated, ad libitum/sCSF-treated, and pair-fed/sCSF-treated animals are presented in Table 4. Resting ($F_{2,23} = 3.63$; *P* = 0.044) and total ($F_{2,23} = 3.83$, *P* = 0.038) CHO-ox in ad libitum/sCSF-treated animals was 45 and 49% higher than in pair-fed/sCSF-treated animals, respectively, whereas only resting Fat-ox in the leptin-treated group was 53% higher than the level in the ad libitum/sCSF group ($F_{2,23} = 3.94$; *P* = 0.035). Although EE was not different among groups over the total 3-h period (5.76 ± 0.19 , 5.62 ± 0.14 , and 5.25 ± 0.15 W/kg lean mass in the ad libitum/sCSF-, ad libitum/leptin-, and pair-fed/sCSF-treated rats, respectively), resting EE in ad libitum/sCSF-treated and leptin-treated animals were higher (13%) than in pair-fed/sCSF-treated animals ($F_{2,23} = 5.43$; *P* = 0.013) (Fig. 4).

In situ hybridization. Figure 5 shows the results of *in situ hybridization* (expressed as %expression of mean value of the ad libitum/sCSF group) of mRNA for CRH

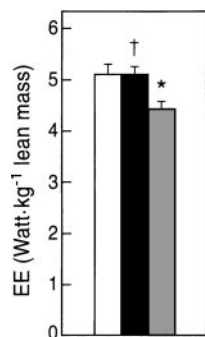


Fig. 4. Resting energy expenditure of ad libitum-feeding rats that received daily intracerebroventricular infusion of sCSF (open bars; *n* = 8) or 3.5 µg leptin (filled bars; *n* = 8) or rats that received intracerebroventricular infusion of sCSF and were pair-fed to leptin-treated animals (shaded bars; *n* = 8). Statistical significance between ad libitum/sCSF-treated group and other groups: **P* < 0.05. Statistical significance between leptin and pair-fed group: †*P* < 0.05.

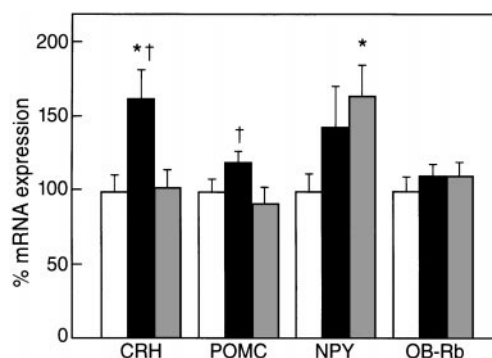


Fig. 5. Expression of mRNA for corticotropin-releasing hormone (CRH) in parvocellular paraventricular hypothalamus and expression of mRNA for neuropeptide Y (NPY), proopiomelanocortin (POMC), and long-form leptin receptor (OB-Rb) in arcuate hypothalamic nucleus of ad libitum-feeding rats that received daily intracerebroventricular infusion of sCSF (open bars; *n* = 8) or 3.5 µg leptin (filled bars; *n* = 8) or rats that received intracerebroventricular infusion of sCSF and were pair-fed to leptin-treated animals (shaded bars; *n* = 8). Levels of mRNA for CRH, NPY, POMC, and OB-Rb were expressed as %mean value of ad libitum/sCSF-treated group. Statistical significance between ad libitum/sCSF group and other groups: **P* < 0.05. Statistical significance between leptin and food-matched/sCSF-treated group: †*P* < 0.05.

in the PVN and mRNA for NPY, POMC, and the long-form leptin receptor in the Arc. Expression of hypothalamic CRH mRNA was increased in leptin-treated animals compared with ad libitum/sCSF-treated (63%) and pair-fed/sCSF-treated (58%) rats. The expression of NPY mRNA in pair-fed/sCSF-treated animals was 64% higher than the level observed in ad libitum/sCSF-treated animals (*P* < 0.05), whereas those of leptin-treated rats were not significantly different from ad libitum controls or pair-fed controls. The expression of POMC mRNA in the Arc was higher (23%) in leptin-treated animals than in pair-fed/sCSF-treated animals. The levels of Ob-Rb mRNA in the Arc were not different among groups.

DISCUSSION

The present study investigated the effect of central leptin administration on ingestive behavior, energy metabolism, and the synthesis of hypothalamic neuropeptides that respond to changes in energy balance. As predicted, i3vt leptin administration substantially reduced daily food intake (by ~50%) and, over a 3-day treatment period, resulted in a reduction in body weight of ~8%. By comparing the results from leptin-treated rats to those in pair-fed animals, we were able to distinguish the metabolic and hypothalamic responses caused by leptin treatment from those that were secondary to reduced food intake. This approach identified a number of metabolic and hypothalamic effects that are directly attributable to leptin action in the brain.

Some of the metabolic effects of leptin treatment can be attributed to food restriction. Relative to ad libitum-fed controls, both the leptin-treated and pair-fed groups had lower levels of plasma leptin, triglycerides, liver glycogen, and body fat content and higher levels of plasma glycerol. By other parameters, however, leptin-

treated rats appeared "fed" despite a marked decrease in food intake. In contrast to pair-fed rats, for example, leptin-treated rats did not exhibit the increase of circulating ketone bodies and fatty acids that occurs when stored triglyceride is hydrolyzed to meet ongoing energy requirements in the face of inadequate caloric intake (41). Another characteristic of a state of negative energy balance is a reduction of basal metabolic rate, presumably the result of diminished sympathetic tone (59). We found that whereas pair-fed animals exhibited a lower resting EE rate at the onset of the dark phase than was detected in ad libitum-fed controls, resting EE in leptin-treated animals was well above the level of the pair-fed animals and, in fact, was indistinguishable from that in the ad libitum controls. Because there were no differences in resting, grooming, or alertness, our data are consistent with previous observations in *ob/ob* and lean mice (25, 32) and provide direct support for the potentially important action (therapeutic, if shown in humans) of leptin in the brain to prevent the fall in metabolic rate that accompanies caloric restriction (16).

Because plasma levels of glycerol (a product of triglyceride hydrolysis) were elevated to a similar degree in leptin-treated and pair-fed animals (respectively, by 143 and 171% relative to ad libitum controls), leptin treatment did not appear to prevent intracellular breakdown of triglycerides. In fact, consistent with other reports (e.g., 21, 25, 29, 34), the leptin-treated animals in the present study, but not pair-fed controls, exhibited an increase in the rate of total body Fat-ox compared with the ad libitum group. In addition, the leptin-treated animals tended to be leaner than their pair-fed controls. Triglyceride hydrolysis and lipid depletion, therefore, appeared to be accentuated by central leptin treatment, and CHO-ox was reduced in pair-fed animals, but not in the leptin-treated animals, relative to ad libitum controls. Thus these results suggest that a combined increase of CHO-ox and Fat-ox contributed to the relatively high resting EE in leptin-treated rats relative to pair-fed animals. This picture of increased EE accompanied by elevated plasma glycerol, but no increase in plasma free fatty acids and ketone levels, suggests a unique effect of leptin on CNS pathways that control metabolism.

The effects of leptin administration on circulating fat fuels in the present study confirm and extend the findings of Shimabukuro et al. (51), who, using rats made hyperleptinemic by adenovirus gene transfer, hypothesized that leptin increases intracellular Fat-ox in tissues (i.e., adipose, liver, pancreas, and muscle), such that lipolysis proceeds without increased plasma levels of free fatty acids. Whereas others have proposed that this occurs via a direct effect in peripheral tissues, our results are the first to demonstrate that these metabolic responses can be mediated indirectly by an action of leptin in the brain. The hypothesis that leptin stimulates intracellular hydrolysis also provides a logical explanation for reduced plasma ketone body levels due to reduced availability of circulating free fatty acids for hepatic ketogenesis. Furthermore, Rossetti et

al. (40) showed that leptin stimulates hepatic gluconeogenesis at the expense of glycogenolysis (yielding no net effect on hepatic glucose output), which potentially would shunt fatty acids into hepatic β -oxidation and away from hepatic ketone body formation. This inhibition of ketogenesis by central leptin provides a potential mechanism linking fasting-induced reductions in circulating leptin with the increased ketogenesis reported by Kolaczynski et al. (27).

A number of studies have shown that in addition to brown adipose tissue, a variety of other peripheral tissues express mitochondrial uncoupling proteins (UCP1–3) enabling energy to be shunted into heat (e.g., 19, 30, 61), and leptin appears to potentiate the synthesis of UCP in most of these tissues (19, 30, 42, 61). It was shown by Cusin et al. (14) that i3vt leptin treatment over a 4-day period prevents the fall in UCP mRNA expression that is associated with reduced caloric intake, demonstrating an effect of CNS leptin on UCP synthesis. Because a state of negative energy balance is associated with diminished sympathetic tone (59) and because leptin has been shown to increase sympathetic nerve traffic (17, 22), central leptin may stimulate the expression of UCPs in peripheral tissues via increased sympathetic outflow, as it does in brown adipose tissue (12). Thus if central leptin activates both intracellular fatty acid oxidation and UCP synthesis within the same peripheral cells, intracellular fatty acids could provide a substrate for increased thermogenesis. This hypothesis intuitively complements the increased glucose turnover reported during both central and peripheral leptin administration in mice (26).

Chronic leptin administration has been shown in other studies (21, 29, 34) to augment body weight loss relative to pair-fed controls. Although weight loss in leptin-treated animals did not exceed that of pair-fed controls in our study, this outcome may have been confounded by the more than twofold increase in the weight of gastrointestinal fill in the leptin-treated versus pair-fed groups. Correcting for the weight of gastrointestinal contents, weight loss of the leptin-treated rats was 32% greater than that of the pair-fed groups relative to ad libitum-fed rats. In fact, whereas leptin-treated rats ate 50% less than ad libitum-fed controls, the gastrointestinal content weight of these two groups was indistinguishable. The leptin effects on gastrointestinal fill are consistent with the findings of Smedh et al. (54), showing that central leptin administration reduces gastric emptying. An inhibitory effect of leptin on gastrointestinal motility is also supported by observations in the *fa/fa* Zucker rat, in which obesity develops due to a mutation of the leptin receptor (35). These animals have increased gastric emptying as well as augmented intestinal transit (33), suggesting that leptin may normally serve to inhibit gastrointestinal motility. Inhibition of gastrointestinal motility would be expected if leptin stimulated sympathetic nervous system outflow to the gastrointestinal tract as is suggested by the observation that leptin increases overall sympathetic nerve traffic (17, 22).

To investigate the CNS mechanisms that mediate leptin's behavioral, metabolic, and gastrointestinal effects, we measured hypothalamic mRNA encoding neuropeptides implicated in energy homeostasis. Consistent with our previous studies, we found that central leptin administration increased CRH mRNA in the hypothalamic PVN. This effect was not due to reduced food intake, because pair-fed animals did not show this response. Because central administration of CRH has anorexigenic and thermogenic effects (7, 39), the data in the present and other studies (37, 45) suggest that leptin's effects could be mediated through activation of hypothalamic CRH neurons. This idea is consistent with a recent study of Uehara et al. (56) demonstrating that the anorexigenic efficacy of central leptin was reduced by central administration of a CRH receptor antagonist. Activation of hypothalamic CRH neurons could also explain the increased SNS outflow (6) and thermogenesis (39) that is observed with leptin administration. It is noteworthy that these stimulatory effects of leptin on CRH signaling coexist with inhibitory effects of leptin on the hypothalamic-pituitary axis (23, 24). These paradoxical observations suggest that leptin may exert distinctly different regulatory effects on discrete subpopulations of CRH neurons in the PVN.

In addition to leptin effects on CRH mRNA, leptin-treated animals also had an increased level of POMC mRNA in the arcuate nucleus relative to the level observed in pair-fed animals. The increase in hypothalamic POMC gene expression is consistent with what we observed after central leptin treatment in rats that were food deprived (46) and supports the hypothesis that melanocortins, which act centrally to reduce food intake, are important mediators of leptin signaling (50).

Previous studies (e.g., Ref. 5) indicate that dramatic weight loss due to caloric restriction or fasting is associated with reduced CRH mRNA levels in the PVN, and this response has been proposed to contribute to the stimulated feeding behavior and reduced SNS outflow that occurs in this setting (6). Although food restriction in the present study was therefore expected to lower hypothalamic CRH gene expression, we found no evidence of reduced CRH mRNA in pair-fed animals, despite weight loss of ~8% relative to ad libitum-fed controls. This observation suggests that weight loss must exceed that experienced by pair-fed rats before CRH mRNA levels are decreased in the PVN (see also Ref. 48). On the other hand, we did find an increase in NPY gene expression in the arcuate nucleus of pair-fed animals relative to ad libitum-fed controls, an effect that is consistent with previous studies (1, 43, 45, 48). However, the leptin-treated animals did not have significantly reduced levels of NPY mRNA relative to the pair-fed group. The effect of leptin to reduce hypothalamic NPY gene expression may therefore be detectable only in animals in which the NPY system has been activated (e.g., when leptin levels are low). Finally, we report in the present study that neither leptin treatment nor food restriction significantly affected long-form leptin receptor mRNA expression in the arcuate

nucleus. Thus, although leptin deficiency in *ob/ob* mice is associated with a two- to threefold increase of expression of leptin receptor mRNA in this brain area (4), our results do not support the hypothesis that leptin administration downregulates leptin receptor gene expression in nonmutant animals with ad libitum access to food.

Perspectives

Among the many factors capable of reducing food intake are an increase in the hepatic oxidation of metabolic fuels, including fat fuels (see Ref. 20), and gastrointestinal distension (15, 36). The present findings, which show that central leptin administration increases fuel oxidation and reduces gastrointestinal clearance, strongly suggest that these metabolic and gastrointestinal effects of leptin contribute to the anorexigenic action of the hormone in the CNS. We hypothesize that leptin activates sympathetic outflow, potentially via an increase in hypothalamic CRH signaling, and this response alters substrate metabolism, metabolic rate, and gastrointestinal function. As shown in Fig. 6, these peripheral effects, in concert with changes in hypothalamic effector pathways that act directly to inhibit food intake, are hypothesized to produce the sustained negative energy balance and depletion of body fat stores characteristic of leptin's action in the brain.

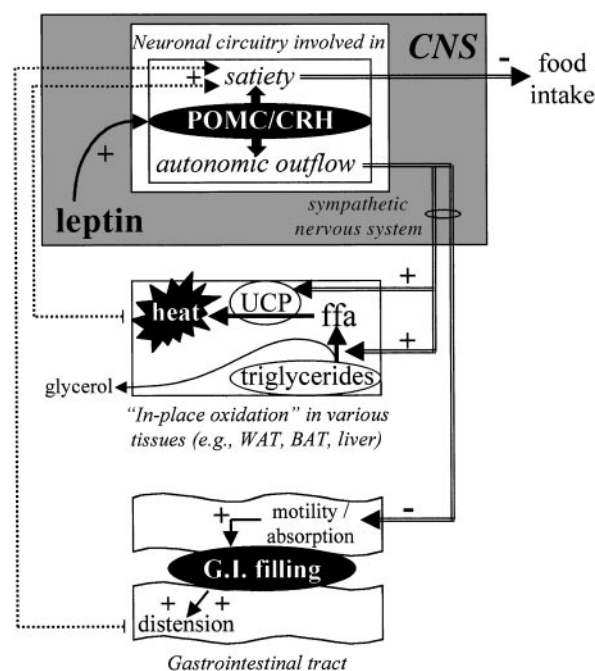


Fig. 6. Summary model of leptin affecting hypothalamic effector pathways that act directly to inhibit food intake and, presumably, via increased sympathetic outflow alter peripheral substrate metabolism and gastrointestinal functioning (double lines). In turn, these peripheral processes elicit feedback signals (dashed lines) that, in concert with brain leptin's direct anorexigenic effect, are hypothesized to sustain negative energy balance and depletion of body fat stores. CNS, central nervous system; GI, gastrointestinal; ffa, free fatty acid; WAT, white adipose tissue; BAT, brown adipose tissue; UCP, uncoupling protein.

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