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Evidence That the Caudal Brainstem Is a Target for the Inhibitory Effect of Leptin on Food Intake

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Three experiments were performed to investigate the hypothesis that leptin action within the caudal brain stem (CBS) contributes to its intake inhibitory effects. The first experiment evaluated the anatomical distribution of leptin receptor mRNA in rat CBS using a sensitive fluorescence *in situ* hybridization method with a riboprobe specific for the long form of the leptin receptor (Ob-Rb). An Ob-Rb mRNA hybridization signal was detected in neurons of several CBS nuclei involved in the control of food intake, including the dorsal vagal complex and parabrachial nucleus. A strong hybridization signal was also obtained from neuronal cell bodies of a number of other structures including the hypoglossal, trigeminal, lateral reticular, and cochlear nuclei; locus ceruleus; and inferior olive. The anatomical profile revealed by fluorescence *in situ* hybridization was in good agreement with immunocytochemical analysis with an antibody specific to Ob-Rb. In a second experiment, exploring the relevance of CBS Ob-Rb to feeding behavior, rats were given a fourth intracerebroventricular (i.c.v.) injection of leptin (0.1, 0.83, or 5.0 μg; n = 9–11/group) or vehicle 30 min before lights-out on three consecutive days The two higher doses reduced food intake significantly at 2, 4, and 24 h after injection and caused significant reductions of body weight. The dose-response profiles for fourth i.c.v. administration were indistinguishable from those obtained from separate groups of rats that received leptin via a lateral i.c.v. cannula. In the last experiment, a ventricle-subthreshold dose of leptin (0.1 μg) microinjected unilaterally into the dorsal vagal complex suppressed food intake at 2, 4, and 24 h. The results indicate that the CBS contains neurons that are potentially direct targets for the action of leptin in the control of energy homeostasis. (*Endocrinology* 143: 239–246, 2002)

SEVERAL OBSERVATIONS SUGGEST that the adipocyte hormone, leptin, inhibits food intake by activating the long-form leptin receptor (Ob-Rb) isoform in the brain, with most attention focused on Ob-Rb expression in the hypothalamus (1). Ob-Rb mRNA (2–6) and protein (7) as well as leptin-binding sites (8, 9) are concentrated in hypothalamic neuronal groups including arcuate, ventromedial, and dorsomedial nuclei. Many of the hypothalamic neurons expressing Ob-Rb also produce one or more neuropeptides that have been clearly linked to the control of intake and energy balance (5, 10, 11). These include NPY, cocaine-amphetamine related transcript, α-MSH (derived from POMC) and agouti-related protein. The projections of these leptin-sensitive neurons (*e.g.*, arcuate to paraventricular nucleus and arcuate to lateral hypothalamus) are central to the contemporary hypothalamic control model (*e.g.*, 12–14). Although evidence has mounted supporting the hypothalamus as an important site for leptin's action on food intake, it is also recognized that leptin is potentially available to all brain areas via the circulation (15), and evidence suggests that leptin receptors are expressed at different levels of the neuraxis (2). It seems premature, therefore, to rule out possible functional contributions of neuronal leptin receptors outside the hypothalamus.

The focus of the present study is the hypothesis that Ob-Rb within the caudal brainstem (CBS) contributes to the anorexic effects of central leptin delivery. A growing literature has established a role for the CBS in the control of energy balance with respect to: 1) mediation of a variety of relevant autonomic reflexes (16, 17); 2) mechanisms or receptors that trigger ingestive effects of a number of pharmacologic and metabolic treatments (18–21); and 3) the ingestive behavioral competence observed in the chronically maintained decerebrate rat (22, 23). There is as yet no direct evidence for intake-relevant action of leptin in the CBS, although two physiological studies suggest that the hindbrain contains a population of neurons that expresses functional leptin receptors. Smedh *et al.* (24) reported that fourth intracerebroventricular (i.c.v.) injection of leptin suppressed gastric emptying in rats, and Zhou and Schneider (25) showed that fourth-i.c.v. leptin reversed fasting-induced anestrus in the hamster. The location of CBS leptin receptors that mediate these effects, however, is not known. Evidence for leptin receptor expression in the dorsal vagal complex (DVC), a region of clear importance to meal size control, has been reported in several immunocytochemical studies (24, 26, 27). The antibodies used in these studies, however, did not distinguish Ob-Rb from other leptin receptor isoforms, such as Ob-Ra, which have markedly attenuated signaling capability. *In situ* hybridization studies have yielded inconsistent

Abbreviations: AP, Area postrema; CBS, caudal brain stem; DMX, dorsal motor nucleus; DVC, dorsal vagal complex; ELF, enzyme-labeled fluorescent; FISH, fluorescence *in situ* hybridization; i.c.v., intracerebroventricular; NTS, nucleus of the solitary tract; Ob-Rb, long-form leptin receptor.

results with respect to Ob-Rb mRNA expression in the DVC (2, 28–30). One study reported negative results for the DVC (28) whereas another group showed Ob-Rb expression in the nucleus of the solitary tract of the mouse but much weaker expression in rat nucleus of the solitary tract (NTS), Ob-Rb expression in rat NTS (29). Elmquist *et al.* (2) found widespread Ob-Rb mRNA expression in the CBS including all three divisions of the DVC (NTS, area postrema [AP], dorsal motor nucleus [DMX]), and parabrachial nucleus, but the density of hybridization signal in these brain stem structures was reported to be relatively low and inconsistent across animals. There is therefore some uncertainty about the location of neurons that could mediate the direct action of leptin in the CBS.

We combined anatomical and functional analyses to address a CBS contribution to the ingestive effects of leptin. We reevaluated the expression of Ob-Rb in the CBS using fluorescence *in situ* hybridization (FISH) (31) and immunocytochemistry with a polyclonal antibody specific to human Ob-Rb (7). We found substantial Ob-Rb expression in the DVC and in other CBS structures. We found significant food intake and body weight reductions after leptin administration to the fourth ventricle and, at a low dose, to the DVC parenchyma. The results taken together are consistent with the hypothesis that brain stem Ob-Rb, perhaps in combination with leptin action in the hypothalamus, contribute to the intake-inhibitory effect of central leptin.

Materials and Methods

Exp 1: Expression of Ob-Rb in CBS

Subjects. Male Wistar rats (specific pathogen-free; 250–280 g) from Simonsen Laboratories (Gilroy, CA) were housed individually under a 12:12 h light-dark cycle in the Animal Research Facility at the Seattle Division of the VA Puget Sound Health Care System. All animal procedures were approved by the Veteran's Affairs, Puget Sound Health Care System and University of Washington Animal Research Committees.

In situ hybridization. Brains for *in situ* hybridization were removed from the cranium of anesthetized rats between 0900 and 1100 h and immediately frozen on dry ice. Coronal cryostat slices (14 μm thick) of brain stem were stored at −70 C. The riboprobe used for specific Ob-Rb mRNA *in situ* hybridization is complementary to the coding region for the c-terminus of the rat Ob-Rb and does not recognize mRNA for any other known leptin receptor splice variants (4, 7). Details of the preparation and labeling of the Ob-Rb mRNA riboprobes used for FISH are described elsewhere (5, 31). Briefly, the Ob-Rb mRNA riboprobes were prepared with biotin-UTP by transcribing cDNA containing the specific coding region for the cytoplasmic tail of the Ob-Rb spice variant (7). The probe was purified using a Qiaquick nucleotide removal kit (QIAGEN, Valencia, CA). To increase the sensitivity of FISH for detection of Ob-Rb mRNA, FISH was amplified by combining the enzyme-labeled fluorescent substrate (ELF) method (Molecular Probes, Inc., Eugene, OR) with tyramide signal amplification (TSA indirect kit, NEN Life Science Products, Boston, MA) (31, 32), in which Ob-Rb mRNA hybridization was visualized in a two-step process. In the first step, the biotin moiety was amplified using a Renaissance TSA indirect kit (NEN Life Science Products) (streptavidin-horseradish peroxidase 1:100) for 30 min at room temperature, followed by biotinyl tyramide (1:50) for 10 min at room temperature. The second step used an ELF-97 mRNA *in situ* hybridization kit no. 2 (Molecular Probes, Inc.). Slides were placed in blocking buffer containing 30 mM Tris (pH 7.4), 150 mM NaCl, 1% BSA, 0.5% Triton X-100, and 1 mM levamisole (Sigma, St. Louis, MO) for 30 min at room temperature, followed by the streptavidin-alkaline phosphatase conjugate (1:50) for 30 min at room temperature. The ELF-AP substrate was prepared according to manufacturer's instructions and applied to each slide for exactly 10 min at room temperature.

Immunocytochemistry. Rats were perfused transcardially under Equithesin anesthesia with cold 4% paraformaldehyde fixative in 0.1 M phosphate buffer, pH 7.4. Brains were removed from the cranium and placed in fresh fixative overnight in the refrigerator, immersed in 0.05 M phosphate buffer containing 25% (wt/vol) sucrose for 1 d, and then frozen on dry ice and sectioned at 5 μm by cryostat. Slide-mounted sections were incubated overnight in a polyclonal antibody directed against the C-terminal region of human Ob-Rb (Linco Research, Inc., St. Charles, MO), diluted 1:1000 in 0.05 M phosphate buffer (pH 7.4, 4 C). Fluorescent visualization of the Ob-Rb antibody binding was done with donkey antigoat IgG conjugated to Cy3 (Jackson ImmunoResearch Laboratories, Inc., West Chester, PA) (7). Immunostaining controls included: 1) omission of primary Ob-Rb antibody; 2) replacement of Ob-Rb antibody with normal rabbit serum at the same concentration; 3) comparison to hypothalamic staining pattern produced by another Ob-Rb antibody that was characterized by immunoblots and immunostaining in a previous study (5); and 4) analysis of immunostaining of the choroid plexus, which expresses Ob-Ra but not Ob-Rb. Absorption controls for immunocytochemistry were not required (33) because the specificity of the antibody for Ob-Rb was shown by Western immunoblots of adipocytes (manufacturer's literature) and rat brain (data not shown).

Microscopy and imaging. Visualization of FISH and immunostaining was done with a Axioplan fluorescence microscope (Carl Zeiss, Thornwood, NY). The red Cy3 fluorescence (immunostaining) was visualized with a conventional rhodamine filter set, whereas the green fluorescence of the ELF alkaline phosphatase substrate reaction product (FISH) was observed with a 365-nm excitation filter, 400-nm dichroic long pass filter, and a 515-nm emission filter (Chroma Technology, Brattleboro, VT). Digital RGB pseudocolored images (10 bit) of the fluorescence preparations were acquired with a C4880 fast-cooled CCD camera (Hamamatsu Corp., Bridgewater, NJ) and the MCID imaging system (Imaging Research, St. Catharines, Ontario) and were exported to Adobe Photoshop as 300 dpi tiff RGB files. The images were processed with pseudocolor palettes that closely matched the intensity and contrast present in the original preparations; no selective contrast enhancement of specific areas or cells was applied to the images.

Exp 2: Delivery of leptin to the fourth and lateral ventricles

Subjects. Male Sprague Dawley rats (n = 57) from Charles River Laboratories, Inc. (Wilmington, MA) weighed between 375 and 525 g at the start of testing. Rats were maintained on a 12-h light, 12-h dark schedule, with food and water available *ad libitum* except for the 75 min before the onset of the dark period. All procedures conformed to standards for animal care at the University of Pennsylvania.

Cannula placement. Rats were anesthetized with ketamine/xylazine (100 mg/kg and 10 mg/kg, im). A guide cannula (22 G, PlasticsOne, Roanoke, VA) was stereotaxically positioned 2 mm above the injection site in either the fourth or the left lateral ventricle. For the fourth i.c.v. placement, the cannula tip was positioned on the midline, 2.5 mm anterior to the occipital suture and 4.5 mm below the dura. The lateral i.c.v. cannula tip was placed 1.6 mm lateral to the midline, 0.9 mm posterior to bregma, and 2.0 mm below the dura. The cannula was fixed to the skull with jeweler's screws and dental cement, and an obturator was inserted. Approximately 2 wk after surgery, cannula placement was verified by measurement of plasma glucose immediately before and 1 h after injection of 5-thio-D-glucose (210 μg in 3 μl isotonic saline). The criterion for an effective cannula was the sympathoadrenal hyperglycemic response (34) of a doubling of plasma glucose concentration above the preinjection baseline level.

Injections. Murine recombinant leptin (Peprotech, Roden Hill, NJ) was dissolved in 5 mM sodium citrate buffer, pH 4. The protein concentration of the solution was determined with BCA colorimetric assay (Pierce Chemical Co., Rockford, IL). The solution was aliquoted and stored at 4 C until time of use, when pH was adjusted to 7.5–8.0 by addition of sodium hydroxide. The leptin concentration of the aliquot was adjusted by the addition of vehicle (1:1 sodium citrate, pH 4: sodium hydroxide, pH 12) to yield leptin doses of 0.03, 0.277, and 1.67 μg/μl. A 3-μl injection

volume was delivered via a 28-gauge injector. Injections into either the lateral or the fourth ventricle were driven by an infusion pump over a 4-min period, 1 min after which the injector was withdrawn and the obturator replaced. Injections were delivered an average of 30 min before the beginning of the dark.

Measurement of food intake and body weight. Rats were removed from their cages and weighed 30 min before the dark period. At the same time, the food dishes and water bottles were weighed. Food, water, and rat were returned to the cage at the beginning of the dark period. On injection days, food intake was also measured 2 and 4 h after lights out.

Experimental design. Testing began between 3 and 4 wk after surgery. Six weight- and age-matched groups of rats (n = 9–11) with fourth or lateral i.c.v. cannula were subjected twice to a 3-d injection protocol during which a given dose of leptin (0.1, 0.83, or 5.0 μg) or vehicle was delivered once daily. For each group, half of the rats received leptin injection for 3 d and then vehicle injections for 3 d. The remaining rats were tested first under vehicle injection conditions followed by leptin treatment. Leptin and vehicle treatment blocks were separated by 3 d without i.c.v. injection.

Data analysis. The fourth-i.c.v. results were analyzed by 3-way, mixed-model, ANOVA (drug [within-subject: leptin *vs.* vehicle] × day within treatment condition [within-subject: d 1, 2, 3] × dose [between-subject: 0.1, 0.83, 5 μg]). A separate ANOVA was run for each of four dependent measures (cumulative intake measures at 2, 4, and 24 h after lights-out, and the 24-h change in body weight). For each ANOVA, an effect of leptin is indicated by a significant main effect of the drug factor. The dose relatedness of the leptin response is indicated by a significant drug × dose interaction. Such interactions were explored via separate two-way ANOVA (drug × day) run at each dose. Comparison between fourth and lateral i.c.v. results was accomplished via four-way ANOVA, with ventricle as the added fourth (between-subjects) factor.

Exp 3: Delivery of leptin to the DVC

Surgery. Subject strain and source, maintenance, and anesthesia were identical to that of Exp 2. Anesthetized male rats (n = 13) were positioned in a stereotaxic instrument. A guide cannula (26 G PlasticsOne) tip was placed 13.8 mm posterior to bregma, 0.5 mm lateral to midline, and 3.7 mm dorsal to the interaural line (a placement 2 mm above the injection site). The cannula was fixed to the skull with jeweler's screws and dental cement, and an obturator was inserted. All procedures conformed to standards for animal care at the University of Pennsylvania.

Verification of parenchymal placements. India ink (0.2 μl) was injected 1.5 mm ventral to the drug injection site just before transcardial perfusion with saline followed by 10% formalin. Brains were then removed and postfixed in a 10% sucrose-formalin solution. Coronal sections (50 μm) were stained with cresyl violet. Where possible, the presence of gliosis was used to determine the site of the injector. In cases in which glial damage was not apparent or determinate, the position of injector placement was assessed in relation to the dye reference. Data from only those animals with verified placements were included in the analysis.

Injections. Leptin and vehicle preparation was the same as described above. A 33-gauge injector (2 mm longer than the guide cannula) was inserted, and a 0.5-μl volume of leptin solution or vehicle was injected over 5 min using a microsyringe pump (Harvard, Holliston, MA). The injector was removed after 1 min. Injections were delivered 30 min before the beginning of the dark period.

Experimental design and analysis. Each rat was subjected twice to a 3-d injection protocol during which leptin (0.1 μg) or vehicle was delivered once daily. Condition (three leptin or three vehicle injections) testing order was counterbalanced across rats. Three days without DVC injection separated the two treatment blocks. A two-way ANOVA (drug × day) was performed for each of four evaluated parameters (2-, 4-, and 24-h intakes, and body weight change).

Results

Expression of Ob-Rb in CBS

The TSA-FISH protocols produced a bright fluorescent reaction product over neuronal perikarya and revealed a widespread distribution of hybridization signal for Ob-Rb mRNA throughout the hindbrain. Identification of the preponderance of cells with Ob-Rb mRNA FISH signal as neurons was unambiguous, based on cell morphology and distribution, although we cannot rule out the possibility that some nonneuronal cells may also have been labeled. At an intermediate level of the DVC (−4.68 mm from interaural line), Ob-Rb mRNA FISH signal was seen in all divisions (AP, NTS and DMX; Fig. 1, A, B, and C). Many cells in the AP were heavily labeled with a bright, fluorescent FISH signal for Ob-Rb. DMX neurons also showed strong Ob-Rb FISH signal in most cells. With comparable magnification, the FISH signal appeared visibly weaker in the NTS than in the DMX although hybridization to Ob-Rb in the NTS was clearly observed. To gauge the relative abundance of NTS neurons with the potential to respond directly to leptin, we counted Ob-Rb mRNA-positive cells in six to eight sections of the NTS from four rats. The results showed that 29.3 ± 4.8% of the neuronal cell bodies in the NTS contained Ob-Rb mRNA FISH signal at detectable levels. Other CBS structures that were observed to express hybridization signal in many neurons included the medial parabrachial nucleus (Fig. 1D), hypoglossal nucleus (Fig. 1E), trigeminal motor nucleus (Fig. 1F), locus ceruleus (Fig. 1G), cochlear nucleus (Fig. 1H), and lateral reticular nucleus (Fig. 1I).

Hybridization signal was absent in all of these regions in negative control sections that were pretreated with RNAase or when the specific riboprobe was omitted. As a positive control, FISH for Ob-Rb mRNA was also performed on sections of the hypothalamus. These sections showed positive FISH for Ob-Rb mRNA in the ARC, ventromedial nucleus, and dorsomedial nucleus (all known to express Ob-Rb) but not in the choroid plexus, which does not express Ob-Rb, or in brain regions in which Ob-Rb expression is believed to be low or absent, such as the caudate nucleus, dentate gyrus, and amygdala. The negative and positive control results were essentially identical to those provided recently by Breininger and Baskin (31), and the reader is referred to that study for representative control images.

Immunocytochemistry of CBS sections with antibodies specific for the cytoplasmic tail of the Ob-Rb splice variant of the leptin receptor produced immunofluorescence in the same regions that were found to contain FISH for Ob-Rb mRNA, including the AP (Fig. 1J), NTS (Fig. 1K), and DMX (Fig. 1L). Similar to the FISH results, the immunostaining was primarily observed in neuronal cell bodies. Visual analysis indicated the numbers of neurons that were Ob-Rb positive in these regions were similar to those observed with Ob-Rb mRNA FISH. Immunostaining was absent in negative control sections that were treated with normal rabbit serum in place of the Ob-Rb antiserum or on which primary antiserum was omitted.

Delivery of leptin to the fourth and lateral ventricles

Fourth i.c.v. injection. Leptin significantly suppressed intake at 2, 4, and 24 h after injection into the fourth ventricle and reduced body weight change, relative to vehicle injection conditions (Fig. 2). At the 2-h food intake, there was an

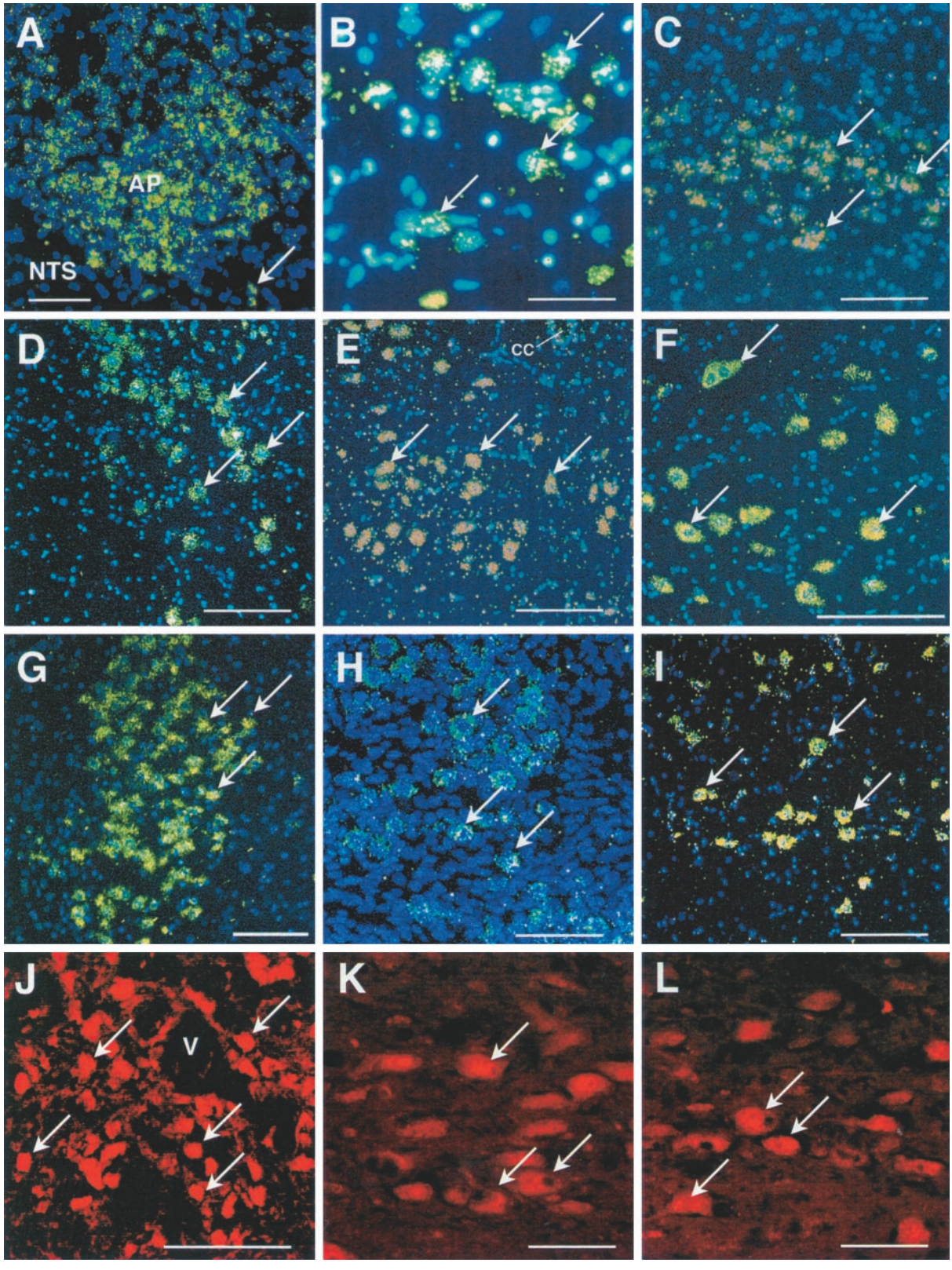

FIG. 1. Expression of Ob-Rb in rat caudal brain stem. A–I, FISH signal for Ob-Rb mRNA. FISH signal appears in cell bodies as a granular, punctate precipitate (*yellow-green* pseudocolor). Cellular nuclei are revealed by *dark blue* fluorescence from the Hoechst nuclear stain. J–L, Immunocytochemical detection of Ob-Rb (no nuclear counterstain). A, AP. A FISH-positive neuron in the subjacent NTS is indicated by *arrow*. *Bar*, 100 μm. B, NTS at level of the AP. Several NTS cells with Ob-Rb mRNA are indicated by *arrows. Bar*, 50 μm C, DMX caudal to the area postrema. Several neurons with Ob-Rb FISH signal are indicated by *arrows. Bar*, 75 μm. D, Medial parabrachial nucleus showing FISH signal

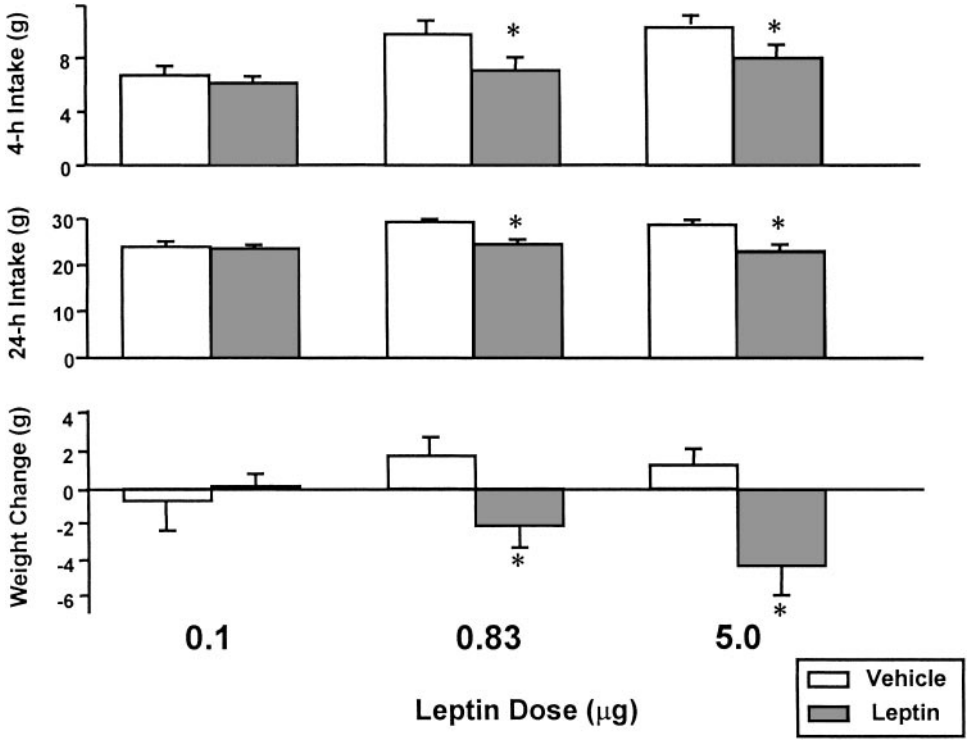

FIG. 2. Average daily 4- and 24-h cumulative intake, and body weight change, in response to leptin or vehicle injections delivered to the fourth ventricle. Daily means (± SEM) are shown for each 3-d injection condition. The *asterisk* indicates a significant difference between values for a given leptin dose and its associated vehicle condition.

overall effect of leptin ($F[1,24] = 19.59$, $P < 0.0002$) that was dose related ($F[2,24] = 8.44$, $P < 0.002$), with *post hoc* analysis showing a significant difference between leptin and vehicle conditions only at the 5.0-μg dose (mean intake: vehicle = 5.8 g; leptin = 4.1 g). For this parameter, as for the 4- and 24-h intake measurements, the magnitude of the drug effect did not vary across the 3 d of the injection protocol. At the 4-h food intake, intake was significantly suppressed by leptin [drug factor ($F[1,24] = 26.09$, $P < 0.00004$; Fig. 2]. A significant effect of dose was obtained ($F[2,24] = 3.56$, $P < 0.05$) along with a marginally significant ($F[2,24] = 3.33$, $P = 0.052$) drug × dose interaction, reflecting the greater intake suppression with leptin (*vs.* vehicle) at the two higher doses. This observation was substantiated by significant *post hoc* results for the 0.83- and 5.0-μg doses (*vs.* the respective vehicle conditions). At the 24-h food intake, the leptin effect on intake ($F[1,24] = 41.21$, $P < 0.00001$) was dose related ($F[2,24] = 8.73$, $P < 0.002$), reflecting significant effects at the two highest doses (Fig. 2, *middle graph).* For body weight change, a significant main effect of leptin to reduce body weight was obtained ($F[1,24] = 9.76$, $P < 0.005$). Dose was not significant as a main effect, but the dose × drug interaction was significant ($F[2,24] = 4.29$, $P < 0.03$). This finding reflects a lowering of body weight at the two higher doses but not at the lowest dose of leptin (Fig. 2, *bottom).* We also found a significant main effect of day ($F([2,48] = 14.88$, $P < 0.00001$). This reflected a loss of weight (−4 g) on d 1, followed by small gains in average weight for d 2 (1 g) and 3 (1.3 g), averaging across drug and dose conditions. There were no significant interactions between day and the drug or dose factors.

Comparison between fourth and lateral i.c.v. results

Administration to a lateral ventricle yielded a remarkably similar profile of effects as that obtained with fourth i.c.v. leptin delivery. There was no significant difference between effect size (drug minus vehicle) as a function of placement for any measurement parameter of dose level (Fig. 3). In the overall ANOVA, there was no main effect of ventricle and no significant interaction involving the ventricle factor.

Delivery of leptin to the DVC

Data for 13 rats with verified injection placements in the DVC are included in these analyses (Fig. 4). The behavioral results are shown in Fig. 5. DVC injection of a dose of leptin that was a subthreshold for food intake suppression when given into either the lateral or fourth ventricle significantly decreased food intake at 2 h (by 24% averaged across days; $F[1,12] = 8.52$, $P < 0.02$), at 4 h (by 12%; $F[1,12] = 7.21$, $P < 0.02$), and at 24 h after injection (by 11%; $F[1,12] = 17.08$, $P < 0.002$). Leptin also caused significant reductions in daily body weight change ($F[1,12] = 21.95$, $P < 0.0006$). Relative to

in neuronal cell bodies, several indicated by *arrows. Bar*, 80 μm. E, Hypoglossal nucleus showing FISH in large neuronal perikarya (several indicated by *arrows*) ventral to the central canal (cc). *Bar*, 100 μm. F, Trigeminal motor nucleus. FISH signal is present in large motor neuron cell bodies, several indicated by *arrows. Bar*, 50 μm. G, Locus ceruleus showing FISH signal in neurons cell bodies (several indicated by *arrows*). *Bar*, 80 μm H, Ventral cochlear nucleus. FISH signal is expressed in neuronal cell bodies, several indicated by *arrows. Bar*, 40 μm. I, Lateral reticular nucleus showing FISH signal in neuronal cell bodies, several indicated by *arrows. Bar*, 75 μm. J, AP. Ob-Rb immunoreactivity in many cell bodies (some indicated by *arrows*). V, Blood vessel. *Bar*, 60 μm. K, NTS. Ob-Rb immunoreactivity in cell bodies (some indicated by *arrows*). *Bar*, 60 μm. L, DMX. Ob-Rb immunoreactivity in cell bodies (some indicated by *arrows). Bar*, 60 μm.

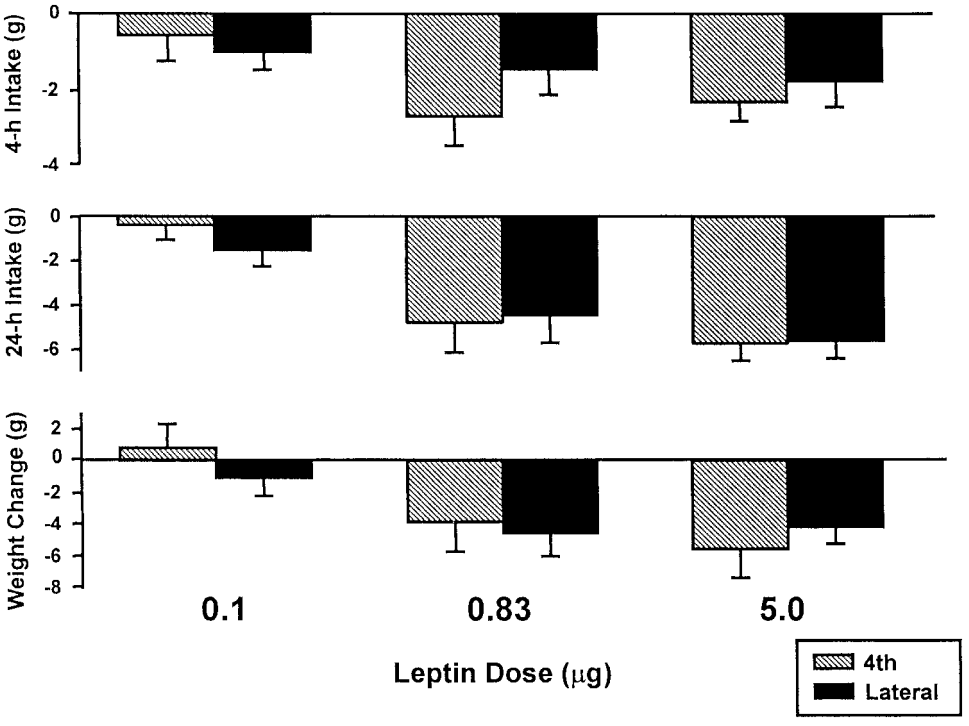

FIG. 3. Comparison between the size of the leptin treatment effects for fourth *(open bars)* and lateral i.c.v. *(striped bars)* injection placements. Values shown are mean (± SEM) differences between leptin dose and associated vehicle outcomes for average daily 4- and 24-h cumulative intake and for body weight change.

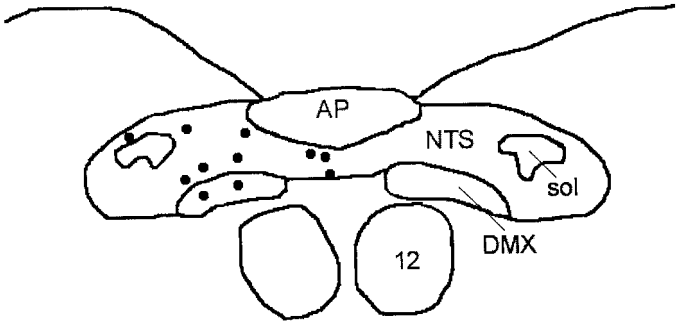

FIG. 4. DVC injection placements. *Black dots* represent the center of the injection placement. Two additional rats had placements 1 mm anterior to this coronal plane within the NTS.

vehicle, rats receiving leptin microinjection into the DVC lost a mean of 3.3 g/d, resulting in a total weight loss of 9.8 g for the 3-d treatment period. For each parameter, there was no day effect and no drug × day interaction.

Discussion

The anatomical and behavioral results reported here support the hypothesis that CBS contains neurons that express Ob-Rb and are targets for the intake inhibitory effects of leptin. Previous reports of leptin receptors in the CBS left some uncertainty about the distribution of Ob-Rb (as opposed to other receptor isoforms) across structures of relevance to intake control (*e.g.* 2, 24, 26, 27, 29). In the present study, we used a modified FISH method with improved sensitivity for detecting neuronal mRNA species of relatively low abundance (such as those encoding most hormone receptors) (31). The mRNA expression results were complemented by immunohistochemistry with an antibody specific for Ob-Rb protein. Across the CBS nuclei examined, we found a high degree of correspondence between results obtained with these two methods. We did not present an exhaustive profile but confirm that the anatomical distribution of Ob-Rb is widespread. Ob-Rb was expressed in several structures that are clearly implicated in the expression or modulation of ingestive behavior including the DVC (*i.e.* most neurons in the DMX and AP and nearly a third of the neurons in NTS), PBN, hypoglossal nucleus, and trigeminal motor nucleus. We also noted Ob-Rb expression in other CBS regions whose possible feeding relevance is not defined or widely discussed, including locus ceruleus, lateral reticular nucleus, and cochlear nucleus. The breadth of the anatomical distribution of Ob-Rb and Ob-Rb mRNA expression described here expands the list of neuronal systems that are potentially leptin responsive and raises the possibility that circulating leptin has direct effects on hindbrain circuits that serve energy homeostasis and perhaps other functions.

The behavioral results reported indicate that delivery of leptin to the CBS gives rise to significant suppression of intake and body weight. We found that fourth i.c.v. leptin administration produced a degree of food intake suppression that is similar to that obtained here and in earlier studies in which lateral i.c.v. effects were evaluated (35, 36). We also sought to determine whether leptin microinjection into a specific CBS site would recapitulate the effects of fourth i.c.v. administration. Because we showed the DVC to contain a relatively high abundance of cells expressing Ob-Rb mRNA and because of its well-documented role in food intake control and autonomic physiology (*e.g.* 19, 21, 37–45), we selected this site as the target for our initial parenchymal injection study. We found significant reductions in short- and longer-term food intake following unilateral DVC injections of leptin at a dose (0.1 μg) that we determined to be subthreshold for the i.c.v. effect. This result is consistent with the

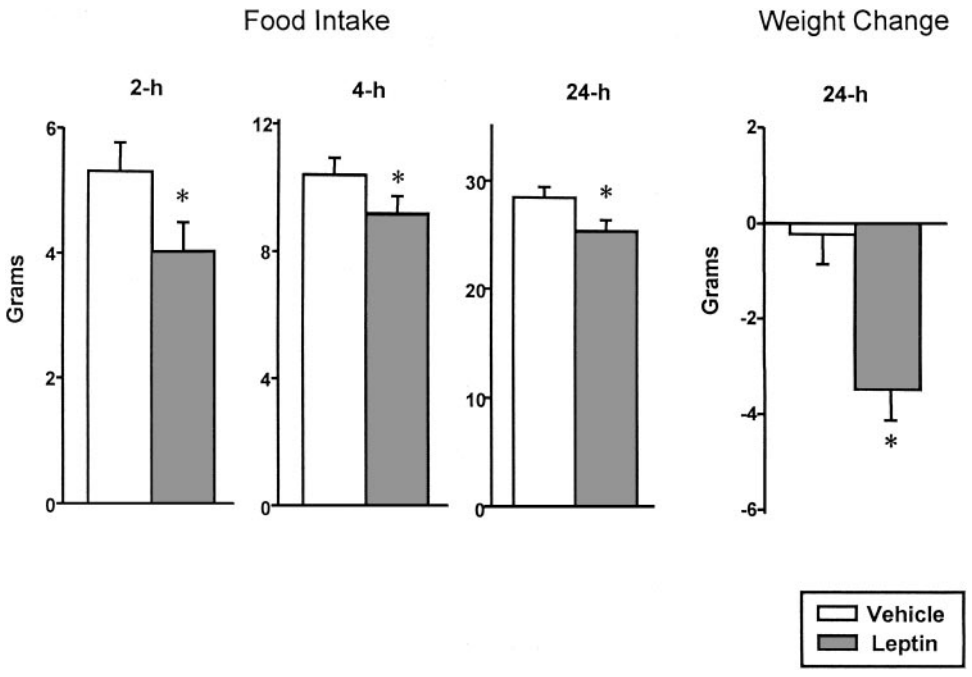

FIG. 5. Average daily 2-, 4- and 24-h cumulative intake, and body weight change, in response to leptin (0.1 μg) or vehicle injections delivered to the DVC. Daily means (± SEM) are shown for each 3-d injection condition. The *asterisk* indicates a significant difference between leptin and vehicle values.

hypothesis that leptin is capable of reducing food intake via a direct action in or near the DVC. In addition, given the proximity of the DVC to the ventricle, it seems reasonable to suggest that stimulation of Ob-Rb in this region also contributes to the effects of fourth i.c.v. leptin delivery. The suggestion could be evaluated in subsequent experiments assessing the consequences of fourth i.c.v. leptin administration for intracellular signals linked to Ob-Rb stimulation (*e.g.* SOCS3 mRNA induction, STAT3 nuclear translocation, PI3K expression) in DVC neurons. Such intracellular signal analyses would also begin to address the possibility that other CBS structures containing Ob-Rb contribute to the effects of fourth i.c.v. leptin.

These results support the hypothesis that widely separated populations of neurons that express Ob-Rb can give rise to similar effects on feeding behavior and body weight. One argument for this contention is the striking similarity of the fourth and lateral i.c.v. response to leptin. If one region were responsible for the i.c.v. effects, we would have expected, rather, the dose-response curve for the more distal placement to be right shifted, with different thresholds and response magnitudes. A more direct source of support for a functionally distributed leptin receptor system is provided by results from parenchymal injection experiments. The anorexic effect we observed following microinjection of leptin into the DVC is similar to that induced by intrahypothalamic leptin administration as reported by Jacob *et al.* (46). They found that local administration of leptin into the ventromedial hypothalamic nucleus, at a daily dose of 0.1 μg bilaterally, reduced intake and body weight but was without effect when delivered to the lateral ventricle. It would appear highly unlikely that the low dose delivered to the DVC could via diffusion have a site of action as distant as the hypothalamus or conversely that a brain stem substrate underlies the effects of hypothalamic microinjection. Taken together, the present and cited results indicate that more than one site in the brain, apparently including at least one in the CBS and one in the hypothalamus can give rise to significant leptin effects on food intake and body weight in the rat.

The results reported here are consistent with the hypothesis that leptin's action in CBS neurons contributes to energy homeostasis. The hypothesis, if correct, is a counterpoint to the currently most widely accepted model for leptin action that focuses almost exclusively on Ob-Rb in the hypothalamus, particularly in the arcuate nucleus. Analysis must now commence addressing the phenotypes of neurons in the brain stem that express Ob-Rb. Given the central role in the hypothalamic model of leptin-sensitive POMC and NPY neurons, it will be interesting to determine whether the NTS POMC and NPY neurons also express Ob-Rb and whether their projections contribute to ingestive effects observed following local administration of leptin. We know that there are neurons in the brain stem that are sensitive to other blood born signals of relevance to the control of energy homeostasis, including cells that express insulin receptor and cells sensitive to changes in blood glucose (37, 47, 48). It will be interesting in this light to determine whether, as in the hypothalamus (49, 50), there are populations of individual neurons that respond to leptin as well as to other interoceptive signals. There are, of course, limits to the parallels that can be drawn between local circuits in the CBS and hypothalamus that are engaged by leptin. Thus, for example leptin receptors are found on agouti-related protein neurons in the arcuate nucleus, whereas in the CBS, receptors but apparently not cell bodies for this peptide have been identified (40). Conversely, whereas Ob-Rb is expressed on GLP-1 neurons in NTS (30), GLP-1 neurons are not found in the hypothalamus (51). In any event, future work in this area will no doubt argue for an expanded model for the feeding-related actions of leptin that encompasses interactions between brain stem and hypothalamic structures and redundant as well as

unique processing functions performed at different levels of the central control system.

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