Evidence that paraventricular nucleus oxytocin neurons link hypothalamic leptin action to caudal brain stem nuclei controlling meal size

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Blevins, James E., Michael W. Schwartz, and Denis G. Baskin. Evidence that paraventricular nucleus oxytocin neurons link hypothalamic leptin action to caudal brain stem nuclei controlling meal size. Am J Physiol Regul Integr Comp Physiol 287: R87–R96, 2004. First published March 25, 2004; 10.1152/ajpregu.00604.2003.—Hindbrain projections of oxytocin neurons in the paravascular paraventricular nucleus (pPVN) are hypothesized to transmit leptin signaling from the hypothalamus to the nucleus of the solitary tract (NTS), where satiety signals from the gastrointestinal tract are received. Using immunocytochemistry, we found that an anorectic dose of leptin administered into the third ventricle (3V) increased twofold the number of pPVN oxytocin neurons that expressed Fos. Injections of fluorescent cholera toxin B into the NTS labeled a subset of pPVN oxytocin neurons that expressed Fos in response to 3V leptin. Moreover, 3V administration of an oxytocin receptor antagonist, -(CH2)5Tyr(Me)2,Orn8]-vasotocin (OVT), attenuated the effect of leptin on food intake over a 0.5- to 4-h period (P < 0.05). Furthermore, to determine whether oxytocin contributes to leptin’s potentiation of Fos activation within NTS neurons in response to CCK, we counted the number of Fos-positive neurons in the medial NTS (mNTS) after 3V administration of CCK before 3V leptin and intraperitoneal CCK-8 administration. OVT resulted in a significant 37% decrease (P < 0.05) in the potentiating effect of leptin on CCK activation of mNTS neuronal Fos expression. Furthermore, 4V OVT stimulated 2-h food intake by 43% (P < 0.01), whereas 3V OVT at the same dose was ineffective. These findings suggest that release of oxytocin from a descending pPVN-to-NTS pathway contributes to leptin’s attenuation of food intake by a mechanism that involves the activation of pPVN oxytocin neurons by leptin, resulting in increased sensitivity of NTS neurons to satiety signals.

brain stem; adiposity; satiety; food intake; oxytocin; CCK

The present study reports data supporting the hypothesis that oxytocin neurons in the paraventricular nucleus (PVN) are a component of a leptin-sensitive signaling circuit between the hypothalamus and central brain stem for the regulation of food intake. The central nervous system (CNS) mechanism for the anorectic effect of leptin is hypothesized to involve neural circuits that connect leptin-responsive neurons in the arcuate nucleus with central brain stem (CBS) neurons in the nucleus tractus solitarius (NTS) (6, 41, 51). It is well established that leptin stimulates arcuate nucleus proopiomelanocortin neurons and inhibits those that coexpress neuropeptide Y and agouti-related peptide (41). Furthermore, neurons in the NTS respond to “satiety signals,” such as cholecystokinin (CCK) and gastric distension, that are generated by the presence of food in the gastrointestinal tract and cause feeding to cease, thereby limiting the amount of food consumed during individual meals (11, 34, 35, 37, 43, 44). The existence of an arcuate nucleus-CBS leptin-sensitive circuit that integrates adiposity signaling with that produced by meal-related satiety signals is supported by evidence that hypothalamic administration of leptin into the third ventricle (3V) produces a reduction of food intake as the result of smaller meal sizes (16, 21). Furthermore, 3V leptin treatment potentiates the satiety effect of CCK and increases the number of brain stem neurons that express Fos protein after peripheral administration of CCK-8 (15), whereas leptin deficiency attenuates the effect of CCK to produce satiety (27, 28). Leptin administration also potentiates the effect of gastric distension to increase c-fos gene expression in the NTS (14).

These findings are consistent with the hypothesis that leptin-activated neurons in the hypothalamus converge on NTS circuits that regulate meal size, although the identities of the neurons comprising such a pathway are not well established. An increasing body of evidence suggests that neurons in the hypothalamic PVN are responsive to leptin and play a role in the relay of anorectic leptin signaling from the arcuate nucleus in the hypothalamus to the NTS and that the effect of this signaling is to enhance satiety signals. For example, PVN neurons express Fos proteins following leptin administration (13, 15), and PVN lesions cause hyperphagia and obesity (23) characterized by attenuated satiety effects of peripherally administered CCK (10). The PVN also contains a diverse population of neuronal cell types expressing an array of peptides that influence energy balance (51). Nevertheless, oxytocin neurons located in the paravascular subdivisions of the PVN (pPVN) are particularly promising candidates for mediating leptin-anorectic signals to the brain stem, as some pPVN oxytocin neurons project directly to the NTS (38, 39) and all oxytocin fibers in the NTS reportedly originate in the pPVN (36). Furthermore, several reports indicate that centrally administered oxytocin reduces food intake (3, 4, 24, 29) and activates Fos protein expression by neurons in NTS subdivisions that are activated by peripheral administration of CCK-8 (31). We recently reported that the oxytocinergic innervation of the NTS is anatomically correlated with the medial (mNTS) and gelatinous (gNTS) subdivisions of the NTS, which are

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particularly sensitive to peripheral CCK-8 treatment (8) and express oxytocin receptors. Furthermore, injection of oxytocin receptor antagonists into a lateral ventricle (3, 4, 24) or the fourth ventricle (4V) (9) stimulates food intake and blunts the satiety effect of CCK-8 (8).

Thus leptin on the one hand has been shown to potentiate the response of the mNTS to CCK satiety signaling, resulting in smaller meals, reduced food intake, and augmented mNTS Fos activation, and pPVN oxytocin neurons are implicated as mediators in this mechanism by both anatomic and pharmacological data. To validate this hypothesis, we investigated three critical questions related to components of the proposed mechanism. First, we conducted immunocytochemical staining studies to demonstrate the feasibility of the hypothesis that 3V leptin administration activates Fos expression in oxytocin neurons in the pPVN. Second, we combined immunocytochemical staining and neuronal retrograde tracing methods to demonstrate that a subset of leptin-activated oxytocin neurons in the pPVN project to the NTS region of the CBS. Third, we determined that blockade of oxytocin action by intracerebroventricular injection of an oxytocin receptor antagonist attenuated leptin’s ability both to reduce food intake and to augment the Fos expression in NTS neurons induced by peripheral CCK-8 administration.

MATERIALS AND METHODS

Animals. The Animal Research Committee of the Seattle Veterans Affairs Puget Sound Health Care System Medical Center approved the current experimental protocols. Adult male Wistar rats (310–409 g body wt) were from Simonsen Laboratories (Gilroy, CA) or Charles River Laboratories (Wilmington, MA). Animals used in testing the effects of leptin on activation of pPVN oxytocin neurons and testing the effects of 3V or 4V administration of the oxytocin receptor antagonist, [d(CH2)2,S.Tyr(Me)2,Orn8]-vasotocin (OVT) on food intake following a 6- or 16-h fast were from Simonsen Laboratories. All other studies used animals from Charles River Laboratories. The animals were housed individually in Plexiglas cages in a temperature-controlled room (22 ± 2°C) under a 12:12-h light-dark cycle (lights off at 1500) and adapted to a 6-h fast before the start of the dark cycle. Animals were fed Purina rat chow pellets (no. 5001). Water was freely available at all times.

Surgical and stereotaxic procedures. The procedure for implantation of the guide cannulas into specific brain sites has been described previously (45). For the immunochemistry studies on leptin induction of Fos in pPVN oxytocin neurons, animals were anesthetized with equithesin (3.4 ml/kg ip) (33). In other studies, animals were anesthetized with a standard ketamine cocktail (1 ml/kg body wt ip) (7). For mounting of cannulas, animals were placed into a stereotoxic apparatus with the incisor bar positioned 3.3 mm below the interaural line. When mounting the 3V cannulas, a 26-gauge guide cannula (18 mm; Small Parts, Miami Lakes, FL) was stereotaxically positioned 1 mm dorsal to the 3V (6.8 mm anterior to the interaural line, 0.05 mm lateral to the midline, and 7.6 mm ventral to the skull surface); for 4V cannulas the coordinates were 3.5 mm posterior to the interaural line, 1.4 mm lateral to the midline, and 6.2 mm ventral to the skull surface. Cannulas were fastened to the surface of the skull with denture acrylic and stainless steel screws. A 33-gauge obturator was inserted into the guide cannula to maintain patency. Ceftriaxone (25 mg/kg; Roche Laboratories, Nutley, NJ) and buprenorphine hydrochloride (0.3 mg/kg im; Reckitt & Colman Pharmaceuticals, Richmond, VA) were administered intraperitoneally after surgery.

Injections of retrograde tracer (cholera toxin subunit B) into CBS. The fluorescent retrograde tracer cholera toxin subunit B (CTB) conjugated to Alexa 488 (Molecular Probes, Eugene, OR) was used to trace PVN projections to the CBS. The Alexa 488-CTB was injected unilaterally into the CBS region occupied by the NTS, dorsal motor nucleus of the vagus (DMV), and area postrema (AP). The goal was to target the mNTS region by using stereotoxic coordinates of −5.5 mm posterior to interaural line, +0.7 mm to the cerebral vein, and −7.1 mm ventral to the surface of the skull. These coordinates for the mNTS were modified from those in the brain atlas of Paxinos et al. (32) on the basis of our postmortem analysis of the injection sites in preliminary studies. A 0.5-μl volume of 0.2% Alexa 488-CTB in saline was injected via a 33-gauge injector connected by 20-μl tubing to a 10-μl Hamilton syringe. Unilateral injections of the Alexa 488-CTB tracer were completed over 60 s; the injector was held stationary for another 60 s and slowly removed over the following 60 s. The guide cannula was held stationary for an additional 5 min and slowly removed during the following 5 min. Each of these animals also received a 3V cannula for leptin injection, as described above. We waited 4 days for retrograde transport of the Alexa 488-CTB to the PVN (12) before injecting the animals with either leptin or vehicle.

Injections into 3V and 4V. Rats were injected through chronic 3V or 4V cannulas, as described above, with the exception that the 33-gauge injector was held stationary at the end of the injection for 2–30 s and the intracerebroventricular injection volume was 2 μl (4V) or 4 μl (3V). Animals were immediately returned to their home cages at the conclusion of each injection protocol. Leptin (Peprotech, Rocky Hill, NJ) was solubilized in a 4 mM sodium citrate (pH 4.0)-saline cocktail and was always given into the 3V 1 h before the start of the dark cycle. OVT (Bachem-Penninsula Laboratories, San Carlos, CA) dissolved in saline was administered into the 3V 30–45 min before leptin administration, or it was given immediately before the start of the dark cycle into either the 3V or 4V, depending on the design of the particular study. Food intake was measured at 0.5, 1, 2, and 4 h after the start of the dark cycle and access to food. Visual examination of the cage bottom revealed negligible spillage, and there were no apparent differences in spillage within or between treatment groups. The results obtained from either leptin or CCK injections from these studies are similar to what others have reported, suggesting that the spillage had minimal impact on the results. Food intake was also measured at 24 h to verify that the animals responded normally to leptin (42).

3V injections of OVT before leptin. Food intake was measured following 3V leptin injection (3.5 μg/2.5 μl) given 1 h before the start of the dark cycle in either the presence or absence of 3V pretreatment with OVT (9,300 pmol given 30–45 min before administration of either leptin or vehicle). This dose of leptin produces a 23–40% reduction in food intake during the 1st h, comparable to the effect that others have reported using a similar paradigm (42). The effects of OVT (0, 93, 930, 9,300 pmol), administered into the 3V under identical conditions to those above (minus the leptin treatment) were examined 48 h before this study to verify that all doses of OVT had no effect on food intake for up to 4 h when administered alone (n = 27 total, 5–8/group). In the subsequent experiment, animals were divided into groups (n = 29 total, 6–9/group) that were matched for body weights and age. Each group received one of four 3V treatments in a 2 × 2 matrix. Group I received the OVT vehicle (saline; i.e., no OVT) before leptin vehicle (i.e., no leptin). Group II received OVT vehicle (no OVT) before leptin administration. Group III received a dose of OVT before the leptin vehicle (i.e., no leptin). Group IV received OVT before leptin (i.e., received both OVT and leptin). Because interpretation of the results of the OVT administration would be ambiguous if the rats failed to show a response to leptin (which occasionally occurred), we established an inclusion requirement of a 20% reduction of food intake within 24 h after leptin treatment in the absence of the OVT either in the actual experiment or in a follow-up study whereby animals in a cross-over within-subjects design received leptin and vehicle. At the completion of this study, all six animals that received vehicle only, five of the eight animals that were initially
injected with leptin in the absence of OVT, five of the seven animals that were initially injected with leptin in the presence of OVT, and six of the eight animals that were initially injected with OVT in the absence of leptin responded to leptin at the conclusion of the study or during the experiment and were used in the final data analysis. Food intake was measured at 0.5, 1, 2, 3, and 4 h after the start of the dark cycle and access to food.

Injection of OVT before leptin and CCK-8. To determine whether endogenous oxytocin signaling contributed to the effect of leptin to potentiate CCK-8-induced Fos expression in NTS neurons, we administered OVT to fasted rats 30–45 min before 5 V leptin, which was given 1 h before injections of CCK-8 (ip), given immediately before the start of the dark cycle and access to food. Food expression was measured by counting Fos-positive neurons within specific regions of the dorsal vagal complex: the gNTS, mNTS, and AP. These regions of the dorsal vagal complex have oxytocin innervation in close vicinity to neurons activated by CCK-8 (8). These four groups were weight- and age-matched and categorized as follows: (group I) OVT vehicle (saline) before leptin vehicle (no leptin) and CCK-8; (group II) OVT vehicle (no OVT) before leptin (3.5 μg/25 μl) and CCK-8 (1.2 nmol = 1.4 μg ip); (group III) OVT (9,300 pmol) before leptin and CCK-8; (group IV) OVT vehicle (no OVT) before leptin vehicle (no leptin) and CCK-8 vehicle (no CCK-8) (n = 18 total, n = 4/2 rats/leptin group, with the hypodiencephalic group including had n = 2 rats). OVT and leptin were administered in a manner identical to that of the previous study, and CCK-8 was given 1 h after leptin administration. The timing of these treatments was designed so that CCK-8 was administered immediately before the start of the dark cycle, when the animals normally begin eating and when CCK-8 has a potent effect on reducing food intake. At 90 min following CCK-8 or vehicle injections, animals were perfused with 4% paraformaldehyde, as described in tissue collection for immunostaining below.

Effect of 3V and 4V administration of OVT. After a 1- to 2-wk period of adaptation to the cannulas and handling, weight- and age-matched groups of animals were fasted for 6 h and received 3V (n = 22 rats) or 4V (n = 20 rats) injections of OVT over a range of doses from 0 (saline control) to 9,300 pmol administered in 2 μl (3V) or 2.5 μl (3V) immediately before the start of the dark cycle (n = 5–7/dose). Food intake was measured at 1, 2, 3, 4, and 24 h after the start of the dark cycle and access to food.

Behavioral assessment of cannula placement. In behavioral studies, the placement of 3V and 4V cannulas was validated by injecting 10 ng of angiotensin II in 1 μl of saline (Sigma, St. Louis, MO) at least 48 h before the start of the feeding experiments. All animals used in the subsequent analysis of the data drank at least 5 ml of water over 30 min. Assessment of 4V cannula placement was done with injections of 15 pmol/2 μl bombesin (Bachem-Peninsula) immediately before the start of the dark cycle in rats that were fasted for 6 h. All animals used in the subsequent analysis of the data reduced their food intake by at least 20% within 1 h, an established criterion for correct placement of these cannulas (2).

Tissue collection for immunostaining. Animals used for immunocytochemistry were returned to their cages immediately after injections. Food was removed to prevent stimulation of brain stem neurons by gastric distension (14, 47). At the conclusion of each study (2–2.5 h after leptin injections and 1–1.5 h from the beginning of the dark cycle), rats were anesthetized with equithesin (3.4 ml/kg ip) and transcardially exsanguinated with saline followed by perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2. Brains were removed and stored overnight in fresh fixative at 4°C and then transferred to 0.1 M phosphate buffer containing 25% sucrose for 24 h. Brains were then frozen by submergence for 20–30 s in isopentane and placed under chilled dry ice. Coronal cryostat sections (14 μm thick) were taken from the hypothalamus and brain stem were mounted on slides and stored at −80°C. Representative sections for immunocytochemical staining of Fos and oxytocin were selected at 126-μm intervals in the classic pPVN region and at 240-μm intervals in the NTS.

The sections were selected with the objective of sampling the rostrocaudal extent of these nuclei at several coronal levels anterior (pPVN: 6.88, 7.2, 7.6, 7.7, 8.08 mm) (33) or posterior (NTS: 3.38, 3.63, 3.87, 4.11, 4.34, 4.58, and 4.83 mm) to the interaural line (32).

Immunocytochemical staining. Slides were washed with 10 mM PBS at room temperature followed by blocking buffer (5% normal goat serum in 10 mM PBS) for 90 min and additional buffer washes. Primary antibodies were 1) rabbit polyclonal anti-Fos, 1:5,000 dilution (Ab-5, Oncogene, San Diego, CA) (28, 46); 2) mouse monoclonal anti-oxytocin, 1:200 dilution (gift of Dr. Ann-Judith Silverman, Columbia University, NY) (20). Oxytocin and Fos antibodies were diluted in 10 mM PBS as a cocktail mixture. After an overnight incubation in primary antibodies at 4°C, slides were washed in 10 mM PBS followed by 1 h in fluorescent second antibodies diluted 1:200 in 10 mM PBS: 1) goat anti-rabbit IgG-Alexa 488 ( Molecular Probes, Eugene, OR) to detect Fos antibodies; 2) goat anti-mouse IgG-Cy3 (Jackson ImmunoResearch, West Grove, PA) to detect oxytocin monoclonal antibody. Slides were washed in 10 mM PBS and cover-slipped using an anti-fading glycerol-based mounting media. Immunostaining specificity controls included replacement of the primary antibody with normal rabbit serum (for polyclonal antibodies) or mouse serum (for the monoclonal antibody) at the same dilutions as the respective primary antibodies. Furthermore, immunocytochemical labeling of the hypothalamus with the oxytocin antibody revealed brightly immunofluorescent cell bodies and axons in the PVN and the supraoptic nucleus (SON), appropriate to the known anatomic distribution of oxytocin cell bodies and axons. Under conditions used in the immunocytochemical staining protocol, immunoreactive Fos protein was concentrated in the nuclei of labeled cells. Specificity of the Fos immunocytochemistry was also confirmed by the highly reproducible increase in nuclear immunoreactive Fos cells in the PVN and SON of rats that were dehydrated for 24 h.

Immunocytochemical data analysis. Slides were analyzed with a Zeiss Axiosplan fluorescence microscope, and all measurements were made with a ×40 objective lens. Identification of anatomic landmarks was assisted by staining cell nuclei with Hoechst 33258 (Sigma), which was added to the mounting medium and observed with a conventional DAPI filter set. The blue fluorescence of the Hoechst 33258 did not interfere with any fluorochromes or tracers used in the staining. Digital RGB images of the fluorescent preparations were acquired with a Hamamatsu (C4880; Tokyo, Japan) fast-cooled charge-coupled device camera and the MCID imaging system (Imaging Research, St Catherines, ON, Canada) and were exported to Photoshop (Adobe, Tucson, AZ). Measurements of Fos expression in oxytocin neurons in classically defined regions of the pPVN, specifically the dorsal cap and medial pPVN regions, were made unilaterally on 6–9 coronal sections per brain (with the exception of one leptin-injected animal that used only four coronal sections because of tissue loss). The analyzed sections, each separated by 126 μm, were located between atlas levels 6.88 and 8.08 mm anterior to the interaural line (33). The classically defined magnocellular pPVN was not included in this analysis, because the magnocellular oxytocin neurons project mainly to hypophysiotropic sites and the neurohypophysis. A neuron was considered to be double labeled if the Fos-positive nucleus was located within the boundary of the oxytocin-stained cytoplasm, as determined by focusing the microscope. On each slide all oxytocin-positive neurons in the pPVN were counted and averaged across all of the PVN sections from each brain. The mean values for each brain were averaged to obtain the mean of the treatment group. The data are expressed as mean numbers of cells counted in microscopic fields (cells/field) sampled with a ×40 microscope objective. The objective was placed visually so as to include all of the oxytocin-positive cells in the pPVN region. This required counting several fields for each brain, but an effort was made to avoid counting the same cells in separate fields. The relative change of Fos expression in leptin-treated vs. control cells was calculated as the percentage increase in the number of Fos-positive oxytocin neurons counted within the 6–9
anatomically matched pPVN levels. Analysis of Fos immunostaining in the NTS following OVT, leptin, and CCK-8 was assessed similarly but bilaterally and at constant magnification using a ×20 objective.

Statistical analysis. One-way analysis of variance (ANOVA) was used to evaluate the effects of leptin on short-term food intake and on Fos expression within the pPVN oxytocin-positive neurons, the effects of 3V administration of OVT on leptin’s inhibition of food intake, the effects of OVT on leptin’s potentiation of CCK-8-induced Fos within the NTS, and the effects of 4V administration of OVT on food intake. Analyses were performed using the statistical program SYSTAT. Data are expressed as means per group ± SE. Differences were considered significant at \( P < 0.05 \). Animals that did not have proper cannula placement were eliminated from the analysis.

RESULTS

Effect of leptin on Fos expression in pPVN oxytocin neurons. To determine the feasibility of detecting the activation of pPVN oxytocin neurons by leptin, we performed double immunostaining for oxytocin and Fos on pPVN sections of rats that were given 3V leptin \(( n = 5 )\) or vehicle \(( n = 4 )\) after a 6-h fast. When viewed with filters for Cy3 fluorescence (red), numerous oxytocin-positive neuronal cell bodies were present in the pPVN. Quantitative analysis showed that the numbers of neurons that had oxytocin immunoreactivity were nearly identical in both the vehicle-treated \((18 ± 4\ cells/field)\) and leptin-treated groups \((18 ± 1\ cells/field)\). When viewed with the filters for Alexa 488 fluorescence (green), we observed abundant Fos-positive cells in the pPVN in both the vehicle-treated and leptin-treated groups; however, these cells were not quantified, because many were nonoxytocin neurons. When the images from Fos immunostaining were merged with those from the oxytocin immunostaining, some oxytocin-positive cells were observed to have Fos-positive nuclei. Examples of Fos-positive oxytocin neurons from a leptin-injected animal are shown in Fig. 1A. The number of pPVN oxytocin neurons that were Fos-positive was relatively small compared with the total population of oxytocin neurons. Nevertheless, a few could be found in each microscopic field sampled in the vehicle-treated animals \((3 ± 1\ cells/field)\). The corresponding number of Fos-positive oxytocin neurons in the pPVN was higher \(( P < 0.01 )\) in the leptin-treated group \((5 ± 1\ cells/field)\). Thus, although the absolute numbers are relatively small, the percentage of oxytocin neurons that showed Fos activation after leptin treatment compared with the controls was doubled in the leptin-treated rats \((29 ± 6\ vs. 15 ± 2\%, \ P < 0.01; \ Fig. 1B)\). These observations suggest that a small cohort of the oxytocin neuronal population in the pPVN is leptin sensitive.

CBS projection of leptin-sensitive oxytocin neurons. These findings raised the question of whether any pPVN oxytocin neurons that show Fos activation in response to leptin treatment actually project to CBS regions involved in regulation of meal size. To determine the feasibility of addressing this question anatomically, we repeated the protocol for activating Fos in the pPVN by 3V leptin injections in three fasted rats that had received an injection of the Alexa 488-labeled CTB retrograde tracer 4 days earlier. Visual analysis of sections from the injection site of each rat showed that the fluorescent CTB was concentrated in the region of the mNTS and gNTS, although some of the tracer had spread to adjacent regions such as the DMV. Fluorescence microscopy revealed that many pPVN neurons contained the CTB retrograde tracer 4 days after CTB injection into the dorsal vagal complex \(( \text{Fig. 1C})\). Double-immunostained sections from these brains with the Fos polyclonal antibody and the oxytocin monoclonal antibody revealed the presence of oxytocin neurons \(( \text{Fig. 1D})\) and Fos-positive neurons that contained the retrograde tracer \(( \text{Fig. 1E})\) in the pPVN. In the latter case, both the immunoreactive Fos and the retrograde tracer were revealed by green Alexa 488 fluorescence. However, it was easy to unambiguously distinguish these different signals because the Fos staining was confined to the nucleus, whereas the retrograde tracer was confined to the cytoplasm of the neuronal cell bodies. When the separate fluorescent images were merged, it was possible to verify by the orange-red pseudocolor of the cytoplasm that some oxytocin neurons (identified by red Cy3 signal in the cytoplasm) projected to the CBS \(( \text{identified by green Alexa 488 signal in the cytoplasm; Fig. 1F})\). Furthermore, merging these images also demonstrated unambiguously that some of these double-labeled oxytocin neurons also showed Fos activation \(( \text{identified by green Alexa 488 signal in the nucleus; Fig. 1F})\), consistent with having been activated by leptin. This result was seen in three animals but not quantified further in this study.

3V injections of OVT before leptin. To determine whether endogenous oxytocin signaling influences the anorectic effect of leptin, we administered OVT or vehicle to 6-h-fasted rats 30–45 min before 3V leptin or vehicle, which was given 1 h before the start of the dark cycle and access to food. If oxytocin signaling contributes to the ability of leptin to reduce food intake, it is expected that the oxytocin receptor antagonist OVT will blunt the effects of leptin to reduce food intake in these animals \(( \text{Fig. 2})\). In the absence of OVT pretreatment \(( \text{group II})\), leptin treatment decreased cumulative food intake by 61, 64, and 37% during the first 0.5, 1, and 24 h, respectively \(( P < 0.05 \), compared with vehicle-injected animals \(( \text{group I})\). Leptin administered in the presence of OVT \(( \text{group IV})\) did not significantly attenuate cumulative food intake at 0.5, 1, and 4 h \(( P > 0.05 \), Administration of OVT followed by the leptin vehicle \(( \text{group III})\) had no effect on cumulative food intake \(( \text{Fig. 2})\). These results reproduced an earlier study under identical conditions in these animals whereby OVT attenuated the effect of leptin \(( \text{group IV})\) to reduce food intake during the first 0.5 h compared with leptin alone \(( \text{group II})\) \((1.6 ± 0.4\ vs. 3.2 ± 0.4\ g, \text{group II vs. group IV, P < 0.01})\). Therefore, the between-subjects comparisons showed significant differences between \( \text{groups II and IV} \) in both studies. A within-subjects analysis of the 0.5-h data from the initial study suggests a similar conclusion \(( P = 0.089)\).

Injection of OVT before leptin and CCK-8 on NTS Fos expression. To determine whether endogenous oxytocin signaling contributes to the effect of leptin to potentiate CCK-8-induced Fos expression in NTS neurons, we administered OVT to rats that had been treated with an intraperitoneal injection of CCK after being given 3V leptin to enhance the effects of CCK on food intake and NTS Fos expression \(( \text{Fig. 3})\). When the data for all neuroanatomically matched sections that were sampled within the gNTS, mNTS, and AP were pooled by treatment group, the results showed that 3V injection of leptin potentiated CCK-8-induced Fos expression within the mNTS \((27 ± 4\ vs. 17 ± 3\ cells/field of the mNTS, \text{group II vs. group I, P < 0.05})\); Fig. 4), gNTS \((8 ± 1\ vs. 3 ± 1\ cells/field of the gNTS, \text{group II vs. group I, P < 0.01})\), and AP \((16 ± 3\ vs. 7.0 ± 2\ cells/field of the AP, \text{group II vs. group I, P < 0.01})\).
Fig. 1. Immunofluorescent detection of leptin activation of Fos expression (green pseudocolor representing Alexa 488 fluorescence) in parvocellular paraventricular nucleus (PVN, pPVN) oxytocin (OXY) neurons (red pseudocolor representing Cy3 fluorescence) that project to the nucleus of the solitary tract (NTS) region of the caudal brain stem. A: coronal section from a leptin-injected animal of pPVN showing immunoreactive Fos protein concentrated in neuronal nuclei and immunoreactive OXY (some of both indicated by labels). Some OXY neurons showed Fos-activated nuclei (arrows), and the distribution of OXY cells that expressed Fos was uneven and quite variable throughout the pPVN. B: quantitative analysis showed that intracerebroventricular (icv) administration of leptin significantly increased the percentage of pPVN OXY neurons that expressed nuclear Fos. C: retrograde transport of fluorescent cholera toxin B subunit (CTB; green pseudocolor) injected unilaterally into the dorsal vagal complex [medial nucleus tractus solitarius (mNTS)/dorsal motor nucleus of the vagus (DMV)] region of the caudal brain stem revealed numerous neuronal cell bodies bilaterally in the pPVN. Scale bar, 100 μm. D-F, triple labeling of OXY, Fos, and CTB in pPVN neurons. D: OXY immunostaining (red cells) of pPVN neurons. Note absence of nuclear staining. E: same field as D, showing double-labeled cells that had Fos immunoreactivity (green fluorescence located in cell nuclei) and CTB retrograde tracer (green fluorescence located in cell cytoplasm; examples indicated by labels). Fos and CTB were unambiguously distinguished by location: Fos in nucleus, CTB in cytoplasm. Note that some cells with CTB did not show nuclear Fos staining (e.g., left center), and many Fos-activated neurons did not contain the CTB retrograde tracer. F: merging of images D and E showed neurons that contained nuclear Fos staining (green) and orange cytoplasm (arrows), indicating pPVN OXY neurons that had CTB retrograde tracer and projected to NTS.
cells/field of the AP, group II vs. group I, P < 0.05). OVT pretreatment reduced the ability of leptin to interact with CCK-8 to induce Fos within the mNTS, but not the gNTS or AP. Thus the number of Fos-positive cells that result from leptin-induced potentiation of CCK-8 action was reduced within the mNTS (27 ± 4 vs. 17 ± 2 cells/field of the mNTS, group II vs. group III, P < 0.05; Fig. 4), but not the gNTS (8 ± 1 vs. 5 ± 1 cells/field of the gNTS, group II vs. group III, P > 0.05; Fig. 4), or AP (16 ± 3 vs. 12 ± 4 cells/field of the AP, group II vs. group III, P > 0.05; Fig. 4). The extent to which OVT attenuated leptin’s potentiation of CCK-induced Fos varied along the rostrocaudal extent of the mNTS, with the medial-to-caudal region of the mNTS appearing to be the most responsive.

Effect of 3V and 4V administration of OVT. To determine whether a deficiency of oxytocin signaling within the CBS

Fig. 2. Effect of OXY receptor antagonist [d-(CH2)5,Tyr(Me)2,Orn8]-vaso-toxin (OVT) on leptin-induced inhibition of food intake following third ventricular (3V) injections. OVT attenuated the effects of leptin to inhibit food intake during the first 4 h. Data represent means ± SE.

Fig. 3. OVT attenuated the potentiation of cholecystokinin (CCK)-8-stimulated Fos expression produced by leptin administration (icv). OVT was injected into the 3V. Fos activation is revealed by concentration of immunoreactive Fos in cell nuclei (Fos+ cells) in the mNTS in each panel. Fos immunostaining was done by Cy3 fluorescence, and images were captured in gray scale and subsequently reversed for clarity in this figure with Photoshop. Images were taken from 1 side of the mNTS, with the 4V shown in top right. A: vehicle-treated control showed few Fos+ cells. B: peripheral injection of CCK-8 produced numerous Fos+ neurons in mNTS. C: leptin administration (icv) before injection of CCK-8 visibly increased the numbers of Fos+ neurons in the mNTS. D: OVT administered (icv) in presence of leptin and CCK-8 treatment markedly attenuated the numbers of Fos+ neurons in mNTS. A-D all at same magnification (bar, 100 μm).
could contribute to the effect of leptin to reduce food intake, we compared food intake after 3V vs. 4V administration of OVT. The results showed that OVT had no effect on food intake when administered alone into the 3V at all doses tested, whereas it potently stimulated food intake when administered into the 4V at much lower doses than those used in the 3V (Fig. 5). Relative to saline, rats that received a 930 pmol dose of OVT into the 4V showed a 62% increase in food intake at 1 h (P < 0.05), and food intake remained elevated by 43% at 2 h (P < 0.05). Even the lowest dose of OVT used (9.3 pmol) increased food intake at 2 h by 40% when administered into the 4V (P < 0.05). Because our results consistently showed that OVT administered alone into the forebrain (3V) was not effective in increasing food intake in rats fasted for 6 h whereas previous reports had indicated that OVT was effective in increasing food intake when administered into the forebrain (lateral ventricle) when the rats had been fasted for 21 h (3, 4), we attempted to determine whether the failure of OVT to stimulate food intake after 3V injections in these protocols could be related to the shorter fast (6 h) that we used in our studies. Therefore, we administered OVT alone into the 3V and measured short-term food intake after administration of various doses of 3V OVT (saline, 9.3, 93, 930, and 9,300 pmol in 2 μl) immediately before the start of the dark cycle after a 16 h fast (n = 10). We used a protocol in which each animal served as its own control and received each dose randomly at 48-h intervals. The results showed that, relative to the effect of saline (no change in food intake), the 930 pmol dose of OVT into the 3V produced a 22% increase in food intake at 2 h [8.9 ± 0.4 vs. 7.3 ± 0.7 g (saline injections), P < 0.05], and the 9,300 pmol dose of OVT produced 21% [7.5 ± 0.4 vs. 6.2 ± 0.8 g (saline injections), P < 0.05] and 25% [9.2 ± 0.7 vs. 7.3 ± 0.7 g (saline injections), P < 0.05] increases in food intake at 1 and 2 h, respectively. OVT at lower doses (9.3 and 93 pmol) had no effect on food intake in the 3V.

**DISCUSSION**

These findings support the hypothesis that leptin inhibition of food intake is mediated, in part, by a neural pathway connecting the PVN of the hypothalamus with CBS areas involved in the control of meal size. Our findings provide new evidence that pPVN oxytocin neurons play a role in this pathway. We demonstrated that oxytocin-like immunoreactivity is present in pPVN neurons that are activated to express Fos by leptin, and that a subset of these pPVN oxytocin neurons project to NTS regions that respond to peripheral CCK-8. Moreover, we report that leptin-induced inhibition of food intake was attenuated by pretreatment with an oxytocin receptor antagonist (OVT) at a dose that had no independent feeding effects. These findings support a role for oxytocin in the CNS effect of leptin to reduce food intake.

Similarly, the effect of leptin to enhance CCK-8-induced activation of NTS neurons was also attenuated by pretreatment with OVT. Specifically, this effect of OVT was detected in the medial NTS, an area that is richly innervated by oxytocin fibers from the PVN but was not seen in adjacent CBS areas that had relatively less oxytocin innervation [e.g., gNTS, AP (8)]. Moreover, we demonstrated that injections of OVT into the 4V stimulated short-term food intake, but 3V injections were ineffective in this same dose range and paradigm. Collectively, these findings support the hypothesis that hypothalamic activation of oxytocin neurons by leptin enhances the efficacy of gut-derived satiety signals to activate key neurons within the NTS that control food intake during individual meals. Thus oxytocin neurons may be a critical link between leptin signaling within the hypothalamus and CBS neurons that regulate meal size.

The findings suggest that oxytocin neurons within the dorsal and medial subdivisions of the PVN are activated by 3V leptin. Our morphological quantitative method sampled a relatively small portion of the pPVN, but the procedure was applied uniformly for all of the animals, so the results can be considered a reflection of the relative change across the pPVN among the experimental groups. In our analysis, we observed many oxytocin neurons in the PVN (including magnocellular neurons) that expressed Fos irrespective of leptin treatment, although only the oxytocin neurons in the pPVN showed a significant increase in Fos expression following 3V treatment. This finding is consistent with a previous study by Elmquist et al. (13), which reported leptin stimulation of Fos expression in parvocellular subdivisions of the PVN. It is possible that some magnocellular PVN neurons were included in this present analysis. However, Fos activation was not detected in magnocellular oxytocin neurons within the SON of leptin-treated animals, a finding consistent with the model of PVN-CBS leptin signaling and with evidence that PVN oxytocin neurons provide the sole source of oxytocin innervation of the NTS (36).
A primary goal of this study was to establish whether it is feasible to detect the presence of pPVN oxytocin neurons that are activated by leptin and, if so, whether any of these neurons project to the NTS. The results support this hypothesis, although future studies are needed to quantify the observation and determine the extent to which this pathway plays a physiological role in food intake and body weight regulation. This is particularly the case for the pPVN oxytocin neuronal population that showed a positive Fos response to leptin and projected to the NTS. An important limitation of the study is that the fluorescent CTB tracer was certainly not confined to the dorsal vagal complex, as the injected CTB certainly spread slightly outside its boundaries (which we confirmed by examining sections of the brain stem injection site with fluorescence microscopy). Thus some of the leptin-activated pPVN oxytocin neurons that contained the CTB tracer may have originated outside the NTS. Future quantitative studies employing this experimental paradigm are needed to characterize the precise brain stem projections of the leptin-activated oxytocin neurons in the pPVN.

These findings do not preclude the possibility that oxytocin-independent signaling pathways contribute to leptin’s downstream signaling to the CBS. Indeed, our observation that leptin’s inhibition of food intake was attenuated but not completely blocked by OVT pretreatment is an indication that nonoxytocinergic neurons may be involved in mediating the feeding effects of leptin. Such nonoxytocin pathways might include other catabolic peptides that are made within the PVN and reported to be activated by leptin, such as corticotropin-releasing hormone (CRH), gastrin-releasing peptide (GRP), and thyrotropin-releasing hormone (TRH). TRH neurons and GRP neurons were reported to be activated after leptin administration into the 3V (1, 18, 22). We reported previously that 3V leptin treatment increased CRH mRNA content in rat PVN (40). Moreover, 3V infusion of a CRH receptor antagonist blocked the effect of leptin to reduce food intake and body weight (48). Interestingly, the potentiation of CCK-induced Fos expression in the NTS by leptin was completely blocked by OVT treatment, a finding that contrasts with the partial blockade of OVT on leptin’s reduction of food intake. The neural circuits that mediate the response of these nonoxytocin PVN neurons to leptin’s anorexic effects require investigation.

In our study, OVT was effective at stimulating food intake when infused into the 4V at doses as low as 9.3 pmol in rats fasted for 6 h, but not in the 3V. In contrast, previous studies in rats found that OVT doses as high as 9,300 pmol were required to stimulate food intake following injections into the lateral ventricles (3, 4). In the latter studies, rats were fasted for 21 h. Furthermore, in our protocol, all doses of OVT, including 9,300 pmol, were ineffective in the 3V when rats were fasted for only 6 h, but both 930 and 9,300 pmol doses were effective at stimulating food intake when administered in the 3V if the rats had been fasted for 16 h. Thus the oxytocin receptor antagonist appears to be more effective in an extended fast, when the animals presumably would be in a state of prolonged leptin deficiency (although leptin levels were not measured in these studies) and, therefore, leptin-stimulated release of oxytocin would also be expected to be reduced, although more studies are required to verify this inference. One limitation of the studies that examined the effects of OVT on leptin’s inhibition of food intake and potentiation of CCK-induced Fos in the NTS was that OVT was administered into the 3V rather than the 4V, closer to the hypothesized site of action of oxytocin within the NTS. Consequently, the role of forebrain oxytocin receptor-driven signaling mechanisms cannot be ruled out in mediating the effects of leptin on food intake or on leptin’s interaction with CCK on Fos induction within the NTS. However, the 4V appears to be more sensitive than the 3V to the effects of OVT on food intake, as none of the OVT doses that we used (up to 9,300 pmol) were effective in the 3V of rats fasted for 6 h whereas doses as low as 9.3 pmol were effective in the 4V. Clearly, additional analyses are required to validate the 4V as the primary site of action of oxytocin, but the initial findings are supportive of this hypothesis.

Recent reports demonstrate that leptin may act directly within the dorsal vagal complex to reduce food intake (17, 19). These reports raise the possibility that oxytocin’s interactions within leptin could take place directly in the brain stem in addition to the hypothalamus. Thus it is possible that leptin could influence the CBS response to satiety signals such as CCK via a direct brain stem action, independent of hypothalamic input, although physiological evidence for such a mechanism remains to be demonstrated. It should also be noted that a transgenic knockout OT/−/− mouse, which has loss of function mutation in the oxytocin gene, reportedly shows normal reduction in food intake in response to CCK-8 administered intraperitoneally (1, 3, and 10 μg/kg) (26), although the interaction of leptin with CCK signaling has not been evaluated. Nevertheless, this finding suggests that nonoxytocin pathways may be able to compensate for the developmental absence of oxytocin.

The findings of the present investigation are consistent with a well-documented role in autonomic regulation for pPVN neurons, along with evidence that NTS neuronal terminals containing oxytocin appear to arise solely from the pPVN (36). Although magnocellular oxytocin neurons contribute to neurohypophysial hormone secretion, they have not been implicated in the direct control of autonomic function. Moreover, oxytocin receptors (25) and oxytocin receptor mRNA are also found in the NTS (5) and adjacent areas such as the DMV (49, 50, 52). Although it remains to be determined whether these CBS oxytocin receptors are expressed by neurons activated by CCK-8, central administration of oxytocin has been shown to induce Fos protein in the same subdivisions of the NTS that are sensitive to peripheral injections of CCK-8 (31). Furthermore, the satiety effects of peripherally injected CCK-8 are attenuated following administration of OVT given intracerebroventricularly (30), and, like CCK-8, oxytocin administration inhibits food intake (29). Our findings that oxytocin receptor antagonism blocks the ability of leptin to potentiate CCK’s activation of Fos within NTS neurons and that food intake increases after administration of OVT into the fourth ventricle are consistent with a model in which the CBS is a key site mediating the inhibitory effects of oxytocin on food intake by acting on neurons that are also sensitive to input from CCK. Taken together, the data suggest that oxytocin-containing neurons are key components of a descending pathway from the pPVN to the NTS, connecting leptin-sensitive hypothalamic neurons to CBS areas that process input from meal-related signals. We propose that this signaling system is a critical component of the mechanism whereby leptin enhances the
ability of satiety signals to activate hindbrain neurons that control meal size.

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