

Divergent Regulation of Proopiomelanocortin Neurons by Leptin in the Nucleus of the Solitary Tract and in the Arcuate Hypothalamic Nucleus

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Proopiomelanocortin (POMC) neurons in the arcuate nucleus (ARC) of the hypothalamus are activated by leptin and mediate part of leptin's central actions to influence energy balance. However, little is known about potential leptin signaling in POMC neurons located in the nucleus of the solitary tract (NTS), the only other known population of POMC neurons. Leptin-responsive neurons do exist in the NTS, but their neurochemical phenotype is largely unknown. The contribution of NTS POMC neurons versus ARC POMC neurons in leptin action is thus undetermined. We show here that in contrast to POMC neurons in the ARC, leptin does not stimulate phosphorylation of signal-transducer and activator of transcription 3 in NTS POMC neurons of POMC-EGFP reporter mice. In addition, leptin does not induce c-Fos expression in NTS POMC neurons unlike ARC POMC neurons. Fasting induces a fall in POMC mRNA in both the ARC and the NTS, but different from the ARC, the reduction in NTS POMC mRNA is not reversed by leptin. We conclude that POMC neurons in the NTS do not respond to leptin unlike ARC POMC neurons. POMC neurons in the hypothalamus may therefore mediate all of leptin's signaling via POMC-derived peptides in the central nervous system. *Diabetes* 55:567–573, 2006

Leptin acts on the brain to regulate a number of processes including energy balance, glucose metabolism, and neuroendocrine function in rodents and in humans (1,2). Several central leptin target sites have been identified, and most studies have been directed toward the hypothalamus, where different groups of neurons express the major signaling form of the leptin receptor (ObRb) (3). One group of leptin-responsive neurons are the proopiomelanocortin (POMC) cells located in

the arcuate nucleus (ARC) of hypothalamus (4,5). POMC neurons produce several neuropeptides derived from the POMC-polypeptide precursor, including the melanocortins α -, β -, and γ -melanocyte-stimulating hormone (MSH), which are ligands for the melanocortin-3 and -4 receptors (MC3/4R) (6,7). A separate population of leptin-responsive cells in the ARC is the agouti-related peptide-producing (AgRP) neurons, which coexpress neuropeptide Y. AgRP is an antagonist of the melanocortin peptides at the MC3/4R (8). In contrast to POMC neurons, AgRP/neuropeptide Y neurons are inhibited by leptin (9). These opposing signaling effects are consistent with the negative actions of leptin on energy balance, since MC3/4R agonists inhibit energy intake while AgRP and neuropeptide Y strongly promote feeding. Together, the POMC, AgRP-, and MC3/4R-expressing cells comprise the core of the central melanocortin system (7).

β -Endorphin, a ligand of the opioid receptors, is also derived from the POMC polypeptide precursor via post-translational processing (10). Compared with the melanocortin system, less is known about the specific role(s) of the opioid system in regulation of energy balance by leptin. However, in contrast to earlier reports, some more recent genetic data suggest that β -endorphin, like the melanocortins, may act to inhibit weight gain (11). It is therefore possible that all these POMC-derived peptides may act in concert to affect body weight and food intake in response to leptin.

It has been clearly demonstrated that leptin can regulate intracellular signaling in hypothalamic POMC neurons, including stimulation of phosphorylation of the signal-transducer and activator of transcription 3 (STAT3) transcription factor (12), induction of suppressor-of-cytokine-signaling 3 (SOCS-3) mRNA, and activation of c-Fos protein expression (13). In addition, leptin positively regulates ARC POMC mRNA levels (14,15), rapidly stimulates neuronal firing rates, and decreases the membrane potential (16), leading to release of mature POMC-derived neuropeptides (17,18).

Leptin also acts directly on several brain regions outside the hypothalamus, including the nucleus of the solitary tract (NTS) in the caudal brainstem, as has been demonstrated by the localization of ObRb mRNA, rapid activation of STAT3 phosphorylation (P-STAT3), induction *SOCS-3* gene expression, and stimulation of c-Fos in this brain region in response to leptin administration in rodents (12,19–21). Furthermore, intra-NTS injection of leptin decreases food intake and body weight of rats (21), as has also been demonstrated by leptin injection into the arcuate of the hypothalamus (22), suggesting a role for both

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AgRP, agouti-related peptide-producing; ARC, arcuate nucleus; CCK, cholecystokinin; IHC, immunohistochemistry; MC3/4R, melanocortin-3 and -4 receptors; MSH, melanocyte-stimulating hormone; NTS, nucleus of the solitary tract; POMC, proopiomelanocortin; STAT3, signal-transducer and activator of transcription 3.

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regions in the regulation of energy intake by leptin. Interestingly, in addition to the ARC, the NTS is the only other site in the brain that contains POMC neurons (23–27). Moreover, the caudal brainstem expresses the MC4R (28,29), and injections of minute amounts of MC4R agonists into the NTS reduce food intake in rodents (30), suggesting a potential role for NTS POMC neurons in regulation of energy balance. Combined, these data open the possibility that POMC neurons in the NTS, like POMC neurons in the ARC, might be leptin responsive and play a role in leptin's effect on energy balance.

The critical role of the central melanocortin system in body weight regulation is evident from pharmacological experiments and from studies of mutations in the *pomc* and the *MC4R* genes, both leading to severe obesity in rodents and humans (31–36). Evidence supporting the regulation and importance of the melanocortin system in leptin's action arises from findings showing that *ob/ob* mice that lack functional leptin, or *db/db* mice that lack *ObRb*, have reduced *pomc* mRNA (14,15), have lower α -MSH peptide levels in the hypothalamus (36), and are hyperphagic and morbidly obese. In addition, fasting of normal mice and rats lead to a reduction in circulating leptin concentrations that is accompanied by a fall in ARC POMC mRNA. This change in POMC mRNA can be prevented by administration of recombinant leptin during the fasting period (14,15). Further support for a role of the melanocortin pathway in leptin action comes from data showing that intracerebroventricular injection of melanocortin receptor antagonists inhibits the effect of leptin to reduce food intake (37,38). Finally, genetic deletion of leptin receptors specifically in POMC neurons leads to increased body weight and reduced hypothalamic POMC mRNA in mice (39). However, none of these studies provide direct evidence that leptin acts solely via *ObRb* on POMC neurons in the ARC of the hypothalamus or whether leptin signaling in POMC neurons in the NTS may also contribute to the regulation of energy balance.

Here, we provide evidence that leptin does not stimulate STAT3 phosphorylation or induce *c-Fos* protein expression in POMC neurons in the NTS of mice. In addition, we show that leptin does not regulate POMC mRNA in the hindbrain as it does in POMC neurons in the hypothalamus. We conclude that POMC neurons in the NTS and in the ARC are differentially regulated by leptin, and that leptin action through POMC-derived peptides in the central nervous system may be mediated entirely via direct effects on POMC neurons in the hypothalamus.

RESEARCH DESIGN AND METHODS

Recombinant mouse leptin was purchased from Dr. E. Parlow (National Institute of Diabetes and Digestive and Kidney Diseases, Torrance, CA). Supplies for immunohistochemistry (IHC) were purchased from Sigma (St. Louis, MO), and the ABC Vectastain Elite kit was from Vector Laboratories (Burlingame, CA). The phospho-specific-(Y705)-STAT3 rabbit antibody was obtained from New England Biolabs (Beverly, MA), the *c-fos* (Ab-2) antibody was from Oncogene Research Products (San Diego, CA), the anti-EGFP antibody was from Abcam (Cambridge, MA), and the donkey anti-rabbit antibody was from Jackson Immuno Research Laboratories (West Grove, PA). Fluorescent donkey anti-goat immunoglobulin conjugates were from Molecular Probes (Eugene, OR) and donkey serum from Invitrogen Life Technologies (Carlsbad, CA).

Male POMC-CRE mice were produced in Dr. Lowell's lab as described earlier (39), and Z/EG-EGFP reporter mice (40) were purchased from Jackson Laboratories (Bar Harbor, ME). POMC-CRE and Z/EG-EGFP mice were crossed to produce POMC-EGFP mice as reported earlier (39) and were studied at 5–6 weeks of age. Wild-type male C57BL6 mice, 5–6 weeks of age, were purchased from Charles River Laboratories (Wilmington, MA). Animals

were housed in a 14/10-h light/dark cycle with ad libitum access to tap water and standard diet, unless otherwise described. The animal procedures were used in accordance with the guidelines and approval of the Harvard Medical School and Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committees.

Leptin stimulation and immunohistochemistry. After 24 h of fasting, POMC-EGFP mice were intraperitoneally injected with leptin (4.0 mg/kg body wt) or vehicle (PBS) and anesthetized 90 min later with ketamine (100 mg/kg body wt) and xylazine (10 mg/kg body wt). Transcardiac perfusion with formalin, removal of the brain, postfixation, and cryoprotection were performed as described earlier (12). Brains were cut in 25- μ m coronal sections, collected in five series, and stored at -20°C until further use. Double P-STAT3 and EGFP IHC was performed as described below. P-STAT3 IHC was done first and as described earlier (12). In brief, free-floating tissue sections were incubated with the P-STAT3 antibody (1:4,000). Sections were incubated with biotinylated anti-rabbit antibody (1:1,000), followed by avidin-biotin–complex labeling, and developed with nickel-diaminobenzidine, generating a brown-black precipitate. Consecutively, fluorescent IHC for EGFP was performed by incubating sections with the anti-EGFP antibody (1:1,000). On the next day, sections were incubated with a fluorescent-labeled secondary antibody generating green fluorescence. For *c-fos* and EGFP double IHC, *c-fos* staining was performed first by incubating sections with the rabbit anti-*c-fos* antibody (1:10,000). Sections were then incubated with biotinylated anti-rabbit antibody (1:1,000), followed by avidin-biotin–complex labeling. Signals were developed with nickel-diaminobenzidine. Fluorescence staining for EGFP was then performed as described above. Results were visualized using either fluorescent light (EGFP) or bright-field light (P-STAT3 and *c-fos*) and captured with a digital camera (AxioCam; Carl Zeiss, Thornwood, NY) mounted on a Zeiss microscope (Axioscope2; Carl Zeiss). Adobe Photoshop software (Adobe, San Jose, CA) was used to merge fluorescence and bright-field photographs via RGB channels to visualize double-labeled cells (41).

Cell counting. One of the five brain series from each animal was subjected to double IHC as described above. Sections were organized in a rostral-to-caudal manner according to the mouse brain atlas (42). All sections in the series were then examined by dark field and fluorescent microscopy to identify single- and double-labeled cells. All brain sections from the hindbrain that contained positive cells were analyzed. Cell counts were obtained from each hemisphere in each section. To obtain estimates for cell numbers in the entire brain region, results from one series were multiplied with five.

Microdissection and real-time PCR. C57BL6 mice were fed ad libitum and treated with vehicle (PBS, intraperitoneally, two times daily) or deprived of food but not water and treated with PBS (50 μ l i.p., two times daily) or deprived of food but not water and treated with leptin (50 μ g in 50 μ l i.p., two times daily). After 48 h, mice were deeply anesthetized (100 mg/kg ketamine and 10 mg/kg xylazine), brains were removed, and 1-mm sagittal sections were cut using a cooled mouse brain matrix (ASI Instruments, Houston, TX). Using landmarks such as the fornix, optic tracts, and the mammillary nucleus, ARC-enriched tissue was obtained using a scalpel under a magnifying glass as described earlier (43). The ARC tissues were snap frozen in liquid nitrogen and stored at -80°C until further use. Tissue from the caudal brainstem that included the entire NTS was dissected from each of the same two 1-mm sagittal sections from the midline of the brain described above. Tissue squares were isolated using the following anatomical parameters: anterior (rostral) margin (bregma ~ -6.5), posterior (caudal) margin (bregma ~ -9.0 , total length 2.5 mm), dorsal margin (dorsal surface of caudal brainstem), and ventral margin (~ 0.7 mm ventral to the dorsal surface of caudal brainstem). The two tissue pieces from the caudal brainstem were combined and frozen in liquid nitrogen and stored at -80°C until further use. Total RNA was isolated from the tissue blocks using RNA STAT60 (Tel-Test, Friedenswood, TX). Five hundred nanograms of total RNA was used for reverse transcription (RT-PCR kit; Clontech, Palo Alto, CA). Quantification of POMC mRNA was carried out by real-time PCR using the Stratagene Mx3000P system. Real-time PCR was performed in a 96-well plate according to the manufacturer's instructions with minor alterations. The primers (Invitrogen, Carlsbad, CA) and probes (Biosearch Technologies, Novato, CA) were designed with the assistance of PrimerExpress software as follows: mPOMCF (5'-ACCTCACCACGGAG AGCA-3'), mPOMCR (5'-GCGAGAGGTCGAGTTTGC-3'), and mPOMCP [5'-6-carboxy-fluorescein (Fam)-TGCTGGCTTCATCCGGG-BHQ-1-3']. PCRs were run in a volume of 25.0 μ l using 1.0 μ l cDNA. A standard curve was generated from duplicate measurements of serial dilutions of arcuate cDNA.

Statistical analyses. All results are given as the means \pm SE, unless otherwise specified. Probabilities of chance differences between groups were calculated by one-way ANOVA (StatView version 5.0.1; SAS Institute, Cary, NC). Means were then compared by post hoc analyses using Fisher's PLSD test.

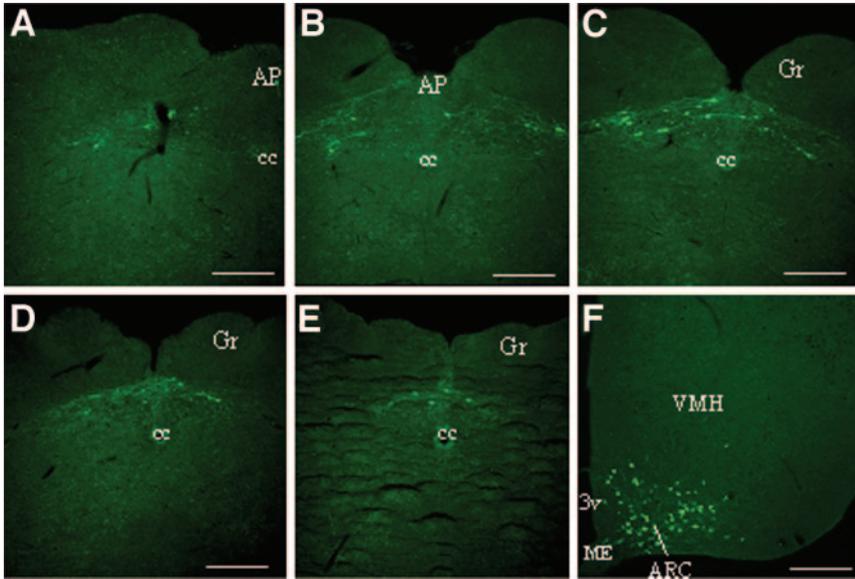


FIG. 1. EGFP neurons are located in the ARC of the hypothalamus and in the NTS of the caudal brainstem of POMC-EGFP reporter mice. Coronal brain sections from the NTS and from the arcuate from POMC-EGFP mice were subjected to IHC for EGFP. Shown are representative microphotographs of EGFP fluorescence-IHC (green) in the NTS and the ARC. *A–E*: Shown are all sections in one series from the NTS of one mouse that contained EGFP-positive neurons (ordered rostrally to caudally). *F*: Shown is a representative microphotograph of EGFP IHC in the ARC of the hypothalamus. Scale bars, 200 μ m. 3v, third ventricle; AP, area postrema; cc, central canal; Gr, gracile nucleus; ME, median eminence; VMH, ventromedial hypothalamus.

RESULTS

While POMC neurons in the arcuate nucleus of the hypothalamus of mice and rats can easily be detected by IHC procedures using antibodies against POMC-derived peptides such as α -MSH or β -endorphin or by *in situ* hybridization methods for POMC mRNA, POMC neurons in the NTS are notoriously difficult to detect by either method due to low POMC expression in individual cells. Our attempts to directly detect α -MSH or β -endorphin immunoreactive cell bodies in the NTS were unsuccessful in both mice and colchicine-treated rats. Yet, POMC neurons were readily detected in the hypothalamus of the same animals using the same antisera. In POMC-EGFP reporter mice, EGFP expression has been demonstrated to colocalize with hypothalamic neurons positive for POMC-derived peptides (16,39). Therefore, to study potential leptin signaling in POMC neurons in the NTS, we took advantage of POMC-EGFP transgenic mice that express high levels of EGFP in the NTS (39).

Since a detailed anatomical description of POMC neurons in the caudal brainstem of the mouse has not previously been reported, we first performed EGFP IHC on sections of the caudal brainstem from POMC-EGFP mice that were ordered in a rostral-to-caudal manner. Figure 1*A–E* shows all sections that contain EGFP-positive neurons in one series from one representative mouse, as demonstrated by fluorescent EGFP IHC. The highest density of EGFP-positive cells is found immediately caudal to the area postrema in the dorsomedial subregions of the NTS (Fig. 1*C*), according to the nomenclature by Herbert et al. (44). Fewer and scattered cells are also detected in the dorsomedial and medial parts of the NTS at the caudal level of the area postrema (Fig. 1*A* and *B*) and in the commissural subnucleus on the NTS (Fig. 1*D* and *E*). No EGFP-positive cells were found rostral or caudal to the regions shown (bregma -7.60 to -8.11 ; Fig. 1*A–E*) (42). On average, 4–5 sections from each series exhibited neurons that were positive for EGFP, and the highest number of positive cells in a single section was 19. In four animals, we counted an average of 38 ± 6 cells in each 1:5 series, which yields an estimated 190 ± 28 POMC-EGFP cells in the entire NTS of the mouse. This localization pattern of POMC neurons and the number of cells per section is similar to that reported earlier using brain

sections from colchicine-treated rats (23–26). As expected, the localization of EGFP cells in the ARC of POMC-EGFP mice also matches the known localization of POMC neurons in the hypothalamus (Fig. 1*F*) (16,39).

We have previously shown the presence of leptin receptor mRNA and leptin-inducible STAT3 tyrosine phosphorylation in the NTS of rats (12,21). These data, as well as the localization of POMC/EGFP neurons in the NTS as shown above, open the possibility of direct leptin signaling in NTS POMC neurons. To address this, we first investigated whether peripheral administration of leptin to POMC-EGFP mice would stimulate STAT3 phosphorylation, a marker of direct leptin action (45), in POMC NTS neurons using double P-STAT3/EGFP IHC. As shown in Fig. 2, leptin readily induced P-STAT3 in the NTS as we have reported earlier in both mice and rats (12,43). No P-STAT3-positive cells were detected in the NTS of PBS-treated mice. P-STAT3-positive cells in leptin-treated animals were almost exclusively localized in the medial subnucleus of the NTS immediately dorsal to the dorsal motor nucleus of the vagus nerve at the level of the area postrema. However, none of the EGFP-positive cells responded to leptin with regard to STAT3 phosphorylation. We showed this in mice treated with leptin for either 60 or 90 min (not shown). Furthermore, we did not find evidence of leptin-inducible c-Fos in NTS POMC neurons by double c-Fos/EGFP IHC in sections from the same mice (Fig. 3). This was also demonstrated in mice treated with leptin for either 60 or 90 min (not shown). The lack of P-STAT3/EGFP or c-Fos/EGFP double-labeled cells was shown in a total of four leptin-treated POMC-EGFP mice. These results are in direct contrast to studies of POMC neurons in the arcuate nucleus of the hypothalamus, where leptin readily induces STAT3 phosphorylation and c-Fos protein expression (12,19) (Fig. 4). In the arcuate nucleus of wild-type C57BL/6 mice, we counted a total of $3,167 \pm 440$ ($n = 3$ animals) POMC neurons using β -endorphin IHC. This number is similar to the $\sim 3,000$ EGFP neurons reported by Cowley et al. (16) in a different strain of POMC-GFP mice. Consistent with these numbers of cells, we counted 3,650 POMC (β -endorphin IHC) neurons in our POMC-EGFP mice ($n = 1$). Furthermore, leptin stimulated STAT3 phosphorylation in $60 \pm 5\%$ of ARC

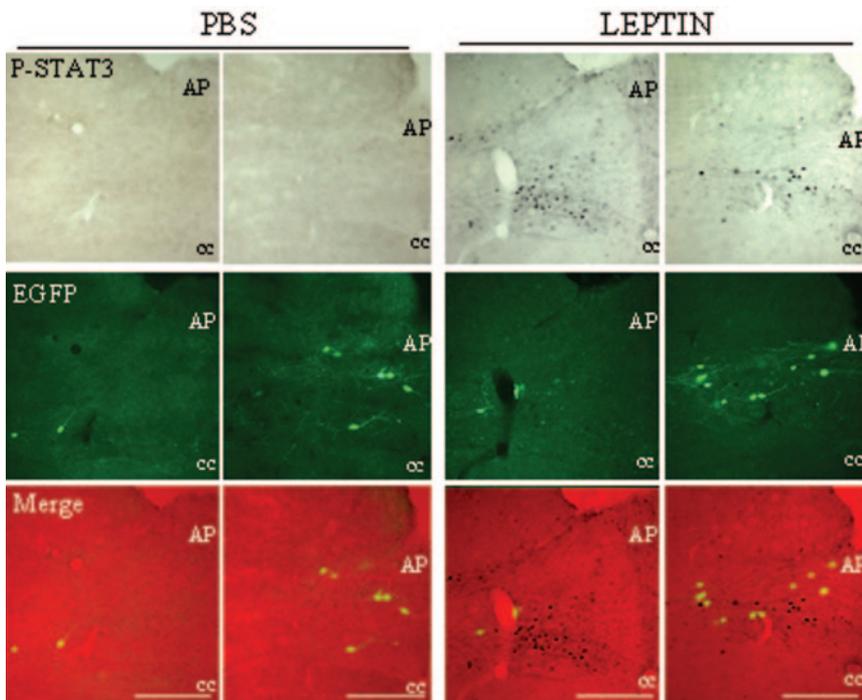


FIG. 2. Leptin does not induce P-STAT3 in POMC/EGFP neurons in the NTS. POMC-EGFP mice were intraperitoneally injected with leptin (*right two columns*) or PBS (*left two columns*). Ninety minutes later, brains were taken and subjected to IHC for P-STAT3 (*top row*) as shown by nickel-diaminobenzidine staining (black-nuclear). The same sections were subjected to fluorescence-IHC (green-cytoplasmic) for EGFP (*middle row*) and merged (*bottom row*) using false colors as described in RESEARCH DESIGN AND METHODS. Shown are representative microphotographs of double IHC for P-STAT3 and EGFP from one PBS- and one leptin-treated mouse, where the *1st* and *3rd columns* show sections that are rostral to the sections shown in *columns 2 and 4*. Scale bars indicate 200 μ m. AP, area postrema; cc, central canal.

POMC neurons ($n = 3$ C57BL6 mice), as determined by double P-STAT3/ β -endorphin IHC. Analysis of one leptin-treated POMC-EGFP mouse yielded a similar percentage by applying double EGFP/P-STAT3 IHC. Combined, these data suggest that leptin does not activate intracellular signaling in POMC neurons in the NTS, in direct contrast to the majority of POMC cells in the ARC nucleus of the hypothalamus.

To further support the data suggesting that POMC neurons in the NTS are not directly responsive to leptin unlike POMC neurons in the ARC, we isolated ARC and NTS tissues by microdissection of brains from three groups of C57BL6 mice. The three groups were fed ad libitum, fasted for 48 h, or fasted for 48 h and injected

twice daily with recombinant leptin during the fasting period. The fed and fasted groups were injected with vehicle (PBS). Messenger RNA was purified, reverse transcribed into cDNA, and subjected to real-time PCR for measurement of POMC mRNA. As shown in Fig. 5, fasting for 48 h results in a fall in ARC POMC mRNA. This decrease in POMC mRNA was prevented by administration of leptin, as has been reported earlier (14,15). Interestingly, the level of POMC mRNA in the NTS also decreased after fasting, but in contrast to the ARC, the change in NTS POMC mRNA after fasting was not prevented by leptin. Taken together, this evidence supports the above data suggesting that POMC neurons in the NTS are not regulated by leptin.

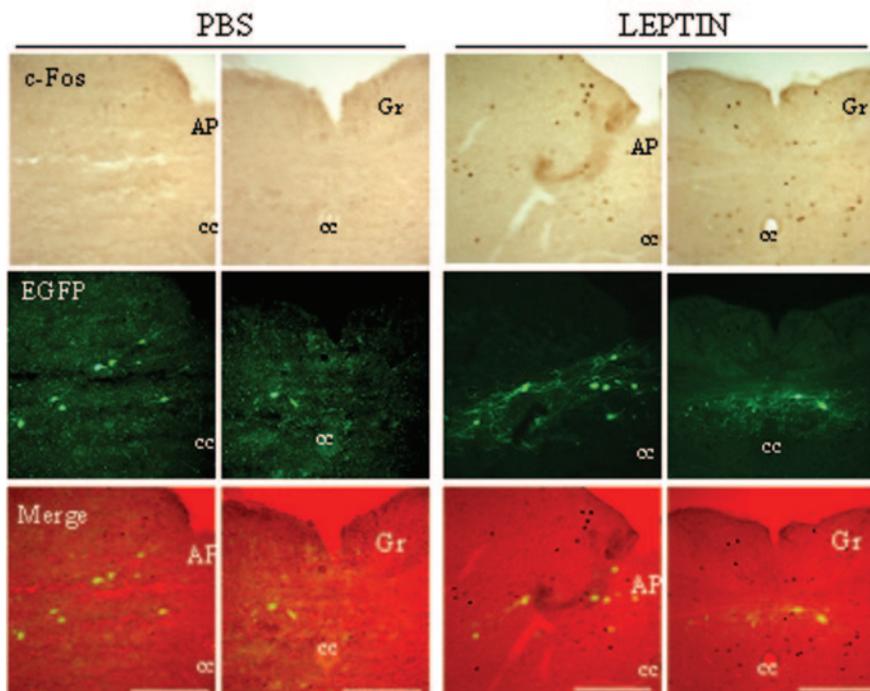


FIG. 3. Leptin does not induce c-Fos in POMC/EGFP neurons in the NTS. POMC-EGFP mice were treated with PBS or leptin as in Fig. 2. Shown is double IHC for c-Fos (brown-nuclear) and fluorescence-IHC (green-cytoplasmic) for EGFP. Representative microphotographs from the NTS are ordered as described under Fig. 2. Scale bars equal 200 μ m. AP, area postrema; cc, central canal; Gr, gracile nucleus.

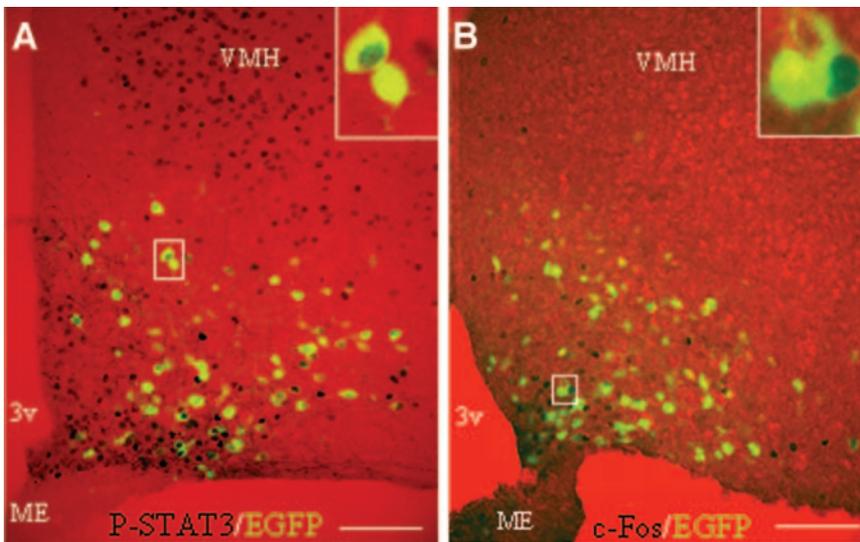


FIG. 4. Leptin induces P-STAT3 and c-Fos in POMC/EGFP neurons in the ARC. POMC-EGFP mice were treated with leptin for 90 min and coronal hypothalamic sections obtained. **A:** Shown is a representative microphotograph of double IHC for P-STAT3 (black-nuclear) and fluorescence-IHC for EGFP (green-cytoplasmic). **B:** Shown is a representative microphotograph of double IHC for c-Fos (black-nuclear) and fluorescence-IHC for EGFP (green-cytoplasmic). High magnification of areas marked in **A** and **B** is shown in *inserts*, depicting a dual-labeled P-STAT3 and EGFP cell and a dual-labeled c-Fos and EGFP cell, respectively. A single-labeled EGFP-positive cell is also shown in each image. Scale bars equal 100 μm . 3v, third ventricle; ME, median eminence; VMH, ventromedial hypothalamus.

DISCUSSION

The hormone leptin signals partly through POMC neurons to influence energy balance. This is supported by several studies, including those showing that hypothalamic POMC neurons express functional leptin receptors and respond to leptin to regulate intracellular signaling and neuronal activity. In addition, genetic deletion of leptin receptors selectively in POMC neurons leads to modest obesity (39). A specific role of the melanocortin system in leptin action is supported by experiments demonstrating that intracerebroventricular administration of pharmacological inhibitors of the central MC3/4R attenuates leptin-induced reduction in food intake (37,38). However, these studies do not provide evidence on whether the effects of leptin

through POMC neurons in the central nervous system to regulate energy balance are mediated entirely via leptin-signaling in POMC cells located in the hypothalamus or whether leptin also acts on POMC-producing neurons located in the caudal brainstem. Here, we provide evidence suggesting that POMC neurons in the nucleus of the tractus solitarius of the caudal brainstem do not respond to leptin in mice, in contrast to the well-documented direct action on POMC neurons in the hypothalamus (45). Combined, these data imply that leptin action through POMC-derived peptides in the central nervous system is exclusively mediated via direct leptin-receptor signaling in POMC neurons located in the hypothalamus and not through POMC cells in the hindbrain.

Our data show that leptin rapidly stimulates cellular STAT3 phosphorylation, a marker of neurons that respond directly to leptin (45), in cells within the NTS. These leptin-responsive cells are located in the medial subnucleus of NTS at the rostral-caudal level of the area postrema. In contrast, the majority of POMC neurons, as measured by expression of EGFP, are concentrated caudal to the area postrema in the dorsomedial- and commissural-subnuclei of the NTS. This expression pattern of POMC neurons is consistent with earlier reports (23–26) showing the location of POMC immunoreactive cells in the NTS of colchicine-treated rats. In POMC-EGFP reporter mice, we counted a total of ~200 EGFP neurons in the entire NTS, a number similar to the ~300 GFP cells reported in the NTS of a different strain of POMC-GFP mice (46). Furthermore, in POMC-EGFP mice and in genetically unmodified mice, we found ~3,100–3,600 POMC neurons (EGFP or β -endorphin immunoreactive cells) in the hypothalamus, a range that is consistent with the ~3,000 GFP-positive cells reported in another line of POMC-GFP mice (16). This number of POMC neurons in the hypothalamus of the mouse is somewhat lower compared with the ~5,600 POMC neurons detected in the hypothalamus of the rat (12).

Consistent with the data showing lack of leptin-inducible STAT3 phosphorylation in POMC neurons in the NTS of POMC-EGFP mice, we did not find any EGFP-positive cells that expressed c-Fos proteins after leptin administration. Both results are in contrast to analyses of POMC neurons in the hypothalamus demonstrating that leptin can induce P-STAT3 and c-Fos in those cells (12,13).

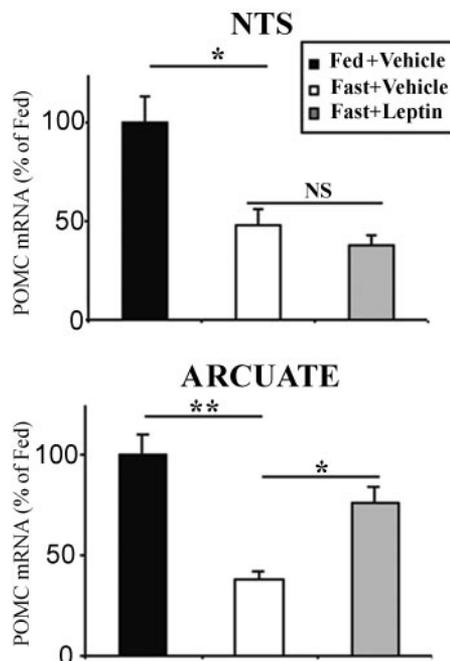


FIG. 5. POMC mRNA is not regulated by leptin in the NTS. C57BL6 mice were fed ad libitum or fasted (48 h) and treated with vehicle (PBS) or fasted (48 h) and treated with leptin (50 μg i.p., two times daily). Arcuate and caudal brainstem tissues were isolated as described in RESEARCH DESIGN AND METHODS. Shown are real-time RT-PCR results for POMC mRNA. Data are means \pm SEM. $n = 10$ mice in each group. NS, not significant; NTS, nucleus tractus solitarius. ** $P < 0.0001$; * $P < 0.005$ (RESEARCH DESIGN AND METHODS).

Moreover, while leptin can stimulate POMC mRNA levels in the hypothalamus of fasted mice, this is not observed in the hindbrain of the same mice. Altogether, our data suggest that POMC neurons in the NTS are not regulated by leptin like POMC cells in the hypothalamus. Consistent with this, *ObRb* mRNA does not appear to colocalize with EGFP-positive neurons in the NTS of the POMC-EGFP mice (J. Lachey, J.K. Elmquist, personal communication).

Interestingly, POMC mRNA levels in the NTS like in the arcuate are reduced in response to fasting. Although NTS POMC mRNA is not affected by leptin under the same experimental paradigm in which ARC POMC mRNA is stimulated by leptin, this result does imply some role of hindbrain POMC neurons in the regulation of energy balance in response to fasting. Since leptin is unlikely to be the factor responsible for the reduction of NTS POMC mRNA during fasting, further studies are needed to explore the mechanism underlying this mRNA regulation. In addition to leptin, POMC neurons in the hypothalamus respond to insulin, glucose, and glucocorticoids (47–49), all factors that change in response to food deprivation. It is therefore possible that the fall in NTS POMC mRNA during fasting could be mediated by one or more of these factors. Finally, since the NTS is the major termination site of vagal sensory input from the gastrointestinal system (50), it is also possible that visceral afferent signals could mediate signaling in NTS POMC neurons, leading to changes in POMC mRNA in response to fasting. Indeed, it has been shown that cholecystokinin (CCK), a gastrointestinal peptide, when given peripherally, induces *c-Fos* expression in POMC neurons in the NTS, an effect likely mediated via the vagus nerve (51,52). Furthermore, inhibition of food intake by CCK is enhanced by leptin (and by insulin) (53). Moreover, CCK-induced satiety (54) is attenuated by pharmacological inhibition of the central MC3/4 receptors, altogether supporting a possible functional connection between leptin, NTS POMC neurons, the melanocortin system, and CCK. Our data, which suggest a lack of direct action of leptin on POMC neurons in the NTS, imply that this leptin-CCK interaction is indirect. This may involve neuronal connections between different subnuclei within the NTS and/or with hypothalamic circuitries. The mechanisms and physiological importance of these interactions as well as a potential role of NTS POMC-derived β -endorphin peptides in response to fasting warrant further studies.

We have previously reported leptin-dependent stimulation of STAT3 phosphorylation in ~1,000 neurons in the NTS of mice (43), supporting the notion that functional leptin receptors are expressed in this region of the hindbrain. This is also consistent with our previous detection of *ObRb* mRNA in the NTS (21) and with earlier data showing leptin-inducible *c-Fos* in the NTS of rats (19), although the latter could be mediated via indirect signaling mechanisms. Indeed, the majority of leptin-inducible *c-Fos* immunoreactive cells in the mouse appear to be located caudal to the population of P-STAT3-positive cells. The low level of *c-Fos* in the medial NTS where the P-STAT3 cells are concentrated suggests that most of the P-STAT3-positive neurons are inhibited by leptin, although further studies are required to demonstrate this directly. Since our data show that the leptin-responsive (P-STAT3) cells in the NTS are not POMC neurons, it forces the question of what is the neurochemical identity of these cells? Leptin receptor mRNA has been reported to colocalize with proglucagon-producing neurons in the NTS of mice (55).

Supporting regulation of hindbrain proglucagon cells by leptin, we have found by real-time PCR that, in contrast to the lack of an effect of leptin on POMC mRNA levels in the NTS, leptin can prevent a fall of proglucagon mRNA in response to fasting (data not shown). Combined, these data suggest that at least a subset of the leptin-responsive cells in the NTS may be proglucagon-producing neurons. Future studies will determine whether these cells represent a significant proportion of leptin responsive cells in the NTS and also elucidate their potential role in leptin action.

In conclusion, we have shown that POMC neurons in the NTS of mice do not respond directly to leptin, in contrast to POMC neurons in the arcuate nucleus of the hypothalamus. This suggests that leptin action in the central nervous system via POMC-derived peptides is mediated exclusively through leptin receptor signaling in POMC neurons located in the hypothalamus. At the same time, we demonstrate that *POMC* gene expression in the NTS is effected by energy status. This finding raises a question for future work: what factors other than leptin mediate the effect of food deprivation on hindbrain POMC neurons? In addition, studies are needed to identify the neurochemical identity of the leptin responsive cells in the medial NTS and whether these cells contribute to regulation of energy balance.

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REFERENCES

- Ahima RS, Saper CB, Flier JS, Elmquist JK: Leptin regulation of neuroendocrine systems. *Front Neuroendocrinol* 21:263–307, 2000
- Kamohara S, Burcelin R, Halaas JL, Friedman JM, Charron MJ: Acute stimulation of glucose metabolism in mice by leptin treatment. *Nature* 389:374–377, 1997
- Elmquist JK, Bjørbaek C, Ahima RS, Flier JS, Saper CB: Distributions of leptin receptor mRNA isoforms in the rat brain. *J Comp Neurol* 395:535–547, 1998
- Cheung CC, Clifton DK, Steiner RA: Proopiomelanocortin neurons are direct targets for leptin in the hypothalamus. *Endocrinology* 138:4489–4492, 1997
- Schwartz MW, Woods SC, Porte D Jr, Seeley RJ, Baskin DG: Central nervous system control of food intake. *Nature* 404:661–671, 2000
- Castro MG, Morrison E: Post-translational processing of proopiomelanocortin in the pituitary and in the brain. *Crit Rev Neurobiol* 11:35–57, 1997
- Cone RD: The central melanocortin system and energy homeostasis. *Trends Endocrinol Metab* 10:211–216, 1999
- Ollmann MM, Wilson BD, Yang YK, Kerns JA, Chen Y, Gantz I, Barsh GS: Antagonism of central melanocortin receptors in vitro and in vivo by agouti-related protein. *Science* 278:135–138, 1997
- Baskin DG, Hahn TM, Schwartz MW: Leptin sensitive neurons in the hypothalamus. *Horm Metab Res* 31:345–350, 1999
- Low MJ: Role of proopiomelanocortin neurons and peptides in the regulation of energy homeostasis. *J Endocrinol Invest* 27:95–100, 2004
- Appleyard SM, Hayward M, Young JI, Butler AA, Cone RD, Rubinstein M, Low MJ: A role for the endogenous opioid beta-endorphin in energy homeostasis. *Endocrinology* 144:1753–1760, 2003
- Munzberg H, Huo L, Nillni EA, Hollenberg AN, Bjørbaek C: Role of signal transducer and activator of transcription 3 in regulation of hypothalamic

- proopiomelanocortin gene expression by leptin. *Endocrinology* 144:2121–2131, 2003
13. Elias CF, Aschkenasi C, Lee C, Kelly J, Ahima RS, Bjørnbæk C, Flier JS, Saper CB, Elmquist JK: Leptin differentially regulates NPY and POMC neurons projecting to the lateral hypothalamic area. *Neuron* 23:775–786, 1999
 14. Schwartz MW, Seeley RJ, Woods SC, Weigle DS, Campfield LA, Burn P, Baskin DG: Leptin increases hypothalamic pro-opiomelanocortin mRNA expression in the rostral arcuate nucleus. *Diabetes* 46:2119–2123, 1997
 15. Mizuno TM, Kleopoulos SP, Bergen HT, Roberts JL, Priest CA, Mobbs CV: Hypothalamic pro-opiomelanocortin mRNA is reduced by fasting and [corrected] in ob/ob and db/db mice, but is stimulated by leptin. *Diabetes* 47:294–297, 1998
 16. Cowley MA, Smart JL, Rubinstein M, Cerdan MG, Diano S, Horvath TL, Cone RD, Low MJ: Leptin activates anorexigenic POMC neurons through a neural network in the arcuate nucleus. *Nature* 411:480–484, 2001
 17. Kim MS, Small CJ, Stanley SA, Morgan DG, Seal LJ, Kong WM, Edwards CM, Abusnana S, Sunter D, Ghatei JL, Bloom SR: The central melanocortin system affects the hypothalamo-pituitary thyroid axis and may mediate the effect of leptin. *J Clin Invest* 105:1005–1011, 2000
 18. Watanobe H, Habu S: Leptin regulates growth hormone-releasing factor, somatostatin, and alpha-melanocyte-stimulating hormone but not neuropeptide Y release in rat hypothalamus in vivo: relation with growth hormone secretion. *J Neurosci* 22:6265–6271, 2002
 19. Elias CF, Kelly JF, Lee CE, Ahima RS, Drucker DJ, Saper CB, Elmquist JK: Chemical characterization of leptin-activated neurons in the rat brain. *J Comp Neurol* 423:261–281, 2000
 20. Hosoi T, Kawagishi T, Okuma Y, Tanaka J, Nomura Y: Brain stem is a direct target for leptin's action in the central nervous system. *Endocrinology* 143:3498–3504, 2002
 21. Grill HJ, Schwartz MW, Kaplan JM, Foxhall JS, Breininger J, Baskin DG: Evidence that the caudal brainstem is a target for the inhibitory effect of leptin on food intake. *Endocrinology* 143:239–246, 2002
 22. Satoh N, Ogawa Y, Katsuura G, Hayase M, Tsuji T, Imagawa K, Yoshimasa Y, Nishi S, Hosoda K, Nakao K: The arcuate nucleus as a primary site of satiety effect of leptin in rats. *Neurosci Lett* 224:149–152, 1997
 23. Joseph SA, Pilcher WH, Bennett-Clarke C: Immunocytochemical localization of ACTH perikarya in nucleus tractus solitarius: evidence for a second opiocortin neuronal system. *Neurosci Lett* 38:221–225, 1983
 24. Schwartzberg DG, Nakane PK: ACTH-related peptide containing neurons within the medulla oblongata of the rat. *Brain Res* 276:351–356, 1983
 25. Yamazoe M, Shiosaka S, Yagura A, Kawai Y, Shibasaki T, Ling N, Tohyama M: The distribution of alpha-melanocyte stimulating hormone (alpha-MSH) in the central nervous system of the rat: an immunohistochemical study. II. Lower brain stem. *Peptides* 5:721–727, 1984
 26. Palkovits M, Eskay RL: Distribution and possible origin of beta-endorphin and ACTH in discrete brainstem nuclei of rats. *Neuropeptides* 9:123–137, 1987
 27. Fodor M, Sluiter A, Frankhuijzen-Sierevogel A, Wiegant VM, Hoogerhout P, De Wildt DJ, Versteeg DH: Distribution of Lys-gamma 2-melanocyte-stimulating hormone- (Lys-gamma 2-MSH)-like immunoreactivity in neuronal elements in the brain and peripheral tissues of the rat. *Brain Res* 731:182–189, 1996
 28. Mountjoy KG, Mortrud MT, Low MJ, Simerly RB, Cone RD: Localization of the melanocortin-4 receptor (MC4R) in neuroendocrine and autonomic control circuits in the brain. *Mol Endocrinol* 8:1298–1308, 1994
 29. Kishi T, Aschkenasi CJ, Lee CE, Mountjoy KG, Saper CB, Elmquist JK: Expression of melanocortin 4 receptor mRNA in the central nervous system of the rat. *J Comp Neurol* 457:213–235, 2003
 30. Williams DL, Kaplan JM, Grill HJ: The role of the dorsal vagal complex and the vagus nerve in feeding effects of melanocortin-3/4 receptor stimulation. *Endocrinology* 141:1332–1337, 2000
 31. Huszar D, Lynch CA, Fairchild-Huntress V, Dunmore JH, Fang Q, Berke-meier LR, Gu W, Kesterson RA, Boston BA, Cone RD, Smith FJ, Campfield LA, Burn P, Lee F: Targeted disruption of the melanocortin-4 receptor results in obesity in mice. *Cell* 88:131–141, 1997
 32. Vaisse C, Clement K, Guy-Grand B, Froguel P: A frameshift mutation in human MC4R is associated with a dominant form of obesity. *Nat Genet* 20:113–114, 1998
 33. Yeo GS, Farooqi IS, Aminian S, Halsall DJ, Stanhope RG, O'Rahilly S: A frameshift mutation in MC4R associated with dominantly inherited human obesity. *Nat Genet* 20:111–112, 1998
 34. Krude H, Biebermann H, Luck W, Horn R, Brabant G, Gruters A: Severe early-onset obesity, adrenal insufficiency and red hair pigmentation caused by POMC mutations in humans. *Nat Genet* 19:155–157, 1998
 35. Yaswen L, Diehl N, Brennan MB, Hochgeschwender U: Obesity in the mouse model of pro-opiomelanocortin deficiency responds to peripheral melanocortin. *Nat Med* 5:1066–1070, 1999
 36. Guo L, Munzberg H, Stuart RC, Nillni EA, Bjørnbæk C: N-acetylation of hypothalamic alpha-melanocyte-stimulating hormone and regulation by leptin. *Proc Natl Acad Sci U S A* 101:11797–11802, 2004
 37. Seeley RJ, Yagaloff KA, Fisher SL, Burn P, Thiele TE, van Dijk G, Baskin DG, Schwartz MW: Melanocortin receptors in leptin effects (Letter). *Nature* 390:349, 1997
 38. Kask A, Rago L, Wikberg JE, Schioth HB: Evidence for involvement of the melanocortin MC4 receptor in the effects of leptin on food intake and body weight. *Eur J Pharmacol* 360:15–19, 1998
 39. Balthasar N, Coppari R, McMinn J, Liu SM, Lee CE, Tang V, Kenny CD, McGovern RA, Chua SC Jr, Elmquist JK, Lowell BB: Leptin receptor signaling in POMC neurons is required for normal body weight homeostasis. *Neuron* 42:983–991, 2004
 40. Novak A, Guo C, Yang W, Nagy A, Lobe CG: Z/EG, a double reporter mouse line that expresses enhanced green fluorescent protein upon Cre-mediated excision. *Genesis* 28:147–155, 2000
 41. Huo L, Munzberg H, Nillni EA, Bjørnbæk C: Role of signal transducer and activator of transcription 3 in regulation of hypothalamic trh gene expression by leptin. *Endocrinology* 145:2516–2523, 2004
 42. Paxinos G, Franklin KBJ: *The Mouse Brain in Stereotaxic Coordinates*. Boston, MA, Elsevier Academic Press, 2004
 43. Munzberg H, Flier JS, Bjørnbæk C: Region-specific leptin resistance within the hypothalamus of diet-induced obese mice. *Endocrinology* 145:4880–4889, 2004
 44. Herbert H, Moga MM, Saper CB: Connections of the parabrachial nucleus with the nucleus of the solitary tract and the medullary reticular formation in the rat. *J Comp Neurol* 293:540–580, 1990
 45. Bjørnbæk C, Kahn BB: Leptin signaling in the central nervous system and the periphery. *Recent Prog Horm Res* 59:305–331, 2004
 46. Cone RD: Anatomy and regulation of the central melanocortin system. *Nat Neurosci* 8:571–578, 2005
 47. Mizuno TM, Makimura H, Silverstein J, Roberts JL, Lopingco T, Mobbs CV: Fasting regulates hypothalamic neuropeptide Y, agouti-related peptide, and proopiomelanocortin in diabetic mice independent of changes in leptin or insulin. *Endocrinology* 140:4551–4557, 1999
 48. Makimura H, Mizuno TM, Isoda F, Beasley J, Silverstein JH, Mobbs CV: Role of glucocorticoids in mediating effects of fasting and diabetes on hypothalamic gene expression. *BMC Physiol* 3:5, 2003
 49. Ibrahim N, Bosch MA, Smart JL, Qiu J, Rubinstein M, Ronnekleiv OK, Low MJ, Kelly MJ: Hypothalamic proopiomelanocortin neurons are glucose responsive and express K(ATP) channels. *Endocrinology* 144:1331–1340, 2003
 50. Blessing WW: *The Lower Brainstem and Bodily Homeostasis*. New York, Oxford University Press, 1997
 51. Fan W, Ellacott KL, Halatchev IG, Takahashi K, Yu P, Cone RD: Cholecystokinin-mediated suppression of feeding involves the brainstem melanocortin system. *Nat Neurosci* 7:335–336, 2004
 52. Appleyard SM, Bailey TW, Doyle MW, Jin YH, Smart JL, Low MJ, Andresen MC: Proopiomelanocortin neurons in nucleus tractus solitarius are activated by visceral afferents: regulation by cholecystokinin and opioids. *J Neurosci* 25:3578–3585, 2005
 53. Baskin DG, Ffiglewicz Lattemann D, Seeley RJ, Woods SC, Porte D Jr, Schwartz MW: Insulin and leptin: dual adiposity signals to the brain for the regulation of food intake and body weight. *Brain Res* 848:114–123, 1999
 54. Gibbs J, Smith GP: Cholecystokinin and satiety in rats and rhesus monkeys. *Am J Clin Nutr* 30:758–761, 1977
 55. Mercer JG, Moar KM, Findlay PA, Hoggard N, Adam CL: Association of leptin receptor (OB-Rb), NPY and GLP-1 gene expression in the ovine and murine brainstem. *Regul Pept* 76:271–278, 1998