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Characterization of Leptin-Responsive Neurons in the Caudal Brainstem

Kate L. J. Ellacott, Ilia G. Halatchev, and Roger D. Cone

Center for the Study of Weight Regulation and Associated Disorders and Vollum Institute, Oregon Health and Science University, Portland, Oregon 97239-3098

The central melanocortin system plays a key role in the regulation of energy homeostasis. Neurons containing the peptide precursor proopiomelanocortin (POMC) are found at two sites in the brain, the arcuate nucleus of the hypothalamus (ARC) and the caudal region of the nucleus of the solitary tract (NTS). ARC POMC neurons, which also express cocaine- and amphetamine-regulated transcript (CART), are known to mediate part of the response to factors regulating energy homeostasis, such as leptin and ghrelin. In contrast, the physiological role(s) of the POMC neurons in the caudal brainstem are not well characterized. However, development of a transgenic mouse expressing green fluorescent protein under the control of the POMC promoter [POMC-enhanced green fluorescent protein (EGFP) mouse] has aided the study of these neurons. Indeed, recent studies have shown significant activation of NTS POMC-EGFP cells by the gut released satiety factor cholecystokinin (CCK). Here we show that peripheral leptin administration induces the expression of phospho-signal transducer and activator of transcription 3 immunoreactivity (pSTAT3-IR), a marker of leptin receptor signaling, in more than 50% of NTS POMC-EGFP neurons. Furthermore, these POMC-EGFP neurons comprise 30% of all pSTAT3-IR cells in the NTS. Additionally, we also show that in contrast to the ARC population, NTS POMC-EGFP neurons do not coexpress CART immunoreactivity. These data suggest that NTS POMC neurons may participate with ARC POMC cells in mediating some of the effects of leptin and thus comprise a novel cell group regulated by both long-term adipostatic signals and satiety factors such as CCK. (*Endocrinology* 147: 3190–3195, 2006)

THE CENTRAL MELANOCORTIN system plays a pivotal role in the regulation of energy homeostasis principally via the action of melanocortin peptides derived from proopiomelanocortin (POMC), and the endogenous antagonist/inverse agonist agouti-related protein (AgRP; 1). Two populations of POMC-immunoreactive neurons exist in the rodent brain, one in the arcuate nucleus of the hypothalamus (ARC) and the other in the caudal portion of the nucleus of the tractus solitarius (NTS) of the hindbrain (2, 3). The POMC neurons of the ARC are also characterized by their expression of the peptide cocaine- and amphetamine-regulated transcript (CART) (4).

ARC POMC neurons mediate a portion of the effects of a number of peripheral hormones involved in the regulation of energy homeostasis including leptin, ghrelin, and insulin (for review see Ref. 5). In the ARC, neuropeptide Y neurons, which coexpress AgRP (6), make synaptic contact with POMC neurons (7), forming a network of neurons capable of producing both orectic and anorectic effects. Leptin, at the level of the ARC neuronal network, acts through leptin receptors present on both populations of neurons (8, 9) to cause a net anorectic effect. The importance of this network in the regulation of energy homeostasis is further confirmed by the findings that ARC POMC and AgRP mRNA levels are regulated by changes in energy balance including fasting (10), diet-induced obesity (11), and exogenous leptin administration (10, 12). Indeed, the basal firing activity of the neuropeptide Y/AgRP neurons is apparently a direct reflection of the metabolic state of the animal (13).

Although the caudal brainstem also contains POMC neurons, their leptin responsiveness has not been reported. Fourth ventricle or direct administration of the synthetic melanocortin agonist, MTII, or the melanocortin 3/4 receptor antagonist, SHU9119, into the dorsal motor nucleus of the vagus modulates food intake in rodents, indicating that melanocortins may act in the hindbrain to regulate energy homeostasis (14–16). However, it is important to note that many of the POMC fibers seen in the hindbrain actually derive from the hypothalamic POMC cell bodies (14, 17, 18). What has been lacking, therefore, for a thorough understanding of the hindbrain melanocortin system is an understanding of the functional role of the NTS POMC cell bodies. One approach to this problem is to characterize the regulatory inputs to the caudal brainstem POMC neurons.

The study of the physiology of POMC neurons has been advanced by the development of a transgenic mouse line expressing enhanced green fluorescent protein (EGFP) under the control of the POMC promoter, the POMC-EGFP mouse (7). This mouse has been especially useful as a putative marker for POMC expression in the caudal brainstem as detection of POMC protein in this region by immunohistochemistry is technically challenging. Recent data have shown that NTS POMC-EGFP neurons are activated by peripheral administration of the known satiety factor, cholecystokinin

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Abbreviations: AgRP, Agouti-related protein; AP, area postrema; ARC, arcuate nucleus of the hypothalamus; CART, cocaine- and amphetamine-regulated transcript; CCK, cholecystokinin; EGFP, enhanced green fluorescent protein; NTS, nucleus of the solitary tract; PBST, PBS containing Triton X-100; POMC, proopiomelanocortin; pSTAT3-IR, phospho-signal transducer and activator of transcription 3 immunoreactivity; TH, tyrosine hydroxylase.

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(CCK) (19, 20), as well as by feeding-induced satiety (20). In the latter study, the satiating actions of CCK were shown to be highly dependent on intact melanocortin 4 receptor signaling, indicating that the central melanocortin system may be involved in mediating a component of the satiety signal (20). Additionally, in an electrophysiological slice preparation, CCK can activate NTS POMC-EGFP neurons both directly and via stimulation of the solitary tract (19), which contains visceral afferents known to express CCK-A receptors (21).

In addition to the hypothalamus, the caudal brainstem has also been implicated in mediating the effects of leptin on energy homeostasis. Leptin receptors are found in the caudal brainstem of rodents (22, 23), and administration of leptin into the fourth ventricle causes a reduction in nocturnal food intake comparable with lateral ventricular administration (23). Additionally, peripheral administration of leptin causes increased expression of phospho-signal transducer and activator of transcription 3 immunoreactivity (pSTAT3-IR), a direct downstream marker of leptin receptor activation, in the caudal brainstem (24, 25). However, the neurochemical identities of the neurons mediating the effects of leptin in the caudal brainstem are not well characterized. In this study we used immunohistochemistry to further characterize neurochemical composition of NTS POMC neurons and examine their responsiveness to peripherally administered leptin.

Materials and Methods

Animals

All animals used in experiments were transgenic C57BL/6J POMC-EGFP (7) or wild-type C57BL/6J mice, 8–12 wk of age (The Jackson Laboratory, Bar Harbor, ME). Animals were housed at 21 ± 2 C with *ad libitum* access to food (Purina rodent diet 5001; Purina Mills, St. Louis, MO) and water. Experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Animal Care and Use Committee of Oregon Health and Science University.

Leptin treatment and tissue fixation

Animals (n = 3–4 per group), *ad libitum* fed, were treated at 1000 h with 0, 1, or 5 mg/kg ip recombinant murine leptin (obtained from Dr. A. F. Parlow, National Hormone and Peptide Program, Torrance, CA) dissolved in sterile saline. Thirty minutes after treatment, mice were deeply anesthetized and underwent tissue fixation via transcardial perfusion with 0.9% saline followed by ice-cold fixative (4% paraformaldehyde in 0.01 M PBS). Brains were postfixed for 2 h in fixative and were then stored overnight in 20% sucrose in PBS as a cryoprotectant before being frozen at −80 C until use. For localization studies, animals were perfused in the absence of leptin treatment.

Antibodies

The following primary and secondary antibody combinations were used: 1:2,000 rabbit anti-CART 55–102 (H-003–62; Phoenix Pharmaceuticals Inc., Belmont, CA) followed by 1:500 donkey antirabbit Alexa 594 or 488 (Molecular Probes Inc., Eugene, OR); 1:5,000 rabbit anti-GFP directly conjugated to Alexa 488 (A-21311; Molecular Probes), and therefore, no secondary detection was required; 1:5,000 mouse antityrosine hydroxylase (TH Mab 318; Chemicon Inc., Temecula, CA) or 1:10,000 mouse anti-TH (Incstar Inc., Stillwater, MN) followed by 1:500 donkey antimouse Alexa 488 (Molecular Probes); 1;3,000 rabbit anti-pSTAT3 (lot 4; Cell Signaling Technology Inc., Beverly, MA) followed by 1:500 donkey antirabbit Alexa 594 (Molecular Probes).

Immunohistochemistry for CART and EGFP

Sections were cut at 30 μm from perfused brains and stored at 4 C, free floating in PBS containing 0.03% sodium azide. Four sets of sections were generated from each hindbrain; thus, each section in a set was approximately 120 μm apart. One set of sections was used to examine CART/EGFP immunoreactivity. After an initial blocking step, 1 h at room temperature in 5% normal donkey serum in PBS containing 0.3% Triton X-100 (PBST), sections were incubated with primary antibody for 24 h at 4 C. All primary antibodies were diluted in 5% normal donkey serum in PBST. After incubation in primary antibody, sections were washed thoroughly in PBS and incubated for 1 h at room temperature with the appropriate secondary antibody, diluted in PBST. After the first primary antibody, the procedure was repeated with another primary/secondary combination for double labeling. Sections were washed thoroughly with PBS between all incubations. At the end of the incubations, the sections were mounted onto gelatin-coated slides, coverslipped using gel-based fluorescence mounting media (Biomeda Corp., Foster City, CA) and viewed under a fluorescence microscope (Axioplan 2; Zeiss Inc., Thornwood, NY). Each secondary antibody was tested in the absence of primary antibody to ensure that there was no cross-reactivity with the tissue.

pSTAT3 Immunohistochemistry

Perfused brains were sectioned at 30 μm through the caudal brainstem and sections were stored, free floating, in PBS containing 0.03% sodium azide at 4 C. Four sets of sections were generated from each hindbrain; thus, each section in a set was approximately 120 μm apart. Immunohistochemistry for pSTAT-3/EGFP, pSTAT-3/TH, or pSTAT-3/CART was carried out on one set of sections per hindbrain. Immunohistochemistry was performed as previously described (25) with slight modifications for immunofluorescence. Briefly, tissue was pretreated with a solution of 1% sodium hydroxide and 1% hydrogen peroxide in water for 20 min at room temperature, followed by 0.3% glycine in PBS for 10 min at room temperature and 0.03% sodium dodecyl sulfate in PBS for 10 min at room temperature. Sections were washed thoroughly with PBS between each step. After the pretreatment steps, sections were incubated for 1 h at room temperature in blocking reagent (5% normal donkey serum in PBST), followed by incubation overnight at 4 C with rabbit anti-pSTAT3 (lot 4; Cell Signaling Technology) diluted 1:3000 in blocking reagent. After incubation in primary antibody, sections were washed thoroughly in PBS and incubated for 1 h at room temperature with donkey antirabbit Alexa 594 (Molecular Probes) diluted 1:500 in PBST.

For double immunohistochemistry in POMC-EGFP animals, the sections were then washed thoroughly with PBS before incubation overnight at 4 C with rabbit anti-GFP directly conjugated to Alexa 488 (Molecular Probes) diluted 1:5000 in blocking reagent. Alternatively, for double immunohistochemistry with CART or TH, sections were incubated with either 1:2000 rabbit anti-CART 55-102 (H-003-62; Phoenix Pharmaceuticals) overnight at 4 C followed by 1:500 donkey antirabbit Alexa 488 (Molecular Probes) for 1 h at room temperature or 1:5,000 mouse anti-TH (Chemicon) or 1:10,000 mouse anti-TH (Incstar Inc., Stillwater, MN) overnight at 4 C followed by 1:500 donkey antimouse Alexa 488 (Molecular Probes) for 1 h at room temperature. After washing, sections were mounted onto gelatin-coated slides, coverslipped using gel-based fluorescence mounting media (Biomeda) and viewed under a fluorescence microscope (Axioplan 2; Zeiss).

Cell counting

For each experiment all pSTAT-3, EGFP, CART, or TH (as appropriate) and double-labeled cells in that set of sections were counted by eye, by two different investigators blinded to the treatments, and results were expressed as the number of cells per section or percentage double labeled, *i.e.* colocalized. Each set of caudal brainstem sections contained five to seven caudal brainstem sections expressing immunopositive cells. In double immunohistochemistry, a cell was determined to be singly labeled when visible only under the fluorescence filter corresponding to the emission wavelength of one of the primary/secondary antibody complexes used, *e.g.* 594 nm and not 488 nm in the case of pSTAT3. When the cell was visible at both 594- and 488-nm filters, it was

deemed to be double labeled. Double-labeled cells were examined at multiple focus levels within the section and at multiple magnifications to ensure that the cell was indeed representative of a single cell labeled with both antibody complexes and not two singly labeled cells in close proximity within different levels of the section. The cells were also examined under a third wavelength not corresponding to the emission wavelength of either of the secondary antibodies to ensure that the immunoreactivity was specific. Each primary antibody used in double immunohistochemistry was also tested by single immunohistochemistry to verify the distribution of the immunoreactivity and ensure that the primary antibodies were not cross-reacting. The secondary antibodies used were also tested in the absence of the primary antibody to ensure that there was no cross-reactivity with the tissue.

Data analysis

Data are expressed as mean ± SE. For parametric data with three or more groups, analysis was carried out using a one-way ANOVA followed by a Dunnett's *post hoc* test.

Results

Peripheral leptin induces pSTAT3 immunoreactivity in NTS POMC-EGFP neurons

In the caudal brainstem of POMC-EGFP mice, EGFP immunoreactivity was seen predominantly in the medial portion of the caudal NTS at the level of the area postrema (AP) (Fig. 1, A–D). However, a small number of cells were seen more rostral or caudal to the level of the area postrema. Additionally, some cells were occasionally seen within the AP itself.

In agreement with the literature (24, 25), peripheral administration of leptin-induced pSTAT3-IR in the caudal NTS (Figs. 1, E–H, and 2: saline, 0 ± 0 cells per section, n = 3; 1 mg/kg, 41 ± 4 cells per section n = 3; 5 mg/kg, 44 ± 8 cells per section, n = 4). The majority of pSTAT3-IR was localized in the medial portion of the caudal NTS at the level of the AP in sections that also contained POMC-EGFP immunoreactivity [compare single immunohistochemistry for POMC-EGFP (Fig. 1, A–D) and pSTAT3-IR (Fig. 1, E–H]; however, some pSTAT3-IR neurons were seen more caudally than POMC-EGFP neurons (Fig. 1E). No pSTAT3-IR was detected in saline-treated animals (Fig. 2B; n = 3, compare with Fig. 2E), and the number of POMC-EGFP cell bodies was not altered by leptin treatment (saline, 19 ± 4 cells/section, n = 3; 1 mg/kg, 20 ± 3 cells/section, n = 3; 5 mg/kg 20 ± 3 cells/section, n = 4). In common with what has been previously shown for the ARC (25), a significant number of POMC-EGFP neurons were found to express pSTAT3-IR in response to leptin administration (Fig. 2, D–I), indicating that they are responsive to peripheral leptin.

Remarkably, over 50% of NTS POMC-EGFP neurons exhibited pSTAT3-IR after leptin treatment (Fig. 2J: 1 mg/kg, 12 ± 2 colocalized cells/section equivalent to 52 ± 3%, n = 3; 5 mg/kg, 11 ± 3 colocalized cells/section equivalent to 56 ± 8%, n = 4). Significantly, of all the pSTAT3-IR seen in the NTS, 30% was found in POMC-EGFP neurons (Fig. 2K: 1 mg/kg, 30 ± 4%, n = 3; 5 mg/kg, 30 ± 6%, n = 4), indicating that the NTS POMC neurons may be responsible for mediating a significant component of the effects of leptin in this brain region.

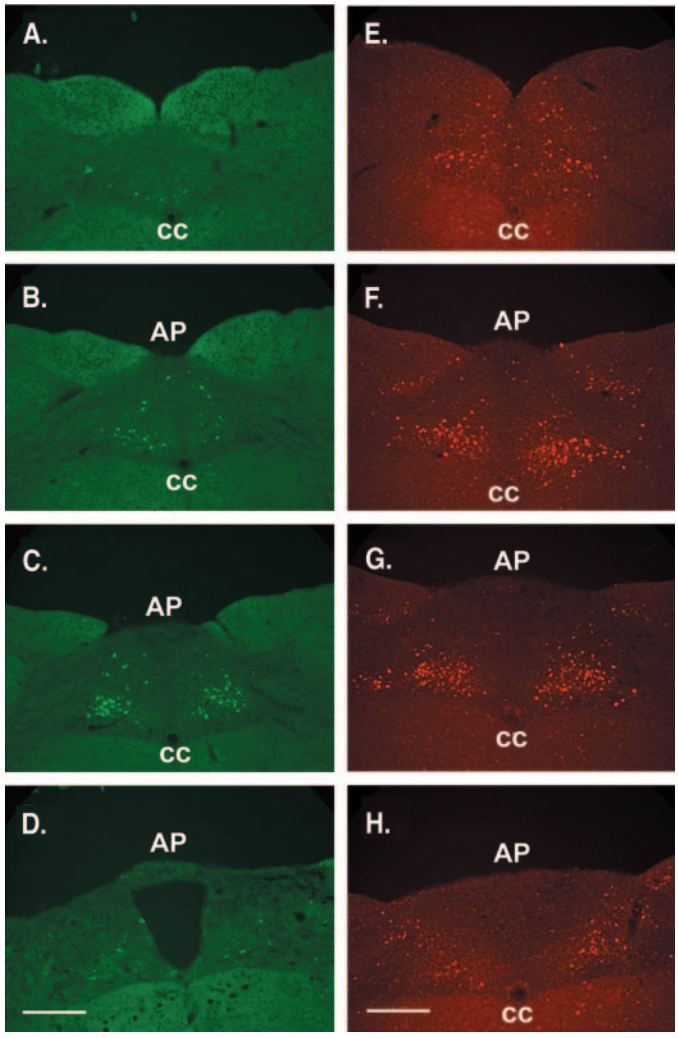

FIG. 1. Comparative distribution of EGFP immunoreactivity in the caudal brainstem of POMC-EGFP mice (A–D, reflecting caudal to rostral progression) and leptin (1 mg/kg ip) induced pSTAT3-IR in the caudal brainstem of wild-type mice (E–H, reflecting caudal to rostral progression). Each antigen was detected by a single immunohistochemistry procedure in different animals. Although as close as possible, anatomical progression shown in A–D is not identical with E–H. *Scale bars*, 160 μm. CC, Central canal; AP, area postrema.

Activation of TH- and CART-positive neurons by leptin

Due to the fact that only 30% of pSTAT3-IR seen after peripheral leptin treatment were found in POMC-EGFP neurons, we performed experiments to more broadly define the neurochemical phenotype of the non-POMC neuronal populations expressing pSTAT3-IR. Early experiments suggest that few TH (Fig. 3, n = 3) or CART (Fig. 4, n = 3) neurons of the caudal brainstem showed expression of pSTAT3 after leptin treatment. Whereas the TH neurons were found close to areas of pSTAT3 expression, few were seen to contain pSTAT3-IR. It has previously been shown that TH and POMC-EGFP are adjacent, but not colocalized, in neurons of the NTS in the POMC-EGFP mouse (19, 20). CART cell bodies were predominantly found more ventrolateral to the pSTAT3-IR.

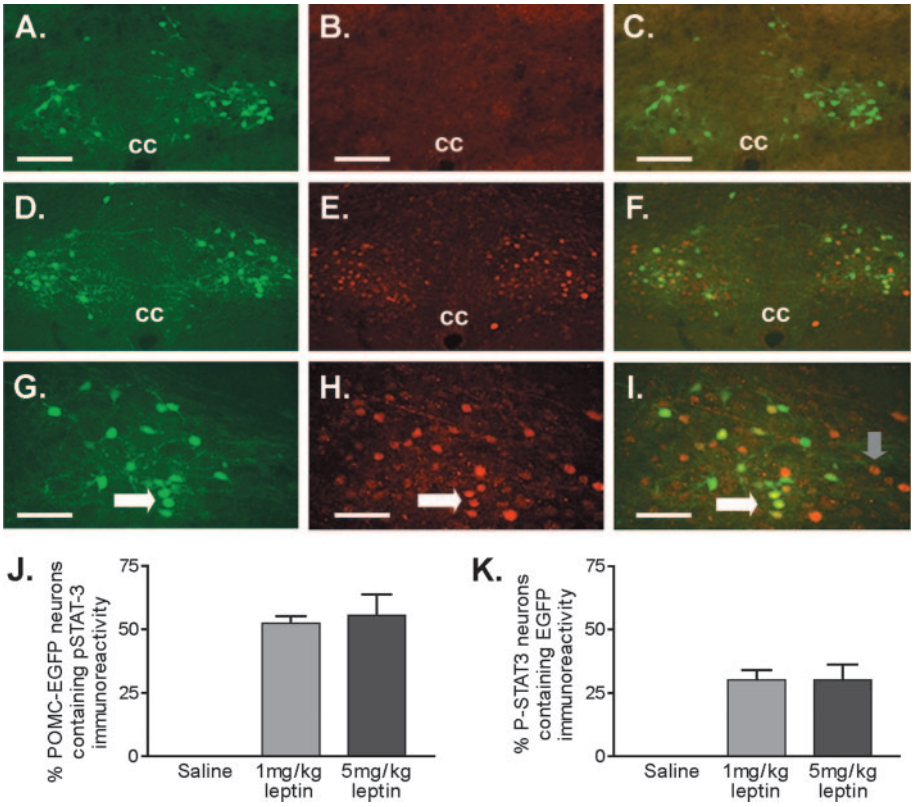

FIG. 2. Double-fluorescence immunohistochemistry for pSTAT3 (B, E, and H) and POMC-EGFP (A, D, and G) in the caudal brainstem. Pictures shown are representative images of the effect seen after treatment with 5 mg/kg leptin ip (n = 4) but also reflect pattern seen after administration of 1 mg/kg leptin ip (n = 3). Peripheral leptin administration results in an up-regulation of pSTAT3-IR (D–F and G–I), compared with saline treatment (A–C). Greater than 50% of all POMC-EGFP neurons are pSTAT3-IR positive (F, I, and J), and approximately 30% of all pSTAT3-IR in the NTS is found in POMC-EGFP positive cells (K). G–I, An enlargement of a portion of panels D–F shown for clarity. *White arrows* indicate examples POMC-EGFP neurons that contain pSTAT3-IR. *Gray arrow* indicates an example of a non-POMC-EGFP neuron that displays pSTAT3-IR. *Scale bars*, 140 μm (A–F) and 70 μm (G–I). CC, Central canal.

Double-fluorescence immunohistochemistry for CART and POMC-EGFP

In the ARC, the anorectic peptide CART shows a high (>90%) level of coexpression with POMC mRNA (4). To address whether this is also the case in the NTS, we performed double-fluorescence immunohistochemistry for CART and GFP in POMC-EGFP mice. Whereas an extensive network of CART-immunoreactive fibers was seen throughout the NTS and AP, CART-immunoreactive cell bodies were seen only in the lateral portion of the nucleus (Fig. 5E). This is in agreement with studies in the rat (26, 27). In contrast to the ARC (Fig. 5, A–C), there was no apparent colocalization of POMC-EGFP and CART cell bodies in the caudal NTS (Fig. 5, D–I, n = 3).

Discussion

ARC POMC neurons have been demonstrated to mediate a portion of the effects of leptin on food intake and body weight (28, 29). In rodents, exogenously administered leptin causes activation of ARC POMC neurons, as measured by the expression of c-Fos (29) and pSTAT3-IR (25). Furthermore, leptin regulates POMC mRNA levels (12) via leptin receptors present on their surface (9). Likewise, the caudal brainstem has also been shown to be capable of mediating an anorectic response to leptin (23). Whereas no specific neuronal population has been implicated in mediating these effects, leptin receptors are expressed in the rodent hindbrain (22, 23). Here we demonstrate that POMC-EGFP neurons of the NTS are unique neurochemically, in that unlike ARC POMC neurons,

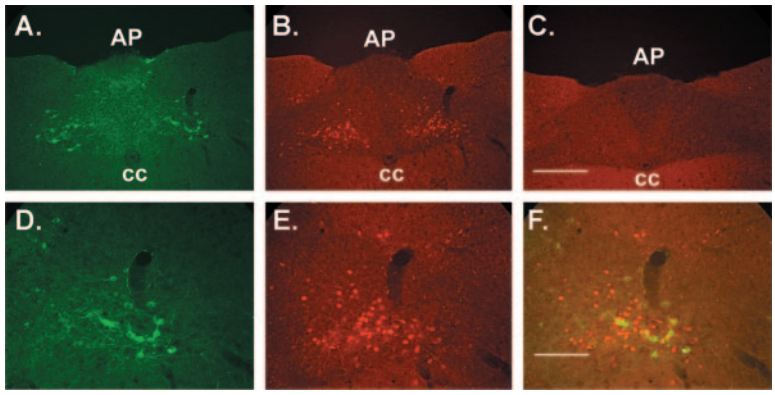

FIG. 3. Double-fluorescence immunohistochemistry for pSTAT3 (B and E) and TH (A and D) in the caudal brainstem after leptin treatment (1 mg/kg ip, n = 3). No pSTAT3-IR was seen after saline administration (C, n = 3), and leptin did not appear to induced pSTAT3 expression in TH neurons (see overlay in F). D–F, An enlargement of a portion of A and B, with F representing an overlay of D and E, shown for clarity. *Scale bar*, 160 μm (A–C); 100 μm (D–F). CC, Central canal; AP, area postrema.

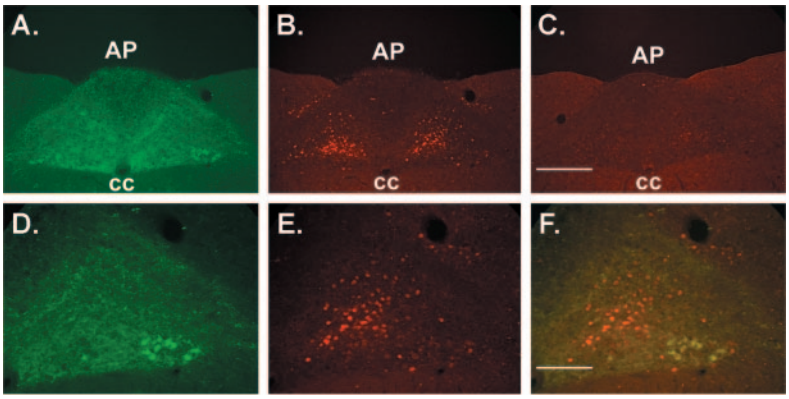

FIG. 4. Double-fluorescence immunohistochemistry for pSTAT3 (B and E) and CART (A and D) in the caudal brainstem after leptin treatment (1 mg/kg ip). No pSTAT3-IR was seen after saline administration (C). Leptin did not appear to induce pSTAT3 expression in CART neurons (see overlay in F). D–F, An enlargement of a portion of A and B, with F representing an overlay of D and E, shown for clarity. *Scale bar*, 160 μm (A–C); 100 μm (D–F). CC, Central canal; AP, area postrema.

they do not coexpress CART. However, in common with ARC POMC neurons, some are directly responsive to peripherally administered leptin, as measured by the expression of pSTAT3-IR. Because pSTAT3 induction is directly linked to leptin receptor activity, it is most likely that a subset of POMC NTS neurons express leptin receptors on their surface; however, the presence of leptin receptors on NTS POMC neurons has yet to be confirmed. The finding that 1 and 5 mg/kg ip leptin produce a similar level of pSTAT3-IR expression in the caudal brainstem suggests that 1 mg/kg produces a maximal activation of the pSTAT3 signaling pathway in the caudal brainstem at 30 min after leptin treatment. Testing other lower doses of leptin or increasing the interval between injection and tissue fixation may reveal a different pattern and or degree of pSTAT3-IR expression.

In a recent paper, another group failed to see activation of caudal brainstem POMC neurons in a distinct mouse POMC-EGFP mouse model (30). However, there are a number of technical differences that may explain these differential findings. First, the leptin response in their model was examined in the fasting state 90 min after treatment, whereas we examined the response in normally fed animals 30 min after treatment. Perhaps more significantly, the mouse models used in the two studies were created in two very different genetic backgrounds. The POMC-EGFP mice in the study by Huo and colleagues were generated on a mixed genetic background consisting of a FVB × 129/SvJ transgenic backcrossed one generation onto the C57BL6/J background, whereas our mice are on pure C57BL6/J background. FVB mice are known to be resistant to diet-induced obesity, whereas the C57BL6/J mice are prone to diet-induced obesity (31). This may reflect an underlying difference in the maintenance of energy homeostasis between these two strains, which may possibly account for some of the differences seen.

In this study we have shown that NTS POMC-EGFP neurons constitute 30% of the neurons expressing pSTAT3-IR

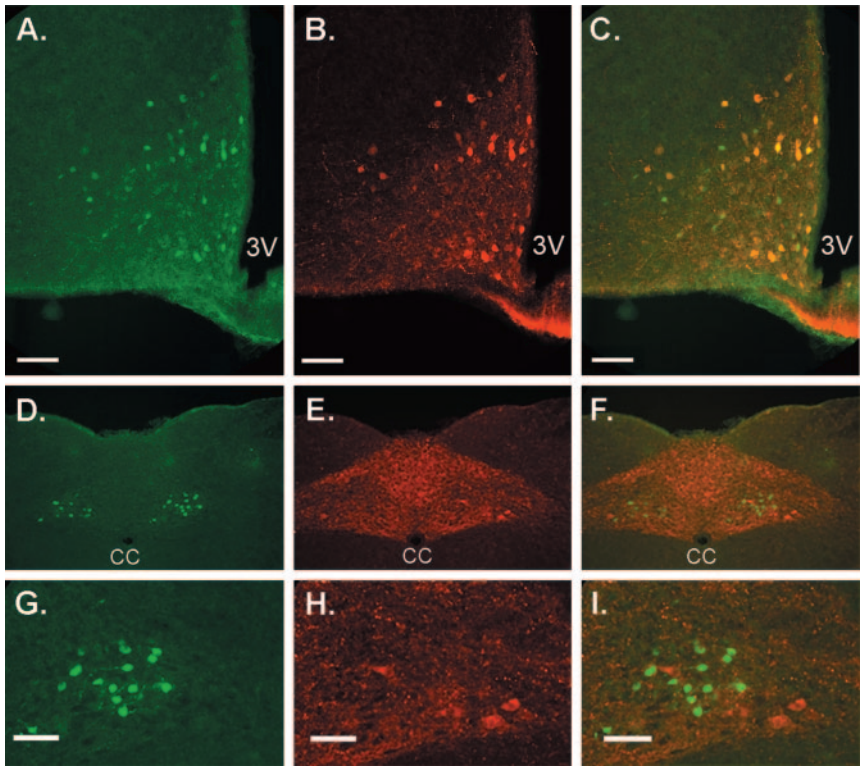

FIG. 5. Double-fluorescence immunohistochemistry for POMC-EGFP (A, D, and G) and CART immunoreactivity (B, E, and H) in the hypothalamus (*upper panels*) and caudal brainstem (*middle* and *lower panels*). G–I, An enlargement of a portion of D–F shown for clarity. POMC-EGFP immunoreactivity shows greater than 90% colocalization with CART immunoreactivity in the hypothalamus (C) but not the caudal brainstem (F–I). *Scale bars*, 60 μm. CC, Central canal; 3V, third ventricle.

after peripheral leptin treatment. The neurochemical phenotype of the other neuronal populations expressing pSTAT3-IR remains to be determined. Based on the sites of expression of pSTAT3-IR, candidates include CART-, glucagon-like peptide-, and TH-expressing neurons. It has been shown previously by us and others (19, 20) that POMC-EGFP and TH do not colocalize in neurons of the caudal NTS. Here we show preliminary data that suggest that neither CART nor TH neurons are significantly activated by leptin treatment. However, due to the extensive CART fiber network detected using immunohistochemistry, additional experiments may be needed to confirm this finding. Additionally, given the fact that leptin is known to exert some effects via the sympathetic nervous system, it is perhaps unexpected that in these experiments pSTAT3-IR was not detected in TH neurons in the caudal brainstem. This result was verified using two commercially available TH antibodies. However, again it will be important to extend these observations by examining different time points and different leptin-dosing regimens.

Overall, this study suggests that in addition to mediating the effects of short-term regulators of energy homeostasis, such as the satiety factor CCK (19, 20), NTS POMC neurons are involved in mediating the effects of the long-term adipostatic factor leptin. The melanocortin circuitry appears to be involved in integrating adipostatic signals, received primarily by the hypothalamus, with satiety and hunger signals received primarily by the caudal brainstem (14, 32). The data presented here suggest that, in addition, NTS POMC neurons may be capable of directly integrating information regarding short-term energy availability and long-term energy storage.

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Address all correspondence and requests for reprints to: Roger D. Cone, Center for the Study of Weight Regulation and Associated Disorders and Vollum Institute, Oregon Health and Science University, 3181 SW Sam Jackson Park Road, Portland, Oregon 97239-3098. E-mail: cone@ohsu.edu.

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