Systemic leptin dose-dependently increases STAT3 phosphorylation within hypothalamic and hindbrain nuclei

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Maniscalco JW, Rinaman L. Systemic leptin dose-dependently increases STAT3 phosphorylation within hypothalamic and hindbrain nuclei. Am J Physiol Regul Integr Comp Physiol 306: R576–R585, 2014.—Leptin released peripherally acts within the central nervous system (CNS) to modulate numerous physiological and behavioral functions. Histochemical identification of leptin-responsive CNS cells can reveal the specific cellular phenotypes and neural circuits through which leptin signaling modulates these functions. Leptin signaling elicits phosphorylation of signal transducer and activator of transcription 3 (pSTAT3), making pSTAT3-immunoreactivity (ir) a useful proxy for identifying leptin-responsive cells. Relatively low systemic doses of leptin (i.e., 10–130 μg/kg body wt) are sufficient to decrease food intake, inhibit gastric emptying, and increase sympathetic activity, but there are no histological reports of central pSTAT3-ir following leptin doses within this range. Considering this, we quantified central pSTAT3-ir in rats after intraperitoneal injections of leptin at doses ranging from 50 to 800 μg/kg body wt. Tissue sections were processed to identify pSTAT3-ir alone or in combination with immunolabeling for cocaine- and amphetamine-regulated transcript (CART), glucagon-like peptide-1 (GLP-1), prolactin-releasing peptide (PrRP), or dopamine-β-hydroxylase (DBH). Leptin doses as low as 50, 100, and 200 μg/kg body wt significantly increased the number of pSTAT3-ir cells in the arcuate nucleus of the hypothalamus (ARC), nucleus of the solitary tract (NTS), and ventromedial nucleus of the hypothalamus, respectively, and also led to robust pSTAT3 labeling in neural processes. The differential dose-dependent increases in pSTAT3-ir across brain regions provide new information regarding central leptin sensitivity. Within the ARC, CART-ir and pSTAT3-ir were often colocalized, consistent with evidence of leptin sensitivity in this neural population. Conversely, within the NTS, pSTAT3 only rarely colocalized with PrRP and/or DBH, and never with GLP-1.

leptin; signal transducer and activator of transcription 3; arcuate nucleus of the hypothalamus; ventromedial nucleus of the hypothalamus; nucleus of the solitary tract; prolactin-releasing peptide; glucagon-like peptide-1; noradrenergic; cocaine- and amphetamine-regulated transcript

LEPTIN, THE PEPTIDE PRODUCT of the obese (Ob) gene, is released from adipose tissue and the gastric epithelium to signal long-term and short-term caloric surfeit, respectively (4, 97). In this capacity, leptin acts as a hormonal feedback signal to promote negative energy balance (37), in part by increasing sensitivity to satiation signals (34, 74, 75, 77, 82) that decrease meal size (35, 36, 57, 59, 70). The strong influence of leptin signaling on energy balance, as well as reproductive, autonomic, and other physiological systems has been clearly demonstrated in rodents with recessive mutations in the gene encoding leptin [ob/ob mice (55)] or its receptor [db/db mice (50) and fa/fa rats (19, 54, 78)]. These rodent strains display marked obesity, hyperphagia, autonomic nervous system dysfunction, infertility, and disruption of hypothalamic-pituitary hormonal signaling (18, 27, 39, 50, 55, 96, 98, 99).

Leptin exerts its behavioral, metabolic, and hormonal effects, at least in part, via direct action within the central nervous system (CNS). Following peripheral release, leptin is unidirectionally transported into the CNS (8), where it can directly access leptin receptors expressed by cells within numerous CNS regions. Leptin receptors are abundantly expressed within the arcuate nucleus of the hypothalamus (ARC), the ventromedial nucleus of the hypothalamus (VMH), and the nucleus of the solitary tract (NTS) (33, 42, 83, 85), three regions that play integral roles in food intake, body weight regulation, glucose homeostasis, and energy metabolism (24, 41, 64, 68, 79, 84, 87). Indeed, acute central administration of leptin is sufficient to robustly decrease food intake and body weight (14, 42, 83), facilitate glucose homeostasis (68), and increase sympathetic nerve activity (17, 26). Furthermore, central leptin signaling is necessary for normal regulation of body weight and adiposity, since neuronal deletion of leptin receptors results in obesity (20), and CNS-specific rescue of leptin receptors in db/db mice ameliorates obesity (60).

Histological identification of leptin-responsive cells in the CNS can provide a wealth of information regarding the specific cellular phenotypes and neural circuits through which leptin signaling modulates physiology and behavior. The long form of the leptin receptor is a member of the cytokine receptor family and is coupled to the janus kinase/signal transducer and activator of transcription (JAK-STAT) pathway (89). Leptin binding to this receptor results in phosphorylation of STAT3 (pSTAT3) (92), making pSTAT3-immunoreactivity (ir) a useful proxy for identifying cells directly responsive to leptin. Centrally administered leptin increases pSTAT3 immunolabeling within the ARC, VMH, and NTS (49). However, given that endogenous leptin is secreted from peripheral tissues, the physiological relevance of centrally administered leptin is unclear.

A robust literature indicates that—similar to central leptin administration—systemic administration of leptin has pronounced effects on food intake, body weight, glucose homeostasis, reproductive function, and sympathetic activity (9, 25, 63, 73, 95). While most studies have used large intraperitoneal or intravenous doses of leptin (i.e., >1 mg/kg body wt) to observe these effects, systemic leptin doses ranging from 10 to 130 μg/kg body wt are sufficient to decrease food intake (10, 14, 23), inhibit gastric emptying (13), and increase sympathetic nervous system activity and lipolysis (86). Western blot and gel-shift assays indicate that peripheral doses of leptin as low as 50–100 μg/kg body wt are sufficient to increase STAT3 phosphorylation centrally (28, 92); however, these techniques...
do not permit identification of leptin-sensitive cellular populations or circuits. Studies that have identified central pSTAT3-positive cells following systemic leptin administration have used doses ranging from 1 to 15 mg/kg body wt (11, 12, 31, 43, 48, 51, 52, 62, 72, 80, 91), much higher than doses required for behavioral, metabolic, and hormonal responses. Central pSTAT3 immunolabeling following systemic leptin doses below 1 mg/kg body wt has not been reported. Considering this, we utilized immunohistochemical antigen retrieval techniques (11, 31, 62, 72) to reveal pSTAT3-ir within anatomically and phenotypically identified populations of CNS cells in rats after peripheral administration of leptin at doses ranging from 50 to 800 μg/kg body wt.

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley rats (Harlan, IN; 225–275 g body wt; n = 34) were housed singly in hanging stainless-steel wire mesh cages in a temperature-controlled room (20–22°C) on a 12:12-h light-dark cycle (lights on at 0700). Rats had ad libitum access to pelleted chow (Purina 5001) and water, except as noted. All experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

Injections and Perusions

Rats were weighed 1 day before leptin administration to determine proper dosage. Rats were deprived of food (but not water) for 16–18 h overnight before leptin or vehicle treatment, since food deprivation increases central responsiveness to systemically administered leptin in mice (11). On the day of the experiment, rats were removed from their home cages between 0830 and 1030 and injected intraperitoneally with 1.0 ml of sterile 0.15 M NaCl containing recombinant rat leptin (H9262) home cages between 0830 and 1030 and injected intraperitoneally (11). On the day of the experiment, rats were removed from their home cages between 0830 and 1030 and injected intraperitoneally with 1.0 ml of sterile 0.15 M NaCl containing recombinant rat leptin (Sigma-Aldrich; L5037) as follows: 0 μg/kg body wt (n = 4), 50 μg/kg body wt (n = 3), 100 μg/kg body wt (n = 8), 200 μg/kg body wt (n = 6), 400 μg/kg body wt (n = 7), and 800 μg/kg body wt (n = 6). Leptin was dissolved in vehicle just before injection, and rats were returned to their home cages immediately after injection.

Ninety minutes after intraperitoneal injection, rats were deeply anesthetized with pentobarbital sodium (39 mg/1.0 ml ip, Fatal Plus Solution; Butler Schein) and perfused transcardially with a brief saline rinse followed by fixative (100 ml of 2% paraformaldehyde and 1.5% acrolein in 0.1 M phosphate buffer, followed by 100 ml of 2% paraformaldehyde alone) (65). Brains were postfixed in situ overnight at 4°C, then removed from the skull and cryoprotected for 24–48 h in 20% sucrose. Brains were blocked and sectioned coronally (35 μm) using a Leica freezing-stage sliding microtome. Sections were collected in six serial sets, and stored at −20°C in cryopreservant solution (94) until immunohistochemical processing.

Immunohistochemistry

pSTAT3 immunolabeling. Immunohistochemical identification of pSTAT3 was used to measure direct leptin signaling in the brain, following a protocol adapted from those described previously (11, 12, 31, 53, 62, 72). Free-floating tissue sections were removed from cryoprotectant storage and rinsed thoroughly in 0.02 M phosphate buffer (PB). Importantly, our pilot studies indicate that this buffer molarity is essential for optimal pSTAT3-ir. Following rinses, tissue was treated with 0.5% sodium borohydride (20 min), 0.3% NaOH + 0.3% H2O2 (20 min), 0.3% glycine (10 min), and 0.03% SDS (10 min; all in 0.02 M PB). Nonspecific binding was prevented with a 20-min incubation in blocking solution (0.02 M PB containing 4% normal donkey serum, 0.4% Triton-X 100, and 1% BSA) before antibody incubation.

Primary and secondary antisera were diluted in blocking solution. Tissue sections were incubated in rabbit anti-pSTAT3 (1:1,000; Cell Signaling, D3A7). The specificity and selectivity of this commercially available antibody have been reported (16, 90). Tissue was incubated in biotinylated donkey anti-rabbit IgG (1:200; Jackson ImmunoResearch), treated with Elite Vectastain ABC reagents (Vector Laboratories), and reacted with diaminobenzidine (DAB) intensified with nickel sulfate to produce a blue-black reaction product.

ARC and NTS neural phenotypes. To begin determining the chemical phenotypes of neurons demonstrating pSTAT3-ir following these relatively low doses of leptin, we assessed pSTAT3-ir within ARC neurons expressing the anorexigenic peptide, cocaine- and amphetamine-regulated transcript (CART) (61). We also assessed pSTAT3-ir within noradrenergic NTS neurons expressing dopamine-β-hydroxylase (DBH) alone or together with prolactin-releasing peptide (PrRP), and in NTS neurons expressing glucagon-like peptide-1 (GLP-1). CART, DBH/PrRP, and GLP-1 neurons were selected for examination because each population has been implicated in central control of body energy balance (61, 64, 79).

To identify pSTAT3-ir within neurons of the ARC that express CART, pSTAT3-labeled forebrain sections from some cases were subsequently incubated in rabbit anti-CART (1:10,000, Phoenix Pharmaceuticals, H-003-62) followed by Alexa Fluor 488-conjugated donkey anti-rabbit IgG (1:300; Jackson Immunoresearch) to produce a green fluorescent cytoplasmic signal.

To localize pSTAT3 within DBH- and PrRP-positive neurons of the NTS, pSTAT3-labeled brain stem sections from some cases were incubated in a cocktail of mouse anti-DBH (1:5,000; Millipore, MAB308) and rabbit anti-PrRP (1:1,000; Phoenix Pharmaceuticals, H-008-52). After this, tissue sets were incubated in a cocktail of Cy3-conjugated donkey anti-rabbit IgG (1:300; Jackson Immunoresearch) and Alexa Fluor 488-conjugated donkey anti-mouse IgG (1:300; Jackson Immunoresearch) to produce red and green fluorescent cytoplasmic signals, respectively.

To localize pSTAT3 within NTS GLP-1-positive neurons, pSTAT3-labeled brain stem sections were incubated in rabbit anti-GLP-1 (1:10,000; Bachem, T-4363) followed by biotinylated donkey anti-rabbit IgG (1:500; Jackson Immunoresearch), Elite Vectastain ABC reagents, and plain DAB to produce a brown cytoplasmic reaction product.

Imaging and Quantification

Quantification of pSTAT3-expressing cells in the ARC, dorsomedial (dm)VMH, and NTS. pSTAT3-labeled tissue was visualized using a 20× objective on an Olympus microscope equipped for bright-field and fluorescent optics, and it was photographed using a digital camera (Hamamatsu Photonics, Hamamatsu, Japan). For all anatomical regions, pSTAT3-positive profiles were quantified on images using Adobe Photoshop CS4 image software. The criterion for counting a cell as pSTAT3-positive was the presence of visible blue-black nuclear immunolabeling, regardless of intensity.

pSTAT3-ir was quantified bilaterally at two rostrocaudal levels of the ARC (~2.00 mm and 2.45 mm caudal to bregma), and bilateral counts were averaged per section. Within the dmVMH, pSTAT3-labeled profiles were quantified bilaterally at a single rostrocaudal level (~2.45 mm caudal to bregma). This level represents the core of the ARC and contained the most robust pSTAT3-ir after our highest peripheral dose of leptin. Within the NTS, pSTAT3-labeled cells were quantified bilaterally at two rostrocaudal levels (~14.36 mm and 14.16 mm caudal to bregma; see Fig. 3) and bilateral counts were averaged per section. In all cases, distance from bregma was approximated on the basis of tissue comparison to a standard rat brain atlas (88).

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Qualitative assessment of pSTAT3-ir in phenotypically identified ARC and NTS neurons. pSTAT3-labeled sections colabeled for CART, ΔβH/PrRP, or GLP-1 were viewed on the Olympus photomicroscope described above. Using a 20× objective, we captured photographic images from a single selected rostrocaudal level of the ARC (~2.00 mm caudal to bregma) or the NTS (~14.36 mm caudal to bregma). Neurons were identified using Adobe Photoshop CS4 image software. Criteria for identifying a neuron as CART-, ΔβH/PrRP-, or GLP-1-positive included clear cytoplasmic labeling and a visible nucleus. Neurons were considered pSTAT3-positive if the nucleus contained pSTAT3 immunolabeling, regardless of intensity.

Statistics

Separate one-way ANOVAs were used to determine the effect of leptin dose (0, 50, 100, 200, 400, and 800 μg/kg body wt) on the number of pSTAT3-positive profiles within the ARC, dmVMH, and NTS. When F values indicated a significant effect, the ANOVA was followed by Fisher’s least significant difference post hoc analyses. Differences were considered significant when P < 0.05.

Preparation of Images

Using Adobe Photoshop software, we adjusted photographic images for optimal brightness and contrast. Images that included both immunoperoxidase and immunofluorescence were altered to generate Fig. 5, B and C (i.e., blue-black NiDAB pSTAT3 labeling photographed in the green color channel was inverted, giving it the appearance of a fluorescent signal). The presence or absence of immunolabeling in images was not digitally manipulated.

RESULTS

The presence of leptin-induced pSTAT3-ir was assessed throughout the full rostrocaudal extent of the brain, from the upper cervical spinal cord through the prefrontal cortex. Systemically administered leptin elicited robust pSTAT3-ir within the ARC, dmVMH, and NTS. Other brain regions contained only scattered, sparse pSTAT3 labeling, even in rats that received the highest leptin dose.

pSTAT3 labeling within the ARC. ANOVA indicated a significant effect of all leptin doses on the number of pSTAT3-positive profiles within the ARC [F (5,28) = 35.712, P < 0.001; Fig. 1D]. While some pSTAT3-ir was observed in the ARC of vehicle-treated rats, all doses of intraperitoneal leptin significantly increased pSTAT3-ir to the same degree (Fig. 1, A–C).

pSTAT3 labeling within the dmVMH. In contrast to the binary pattern of STAT3 phosphorylation observed in the ARC (i.e., similar increases in pSTAT3-ir after all leptin doses), the dmVMH displayed a leptin dose-dependent increase in pSTAT3 labeling (Fig. 2, A–C). ANOVA indicated a significant effect of leptin dose on the number of pSTAT3-ir profiles within the dmVMH [F (5,28) = 78.057, P < 0.001]. pSTAT3-ir was negligible in vehicle-treated rats and in rats treated with 50 or 100 μg/kg body...
wt leptin, whereas higher doses elicited marked increases in the number of pSTAT3-ir profiles (Fig. 2D).

**pSTAT3 labeling within the NTS.** The rostrocaudal extent of the NTS was examined for pSTAT3-ir to evaluate subregional effects of systemic leptin. Even in rats receiving the highest leptin dose (800 μg/kg body wt), pSTAT3-ir within the NTS was largely limited to the medial subnucleus of the caudal “visceral” NTS, with peak immunolabeling at the rostrocaudal level of the area postrema (Fig. 3). In each rat, STAT3 phosphorylation was quantified in two tissue sections through this level of the NTS (i.e., 14.36 and 14.16 mm caudal to bregma) (Fig. 3). ANOVA indicated a significant effect of leptin dose on the number of pSTAT3-ir profiles within the NTS at these rostrocaudal levels [F (5,28) = 11.282, P < 0.001]. pSTAT3-ir was negligible in vehicle-treated rats, whereas systemic leptin elicited a dose-dependent increase in pSTAT3-ir that reached a plateau at the 200 μg/kg body wt dose (Fig. 4).

**pSTAT3 immunolabeling in phenotypically identified neurons.** Consistent with a previous report using peripheral leptin administration in rats (51), pSTAT3 within the NTS was not colocalized in any GLP-1 neurons (Fig. 5A). pSTAT3-ir was colocalized in a subset of DβH- and DβH/PrRP-positive neurons of the caudal NTS (Fig. 5B). However, qualitative assessment indicated that the majority of DβH- and DβH/PrRP-positive neurons did not colocalize pSTAT3, and vice versa. Within the ARC, many CART-expressing neurons were also pSTAT3-positive (Fig. 5C), consistent with reports that peripheral leptin activates these neurons (21, 30).

**pSTAT3 immunolabeling within neural processes.** We observed dose-dependent increases in cytoplasmic pSTAT3-ir within neural processes in the ARC, dmVMH, and NTS. Labeling within the ARC appeared to remain within the anatomic boundaries of the nucleus. Conversely, pSTAT3-positive dmVMH processes projected radially in all directions away from the core of the nucleus, with particularly prominent labeling located ventral to the dmVMH within the internuclear area separating the VMH from the ARC (Fig. 2C). Labeling of neural processes within the NTS was most prevalent within the medial subnucleus but was also observed within the commissural subnucleus and extending into the area postrema (Figs. 3 and 4, B and C).

**DISCUSSION**

Leptin plays a critical role in regulating energy balance and other physiological functions (15, 38, 71), and leptin signaling
pathways that underlie these effects include direct activation of leptin receptors in brain stem and hypothalamic nuclei (45, 68, 81). To better understand how systemically released leptin acts on the brain to modulate physiology and behavior, it is necessary to localize and phenotypically identify leptin-responsive cells within the CNS. The present study is the first to immunohistochemically visualize pSTAT3 in rats after relatively low doses of systemic leptin. Doses used in the present study are known to elicit behavioral and physiological effects after systemic administration (13, 14, 86) and are much lower than systemic doses used previously to induce central pSTAT3 labeling. We report increased pSTAT3-ir after peripheral doses of leptin as low as 50 μg/kg body wt, well within the range of leptin doses that alter physiology and behavior (13, 14, 86). We also conducted the first reported dose-response assessment of leptin-induced pSTAT3 in several brain regions, providing new information regarding leptin sensitivity within and between central nuclei. Our results demonstrate that intraperitoneal leptin at doses ranging from 50 to 800 μg/kg body wt elicits unique and dose-dependent patterns of STAT3 phosphorylation in the ARC, dmVMH, and NTS.

Arcuate nucleus of the hypothalamus. It is well established that the ARC plays a critical role in energy balance by transducing circulating signals, including the adiposity signal leptin, into neural responses (21, 22, 29). Leptin receptors are abundantly expressed within the ARC, and numerous studies have reported dense ARC pSTAT3-ir in rodents after high peripheral doses of leptin (11, 62, 91). However, our study is the first to assess leptin-induced pSTAT3-ir within the ARC after leptin doses lower than 1 mg/kg body wt. Our results demonstrate that the ARC displays moderate pSTAT3-ir in vehicle-treated rats, presumably the result of endogenous leptin or other signaling factors that phosphorylate STAT under these conditions. Interestingly, the number of pSTAT3-positive ARC cells increased to maximal levels following the lowest leptin dose used in the present study (50 μg/kg body wt), a dose 100–300 times lower than those previously shown to be effective (48, 62, 91). Higher leptin doses elicited no further increase in pSTAT3-ir. These results support the view that the ARC is unusually sensitive to systemically administered leptin, probably due to high rates of peripheral-to-central leptin transport at this site (7) and likely tied to the ARCs critical role in controlling body energy homeostasis.

Since leptin receptor activation within the ARC decreases food intake and body weight (81), we investigated the ability of peripheral leptin to phosphorylate STAT3 within ARC neurons that have a known anorexigenic role. ARC neurons that express both proopiomelanocortin (POMC) and CART (30) drive anorexigenic responses to leptin via downstream central targets (2, 5, 61). As predicted, many CART-positive ARC neurons colocalized pSTAT3 after 400 μg/kg body wt leptin. This finding is consistent with evidence that POMC/CART neurons contribute to the decreased food intake (14), inhibition of gastric motility (13), and increased sympathetic outflow (86) observed following peripheral doses of leptin similar to those used here.

Within the ARC, maximal pSTAT3-ir was observed in rats that received the lowest leptin dose (i.e., 50 μg/kg body wt). It will be important in future studies to identify the threshold dose for increased STAT3 phosphorylation within the ARC and to

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Fig. 3. Images of pSTAT3 labeling through the rostrocaudal extent of the caudal nucleus of the solitary tract (NTS) in a representative rat after intraperitoneal leptin at a dose of 800 μg/kg body wt. pSTAT3 immunoreactivity was located primarily within the medial subnucleus of the caudal NTS. Numbers in each panel represent relative distance from bregma. AP, area postrema; DMX, dorsal motor nucleus of the vagus nerve. Scale bar = 200 μm, which applies to all panels.
explore dose-related pSTAT3 responses among phenotypically identified neural populations.

**Ventromedial nucleus of the hypothalamus.** The VMH participates in the control of feeding and energy balance (46), and recent evidence indicates that it does so, in part, through direct leptin signaling. The VMH expresses the leptin receptor (33, 83), and intra-VMH leptin injection facilitates blood glucose homeostasis (68), increases sympathetic outflow (81), and decreases food intake (56). Moreover, genetic deletion of the leptin receptor within a subset of VMH neurons that express steroidogenic factor-1 (SF-1) results in increased body weight and susceptibility to diet-induced obesity (24). The downstream targets through which VMH neurons modulate energy balance remain under investigation (32, 87).

Following peripheral administration of leptin, dose-dependent increases in pSTAT3-ir were observed within the VMH. pSTAT3-ir was present predominantly within the dorsomedial subregion of the VMH, although labeling was also observed in the central and ventrolateral subregions. Within the dmVMH, doses of leptin at or below 100 \(\mu g/kg\) body wt elicited negligible pSTAT3-ir, whereas dose-related increases in STAT3 phosphorylation were observed after administration of leptin at 200, 400, and 800 \(\mu g/kg\) body wt. The significant response to leptin at the 200 \(\mu g/kg\) dose is especially noteworthy, as this dose is 25 times lower than the lowest peripheral dose previously reported to increase pSTAT3-ir within the VMH (91). No plateau in pSTAT3-ir was observed within the VMH in the present study, suggesting that higher doses of leptin might induce additional STAT3 phosphorylation in this brain region.

The chemical phenotypes of VMH neurons sensitive to leptin at these doses are unclear, although they likely include SF-1 neurons that colocalize pituitary adenylate cyclase-activating polypeptide, based on evidence that these neurons mediate some of leptin’s hypophagic effects (24, 44).

**Nucleus of the solitary tract.** A growing literature has implicated neurons of the NTS in the changes in energy balance produced by central leptin signaling (for review, see Ref. 40). Recent studies show that leptin signaling in the NTS is not only sufficient to decrease food intake and body weight (42), but is necessary for maintenance of normal energy balance in freely feeding rats (45). Previous reports indicate that systemic leptin doses between 1 and 5 mg/kg body wt are sufficient to elicit STAT3 phosphorylation within the NTS (31, 51, 72, 91). No study, however, has investigated whether lower doses of leptin—sufficient to alter autonomic, physiological, and behavioral output (10, 13, 14, 86)—act directly on neurons of the NTS.

The present results demonstrate significantly elevated STAT3 phosphorylation within the NTS in rats after peripheral...
administration of leptin at doses as low as 100 \( \mu g/kg \) body wt, which is 10–50 times lower than doses used previously. pSTAT3-ir within the NTS displayed a leptin dose-response effect that was intermediate to effects observed within the ARC and the dmVMH. Some baseline pSTAT3-ir was present within the NTS in vehicle-treated rats, and the ability of leptin to increase pSTAT3-ir reached a plateau at the 200 \( \mu g/kg \) body wt dose. Increased pSTAT3-ir was confined to the caudal "visceral" NTS, a region known for its role in satiation and visceral sensory processing (64). Consistent with a previous report using systemic leptin administration (51), most of the pSTAT3-ir observed in the present study was located within the medial subnucleus (mNTS), which receives digestive related vagal sensory input (1, 69). Intra-mNTS leptin injections suppress food intake and motivation for food seeking (42, 58), while virus-mediated knockdown of mNTS leptin receptors increases food intake, meal size, body weight, and adiposity (45, 59). However, the neural circuits underlying these effects remain unclear.

The majority of pSTAT3-ir within the caudal NTS was localized in phenotypically unidentified neurons. We were somewhat surprised to observe relatively few DBH- and DBH/PrRP-positive neurons among those expressing pSTAT3-ir, because these neurons together comprise the A2 noradrenergic cell group, which contributes importantly to food intake and meal size control in rats (64, 79). GLP-1 neurons were never pSTAT3-positive, consistent with a previous report in rats, but in contrast to histological and electrophysiological results in mice (47, 51). This is not to say, however, that leptin does not affect A2 or GLP-1-expressing neurons in rats. Leptin exerts a strong depolarizing effect on glutamatergic vagal afferents that synapse directly onto neurons of the NTS (3, 74, 76), providing a potential route through which leptin receptors might modulate visceral afferent signaling to caudal NTS neurons, including A2 and GLP-1 neurons.

Dose-response summary. To our knowledge, this report is the first to describe differential dose-related sensitivity of central pSTAT3-ir responses to leptin. The ARC displayed the greatest sensitivity, with maximal pSTAT3 labeling observed after the lowest leptin dose administered (i.e., 50 \( \mu g/kg \) body wt), while pSTAT3-ir within the NTS did not reach maximal levels until rats were dosed with leptin at 200 \( \mu g/kg \) body wt. Both the ARC and the NTS displayed leptin dose-related plateaus in pSTAT3-ir, evidence that higher doses would be unlikely to further increase pSTAT3-ir in these regions. Phosphorylation of STAT3 within the dmVMH, however, increased progressively through the highest dose administered (i.e., 800 \( \mu g/kg \) body wt) without reaching an evident plateau, suggesting that higher doses might produce additional increases in pSTAT3-ir within the VMH.

Cytoplasmic pSTAT3 immunoreactivity. In addition to nuclear labeling, strong pSTAT3-ir was present within neural processes. Leptin receptors are expressed on both proximal and distal dendrites, where STAT3 phosphorylation occurs before translocation to the nucleus (6, 43, 93). Thus, neuritic labeling likely represents dendritic pSTAT3 that has yet to undergo nuclear translocation. Because pSTAT3 accumulates in the cell’s nucleus but not in the cytoplasm, it is likely that visualization of dendritic pSTAT3-ir requires more sensitive immunohistochemical and optic techniques than are commonly used (43). Our report is not the first to show dendritic pSTAT3 labeling (43, 48, 49, 80), but immunolabeling in the present study is significantly more pronounced than previously documented.

Importantly, the dendritic pSTAT3 labeling in our material closely matches established dendritic patterns of neurons within each brain region. Golgi staining reveals that ARC neuronal dendrites remain largely within the anatom-
tical boundaries of the ARC (66), whereas VMH neuronal dendrites radiate in all directions, including ventrally directed dendritic branches that extend into the internuclear area separating the ARC from VMH (66, 67). These dendritic patterns precisely match the pSTAT3 labeling we observed within the medial hypothalamus. Within the NTS, dendritic pSTAT3 immunolabeling was confined predominantly to the medial subnucleus but was also observed in processes extending medially into the commissural subnucleus and dorsally into the area postrema. This pattern of labeling reflects the organization of NTS neuronal dendrites (31) and is consistent with dendritic pSTAT3 labeling reported within the NTS of rats that received a much higher systemic dose of leptin (43, 48).

**Perspectives and Significance**

Since its discovery 20 years ago, much has been learned about leptin and leptin receptor-mediated regulation of physiology and behavior. However, much remains to be discovered about leptin and leptin receptor-mediated regulation of physiological processes extending medially into the commissural subnucleus and dorsally into the area postrema. This pattern of labeling reflects the organization of NTS neuronal dendrites (31) and is consistent with dendritic pSTAT3 labeling reported within the NTS of rats that received a much higher systemic dose of leptin (43, 48).

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