Neurochemical Characterization of Body Weight-Regulating Leptin Receptor Neurons in the Nucleus of the Solitary Tract

Alastair S. Garfield, Christa Patterson, Susanne Skora, Fiona M. Gribble, Frank Reimann, Mark L. Evans, Martin G. Myers, Jr., and Lora K. Heisler

Department of Pharmacology (A.S.G., S.S., L.K.H.) and Cambridge Institute for Medical Research (F.M.G., F.R.), University of Cambridge, Cambridge CB2 1PD, United Kingdom; Division of Metabolism, Endocrinology, and Diabetes (C.P., M.G.M.), Department of Internal Medicine, University of Michigan, Ann Arbor, Michigan 48109; and Department of Medicine and Institute of Metabolic Science (M.L.E.), University of Cambridge, Addenbrooke’s Hospital, Cambridge CB2 0QQ, United Kingdom

The action of peripherally released leptin at long-form leptin receptors (LepRb) within the brain represents a fundamental axis in the regulation of energy homeostasis and body weight. Efforts to delineate the neuronal mediators of leptin action have recently focused on extrahypothalamic populations and have revealed that leptin action within the nucleus of the solitary tract (NTS) is critical for normal appetite and body weight regulation. To elucidate the neuronal circuits that mediate leptin action within the NTS, we employed multiple transgenic reporter lines to characterize the neurochemical identity of LepRb-expressing NTS neurons. LepRb expression was not detected in energy balance-associated NTS neurons that express cocaine- and amphetamine-regulated transcript, brain-derived neurotrophic factor, neuropeptide Y, nesfatin, catecholamines, γ-aminobutyric acid, prolactin-releasing peptide, or nitric oxide synthase. The population of LepRb-expressing NTS neurons was comprised of subpopulations marked by a proopiomelanocortin-enhanced green fluorescent protein (EGFP) transgene and distinct populations that express proglucagon and/or cholecystokinin. The significance of leptin action on these three populations of NTS neurons was assessed in leptin-deficient Ob/Ob mice, revealing increased NTS proglucagon and cholecystokinin, but not proopiomelanocortin, expression. These data provide new insight into the appetitive brainstem circuits engaged by leptin. (Endocrinology 153: 4600–4607, 2012)
abolic dysfunction and obesity observed of globally LepR-deficient mice (3–5). These genetic studies suggest critical roles for extrahypothalamic LepRb populations in the regulation of energy balance by leptin. Indeed, recent investigations have revealed that leptin action in the nucleus of the solitary tract (NTS) and the ventral tegmental area contributes to the regulation of feeding (6–8).

The brainstem NTS represents an integrative node through which numerous peripherally derived appetitive signals are routed. Hormonal and mechanical inputs converge within the NTS to regulate the activity of a number of neurochemically distinct neuronal populations. LepRb-expressing neurons within the caudal NTS are most abundant in the medial portion at the level of the area postrema, an area known to participate in the regulation of appetitive state and consumptive behavior. Brainstem administration of exogenous leptin suppressed feeding in rodents (9). More recently, RNA interference-mediated knockdown of LepRb mRNA within the adult rat NTS and Cre-mediated ablation of LepRb in the mouse brainstem promoted hyperphagia, accelerated weight gain, and increased adiposity, revealing the physiological significance of this population of LepRb-expressing neurons to leptin’s energy balance functions (6, 8).

Understanding the NTS systems engaged by leptin that may be salient to the regulation of appetite and body weight requires defining their neurotransmitter/neuropeptide content. Here we employ multiple transgenic and histological techniques to reveal the neurochemical phenotype of LepRb-expressing neurons within the NTS.

Materials and Methods

Animals

Proopiomelanocortin (POMC)-enhanced green florescent protein (EGFP) (kindly provided by Profs. Richard Simerly and Malcolm Low), neuropeptides Y (NPY)-green florescent protein (GFP) (kindly provided by Prof. Jeffrey Friedman), proglucagon (PGP)-yellow fluorescent protein (YFP), LepRb-Cre::enhanced yellow fluorescent protein (EYFP) and LepRb-Ires-Cre::tdTOMATO (tdTOM) (kindly provided by Profs. Joel Elmquist and Jeffrey Friedman), and cholecystokinin (CCK)-Ires-Cre::EYFP mice were generated and maintained as previously described (3, 10–13). Animals were provided with standard laboratory chow and water ad libitum in a light- and temperature-controlled environment. POMC-EGFP/LepRb-Ires-Cre::tdTOM and NPY-GFP/LepRb-Ires-Cre::tdTOM double-reporter lines were generated by intercrossing homozygous POMC-EGFP or NPY-GFP males with LepRb-Ires-Cre::tdTOM females. All procedures performed were in accordance with the United Kingdom Animals (Scientific Procedures) Act, 1986, with appropriate ethical approval (United Kingdom) or National Institutes of Health guidelines on animal care and use and with the approval of the University Committee on Use and Care of Animals (United States).

Tissue preparation

Mice were anesthetized with pentobarbitone (50 mg/kg ip) and transcardially perfused with diethylpyrocarbonate-treated PBS followed by 10% neutral buffered formalin (Sigma Chemical Co., St. Louis, MO), cryoprotected in 20% sucrose, and sectioned coronally on a freezing sliding microtome at 25 μm.

Dual immunofluorescent histochemistry

As previously described (14), sections were washed in PBS before blocking in 0.5% BSA/0.5% Triton X-100 in PBS for 1 h at room temperature. Tissue was incubated overnight at room temperature in blocking buffer containing the following primary antibodies (diluted 1/1000): goat α-GFP (to detect GFP, EGFP and EYFP) (Abcam, Cambridge, MA), rabbit α-red fluorescent protein (to detect tdTOM) (Rockland Immunocchemicals, Gilbertsville, PA), rabbit α-nesfatin (Phoenix Pharmaceuticals, Burlingame, CA), mouse α-tyrosine hydroxylase (TH) (Chemicon, Temecula, CA), goat α-choline acetyltransferase (ChAT; Millipore, Billerica, MA), or rabbit α-nitric oxide synthase (nNOS; Immunostar, Hudson, WI). Sections were washed in PBS then incubated in blocking buffer containing appropriate secondary antibody (1/1000, Alexa Fluor; Molecular Probes, Eugene, OR) for 1 h. Sections were mounted onto microscope slides and coverslipped in an aqueous mounting medium (Vectorstain; Vector Laboratories, Burlingame, CA). Double-labeled cells were recorded if both fluorescent signals were present in the same cell and each alone clearly defined a cellular shape.

Leptin-induced phosphorylated signal transducer and activator of transcription 3 (pSTAT3) immunohistochemistry

LepRb-Cre-EYFP and LepRb-Ires-Cre::tdTOM mice were injected during the light cycle with 5 mg/kg mouse recombinant leptin (ip) (Merck, Whitehouse Station, NJ) and food removed. Animals were perfused 2 h later and the tissue processed as detailed above. pSTAT3 immunohistochemistry using a rabbit anti-pSTAT3 antibody (1/250; Cell Signaling Technology, Danvers, MA) was conducted as previously described (3). Sections were then extensively washed in PBS and processed for either GFP or red fluorescent protein immunofluorescence histochemistry as described above.

Dual in situ hybridization and immunohistochemistry

In situ hybridization was conducted as previously described (14). Radiolabeled riboprobes specific to the mRNA sequences of PPG (15), cocaine- and amphetamine-regulated transcript (CART) (15), CCK (16), glutamate decarboxylase-67 (GAD67) (17), brain-derived neurotrophic factor (BDNF; designed to 1172–1467 bp of the mouse cDNA), and prolactin-releasing peptide (PrRP; designed to the whole mouse coding region) were used to detect gene expression. Linearized recombinant plasmids were subjected to in vitro transcription with a T7 RNA polymerase (Ambion Inc., Austin, TX) in the presence of 35S-labeled UTP. cRNA riboprobes were diluted to 2 × 107 cpm/ml in hybridization solution. Tissue was washed in PBS before commencement of the immunohistochemistry protocol. Sections were treated for 30 min in 0.3% H2O2 in PBS, rinsed in PBS, and blocked in 0.5% BSA/0.5% Triton X-100 in PBS for 1 h. Sections
were incubated in blocking buffer containing goat α-GFP antibody (1/1000; Abcam) overnight. Sections were washed in PBS and a biotinylated donkey antigoat secondary antibody (Vector Laboratories) applied at 1/1000 in blocking buffer for 1 h. Sections were then washed in PBS and incubated for 1 h in VectaStain ABC reagent and chromogenic detection conducted using 3,3′-diaminobenzidine reagent (Vector Laboratories). Sections were mounted onto microscope slides and air dried. Slides were dipped in photographic emulsion (Kodak, Rochester, NY) and stored at 4°C for 2 wk before being developed in D-19 developer and fixer (Kodak). Double-labeled cells were recorded if GFP-positive cell bodies contained overlying black grains that were in a quantity greater than three times background and conformed to the shape of the GFP-immunoreactive (IR) cell bodies.

Analysis
Sections containing NTS were assigned a bregma level and boundaries of the NTS delineated based on neuro-architecture and the Mouse Brain Atlas (18). The number of LepRb-expressing neurons, neurochemically defined neurons, and double-labeled neurons falling within the defined regions were counted and expressed as a percentage of total LepRb-expressing neurons in that section. Data are presented as mean ± SEM (n = 3–4 per study).

Results
LepRb NTS subregional localization and validation of LepRb reporter lines
Two reporter lines, LepRb-Ires-Cre::tdTOM and LepR-Cre::EYFP, were used to perform a characterization of LepRb expression within the NTS. The overall distribution of tdTOM-IR and GFP-IR (EYFP) cells was consistent in both lines and extended across the rostral-caudal extent of the NTS, although in general, the LepR-Cre-EYFP line labeled more cells than the LepRb-Ires-Cre::tdTOM line (Fig. 1). In both lines, LepRb-expressing neurons within the NTS were most abundant at the level of the area postrema. Reporter detection of LepRb-expressing cells was also found within most subdomains of the NTS but was highest in the medial and, to a lesser extent, ventrolateral regions; the commissural portion was typically devoid of labeled cells.

To validate reporter expression with endogenous LepRb, we mapped and quantified colocalization of tdTOM-IR and GFP-IR with leptin-induced pSTAT3-IR. Colocalization of tdTOM-IR and GFP-IR with pSTAT3-IR also revealed a high degree of consistency between both reporter lines. Within a discrete subregion of the NTS, the rostral portion, very few tdTOM-IR or GFP-IR neurons colabeled with pSTAT3-IR (Fig. 1; bregma −6.84 mm, tdTOM-IR 6.3 ± 2.6% and GFP-IR 4.1 ± 2.9%; bregma −7.20 mm, tdTOM-IR 10.0 ± 1.4% and GFP-IR 25.0 ± 6.0%), highlighting a localized site of potential ectopic reporter expression or LepRb neurons of lower sensitivity to exogenous leptin administration. However, in the remainder of the NTS (bregma −7.56 mm to the caudal extent of the NTS), pSTAT3-IR was detected in 70.1 ± 1.9% tdTOM-IR neurons and 80.1 ± 1.9% of GFP-IR neurons (Fig. 1). These data support the utility of both reporter lines in LepRb characterization in the NTS including bregma −7.56 mm to the caudal extent, where the majority of NTS LepRb reside and known energy balance-associated neuromodulators are also expressed.

Identification of first-order NTS leptin-responsive neurons
To identify the first-order energy balance-associated NTS neurons directly modulated by leptin, we analyzed the chemical phenotype of LepRb-expressing neurons
(bregma −7.56 mm to the caudal extent of the NTS). In light of leptin’s regulation of arcuate nucleus POMC and NPY neurons (10), we examined whether the NTS represents another site of functional convergence for these systems. To facilitate analysis, the LepRb-Ires-Cre::tdTOM line was crossed with the POMC-EGFP or NPY-GFP lines. We observed that 26.0 ± 7.8% of LepRb-Ires-Cre::tdTOM-IR neurons were colocalized POMC-EGFP-IR cells within the medial NTS at the level of the area postrema (Fig. 2A and Table 1). In contrast, LepRb neurons did not colocalize with cells expressing NPY-GFP-IR (Fig. 2B).

Additional neurochemical profiling demonstrated the lack of LepRb in several other NTS neuronal populations implicated in energy balance, including those expressing TH (a catecholamine marker, Fig. 2C), nesfatin (Fig. 2D), ChAT (a cholinergic marker, Fig. 2E), nNOS (Fig. 2F), PrRP (Fig. 2G), GAD67 (a γ-aminobutyric acid (GABA)-

### TABLE 1. Quantification of LepRb-expressing NTS neurons

<table>
<thead>
<tr>
<th>Bregma</th>
<th>−7.56</th>
<th>−7.64</th>
<th>−7.76</th>
<th>−7.92</th>
<th>Total</th>
<th>%</th>
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<tr>
<td>POMC</td>
<td>14/40</td>
<td>5/18</td>
<td>3/18</td>
<td>0/11</td>
<td>20/77</td>
<td>26</td>
</tr>
<tr>
<td>PPG</td>
<td>22/40</td>
<td>18/22</td>
<td>7/14</td>
<td>5/7</td>
<td>52/83</td>
<td>62</td>
</tr>
<tr>
<td>CCK</td>
<td>18/42</td>
<td>12/26</td>
<td>4/10</td>
<td>6/13</td>
<td>40/91</td>
<td>44</td>
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The average number of LepRb-expressing neurons positive for POMC, PPG, or CCK per total LepRb within that level of the NTS (n = 3–4).

FIG. 2. Neurochemical profiling of LepRb-expressing cells of the caudal NTS. A–F, Double-immunofluorescent analysis of LepRb-expressing neurons in compound LepRb-Ires-Cre::tdTOM/POMC-EGFP and LepRb-Ires-Cre::tdTOM/NPY-GFP mice revealed colocalization of LepRb-expressing neurons (red) with POMC-expressing (green; A) but not NPY-expressing (green; B) neurons of the NTS. Immunohistological analysis in LepRb-Cre::EYFP mice also demonstrated these cells to be negative for TH (C), nesfatin (D), ChAT (E), nNOS (F). G–L, Dual-immunohistological and in situ hybridization analysis demonstrated the absence of LepRb coexpression (brown cytoplasmic stain) with PrRP (G), GAD67 (H), BDNF (I), or CART (J) mRNA (black grains). However, LepRb neurons were found to express PPG/GLP1 (K) and CCK (L) mRNA. M–O, Population analysis of NTS LepRb-expressing neurons demonstrated that POMC-EGFP neurons did not express PPG/GLP1 (M) or CCK (N) but that a proportion of PPG/GLP1-YFP NTS neurons also exhibited CCK expression (O). Arrows represent single-labeled cells and arrowheads colocalized cells. cc, Central canal. Scale bar in A applies to A–I and represents 50 μm. Scale bar in A inset applies to insets in K, L, and O and represents 25 μm.
ergic marker, Fig. 2H, BDNF (Fig. 2I), or CART (Fig. 2J). In contrast, and located more caudally than the POMC/LepRb population, 62.6 ± 6.4% of LepRb-Cre::EYFP-IR neurons were found to express PPG mRNA [or glucagon-like peptide-1 (GLP1)] (Fig. 2K and Table 1), and 43.9 ± 1.4% expressed CCK mRNA (Fig. 2L and Table 1).

We further characterized these three populations of LepRb-expressing NTS neurons to determine whether they represent distinct or overlapping populations. We assessed PPG/GLP1 coexpression with POMC through PPG mRNA labeling in the POMC-EGFP line (Fig. 2, M and N) and examined CCK coexpression with POMC and PPG/GLP1 using CCK mRNA expression in the POMC-EGFP line (Fig. 2N) or PPG-YFP (Fig. 2O) line. We observed no overlap between POMC-EGFP and either PPG/GLP-1 or CCK, suggesting that POMC-EGFP neurons represent a discrete subpopulation of LepRb NTS neurons, although we observed some overlap between PPG/GLP1 and CCK.

**Effects of leptin deficiency on leptin-responsive NTS neuropeptide expression**

The significance of leptin signaling to the physiological function of these three neuronal populations was investigated through the assessment of gene expression in congenitally leptin-deficient Ob/Ob mice. Radioactive in situ hybridization followed by densitometry analysis demonstrated a substantial increase of PPG mRNA (Fig. 3, D–F; 100%, t = 3.9, P < 0.05) and CCK mRNA (Fig. 3, G–I; 346%, t = 3.6, P < 0.05) but not POMC mRNA (Fig. 3, A–C) specifically within the NTS of Ob/Ob mice compared with wild-type controls. These data reveal that loss of leptin tone in Ob/Ob mice is important to the transcriptional regulation of PPG/GLP1 and CCK, but not POMC.

**Discussion**

Leptin action via LepRb-expressing NTS neurons plays a crucial role in the physiological regulation of energy balance (6, 8, 9). In an effort to define the neurotransmitter/neuropeptide content of first-order LepRb neurons in this brain region, we performed a comprehensive neurochemical profile of NTS LepRb neurons. Using multiple transgenic reporter lines to facilitate analysis, we report LepRb colocalization with POMC-EGFP neurons, with neurons that express PPG/GLP1 and with neurons that express...
CCK but not with other energy balance-associated populations that contain NPY, BDNF, CART, nesfatin, catecholamines, GABA, acetylcholine, PrRP, or nNOS.

Validation analysis of the LepRb-reporter lines revealed a high degree of colocalization with endogenous LepRb in all regions of the NTS except the rostral portion. This identifies either a discrete rostral site of ectopic expression in both reporter lines or endogenous LepRb that are less sensitive to exogenous leptin administration. Regardless, none of the rostral reporter-labeled cells coexpressed the chemical markers investigated here. We conclude that the data presented provide insight into the mediators that directly respond to leptin in the NTS in the regulation of energy balance.

Consistent with the present results, the detection of leptin-stimulated STAT3 phosphorylation (a marker of LepRb activation) has been used previously to indicate the absence of LepRb expression on neurochemically defined NTS neurons, such as those expressing CART (19), TH/catecholamines (19), and GABA (20), and their presence on glutamatergic (20) and POMC (19) neurons. Our results demonstrating the lack of LepRb in NTS NPY, nesfatin, acetylcholine, nNOS, and BDNF neurons are novel. The lack of LepRb in NTS BDNF neurons is unexpected given that NTS BDNF expression is influenced by nutritional state and exogenous leptin administration (21). Thus, leptin regulation of NTS BDNF is likely to be indirect, perhaps via local projections from neighboring NTS LepRb neurons. That we find no support for LepRb expression on PrRP neurons is in contrast to previous studies (22, 23). It is possible that the approximately 7% of mouse NTS PrRP neurons reported to exhibit leptin-induced pSTAT3-IR (22) are not represented by the reporter lines used. However, evidence that NTS PrRP neurons are also catecholaminergic (23) supports our observations, because LepRb-expressing neurons have been demonstrated to be TH negative (19) (Fig. 2C).

Due to low endogenous POMC expression in the NTS, NTS POMC neurons are typically reliably detected only at the single-cell level through transgenic methods of visualization. The combined use of POMC-EGFP and LepRb-Ires-Cre::tdTOM reporter alleles thus facilitated the direct detection of NTS POMC-EGFP/LepRb colocalization. The majority of EGFP/tdTOM-positive cells were localized to the NTS where approximately 26% of LepRb-expressing neurons were POMC-EGFP positive. These findings are consistent with previous observations of leptin-induced pSTAT3-IR in POMC-EGFP cells (19) but are in contrast to a report of a lack of leptin action in NTS POMC neurons using a Cre-driven POMC reporter line (24), now known to also label nonfated POMC cells (25). However, we demonstrate that leptin deficiency does not alter endogenous POMC mRNA expression in Ob/Ob mice. The very low expression of POMC mRNA in the NTS suggests that POMC-derived peptides may not significantly contribute to neurotransmission by the POMC-EGFP/LepRb neurons, consistent with the lack of regulation of NTS POMC mRNA by leptin deficiency or peripheral leptin administration (24). Thus, although a proportion of NTS LepRb neurons are coexpressed with POMC-EGFP, and these are clearly distinct from PPG/GLP-1 and CCK neurons, the relevant leptin-regulated neuropeptides and/or neurotransmitters in the LepRb/POMC-EGFP neurons of the NTS is unclear.

A second population of leptin-responsive neurons express PPG/GLP1 within the lateral portion of the medial NTS, consistent with a previous report (26–28). Our transgenic approach to LepRb visualization suggests PPG/GLP1 neurons represent the largest neurochemically defined subpopulation of LepRb-expressing cells, accounting for approximately 60% of NTS LepRb. This observation is consistent with recent studies of NTS PPG/GLP1 cells that suggest them to be the primary effector of NTS LepRb-regulated energy balance in mice (8). Concordantly, NTS PPG mRNA expression was significantly increased in Ob/Ob mice, corroborating previous observations made in the obese Zucker rat (a rat model of leptin insufficiency), which also exhibits elevated NTS PPG expression (29).

As peripherally synthesized satiety factors, CCK and leptin act synergistically at vagal afferents to suppress food consumption (1). However, in addition to its synthesis in I-cells of the small intestine, CCK is abundantly expressed within the brain where it serves as a peptidergic neurotransmitter. We now report that approximately 40% of NTS LepRb neurons express CCK; these neurons are clustered caudal to the area postrema, in a distribution that partially overlaps with the distribution of PPG/GLP-1 LepRb neurons. Furthermore, NTS CCK expression is increased in the absence of leptin signaling. Although previous analyses of CCK mRNA levels in Ob/Ob whole brain failed to identify significant changes (1, 30), more discrete neuroanatomical investigation now demonstrates marked up-regulation in the Ob/Ob NTS. That we observed no overt changes in CCK expression in any other site of the Ob/Ob brain (data not shown) suggests that NTS CCK neurons may be of particular relevance to energy homeostasis and that leptin directly influences central CCK function.

The finding that LepRb neurons expressing PPG/GLP-1 and CCK are codistributed and that a proportion of PPG/GLP-1 neurons contain CCK suggests that a subpopulation of NTS LepRb PPG/GLP-1 neurons coexpress CCK. Indeed, because NTS POMC-EGFP neurons (26%
of NTS LepRb neurons do not express either PPG/GLP-1 or CCK (62 and 44% of NTS LepRb neurons, respectively), some overlap between LepRb PPG/GLP-1 and LepRb CCK neurons must exist.

Although the data presented here consolidate and expand upon the understanding of leptin-engaged NTS circuits, it is possible that the absence of LepRb expression on CART, BDNF, GABA, or PrRP neurons may be influenced by the sensitivity of the in situ hybridization method. However, unlike previously employed PCR techniques, in situ hybridization affords neuroanatomical specificity, enabling a highly focused assessment of NTS LepRb neuron populations.

The cellular heterogeneity of the NTS raises questions as to the neurochemical identity of the neurons responsible for promoting the physiological response to LepRb activation and the pathological consequences of LepRb inactivation. However, until now, no comprehensive neurochemical characterization of these neurons has been undertaken. Here we demonstrate this population to be comprised of POMC-, PPG/GLP1-, and CCK-expressing cells. These data provide valuable insight into the brainstem pathways through which leptin’s energy balance functions are implemented.

Acknowledgments

Address all correspondence and requests for reprints to: Lora Heisler, University of Cambridge, Department of Pharmacology, Tennis Court Road, Cambridge CB2 1PD, United Kingdom. E-mail: lkh30@medschl.cam.ac.uk.

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Present address for S.S.: Research Institute of Molecular Pathology, Vienna, Austria.

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