Leptin receptor–expressing nucleus tractus solitarius neurons suppress food intake independently of GLP1 in mice

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Introduction

The nucleus tractus solitarius (NTS) of the caudal brainstem receives and integrates information from the gut and elsewhere in the periphery to inhibit food intake (1–3). Given the function of the adipose-derived signal of energy repletion, or leptin, in the control of energy balance and suggestions of important roles for leptin action in the NTS (1, 2, 4, 5), leptin receptor–expressing (LepRb-expressing) neurons of the NTS (LepRbNTS neurons) are of particular interest. Leptin augments the suppression of food intake by the vagal and/or hindbrain action of gut peptides (4, 6–8), and ablation of LepRb in the NTS of mice or rats increases meal size and tends to increase body weight, especially in animals fed a high-calorie diet (HCD) (9, 10). The mechanisms of action by which LepRbNTS neurons modulate feeding remain unclear, however.

Agonists for the glucagon-like peptide-1 receptor (GLP1R) act via the CNS to suppress food intake (11–13). The expression of preproglucagon (Ppg), which encodes the precursor peptide for GLP1 (as well as glucagon and GLP2), is restricted to the a cells in the pancreatic islets, L cells in the gut, and a small set of neurons in the hindbrain, primarily in the NTS (14). Because GLP1R agonists represent promising medical therapies to reduce food intake and treat obesity, a great deal of research has focused on defining the potential therapeutic utility of GLP1NTS cells and their downstream targets (15–17).

The early developmental ablation of Glp1r or Ppg in mice minimally alters energy balance or food intake (11–13). The expression of preproglucagon (Ppg), which encodes the precursor peptide for GLP1 (as well as glucagon and GLP2), is restricted to the a cells in the pancreatic islets, L cells in the gut, and a small set of neurons in the hindbrain, primarily in the NTS (14). Because GLP1R agonists represent promising medical therapies to reduce food intake and treat obesity, a great deal of research has focused on defining the potential therapeutic utility of GLP1NTS cells and their downstream targets (15–17).

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GLP1 activity at sites within the CNS minimally alters food intake under normal conditions (11, 23–28). Several food intake–suppressing stressors (including large volume loads in the stomach and chronic variable stress) activate GLP1NTS cells, however, and interference with CNS GLP1 action or GLP1NTS cells attenuates the acute anorexic response to these stressors (17, 26). Thus, GLP1NTS cells may modulate food intake mainly in response to particularly strong or stressful stimuli.

While interfering with endogenous GLP1/GLP1R action minimally impacts food intake, the activation of GLP1NTS cells decreases feeding (16, 17), suggesting that the activation of these cells could provide a useful treatment for obesity. GLP1 could also contribute to the function of GLP1NTS and/or LepRbNTS cells. Here, we have investigated the suppression of food intake by GLP1NTS and LepRbNTS cells and determined the roles for GLP1 signaling in the suppression of food intake by these neuronal populations. We found that the activation of LepRbNTS neurons mediates the robust and durable suppression of food intake independently of GLP1 signaling. These findings reveal the dominance of GLP1-independent signals for the suppression of food intake by the NTS.

**Results**

**Ablation of Ppg in the NTS fails to alter energy balance.** While LepRbNTS cells are distinct from NTS cells that express cholecystokinin (CCK), prolactin-releasing hormone (PRLH), tyrosine hydroxylase (TH) (Figure 1, A–C), and calcitonin receptor (29) and do not colocalize with cholinergic neurons of the adjacent dorsal motor nucleus of the vagus (DMV) (Figure 1D); GLP1NTS cells represent a subset of LepRbNTS cells (Figure 1E) (4, 6, 10). Because LepRbNTS cells tend to be activated by feeding (Figure 1, F–H) and are thought to synergize with gut signals that participate in the control of food intake (1, 2, 4, 5, 8) — and because GLP1R agonists act in the brain to suppress food intake (13) — we sought to understand the potential role for NTS GLP1 in the control of energy homeostasis by LepRbNTS and GLP1NTS cells.

We ablated Ppg in the NTS by crossing Ppgcre onto the Leprcre or PpgCre (a BAC transgenic mouse with an integration site remote from the endogenous Ppg locus and that demonstrates NTS-specific cre expression; ref. 16) backgrounds (Figure 2A). Leprcre;PpgCre (PpgleprKO) mice demonstrated undetectable GLP1-immunoreactivity (GLP1-IR) in the NTS, as expected, since GLP1-containing NTS neurons in the mouse contain LepRb (Figure 1E and Figure 2, B and C) (6). Similarly, PpgCre;PpgCre (PpgGLP1NTSKO) animals exhibited no GLP1-IR in the NTS (Figure 2D). Note that, while most of our studies are not powered to detect sex differences, we have provided data broken down by sex in Supplemental Figures 1 and 2 (supplemental material available online with this article; https://doi.org/10.1172/jci.insight.134359DS1). Neither of these lines demonstrated altered body weight, food intake, or body composition compared with control animals at baseline, however (Figure 2, E–J, and Supplemental Figure 1, A–H), consistent with the lack of body weight changes in the Glp1-null mouse (20).

Adult ablation of NTS Ppg in rats can alter food intake and energy balance (30), suggesting that early developmental ablation of Ppg or Glp1r might provoke developmental compensation that could mask a potential phenotype due to the loss of Ppg in the NTS. Thus, we also ablated Ppg in adult (8–12 weeks of age) mice by the bilateral injection of an mCherry-tagged AAVcre into the NTS of Ppgfl/fl mice (PpgAAVNTSKO mice) (Figure 3A). As a control, we injected AAVGFP bilaterally into the NTS of littermate mice. As expected, the injection of AAVcre, but not AAVGFP, into the NTS ablated GLP1-IR in PpgAAVNTSKO mice (Figure 3, B and C). Although we studied these mice for 9 weeks on chow diet and an additional 8 weeks on HCD, PpgAAVNTSKO mice displayed no alterations in body weight, food intake, or body composition compared with their controls (Figure 3, D–F, and Supplemental Figure 1I). Thus, the ablation of GLP1 from the NTS in adult mice, as during development, does not detectably alter energy balance.

**LepRbNTS neurons suppress food intake more effectively than GLP1NTS neurons.** To compare the food intake–suppressing potential of GLP1NTS and LepRbNTS neurons, we bilaterally injected AAVFm-Dq into the NTS of Ppgcre or Leprcre mice to cre-dependently express the Gq-coupled (activating, hM3Dq) Designer Receptors Exclusively Activated by Designer Drugs (DREADD) in GLP1NTS and LepRbNTS cells (LepRbNTS-Dq and GLP1NTS-Dq mice, respectively). DREADD-hM3Dq expression in neurons permits their activation by the injection of clozapine N-oxide (CNO) (which is metabolized to produce the DREADD ligand) (31, 32). We used the post hoc detection of mCherry (which is fused to hM3Dq in AAVFm-Dq) and FOS-IR to ensure that we analyzed only mice with robust bilateral NTS hM3Dq expression. As expected, CNO promoted FOS accumulation in mCherry-expressing cells of the NTS in both lines (Figure 4, A and B).
We examined the ability of CNO injection to acutely suppress food intake following an overnight fast and at the onset of the dark cycle with exposure to normal chow or HCD (Figure 4, C–E, and Supplemental Figure 1, J–L). We found that the activation of GLP1 NTS or LepRbNTS cells similarly reduced food intake over the first 6 hours following an overnight fast. While the activation of GLP1NTS or LepRbNTS cells both almost entirely abrogated food intake for the first 2 hours at the onset of the dark cycle, by 4 hours, the food intake-suppressing effect of GLP1 NTS neuron activation attenuated substantially compared with LepRbNTS cells. For animals provided with HCD at the onset of the dark cycle, the activation of LepRb NTS cells suppressed food intake more effectively than did the activation of GLP1NTS neurons at all time points. Thus, the acute activation of LepRb NTS cells suppresses food intake more effectively and more durably than does the activation of GLP1NTS cells.

We also examined the long-term suppression of food intake and body weight in LepRbNTS-Dq and GLP1NTS-Dq mice subjected to twice-daily injections of CNO over 4 days (Figure 4, F and G, and Supplemental Figure 1, M and N). For LepRbNTS-Dq mice, this resulted in a sustained, approximately 50% decrease in food intake for all 4 days of the treatment, resulting in the maintenance of an approximately 5% weight loss for the duration of the treatment. In contrast, this prolonged CNO treatment of GLP1NTS-Dq mice detectably decreased food intake (by ~25%) only for the first day of treatment — after which, food intake and body weight reverted to baseline, despite ongoing CNO administration. Thus, LepRbNTS cells provoke a stronger and longer-lasting anorectic response compared with GLP1NTS cells, consistent with the larger number of LepRbNTS cells compared with GLP1NTS neurons.

Ppg ablation attenuates the suppression of food intake by GLP1NTS neurons. To determine the potential role for GLP1 in the suppression of food intake by GLP1NTS neurons, we bilaterally injected the cre-inducible AAVFlex-Dq into either Ppgcre or PpgGLP1-NTSKO (PpgGLP1-NTSKO–Dq) mice and examined their response to CNO (Figure 5). While CNO promoted the accumulation of FOS in the NTS of both GLP1NTS-Dq and PpgGLP1-NTSKO–Dq mice (Figure 5, A and B), CNO failed to decrease food intake in PpgGLP1-NTSKO–Dq mice following an overnight fast or at the onset of the dark cycle in mice fed chow or HCD (Figure 5, C and D, and Supplemental Figure 2, A and B). Similarly, CNO failed to decrease body weight in
Thus, in the absence of GLP1, other neurotransmitters that are expressed in GLP1 NTS cells are insufficient to mediate the suppression of food intake; GLP1 is required for the suppression of food intake by GLP1 NTS neurons. Ppg expression contributes minimally to the suppression of food intake by LepRbNTS neurons. To understand the potential contribution of GLP1 signaling to food intake suppression by LepRbNTS cells, we bilaterally injected the cre-inducible AAVFlex-Dq into either Leprcre or PpgLepRbKO (PpgLepRbKO-Dq) mice and examined their response to CNO treatment (Figure 6). As expected, CNO stimulated FOS-IR in the NTS of both LepRbNTS-Dq and PpgLepRbKO-Dq mice (Figure 6, A and B). Unlike the absent food intake suppression observed in PpgGLP1-NTSKO–Dq mice, however, CNO stimulated similar suppression of food intake in PpgLepRbKO-Dq and LepRbNTS-Dq mice (Figure 6, C–F, and Supplemental Figure 2, E–H). There was no difference in food intake suppression between LepRbNTS-Dq and PpgLepRbKO-Dq mice at the onset of the dark cycle (although there was a small attenuation of food intake suppression in chow-fed PpgLepRbKO-Dq mice at the onset of the dark cycle). Furthermore, there was no difference between LepRbNTS-Dq and PpgLepRbKO-Dq mice in the ability of CNO to decrease food intake and body weight during 4 days of twice-daily CNO treatment. Thus, while GLP1 is required for the suppression of food intake by GLP1NTS cells, and GLP1NTS cells represent a subset of LepRbNTS cells, GLP1 is not required for the suppression of food intake by LepRbNTS cells. Thus, GLP1-independent pathways dominate over GLP1 for the suppression of food intake by the NTS.
**Discussion**

Our data reveal that, despite the lack of effect of NTS Ppg ablation on energy balance in mice, Ppg is required for the suppression of food intake mediated by the activation of mouse GLP1NTS cells. However, GLP1NTS cells represent a subset of mouse LepRbNTS cells. Furthermore, LepRbNTS cells more strongly and durably inhibit food intake than GLP1NTS cells, while Ppg does not meaningfully contribute to the suppression of food intake by LepRbNTS cells. Thus, non-Ppg-derived neurotransmitters in the...
non-GLP1NTS subpopulation of LepRbNTS cells dominate over GLP1-derived signals for the suppression of feeding.

The DREADD-mediated activation of NTS neuronal populations, which we have employed here, provides a robust assay for the function of these cell types and the mechanisms by which they alter feeding behavior. LepRbNTS cells are acetylated by acute refeeding, suggesting that they receive inputs from the gut (e.g., via the vagus and/or gut peptides) as previously proposed (8) and that the activation of these cells mimics the postprandial function of them. While these represent pharmacologic manipulations designed to test the functional output of maximally activating a cell type/circuit, our findings that LepRbNTS and non-GLP1 signals play a more prominent role in food intake suppression than GLP1 and GLP1NTS neurons are consistent with findings that interfering with endogenous GLP1/GLP1R action (18–20) minimally alters
food intake and energy balance, while interfering with leptin action via LepRbNTS cells increases food intake and body weight (10, 33).

Because endogenous GLP1 contributes relatively little to the control of food intake and body weight while pharmacologic GLP1R agonists effectively suppress food intake and body weight, it is possible that pharmacologic GLP1R agonists may act via different mechanisms than endogenous NTS GLP1. For instance, peripherally administered GLP1R agonists may act via brain structures that are more accessible to the circulation. Indeed, the finding that glutamatergic Glp1r neurons distinct from several hypothalamic populations of Glp1r neurons mediate the anorectic effect of liraglutide suggests that Glp1r neurons in the area postrema (AP) may mediate this effect of GLP1R agonists (12, 13, 28). In contrast, the minimal AP FOS-IR following DREADD-mediated activation of LepRb NTS cells suggests that these cells contribute little (if at all) to the activation of AP GLP1R cells. Thus, the neural targets for peripherally applied pharmacologic GLP1R agonism likely differ from those engaged by NTS-derived GLP1.

Importantly, however, our data do not rule out the possibility of changes in meal size or frequency resulting from the ablation of Ppg in the NTS. While we have not examined a potential role for NTS Ppg or GLP1NTS cells in glucose homeostasis, the DREADD-mediated inhibition of LepRbNTS cells failed to alter glucose tolerance (Supplemental Figure 3). In the future, it will be interesting to examine the long-term effects of inhibiting these cells.

Interestingly, the NTS Ppg system in rats may differ in important ways from that of the mouse. For instance, interfering (postnatally) with endogenous CNS GLP1/GLP1R signaling in the rat increases food intake and...
food intake and body weight (30). While GLP1NTS cells represent a subset of LepRbNTS cells in the mouse, GLP1- and LepRb-containing cells are distinct in the rat NTS. Thus, because interfering with leptin action on rat LepRbNTS neurons also increases food intake and body weight, non-GLP1 NTS neurotransmitters in rat LepRbNTS cells participate in the control of food intake and energy balance, as in the mouse. The fact that non-GLP1 neurotransmitters must mediate substantial components of the NTS-mediated control of food intake and energy balance suggests the importance of understanding roles for NTS neurotransmitters other than GLP1 for the control of food intake. In the future, it will be important to identify the non-GLP1 neurotransmitters by which LepRbNTS cells contribute to the control of food intake and body weight, and to compare these across species.

Methods

Animals. Mice were bred in our colony in the Unit for Laboratory Animal Medicine at the University of Michigan. Male and female C57BL/6 mice were used in experiments. Mice were provided with ad libitum food and water. Food intake and body weight were measured in male and female mice at 3 months of age, and mice were housed in temperature-controlled rooms on a 12-hour light-dark cycle.

We purchased male and female C57BL/6 mice from the Jackson Laboratory. LepRbcre, Ppgcre, and Ppgflm mice have been described (16, 20, 34, 35) and were propagated by intercrossing homozygous mice of the same genotype. Cckcre mice were purchased from the Jackson Laboratory (stock no. 012706). LepRbcre and Cckcre were bred to the Rosa26eGFP-L10a background (36) to generate LepRbGFP and CckeGFP reporter lines, respectively.
Ppgfl mice were crossed twice onto the Leprcre or Ppgcre background to generate Leprcre/cre;Ppgfl/+ or Ppgcre/cre;Ppgfl/+ animals, which were intercrossed to generate Ppgcre/cre;Ppgfl/+, Ppgfl/+, and littermate control mice. For all studies, animals were processed in the order of their ear tag number, which was randomly assigned at the time of tailing (before genotyping).

Viral reagents and stereotaxic injections. AAVFlies-M3Dq, AAVGFP, and AAVcre-mCherry were generated as previously described and prepared by the University of North Carolina Vector Core (Chapel Hill, North Carolina, USA) and the University of Michigan Vector Core.

For injections, following the induction of isoflurane anesthesia and placement in a stereotaxic frame, the skulls of adult Leprcre/cre, PpgLepRbKO, Ppgcre/cre, PpgPpg-NTSKO, or Ppgfl/fl mice were exposed. The obex was set as reference point for injection. After the reference was determined, a guide cannula with a pipette injector was lowered into the approximate NTS coordinates, which was anterior/posterior, –0.2; medial/lateral, ±0.2; dorsal/ventral, –0.2 from the obex, and 100 nL of virus was injected by using a picospritzer at a rate of 5–30 nL/minutes with pulses. Five minutes following injection, to allow for adequate dispersal and absorption of the virus, the injector was removed from the animal; the incision site was closed and glued. The mice received prophylactic analgesics before and after surgery. The mice injected with AAVFlies-M3Dq and their control were allowed at least 3 weeks to recover from surgery before experimentation.

For the viral KO, we performed post hoc IHC to examine the expression of the viral reporter and GLP1-IR. Animals with robust bilateral reporter expression (and lack of GLP1-IR for the KOs) were deemed hits; other animals were excluded from analysis. For the DREADD studies, we examined reporter expression and FOS-IR following CNO administration and perfusion at the time of euthanasia. Animals with robust bilateral reporter expression and FOS-IR were considered hits; other animals were excluded from analysis.

Phenotypic studies. Animals were singly housed from the time of weaning (PpgLepRbKO and PpgPpg-NTSKO) or beginning 7 days after surgery (PpgAAV-NTSKO). Food intake and body weight were monitored weekly.

For stimulation studies, KO mice, DREADD-expressing mice, or their controls that were either at least 2 months old or 1 month past surgery, and they were treated with saline or CNO (4936, Tocris) at the onset of dark cycle; subsequent food intake was monitored. For chronic food intake and body weight changes, mice were treated with saline (i.p., bid) for 2–3 days prior to injecting saline or CNO (1 mg/kg, i.p., bid) for 4 days (injections were given at ~5:30 p.m. and ~8 a.m.). Mice were subsequently subjected to saline injections for another 2–3 days to monitor recovery.

Perfusion and IHC. Mice were anesthetized with isoflurane and transcardially perfused with PBS followed by 10% buffered formalin. Brains were removed, placed in 10% buffered formalin overnight, and dehydrated in 30% sucrose for 1 week. With use of a freezing microtome (Leica), brains were cut into 30-μm sections. Sections were treated sequentially with 1% hydrogen peroxide/0.5% sodium hydroxide, 0.3% glycine, 0.03% sodium dodecyl sulfate, and blocking solution (PBS with 0.1% triton, 3% normal donkey serum; MilliporeSigma). The sections were incubated overnight at room temperature in rabbit anti-pSTAT3 (Cell Signaling Technology, 9145L; 1:250) and exposed the next day with either biotinylated (1:200 followed by avidin-biotin complex [ABC] amplification and DAB reaction) or fluorescent secondary antibody (Molecular Probes, 1:200) to visualize proteins. Immunofluorescent staining was performed using primary antibodies (FOS, 2250, Cell Signaling Technology; dsRed, 632496, Takara, 1:1000; GLP1, T-4363.0030, Peninsula Laboratories International Inc., 1:1000; TH, NB300-109, Novus Biologicals, 1:1000; choline acetyltransferase, AB144P, MilliporeSigma, 1:500; and prolactin-releasing peptide, H-008-52, Phoenix Pharmaceuticals, 1:1000); antibodies were reacted with species-specific Alexa Fluor–488, –568, or –647 conjugated secondary antibodies (Thermo Fisher Scientific, 1:200). Images were collected on an Olympus BX53F microscope. Images were pseudocolored using Photoshop software (Adobe) or Image J (NIH).

Statistics. Data are reported as mean ± SEM. Statistical analyses of physiologic data were performed with Prism software (version 7). Two-way ANOVA, or paired or unpaired 2-tailed t tests, were used as indicated in the text and figure legends; P < 0.05 was considered statistically significant.

Study approval. The animal procedures performed were approved by the University of Michigan Committee on the Use and Care of Animals in accordance with Association for the Assessment and Approval of Laboratory Animal Care and NIH guidelines.
Author contributions

WC, EN, CH, KR, AM, BK, JM, and KSK researched and analyzed data and proofread the manuscript. WC, DPO, RJS, DS, CJR, and MGM designed experiments and wrote and edited the manuscript. MGM is the guarantor of the manuscript.

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