Liraglutide Modulates Appetite and Body Weight Through Glucagon-Like Peptide 1 Receptor–Expressing Glutamatergic Neurons

Jessica M. Adams,[1] Hongjuan Pei,[1] Darleen A. Sandoval,[2] Randy J. Seeley,[2] Rui B. Chang,[3] Stephen D. Liberles,[3] and David P. Olson[1]

Diabetes 2018;67:1538–1548 | https://doi.org/10.2337/db17-1385

Glucagon-like peptide 1 receptor (GLP-1R) agonists are U.S. Food and Drug Administration–approved weight loss drugs. Despite their widespread use, the sites of action through which GLP-1R agonists (GLP1RAs) affect appetite and body weight are still not fully understood. We determined whether GLP-1Rs in either GABAergic or glutamatergic neurons are necessary for the short- and long-term effects of the GLP1RA liraglutide on food intake, visceral illness, body weight, and neural network activation. We found that mice lacking GLP-1Rs in *vGAT*-expressing GABAergic neurons responded identically to controls in all parameters measured, whereas deletion of GLP-1Rs in *vGlut2*-expressing glutamatergic neurons eliminated liraglutide-induced weight loss and visceral illness and severely attenuated its effects on feeding. Concomitantly, deletion of GLP-1Rs from glutamatergic neurons completely abolished the neural network activation observed after liraglutide administration. We conclude that liraglutide activates a dispersed but discrete neural network to mediate its physiological effects and that these effects require GLP-1R expression on glutamatergic but not GABAergic neurons.

The incretin glucagon-like peptide 1 (GLP-1) is produced mainly in intestinal L cells and a discrete population of hindbrain neurons. GLP-1 is released from the intestine after a meal and acts through its receptor (GLP-1R) to increase insulin and decrease glucagon secretion in a glucose-dependent manner (1). As such, long-acting synthetic GLP-1R agonists (GLP1RAs) are useful therapeutic agents to treat type 2 diabetes (2). Of note, long-acting GLP1RAs, such as liraglutide, not only improve blood glucose homeostasis but also cause significant weight loss (3). In rodents and humans, the weight loss is associated with a reduction in food intake with little effect on energy expenditure (4,5). Despite these clinically significant effects on body weight, the cellular mechanisms by which GLP1RAs modulate feeding are unclear, and understanding these mechanisms remains an important research goal.

GLP-1R is expressed in the pancreas, heart, kidney, gastrointestinal tract, and brain (1). We and others have shown that GLP-1R–expressing cells in the central nervous system (CNS) are required for the full anorectic and body weight effects of peripherally administered liraglutide in both mice and rats (6,7). GLP-1Rs are distributed across the CNS, including in areas known to be critical for the regulation of food intake and body weight (8,9). The fact that local microinfusion of GLP1RAs into numerous CNS nuclei is sufficient to decrease food intake (10) suggests that many GLP-1R–expressing neuronal populations contribute to the anorectic effects of peripherally administered liraglutide. To date, genetic deletion of GLP-1R from specific brain nuclei known to be involved in energy balance has shown that GLP-1Rs in the paraventricular hypothalamus (PVH), arcuate nucleus, and ventromedial nucleus of the hypothalamus are not necessary for the anorectic effects of peripherally administered GLP1RAs (11–13).

These results are consistent with a model in which the anorectic effects of GLP1RAs are distributed across the CNS rather than contained in a single anatomic site (10). Because cell-specific targeting of GLP-1R expression

[1]Department of Pediatrics, University of Michigan, Ann Arbor, MI
[2]Department of Surgery, University of Michigan, Ann Arbor, MI
[3]Department of Cell Biology, Harvard Medical School, Boston, MA
Corresponding author: David P. Olson, dpolson@med.umich.edu.

Received 15 November 2017 and accepted 6 May 2018.

This article contains Supplementary Data online at http://diabetes.diabetesjournals.org/lookup/suppl/doi:10.2337/db17-1385/-/DC1.

has yet to identify critical neural populations required for GLP1RA action, we used established neuron-specific Cre lines to target broad and mostly nonoverlapping neuronal populations: *vGlut2*-expressing glutamatergic and *vGAT*-expressing GABAergic neurons (14). With the use of Cre-dependent reporter animals, we examined and cataloged the brain regions and neuronal subtypes activated by liraglutide and found a discrete pattern of neural activation that encompasses both glutamatergic and GABAergic neurons. We then deleted GLP-1R from each population and examined the behavioral and physiological outcomes at baseline and in response to short- and long-term liraglutide treatment in both lean and obese mouse models. We found that GLP-1Rs expressed in GABAergic neurons are dispensable for liraglutide-induced anorexia, weight loss, and neural network activation, whereas GLP-1Rs in glutamatergic neurons are required for these effects.

RESEARCH DESIGN AND METHODS

Animals

Glp1r-flox (6), *vGAT*-Cre (14), *vGlut2*-Cre (14), *Glp1r*-Cre (15), and *L10*-GFP reporter (16) mice have been described previously (see Supplementary Data for breeding strategies). All studies were approved by the University of Michigan institutional animal care and use committee.

Immunohistochemistry

Pair-housed *vGAT*-GFP, *vGlut2*-GFP, and *Glp1r*-GFP (n = 7–8 per genotype and treatment, sexes combined) and singly housed male *Glp1r*-flox, vGAT$^{\Delta Glp1r}$, and vGlut2$^{\Delta Glp1r}$ (n = 5 per genotype and treatment) were used for Fos analysis. Mice were fasted at 9:00 A.M. and subcutaneously injected with either liraglutide (400 μg/kg; Novo Nordisk) or saline (10 mL/kg body weight) at 11:00 A.M. At 1:00 P.M., mice were perfused and brains processed for immunostaining as previously described (17) (see Supplementary Data for antibodies used and protocol).

Microscopy and Image Analysis

Coordinates and landmarks for regions of interest were identified by counterstaining and the mouse brain atlas (18) (Supplementary Table 1). For each nucleus, counts from the left and right side were added, and then those from each coronal plane were averaged to yield one count per nucleus per mouse (see Supplementary Data regarding image acquisition and analysis).

Model Characterization

Knockdown of *Glp1r* in the hypothalami of vGAT$^{\Delta Glp1r}$ and vGlut2$^{\Delta Glp1r}$ mice was confirmed by semiquantitative real-time PCR (RT-PCR). Hypothalamic tissue was microdissected from male mice in the long-term liraglutide study (see below), with treatment groups collapsed (n = 13–14 per genotype) and processed and analyzed as previously described (19) (Supplementary Data).

For in situ hybridization (ISH), *Glp1r*-flox, vGAT$^{\Delta Glp1r}$, and vGlut2$^{\Delta Glp1r}$ mice were decapitated under anesthesia; whole brains were dissected, flash-frozen in isopentane chilled on dry ice, and stored at −80°C. Sixteen-micrometer-thick cryostat coronal sections were thaw-mounted to slides and stored at −80°C. Slides were processed for ISH using RNAScope per the manufacturer's protocol (Advanced Cell Diagnostics), and the multiplex fluorescent assay (320850) was used to visualize *Glp1r* (418851) and *Cre* (312281-C3) probes using Amp 4 Alt-A.

Animal Studies

Dosages of liraglutide were chosen on the basis of previous studies (6). For the longitudinal study, mice (n = 10 per sex and genotype) were singly housed at age 4 weeks, and body weight and food intake were monitored weekly. At age 13–15 weeks, mice were fasted at 10:00 A.M. and then subcutaneously injected with either liraglutide (400 μg/kg) or saline at 5:00 P.M. Preweighed food was given at 6:00 P.M., and food intake was measured 1, 2, 4, and 24 h later. Body weight was measured at the 24-h time point. One week later, all mice were given the opposite treatment (saline or liraglutide) in a crossover design, and the study was repeated. At age 15–17 weeks, mice were fasted at 9:00 A.M., and basal fasting blood glucose was measured at 1:00 P.M. with a glucometer (Contour Next EZ; Bayer). Mice were then injected with liraglutide (400 μg/kg) or saline as above and blood glucose measured at 3:00 P.M. The crossover treatment was performed 1 week later. At age 18 weeks, brains from the male *Glp1r*-flox, vGAT$^{\Delta Glp1r}$, and vGlut2$^{\Delta Glp1r}$ mice were collected for Fos immunohistochemistry (IHC) analysis.

For the long-term liraglutide study, male *Glp1r*-flox, vGAT$^{\Delta Glp1r}$, and vGlut2$^{\Delta Glp1r}$ mice (n = 6–8 per genotype and treatment) were weaned into group housing with standard rodent chow. At age 5 weeks, the food was switched to high-fat chow (D12451; Research Diets), and the mice were singly housed at age 9 weeks. At age 11 weeks, mice were randomly assigned to either the saline control or liraglutide group and subcutaneously injected once per day (5:00 P.M.) for 2 weeks using a previously described ascending dosing schedule (6). Body weight and food intake were measured daily. On day 15, mice were decapitated under anesthesia and the hypothalamus was isolated, snap-frozen on dry ice, and stored at −80°C for semiquantitative RT-PCR analysis (see above). Gonadal and perirenal fat pads were removed and weighed. To assay conditioned taste avoidance (CTA), male and female *Glp1r*-flox, vGAT$^{\Delta Glp1r}$, and vGlut2$^{\Delta Glp1r}$ mice (n = 8 per genotype and treatment) were singly housed at age 10–12 weeks, and the assay was performed as previously described (20) (Supplementary Data).

Statistics

Data are presented as mean ± SEM, and statistical analyses were performed with GraphPad Prism 7 software. Specific tests performed for each experiment, along with n values, are included in the figure legends. Post hoc tests after ANOVA were performed only if the interaction between the two variables was significant at $P < 0.05$.

RESULTS

Liraglutide Activates Both GABAergic and Glutamatergic Neurons

To characterize the subtypes of neurons activated by liraglutide, we generated *vGAT*- and *vGlut2*-GFP mice to label GABAergic and glutamatergic neurons, respectively (Fig. 1*A*). We then performed IHC for Fos, an early marker of neural activation, after liraglutide or saline injection. Representative images from brain regions known to be activated by GLP1RAs (21,22) show specific GFP expression in the GABAergic central amygdala (CeA) versus the glutamatergic basolateral amygdala and lateral parabrachial nucleus (lPBN) (Fig. 1*B* and *C*). Liraglutide induced a significant increase in Fos+ cells in five regions: CeA, lPBN, bed nucleus of the stria terminalis (BNST), caudal nucleus of the solitary tract (cNTS), and area postrema (AP) (Fig. 1*D–H*). No effect of liraglutide on Fos activation in the lateral septum, paraventricular thalamus, PVH, arcuate, ventromedial hypothalamus, lateral dorsal tegmental nucleus, or rostral NTS were found (Supplementary Fig. 1).

The CeA and BNST are largely GABAergic regions, and 93.6 ± 1.2% and 88.9 ± 2.7%, respectively, of the Fos+ neurons in these regions were *vGAT*-GFP+. Conversely, in the glutamatergic lPBN, 95.4 ± 1.1% of Fos+ neurons were *vGlut2*-GFP+. The cNTS and AP were more heterogeneous

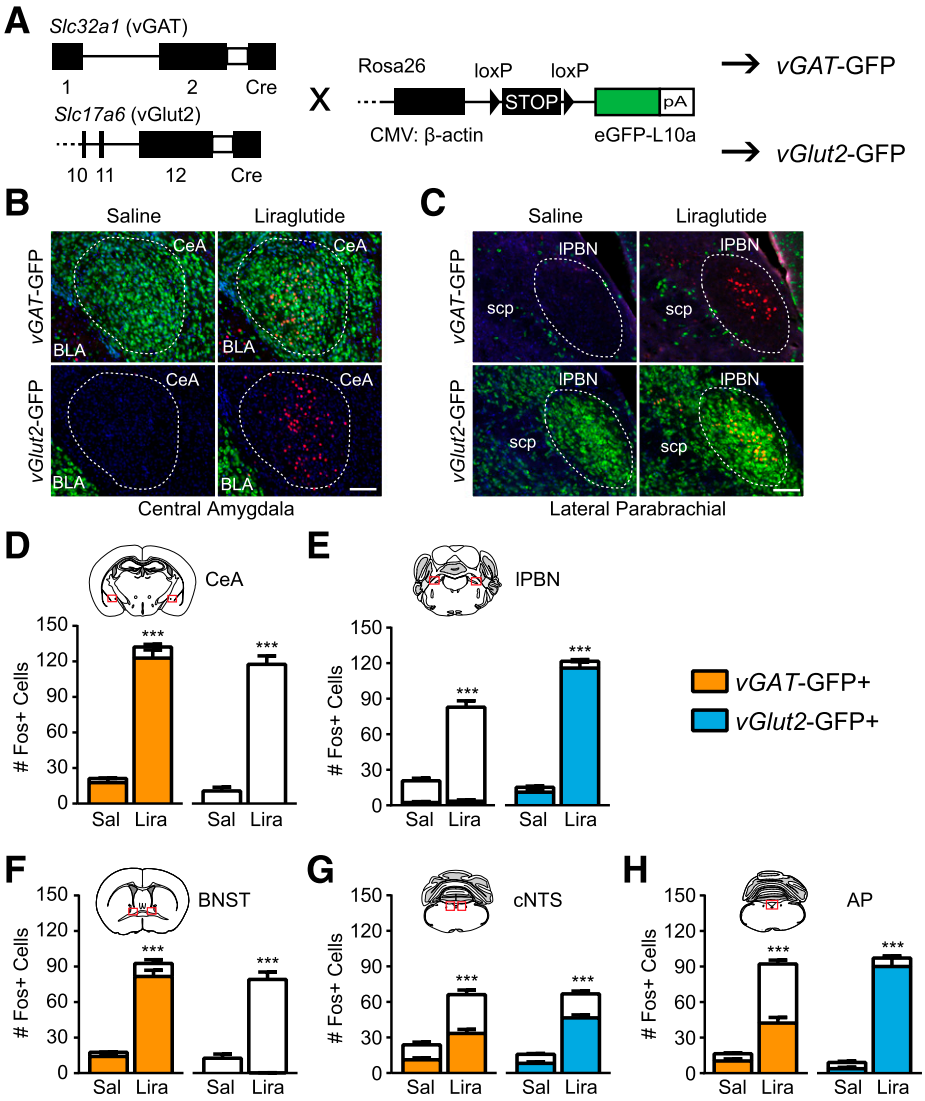

Figure 1—Characterization of neuronal subtypes activated by liraglutide. *A*: Breeding schematic with genetic constructs used to generate *vGAT*-GFP and *vGlut2*-GFP mice. *B* and *C*: Representative images from the CeA (*B*) and lPBN (*C*), showing Fos IHC (red) after saline or liraglutide injection in *vGAT*-GFP (green) and *vGlut2*-GFP (green) neurons. Blue is DAPI. Scale bars = 100 μm. Regions of interest for quantification are highlighted by a dotted line. *D–H*: Quantification of Fos+ neurons per section in five regions. The proportion of Fos+ neurons positive for *vGAT*-GFP or *vGlut2*-GFP is indicated ($n \geq 6$). Data were analyzed by unpaired Student *t* test between drug treatments. White bars indicate GFP-negative neurons. ***$P < 0.001$. BLA, basolateral amygdala; CMV, cytomegalovirus; eGFP, enhanced green fluorescent protein; Lira, liraglutide; Sal, saline; scp, superior cerebellar peduncle.

in the expression of *vGlut2*- and *vGAT*-GFP. In the cNTS, 50.9 ± 3.5% of Fos+ cells were *vGAT*-GFP+, and 69.9 ± 2.8% were *vGlut2*-GFP+. In the AP, 45.4 ± 3.3% of Fos+ cells were *vGAT*-GFP+, and 93.2 ± 1.5% were *vGlut2*-GFP+. These combined percentages exceed 100, suggesting that a fraction of cNTS and AP neurons express both *vGAT* and *vGlut2* during development. Thus, peripheral liraglutide injection leads to activation of specific brain regions containing both GABAergic and glutamatergic neurons, consistent with a model of distributed GLP1RA action (10).

GLP-1R–Expressing Glutamatergic Neurons Mediate the Short-term Feeding Effects of Liraglutide

To test the role of GLP-1Rs expressed in GABAergic versus glutamatergic neurons in response to GLP1RAs, we generated mice lacking *Glp1r* in either *vGlut2*- or *vGAT*-expressing cells (Fig. 2*A*). In the hypothalamus, *Glp1r* mRNA expression levels were 45.3 ± 5.1% and 50.2 ± 5.3% of controls in vGAT$^{\Delta Glp1r}$ and vGlut2$^{\Delta Glp1r}$ mice, respectively (Fig. 2*B*). To confirm that this knockdown was cell-type specific, we performed double ISH for *Glp1r* and *Cre* transcripts in the liraglutide-responsive AP, which comprises both glutamatergic and GABAergic cell types. *Glp1r*-flox control mice had robust expression of *Glp1r* and no *Cre* throughout the AP. In vGAT$^{\Delta Glp1r}$ mice, no overlap of *Cre* and *Glp1r* in the AP was found. In vGlut2$^{\Delta Glp1r}$ mice, we observed many neurons expressing *Cre*, but *Glp1r* expression was absent throughout the AP (Fig. 2*C*). The absence of *Glp1r* from the AP of vGlut2$^{\Delta Glp1r}$ mice is consistent with transient developmental *vGlut2* expression across this region.

vGAT$^{\Delta Glp1r}$ and vGlut2$^{\Delta Glp1r}$ mice exhibited normal body weight, food intake, and blood glucose at baseline (Supplementary Fig. 2). We then assessed the response of these genotypes to GLP1RA administration with regard to food intake, body weight, and blood glucose. After liraglutide injection, control and vGAT$^{\Delta Glp1r}$ mice of both sexes decreased their 24-h food intake by 60–70% compared with saline injection. Liraglutide produced a significant but dramatically attenuated anorectic effect in vGlut2$^{\Delta Glp1r}$ mice, with 24-h food intake decreasing by only 15–20% compared with saline treatment (Fig. 3*A*). Of note, liraglutide-induced anorexia was delayed in vGlut2$^{\Delta Glp1r}$ mice relative to vGAT$^{\Delta Glp1r}$ and control mice (Supplementary Fig. 3), suggesting that some aspect of the effects of GLP1RAs may be mediated by non-*vGlut2*–expressing neurons. These results indicate that GLP-1Rs in glutamatergic neurons are required for the full anorectic effect of liraglutide.

Twenty-four hours after liraglutide injection, control and vGAT$^{\Delta Glp1r}$ mice lost ~5–6% of their body weight, whereas vGlut2$^{\Delta Glp1r}$ mice maintained normal body weight (Fig. 3*B*). Liraglutide lowered blood glucose equally in males of all three genotypes, but in females, only control and vGAT$^{\Delta Glp1r}$ mice responded (Supplementary Fig. 4). Because liraglutide is known to cause visceral illness, we tested whether this effect requires GLP-1R in GABAergic

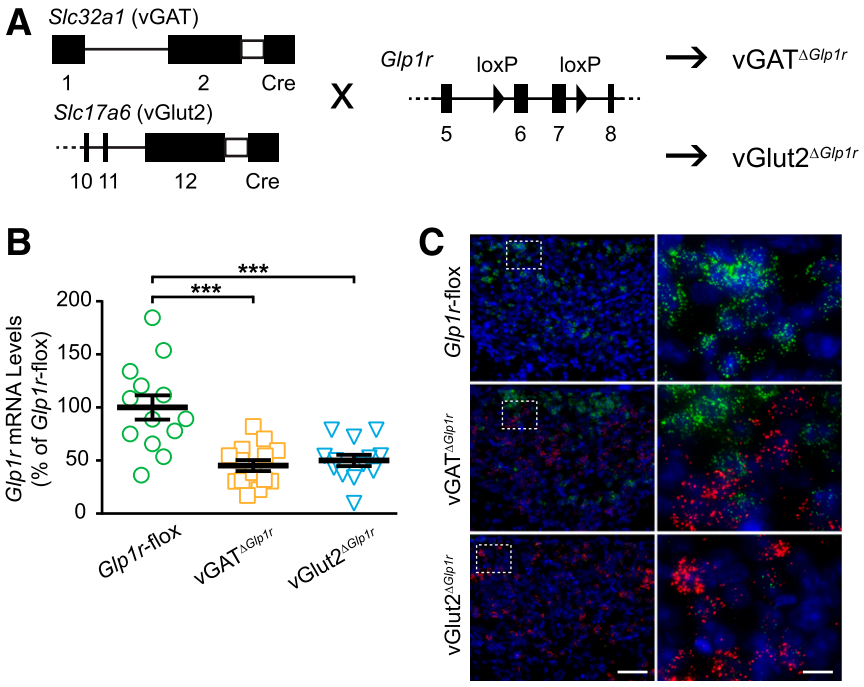

Figure 2—Generation and validation of vGAT$^{\Delta Glp1r}$ and vGlut2$^{\Delta Glp1r}$ mice. *A*: Breeding schematic with genetic constructs. *B*: Semiquantitative RT-PCR of *Glp1r* in the hypothalamus of male mice ($n \geq 13$ per group). Data analyzed by one-way ANOVA. ***$P < 0.001$ by Tukey multiple comparisons test. *C*: Representative ISH images showing efficient knockdown of *Glp1r* (green) in cells containing *Cre* recombinase (red) in the AP. Blue is DAPI. Inset in left panels are shown at higher magnification in the right panels. Scale bars = 50 μm (left panels) and 10 μm (right panels).

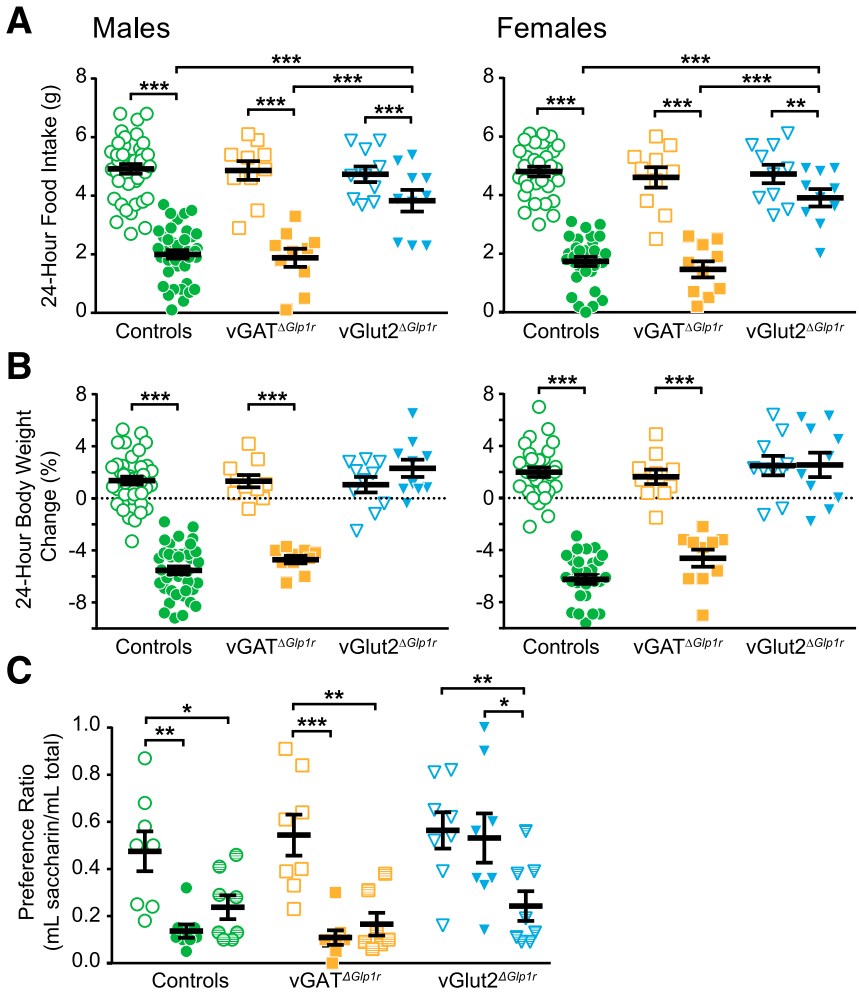

Figure 3—Short-term effects of liraglutide in vGAT$^{\Delta Glp1r}$ and vGlut2$^{\Delta Glp1r}$ chow-fed mice. Males and females are displayed separately (*A* and *B*) and combined (*C*). Change in total food intake (*A*) and body weight (*B*) 24 h after liraglutide or saline control injection. CTA test results (*C*) display the taste preference for saccharin after pairing with either saline, liraglutide, or lithium chloride (positive control). $n \geq 10$ (*A* and *B*) and $n = 8$ (*C*). Repeated-measures two-way ANOVA (*A* and *B*) or standard two-way ANOVA (*C*) was performed followed by Sidak (*A* and *B*) or Tukey (*C*) multiple comparisons test. Open symbols indicate saline treatment, partially filled symbols indicate lithium chloride treatment, and closed symbols indicate liraglutide treatment. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

or glutamatergic neurons by using a CTA assay. After learning to associate a novel taste (saccharin water) with lithium chloride (positive control), mice of all genotypes demonstrated a decreased preference for the saccharin water, indicating that the lithium chloride was aversive (Fig. 3*C*). In contrast, liraglutide induced CTA in control and vGAT$^{\Delta Glp1r}$ but not vGlut2$^{\Delta Glp1r}$ mice, thus revealing that GLP-1Rs in glutamatergic neurons are also necessary for the visceral illness response to liraglutide.

Long-term Liraglutide-Induced Weight Loss Is Mediated by GLP-1R–Expressing Glutamatergic Neurons

To model the clinical use of liraglutide as an obesity therapy, we fed a second cohort of male *Glp1r*-flox, vGAT$^{\Delta Glp1r}$, and vGlut2$^{\Delta Glp1r}$ mice a high-fat diet to induce obesity. After 6 weeks, all three genotypes had gained significant weight (15–20%) compared with genotype- and age-matched controls from the previous study, with no differences between groups (*Glp1r*-flox 29.5 ± 0.6 g, vGAT$^{\Delta Glp1r}$ 29.6 ± 0.6 g, vGlut2$^{\Delta Glp1r}$ 31.2 ± 0.7 g; not significant by one-way ANOVA).

Obese mice were then treated daily with liraglutide or saline for 14 days. As seen in the short-term studies, *Glp1r*-flox control and vGAT$^{\Delta Glp1r}$ mice showed a decrease in food intake and body weight 24 h after liraglutide injection, whereas no effect was observed in vGlut2$^{\Delta Glp1r}$ mice. Body weights of *Glp1r*-flox control and vGAT$^{\Delta Glp1r}$ mice plateaued at ~90% of original weight after 3 days of liraglutide treatment, whereas saline-treated mice steadily gained weight (Fig. 4*A*). In contrast, liraglutide had no effect on weight gain in vGlut2$^{\Delta Glp1r}$ mice. The sustained body weight loss in *Glp1r*-flox and vGAT$^{\Delta Glp1r}$ mice was accompanied by a significant but transient drop in food intake that returned to normal after 4 days of treatment (Fig. 4*B*). Food intake of vGlut2$^{\Delta Glp1r}$ mice was not affected by

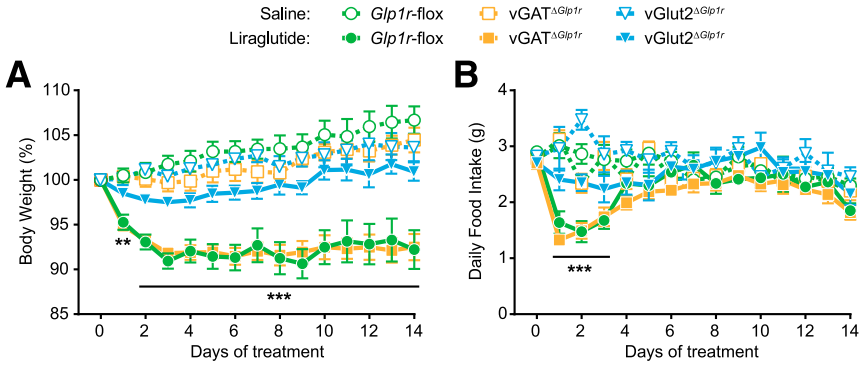

Figure 4— Long-term effects of liraglutide in vGAT$^{\Delta Glp1r}$ and vGlut2$^{\Delta Glp1r}$ high-fat diet–fed male mice. Body weight change from baseline (*A*) and daily food intake (*B*) during a 14-day treatment with once daily liraglutide or saline control injection. All mice were fed a 45% high-fat diet 6 weeks before and during the study ($n \geq 6$). Data were analyzed by repeated-measures two-way ANOVA followed by Dunnett multiple comparisons test. $^{**}P < 0.01$, $^{***}P < 0.001$ at each time point compared with saline-treated controls of the same genotype.

liraglutide during the study. Long-term liraglutide reduced visceral fat pad weights in *Glp1r*-flox controls and vGAT$^{\Delta Glp1r}$ mice but had no effect on fat pad weights in vGlut2$^{\Delta Glp1r}$ mice (Supplementary Fig. 5). Together, these results suggest that GLP-1R–expressing glutamatergic neurons mediate both the short-term anorexia and the long-term weight loss effects of liraglutide.

Liraglutide Activates a Neuronal Network Through GLP-1R–Expressing Glutamatergic Neurons

To identify neural responses associated with these different behavioral phenotypes, we examined Fos IHC after saline and liraglutide treatment in male *Glp1r*-flox, vGAT$^{\Delta Glp1r}$, and vGlut2$^{\Delta Glp1r}$ mice. In both *Glp1r*-flox controls and vGAT$^{\Delta Glp1r}$ mice, liraglutide treatment significantly increased Fos+ cells in the same regions identified previously (i.e., CeA, BNST, lPBN, cNTS, AP). Somewhat surprisingly, Fos activation in all of these regions (both glutamatergic and GABAergic) was completely absent in vGlut2$^{\Delta Glp1r}$ mice (Fig. 5). These data suggest that a population of glutamatergic GLP-1R–expressing neurons are activated directly by liraglutide and then engage a downstream neural network that is both glutamatergic and GABAergic to elicit potent physiological effects.

Glp1r–Expressing Neurons in the lPBN and AP Are Activated by Liraglutide

To identify which GLP-1R–expressing neurons are directly activated by liraglutide, we generated *Glp1r*-GFP mice (Fig. 6*A*). The pattern of *Glp1r*-GFP expression across the brain generally agreed with that reported for *Glp1r* expression using other methods (8,9) (Supplementary Fig. 6). *Glp1r*-GFP mice were injected with saline or liraglutide for subsequent Fos analysis. Although *Glp1r*-GFP+ neurons were found in all five identified regions of interest, most were not activated by liraglutide. In the CeA, BNST, and cNTS, only 9.4 ± 1.1%, 10.7 ± 1.0%, and 9.4 ± 1.2%, respectively, of Fos+ neurons also expressed *Glp1r*-GFP. On the other hand, 28.6 ± 2.2% and 84.3 ± 1.3% of lPBN and AP Fos+ neurons, respectively, were *Glp1r*-GFP+ (Fig. 6*B* and *C*). Thus, the lPBN and AP contained the highest percentage of liraglutide-activated Fos+ neurons that also expressed *Glp1r*-GFP, suggesting that glutamatergic neurons in these regions are critical for GLP1RA actions.

DISCUSSION

Consistent with previously published data on other GLP1RAs (21–24), we found that peripheral liraglutide activates a rather limited network of cells in the BNST, CeA, lPBN, cNTS, and AP. Of the five liraglutide-activated nuclei that we identified, two (BNST, CeA) are primarily composed of *vGAT*-positive neurons, one (lPBN) almost exclusively is composed of *vGlut2*-positive neurons, and two (cNTS, AP) contain a mixture of both cell types. To test the role of these neuron groups in mediating the effects of liraglutide, we deleted *Glp1r* specifically in neurons expressing *vGAT* or *vGlut2*. We found that mice lacking GLP-1R in *vGlut2*-expressing neurons had a blunted anorectic response to liraglutide and did not lose body weight after short- or long-term treatment, whereas loss of *Glp1r* from *vGAT*-expressing neurons had no effect on liraglutide responsiveness. These data indicate that GLP-1Rs in *vGlut2*-expressing neurons are required for the majority of the anorectic and weight loss effects of liraglutide. Furthermore, we found that GLP1RA administration was associated with a distinct pattern of neuronal activation across both glutamatergic and GABAergic cell groups that depends on a direct effect of liraglutide on GLP-1R–expressing *vGlut2*+ neurons.

Nausea is a common early side effect of GLP1RAs in humans and may contribute to the initial appetite loss and subsequent weight loss (25). In rodents, drug-induced visceral illness can be inferred by the formation of a CTA to a novel taste paired with the drug (with the caveat that rats will sometimes exhibit CTA even to substances that they find rewarding, such as amphetamines [26]) and/or by pica (consumption of a nonnutritive clay after drug

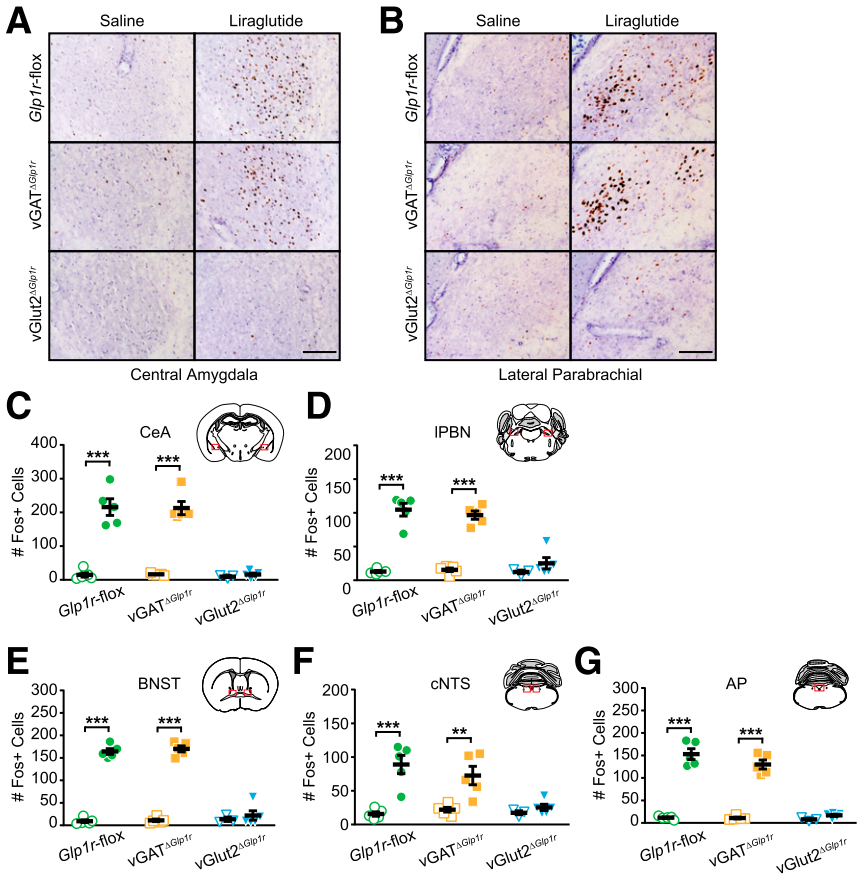

Figure 5— Neuronal activation in vGAT$^{\Delta Glp1r}$ and vGlut2$^{\Delta Glp1r}$ mice after liraglutide injection. *A* and *B*: Representative images from the CeA (*A*) and lPBN (*B*) showing Fos IHC (brown) after saline or liraglutide injection. Sections were counterstained with hematoxylin. Scale bars = 100 μm. *C–G*: Quantification of Fos+ neurons in each of five brain regions ($n \geq 3$). Data were analyzed by two-way ANOVA followed by Tukey multiple comparisons test. Open symbols indicate saline treatment, and closed symbols indicate liraglutide treatment. $^{**}P < 0.01$, $^{***}P < 0.001$.

treatment). Long-term liraglutide treatment induced pica in rats only at the onset of treatment, mirroring reports of nausea in patients (27). Although well characterized in rats, the validity of the pica assay has been questioned in mice (28). In both rats and mice, peripheral administration of GLP1RAs also led to the formation of a CTA that is mediated by GLP-1R–expressing cells in the brain (6,21,27). Some evidence has suggested that the nausea and anorexia caused by GLP1RAs are achieved by separate and dissociable mechanisms. For example, in rats, direct administration of GLP1RAs into the fourth ventricle (29), the PVH (30), and the nucleus accumbens (31) reduces food intake without inducing CTA, although this may be dose dependent.

The CeA has been implicated as a neural mediator for the aversive effects of GLP1RAs because GLP1RA infusion into the CeA induced CTA without reducing food intake (27,29). The current results suggest that the CeA is not a direct site of action for peripherally administered liraglutide (although it could be a downstream target) because mice lacking *Glp1r* in the GABAergic CeA still formed a CTA to liraglutide. On the other hand, bona fide species differences between mice and rats, particularly pertaining to the GLP-1 system and CTA, have been identified previously (32). Thus, we cannot exclude the possibility that the CeA is an important direct site for GLP-1–induced aversion in rats but not in mice. Nevertheless, the current results suggest that although the sites of action may be separable, both the aversive and the anorectic effects of liraglutide require GLP-1R expression on *vGlut2*+ neurons.

Studies that used various methodological approaches in rats have implicated vagal GLP-1Rs in the anorectic effect of intraperitoneally administered GLP1RAs (7,33,34). However, rats subjected to subdiaphragmatic vagal afferent deafferentation responded normally to intravenous and subcutaneous liraglutide injection (35,36). Taken together, these studies suggest that the sites (peripheral vs. central) of action of GLP1RAs may differ depending on the route of drug administration as well as on the dosage. The current study was not specifically designed to test the role of vagal versus central GLP-1R. Because GLP-1R–expressing neurons in the nodose ganglion are glutamatergic (15) and would be deleted in vGlut2$^{\Delta Glp1r}$ mice, a contribution of vagal afferent glutamatergic GLP-1R–expressing neurons in the anorectic effect of liraglutide cannot be fully excluded. However, mice lacking GLP-1Rs on vagal

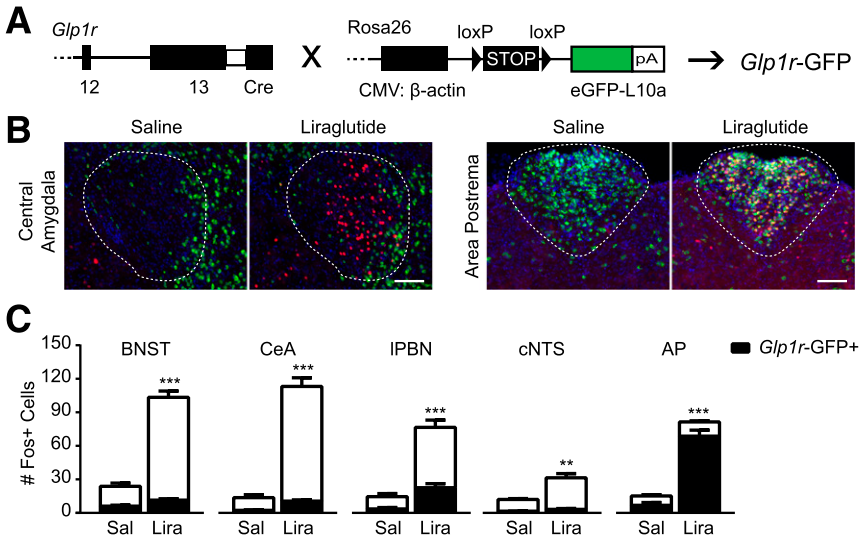

Figure 6—Identification of liraglutide-activated neurons expressing *Glp1r*. *A*: Breeding schematic with genetic constructs used to generate *Glp1r*-GFP mice. *B*: Representative images from the CeA and AP showing Fos IHC (red) after saline or liraglutide injection in *Glp1r*-GFP (green) neurons. Blue is DAPI. Scale bars = 100 μm. Regions of interest for quantification are highlighted by a dotted line. *C*: Quantification of Fos+ neurons per section in each region. In each column, the proportion of Fos+ neurons positive for *Glp1r*-GFP is indicated in black ($n \geq 6$). Data were analyzed by unpaired Student *t* test between drug treatments. White bars indicate GFP-negative neurons. $^{**}P < 0.01$, $^{***}P < 0.001$. CMV, cytomegalovirus; eGFP, enhanced green fluorescent protein; Lira, liraglutide; Sal, saline.

afferents responded normally to the same dose of subcutaneous liraglutide used in this study (6), underscoring the importance of central GLP-1R–expressing neurons in our paradigm.

GLP-1–expressing neurons in the brainstem are glutamatergic and project to the hypothalamus (37,38). Activation of brainstem GLP-1 neurons induces Fos expression in hypothalamic regions (39), suggesting that the hypothalamus mediates some of the effects of endogenous brainstem-derived GLP-1. However, the role of the hypothalamus in the physiological response to peripherally administered GLP1RAs is less clear. Some studies have found hypothalamic neuronal activation after short-term peripheral GLP1RA injection, whereas others, including the current study, have not (21,22,40). Mice with GLP-1R deleted from the majority of the hypothalamus (Nkx2.1-Cre), the PVH (Sim1-Cre), or the ventromedial nucleus (SF1-Cre) had normal anorectic responses to peripherally administered GLP1RAs (11–13). In addition, decerebrate rats, in which hindbrain-forebrain connectivity is severed, reduced their food intake comparably with control rats after peripheral GLP1RA administration (41). The current data support a model in which hypothalamic GLP-1Rs are not required for the anorectic effects of peripherally administered GLP1RAs.

In this study, mice lacking GLP-1R in either GABAergic or glutamatergic neurons did not differ from controls in body weight or food intake under basal conditions or when fed a high-fat diet, suggesting that GLP-1R in these neural populations is not necessary for normal appetite and body weight regulation. Indeed, whole-body *Glp1r*-knockout as well as pan-neuronal *Glp1r*-knockout mice exhibit normal food intake and body weight (6,42). However, the possibility of developmental compensation for loss of this receptor should not be overlooked. In rats, intracerebroventricular injection of a specific GLP-1R antagonist, exendin(9-39) has been shown to increase food intake in the short and long term, suggesting that neuronal GLP-1Rs contribute to normal feeding behavior (43,44). Site-specific manipulations, such as antagonist microinfusion or RNA interference–mediated *Glp1r* knockdown are further unraveling the roles of specific *Glp1r*-expressing populations in normal physiology (45–47). Future studies in mice should use site-specific viral delivery for Cre-mediated excision of *Glp1r* in adult mice or tamoxifen-inducible mouse strains to circumvent developmental compensation.

Endogenous GLP-1 can regulate feeding in the short term by signaling satiety as well as by inhibiting gastric emptying (34,48). Because synthetic GLP1RAs have been engineered for prolonged biological activity, it is plausible that the actions of GLP1RA will mimic those of endogenous GLP-1 in addition to inducing visceral illness. In the current study, control animals ate significantly less food within 1 h of liraglutide injection, and this effect was maintained for 24 h. Of note, vGlut2$^{\Delta Glp1r}$ mice also exhibited a blunted food intake reduction at the 4- and 24-h time points after liraglutide administration, suggesting a role for nonglutamatergic GLP-1R–expressing cells in some aspect of the anorectic response. Future meal pattern analyses and gastric emptying studies will clarify the role of specific neuronal subsets in these physiological responses.

Long-term liraglutide administration decreased food intake for only 3 days in control and vGAT$^{\Delta Glp1r}$ mice, whereas body weight was reduced throughout the study.

Persistent weight reduction in the setting of transient anorexia suggests establishment of a new body weight set point. This phenomenon is seen with multiple weight loss regimens (e.g., lorcaserin [49], vertical sleeve gastrectomy [50]), and the mechanism underlying this shared response requires additional investigation. Future studies are needed to determine whether this transient anorexia reflects cellular desensitization or changes in neuronal response patterns after long-term liraglutide administration.

Liraglutide induces a characteristic pattern of Fos expression in a mixture of GABAergic and glutamatergic cell groups; moreover, this pattern requires GLP-1Rs on *vGlut2*+ but not *vGAT*+ neurons, which supports the hypothesis that a population of GLP-1R–expressing glutamatergic neurons serves as a gateway to transduce the signal of peripheral liraglutide into the brain. To identify the critical population of neurons, we treated *Glp1r*-GFP reporter mice with liraglutide and examined Fos activation specifically in *Glp1r*+ cells. We found that the greatest overlap of GLP-1R-GFP expression and Fos activation was within the AP and to a lesser extent in the lPBN, suggesting that glutamatergic cells in these two brain regions may be particularly important for liraglutide action.

The AP is a sensory circumventricular organ known to play important roles in autonomic output, nausea, and the response to emetic drugs (51). It is activated in response to peripheral administration of GLP1RAs (24,52), and liraglutide binds the AP in a GLP-1R–dependent manner (35). The current results suggest that the AP is an important site mediating the anorectic effects of GLP1RAs. In contrast, AP lesions in rats did not decrease the short-term anorectic or long-term weight loss effects of GLP1RAs (35,53). Although the apparent contradiction of these results with the current study might be explained by differences in drug, dose, species, and/or experimental paradigm, this discrepancy suggests the existence of both AP-dependent and AP-independent responses to peripheral GLP1RAs. The lPBN could be a candidate site for AP-independent actions of GLP1RAs because *1*) it is glutamatergic, *2*) it expresses GLP-1R and is activated by peripheral GLP1RAs (23,24), and *3*) direct lPBN infusion of exendin-4 decreases food intake and body weight (46). The NTS also has been identified as a potential site of action of GLP1RAs (10). Although the current study cannot completely exclude an NTS contribution, we found little liraglutide-induced Fos in NTS *Glp1r*-GFP cells. Furthermore, ISH revealed very few neurons expressing *Glp1r* mRNA in this region (data not shown). Determining the relative contribution of each of these regions to GLP1RA action will require future nucleus-specific manipulations.

In conclusion, we have used genetic mouse models to clarify the mechanisms whereby liraglutide decreases food intake and body weight. We found that short-term peripheral liraglutide injection activates several brain regions that comprise both GABAergic and glutamatergic neurons. By inactivating *Glp1r* in each population, we determined that liraglutide acts through GLP-1R exclusively expressed on glutamatergic, *vGlut2*-expressing neurons to activate a specific neuronal circuit, induce visceral illness, and reduce food intake and body weight in both the short and the long term. Moreover, GLP-1R expressed in GABAergic, *vGAT*-expressing neurons is not required for these effects. GLP-1R–expressing neurons in the AP and lPBN may be important direct targets through which peripherally administered liraglutide engages the CNS. Understanding the neural mechanisms by which liraglutide exerts its beneficial effects not only will help to elucidate its mechanism of action but also may reveal neural circuit components that might be exploited for more effective weight loss therapies.

Acknowledgments. The authors acknowledge the assistance of all the members of D.P.O.'s laboratory (University of Michigan), especially Korri Burnett and Allen Zhu, for mouse colony maintenance and genotyping.

Funding. These studies were funded by the following National Institute of Diabetes and Digestive and Kidney Diseases grants: T32-DK-101357 (to J.M.A.), DK-082480 (to D.A.S.), DK-093848 and DK-107652 (to R.J.S.), DK-103703 (to S.D.L.), and DK-104999 (to D.P.O.).

Duality of Interest. J.M.A., D.A.S., R.J.S., and D.P.O. received funding from Novo Nordisk. This work was supported in part by Novo Nordisk, which also supplied the liraglutide. The investigators also received support from Boehringer Ingelheim (to D.A.S. and R.J.S.), Ethicon Endo-Surgery (to D.A.S. and R.J.S.), MedImmune (to D.A.S., R.J.S., and D.P.O.), Sanofi (to R.J.S.), Janssen/Johnson & Johnson (to R.J.S.), Kallyope (to R.J.S.), and Zafgen (to R.J.S.). D.A.S. has served on a scientific advisory board for Novo Nordisk. R.J.S. has served on scientific advisory boards for Ethicon Endo-Surgery, Daiichi Sankyo, Janssen, Orexigen, Novartis, Nestlé, Takeda, Boehringer Ingelheim, Kallyope, Scohia, Sanofi, and Novo Nordisk. R.J.S. is also a paid speaker for Ethicon Endo-Surgery. No other potential conflicts of interest relevant to this article were reported.

Author Contributions. J.M.A. and H.P. performed experiments and acquired the data. J.M.A., D.A.S., R.J.S., and D.P.O. designed the research studies. J.M.A. and D.P.O. analyzed and interpreted the data and wrote the manuscript. D.A.S., R.J.S., R.B.C., and S.D.L. provided reagents. D.A.S., R.J.S., and S.D.L. reviewed and edited the manuscript. D.P.O. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Prior Presentation. Parts of this study were presented at the Keystone Symposia Conference, Copenhagen, Denmark, 9–13 May 2017.

References

1. Holst JJ. The physiology of glucagon-like peptide 1. Physiol Rev 2007;87:1409–1439

2. Meier JJ. GLP-1 receptor agonists for individualized treatment of type 2 diabetes mellitus. Nat Rev Endocrinol 2012;8:728–742

3. Pi-Sunyer X, Astrup A, Fujioka K, et al.; SCALE Obesity and Prediabetes NN8022-1839 Study Group. A randomized, controlled trial of 3.0 mg of liraglutide in weight management. N Engl J Med 2015;373:11–22

4. van Can J, Sloth B, Jensen CB, Flint A, Blaak EE, Saris WHM. Effects of the once-daily GLP-1 analog liraglutide on gastric emptying, glycemic parameters, appetite and energy metabolism in obese, non-diabetic adults. Int J Obes 2014;38:784–793

5. van Bloemendaal L, Ten Kulve JS, la Fleur SE, Ijzerman RG, Diamant M. Effects of glucagon-like peptide 1 on appetite and body weight: focus on the CNS. J Endocrinol 2014;221:T1–T16

6. Sisley S, Gutierrez-Aguilar R, Scott M, D'Alessio DA, Sandoval DA, Seeley RJ. Neuronal GLP1R mediates liraglutide's anorectic but not glucose-lowering effect. J Clin Invest 2014;124:2456–2463

7. Kanoski SE, Fortin SM, Arnold M, Grill HJ, Hayes MR. Peripheral and central GLP-1 receptor populations mediate the anorectic effects of peripherally administered GLP-1 receptor agonists, liraglutide and exendin-4. Endocrinology 2011;152:3103–3112
8. Merchenthaler I, Lane M, Shughrue P. Distribution of pre-pro-glucagon and glucagon-like peptide-1 receptor messenger RNAs in the rat central nervous system. J Comp Neurol 1999;403:261–280
9. Cork SC, Richards JE, Holt MK, Gribble FM, Reimann F, Trapp S. Distribution and characterisation of Glucagon-like peptide-1 receptor expressing cells in the mouse brain. Mol Metab 2015;4:718–731
10. Kanoski SE, Hayes MR, Skibicka KP. GLP-1 and weight loss: unraveling the diverse neural circuitry. Am J Physiol Regul Integr Comp Physiol 2016;310:R885–R895
11. Ghosal S, Packard AEB, Mahbod P, et al. Disruption of glucagon-like peptide 1 signaling in Sim1 neurons reduces physiological and behavioral reactivity to acute and chronic stress. J Neurosci 2017;37:184–193
12. Burmeister MA, Ayala JE, Smouse H, et al. The hypothalamic glucagon-like peptide 1 receptor is sufficient but not necessary for the regulation of energy balance and glucose homeostasis in mice. Diabetes 2017;66:372–384
13. Burmeister MA, Brown JD, Ayala JE, et al. The glucagon-like peptide-1 receptor in the ventromedial hypothalamus reduces short-term food intake in male mice by regulating nutrient sensor activity. Am J Physiol Endocrinol Metab 2017;313:E651–E662
14. Vong L, Ye C, Yang Z, Choi B, Chua S Jr., Lowell BB. Leptin action on GABAergic neurons prevents obesity and reduces inhibitory tone to POMC neurons. Neuron 2011;71:142–154
15. Williams EK, Chang RB, Strochlic DE, Umans BD, Lowell BB, Liberles SD. Sensory neurons that detect stretch and nutrients in the digestive system. Cell 2016;166:209–221
16. Krashes MJ, Shah BP, Madara JC, et al. An excitatory paraventricular nucleus to AgRP neuron circuit that drives hunger. Nature 2014;507:238–242
17. Sutton AK, Pei H, Burnett KH, Myers MG Jr., Rhodes CJ, Olson DP. Control of food intake and energy expenditure by Nos1 neurons of the paraventricular hypothalamus. J Neurosci 2014;34:15306–15318
18. Paxinos G, Franklin KBJ. *The Mouse Brain in Stereotaxic Coordinates*. Amsterdam, Elsevier Academic Press, 2008
19. Adams JM, Otero-Corchon V, Hammond GL, Veldhuis JD, Qi N, Low MJ. Somatostatin is essential for the sexual dimorphism of GH secretion, corticosteroid-binding globulin production, and corticosterone levels in mice. Endocrinology 2015;156:1052–1065
20. Ding HK, Teixeira CM, Frankland PW. Inactivation of the anterior cingulate cortex blocks expression of remote, but not recent, conditioned taste aversion memory. Learn Mem 2008;15:290–293
21. Baraboi ED, St-Pierre DH, Shooner J, Timofeeva E, Richard D. Brain activation following peripheral administration of the GLP-1 receptor agonist exendin-4. Am J Physiol Regul Integr Comp Physiol 2011;301:R1011–R1024
22. Parker JA, McCullough KA, Field BCT, et al. Glucagon and GLP-1 inhibit food intake and increase c-fos expression in similar appetite regulating centres in the brainstem and amygdala. Int J Obes 2013;37:1391–1398
23. Yamamoto H, Lee CE, Marcus JN, et al. Glucagon-like peptide-1 receptor stimulation increases blood pressure and heart rate and activates autonomic regulatory neurons. J Clin Invest 2002;110:43–52
24. Baggio LL, Huang Q, Brown TJ, Drucker DJ. A recombinant human glucagon-like peptide (GLP)-1-albumin protein (albugon) mimics peptidergic activation of GLP-1 receptor-dependent pathways coupled with satiety, gastrointestinal motility, and glucose homeostasis. Diabetes 2004;53:2492–2500
25. Buse JB, Rosenstock J, Sesti G, et al.; LEAD-6 Study Group. Liraglutide once a day versus exenatide twice a day for type 2 diabetes: a 26-week randomised, parallel-group, multinational, open-label trial (LEAD-6). Lancet 2009;374:39–47
26. Parker LA, Rana SA, Limebeer CL. Conditioned nausea in rats: assessment by conditioned disgust reactions, rather than conditioned taste avoidance. Can J Exp Psychol 2008;62:198–209
27. Kanoski SE, Rupprecht LE, Fortin SM, De Jonghe BC, Hayes MR. The role of nausea in food intake and body weight suppression by peripheral GLP-1 receptor agonists, exendin-4 and liraglutide. Neuropharmacology 2012;62:1916–1927
28. Liu YL, Malik N, Sanger GJ, Friedman MI, Andrews PLR. Pica–a model of nausea? Species differences in response to cisplatin. Physiol Behav 2005;85:271–277
29. Kinzig KP, D'Alessio DA, Seeley RJ. The diverse roles of specific GLP-1 receptors in the control of food intake and the response to visceral illness. J Neurosci 2002;22:10470–10476
30. McMahon LR, Wellman PJ. PVN infusion of GLP-1-(7-36) amide suppresses feeding but does not induce aversion or alter locomotion in rats. Am J Physiol 1998;274:R23–R29
31. Dossat AM, Lilly N, Kay K, Williams DL. Glucagon-like peptide 1 receptors in nucleus accumbens affect food intake. J Neurosci 2011;31:14453–14457
32. Lachey JL, D'Alessio DA, Rinaman L, Elmquist JK, Drucker DJ, Seeley RJ. The role of central glucagon-like peptide-1 in mediating the effects of visceral illness: differential effects in rats and mice. Endocrinology 2005;146:458–462
33. Abbott CR, Monteiro M, Small CJ, et al. The inhibitory effects of peripheral administration of peptide YY(3-36) and glucagon-like peptide-1 on food intake are attenuated by ablation of the vagal-brainstem-hypothalamic pathway. Brain Res 2005;1044:127–131
34. Krieger JP, Arnold M, Pettersen KG, Lossel P, Langhans W, Lee SJ. Knockdown of GLP-1 receptors in vagal afferents affects normal food intake and glycemia. Diabetes 2016;65:34–43
35. Secher A, Jelsing J, Baquero AF, et al. The arcuate nucleus mediates GLP-1 receptor agonist liraglutide-dependent weight loss. J Clin Invest 2014;124:4473–4488
36. Rüttimann EB, Arnold M, Hillebrand JJ, Geary N, Langhans W. Intrameal hepatic portal and intraperitoneal infusions of glucagon-like peptide-1 reduce spontaneous meal size in the rat via different mechanisms. Endocrinology 2009;150:1174–1181
37. Zheng H, Stornetta RL, Agassandian K, Rinaman L. Glutamatergic phenotype of glucagon-like peptide 1 neurons in the caudal nucleus of the solitary tract in rats. Brain Struct Funct 2015;220:3011–3022
38. Llewellyn-Smith IJ, Reimann F, Gribble FM, Trapp S. Preproglucagon neurons project widely to autonomic control areas in the mouse brain. Neuroscience 2011;180:111–121
39. Gaykema RP, Newmyer BA, Ottolini M, et al. Activation of murine preproglucagon-producing neurons reduces food intake and body weight. J Clin Invest 2017;127:1031–1045
40. Baggio LL, Huang Q, Brown TJ, Drucker DJ. Oxyntomodulin and glucagon-like peptide-1 differentially regulate murine food intake and energy expenditure. Gastroenterology 2004;127:546–558
41. Hayes MR, Skibicka KP, Grill HJ. Caudal brainstem processing is sufficient for behavioral, sympathetic, and parasympathetic responses driven by peripheral and hindbrain glucagon-like-peptide-1 receptor stimulation. Endocrinology 2008;149:4059–4068
42. Scrocchi LA, Brown TJ, MaClusky N, et al. Glucose intolerance but normal satiety in mice with a null mutation in the glucagon-like peptide 1 receptor gene. Nat Med 1996;2:1254–1258
43. Turton MD, O'Shea D, Gunn I, et al. A role for glucagon-like peptide-1 in the central regulation of feeding. Nature 1996;379:69–72
44. Meeran K, O'Shea D, Edwards CMB, et al. Repeated intracerebroventricular administration of glucagon-like peptide-1-(7-36) amide or exendin-(9-39) alters body weight in the rat. Endocrinology 1999;140:244–250
45. Hayes MR, Bradley L, Grill HJ. Endogenous hindbrain glucagon-like peptide-1 receptor activation contributes to the control of food intake by mediating gastric satiation signaling. Endocrinology 2009;150:2654–2659
46. Alhadeff AL, Baird JP, Swick JC, Hayes MR, Grill HJ. Glucagon-like peptide-1 receptor signaling in the lateral parabrachial nucleus contributes to the control of food intake and motivation to feed. Neuropsychopharmacology 2014;39:2233–2243
47. Barrera JG, Jones KR, Herman JP, D'Alessio DA, Woods SC, Seeley RJ. Hyperphagia and increased fat accumulation in two models of chronic CNS glucagon-like peptide-1 loss of function. J Neurosci 2011;31:3904–3913

48. Williams DL, Baskin DG, Schwartz MW. Evidence that intestinal glucagon-like peptide-1 plays a physiological role in satiety. Endocrinology 2009;150:1680–1687
49. Thomsen WJ, Grottick AJ, Menzaghi F, et al. Lorcaserin, a novel selective human 5-hydroxytryptamine2C agonist: in vitro and in vivo pharmacological characterization. J Pharmacol Exp Ther 2008;325:577–587
50. Stefater MA, Pérez-Tilve D, Chambers AP, et al. Sleeve gastrectomy induces loss of weight and fat mass in obese rats, but does not affect leptin sensitivity. Gastroenterology 2010;138:2426–2436, 2436.e1–2436.e3
51. Price CJ, Hoyda TD, Ferguson AV. The area postrema: a brain monitor and integrator of systemic autonomic state. Neuroscientist 2008;14:182–194
52. Yamamoto H, Kishi T, Lee CE, et al. Glucagon-like peptide-1-responsive catecholamine neurons in the area postrema link peripheral glucagon-like peptide-1 with central autonomic control sites. J Neurosci 2003;23:2939–2946
53. Baraboi ED, Smith P, Ferguson AV, Richard D. Lesions of area postrema and subfornical organ alter exendin-4-induced brain activation without preventing the hypophagic effect of the GLP-1 receptor agonist. Am J Physiol Regul Integr Comp Physiol 2010;298:R1098–R1110