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# Knockdown of GLP-1 Receptors in Vagal Afferents Affects Normal Food Intake and Glycemia

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Nutrient stimulation of enteroendocrine L cells induces the release of the incretin and satiating peptide glucagonlike peptide 1 (GLP-1). The vagus nerve innervates visceral organs and may contribute to the mediation of aut-derived GLP-1's effects on food intake, energy homeostasis, and glycemic control. To test the hypothesis that vagal afferent neuron (VAN) GLP-1 receptors (GLP-1Rs) are necessary for these effects of endogenous GLP-1, we established a novel bilateral nodose ganglia injection technique to deliver a lentiviral vector and to knock down VAN GLP-1Rs in male Sprague Dawley rats. We found that a full expression of VAN GLP-1Rs is not necessary for the maintenance of longterm energy balance in normal eating conditions. VAN GLP-1R knockdown (kd) did, however, increase meal size and accelerated gastric emptying. Moreover, postmeal glycemia was elevated and insulin release was blunted in GLP-1R kd rats, suggesting that VAN GLP-1Rs are physiological contributors to the neuroincretin effect after a meal. Collectively, our results highlight a crucial role for the VANs in mediating the effects of endogenous GLP-1 on food intake and glycemia and may promote the further development of GLP-1-based therapies.

Glucagon-like peptide 1 (GLP-1) is an incretin and satiating hormone that has provided new tools for the pharmacotherapy of obesity and diabetes (1,2). Yet, despite the clinical effectiveness of GLP-1-based drugs in ameliorating the symptoms of type 2 diabetes, the role of endogenous GLP-1 in the control of energy intake and glucose homeostasis is not fully understood. Vagal afferent neurons (VANs) express GLP-1 receptors (GLP-1Rs) (3,4) and terminate in the lamina propria of the intestinal mucosa as well as in the wall of the hepatic portal vein

Corresponding author: Jean-Philippe Krieger, jean-philippe-krieger@ethz.ch. Received 15 July 2015 and accepted 16 September 2015. signals to the brain and, hence, mediate satiating and glucoregulatory responses. Previous studies using lesioning approaches have implicated the vagus nerve in the effects of peripherally administered GLP-1 on food intake and glycemia (reviewed in 6,7). In more recent studies, sudiaphragmatic vagal deafferentation (SDA) in rats clearly attenuated the acute eating-inhibitory effect of intraperitoneally (IP) infused GLP-1 (8) and exendin-4 (Ex-4), a GLP-1R agonist (9). Moreover, unlike Sham-operated rats, SDA rats failed to show a GLP-1R-mediated incretin response (10). Based on these findings, it is reasonable to hypothesize that endogenous gut-derived GLP-1 could activate GLP-1Rs on VANs in a paracrine-like fashion to reduce food intake, limit gastric emptying, and trigger a neural component of the incretin effect. Disruption of this endogenous GLP-1 signaling mechanism in the VANs due to genetic or environmental factors may contribute to the pathophysiology of obesity and diabetes. Hence, we examined the physiological role of VAN GLP-1Rs in the control of food intake and regulation of glucose homeostasis by generating a specific knockdown (kd) of VAN GLP-1R expression in rats. Our approach is based on the delivery of a short hairpin (sh)RNA construct targeting the GLP-1R mRNA transcript by injecting a lentiviral vector (LV) bilaterally into the nodose ganglia (NG) of rats. Using RNA interference to manipulate gene expression in a tissue-specific manner, we report that VAN GLP-1Rs 1) are required for the physiological control of meal size and gastric emptying but not for the regulation of long-term energy intake and body weight, 2) are necessary for the full effects of acute IP GLP-1 and Ex-4 administration on eating and gastric emptying, and 3) mediate a neural component of GLP-1's incretin effect that is

the work is not altered.

(HPV) (5). VANs may therefore relay the gut GLP-1-derived

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physiologically relevant for the postprandial regulation of blood glucose.

Collectively, our findings establish the VANs as a major mediator of endogenous GLP-1's short-term effects on eating, gastric emptying, and glycemia. They also indicate, however, that the reduction of GLP-1R expression in VANs is not sufficient to promote obesity under normal eating conditions.

## **RESEARCH DESIGN AND METHODS**

### Animals and Housing

Male Sprague Dawley rats (Charles River) were individually housed (21  $\pm$  1°C, 55  $\pm$  5% relative humidity) with a 12/12 h dark/light cycle. Unless otherwise noted, animals had ad libitum access to water and standard chow (Kliba 3433, energy density 3.13 kcal/g). All experimental procedures were approved by the Zurich Cantonal Veterinary Office.

#### Lentivirus-Mediated shRNA Interference

pLKO.1-puro vectors expressing turbo green fluorescent protein (GFP) and the U6 promoter-driven shRNA sequence targeting the rat GLP-1R mRNA or a nontarget shRNA sequence were obtained from Sigma-Aldrich. Efficiency of the GLP-1R-targeting shRNA construct was verified in vitro in INS-1E cells (a GLP-1R expressing rat pancreatic  $\beta$ -cell line, generously provided by P. Maechler and C.B. Wollheim, University of Geneva). GLP-1R-targeting (LV-shGLP-1R) or control (LV-shCTL) lentiviral particles were produced in human embryonic kidney 293T cells using the pMD2.G and psPAX2 plasmids (gifts from D. Trono, École Polytechnique Fédérale de Lausanne; cat. no. 12259 and 12260; Addgene) and concentrated to 10<sup>10</sup> particles/mL using 8% PEG6000 (Millipore) and resuspended in PBS.

#### Surgery

Rats (290–340 g on surgery day) were anesthetized by an IP injection of ketamine (88 mg/kg; Ketalar, Cantonal Pharmacy Zurich) and xylazine (5 mg/kg; Rompun 2%, Cantonal Pharmacy Zurich), and NG were exposed. A glass capillary (50  $\mu$ m tip) was used to administer 1.5  $\mu$ L viral solution into each NG with a Picospritzer III injector (Parker Hannifin). To ensure expression of the viral constructs, we allowed animals to recover for 20 days. IP and HPV catheters were implanted as previously described (complete description in 11). Intracerebroventricular (ICV) cannulas were implanted in the fourth ventricle (stereotaxic coordinates: 2.5 mm posterior to lambda, 0 mm lateral to mid-line, 5 mm below skull surface), and placement was verified functionally with infusion of 5-thioglucose (Sigma-Aldrich) using a 2.5-mm injector (210  $\mu$ g/rat) and anatomically postmortem.

#### **Tissue Collection**

Animals received an IP injection of pentobarbital (100 mg/kg; Cantonal Pharmacy Zurich), and NG, brain, and pancreas were immediately collected. For gene and protein analysis, tissues were frozen in liquid nitrogen and stored at  $-80^{\circ}$ C. For GFP visualization, NG were fixed for 2 h in 4% paraformaldehyde and 25% sucrose solution in PBS and cut at 10  $\mu$ m in a cryostat and mounted on glass slides.

#### Gene Expression and Protein Analysis

The nucleus tractus solitarii (NTS) and the hypothalamic dorsomedial, paraventricular, and arcuate nuclei were mircropunched using anatomical landmarks, and NG from the same animal were pooled before RNA and proteins were extracted using Trizol (Life Technologies). RT quantitative PCR was performed using SybR Green on a OneStep Plus instrument (Applied Biosystems), and results were analyzed using the 2ddCt method. A Western blot was performed to detect the GLP-1R protein (1:400; rabbit antibody 39072, Abcam) using  $\beta$ -actin as reference (1:3,000; mouse antibody AC-74, Sigma-Aldrich).

#### Drugs

GLP-1(7-36)amide (Bachem H-6795), Ex-4 (Bachem H-8730), and cholecystokinin octapeptide (CCK) (Bachem H-2080) were resuspended in sterile PBS and administered at doses of 33  $\mu$ g/kg IP (GLP-1), 0.3  $\mu$ g/kg IP (Ex-4), and 4  $\mu$ g/kg IP. (CCK) via catheters. Ex-4 was administered into the fourth ventricle at a dose of 0.3  $\mu$ g/rat. Rats were habituated to IP or ICV injections with vehicle solutions on three occasions before experiments.

#### Food Intake Measurement and Meal Pattern Analysis

Food was available through a niche and placed on scales (XS4001S; Mettler-Toledo) for continuous measurement (described in 12). Meal patterns were analyzed with custom software (LabX meal analyzer 1.4, Mettler-Toledo). Data are presented as average of 3 days. For food intake experiments after IP GLP-1, rats were fasted overnight and received a 3-g premeal 1 h before dark onset to allow GLP-1R trafficking to the VAN membrane (4). For IP CCK and Ex-4 or ICV Ex-4, rats were fasted for 4 h before dark onset. In all cases, rats received IP or ICV injections right before dark onset and were brought immediately to their home cages.

#### **Gastric Emptying Assay**

One week prior to the experiment, rats were habituated to test meals and restricted feeding schedule (test meal at dark onset, ad libitum food access from 3 to 8 h after dark onset, and food deprivation otherwise). On experimental days, rats received a 4-g chow meal containing 1% (wt/wt) paracetamol (4-acetamido-phenol, Sigma-Aldrich) and 0.25% (wt/wt) saccharin (Sigma-Aldrich). IP or ICV injections were given 5 min prior to the test meal. Baseline tail vein blood was taken 30 min prior to test meal onset, and postmeal blood was collected according to the scheduled time points. Paracetamol concentrations were measured with a commercial kit (K8002; Cambridge Life Sciences).

#### **Indirect Calorimetry**

Measurements were conducted in an open-circuit calorimetry Phenomaster system (TSE) after 5 days of habituation. Data are presented as 1 h time bins averaged over 3 days.

### Plasma Analysis After Test Meal

Blood was sampled from HPV catheters in unrestrained animals 30 min prior to (baseline) and according to the scheduled time points after the beginning of a 5-g chow test meal. Glucose was measured twice using AccuCheck (Roche), and 150  $\mu$ L blood was immediately mixed with EDTA (Titriplex, Merck), aprotinin (Sigma-Aldrich), and DPP-IV inhibitor (Millipore) before centrifugation and storage of the plasma at  $-80^{\circ}$ C. Total active GLP-1, insulin, and glucagon were measured simultaneously using an immunoassay (MesoScale Discovery multispot K15171C).

#### **Oral Glucose Tolerance Test**

Rats food-deprived for 16 h and adapted to gavage received an oral bolus of 40% glucose solution (2 g/kg). Blood samples for glucose and insulin were taken from tail vein at baseline and 15, 30, 60, 90, and 120 min after the oral glucose bolus. Insulin was measured using an immunoassay (MesoScale Discovery single spot for mouse/rat K152BZC).

#### **Statistical Analysis**

Data normality was verified using the Shapiro-Wilk (when  $n \ge 7$ ) and the Kolmogorov-Smirnov (when  $n \le 6$ ) tests, and homoscedasticity was checked by visualizing the distribution of residuals. Nonparametric tests were used otherwise. When data distribution was compatible with

normality, outliers were detected using the Grubb test. Differences were analyzed by a Student t test for unpaired normally distributed values of equal variance (Figs. 1C, 1E-G, 2D, 3A-I, 3K-L, and 5A and B), or a Mann-Whitney U test for unpaired comparison of non-normally distributed data (Figs. 1D and 3L) using GraphPad Prism (version 6.05). Where the dependent variable was affected by two factorsone within-subject factor (time or injection) and one betweensubject factor (surgery group)—the data were analyzed with a mixed ANOVA (Figs. 2A-C, 3J, 4A-F, and 5C-H) using SAS (version 9.3). When the main effect or interaction terms were significant, post hoc analyses using the Bonferroni correction were performed. Data are presented as mean  $\pm$  SEM. P values <0.05 were considered significant. ns indicates that statistical significance was not reached. All graphs were generated using GraphPad Prism (version 6.05).

#### RESULTS

#### Histological Confirmation of Viral Infection and Quantification of In Vivo GLP-1R kd

Three weeks after bilateral NG injection of a lentivirus containing a GLP-1R-targeting shRNA construct (Fig. 1*A*), infection of VANs was confirmed by visualizing GFP expression in NG sections (Fig. 1*B*). GLP-1R mRNA expression in NG was reduced by 52.5% in GLP-



**Figure 1**—NG injection of a GLP-1R-targeting lentivirus led to a specific reduction in GLP-1R expression in the VANs. *A*: Schematic representation of lentiviral injection site in a rat NG. *B*: Visualization of GFP expression in the NG of a rat injected with GLP-1R-targeting lentiviral particles (representative picture). Scale bar, 50  $\mu$ m. *C*: Relative expression of GLP-1R mRNA in the NG of control and GLP-1R kd rats (n = 10/8; Student *t* test, P < 0.0001). *D*: Relative expression of the GLP-1R protein levels in the NG of control and GLP-1R kd rats (n = 5/5; Mann-Whitney *U* test, P < 0.01) as measured by the relative intensity of the GLP-1R detection band normalized by the intensity of GLP-1R mRNA in the pancreas (n = 6/7; Student *t* test, not statistically significant [ns]), nucleus tractus solitarii (NTS) (n = 7/7; Student *t* test, ns), and arcuate (n = 7/7; Student *t* test, ns), paraventricular (PVH) (n = 7/6; Student *t* test, ns), and dorsomedial (DMH) (n = 7/7; Student *t* test, ns) mRNA in the NG of control and GLP-1R kd rats. *G*: Percent decrease in 1 hood intake after injection of CCK (4  $\mu$ g/kg IP) relative to vehicle injection (n = 6/6; Student *t* test, ns) of control and GLP-1R kd rats. \*Significant difference between the control and GLP-1R kd groups (P < 0.05).



**Figure 2**—VAN GLP-1Rs are not required for normal body weight, daily food intake, and energy expenditure. (*A*) Body weight [n = 11/11; ANOVA, group F(1,20) = 0.26, not statistically significant (ns)] and (*B*) daily food intake [n = 11/11; ANOVA, group F(1,20) = 1.10, ns] of control and GLP-1R kd rats maintained on chow after surgical injection. (*C*) Twenty-four hour time course [n = 8/7; ANOVA, group F(1,13) = 0.08, ns] and (*D*) cumulative energy expenditure (EE) (n = 8/7; Student *t* test, ns) in control and GLP-1R kd rats fed ad libitum with chow. AUC, area under the curve.

1R kd rats compared with control rats injected with control lentiviral particles containing nonspecific target shRNA (Fig. 1C). In addition, reduction of GLP-1R protein was confirmed using Western blot from NG protein extracts of control and GLP-1R kd rats (Fig. 1D). GLP-1R expression was unchanged in the pancreas, where GLP-1R activation improves insulin secretion, as well as in the key GLP-1Rexpressing regions in the brain involved in the control of food intake and glucose homeostasis (Fig. 1E), indicating tissue specificity of the LV-mediated gene kd approach. Moreover, VAN genes involved in the control of food intake, such as the CCK A receptor (CCKaR), leptin receptor (LepR), and peroxisome proliferator-activated receptor  $\gamma$  (PPARg), were similarly expressed in the NG of control and GLP-1R kd rats (Fig. 1F), suggesting that the GLP-1R shRNA construct used was specific. Finally, we measured food intake 30 min after an IP injection of CCK and found a 25-40% reduction (9,13) in both groups, demonstrating the preservation of VAN functional integrity (Fig. 1G). Together, these results indicate that the LV-mediated delivery of an shRNA construct by bilateral NG injection is tissue specific, targetspecific, and does not impair vagal afferent function.

# Endogenous GLP-1R Signaling in the VANs Is Not Required for Normal Long-term Energy Balance

Body weights of control and GLP-1R kd rats remained similar during the entire course of the experiments when fed ad libitum (Fig. 2*A*). Moreover, energy balance remained undisturbed as documented by similar daily chow intake (Fig. 2*B*) and daily energy expenditure (Fig. 2*C* and *D*).

#### Endogenous GLP-1R Signaling in the VAN Controls Meal Size and Gastric Emptying

Measurements of undisturbed meal patterns showed that GLP-1R kd induced increases in meal size (Fig. 3A) and meal duration (Fig. 3D). Although these changes were significant over 24 h, the increase in meal size and duration was only evident during the dark phase with no significant differences in the light phase (Fig. 3B, C, E, and F). Consistent with the longterm daily food intake measurements (Fig. 2A), 24-h food intake was not affected by the kd during the period of meal pattern measurements (data not shown): this was mainly due to a compensatory decrease in the number of meals in the GLP-1R kd rats (Fig. 3G), which was also evident only during the dark phase (Fig. 3H and I). Moreover, the rate of gastric emptying after a meal, as measured by the appearance of paracetamol in the plasma after a test meal, was enhanced in GLP-1R kd rats compared with controls (Fig. 3J). Finally, in line with their ad libitum meal pattern, GLP-1R kd rats showed an increase in food intake during the 1-h refeeding period after a 16-h fast (Fig. 3K), which was associated with an amplified peak of energy expenditure during this period (Fig. 3L).

#### Endogenous GLP-1 Signaling in the VAN Is Required for the Effects of IP GLP-1 and Ex-4 but Not ICV Ex-4 on Food Intake and Gastric Emptying

According to previous studies (for review, see 6,7), GLP-1 and low-dose Ex-4 require intact VAN to exert their full



**Figure 3**—VAN GLP-1Rs controlled the size and the gastric emptying of a meal. Average meal size (*A*) over 24 h, (*B*) 12-h dark phase and (*C*) 12-h light phase (n = 8/7; Student *t* tests, respectively, P < 0.05, P < 0.05, not statistically significant [ns]) in ad libitum–fed control and GLP-1R kd rats. Average meal duration (*D*) over 24 h, (*E*) 12-h dark phase, and (*F*) 12-h light phase (n = 8/7; Student *t* tests, respectively, P < 0.05, P = 0.07, ns) in ad libitum–fed control and GLP-1R kd rats. Number of meals (*G*) in 24 h, (*H*) 12-h dark phase, and (*I*) 12-h light phase, and (*I*) 12-h light phase, and (*I*) 12-h light phase (n = 8/7; Student *t* tests, respectively, P < 0.05, P < 0.01, ns) in ad libitum–fed control and GLP-1R kd rats. Number of meals (*G*) in 24 h, (*H*) 12-h dark phase, and (*I*) 12-h light phase (n = 8/7; Student *t* tests, respectively, P < 0.05, P < 0.01, ns) in ad libitum–fed control and GLP-1R kd rats. (*J*) Plasma paracetamol concentrations of control and GLP-1R kd rats after a 4-g powdered chow test meal containing 1% paracetamol (wt/wt) [n = 9/9; ANOVA, group F(1,16) = 17.98, P < 0.0001; group × time F(6,96) = 4.71, P < 0.0001]. (*K*) Food intake (n = 10/10; Student *t* test, P < 0.05) and (*L*) peak of energy expenditure (EE) in the first hour of ad libitum refeeding with chow after a 16-h fast (n = 8/7; Mann-Whitney *U* test, P < 0.05). "Significant difference between the control and GLP-1R kd groups (P < 0.05).

inhibitory effects on short-term food intake. Hence, we further tested whether the satiating effects of exogenous GLP-1 and Ex-4 were attenuated in GLP-1R kd rats. Indeed, IP injections of GLP-1—at a dose that elevates intestinal lymph GLP-1 similar as a meal (14)—or low-dose Ex-4 failed to significantly reduce 1-h food intake in GLP-1R kd rats (Fig. 4A and B). In a similar design, we tested whether GLP-1Rs in the VAN mediate the GLP-1– or Ex-4–induced inhibition of gastric emptying using the paracetamol test. IP GLP-1 and Ex-4 failed to inhibit gastric emptying in the GLP-1R kd group as shown by early appearance of paracetamol in the plasma 20 min after a test meal (Fig. 4D and E). To test whether GLP-1R expressed on peripheral or central terminals of vagal afferents mediate these effects, we performed a fourth ICV injection of Ex-4. In contrast to the attenuated effects of IP Ex-4, the fourth ICV injection of Ex-4 showed the full expression of the inhibitory effects on eating and gastric emptying in kd rats (Fig. 4C and F). These observations are consistent with the idea that peripheral but not central GLP-1Rs expressed on VAN terminals mediate the effects of IP-infused GLP-1 on food intake and gastric emptying. Together, these results indicate that activation of GLP-1Rs on the VANs in the gut mediates the



**Figure 4**—VAN GLP-1R kd attenuated the effects of IP GLP-1 and Ex-4, but not ICV Ex-4, on food intake and gastric emptying. (*A*) One-hour food intake after GLP-1 (33  $\mu$ g/kg IP; *n* = 7/8), (*B*) Ex-4 (0.3  $\mu$ g/kg IP; *n* = 12/11), and (*C*) Ex-4 (0.3  $\mu$ g ICV; *n* = 6–8). Plasma paracetamol concentrations of control and GLP-1R kd rats 20 min after allowed access to a 4-g powdered chow test meal containing 1% paracetamol (w/w) and injected with (*D*) GLP-1 (33  $\mu$ g/kg IP; *n* = 7/7), (*E*) Ex-4 (0.3  $\mu$ g/kg IP; *n* = 7/7), and (*F*) Ex-4 (0.3  $\mu$ g ICV; *n* = 6–8). For all results, ANOVA was followed by post hoc comparisons. Different letters indicate a significant difference between two groups after post hoc Bonferroni-corrected comparisons (*P* < 0.05). Veh, vehicle.

inhibitions of eating and gastric emptying induced by IP GLP-1 or low-dose Ex-4.

#### Endogenous GLP-1 Signaling in the VANs Is Required for Normal Glycemia After a Mixed-Nutrient Meal but Not After an Oral Glucose Challenge

GLP-1Rs on vagal afferents have been implicated in the neuroincretin effects of endogenous GLP-1. We measured HPV blood glucose in the fasted and fed conditions in control and GLP-1R kd rats. After an overnight fast (16 h), HPV blood glucose was not different between GLP-1R kd and control rats (Fig. 5A), whereas kd rats showed a higher blood glucose than controls in the fed state (2 h of fasting after ad libitum food access 5 h into the dark phase) (Fig. 5B). This indicated that GLP-1Rs in the VAN are necessary for the full incretin effect of meal-induced GLP-1. To test this hypothesis, we measured HPV blood glucose (Fig. 5C), plasma insulin (Fig. 5D), GLP-1 (Fig. 5E), and glucagon (Fig. 5F) after a 16-h fast followed by a 5-g chow test meal. HPV blood sampling was chosen to allow for the concomitant measurement of the meal-induced increase in HPV GLP-1 levels (which is subject to a rapid degradation by dipeptidyl peptidase-4 in the systemic circulation and in the liver). The test meal elevated HPV blood glucose in both groups but resulted in a higher postmeal blood glucose level in the GLP-1R kd rats. Interestingly, postmeal HPV GLP-1 and glucagon were similar in both groups, but insulin appearance in the HPV was blunted in the GLP-1R kd group. An oral glucose tolerance test, however, did not reveal differences in plasma glucose and insulin levels between control and kd rats (Fig. 5G and H)

### DISCUSSION

It has long been hypothesized that VANs could control eating behavior by serving as a key mediator of nutritional cues from the intestine to the brain (6,7). Vagal lesioning methods (including SDA, the most specific method for the disconnection of subdiaphragmatic vagal afferents [15]) provided initial evidence for the role of the VANs in mediating peripheral exogenous GLP-1 effects on food intake and glycemia (9,16). These methods, however, resulted in a complete impairment of VAN signaling and function, and they did not specifically test the role of VANs in the effects of endogenous GLP-1. Therefore, the role of VAN GLP-1R signaling in mediating the effects of endogenous GLP-1 on energy homeostasis has been difficult to elucidate. Lately, vagal-specific genetic deletions of receptors involved in nutrient sensing have been attempted in mouse models using Phox2b or Nav1.8 genes, whose promoters drive cre-recombinase expression (17-19). The cre expression in these mouse models is, however, not limited to the VANs and extends to the spinal afferents and brainstem (20,21). Moreover, for a tightly controlled



**Figure 5**—VAN GLP-1R kd disturbed postmeal glycemia and insulinemia but did not impair tolerance of an oral glucose bolus. HPV glucose (*A*) after an overnight fast (n = 8/8; Student *t* test, not statistically significant [ns]) or (*B*) 2 h after food deprivation of ad libitum–fed animals (n = 6/6; Student *t* test, P < 0.05). HPV (*C*) blood glucose [n = 8/8; ANOVA; group F(1,14) = 28.4, P < 0.0001; group × time F(5,70) = 3.440, P < 0.01], (*D*) plasma insulin [n = 8/8; ANOVA; group F(1,14) = 0.012, ns; group × time F(5,70) = 2.90, P < 0.05], (*E*) GLP-1 [n = 8/8; ANOVA; group F(1,14) = 0.73, ns; group × time F(5,70) = 0.68, ns], and (*F*) glucagon [n = 8/8; ANOVA; group F(1,14) = 0.13, ns; group × time F(5,70) = 0.29, ns] after a chow test meal. Tail vein (*G*) blood glucose [n = 7/7; ANOVA; group F(1,12) = 0.30, ns; group × time F(6,72) = 1.29, ns] and (*H*) plasma insulin [n = 7/7; ANOVA; group F(1,12) = 0.16, ns; group × time F(4,48) = 1.12, ns] after an OGTT (2 g/kg). \*Significant difference between the control and GLP-1R kd groups (P < 0.05).

system such as eating behavior, gene deletion approaches are suspected to yield compensatory mechanisms during development to maintain overall energy balance (18,22,23). To overcome these obstacles, we used the bilateral delivery of an shRNA-expressing viral vector into the NG to accomplish an inducible molecular manipulation of VAN function in adult rats. To our knowledge, NG injection has so far been limited to nonsurvival administration of compounds for electrophysiological recordings (24) and, more recently, to the unilateral delivery of viral tracers and optogeneticsrelated tools (25). Here, we demonstrate that bilateral NG injection of a viral-mediated shRNA yields a specific and long-lasting reduction of GLP-1R expression in the VANs.

When fed ad libitum, control and GLP-1R kd rats had similar body weights over the entire course of the experiment. Also, daily food intake was unchanged, which, together with the unchanged energy expenditure, demonstrates that a reduction of GLP-1R expression in VANs does not chronically disturb energy balance. Overnight fasting and refeeding, however, led to a much larger food intake and peak of energy expenditure in the GLP-1R kd rats, presumably due to an increased meal-induced thermogenesis. A major caveat of the viral-mediated GLP-1R kd approach is that the reduction of GLP-1R expression in the VAN is partial. Therefore, it cannot be excluded that the remaining expression of GLP-1R in the VAN accounted for the absence of chronic changes in energy homeostasis. Nevertheless, this negative phenotype in the body weight and daily food intake in our kd model is in accordance with the GLP-1R  $kd^{\Delta Phox2b}$  mouse model (17).

Previous data using surgical lesions concluded that vagal afferents are needed for the full expression of IP-injected GLP-1 or acute Ex-4 effects on food intake (8,9,16,26). Given that GLP-1 is cleared from plasma within minutes (27,28), these results suggested that endogenous GLP-1 released from intestinal L cells activates GLP-1R located on intestinal VANs in a paracrine-like fashion. Here, we present strong evidence that GLP-1Rs in the VANs in fact mediate the satiating and gastric emptying–inhibiting effects of endogenous GLP-1, as well as IP-infused GLP-1 and Ex-4.

Our data specifically confirm the importance of VAN GLP-1R signaling in the short-term control of eating by endogenous GLP-1. GLP-1R kd specifically delayed nocturnal meal termination (satiation) without affecting postmeal satiety, consistent with a paracrine effect of endogenous GLP-1 on VAN GLP-1Rs. It stands to reason that the effects are mostly nocturnal, when rats consume most of their calories, because intestinal GLP-1 is being secreted via luminal nutrient stimulation. Moreover, recent data demonstrated a circadian rhythm for GLP-1 (29), with a maximal GLP-1 release upon glucose stimulation before dark onset. The effects of the GLP-1R kd may therefore be magnified during the early dark phase when circulating GLP-1 levels are high. Finally, GLP-1Rs in the VAN are internalized during fasting and translocated to the membrane in the fed state (4). The absence of an effect on

meal size and duration during the light phase may therefore be due to the internalization of GLP-1Rs in the VAN when food intake is low and intermeal intervals are prolonged.

In addition, we demonstrated that VAN GLP-1R expression is necessary for the normal gastric emptying of a meal, as hypothesized from earlier studies (27,30,31), consistent with a paracrine effect of endogenous GLP-1 on VAN GLP-1Rs. Whether the GLP-1–induced reduction in gastric emptying is a major mechanism by which endogenous GLP-1 controls meal size is unclear. Further experiments should test whether the eating-inhibitory effect of GLP-1 on meal size is still present in sham-fed animals in which normal gastric emptying is prevented by a gastric fistula.

Surprisingly, despite the consistent and lasting increase in meal size, daily food intake was not altered by VAN GLP-1R kd, which was the result of a compensatory decrease in meal number. Given the fact that IP administration of GLP-1 induces short-term satiation by a reduction in meal size (8,32), it appears that the increase in meal size is the primary effect of VAN GLP-1R kd, reflecting a specific satiating effect of endogenous GLP-1 via a paracrine action. The decrease in meal number is likely a secondary, compensatory mechanism. It is also possible to speculate that bigger meals in VAN GLP-1R kd rats trigger the release of other eating-inhibitory hormones (e.g., CCK, peptide tyrosine tyrosine) whose signaling remains intact and delay the rise in ghrelin. Moreover, VAN GLP-1R kd may lead to compensatory changes in neuronal activity of the dorsal vagal complex (N. Gafner, J.-P. Krieger, S.J. Lee, unpublished observations), that, in turn, may prolong the intermeal interval and hence decrease meal number. Together, this strengthens the classical view that VAN GLP-1Rs mainly mediate shortterm satiation and may be one of several redundant eating control mechanisms comprising the gut-brain axis (7,9,16,33).

Activation of pancreatic GLP-1Rs by gut-derived endogenous GLP-1 is considered to be the classical mechanism of GLP-1's incretin effect. Several findings in rodents and humans, however, suggest an additional involvement of a neural pathway in the GLP-1R-dependent release of insulin (34-38). Our findings demonstrate that vagal GLP-1R signaling is necessary for the normal control of glycemia after eating. Interestingly, the elevated postmeal glycemia in GLP-1R kd rats was concomitant with lower levels of HPV insulin 15 min after the beginning of the meal without changes in GLP-1 or glucagon levels. These results indicate that endogenous meal-induced GLP-1 acts on VAN GLP-1Rs to control postmeal glycemia via a neural component of the incretin effect. Moreover, recent data showed that IP infusion of atropin, a blocker of muscarinic receptors, reduces the insulin response after intravenous coinfusion of glucose and GLP-1 (39). Together with our findings, this supports the idea that ascending VANs and descending pancreatic efferents form a "gutbrain-pancreas" axis mediating some of the effects of intestinal GLP-1 on insulin secretion. Intriguingly, after an oral glucose tolerance test (OGTT), no significant differences in blood glucose or plasma insulin were seen between the two groups. Several differences between the use of a test meal or a glucose bolus could explain this discrepancy. First, an OGTT is thought to induce the release of GLP-1 with a different amplitude and/or time course than a solid meal ingested over several minutes (40). As vagal lesion studies indicated that high levels of circulating GLP-1 can exert a VAN-independent effect (8,41), it is plausible that higher GLP-1 levels after the OGTT mask the role of VAN GLP-1Rs. Second, glucose measurements from HPV blood sampling may not capture the hepatic effect on glycemia. The contribution of the liver to the glucoregulatory effect of GLP-1 via both insulin-dependent and insulin-independent mechanisms is indeed receiving increasing support (42-44), and it is consequently possible that HPV blood glucose and insulin levels differ from systemic blood values. Finally, the stress caused by the OGTT procedure (gavage and tail vein sampling) may have masked the difference in glucose levels between GLP-1R kd and controls. Corticosterone has a powerful effect on glucose levels because it inhibits insulin secretion and increases hepatic gluconeogenesis. Therefore, a stress-free HPV sampling (voluntary meal followed by unrestrained blood sampling through HPV catheter) may be a more accurate way to differentiate VAN GLP-1R kd effects on glucose/hormonal changes than tail vein sampling.

Together, our findings demonstrate a crucial role for a vagal pathway in the maintenance of normal eating behavior and postprandial glycemia by endogenous GLP-1. Recent studies, however, have shown that GLP-1R agonists, such as liraglutide, exert their body weight and glucose-lowering effects independent of vagal afferents (45) and do not require VAN GLP-1Rs (17). Most likely, GLP-1R agonists do not access VAN GLP-1Rs when administered subcutaneously. Instead, GLP-1R agonist effects on body weight may be mediated by the activation of central GLP-1Rs (45). Based on our data, it is, however, possible to consider the vagus nerve as a target organ to modulate satiation and glycemia.

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manuscript. S.J.L. participated in the study conception and design, participated in the experiments/data interpretation, and edited and reviewed the manuscript. S.J.L. 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.

#### References

 Vilsbøll T, Christensen M, Junker AE, Knop FK, Gluud LL. Effects of glucagonlike peptide-1 receptor agonists on weight loss: systematic review and metaanalyses of randomised controlled trials. BMJ 2012;344:d7771

2. FDA approves weight-management drug Saxenda [article online], 2014. Available from http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ ucm427913.htm. Accessed 10 June 2015

3. Nakagawa A, Satake H, Nakabayashi H, et al. Receptor gene expression of glucagon-like peptide-1, but not glucose-dependent insulinotropic polypeptide, in rat nodose ganglion cells. Auton Neurosci 2004;110:36–43

4. Ronveaux CC, de Lartigue G, Raybould HE. Ability of GLP-1 to decrease food intake is dependent on nutritional status. Physiol Behav 2014;135:222–229

5. Berthoud HR, Neuhuber WL. Functional and chemical anatomy of the afferent vagal system. Auton Neurosci 2000;85:1-17

 Ronveaux CC, Tomé D, Raybould HE. Glucagon-like peptide 1 interacts with ghrelin and leptin to regulate glucose metabolism and food intake through vagal afferent neuron signaling. J Nutr 2015;145:672–680

 Krieger JP, Langhans W, Lee SJ. Vagal mediation of GLP-1's effects on food intake and glycemia. Physiol Behav. 3 June 2015 [Epub ahead of print]. DOI: 10.1016/j.physbeh.2015.06.001

8. 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

9. Labouesse MA, Stadlbauer U, Weber E, Arnold M, Langhans W, Pacheco-López G. Vagal afferents mediate early satiation and prevent flavour avoidance learning in response to intraperitoneally infused exendin-4. J Neuroendocrinol 2012;24:1505–1516

10. Hayes MR, Kanoski SE, De Jonghe BC, et al. The common hepatic branch of the vagus is not required to mediate the glycemic and food intake suppressive effects of glucagon-like-peptide-1. Am J Physiol Regul Integr Comp Physiol 2011; 301:R1479–R1485

11. Surina-Baumgartner DM, Langhans W, Geary N. Hepatic portal insulin antibody infusion increases, but insulin does not alter, spontaneous meal size in rats. Am J Physiol 1995;269:R978–R982

12. Karimian Azari E, Leitner C, Jaggi T, Langhans W, Mansouri A. Possible role of intestinal fatty acid oxidation in the eating-inhibitory effect of the PPAR- $\alpha$  agonist Wy-14643 in high-fat diet fed rats. PLoS One 2013;8:e74869

 Klarer M, Arnold M, Günther L, Winter C, Langhans W, Meyer U. Gut vagal afferents differentially modulate innate anxiety and learned fear. J Neurosci 2014; 34:7067–7076

14. Arnold M, Thurnherr A, Dai Y, Graber M, Pacheco-López G, Langhans W. Intraperitoneal (IP) glucagon-like peptide-1 (GLP-1) injections and meals in rats increase intestinal lymphatic GLP-1 similarly (Abstract). Appetite 2012;59:e3

15. Norgren R, Smith GP. A method for selective section of vagal afferent or efferent axons in the rat. Am J Physiol 1994;267:R1136-R1141

16. 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

17. 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

 de Lartigue G, Ronveaux CC, Raybould HE. Deletion of leptin signaling in vagal afferent neurons results in hyperphagia and obesity. Mol Metab 2014;3: 595–607

19. Liu C, Bookout AL, Lee S, et al. PPAR  $\!\gamma$  in vagal neurons regulates high-fat diet induced thermogenesis. Cell Metab 2014;19:722–730

20. Scott MM, Williams KW, Rossi J, Lee CE, Elmquist JK. Leptin receptor expression in hindbrain Glp-1 neurons regulates food intake and energy balance in mice. J Clin Invest 2011;121:2413–2421

21. Stirling LC, Forlani G, Baker MD, et al. Nociceptor-specific gene deletion using heterozygous Na\_v1.8-Cre recombinase mice. Pain 2005;113:27–36

22. Erickson JC, Clegg KE, Palmiter RD. Sensitivity to leptin and susceptibility to seizures of mice lacking neuropeptide Y. Nature 1996;381:415–421

23. Qian S, Chen H, Weingarth D, et al. Neither agouti-related protein nor neuropeptide Y is critically required for the regulation of energy homeostasis in mice. Mol Cell Biol 2002;22:5027–5035

24. Calik MW, Radulovacki M, Carley DW. A method of nodose ganglia injection in Sprague-Dawley rat. J Vis Exp 2014;93:e52233

25. Chang RB, Strochlic DE, Williams EK, Umans BD, Liberles SD. Vagal sensory neuron subtypes that differentially control breathing. Cell 2015;161: 622–633

26. 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

27. Schjoldager BT, Mortensen PE, Christiansen J, Orskov C, Holst JJ. GLP-1 (glucagon-like peptide 1) and truncated GLP-1, fragments of human proglucagon, inhibit gastric acid secretion in humans. Dig Dis Sci 1989;34:703–708

 Deacon CF, Pridal L, Klarskov L, Olesen M, Holst JJ. Glucagon-like peptide 1 undergoes differential tissue-specific metabolism in the anesthetized pig. Am J Physiol 1996;271:E458–E464

29. Gil-Lozano M, Mingomataj EL, Wu WK, Ridout SA, Brubaker PL. Circadian secretion of the intestinal hormone GLP-1 by the rodent L cell. Diabetes 2014;63: 3674-3685

30. Anvari M, Paterson CA, Daniel EE, McDonald TJ. Effects of GLP-1 on gastric emptying, antropyloric motility, and transpyloric flow in response to a nonnutrient liquid. Dig Dis Sci 1998;43:1133–1140

 Imeryüz N, Yeğen BC, Bozkurt A, Coşkun T, Villanueva-Peñacarrillo ML, Ulusoy NB. Glucagon-like peptide-1 inhibits gastric emptying via vagal afferentmediated central mechanisms. Am J Physiol 1997;273:G920–G927

32. Williams DL, Baskin DG, Schwartz MW. Evidence that intestinal glucagonlike peptide-1 plays a physiological role in satiety. Endocrinology 2009;150: 1680–1687

 Lenard NR, Berthoud HR. Central and peripheral regulation of food intake and physical activity: pathways and genes. Obesity (Silver Spring) 2008;16(Suppl. 3): S11–S22

34. Balkan B, Li X. Portal GLP-1 administration in rats augments the insulin response to glucose via neuronal mechanisms. Am J Physiol Regul Integr Comp Physiol 2000;279:R1449–R1454

35. Ahrén B. Sensory nerves contribute to insulin secretion by glucagon-like peptide-1 in mice. Am J Physiol Regul Integr Comp Physiol 2004;286:R269-R272

 Nishizawa M, Nakabayashi H, Uehara K, Nakagawa A, Uchida K, Koya D. Intraportal GLP-1 stimulates insulin secretion predominantly through the hepatoportalpancreatic vagal reflex pathways. Am J Physiol Endocrinol Metab 2013;305:E376– E387

37. Fujiwara K, Gotoh K, Chiba S, et al. Intraportal administration of DPP-IV inhibitor regulates insulin secretion and food intake mediated by the hepatic vagal afferent nerve in rats. J Neurochem 2012;121:66–76

 Vahl TP, Tauchi M, Durler TS, et al. Glucagon-like peptide-1 (GLP-1) receptors expressed on nerve terminals in the portal vein mediate the effects of endogenous GLP-1 on glucose tolerance in rats. Endocrinology 2007;148:4965–4973 39. Ahlkvist L, Ahrén B. Inhibiting the cholinergic effector system of the vagus nerve reduces the insulinotropic effects of both GLP-1 and GIP (Abstract). Diabetologia 2014;57:223

40. Calanna S, Christensen M, Holst JJ, et al. Secretion of glucagon-like peptide-1 in patients with type 2 diabetes mellitus: systematic review and metaanalyses of clinical studies. Diabetologia 2013;56:965–972

41. Zhang J, Ritter RC. Circulating GLP-1 and CCK-8 reduce food intake by capsaicin-insensitive, nonvagal mechanisms. Am J Physiol Regul Integr Comp Physiol 2012;302:R264–R273

42. Schwartz MW, Seeley RJ, Tschöp MH, et al. Cooperation between brain and islet in glucose homeostasis and diabetes. Nature 2013;503:59–66

43. Scarlett JM, Schwartz MW. Gut-brain mechanisms controlling glucose homeostasis. F1000Prime Rep 2015;7:12

44. Ahrén B. Hepato-incretin function of GLP-1: novel concept and target in type 1 diabetes. Diabetes 2015;64:715–717

45. 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