Knockdown of GLP-1 receptors in vagal afferents affects normal food intake and glycemia

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ABSTRACT

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

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 (VAN) express GLP-1R (3,4) and terminate in the lamina propria of the intestinal mucosa as well as in the wall of the hepatic portal vein (HPV) (5). VAN may therefore relay the gut GLP-1-derived 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 (see (6) and (7) for review). 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-1R on VAN 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 VAN due to genetic or environmental factors may contribute to the pathophysiology of obesity and diabetes. Hence, we examined the physiological role of VAN GLP-1R 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 shRNA construct targeting the GLP-1R mRNA transcript by injecting a lentiviral vector 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-1R 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; 3) mediate a neural component of GLP-1’s incretin effect that is physiologically relevant for the post-prandial control of blood glucose.

Collectively, our findings establish the VAN 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 VAN is not sufficient to promote obesity under normal eating conditions.

MATERIAL AND METHODS

Animals and housing

Male Sprague Dawley rats (Charles River) were individually housed (21±1°C, 55±5% HR) 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 Zürich Cantonal Veterinary Office.

Lentivirus-mediated short hairpin RNA interference

pLKO.1-puro vectors expressing turboGFP and the U6 promoter-driven shRNA sequence targeting the rat GLP-1R mRNA or a non-target shRNA sequence were obtained from Sigma-Aldrich. Efficiency of the GLP-1R-targeting shRNA construct was verified in vitro in INS-1E cells (Prof. Maechler and Prof. Wollheim, Geneva University). GLP1-R-targeting or control lentiviral particles were produced in HEK 293T cells using the pMD2.G and psPAX2 plasmids (Prof. Trono, Addgene #12259 and 12260) and concentrated to 10^10 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, Kanthonsapotheke Zürich) and xylazin (5 mg/kg, Rompun 2%, Kantonsapotheke Zürich) and nodose ganglia (NG) were exposed. A glass capillary (50 μm tip) was used to administer 1.5 μL of viral solution into each NG with a Picospritzer III injector (Parker Hannifin). To ensure expression of the viral constructs, animals were allowed to recover for 20 days. IP and HPV catheters were implanted as previously described (see (11) for complete description). Intracerebroventricular (ICV) cannulas were implanted in the 4th ventricle (stereotaxic coordinates: 2.5 mm posterior to lambda, 0 mm lateral to midline, 5 mm below skull surface) and placement was verified functionally with infusion of 5-thioglucose (Sigma-Aldrich) using a 2.5 mm injector (210 μg/rat) and anatomically post-mortem.

Tissue collection

Animals received an IP injection of pentobarbital (100 mg/kg, Kantonsapotheke Zürich) and NG, brain and pancreas were immediately collected. For gene and protein analysis tissues were frozen in liquid nitrogen and stored at -80°C. For GFP visualization, NG were fixed for 2 h in 4% paraformaldehyde, 25% sucrose solution in PBS and cut at 10 μm in a cryostat and mounted on glass slides.

Gene expression and protein analysis

The nucleus tractus solitarii (NTS), the hypothalamic dorsomedial, paraventricular, and arcuate nuclei (DMH, PVH and Arc, respectively) were micropunched using anatomical landmarks, and NG from the same animal were pooled before RNA and proteins were extracted using Trizol (Life Technologies). RT-qPCR 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 (Rabbit antibody 39072, 1:400, Abcam) using β-actin as reference (Mouse antibody AC-74, 1:3000, Sigma-Aldrich).

**Drugs**

GLP-1(7-36)amide (GLP-1, Bachem H-6795), exendin-4 (Ex-4, Bachem H-8730) and cholecystokinin octapeptide (CCK, Bachem H-2080) were resuspended in sterile PBS and administered at doses of 33 μg/kg (GLP-1), 0.3 μg/kg (Ex-4) and 4 μg/kg (CCK) via IP catheters. Ex-4 was administered into the 4th ventricle at a dose of 0.3 μg/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 (see (12) for description). 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 pre-meal 1 h before dark onset to allow GLP-1R trafficking to the VAN membrane (4). For IP CCK, 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, food
deprivation otherwise). On experimental days, rats received a 4 g chow meal containing 1% (w/w) paracetamol (4-acetamido-phenol, Sigma-Aldrich) and 0.25% (w/w) 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 post-meal blood was collected according to the scheduled timepoints. Paracetamol concentrations were measured with a commercial kit (Cambridge Life Sciences K8002).

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 timepoints after the beginning of a 5 g chow test meal. Glucose was measured twice using AccuCheck (Roche) and 150 μL of 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°C. Total active GLP-1, insulin and glucagon were measured simultaneously using an immunoassay (MesoScale Discovery multi-spot K15171C).

OGTT

16 h-food deprived rats 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≥7) and the Kolmogorov-Smirnov (when n≤6) tests and homoscedasticity was checked by visualizing the distribution of residuals. Non-parametric tests were used otherwise. When data distribution was compatible with normality, outliers were detected using the Grubb’s test. Differences were analyzed by a Student t-test for unpaired normally distributed values of equal variance (Fig. 1 C, E, F, G; Fig. 2 D; Fig 3 A-I, K; Fig. 5 A, B), or a Mann-Whitney test for unpaired comparison of non normally distributed data (Fig. 1 D; Fig. 3 L) using GraphPad Prism (version 6.05 for Windows). Where the dependent variable was affected by two factors – one within-subject factor (time or injection) and one between-subject factor (surgery group), the data were analyzed with a mixed ANOVA (Fig. 2 A-C; Fig. 3 J; Fig. 4; Fig. 5 C-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 ± SEM. P-values < 0.05 were considered significant. 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. 1A), infection of VAN was confirmed by visualizing GFP expression in NG sections (Fig. 1B). GLP-1R mRNA expression in NG was reduced by 52.5% in GLP-1R kd rats compared to control rats injected with LV-shCTL containing non-specific 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-1R expressing 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 cholecystokinin 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 the GLP-1R shRNA construct employed was specific. Finally, we measured food intake 30 min following 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 LV-mediated delivery of a shRNA construct by bilateral NG injection is tissue-specific, target-specific and does not impair vagal afferent function.

Endogenous GLP-1R signaling in the VAN 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. 2A). Moreover, energy balance remained undisturbed as documented by similar daily chow intake (Fig. 2B) and daily energy expenditure (Fig. 2C, 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 hours, the increase in meal size and duration was only evident during the dark phase.
with no significant differences in the light phase (Fig. 3B, 3C, 3E and 3F). Consistent with the
long-term 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, 3I). 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 to 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), associated with an amplified peak of energy
expenditure during the 1 h refeeding 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 (see (6,7) for review), GLP-1 and low-dose Ex-4 require intact
VAN to exert their full 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, B). In a similar design, we tested whether GLP-1R 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 minutes after a test meal (Fig. 4D, E). To test
whether these effects of GLP-1R kd are mediated by receptors expressed on peripheral or
central terminals of vagal afferents, we performed 4th ventricular (4th ICV) injection of Ex-4.
In contrast to the attenuated effects of IP Ex-4, 4th ICV injection of Ex-4 showed the full
expression of the inhibitory effects on eating and gastric emptying in kd rats (Fig. 4C, F), consistent with the idea that peripheral but not central GLP-1R 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-1R on the VAN in the gut mediates the inhibitions of eating and gastric emptying induced by IP GLP-1 or low-dose Ex-4.

Endogenous GLP-1 signaling in the VAN is required for normal glycemia after a mixed-nutrient meal but not after oral glucose challenge

GLP-1R 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-1R 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 DPP-IV in the systemic circulation and in the liver). The test meal elevated HPV blood glucose in both groups but resulted in a higher post-meal blood glucose level in the GLP-1R kd rats. Interestingly, post-meal 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 GTT, however, did not reveal differences in plasma glucose and insulin levels between control and kd rats (Fig. 5G, H)
DISCUSSION

It has long been hypothesized that VAN 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 VAN 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 VAN 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 VAN and extends to the spinal afferents and brainstem (20,21). Moreover, for a tightly controlled 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 a 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 optogenetics-related 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 VAN.
When fed *ad libitum*, body weights of control and GLP-1R kd rats were similar 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 VAN 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 kdΔPhox2b mouse model (17).

Previous data using surgical lesions concluded that vagal afferents are needed for the full expression of IP-injected GLP-1 or Ex-4 effects on food intake (8,9,16,26). Considering the fact 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 VAN in a paracrine-like fashion. Here, we present strong evidence that GLP-1R in the VAN 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 post-meal satiety, consistent with a paracrine effect of endogenous GLP-1 on VAN GLP-1R. 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-1R 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-1R 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-1R. 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 if the eating-inhibitory effect of GLP-1 on meal size is still present in animals where gastric emptying is eliminated by 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, PYY) 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 (unpublished observations), that, in turn, may prolong the intermeal interval and hence decrease meal number. Together, this strengthens the classical view that VAN GLP-1R mainly mediate short-term satiation and may be one of several redundant eating control mechanisms comprising the gut-brain axis (7,9,16,33).
Activation of pancreatic GLP-1R 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 post-meal glycemia in GLP-1R kd rats was concomitant with lower levels of HPV insulin 15 minutes 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-1R to control post-meal 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 co-infusion of glucose and GLP-1 (39). Together with our findings, this supports the idea that ascending VAN and descending pancreatic efferents form a «gut-brain-pancreas» axis mediating some of the effects of intestinal GLP-1 on insulin secretion. Intriguingly, after an 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, 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 OGTT mask the role of VAN GLP-1R. Second, glucose measurements from HPV blood sampling may not reflect the incretin effect of GLP-1R signaling in the liver. The contribution of the liver to 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 post-prandial 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-1R (17). Most likely, GLP-1R agonists do not access VAN GLP-1R when administered subcutaneously. Instead, GLP-1R agonist effects on body weight may be mediated by the activation of central GLP-1R (45). Based on our data, it is, however, possible to consider the vagus nerve as a target organ to modulate satiation and glycemia.
AUTHORS CONTRIBUTIONS

Krieger J.-P. participated in the study conception and design, conducted the experiments, analyzed the data and wrote the manuscript.

Arnold M. set up and performed the nodose ganglion injections.

Grossi Pettersen K. participated in the experiments and data interpretation.

Langhans W. participated in the study conception and design, participated in data interpretation and edited/reviewed the manuscript.

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

The authors thank Dr M. Hayes (University of Pennsylvania), Dr R. Burcelin (INSERM Toulouse) and Dr T. Lutz (University of Zürich) for their scientific advice while preparing this study. R. Clara, S. Fedele, N. Jejelava, S. Kaufman, R. Kästli, M. Klarer and M. Labouesse (ETH Zürich) are acknowledged for their precious help during animal experiments and data analysis. We thank Dr C. Boyle (University of Zürich) for support and advice related to indirect calorimetry measurements.
FIGURE LEGENDS

Figure 1: NG injection of a GLP-1R-targeting lentivirus led to a specific reduction in GLP-1R expression in the VAN

(A) Schematic representation of lentiviral injection site in a rat nodose ganglion

(B) Visualization of GFP expression in the nodose ganglion of a LV-shGLP-1R injected rat, representative picture. Scale bar, 50 μm

(C) Relative expression of GLP-1R mRNA in the nodose ganglia 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 nodose ganglia of control and GLP-1R kd rats (n=5/5; Mann-Whitney test, P<0.01) as measured by the relative intensity of the GLP-1R detection band normalized by the intensity of the β-actin band, with representative examples. A dotted line indicates where non-contiguous bands were grouped.

(E) Relative expression of GLP-1R mRNA in the pancreas (n=6/7; Student t-test, ns), NTS (n=7/7; Student t-test, ns), Arc (n=7/7; Student t-test, ns), PVH (n=7/6; Student t-test, ns) and DMH (n=7/7; Student t-test, ns) of control and GLP-1R kd rats

(F) Relative expression of LepR (n=6/7; Student t-test, ns), CCKaR (n=6/7; Student t-test, ns) and PPARg (n=6/7; Student t-test, ns) mRNA in the nodose ganglia of control and GLP-1R kd rats

(G) Percentage of decrease in 1 h food intake after IP injection CCK (4 μg/kg) relative to vehicle injection (n=6/6; Student t-test, ns) of control and GLP-1R kd rats

* indicates a significant difference between the control and GLP-1R kd groups (P<0.05)

Figure 2: VAN GLP-1R 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, 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) 24 h time course (n=8/7; ANOVA, group F(1,13)=0.08, ns) and (D) cumulative energy expenditure (n=8/7; Student t-test, ns) in control and GLP-1R kd rats fed ad libitum with chow.

Figure 3: VAN GLP-1R controlled the size and the gastric emptying of a meal

(A) Average meal size 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, ns) in ad libitum-fed control and GLP-1R kd rats.

(D) Average meal duration 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.

(G) Number of meals in 24 h, (H) 12 h dark phase, (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 (w/w) (n=9/9; ANOVA, group F(1,16)=17.98, P<0.0001; group x 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 in the first hour of ad libitum refeeding with chow after a 16 h fast (n=8/7; Mann-Whitney test, P<0.05).

* indicates a significant difference between the control and GLP-1R kd groups (P<0.05).

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.
1 h food intake after (A) IP GLP-1 (33 μg/kg; n=7/8), (B) IP Ex-4 (0.3 μg/kg; n=12/11) and (C) ICV Ex-4 (0.3 μg; n=6-8).

Plasma paracetamol concentrations of control and GLP-1R kd rats 20 minutes after allowing access to a 4 g powdered chow test meal containing 1% paracetamol (w/w) and injected with (D) IP GLP-1 (33 μg/kg; n=7/7), (E) IP Ex-4 (0.3 μg/kg; n=7/7) and (F) ICV Ex-4 (0.3 μg; 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).

Figure 5: VAN GLP-1R kd disturbed post-meal glycemia and insulinemia but did not impair tolerance of an oral glucose bolus.

HPV glucose after (A) an overnight fast (n=8/8; Student t-test, 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 x time F(5,70)=3.440, P<0.01), (D) plasma insulin (n=8/8; ANOVA; group F(1,14)=0.012, ns; group x time F(5,70)=2.90, P<0.05), (E) GLP-1 (n=8/8; ANOVA; group F(1,14)=0.73, ns; group x time F(5,70)=0.68, ns) and (F) glucagon (n=8/8; ANOVA; group F(1,14)=0.13, ns; group x 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 x time F(6,72)=1.29, ns) and (H) plasma insulin (n=7/7; ANOVA; group F(1,12)=0.16, ns; group x time F(4,48)=1.12, ns) after an OGTT (2 g/kg).

* indicates a significant difference between the control and GLP-1R kd groups (P<0.05).
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Nerve IX
Pharyngeal nerve
Superior laryngeal nerve

CNS projections
Peripheral projections

Nodose ganglion
Lentivirus injection site
Pancreas NTS Arcuate PVH DMH

GLP-1R mRNA expression relative to control
Control GLP-1R kd

GLP-1R protein bands / β-actin
Control GLP-1R kd

% reduction in food intake compared to vehicle injection
Control GLP-1R kd
**Figure 1:**

A. HPV blood glucose (mmol/L) comparison between Control and GLP-1R kd.

B. Bar graph showing the difference in HPV blood glucose levels between Control and GLP-1R kd.

C. Graph illustrating the effect of a test meal on HPV blood glucose levels, with * indicating statistical significance.

D. Graph depicting the change in HPV blood glucose levels over time following a test meal, with * indicating statistical significance.

E. Graph showing the effect of a glucose bolus on HPV plasma GLP-1 levels.

F. Graph illustrating the change in HPV plasma GLP-1 levels over time following a glucose bolus.

G. Graph comparing blood glucose levels between Control and GLP-1R kd groups.

H. Graph showing the effect of a glucose bolus on insulin secretion, comparing Control and GLP-1R kd groups.

Diabetes