Doctoral Thesis

Role of the Vagus Nerve in the Regulation of Energy Homeostasis and Glycemia by Intestinal Glucagon-Like Peptide-1

Author(s):
Krieger, Jean-Philippe

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Role of the Vagus Nerve in the Regulation of Energy Homeostasis and Glycemia by Intestinal Glucagon-Like Peptide-1

A thesis submitted to attain the degree of
DOCTOR OF SCIENCES of ETH ZURICH
(Dr. sc. ETH Zurich)

presented by
JEAN-PHILIPPE KRIEGER
Ingénieur diplômé de l’Institut des Sciences et Industries du Vivant et de l’Environnement
AgroParisTech (Paris, France)
born on 29.03.1987
citizen of France

Prof. Dr. Wolfgang Langhans, examiner
Prof. Rémy Burcelin, co-examiner
Dr. Matthew Hayes, co-examiner
Dr. Shin Jae Lee, co-examiner
Prof. Dr. Thomas Lutz, co-examiner

2016
« People ask me, 'What is the use of climbing Mount Everest?' and my answer must at once be, 'It is of no use.' There is not the slightest prospect of any gain whatsoever. Oh, we may learn a little about the behaviour of the human body at high altitudes, and possibly medical men may turn our observation to some account for the purposes of aviation. […]

If you cannot understand that there is something in man which responds to the challenge of this mountain and goes out to meet it, that the struggle is the struggle of life itself upward and forever upward, then you won’t see why we go. What we get from this adventure is just sheer joy. And joy is, after all, the end of life. »

George Mallory

Climbing Everest
ACKNOWLEDGMENTS

WARNING: The acknowledgment section usually is the most carefully read section of an entire thesis, so I will try my best not to forget anyone. If you don’t find yourself in the next lines, let me know and I will offer profuse apologies, blame it on my temporary exhaustion and get you a new copy of the thesis with your name on it.

It has been a busy, rich and fruitful journey. Now is the time to do what I could so rarely do during my busy doctoral life: take the time to say thank you and acknowledge those who contributed to this work, either by their scientific inputs, experimental help, administrative support, adventurous spirit, craziness, food supply, friendship and love.

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Moreover, I am also extremely grateful to Dr. Shin Lee. Thank you for your guidance and critical inputs along the way. We almost started in this laboratory at the same time and I can say that our work together and our constant interactions really shaped my scientific thinking. Especially, thank you for opening my mind to new approaches and showing me that we can always go one step further. The work that follows is also yours and it’s needless to say I could not have done it without you.

I would like to thank Pr. Thomas Lutz, Pr. Rémy Burcelin and Dr. Matthew Hayes for following my work over the last years, taking the time to read my thesis, and being part of my thesis committee.

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surgery room for my project. Your hard work and constant willingness to improve will always be remembered.

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I am also extremely grateful to the former members of our lab, especially Elnaz, Guggi, Ulli and Sandra for their inspirational work and the fun moments spent together. Thanks to Urs for making science fun and a little less serious, and to the members of his lab for providing us with lots of ideas and new behavioral paradigms.

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thesis.

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responsible for where I am today.

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PM, Delphine …

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pursuing the path that I chose. Thank you to my brother Olivier for really saving the world
while I was just “playing with pipettes in Switzerland”.

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<td>acetyl-coA carboxylase</td>
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<td>AgRP</td>
<td>agouti-related peptide</td>
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<td>AMPK</td>
<td>adenosine monophosphate-activated protein kinase</td>
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<td>ANOVA</td>
<td>analysis of variance</td>
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<td>AP</td>
<td>area postrema</td>
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<td>Arc</td>
<td>arcuate nucleus of the hypothalamus</td>
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<td>ATP</td>
<td>adenosine triphosphate</td>
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<td>AUC</td>
<td>area under the curve</td>
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<td>B3AR</td>
<td>β3-adrenergic receptor</td>
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<td>BAT</td>
<td>brown adipose tissue</td>
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<td>BBB</td>
<td>blood-brain barrier</td>
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<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
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<td>CART</td>
<td>cocaine- and amphetamine-regulated transcript</td>
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<td>CCK</td>
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<td>CCKaR</td>
<td>cholecystokinin a receptor</td>
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<td>CeA</td>
<td>central nucleus of amygdala</td>
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<td>CHB</td>
<td>common hepatic branch</td>
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<td>CHBX</td>
<td>common hepatic branch vagotomy</td>
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<td>clozapine-N-oxide</td>
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<td>CNS</td>
<td>central nervous system</td>
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<td>CREB</td>
<td>cAMP response element binding protein</td>
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<td>CVO</td>
<td>circumventricular organs</td>
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<td>DIO</td>
<td>diet-induced obese</td>
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<td>DMEM</td>
<td>Dulbecco's modified Eagle Medium</td>
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<td>DMH</td>
<td>dorsomedial hypothalamus</td>
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<td>dorsal motor nucleus of the vagus nerve</td>
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<td>DPP-IV</td>
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<td>DREADD</td>
<td>designer receptor exclusively activated by designer drugs</td>
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<td>DRG</td>
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<td>ENS</td>
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<td>GABA</td>
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<td>GIP</td>
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<td>pseudorabies virus</td>
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<td>vagal efferent neurons</td>
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<td>vagus nerve</td>
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<td>VNS</td>
<td>vagus nerve stimulation</td>
</tr>
<tr>
<td>VO₂</td>
<td>rate of oxygen consumption</td>
</tr>
<tr>
<td>vWAT</td>
<td>visceral white adipose tissue</td>
</tr>
<tr>
<td>WAT</td>
<td>white adipose tissue</td>
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<tr>
<td>YFP</td>
<td>yellow fluorescent protein</td>
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ABSTRACT

In a world where 600 million adults are obese, understanding this condition and the associated comorbidities has become urgent. Because obesity is due to an imbalance between energy intake and energy expenditure, our field of research aims at providing the fundamental knowledge of how the energy balance is normally regulated and how this regulation may fail in pathological states. In this context, the role of the so-called “gut-brain axis” in the control of food intake has received increasing attention. Indeed, the mechanical, metabolic or hormonal signals generated in the periphery are centrally integrated and translated into an appropriate eating behavior. In this gut-brain communication, the vagus nerve (VN) plays an essential role. It consists of a bidirectional neuronal route, conveying bottom-up information from the gastrointestinal tract about food quantity and composition, and exerting a top-down control on gastrointestinal motility and secretion.

One of the intestinal peptides interacting with the VN is the glucagon-like peptide-1 (GLP-1). Originally identified as an incretin hormone, GLP-1 has been found to inhibit eating and gastric emptying. Based on these findings, GLP-1 receptor (GLP-1R) agonists have been developed and are promising pharmacological tools in the treatment of obesity and diabetes. In the present thesis, we dissect the molecular and functional interaction between endogenous intestinal GLP-1 and the VN in the control of food intake, energy expenditure and glycemia.

Initially, we reviewed the available literature (CHAPTER 2) testing the hypothesis that vagal afferent neurons (VAN) mediate the effects of intestinal GLP-1 on food intake and glycemia. This literature review revealed several important gaps and remaining questions to be addressed.

First, the previously-available evidence supporting a role for the vagus nerve in mediating the effects of intestinal GLP-1 on food intake and glycemia were based on methods that lesioned the VN. The results of lesion studies are difficult to interpret because the lesions affect both VAN and vagal efferents neurons (VEN) and may mask potential antagonist effects of different vagal fibers. Hence, the exact role of VAN GLP-1R in mediating the effects of endogenous intestinal GLP-1 was still unclear and required a new experimental approach. We set up a bilateral nodose ganglion (NG) injection technique to deliver a lentiviral vector and knock down GLP-1R in VAN via RNA interference. Using this approach, we showed that GLP-1R in VAN are necessary for the normal control of meal size, gastric emptying and post-meal glycemia (CHAPTER 3).
Second, we further investigated potential downstream mediators of the GLP-1 signal within VAN and their relevance for the control of food intake and gastric emptying. Multiple neuropeptides have been identified in the NG and some of them, including the Cocaine- and Amphetamine-Regulated Transcript (CART) had already been implicated in the eating-inhibition induced by cholecystokinin (CCK). We screened several neuropeptides in the NG for their transcriptional response to GLP-1 in a primary VAN culture model and found that only CART expression was modulated. We further showed that GLP-1R activation induces VAN CART expression in vivo. Moreover, using two models of CART inhibition, we demonstrated that a full CART expression in VAN and CART release in the NTS are necessary for the full eating and gastric emptying inhibitory effects induced by intraperitoneal GLP-1 (CHAPTER 4). These results implicate for the first time CART as a neuropeptide transmitting the signal of GLP-1 to the brainstem. The function and neuronal targets of CART within the NTS remain to be further investigated.

Additionally, the idea that endogenous peripheral GLP-1 modulates energy expenditure was largely unexplored. We set up a protocol to investigate the effects of peripheral GLP-1R activation on energy expenditure in wild-type rats while controlling for food intake and gastric emptying, two potential confounding factors. We found that peripheral GLP-1R activation induced a transient decrease of whole-body energy expenditure, accompanied by a decrease in interscapular brown adipose tissue (iBAT) temperature. Moreover, upon high-fat diet exposure, reduced GLP-1R expression in VAN increased energy expenditure and BAT thermogenesis, and alleviated the development of HFD-induced obesity and glucose intolerance. Together with a retrograde tracing study from the iBAT and central gene expression analysis, this led us to propose a new vagally-mediated pathway by which endogenous peripheral GLP-1 tunes down iBAT activity after a meal (CHAPTER 5).

Considered together, these findings provide novel mechanistic and functional data about the gut-to-brain communication in the control of energy balance. Beyond the classic view that VAN are involved in the acute mediation of satiation signals, our findings indicate that vagal GLP-1R also play a role in the acute control of glycemia and the chronic control of energy expenditure. Our results also raised new questions about the exact role of vagally-expressed neuropeptides in the control of energy balance and their contribution to the development of obesity. Moreover, based on our findings, we developed the novel idea of a specific time window at the onset of diet-induced obesity, in which HFD-induced secretion of endogenous intestinal GLP-1 may amplify the development of an obese phenotype (CHAPTER 6).

Eventually, our findings and the questions that they raised may contribute to the fundamental knowledge needed for the further development of GLP-1-based or vagal-targeting therapies.
RESUME

Dans le monde, plus de 600 millions d'individus sont obèses; la compréhension des mécanismes de cette pathologie, ainsi que celle des maladies qui lui sont associées, est devenue urgente. Le développement de l'obésité est dû à un déséquilibre quantitatif et chronique entre l'énergie ingérée et l'énergie dépensée. Notre domaine de recherche s'efforce donc d'éclaircir les mécanismes de contrôle de la balance énergétique, et de comprendre comment un dérèglement de ces mécanismes aboutit à l'obésité. Dans ce contexte, le rôle que joue l'« axe intestin-cerveau » dans le contrôle de la prise alimentaire est devenu un thème de recherche incontournable. En effet, les signaux mécaniques, métaboliques ou hormonaux générés en périphérie sont intégrés au niveau du cerveau et aboutissent à la formation d'un comportement alimentaire approprié. Dans cette communication entre l'intestin et le cerveau, le nerf vague joue un rôle essentiel. Le nerf vague est une voie neuronale bidirectionnelle qui, d'une part, transmet de manière ascendante des informations du tube digestif concernant la quantité et la qualité des aliments ingérés et, d'autre part, exerce un contrôle descendant sur les propriétés contractiles et sécrétrices de l'estomac et l'intestin.

Le « glucagon-like peptide-1 » (GLP-1) est un des signaux peptidiques provenant de l'intestin et interagissant avec le nerf vague. GLP-1 fut identifié en premier lieu comme une hormone amplifiant la sécrétion d'insuline induite par l'élévation du glucose plasmatique. Plus tard, de nombreux autres effets métaboliques de cette hormone ont été découverts, notamment son rôle dans la réduction de l'appétit et la régulation de la vidange gastrique. En se basant sur ces effets endogènes, des agonistes du récepteur au GLP-1 ont été développés et sont désormais à la base de prometteurs traitements contre l'obésité et le diabète. Dans cette thèse, nous analysons en détail les interactions moléculaires et fonctionnelles entre le nerf vague et le GLP-1 intestinal dans le contrôle de l'homéostat énergétique et glucidique.

Nous débutons par une revue de la littérature scientifique (CHAPITRE 2) testant l'hypothèse selon laquelle les afférences vagales sont des intermédiaires indispensables aux effets du GLP-1 intestinal sur la prise alimentaire et la glycémie. Cette revue de la littérature a révélé d'importants manques dans notre compréhension des interactions GLP-1/nerf vague et souligne les questions en suspens auxquels nous nous sommes efforcés de répondre par une série d’approches expérimentales.

Tout d'abord, les études disponibles concernant ce sujet sont basées principalement sur des lésions du nerf vague. Les études basées sur des lésions sont difficiles à analyser de manière précise car les afférences comme les efférences vagales sont coupées et cela
masque la potentielle complexité des effets des différentes fibres vagales. Par conséquent, le rôle exact des récepteurs au GLP-1 présents sur les afférences vagales restait à éclaircir par le biais d’une nouvelle approche expérimentale. Pour cela, nous avons développé une technique d’injection bilatérale dans le ganglion pléxiforme afin d’y délivrer un vecteur viral et de réduire l’expression des récepteurs au GLP-1 par interférence ARN. Grâce à cette approche, nous avons pu montrer que les récepteurs au GLP-1 présents sur les afférences vagales sont en effet nécessaires au contrôle normal de la satiété, de la vidange gastrique, et de la glycémie post-prandiale (CHAPITRE 3).

Dans un second temps, nous souhaitions investiguer l’implication des différents neuropeptides exprimés par les fibres vagales dans le contrôle de la prise alimentaire et de la vidange gastrique par GLP-1. En effet, de nombreux neuropeptides ont été détectés dans le ganglion pléxiforme et certains d’entre eux, tels que le “Cocaine- and Amphetamine-Regulated Transcript” (CART), ont déjà été impliqués dans l’effet satiétogène de la cholécystokinine. En revanche, l’implication de tels neuropeptides dans le signal vagal de GLP-1 était une thématique inexplorée. Nous avons étudié la réponse transcriptionnelle de plusieurs neuropeptides à l’application de GLP-1 dans une culture primaire d’afférences vagales et avons détecté que seule l’expression de CART était modulée. Nous avons par la suite vérifié que l’activation des récepteurs au GLP-1 induisait également l’expression de CART in vivo. De plus, en utilisant deux modèles distincts d’inhibition de CART, nous avons démontré que l’expression de CART par les afférences vagales ainsi que la sécrétion de CART dans le noyau du tractus solitaire (NTS) sont nécessaires aux effets sur la satiété et la vidange gastrique d’une injection intra-péritonéale de GLP-1 (CHAPITRE 4). Ces résultats impliquent pour la première fois CART en tant que transmetteur du signal vagal de GLP-1 vers le tronc cérébral. Les cibles neuronales ainsi que la fonction exacte de CART dans le NTS restent à investiguer.

De plus, l’idée que GLP-1 puisse moduler la dépense énergétique restait également peu étudiée. Nous avons initialement développé un protocole d’étude des effets de l’activation périphérique des récepteurs au GLP-1 sur la dépense énergétique de rats (phénotype sauvage) tout en contrôlant la prise alimentaire et la vidange gastrique, deux potentiels facteurs de confusion. Nous avons constaté que l’activation des récepteurs au GLP-1 périphérique conduisait à une diminution de la dépense énergétique, accompagnée d’une diminution de la température du tissu adipeux brun interscapulaire (iBAT). Nous avons par la suite montré qu’un knockdown du récepteur au GLP-1 dans les afférences vagales, pendant l’exposition à un régime riche en gras, engendrait une augmentation de la dépense énergétique et de l’activité du iBAT. Ces changements étaient suffisants pour freiner le développement d’un phénotype obèse et l’apparition de l’intolérance au glucose. En
complément, un marquage rétrograde des neurones provenant du iBAT et l’analyse de l’expression centrale de gènes impliqués dans le métabolisme énergétique nous permettent, au final, de proposer un nouvelle route neuronale par laquelle le GLP-1 intestinal réduit l’activité du iBAT après un repas (CHAPITRE 5).

Pris dans leur ensemble, nos résultats apportent de nouvelles connaissances sur les aspects mécanistiques et fonctionnels de la communication intestin-cerveau. Au-delà de la vue classique selon laquelle les afférence vagales sont impliqués dans les signaux de satiété à court terme, nous montrons également qu’elles sont impliquées dans la régulation de la glycémie et dans le contrôle chronique de la dépense énergétique. Nos résultats posent également la question du rôle des neuropeptides dans les afférences vagales et de leur contribution au développement de l’obésité. De plus, nos résultats nous ont permis de formuler l’hypothèse selon laquelle le développement de l’obésité (par opposition à une obésité établie) consiste en une fenêtre spécifique durant laquelle une importante sécrétion de GLP-1 par l’intestin peut participer à l’aggravation du phénotype obèse (CHAPITRE 6).

Finalement, nos résultats et les questions nouvelles qu’ont soulevées ces expériences permettront d’apporter des connaissances fondamentales au développement de thérapies ciblant GLP-1 et le nerf vague.
CHAPTER 1: GENERAL INTRODUCTION

OBESITY AND THE CONTROL OF ENERGY BALANCE BY THE VAGUS NERVE AND THE GLUCAGON-LIKE PEPTIDE-1
1 The worldwide obesity epidemic: trends, causes and possible solutions

Research articles and talks on energy homeostasis are often introduced with the statement that obesity is on the rise. The use of this introductory statement implies that our field of research is dedicated to provide the fundamental knowledge that, in fine, will help to alleviate obesity and its consequences. Hence, to introduce my work, I wanted to relay the current views on trends, causes, and consequences of the obesity epidemic. Furthermore, I will discuss how the approach of our laboratory, focusing on the control of food intake, can help understand and tackle the obesity epidemic.

1.1 The epidemiology of obesity: a short overview of current trends

Obesity itself is defined by an increase in the amount of body fat, approximated at the population level by an elevated body mass index (BMI). In 2014, more than 1.9 billion adults were overweight (BMI > 25) and 600 million were obese (BMI > 30), representing 39% and 13% of the adult world population, respectively (1). Moreover, these figures have doubled since 1980 and this strong upward trend seems even more alarming than the worldwide prevalence itself. The rise of obesity is a major public health concern because obesity is strongly associated with an impressive array of comorbidities (including type 2 diabetes mellitus, hypertension, coronary heart disease, dyslipidemia, … (2)) and, as a result, with a reduction in life expectancy (3). Forecast models predict that the life-shortening effects of obesity-associated diseases may reverse the increase in life expectancy induced by medical advancements in the United States (US) during the 21st century (4).

The American continent, as well as Europe and the Eastern Mediterranean area, show worldwide the highest prevalence of obesity (Fig. 1). It would however be misleading to consider obesity a high-income country problem: indeed, in low- and middle-income countries (as classified by the World Bank), where the prevalence of overweight and obesity
is still relatively low, obesity is rising dramatically, especially in large urban areas and among children. Moreover, in all regions (as defined by the World Health Organization), the obesity rates are higher among women than men (1).

![Figure 1: Prevalence of obesity by sex and regions as defined by the World Health Organization (1)](image)

Most of the key facts of the prevalence of overweight and obesity are based on the classification of individuals in discrete BMI ranges (i.e., overweight: 25 to 30, mildly obese: 30 to 35, etc). This does not present a full picture of the overall distribution of individuals BMI within the population and how this distribution evolved in recent years. Indeed, a slight shift of the population weight could have dramatically increased the number of individuals falling into the superior BMI category. This data presentation in BMI groups has been debated and, in some cases, accused to artificially magnify the problem (5). Figure 2 presents a less often seen, more complete picture of the BMI shifts at the population level. It compares the BMI distribution of adults and adolescents in the United Stated between the 1976-1980 and a 1999-2004 National Health and Nutrition Examination Surveys (NHANES). This presentation shows that, both for adults and adolescents, the distribution has shifted to the right, with a greater shift for the upper BMI percentiles (6).

Together, these data indicate that obesity is indeed on the rise and that a better understanding of the causes of such a profound trend is needed.
Figure 2: Change in the distribution of BMI between the 1976-80 and the 1999-2004 NHANES in adults (20-74 years old; left) and children and adolescents (6-19 years old; right) in the US (6)

It is, however, important to emphasize that these data are of epidemiological nature and are thus, limited in two ways. First, they may not translate at the individual level. This is exemplified by the case of “healthy” obese individuals, i.e., individuals that are obese but do not show the epidemiologically-associated symptoms of one or several comorbidities. When these healthy obese subjects are followed long enough, comorbidities may eventually develop (7), thus reconciliating epidemiological and individual data. Second, epidemiological data do not show causality, and understanding the true causes of obesity requires other approaches. In the next paragraph, we will try to apprehend the complexity of the causes leading to obesity.

1.2 A complex network of causes leads to obesity

Looking at obesity from a medical point of view may give the impression that obesity is a rather moncausal disease that can be traced to a simple disruption of the energy balance. It is, however, a more complex network of causes that drives the obesity epidemic.

1.2.1 One cause, several causes, layers of influences

Initially reserved for infectious diseases, the word “epidemic” is now widely used to also refer to non-communicable diseases (NCD) that are spreading rapidly on a worldwide scale. Although the same word is used to describe both infectious diseases and NCD, causes of NCD are thought to reside in a more complex network of causes.
Egger (8) proposes to conceptually divide the causes of a NCD into several “layers of influence” (see Fig. 3): risk factors are the most immediate causes of the disease. They are, in turn, influenced by proximal (i.e., nutrition, sedentary time), medial (i.e., food and exercise environments) or distal (i.e., economics, social context) determinants.

### 1.2.2 Illustrating the multifactorial origin of obesity

As an exhaustive list of all proposed causes for the rise in obesity would be beyond the scope of this introduction, we chose to illustrate these “layers of influence” with a few examples, starting with the more distal determinants.

Economic aspects may be one of the most tangible distal determinants of the rise in obesity. As mentioned in a very complete review by J. Cawley (9), the first simple economic determinant of obesity could be a differential evolution of the cost of energy-dense vs. nutritious foods: a striking example is the dramatic decrease in the prices of sugar and sweetened beverages between 1990 and 2007 in the US (2L bottle of Coca Cola -34.9%). Meanwhile, the prices of fresh fruits and vegetables steadily rose (10). Moreover, the NHANES 2005-2008 showed a negative correlation between weight and income, especially among women (11), supporting the idea that the increasing cost of food, together with low income, may impact food choices and caloric intake. Whether these changes in prices were large enough to contribute to the rise of obesity in the US is however debated (9).

Typical medial or proximal determinants of obesity are often grouped under the term of “lifestyle”. The emergence of modern western lifestyles, including a shift in nutrition and exercise habits, seems indeed to temporally correlate with the emergence of obesity as an...
issue at the population level (12). To illustrate the diversity of these so-called lifestyle factors, we can use Egger and Dixon’s attempt to summarize them under the acronym “NASTIE ODOURS”: Nutrition, (In)Activity, Stress, Technology-induced pathologies, Inadequate sleep, Environment, Occupation, Drugs/smoking/alcohol, Over/underexposure, Relationships, Social factors (8).

Moreover, genetic factors play a role in the variation of body fatness. Early evidence came from family, twin or adoption studies, in which the contributions of the environmental and genetic factors could be differentiated and quantitatively estimated (see (13) for review). Based on these studies, 40-70% of the BMI variability between individuals has been attributed to genetics. With the technical advances of genome sequencing, genetic polymorphism of large populations can be correlated with BMI in genome-wide association studies (GWAS). This new approach led to the identification of around 100 loci linked to an obese phenotype, explaining around 21% of BMI (14). Consequently, GWAS further emphasize the strong genetic component in BMI variations. The discrepancy between the contribution of genetic factors seen in GWAS (21%) and in family studies (40 - 70%) may, however, indicate that a large component of the genetic influence on BMI is due to non-common variants, that are thus not found in large population screening.

Together, these determinants of obesity illustrate the multifactorial and complex nature of the pathology of obesity.

1.3 Current approaches used to tackle obesity

In this section, we will briefly review the panel of existing approaches currently being available. This will underline why understanding the control of food intake (i.e., the topic of our laboratory) is relevant for obesity.

1.3.1 “Lifestyle” interventions

Many clinical interventions have been designed to promote healthy nutrition and exercise and induce weight loss in overweight or obese individuals. A meta-analysis of several of
these interventions, however, indicates only a modest weight loss (< 5 kg) after 2 to 3 years (15). The reasons for this limited long-term weight loss are unclear but do not seem to be linked with a low long-term compliance to the new dietary and exercise habits (15). Interestingly, despite the relatively small weight loss, improvements in cardiovascular outcomes and glucose metabolism persist for years after the initial intervention (15,16), suggesting a weight-independent benefit of the lifestyle interventions.

In addition, broad multi-approach programs have been designed to prevent the development of overweight and obesity. The EPODE program (French for « Together Let’s prevent childhood obesity ») for example, uses an approach based on social marketing and help of local communities to promote exercise and healthy nutrition in children (17), thus attempting to soften the effects of economic and lifestyle determinants. The diversity of the actions undertaken, the numbers of actors involved and their long-term engagement are key factors for the success of such programs. The main drawbacks of these programs are their cost and the relative difficulty to monitor their impact (9).

1.3.2 Weight-reducing drugs

Where prevention and lifestyle interventions have not sufficed, medical solutions have emerged. A first category of medical help can be found in weight-reducing drugs. Some of these drugs primarily reduce the absorption of calories, such as Orlistat, the most common anti-obesity drug. Orlistat inhibits the pancreatic lipase and, thus, the absorption of food-derived triglycerides in the intestine. Another class of drugs is designed to mimic or target appetite-controlling pathways, such as rimonabant (a cannabinoid receptor 1 antagonist), pramlintide (a synthetic amylin analog), or the glucagon-like peptide-1 receptor agonists (exenatide, liraglutide, …). A historical perspective indicates that the toxicity of appetite-regulated drugs needs to be carefully reviewed, because several of them were withdrawn due to life-threatening side effects, including alterations of the cardiovascular functions (such as sibutramin or the fenfluramine-phentermine combination).
It is however interesting to note that pharmacological interventions with the above-mentioned drugs reduce body weight by 5-10 kg after 2 years in overweight and obese individuals (15), indicating an improved weight loss efficacy compared to a diet-only therapy.

1.3.3 Bariatric surgery

Bariatric surgery, which comprises several techniques reorganizing the anatomy of the gastrointestinal (GI) tract, is regarded as the most effective weight loss strategy for obese individuals (18,19). Indeed, techniques such as the Roux-en-Y gastric bypass surgery, which reduces the stomach to a pouch and bypasses the duodenum and a part of the jejunum, results in a rapid weight loss and in the resolution of diabetes, and shows a greater maintenance of weight loss than any other method to date (18). Although irreversible and linked with the inherent risks of an invasive surgery, the number of such interventions has peaked in the early 2000’s and may have stabilized today between 100,000 and 200,000 bypass surgeries per year in the US (20,21). Surprisingly, the mechanisms of the effectiveness of bypass surgeries are relatively unclear, and research projects identifying these mechanisms may lead to new efficient therapies.

1.4 Understanding the control of food intake to tackle obesity.

The first law of thermodynamics states that the energy consumed must be either expended, excreted or stored. This indicates that obesity, i.e., the storage induced by excess energy intake, can theoretically be reduced by a change in the energy balance and, in particular, the energy intake. As mentioned earlier, drugs, surgeries or lifestyle interventions aim to treat obesity via, at least partly, a reduction in the energy consumed by individuals. To provide the fundamental knowledge needed for this medical approach, our laboratory has focused on the physiological mechanisms that control food intake, as well as on the potential factors that may override a normal energy balance during obesity.

The science behind eating behavior was first dominated by the view that the utilization of glucose was a primary factor in the initiation and termination of meals (22) and that specific hypothalamic brain areas were responsible for one particular aspect of eating behavior (23).
In the next 40 years, a much more complex picture has emerged: according to this contemporary view, a complex central neuronal network responds to a spectrum of signals emanating from the GI tract (hormonal, neuronal, metabolic) and adipose tissue. The central command also integrates signals from the external environment or learned eating habits such as food cues and, in fine, translates them into an appropriate behavioral response.

A first set of signals that control food intake are those that are generated during meals and influence satiation (i.e., meal termination) or satiety (i.e., meal intervals). Typically, these signals are of mechanical nature (distention of the stomach) or are peptides secreted by the gut (e.g., cholecystokinin, CCK (24)). Moreover, hormones secreted in proportion to body fat, such as insulin and leptin, act as adiposity signals. They gain access to the hypothalamic and hindbrain neurons influencing food intake and energy homeostasis, thus exerting a longer-term control on these parameters via the modulation of autonomic and neuroendocrine pathways (25). It is generally assumed that an inability of the brain to integrate these adiposity signals contributes to the development of an obese phenotype (26).

Among the central circuit integrating satiety and adiposity signals, neurons of the arcuate nucleus of the hypothalamus (Arc) were broadly studied for their role in the neural control of energy homeostasis. Briefly, one population of neurons expresses the proopiomelanocortin (POMC) gene and stimulates a melanocortin signaling pathway to exert a catabolic action. A second set of neurons express the orexigenic neuropeptides agouti-related peptide (AgRP) and neuropeptide-Y (NPY) to induce anabolic processes, including the stimulation of appetite. There is evidence that the action of adiposity signals relies on the activation of the POMC neurons while suppressing the activity of the NPY/AgRP neurons (see (25) for review).

It would, however, be too simplistic to assume that this neuronal circuit in the Arc integrates all peripheral and environmental signals. A whole field of research still investigates the roles of a broad array of brain areas in the control of energy intake and expenditure, and it would
be beyond the scope of this introduction to detail all of them (see (27) for a more complete overview of brain circuits).

In brief, the mechanisms that control food intake have progressively been unraveled in the last 60 years (see Fig. 4). Most notably, the role of the peripheral signals has been better characterized, with more than 30 gut peptides identified (28). Nevertheless, how exactly peripheral signals get into the brain, are integrated and translated into eating behavior, is still largely unclear. In particular, the deregulation of this well-integrated system that may lead to obesity remains to be fully discovered.

Figure 4: A model summarizing the control of energy intake by the integration of peripheral signals by hypothalamic and hindbrain circuits (26)
Abbreviations: ARC, arcuate nucleus of the hypothalamus; CCK, cholecystokinin; GI, gastrointestinal; LHA, lateral hypothalamus; NPY, neuropeptide Y; NTS, nucleus of the tractus solitarii; PFA, perifornical area; POMC, proopiomelanocortin; PVN, paraventricular nucleus of the hypothalamus

In summary, we highlighted in this section the current rise in worldwide obesity and the complexity of causes that drive this trend. Because obesity is caused by an imbalance between energy intake and energy expenditure, our lab’s research aims at providing the
fundamental knowledge of how eating is normally controlled and how this system can get deregulated.

In the next sections, I will introduce the two systems that will be further studied in the subsequent chapters. Consecutively, I will describe how the vagus nerve (VN) and the gut hormone glucagon-like peptide-1 (GLP-1) are involved in the control of eating, the regulation of glycemia and the control of energy expenditure.

2 The vagus nerve, a bidirectional information highway linking brain and periphery for the regulation of body homeostasis

The gut-brain axis is a conceptual entity that names all information that is conveyed from the GI tract to the brain. In the context of eating, the signals conveyed by the gut-brain axis inform about food amount, composition and energy content. The VN has been recognized as a major neuronal component of the gut-brain axis in the control of food intake (29). Before explaining its role in more details, I will briefly describe the anatomy of the VN as well as some of its other classic functions unrelated to eating.

2.1 Anatomy of the vagus nerve

The VN (cranial nerve X) innervates all organs in the thoracic and abdominal cavities. Its complicated journey between the brain and the abdomen earned the VN the name of « wanderer ». Vagal efferent neurons (VEN) primarily arise from the nucleus ambiguus and the dorsal motor nucleus of the VN (DMV), whereas vagal afferent neurons (VAN) mainly project to the nucleus tractus solitarii (NTS). Numerous rootlets exit the medulla and join before exiting the jugular foramen. VN fibers then pass through two ganglia, where the afferent cell bodies are located (jugular/superior and nodose/inferior ganglion), and separate in several branches (auricular, meningeal, sympathetic, pharyngeal and laryngeal branches). The remaining VN fibers travel along the carotid artery and form the laryngeal nerve or send branches to the esophagus, heart and lungs. Finally, after passing the diaphragm, VN fibers
innervate all abdominal organs and related ganglia or plexuses (30). The ventral (left) and dorsal (right) trunks separate into several branches: the gastric branches, the celiac branches and the common hepatic branch. Gastric branches innervate the stomach and proximal intestine whereas celiac branches innervate the small intestine, cecum and colon (31). The common hepatic branch only originates from the ventral trunk. It innervates not only the liver and the hepatic portal vein (HPV), but also the stomach, pancreas and proximal intestine (31) (see Fig. 5).

As mentioned above, the VN contains VAN and VEN. VAN are pseudounipolar neurons whose cell bodies reside in the nodose ganglia (NG). They are involved in the sensing of numerous peripheral stimuli (mechanical, chemical, nociceptive, …) and relay the corresponding signals to the dorso-vagal complex in the hindbrain. Preganglionic VEN have their cell bodies in the DMV and project to postganglionic neurons located in close proximity to the target organs. VEN mostly convey motor signals to the periphery and constitute a large part of the parasympathetic nervous system. In the VN, the number of afferents greatly outweighs the number of efferents, with 60 to 80% of vagal neurons being afferents coming from visceral organs (30). The absolute number of vagal neurons has been estimated to be around 16,000 to 18,000 and to be conserved across species (32).

The VN contains A- (large, myelinated), B- (intermediate, lightly myelinated) and C-fibers (small, unmyelinated) but most of the fibers (60-80%) are afferent C-fibers from visceral organs (30). VEN use acetylcholine as a major neurotransmitter, whereas VAN are mostly glutamatergic. A wide array of neurotransmitters and neuropeptides, however, are expressed in the NG, but their roles are still unclear (32).
Chapter 1: Obesity, the vagus nerve and the glucagon-like peptide-1

Figure 5: Schematic representation of the anatomy of the vagus nerve (adapted from (31)).
Cervical and thoracic branches have been simplified by the author for clarity but are all bilateral. Subdiaphragmatic branches have been highlighted over the original figure: hepatic branch (yellow), gastric branch (green) and celiac branch (green).
Abbreviations for periphery: abd, abdominal; ac, celiac artery; access, accessory; agd, right gastric artery; ags, left gastric artery; ahc, common hepatic artery; al, splenic artery; ams, superior mesenteric artery; auric, auricular; br(s), branch(es); cel, celiac; commun, communicating; dep, depressor; gangl, ganglion; hep, hepatic; laryng, laryngeal; la, larynx; mening, meningeal; mes, mesenteric; n, nerve; ph, pharynx; reccur, reccurent; sup, superior; tr, trachea; v,vein.
Abbreviations for the brain: amb, nucleus ambiguus; dmnX, dorsal motor nucleus of the vagus nerve; NTS, nucleus of the tractus solitarii; AP, area postrema.
2.2 Overview of vagus nerve homeostatic functions

The VN functions as an « unconscious inner brain », integrating peripheral signals via VAN and providing metabolic feedback regulation via VEN (30). With its broad neuronal network, the VN plays a role in the regulation of numerous organ functions, such as breathing, heart rate, blood pressure and vascular stiffness (30). It would be beyond the scope of this introduction to detail the role of the VN in regulating all these functions but I can, as an example, briefly mention the predominant role of the VN in mediating two well-described reflexes. First, the cough reflex is largely mediated by VAN inputs from the larynx and trachea via activation of so-called cough receptors on C and Aδ fibers. The appropriate response to this stimulation originates in the NTS: the nucleus ambiguous is activated and efferent fibers controlling respiratory and pharyngolaryngeal muscles generate the cough (33). Moreover, a second well-described vagal loop is the Hering-Breur reflex, also known as respiratory sinus arrhythmia: sensing of bronchial stretch by VAN is relayed in the NTS to activate cardiac vagal motor neurons. This results in the heart rate variation due to respiration (33).

In the next paragraphs, I will describe the role of the VN in the following physiological functions: the control of eating and energy expenditure, as well as the regulation of glucose metabolism.

2.3 Role of the vagus nerve in the control of eating and post-absorptive mechanisms

As mentioned earlier, the VN densely innervates the GI tract. VAN sense post-absorptive mechanical and chemical signals relevant for the control of food intake, and VEN are involved in the control of digestion and absorption of nutrients. Both VAN and VEN play a role in the control of eating at several levels. In the stomach, they mostly relay mechanical stimuli and control gastric secretion; in the intestine, VAN are mostly involved in the chemosensing of gut peptides.
2.3.1 Vagal sensing and control of gastric motility and secretion

Electrophysiological and physiological evidence suggests that VAN convey information about the volume of food ingested via gastric distension. Indeed, gastric loads were shown to activate VAN (34) and induce neuronal activation in the NTS and DMV (35). Moreover, infused gastric loads of identical volumes but of different nutritional content were associated with similar firing rates of gastric VAN (36) and similar eating responses (37). Most mechanosensitive VAN terminals innervating the stomach are intraganglionic laminar endings (IGLE) located in the connective “capsule” of the myenteric plexus (38), and intramuscular arrays (IMA) located in the stomach longitudinal and circular muscle layers (39). Although structurally different, both terminals respond to the mechanical cues generated by stretch and contraction of the stomach muscle layers (40,41). How they functionally differ is, however, still unclear (42). A less abundant subgroup of gastric VAN terminates in the gastric mucosa and is sensitive to touch (41,43), i.e., to the direct contact with a gastric load, rather than gastric distension. Together, these structural and functional observations led to the view that gastric VAN primarily integrate the mechanical signals induced by a gastric load.

Several observations suggested that VE are involved in the coordination of secretion and motility of the GI tract. First, vagotomy acutely impairs the reservoir function of the stomach (44) and differentially modulate the gastric emptying of solids and fluids (de Lartigue, unpublished). Moreover, numerous GI pathologies in which gastric motility or secretion is impaired (gastroparesis, esophageal reflux, …) are associated with dysfunctional vagal reflexes (45). Two distinct pathways in the parasympathetic postganglionic neurons mediate the effects of gastric VEN on gastric motility. The first is an excitatory cholinergic pathway that results in the activation of muscarinic cholinergic receptors in the gastric smooth muscle and eventually increases gastric motility and secretion. The second pathway is a non-cholinergic, non-adrenergic pathway that inhibits gastric function via the release of nitric
oxide and or vasoactive intestinal neuropeptide (46). Together, these two excitatory and inhibitory pathways provide the possibility of a precise descending control of gut motility.

Preganglionic DMV neurons projecting to the stomach show spontaneous tonic activity in a pacemaker-like fashion (47) but are under constant modulation by humoral or descending CNS inputs and via VAN inputs (via a relay in the NTS) (see (48) and (45) for review). Neuronal projections from the NTS to the DMV (mostly glutamatergic, GABAergic and catecholaminergic inputs) directly link VAN to VEN, providing the neuronal substrate for so-called « vago-vagal reflexes ». Several vago-vagal reflexes have been studied and are relevant for the maintenance of the regional coordination of food transit. The gastric accommodation reflex, for example, causes a reduction in pressure of the stomach corpus when the antrum is stretched (49). This allows for filling of the stomach without an increase in gastric pressure and, thus, accommodates various meal sizes. Moreover, it induces a retropropulsion of the chyme that greatly participates in the mechanical disruption of food particles during digestion. This reflex is initiated by gastric vagal mechanoreceptors from the antrum that cause, after a relay in the NTS, the inhibition of DMV neurons maintaining gastric tone in the corpus (50). Vago-vagal reflexes also coordinate gastric secretion during the gastric phase of the digestion: VEN cholinergic inputs to the stomach stimulate H+ release by parietal cells. This acid release is further amplified by the vagally-induced release of histamine from ECL cells. Moreover, among other contributors, VEN induce the release of gastrin from the G-cells and attenuate the inhibition of gastrin release by somatostatin. Gastrin, in turn, stimulates H+ secretion from parietal cells and the release of peptidases by chief cells. Together, these vagally-mediated mechanisms coordinate the secretion of acids and enzymes needed for the chemical processing of the ingested food.

In summary, gastric VEN activation via vago-vagal reflexes results in:

a/ the regional contraction and relaxation of the stomach, which facilitates the mechanical disruption of the food and allows its transit to the duodenum for further nutrient absorption
b/ the control of acid secretion and the release of digestive enzymes resulting in the chemical breakdown of food particles.

2.3.2 Vagal chemosensing in the intestine

Several early observations support the view that intestinal VAN are predominantly involved in chemosensing rather than mechanosensing. First, although the several types of mechanoreceptors described in the stomach (IMA, IGL E and mucosal afferents) are also present in the small intestine, their abundance is greatly reduced (51). Moreover, nutrient infusions into the proximal small intestine elicited a nutrient-specific pattern of activation in intestinal VAN (52).

As demonstrated by tracing studies, VAN are mainly present within the crypts and villi of the lamina propria (Fig. 6) (53). They are therefore in close proximity to the intestinal epithelial cells, but not directly exposed to luminal contents. It is therefore unlikely that their principal role is to sense luminal nutrients (51). They are, however, ideally positioned to sense the gut peptides released by enteroendocrine cells (54). Moreover, intestinal VAN express receptors for a wide spectrum of gut peptides (55). Due to the proximity between enteroendocrine cells and VAN terminals, the classical view is that VAN sense gut peptides in a paracrine-like fashion. Moreover, it was recently reported that some enteroendocrine cells show long axon-like processes called « neuropods » (Fig. 6) (56). This opened the possibility that enteroendocrine cells form synapses with sensory fibers. The in vivo functionality of these neuropods and how exactly they connect to VAN remains to be further explored.

Gut-derived CCK is the best-characterized example of mediation of a gut peptide’s effects on food intake and gastric motility by VAN. Indeed, VAN express the CCKa receptor (CCKaR), CCK stimulates VAN discharge and VAN lesions block the effects of CCK on food intake and gastric motility. Moreover, CCK regulates the expression of certain receptors and neuropeptides in VAN to encode the nutritional status and modulate the capacity of food stimulation (see (58) for review). There is also substantial evidence that VAN mediate the
effects of other anorectic peptides, including the peptide YY (59) and the glucagon-like peptide-1 (see below and chapter 2).

To summarize, intestinal VAN mostly encode nutrient composition via the sensing of gut peptides and are thus involved in the control of FI. While gastric VEN are clearly involved in the control of gastric motility and secretion, the role of intestinal VEN is unclear. Indeed, the enteric nervous system (ENS) is the main regulator of intestinal motility but a functional interaction between VEN and ENS is probable. To which extent VEN can control intestinal motility is, however, unknown.

2.4 Role of the vagus nerve in the control of glucose homeostasis

This section will discuss the current literature describing the role of the VN in glucoregulatory processes in the pancreas and the liver.

First, VAN terminating in the hepatoportal bed are implicated in the control of glucose homeostasis. Indeed, a glucosensory site localized in the HPV, upstream of the hepatic hilus (60) is connected to VAN (61). These afferents fire upon glucose stimulation in a concentration-dependent manner (62) and project to the NTS (63), which makes neural
connections to hypothalamic glucoregulatory centers (64). The exact role of VAN in this process has, however, not been fully established, mainly due to the use of unspecific lesioning techniques (common hepatic branch vagotomy). In the next chapter, we will discuss the possibility that VAN can mediate an effect on glucose homeostasis initiated by the activation of GLP-1 receptors. The activation of the VAN of the hepatoportal bed has been shown to increase whole-body glucose utilization by both insulin-dependent and – independent mechanisms (65). Briefly, recent findings support the idea of a gut-to-brain-to-periphery axis controlling glucose homeostasis via a VEN feedback on hepatic glucose production (hepato-incretin axis) (66) and pancreatic islet insulin release (neuroincretin effect) (67).

2.5 Role of the vagus nerve in the control of energy expenditure

Indications for a role of the VN in the control of energy expenditure come in part from animal and human studies involving electrical VN stimulation (VNS). Indeed, stimulation of the cervical VAN decreased brown adipose tissue (BAT) temperature and indicators of energy expenditure in anesthetized rats (68,69). Conversely, auricular VNS in diet-induced obese rats induced weight loss without changes in daily intake, indicative for an increased energy expenditure (70). In humans, a temporary interruption of VNS in epileptic patients led to a reduction in energy expenditure, correlating with a reduced BAT activity (71). On the other hand, another study involving chronic subdiaphragmatic VNS in pigs showed no changes in energy expenditure, although the measurement was technically limited (72). These apparently contradictory findings may be explained by the use of slightly different models of VNS. Moreover, several molecular manipulations of VAN in mice, have led to changes in energy expenditure. Indeed, downregulation of the nuclear receptors peroxisome proliferator-activated receptor γ (PPARγ) and liver X receptor α and β (LXRA/β) in paired-like homeobox 2b (Phox2b)-expressing neurons amplified the high-fat diet (HFD)-induced increase in energy expenditure (73,74). Although there are concerns with respect to the use of Phox2b as a marker of VAN (75), these results support the idea that disrupting vagal sensing can
impact whole-body energy expenditure. A recurrent finding, though, is the correlation between changes in whole-body energy expenditure and interscapular BAT activity (69-71). How VAN inputs to the NTS affect the sympathetic outputs to BAT is largely unknown (68) and remains to be further investigated.

3 The glucagon-like peptide-1, a multifaceted peptide involved in energy homeostasis

3.1 Secretion of intestinal GLP-1

Intestinal GLP-1 is secreted by enteroendocrine L-cells. Such cells are found all along the intestine; within the small intestine, the highest density of L-cells is found in the ileum (76,77). The peptide GLP-1 is encoded by the preproglucagon gene and further post-translationally processed by the prohormone convertase PC1/3 (78). GLP-1 is found in vivo in several forms but only the GLP-1(7-36) and GLP-1(7-37) are believed to be active: both forms were shown to have similar biological effects and kinetics in humans, although the GLP-1(7-36) form is predominant (79). L-cells release GLP-1 upon nutrient stimulation: the current view is that several G-protein coupled receptors (GPCRs) expressed by the L-cells sense the digestion products of carbohydrates, lipids and proteins (80). The intracellular signals generated by the binding of these compounds to the receptors interact with glucose utilization and cation transporters to induce a calcium efflux from the endoplasmic reticulum, with the resulting increase in intracellular calcium triggering GLP-1 release (80) (Fig. 7). Moreover, recent findings indicate the possibility that certain fatty acids or bacterial compounds induce the release of GLP-1 via a metabolic effect on L-cells, involving mitochondrial uncoupling and changes in ATP production (81,82). After its release, GLP-1 is rapidly inactivated by the enzyme dipeptidyl peptidase-IV (DPP-IV) via N-terminal degradation at the alanine position 2 (83). Plasmatic and liver concentrations of DPP-IV are high, thus limiting the half-life of GLP-1 in the systemic circulation (84).
The GLP-1 receptor (GLP-1R) is a 7 transmembrane-spanning heterotrimeric GPCR class B (85). It is expressed in many tissues, including pancreatic α-, β-, and δ-cells but also in heart, muscles, white adipose tissue, stomach, skin and numerous brain regions (86). The GLP-1R has been shown to couple to multiple intracellular downstream pathways (adenylate cyclase, phospholipase C (PLC), phosphokinase A or C (PKA, PKC), phosphoinositide 3-kinase (PI3K), mitogen-activated protein kinase (MAPK), ...) depending on the cell type studied (87,88) and can thus be either a Gs-, Gi-, Gq- or Go-coupled receptor (89). Desensitization has been observed in vitro in β-cell models (90,91), but, in vivo, subchronic GLP-1R agonist treatment was not associated with a reduction of the GLP-1R-dependent insulin response (92). Nevertheless, internalization of the ligand/receptor complex and trafficking between membrane and intracytoplasmic locations were observed in vivo in the brain (93) and in the NG (94). These observations led to the idea that, in certain tissues, GLP-1R sensitivity can be temporarily modulated by previous GLP-1R activation and the nutritional status.
3.2 The biological functions of GLP-1 in glucose and energy homeostasis

3.2.1 Peripheral GLP-1’s effects on glucose homeostasis

GLP-1 was initially identified as an incretin hormone both in rats and humans (95,96): together with the gastric inhibitory peptide (GIP), GLP-1 enhances the glucose-induced insulin secretion and, thus, limits postprandial glucose excursion. The insulinotropic effect of GLP-1 is at least partly mediated by the direct binding of GLP-1 to its receptor on the pancreatic β-cells (97). The β-cell GLP-1R activates the adenylate cyclase, via a stimulatory G-protein, and increases intracellular cAMP levels. A broad array of intracellular pathways are then activated by PKA and the exchange protein activated by cAMP-2 (Epac2) that lead to β-cell membrane depolarization, increases in intracellular ATP and Ca^{2+} and, eventually, to the release of insulin storage vesicles (see (98) for review). Importantly, the insulinotropic action of GLP-1 is strictly glucose-dependent, as shown by the absence of insulin secretion in islets perfused with low glucose levels (99). Beyond the enhancement of glucose-induced insulin secretion, GLP-1R activation on the β-cells also promotes insulin synthesis at the transcriptional (translocation of the cAMP responsive binding element protein (CREB) promoter to the nucleus) and post-transcriptional level (enhancement of mRNA stability) (100). Moreover, GLP-1R activation increases β-cell mass in vitro and in vivo, via increased β-cell proliferation and reduced β-cell apoptosis (101,102). This protective mechanism may involve an intra-islet secretion of GLP-1 by α-cells, shifting their prohormone convertase expression profile from PC2 (leading to glucagon synthesis) to PC1/3 (67). In the pancreatic islets, GLP-1 also inhibits glucagon secretion by α-cells and stimulates somatostatin secretion by δ-cells, likely via a direct effect on their GLP-1R (103). In vitro evidence also suggests that GLP-1 controls glucose homeostasis via direct insulin-independent effects on muscles, adipose tissue and liver (see (86) for review). In vivo, it is difficult to highlight potential direct GLP-1 effects on glucose homeostasis because insulin, glucagon and somatostatin secretions are affected by the pharmacological administration of GLP-1. Experiments in humans undergoing an euglycemic hyperinsulinemic clamp together with a
normalization of somatostatin and glucagon levels have not shown insulin-independent effects of GLP-1 on glucose infusion rates (104). Further studies suggested little to no acute or chronic insulin-independent effects of GLP-1 on glucose turnover at the whole-body level (97,105).

3.2.2 Peripheral effects of GLP-1 on eating

The effects of endogenous GLP-1 and exogenous GLP-1R agonists on eating will be further discussed along the thesis. Briefly, I would like to provide here evidence that endogenous GLP-1 inhibits eating. Peripheral administration of GLP-1 or GLP-1R agonists reduced food intake in a variety of species, including rodents, primates and humans (106-108). The classic view is that endogenous intestinal GLP-1 is mainly implicated in meal termination: indeed, GLP-1 secretion is rapidly induced by luminal nutrient stimulation and GLP-1 HPV concentrations rise already during a meal, which is consistent with an intra-meal effect. Moreover, acute intraperitoneal (IP) injections of exendin-4 (Ex-4, a GLP-1R agonist) or GLP-1, shortly before or during a meal, reduced food intake via an effect on meal size (i.e., by inducing satiation) (109,110). The mechanisms by which endogenous intestinal GLP-1 reduces meal size have not been fully elucidated (see (111) for review). An endocrine effect on brain regions expressing GLP-1R and involved in the control of eating has received some experimental support (112). The very limited GLP-1 half-life in the plasma, however, is more consistent with a paracrine effect on VAN. In the next chapter, the potential vagal mediation of GLP-1’s effects on food intake and glycemia will be further discussed. Intestinal GLP-1 is also thought to exert a potent control on gastric acid secretion and motility via the VN (113). Slowing nutrient transit in the stomach, in turn, affects glucose homeostasis (slower nutrient absorption). Moreover, it likely increases the duration of nutrient interactions with vagal mechanoreceptors and may therefore participate in the eating inhibition induced by GLP-1.
Off note, diverse other organs have been shown to express the GLP-1R (heart, bones, kidney, testis, …) and a broad spectrum of functions have been reported. It is beyond the scope of this introduction to detail them, but the most studied non-classic functions involve cardioprotection (see (114) for review) and neuroprotection (see (115) for review) (Fig. 8).

Figure 8: overview of the known effects of GLP-1 on eating, glucose homeostasis, cardiac and brain functions (86)

3.2.3 Evidence for an effect of GLP-1 on energy expenditure

There is evidence indicating that direct activation of central GLP-1R in the ventromedial hypothalamus (VMH) increases energy expenditure via the activation of BAT thermogenesis (116). Whether this mechanism is relevant for the control of energy expenditure by endogenous GLP-1 has not been established. In addition, the effect of peripheral GLP-1R activation on energy expenditure has scarcely been studied. Conflicting reports regarding the effects of GLP-1 on energy expenditure can be found in the literature (Table 1). Briefly, elevated circulating GLP-1 levels have been associated with increased energy expenditure in mice (117) and humans (118). This has been supported by the fact that central as well as
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Peripheral administration of GLP-1 or GLP-1R agonists acutely increased VO₂ or heat production in mice (116), rats (119-121) and humans (121). Inversely, others reported no change (122) or an acute decrease in VO₂, heat production or diet-induced thermogenesis in mice (123), hamsters (124) and humans (125,126) (Table 1). These discrepancies may be explained by the different doses, agonists (GLP-1, Ex-4, liraglutide) or models (mice, rats, hamsters, humans) used in these studies. More importantly, the different routes of administration used (IP, intravenous (IV) or subcutaneous (SC)) may provide access to distinct GLP-1R populations and, hence, contribute to the conflicting reports.

3.3 The central GLP-1 system: effects and interaction with the periphery

There is early evidence for the presence of glucagon-like peptides in the mammalian brain (127,128). More recently, the cell bodies of PPG-expressing neurons have been localized in the NTS, the reticular formation and the olfactory bulb (129-131). PPG-expressing neurons project to most of the known GLP-1R-expressing brain areas (54,132), with an important density of projections to the paraventricular and dorsomedial nuclei of the hypothalamus (PVN and DMH) (133). Data from a mouse model expressing the yellow fluorescent protein (YFP) in PPG-expressing cells suggested that GLP-1 neurons in the olfactory bulb are mainly local interneurons, and that most of the projections seen in the brain originate from brainstem PPG neurons (132,134). PPG neuronal projections are found in many GLP-1R-expressing brain areas, suggesting a functional role for the central GLP-1 system. Several studies (reviewed in (135)) used the central administration of GLP-1 or GLP-1R agonists to test the potential functions of central GLP-1R populations: the range of response observed was strikingly similar to the effects of peripheral GLP-1, i.e., notably a suppression of food intake and an improved glucose tolerance. Pharmacological activation of brain GLP-1R, however, is not a direct assessment of the physiological role of the central GLP-1 system. More recently, a PPG knockdown in the NTS of rats via RNA interference indicated a physiological role for NTS PPG neurons to regulate HFD-induced weight gain and glucose intolerance (136). Moreover, an activation of Phox2b neurons in the NTS (among which are
### Table 1: Summary of available evidence that central or peripheral GLP-1R modulate whole-body energy expenditure and its surrogate markers (Krieger 2015, unpublished)

<table>
<thead>
<tr>
<th>Model</th>
<th>Drug</th>
<th>Route</th>
<th>Dose</th>
<th>Effects described</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mice</td>
<td>Ex4</td>
<td>ICV</td>
<td>1 µg</td>
<td>Reduced VO2 / Reduced RER</td>
<td>Baggio 2004 (123)</td>
</tr>
<tr>
<td>Mice</td>
<td>Liraglutide</td>
<td>ICV</td>
<td>3 µg</td>
<td>Increased EE</td>
<td>Beiroa 2004 (116)</td>
</tr>
<tr>
<td>Rat</td>
<td>GLP-1</td>
<td>ICV</td>
<td>1 µg</td>
<td>Increased VO2 (for 2h, in lean but not in obese rats)</td>
<td>Hwa 1998 (119)</td>
</tr>
<tr>
<td>Rats</td>
<td>GLP-1</td>
<td>ICV</td>
<td>3 nmol</td>
<td>Increased VO2 (still occurs in vagotomized rats)</td>
<td>Osaka 2005 (120)</td>
</tr>
<tr>
<td>Humans</td>
<td>GLP-1</td>
<td>IV (post meal)</td>
<td>50 pmol/kg/h</td>
<td>Decreased EE and DIT</td>
<td>Flint 2000 (125)</td>
</tr>
<tr>
<td>Humans</td>
<td>GLP-1</td>
<td>IV (post meal)</td>
<td>50 pmol/kg/h</td>
<td>Decreased EE and DIT</td>
<td>Flint 2001 (126)</td>
</tr>
<tr>
<td>Humans</td>
<td>GLP-1</td>
<td>IV (180' infusions)</td>
<td></td>
<td>Increased VO2, VCO2, EE</td>
<td>Shalev 1997 (121)</td>
</tr>
<tr>
<td>Rats</td>
<td>GLP-1</td>
<td>IV</td>
<td>0.2 to 5 nmol</td>
<td>Increased VO2 and increased body temperature</td>
<td>Osaka 2005 (120)</td>
</tr>
<tr>
<td>Mice</td>
<td>Ex4</td>
<td>IP</td>
<td>1.5 µg</td>
<td>Reduced VO2 / Reduced RER (for about 2h)</td>
<td>Baggio 2004 (123)</td>
</tr>
<tr>
<td>Rats</td>
<td>Ex4 / Ex9</td>
<td>SC</td>
<td>5 / 50 µg/kg</td>
<td>Reduced EE during fasting</td>
<td>Abegg 2013 (122)</td>
</tr>
<tr>
<td>Mice</td>
<td>DPP IV -/-</td>
<td></td>
<td></td>
<td>Increased VO2 (throughout the day)</td>
<td>Conarello 2002 (117)</td>
</tr>
<tr>
<td>Humans</td>
<td>Correlative study</td>
<td></td>
<td></td>
<td>Fasting levels of GLP-1 are positively correlated with basal EE, independent of body composition</td>
<td>Pannacciuli 2006 (118)</td>
</tr>
</tbody>
</table>

Abbreviations: DIT, diet-induced thermogenesis; DPP IV -/-, dipeptidyl-peptidase IV knockout mice; EE, energy expenditure; Ex-4, exendin-4; Ex-9, exendin-9; ICV, intracerebroventricular; IP, intraperitoneal; IV, intravenous; RER, respiratory exchange ratio; SC, subcutaneous.
the PPG neurons) using “Designer Receptors Exclusively Activated by Designer Drugs” (DREADD) supported a role for PPG neurons in reducing the intake of palatable foods (137). Further experiments involving site- and time-specific manipulations of central GLP-1R are however needed to critically examine the physiological relevance of endogenous central GLP-1 and the roles of distinct GLP-1R populations.

As mentioned earlier, the functions of central and peripheral GLP-1 seem redundant. Little is known, though, about the interplay between intestinal and central GLP-1 (138) and whether the peripheral release of GLP-1 in a postprandial situation induces the release of central GLP-1 is still unclear. The possibility that peripheral GLP-1 directly activates PPG neurons, via GLP-1R located on dendrites in the area postrema (AP) for example, is very unlikely. Indeed, PPG neurons do not express the GLP-1R and are not electrically activated by GLP-1 (139). Alternatively, GLP-1-sensitive neurons in the AP may serve as a relay for the activation of PPG neurons: GLP-1R expressing neurons in the AP, however, are mostly catecholaminergic neurons and recent results indicate that NTS PPG neurons do not receive catecholaminergic inputs (140). Peripheral GLP-1 may activate PPG neurons via VAN, because PPG neurons receive direct monosynaptic glutamatergic inputs from VAN (139). Unpublished observations (from Lutz and Vrang), however, did not show co-localization between Ex-4-induced c-Fos and GLP-1 immunoreactivity in the NTS of rats. In brief, although it is tempting to think that peripheral GLP-1 can stimulate central GLP-1 secretion, there is limited functional and mechanistic evidence to support this idea. The peripheral and central GLP-1 systems may be two separate entities with converging functions. Other peripheral signals activate PPG neurons: indeed, the electrical activity of these neurons is modulated by other peripheral signals such as CCK, leptin (139) and gastric distension (141). The current understanding of how central GLP-1 neurons interact with the periphery and exert their functions was summarized by (142) (see Fig. 9).
3.4 The GLP-1R agonists for anti-diabetes and anti-obesity therapies

While the studies presented in this thesis mainly investigate the mechanisms by which endogenous GLP-1 exerts its functions, it is important to note that GLP-1R agonists are currently used as anti-diabetes and anti-obesity treatments, and that their action does not necessarily mimic the mechanisms of action of endogenous GLP-1. The differences between endogenous GLP-1 and the pharmacological administration of GLP-1R agonists are highlighted.

Several pharmaceutical companies have tested numerous GLP-1R agonists for their ability to improve glucose tolerance in type 2 diabetic patients. These GLP-1R agonists are either short-acting (e.g., exenatide, also called Ex-4, or lixisenatide) or long-acting compounds (e.g., liraglutide, albiglutide, dulaglutide, …) (143). It is generally conceptualized that short-acting compounds primarily have a positive effect on glycemia via a strong inhibition on gastric emptying combined with an insulinotropic and glucagonostatic action. Long-acting
compounds, however, seem to exert a more profound effect on glucose homeostasis. Indeed, chronic administration of GLP-1R agonists improved glucose tolerance in obese individuals, via mechanisms that involve an improved glucose-induced insulin secretion and reduced glucagon secretion, but also an improved capacity of resistant β-cells to secrete insulin and the improvement of β-cell mass (144). Reports that chronic administration of GLP-1R agonists induced weight loss in obese patients led to the recent FDA approval of liraglutide as a weight loss drug (145). The mechanisms, however, are less clear. In a recent study, Secher and colleagues showed that liraglutide has direct access to the brain and induces weight loss that is mediated via GLP-1R in the Arc (93).

This study, among others, highlights the idea that the mechanisms by which GLP-1R agonists inhibit eating and regulate glucose homeostasis, may be distinct from the mechanisms by which endogenous peripheral GLP-1 fulfills similar functions. In other words, pharmacological administration of GLP-1R agonists may bypass the normal route by which intestinal GLP-1 signals the brain and may therefore rather mimic several aspects of the central GLP-1 system.

In summary, we can conclude that endogenous peripheral and central GLP-1 exert several physiological functions including the control of food intake, the regulation of glycemia and energy expenditure. The physiological mechanism by which endogenous peripheral GLP-1 signals the brain is unclear and may not be recapitulated by the use of GLP-1R agonists. Understanding this physiological mechanism may provide a new approach to target the GLP-1 system.

### 4 Aims of the thesis

Early in my thesis, I was intrigued and interested in the concept of a gut-brain axis controlling the energy balance. I rapidly focused on the possibility that the VN, specifically VAN, can mediate the effects of peripheral endogenous GLP-1 on food intake, glycemia and energy expenditure. Therefore, I structured my thesis around the following aims.
The first aim was to evaluate the available evidence that the VN mediates the effects of peripheral endogenous GLP-1 on food intake and glycemia. Indeed, a broad array of studies points towards a role of VAN in mediating the endogenous peripheral GLP-1 signal to the brain. Nonetheless, these studies only provide indirect evidence for a role of VAN GLP-1R signaling in the normal control of eating and regulation of glycemia. The article found in **CHAPTER 2** reviews these studies.

This literature review showed that the experimental methods used to date did not specifically test the physiological role of VAN GLP-1R in energy balance. Therefore, the second aim of my thesis was to develop a new method to specifically test whether VAN relay a GLP-1 signal to the brain relevant for the control of food intake (including gastric emptying) and glycemia. Thus, in **CHAPTER 3**, I present the bilateral delivery of a lentiviral vector into the NG as a way to specifically knock down GLP-1R in rat VAN. Building on this new method, we critically examined the physiological role of the endogenous gut-derived GLP-1 acting on vagal GLP-1R to control food intake and participate in gastric emptying and glucose homeostasis.

As mentioned earlier, the presence of numerous neuropeptides has been reported in the NG, and some of these peptides have been implicated in the control of food intake. Whether a vagally-mediated GLP-1 signal involves such neuropeptides is, however, unknown. Based on our previous results, we hypothesized that the effects of GLP-1R activation in the VAN may rely on the expression and the release of one of these neuropeptides. **CHAPTER 4** presents our current results showing that CART is a necessary mediator of the control of food intake and gastric emptying by GLP-1.

In the initial literature review presented in Chapter 2, I did not comment on a role of the VN in the effects of GLP-1 on energy expenditure because the role of peripheral GLP-1 on energy expenditure itself was understudied and remained unclear. Hence, the fourth aim of the study was to investigate whether endogenous peripheral GLP-1 can modulate energy expenditure and if so, whether this is vagally-mediated. Moreover, based on preliminary reports indicating
a modulation of BAT activity by VNS or central GLP-1 activation, we also investigated the role of the BAT in a potential modulation of energy expenditure by peripheral GLP-1. Overall, in CHAPTER 5, we present evidence that, with high-fat diet exposure, the activation of VAN GLP-1R reduces energy expenditure and BAT activity and that a specific attenuation of this gut-BAT crosstalk can alleviate the course of HFD-induced obesity.

In summary, this thesis aims at providing a clearer view on the vagal mediation of GLP-1’s effects in the control of energy balance, both in a physiological and obesogenic context.

The present thesis comprises research articles originally written for peer-reviewed journal publications. The articles are presented in the form in which they have been published (CHAPTERS 2 and 3) or in a form that reflects our current understanding of ongoing studies (CHAPTERS 4 and 5). A more detailed description of the research aims can be found in each chapter, as well as a discussion of the findings. Eventually, considering these articles together, new scientific questions emerged, that we will discuss in CHAPTER 6.
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CHAPTER 2:

VAGAL MEDIATION OF GLP-1’S EFFECTS ON FOOD INTAKE AND GLYCEMIA

AUTHORS

Krieger JP, Langhans W, Lee SJ

Physiology and Behavior Laboratory, ETH Zürich, Switzerland

STATUS

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ABSTRACT

Nutrient stimulation of the enteroendocrine L-cells induces the release of the glucagon-like peptide-1 (GLP-1), an incretin and satiating peptide. Due to its short half-life, meal-induced GLP-1’s effects on food intake and glycemia are likely to be mediated in part by a paracrine signaling mechanism near the site of release. Early and recent findings from vagus nerve (VN) lesion studies scrutinized in this review strongly support an important role of the VN in mediating GLP-1’s effects. Peripheral GLP-1 or GLP-1R agonist treatment failed to elicit the full satiating effects and maintain glucose homeostasis in various lesion models. The potential mechanisms underlying the vagal GLP-1R mediated satiation and glycemic control presumably involve the activation of caudal brainstem neurons via glutamatergic signaling, which activate a vagal reflex loop or/and relay the information to higher brain centers. Recent studies also presented here, however, diminish the relevance of the VN for the pharmacological intervention of obesity and diabetes with chronic GLP-1R agonist treatments, suggesting that endogenous intestinal GLP-1 and GLP-1R agonists may activate different GLP-1R populations. Finally, lesion-based approaches are limited and new technical approaches are discussed to improve the understanding of vagal GLP-1R functions in maintaining normal energy balance and its relevance in pharmacological interventions.
1 Introduction

Eating stimulates the release of glucagon-like peptide-1 (GLP-1) from intestinal L-cells (see (1-4) for review). GLP-1 is mainly known for its incretin effect, i.e., for its ability to enhance glucose-induced insulin secretion (5-7), and for its satiating effect, which is predominantly expressed as a reduction in meal size (see (8) for review). Once in the blood stream, GLP-1 is subject to rapid degradation by the enzyme dipeptidyl-peptidase-4 (DPP-IV) (9,10), which is also present in the liver (11). This limits the amount of active GLP-1 reaching the systemic circulation and its potential access to the brain and to remote areas in the periphery. It therefore stands to reason that a paracrine mechanism, i.e., the activation of GLP-1 receptors (GLP-1R) located in the vicinity of L-cells, can mediate some of GLP-1’s effects on eating and glycemia. The vagus nerve has initially been proposed to relay the GLP-1 initiated signals to the brain: vagal afferent neurons (VAN) terminate in the lamina propria of the intestinal mucosa (12), express GLP-1R (2,13) and are activated in response to GLP-1 (2,14,15), giving them both an ideal position and the cellular machinery to sense L-cell-released GLP-1, and to relay the resulting signals to the brain. Moreover, central terminals of VAN are mainly found in the nucleus tractus solitarii (NTS), an area that is known to integrate many energy-related signals from the periphery and descending neural signals from the forebrain (see (8,16) for review). Numerous results reviewed below further implicate the VN in the mediation of endogenous GLP-1’s effects on eating and glycemia. Paradoxically, recent data investigating the mode of actions of long-lasting GLP-1R agonists such as liraglutide in appetite reduction and glycemic control question the role of VAN in mediating endogenous GLP-1 effects on the maintenance of energy balance. This review aims at dissecting these findings in order to distinguish between the physiological and pharmacological relevance of vagal GLP-1R activation. Moreover, the mechanisms by which vagal GLP-1R activation decreases food intake and controls glycemia, including VAN GLP-1R signaling and central relays, are unclear. We will discuss recent findings and present a
brief overview of potential pathways involved in vagal GLP-1R effects on food intake and glycemia. Finally, we emphasize that previous studies have often used unspecific lesion techniques to demonstrate the necessity of the VN in mediating GLP-1’s effects on food intake and glycemia, and we therefore advocate for further careful investigation of the relationship between GLP-1 and the VN.

2 Vagal GLP-1R activation in the control of food intake

2.1 Necessity of vagal afferents

Early studies reported that peripheral administration of GLP-1 promoted satiation and reduced energy intake in humans and rats (17-20), raising the possibility that GLP-1 therapy could be effective in treating obesity. Therefore, many laboratories attempted to unravel the mechanisms of peripheral GLP-1 mediated satiation (21-23). The eating-inhibitory effect of intraperitoneally (IP) injected GLP-1 was blocked by IP, but not by intracerebroventricular (ICV) administration of the GLP-1R antagonist, exendin-(9-39) (Ex-9) (24), suggesting that the effect of peripheral GLP-1 on food intake is mediated by GLP-1R in the periphery. Given the short half-life and the generally low concentration of GLP-1 in the systemic circulation, the hypothesis that a neural pathway mediates peripheral GLP-1’s effects on food intake has been explored. A number of studies (summarized in Table 1) used a combination of vagal manipulation with activation or blockade of peripheral GLP-1R. The inhibitory effect on food intake of low doses of IP exendin-4 (Ex-4, a GLP-1R agonist) was lost in mice pretreated with capsaicin (25), suggesting the involvement of sensory fibers. Moreover, bilateral total subdiaphragmatic vagotomy (VGX) attenuated the reduction of food intake induced by IP injection of GLP-1 in rats (26), suggesting that the eating-inhibitory effect of peripheral GLP-1 requires an intact VN. More recent data in patients with VGX and pyloroplasty extended this conclusion to humans, demonstrating that an intact VN is needed for the inhibition of food intake by intravenous (IV) GLP-1 (27). The VGX procedure, however, lesions VAN as well as vagal efferent neurons (VEN), and the absence of efferent projections from the brainstem
can significantly alter gut motility, exocrine and endocrine secretion and, subsequently, eating behavior (28). Subdiaphragmatic vagal deafferentation (SDA), the most complete and selective vagal deafferentation method available to date (29-31), leaves about 50% of the VEN intact and, hence, largely avoids the strong side effects of VGX. Studies using SDA allow for a markedly improved interpretation of the lesion-induced attenuation of GLP-1’s effects on food intake (32-35). Altogether, they indicate the necessity of VAN in mediating peripheral GLP-1 satiation, consistent with the idea that intestinal GLP-1 can induce meal termination via activation of vagal GLP-1R.

### 2.2 Roles of vagal subbranches

GLP-1R are expressed on vagal afferent fibers innervating the hepatoportal bed (i.e., on the common hepatic branch - CHB) as well as on fibers innervating the gastrointestinal (GI) tract (i.e., the CHB as well as the gastric and celiac branches) (12). Therefore, the eating-inhibitory effects of intestinal GLP-1 could either be mediated by GLP-1R signaling through the CHB of the VN (endocrine action) or by the vagal afferent branches innervating the intestine (paracrine action). One way to address this question is to administer GLP-1 via different routes. Indeed, IP injection of GLP-1 appears to provide an easy access to the lamina propria of the intestinal mucosa, where VAN innervating the GI tract terminate. An infusion of GLP-1 into the hepatic portal vein (HPV), however, should mainly activate GLP-1R present in the hepatoportal bed, i.e. on the CHB. To differentiate these possible mechanisms of eating inhibition by peripheral GLP-1 infusions, a previous study from our laboratory used a combination of remotely controlled intrameal GLP-1 infusions (IP or HPV) and SDA, and showed that intact vagal afferents are required for the inhibition of eating after IP, but not after HPV, GLP-1 infusions (35), as previously suggested by other studies (14,36). Whether the CHB of the VN is required for the reduction of food intake after IP GLP-1 was directly addressed by Hayes and colleagues. In this study, rats showed a blunted eating-inhibitory response to IP GLP-1 after SDA, but not after a selective ablation of the CHB (CHBX), indicating that IP injected GLP-1 does not control food intake
### Table 1: Summary of animal studies investigating the role of the vagus nerve in mediating GLP-1’s effects on food intake by combining vagal manipulation with peripheral GLP-1R activation or blockade. **Abbreviations:** ICV: intracerebroventricular, IP: intraperitoneum, JV: jugular vein, HPV: hepatic portal vein, SC: subcutaneous, VC: vena cava; CHBX: common hepatic branch vagotomy, SDA: subdiaphragmatic vagal deafferentation, VGX: vagotomy

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via the activation of vagal GLP-1R in the hepatoporal bed (33). Consistent with this interpretation, HPV and vena cava infusions of GLP-1 reduced meal size similarly (34). These findings collectively suggest that an acute IP injection of GLP-1 reduces food intake via an activation of GLP-1R located on VAN of gastrointestinal but not hepatic origin. As GLP-1 released during meals is likely to activate GLP-1R present on VAN terminals in the lamina propria, it stands to reason that GLP-1 released during meals reduces food intake via a similar mechanism. These findings also suggest, however, that high systemic blood levels of GLP-1 or of its DPP-IV-resistant agonists reduce food intake via a different mechanism that may involve central GLP-1R activation.

Recent studies challenged the prevailing view that vagal afferents innervating the GI tract are the main neural pathway mediating peripheral GLP-1 effects on food intake. Injection of a DPP-IV inhibitor in the HPV that raises HPV (but not systemic) GLP-1 levels within the physiological range have been shown to reduce 1 h and 12 h food intake (37). Moreover, this effect required an intact CHB. These findings can only be reconciled by the idea that pharmacological inhibition of DPP-IV might raise HPV GLP-1 in a more pronounced and sustained way in contrast to IP or HPV injection of GLP-1; the duration of GLP-1 elevation in the HPV may be a critical factor for the activation of the CHB.

2.3 Mediation of acute vs chronic effects of GLP-1R agonists by the vagus nerve

The use of GLP-1R agonists to treat obesity is looming above the horizon. It is therefore important to ask whether vagal afferents are also required for the eating-inhibitory and anti-obesity effects of long-lasting GLP-1R agonists. Two studies examined the acute effects of IP infusions of variable doses of Ex-4 or liraglutide on food intake (1 to 24 h) in SDA rats (32,34). Together, these studies demonstrated that the lesion of vagal afferents either decreased the sensitivity (i.e. the threshold dose needed to decrease food intake) or blunted the short-term effect of GLP-1R agonists on food intake. This suggests that vagal afferents are needed for the full expression of the acute effects of GLP-1R agonists on food intake.
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GLP-1R agonists are, however, administered chronically at pharmacological doses and the same conclusion may not hold for the chronic effects of GLP-1R agonist on food intake and body weight. In fact, food intake and body weight were decreased to the same extent in Sham and SDA rats after a 14-day treatment with subcutaneous (SC) liraglutide (38). Moreover, a genetic deletion of the glp-1r gene in paired-like homeobox 2b (Phox2b)-expressing visceral nerves did not affect the effects of liraglutide on short-term (4 h and 24 h) chow intake or 14-day cumulative food intake and body weight change (39). These data suggest that vagal afferents do not play a major role in mediating the chronic changes in food intake and body weight after pharmacological administration of GLP-1R agonists.

2.4 Summary

Together, these results advocate for prudence when it comes to make statements about the role of vagal GLP-1R activation in controlling food intake.

a) There is strong evidence indicating that endogenous GLP-1 released during meals reduces meal size by activating GLP-1R located on intestinal vagal afferents.

b) This mechanism may be mimicked by IP injected GLP-1 and also be the basis for the acute effects of GLP-1R agonists.

c) High levels of circulating GLP-1, which can be achieved by intravenous infusions of GLP-1 or pharmacological inhibition of DPP-IV activity, reduce food intake by recruiting other mechanisms distinct from vagal GLP-1R activation.

c) Chronic pharmacological administration of liraglutide may exert its eating-inhibitory and body weight lowering effects via non-vagal mediated mechanisms. It is possible to speculate from the available data that the eating-inhibitory effects of central GLP-1R activation dominate that of the vagal activation in the context of pharmacological intervention. Indeed, central GLP-1R activation elicits multiple behavioral effects beyond the homeostatic control of food intake, including reward suppression and activation of stress responses (40-45).
Further research is needed to understand which central GLP-1R population mediates the effects of GLP-1R agonists on food intake.

3 Vagal GLP-1R activation and the neuroincretin effect

3.1 The vagus nerve can mediate GLP-1 effects on post-prandial glycemia

The interaction between autonomic nerves and incretin hormones to control islet function has been conceptualized as the “neuroincretin effect” (14,46,47). Although the neuroincretin effect of GLP-1 has been reviewed elsewhere (47-49), we aimed at reviewing both early and recently published data involving a manipulation of the VN, together with GLP-1R activation or blockade (Table 2), through the prism of the physiology/pharmacology dichotomy.

Early findings showing an increase in the discharge rate of hepatic VAN and pancreatic VEN upon GLP-1 stimulation provided evidence for a vagal mediation of the neuroincretin effect of GLP-1 (14). In a study published by Imeryüz et al., a SC injection of GLP-1 did not change blood glucose levels in rats treated with perivagal application of capsaïcin or Sham rats (21). In the same study, capsaïcin-treated rats, however, showed an early hyperglycemia after a glucose test meal compared to Sham animals, consistent with the idea that activation of VAN by prandially released GLP-1 could play a role in the control of post-meal blood glucose. Human data showed that post-meal insulin secretion was unaffected by IV GLP-1 in VGX patients with pyloroplasty, but was suppressed in healthy controls (27). In addition, the same group demonstrated that gastrointestinal-mediated glucose disposal was reduced in VGX patients with pyloroplasty (50), suggesting that a vagal mediation of GLP-1’s effects on glycemia is potentially relevant to control post-meal blood glucose in humans.

3.2 Roles of vagal subbranches

More specifically, because Nakabayashi and Nishizawa (14) addressed the hepatic vagal afferents as a potential route for the mediation of GLP-1’s neuroincretin effect, later reports focused on whether this «portal sensor» is indeed involved. A series of studies tested the
role of GLP-1R localized on VAN terminals of the CHB by performing HPV injections of GLP-1 (51,52), DPP-IV inhibitor (37) or a GLP-1R antagonist (52,53). Together, these studies demonstrated that a local elevation of HPV GLP-1 could reduce blood glucose after an OGTT or after IV glucose infusion and that this glucose lowering effect was lost after lesion of the CHB. These studies assumed that GLP-1 concentrations in the HPV after a meal activate GLP-1R on the sensory terminals in the hepatoportal bed (the CHB) and generate neural signals to the dorsal vagal complex to regulate blood glucose levels. Prandially released GLP-1, however, could also activate GLP-1R located on intestinal VAN terminals. To compare the role of paracrine (intestinal) vs endocrine (hepatic) GLP-1R signaling in glucose regulation, Hayes and colleagues compared the glucose responses to an OGTT after IP Ex-9 pretreatment in Sham, CHBX or SDA rats (33). While IP Ex-9 treatment increased blood glucose levels similarly in CHBX and Sham rats, it failed to elicit a further increase in glucose response in SDA rats. This suggests that the CHB is not required to mediate the glucose lowering effects of GLP-1.

It may be possible to reconcile these apparently inconsistent findings by conceptualizing the GLP-1 sensing of intestinal and hepatic afferents as a collaborative integrated system. After a meal, high local concentrations of GLP-1 in the vicinity of intestinal vagal afferent terminals initiate the neuroincretin effect via a vagal pancreatic reflex pathway. Then, if the meal leads to high portal GLP-1 concentrations, hepatic afferents may play an additive role to further maintain the glucose-lowering effect of vagal GLP-1R activation. As we speculated above for the control of food intake, it is plausible that hepatic afferents require a sustained activation to contribute to the regulation of prandial glucose. In summary, whereas the exact role of different vagal branches is still unclear, it is reasonable to assume that the VN contributes to GLP-1’s neuroincretin effect.
Table 2: Summary of animal studies investigating the role of the vagus nerve in mediating GLP-1’s effects on glycemia by combining vagal manipulation with peripheral GLP-1R activation or blockade. Abbreviations: IP: intraperitoneal, IV: intravenous, JV: jugular vein, HPV: hepatic portal vein, SC: subcutaneous; CHBX: common hepatic branch vagotomy, SDA: subdiaphragmatic vagal deafferentation, VGX: vagotomy.
3.3 Relevance of the vagal neuroincretin effect of GLP-1

The incretin effect of circulating GLP-1 is mediated in large part by the direct activation of GLP-1R on pancreatic β-cells (5-7). An additional vagal component of GLP-1’s effects on glycemia is generally conceptualized as a “gut-to-brain-to-pancreas” pathway that results in an increase in insulin release in addition to the conventional “incretin” effects (47,48). A few studies addressed this topic by measuring insulin after a glucose challenge in the context of vagal manipulation. The glucose-stimulated insulin secretion induced by intravenous infusion of GLP-1 was attenuated by the ganglionic blocker chlorisondamine (54), by capsaïcin pretreatment (55) or by CHBX (51). Similarly, the effect of an HPV administration of a DPP-IV inhibitor on glucose-stimulated insulin secretion was attenuated by VGX (37). Moreover, IP infusion of atropin, a blocker of the muscarinic receptors, reduced the insulin response after intravenous co-infusion of glucose and GLP-1 (56). This further suggests that the activation of vagal afferent GLP-1R signaling increases insulin secretion through a descending signal mediated by pancreatic vagal efferents.

Interestingly, quantifications of the neuroincretin effect, defined as the supplementary insulin secretion induced by the activation of vagal GLP-1R, ranged from 0 to 60% (51,55). It seems to be clear from these studies that the neuroincretin effect is relevant only when small (physiological) doses of GLP-1 are used and that insulin secretion does not increase in a dose dependent manner. Mice with a genetic deletion of the \( glp1r \) in visceral nerves, however, did not show any significant impairment in glucose tolerance or insulin secretion (39). Moreover, pancreatic re-expression of the human GLP-1R in \( glp-1r^{-/-} \) mice (Pdx1-hGLP1R:glp1r\(^{-/-}\)) was sufficient to normalize glucose tolerance after an OGTT or an IPGTT (57), suggesting that VAN GLP-1R are not crucial for glycemic control. It is, however, possible that the relatively large bolus of glucose used in these studies raised circulating GLP-1 values much higher than the post-meal circulating GLP-1 levels, consequently masking the vagally-mediated component of the incretin effect.
Moreover, some studies have reported changes in blood glucose after activation or blockade of portal GLP-1R without significant changes in insulin levels compared to vehicle injections (52,53,58). These findings are consistent with the idea that GLP-1 can affect glycemia in an insulin-independent manner (59-61), and fit studies showing that GLP-1R activation can produce a beneficial effect on glucose regulation in Type 1 diabetes patients, despite their inherent lack of insulin production (62-67). More recent studies have provided experimental support for this idea: GLP-1R blockade by Ex-9 or loss of GLP-1 action in glp-1r KO mice increased glucose levels in streptozotocin animals with no glucagon signaling (68,69).

Evidence presented above and other findings also support the idea that GLP-1 in the HPV stimulates VAN there and enhances glucose disposal (14,70). Nevertheless, whether GLP-1 acts directly on hepatocytes to regulate hepatic glucose production or whether its effect is executed through autonomic nerves is currently unknown. The idea that a “gut-brain-liver” axis controls glucose homeostasis by both insulin-dependent and independent mechanisms is getting increasing experimental support, but an unequivocal demonstration of this pathway requires further research (see (71-73) for review).

Finally, sustained improvements in glucose tolerance were seen in the mouse model of glp1r KO in visceral nerves after chronic liraglutide administration, indicating no requirements of vagal GLP-1R in blood glucose regulation by GLP-1R agonists (39). This finding further suggests that the mechanism by which GLP-1R agonists exert their chronic effects on glucose tolerance does not require intact GLP-1R signaling in the visceral nerves.

3.4 Summary

Altogether, the available evidence suggests that:

a) peripheral GLP-1 can activate a reflex loop that involves subdiaphragmatic VAN and pancreatic VEN to further enhance the direct effect of GLP-1 on pancreatic beta-cells and to increase glucose-stimulated insulin secretion. The exact roles of vagal subbranches, as well as their physiological relevance, remains to be further investigated.
b) more specific approaches manipulating GLP-1R populations in a tissue specific manner indicate no involvement of vagal receptors in the control of blood glucose after glucose challenges (OGTT or IPGTT). These studies, however, did not investigate these parameters in the context of a physiological mixed-nutrient meal.

c) recent data raise the possibility that GLP-1 can decrease hepatic glucose production through insulin-dependent mechanisms that may involve VAN.

d) chronic liraglutide treatment improves glycemia in the absence of vagal GLP-1R signaling, but further research is needed to confirm that the VN is not involved in the beneficial effects of chronic GLP-1R agonist treatment.

4 Putative mechanisms of vagal GLP-1R effects on food intake and glycemia

Whereas the studies reviewed above provide strong evidence that GLP-1 can reduce food intake and glycemia through VAN GLP-1R activation, the mechanisms by which it does so remain poorly understood. Indeed, there are two important points to be considered: 1) the brain circuits that process the satiation signals generated by vagal GLP-1R activation remain unclear; 2) the neurotransmitters/neuropeptides involved in mediating the effects of VAN GLP-1R activation are still unknown. In this section, we will review the current knowledge related to this topic and propose putative mechanisms that could link VAN GLP-1R activation with the behavioral outputs governed by the central processing of peripheral signals.

4.1 Central pathways activated by vagal GLP-1R activation

VAN terminals project to the caudal brainstem where they make synaptic connections to second order neurons in the NTS (12). Glutamate is considered to be the major neurotransmitter of VAN, and numerous findings have implicated the release of glutamate in the NTS in the control of eating (see (74) for review). Therefore, it is reasonable to speculate that VAN GLP-1R activation results in the activation of NTS neurons via glutamatergic
synaptic transmission, initiating the processing of the eating-inhibitory signal in various brain areas including hypothalamic and forebrain nuclei. Indeed, VGX blunted the c-fos mRNA expression induced by IP Ex-4 injection in the medial part of the NTS (75). The neurochemical phenotype of the NTS neurons receiving vagal GLP-1R mediated signals is currently unknown. One potential candidate are catecholamine (CA) neurons in the caudal brainstem that are linked to the control of autonomic processes, food intake and stress (76). CA neurons in the NTS receive direct monosynaptic inputs from visceral afferent neurons and are activated by IV administration of GLP-1R agonists (77,78). Therefore, based on their relevance and anatomical location, it is likely that CA neurons are key players in relaying the VAN GLP-1R signal to higher brain centers.

Peripheral GLP-1R agonist treatment induces neuronal activation in multiple other brains sites in the ascending visceral afferent pathway, including the parabrachial nucleus (PBN), the paraventricular nucleus of the hypothalamus (PVN), and the central nucleus of amygdala (CeA) (32,75). It is important to consider that the long lasting GLP-1R agonists can bind to central GLP-1R in leaky brain areas such as the arcuate nucleus of the hypothalamus (Arc) and in circumventricular organs such as the area postrema (AP) (38). They can therefore indirectly activate other brain areas such as the ones mentioned above. Nonetheless, the c-Fos expression pattern after HPV administration of a DPP-IV inhibitor (that raises HPV, but not systemic GLP-1 levels) is to a great extent similar to the expression pattern induced by IP Ex-4 (37). In the same study, VGX blocked the DPP-IV inhibitor-induced c-Fos expression in all brain areas including the NTS, PVN, PBN, and CeA, suggesting that vagal GLP-1R stimulation is required for the activation of brainstem and forebrain neurons linked to ascending visceral pathways.

Abbott et al. provided evidence for a role of the vagal-brainstem-hypothalamic pathway in the effects of GLP-1 on food intake by interrupting the connection between caudal brainstem and hypothalamus using midbrain knife cuts (26). The PVN is one of the major hypothalamic sites receiving afferent projections from the NTS involved in the control of food intake and energy
expenditure (79-81). The c-Fos activation in the PVN after IP Ex-4 was attenuated in VGX (75) as well as in SDA rats (32). This decrease in PVN neuronal activation was associated with an attenuated acute eating-inhibitory response to IP Ex-4 (32). This finding is consistent with the idea that the PVN modulates food intake by integrating neural signals generated in the brainstem as well as the hypothalamus in response to nutritional and hormonal factors (82,83). Moreover, lesions (84,85) or a functional disruption of the PVN (86,87) both lead to severe hyperphagia and obesity. Altogether, the available evidence strongly suggests that the PVN is a major hypothalamic site mediating the eating-inhibitory effects of peripheral GLP-1R activation.

To challenge this idea, Hayes and colleagues interrupted the forebrain-caudal brainstem communication by generating supracollicular decerebrated rats and tested the effects of peripheral GLP-1R stimulation on eating behavior in these animals. Ex-4 decreased food intake and gastric emptying similarly between control and chronic decerebrated rats (88), suggesting that the caudal brainstem is sufficient to mediate these effects of peripheral GLP-1 and that the hypothalamic/forebrain processing is not necessary. In support of this interpretation, the existence of a monosynaptic vago-vagal input output circuit in the NTS and dorsal motor nucleus of the vagus (DMV) provides additional evidence for the sufficiency of the caudal brainstem for the effect of control of food intake under peripheral GLP-1R stimulation on eating (89). The contradictory findings of the midbrain knife cut and decerebrated rat models are difficult to reconcile, but the choice of drug, GLP-1 (100 nmol/kg) vs. Ex-4 (1.2 - 3 µg/kg) may play a role. It is possible that the dose of Ex-4 used in this study induced visceral malaise and a taste aversion that contributed to the eating suppression (90). Furthermore, given the high lipid solubility of Ex-4, it is possible that it exerted independent effects on forebrain circuits to inhibit eating without the brainstem connections. Future studies are needed to further address the necessity of the brainstem-forebrain connection in processing the vagal GLP-1R mediated inhibition of eating.
Whether activation of VAN GLP-1R initiates effects of food intake and glycemia through a common neuronal pathway remains an open question. A recent study using visceral and neuronal glp-1r KO mouse models disregarded the importance of GLP-1R in VAN and in the CNS in the effects of liraglutide on glucose homeostasis (39). Although the role of VAN GLP-1 signaling in glucose homeostasis is still controversial (57), it is generally thought that meal-induced GLP-1 secretion leads to the activation of vago-vagal reflexes that control insulin secretion indirectly (see above and (91)). Moreover, as mentioned earlier, the possibility that the VN can also mediate an insulin-independent effect of GLP-1 on endogenous glucose production is getting increasing attention (71-73). The exact mechanism of the neural effects of GLP-1 on glycemia, including its central processing, still needs to be explored.

4.2 Neuropeptides mediating vagal GLP-1R effects in VAN

Due to limited viscerotopic organization of NTS neurons receiving vagal inputs (92), it is unlikely that glutamatergic transmission alone would permit an unambiguous transmission of vagal-branch specific information of gut-derived factors to the brainstem. Recently, vagally-expressed neuropeptides have been found to play important roles in meal-induced satiation and diet–induced obesity (74). The nodose ganglia (NG) express notably the cocaine- and amphetamine-regulated transcript (CART) (93-95) and melanin-concentrating hormone (MCH) (96-98). Both neuropeptides are also abundantly expressed in other brain areas involved in the control of food intake. There is evidence that the expression of CART and MCH in the NG is differentially regulated by the nutritional status and cholecystokinin (CCK). Indeed, CART co-localizes with CCK1a receptors (CCK1aR) in VAN (94) and its expression is induced in, and the peptide is released from, cultured primary VAN in response to CCK (93). Moreover, leptin and ghrelin potenti ate or inhibit this effect of CCK on CART expression (93,99), raising the possibility that the VN is an important hub for the integration of gastric, intestinal and adiposity signals. The CART regulation by CCK has enhanced our understanding of the cellular mechanisms in VAN, which can be applied to the studies of other vagally-mediated satiating signals. Currently, no information is available regarding a
neuropeptide expression under the control of GLP-1R signaling. It has been shown, however, that the GLP-1R localization in the plasma membrane is nutritionally regulated and increased in the NG by eating (100). Furthermore, GLP-1R expression is down-regulated in the NG of rats prone to diet-induced obesity (DIO) fed with a high-fat diet (101), with a concomitant decrease in circulating GLP-1 levels (24,101,102). This suggests an impaired GLP-1R signaling that may contribute to the hyperphagia and obesity phenotypes of DIO rats (24). Based on these findings, it is imperative to identify the neuropeptide(s) mediating the vagal GLP-1R signaling and investigate the molecular events associated with the impaired GLP-1R functions in obesity.

5 Methodological obstacles to overcome: suggestions for future vagal manipulations

As mentioned briefly in the introduction, the research into the vagal mediation of GLP-1 effects has been hampered by a few technical caveats. In this last part, we aim at highlighting the limitations of the techniques described above in order to suggest alternative approaches for future vagal manipulations.

5.1 Lesion-based approaches and genetic manipulations of the vagus nerve

Until recently, manipulations of the VN could only be achieved by a lesion-based approach, such as capsaïcin treatment or different variants of vagotomy. Although surgical techniques have been modified to increase the specificity to VAN or VEN (29) or to a specific branch of the VN, lesion approaches result in a non-specific impairment of the vagal neurons, compromising a broad range of signaling. An example can be seen in SDA rats: food intake and gastric emptying rate is already impaired in SDA compared to Sham rats (unpublished data - 50% of the efferents are also sectioned). Consequently, the question remains whether the SDA model is ideal for assessing the essential role of the vagus nerve in mediating GLP-
effects on food intake because food intake may already be limited by unspecific impairments of vagal function.

Moreover, a lesioning approach also impairs other pathways involved in the control of food intake, such as CCK satiation. To overcome this lack of specificity, many studies combined a vagal manipulation with an exogenous infusion of GLP-1 or one of its agonists/antagonists. This approach, however, presents the problems of choosing the right dose and route of administration that can simulate the nutrient-induced release of GLP-1. Whereas IP administration targets the activation of gastro-intestinal GLP-1R populations, more targeted IV infusions - such as HPV – may help dissecting out the role of CHB, but do not seem to be the ideal model to study the intestinal release of GLP-1 (35,103). Moreover, as mentioned above, the use of pharmacological doses of GLP-1 or GLP-1R agonists rather than physiological doses of GLP-1 increase the likelihood of direct activation of central GLP-1R and may mask vagal-specific effects.

Recently, a study has overcome the need of vagal lesioning by performing a genetic deletion of the \textit{glp1r} gene based on the \textit{cre-lox} recombination system in mice (39). Vagal-specific genetic deletions published so far were based on the expression of a \textit{cre}-recombinase driven by a \textit{Phox2b} (39,104) or \textit{Nav1.8} (105) promotor. Expression of these two genes is, however, not restricted to the NG (106,107). Hence, the use of their promoters can drive non vagal \textit{cre} expression and potential non specific \textit{glp1r} deletion, which could complicate interpretation.

\subsection*{5.2 Proposed approaches for future studies of vagal signaling in the control of eating and glycemia}

Based on these limitations, we propose a lesion-free technique that could specifically target GLP-1R on VAN. First, as just mentioned, the main limitation of the \textit{cre-lox} approach in this context is whether or not it drives \textit{cre} recombinase expression in a VAN-specific manner. Consequently, it is reasonable to suggest that gene expression screening of VAN is needed to identify candidate genes specific to these neurons. Another approach could involve the delivery of viral vectors to unlock a new spectrum of vagal-specific genetic manipulations.
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The NG contains the cell bodies of VAN and hence is a well-suited delivery site (108). Whether viral vector delivery could potentially be efficient at manipulating gene expression in VAN, and which type of viral vectors are suitable for these neurons, has not being investigated yet. To be more specific, this approach can be used for the delivery of short hairpin RNA (shRNA)-based vectors and opens the door for optogenetics or DREADD (Designer Receptors Exclusively Activated by Designer Drugs) manipulations of the VN.

Furthermore, the field of vagus nerve stimulation (VNS) has recently received attention in relation to the control of eating and the regulation of body weight (see (109) for review). Approaches based on this technique could allow modulating vagal activity, which could be of interest not only for therapeutic, but also for experimental purposes. None of these approaches, however, distinguishes between the different vagal branches, i.e., receptor populations. Consequently, further research is needed to overcome this methodological obstacle in investigating vagal branch-specific functions. Altogether, considering these perspectives will further enhance our understanding of the role of the VN in the control of food intake and glucose homeostasis.

6 Concluding comments

In summary, evidence presented in this review strongly suggests that the contribution of the VN to mediating GLP-1 effects on food intake and glycemia is context specific. Indeed, although not being fully demonstrated, it is commonly assumed that the VN is a key player in mediating the effects of intestinal GLP-1 on meal size and post-meal control of blood glucose. The importance of this vagal pathway is, however, diminished when levels of circulating GLP-1 are markedly elevated, such as after an IV GLP-1 infusion, or after DPP-IV inhibitor treatment (see summary figure). Moreover, some reports indicate that the acute effects of long-lasting GLP-1R agonists on food intake are at least in part vagally mediated, whereas the weight loss and improved glucose tolerance after chronic treatments do not seem to require vagal afferents. The mechanisms involved in the beneficial effects of chronic
agonist treatments are still investigated, but evidence indicates a crucial involvement of central nervous system GLP-1R populations, especially in the hypothalamus, in appetite reduction (38,39). Despite the recent reports questioning a role of the VN in mediating GLP-1R agonist actions, further research is needed to address whether VAN GLP-1R activation leads to appetite reduction and weight loss without the side effects normally associated with GLP-1R agonist treatment in humans (110).

In addition, this review highlighted the fact that the central mechanisms mediating the effects of vagal GLP-1R activation on food intake and glucose homeostasis, as well as the GLP-1R VAN signaling, are not yet understood. We advocate further research to provide a complete understanding of the neural mechanisms that relay intestinal GLP-1 signal to the brain and induce satiation and glucoregulatory responses. Ideally, future studies should avoid lesion-based approaches for the benefit of specific molecular manipulation of vagal GLP-1R signaling.

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Summary figure:
Meal-induced release of intestinal GLP-1 can access GLP-1R on vagal afferents of gastrointestinal (a) and hepatic origins (b). Vagal information is processed in the brainstem to activate a vago-vagal loop and/or higher brain centers (c). Vagal efferent projections to the stomach and pancreas control gastric emptying and participate in glucose-induced insulin production (d), supplementary to the direct incretin GLP-1 effect on pancreatic cells (e). New lesion-free approaches are needed to provide an unequivocal demonstration of this pathway.

GLP-1R agonists have been shown to require vagal afferents to exert their acute effects on food intake when administered IP (a). Their chronic pharmacological effects on body weight and glucose tolerance, however, seem to be primarily mediated by a direct activation of central GLP-1R (f). Which central GLP-1R population mediates these effects requires further investigation.
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CHAPTER 3:

KNOCKDOWN OF GLP-1 RECEPTORS IN VAGAL AFFERENTS AFFECTS NORMAL FOOD INTAKE AND GLYCEMIA

AUTHORS


Physiology and Behavior Laboratory, ETH Zürich, Switzerland

STATUS

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Chapter 3: GLP-1R knockdown in vagal afferent neurons, food intake and glycemia

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 exenin-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.

**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, Kantsponsapotheke 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 2-ddCt method. A western blot was performed to detect the GLP-1R protein (Rabbit antibody 39072, 1:400, Abcam) using ß-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).

**Oral glucose tolerance test**

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 γ (PPARγ), 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).
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. The dotted lane 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), CCKαR (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)

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, 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).

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

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
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)
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 VAN 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).

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) 1 h food intake after 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). (D) 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 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)
Chapter 3: GLP-1R knockdown in vagal afferent neurons, food intake and glycemia

Figure 5: VAN GLP-1R kd disturbed post-meal glycemia and insulinemia but did not impair tolerance of an oral glucose bolus
(A) HPV glucose after 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). (C) HPV 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. (G) Tail vein 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)

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 paired-like homeobox 2b (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 kd^ΔPhox2b 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.

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CHAPTER 4:

THE COCAINE- AND AMPHETAMINE-REGULATED TRANSCRIPT MEDIATES THE SATIATING EFFECTS OF GLP-1 IN VAGAL AFFECTENT NEURONS

AUTHORS

Krieger JP\textsuperscript{1}, Arnold M\textsuperscript{1}, Langhans W\textsuperscript{1}, de Lartigue G\textsuperscript{2}, Lee SJ\textsuperscript{1}

\textsuperscript{1} Physiology and Behavior Laboratory, ETH Zürich, Switzerland

\textsuperscript{2} John B. Pierce Laboratory, New Haven, USA

STATUS

In preparation
ABSTRACT

GLP-1 is an incretin and satiation hormone produced in the intestine in response to eating. Using a rat model with a specific knockdown (kd) of GLP-1 receptors (GLP-1R) in vagal afferent neurons (VAN), we previously demonstrated that VAN GLP-1R are required for endogenous GLP-1-induced satiation. The molecular mechanism by which VAN GLP-1R activation decreases food intake remains unknown. We screened the expression of neuropeptides involved in the control of food intake in a primary culture of VAN and found that the Cocaine- and Amphetamine-Regulated Transcript (CART) mRNA and protein levels were upregulated in response to GLP-1 stimulation in a GLP-1R dependent manner. CART was co-localized in GLP-1R expressing cells in the rat nodose ganglia (NG), and intraperitoneal (IP) GLP-1 (10 nmol/kg) was sufficient to induce CART mRNA and protein expression. We therefore hypothesized that CART mediates the eating-inhibitory effects of vagal GLP-1R activation and tested this using two rat models of CART inhibition. First, we reduced VAN CART expression using a lentiviral-mediated CART kd. Second, we blocked CART signaling in the nucleus tractus solitarii (NTS) by administering a CART antibody into the NTS. In both models, CART inhibition abolished IP GLP-1-induced (10 nmol/kg) satiation and attenuated GLP-1-induced gastric emptying. We conclude that GLP-1 induces CART transcription and synthesis in VAN and that CART released from VAN into the NTS mediates the physiological effects of GLP-1 on satiation and gastric emptying.
Chapter 4: CART mediates the effects of GLP-1 in vagal afferent neurons

INTRODUCTION

Vagal afferent neurons (VAN) are key components of the gut-brain axis, conveying mechanical and chemical signals from the gastrointestinal tract to the brain. They participate in the normal control of food intake and body weight (see (1) for review) and can respond to nutritional challenges (2). Recently, a series of studies using viral or transgenic approaches began to unravel the molecular mechanisms by which VAN can modulate energy balance (3-7). Specifically, we demonstrated that intestinal glucagon-like peptide-1 (GLP-1) acts in a paracrine fashion on VAN GLP-1 receptors (GLP-1R) to induce satiation and to slow gastric emptying (7). The vagal and central mechanisms by which VAN GLP-1R activation decreases food intake remain, however, unclear. Substantial evidence indicates that glutamate is the main neurotransmitter involved in the transmission of sensory information from VAN to the nucleus tractus solitarii (NTS, see (8) for review). Yet VAN also express a broad array of peptide-based neurotransmitters, such as Cocaine- and Amphetamine-Regulated Transcript (CART, (9)), Melanin-Concentrating Hormone (MCH, (10)), substance P (subP, (11,12)) and neurokinin A (11,13). Their roles in the vagal control of food intake is however largely unknown (8). Among these neuropeptides, CART has received attention due to its broad role in the control of food intake. Indeed, since its original discovery (14), CART has been detected in many brain areas involved in the control of eating (see (15) for review), and intracerebroventricular (ICV) injections of the CART peptide or the CART antibody have highlighted an anorexigenic property of CART in the CNS (16-18). CART is also expressed in nearly half of the neurons of the medial and caudal nodose ganglion (NG) (9,19,20) and vagal CART positive fibers terminate in the commissural and medial NTS (16,19). Several studies demonstrated that CART is a mediator of the eating-inhibitory effects of cholecystokinin (CCK) in VAN (9,20-22). Indeed, CART is colocalized with CCKa receptors in the NG (9), and IP administration of CCK increased CART protein expression in the NG (20). Moreover, in cultured VAN, administration of CCK induced CART expression and release.
and this effect was modulated by leptin and ghrelin (21,23). Finally, silencing CART in vivo in VAN blocked the effects of IP-infused CCK and leptin on short-term food intake (22).

In this study, we hypothesized that vagally expressed neuropeptides play an essential role in the eating inhibition induced by VAN GLP-1R activation. We initially screened a set of neuropeptides for their transcriptional response to GLP-1 stimulation in primary rat VAN cultures and found that CART was upregulated by GLP-1 via the GLP-1R. Consequently, we further tested the idea that CART in VAN is a necessary mediator of GLP-1’s effects on food intake and gastric emptying and present here in vitro and in vivo evidence that support this hypothesis.

**METHODS**

**Primary culture of vagal afferent neurons**

Primary cultures of rat VAN were carried out as previously described (20). Immediately after collection, NG were digested for 2 h with collagenase type Ia (Roche) (2 mg/mL) in 37°C HBSS (Sigma-Aldrich), dissociated with coated glass pipettes (Sigma-coat, Sigma-Aldrich), and plated in poly-L-lysin-coated chamber slides (Millicell EZ Slide, Millipore). Cultures were grown for 72 h in 4.5 g/L DMEM with 10% bovine serum. For experiments, VAN cultures were transferred to a serum-free medium (20% glucose in cell-culture grade water) for 2 h and incubated for 1 h with GLP-1 (10 nM final concentration, Bachem) or PBS. The GLP-1R antagonist exendin-(9-39) (Ex-9, 100 nM final concentration, Bachem) was added 30 min prior to GLP-1 or PBS. VAN mRNA was extracted using Trizol according to the manufacturer’s protocol (Life Technologies) and levels of the calcitonin (calc), neuropeptide y (npy), pro-melanin-concentrating hormone (pmch), substance P (subP) and CART (cart) genes were measured using RT-qPCR. For immunocytochemistry, VAN cultures were fixed for 30 min with 4% PFA and stained for CART (Rabbit anti-CART(55-102), H-003-62, Phoenix Pharmaceuticals, 1:1K; Cy-3-conjugated donkey anti-rabbit, 711-165-152, Jackson ImmunoResearch, 1:400).
Immunohistochemistry in the nodose ganglia

NG were collected and immediately fixed for 2 h in 4% PFA, and immersed in a 25% sucrose PBS solution overnight. NG were sectioned at 10-14 µm in a cryostat (CM 1950, Leica) and placed on polysine-coated microscope slides (Menzel, ThermoScientific). The co-immunostaining for CART and GLP-1R was performed in a three-step procedure. First, the GLP-1R staining was carried out (Rabbit anti-GLP-1R, ab39072, Abcam, 1:1K; Alexa 488-conjugated donkey anti-rabbit, A21206, Invitrogen, 1:400). Second, the GLP-1R detection complex was protected by successive incubation with rabbit serum followed by F(Ab) fragment (Donkey anti-rabbit F(ab) fragment, 711-007-003, Jackson ImmunoResearch, 40 µg/mL). Third, the CART staining was carried out as described above. To test the induction of CART by vagal GLP-1R activation, NG were collected from fasted rats (24 h fasting) and refed rats (24 h fasting + 4 h ad libitum refeeding) or after an IP GLP-1 injection (24 h fasting + 1 h after injection of IP GLP-1 10 nmol/kg, Bachem). NG were fixed and stained for CART as described above.

CART knockdown in the nodose ganglion

A shRNA sequence targeting the rat CART mRNA or a non-target shRNA sequence were driven by a U6 promoter in a pLKO.1-puro vectors expressing turboGFP (Sigma-Aldrich). The efficiency of the CART-targeting shRNA construct was verified in vitro in GH3 cells (CCL 82.1, ATCC) after sorting turboGFP-expressing cells (FACS Aria III FCF, Flow Cytometry Facility, University of Zurich). CART-targeting (LV-shCART) or control (LV-shCTL) lentiviral particles were produced in HEK 293T cells using the pMD2.G and psPAX2 plasmids (gifts from Dider Trono, Ecole Polytechnique Fédérale de Lausanne; cat. no. 12259 and 12260) and concentrated to 10^{10} particles/mL using 8% PEG6000 (Millipore) in 0.3M NaCl and resuspended in PBS. Rats (290-340 g on surgery day) were anesthetized by an IP injection of ketamine (88 mg/kg, Ketalar, Zurich Cantonal Pharmacy) and xylazin (5 mg/kg, Rompun 2%, Zurich Cantonal Pharmacy) and NG were exposed. A custom-made glass capillary (50 µm tip) was used to administer 1.5 µL of viral solution (LV-shCTL or LV-shCART) into each node.
NG with a Picospritzer III injector (Parker Hannifin). To ensure expression of the viral constructs, animals were allowed to recover for 20 days after surgery. Rats were handled and habituated to IP injections of NaCl prior to the experiment. Animals had *ad libitum* access to water and chow food (3433, Kliba-Nafag) unless otherwise noted.

**NTS cannulation**

Rats (290-340 g on surgery day) were anesthetized by an IP injection of ketamine and ketamine as described above and placed in a stereotaxic apparatus. Cannulas (PlasticOne) were implanted in the NTS (stereotaxic coordinates: 1.0 mm posterior to occipital crest, 0.7 mm lateral to midline, 5.9 mm below skull surface). Animals were allowed to recover for 10 days and habituated to the cannula manipulation and to the NTS injection procedure before experiments. Animals had *ad libitum* access to water and chow (3433, Kliba-Nafag) unless otherwise noted. Cannula placement was verified by dye injection (500 nL) and post-mortem anatomical visualization of the solution spread.

**Effect of IP GLP-1 on food intake in the CART inhibition models**

The effect of IP GLP-1 on short-term food intake was assessed in a similar manner in two experiments, in CART knockdown (kd) rats and in wild-type rats receiving a CART antibody injection. Animals were habituated to acrylic cages in which powdered chow (3436, Kliba-Nafag) was available through a niche in feeding cups placed on scales (XS4001S, Mettler-Toledo) for continuous measurement of meal patterns (see (24) for detailed description). After 16 h of food deprivation, animals were allowed access to a pre-meal composed of 3 g powdered chow and 3 mL water 30 min before dark onset. A pre-meal allows GLP-1R trafficking to the VAN membrane (25). Only animals that finished the pre-meal within 10 min were included in the experiments. In the CART kd experiment, rats were IP injected with GLP-1 (10 nmol/kg) or a vehicle solution 5 min before dark onset. In the CART antibody experiment, rats were first injected with a CART antibody solution (1:500 in PBS, 500 nL over 30 sec) or a vehicle solution using an electronically controlled pump (UltraMicroPump UMP3 with SYS-Micro4 controller, World Precision Instruments) 15 min prior to dark onset. Rats
subsequently received an IP injection of GLP-1 (10 nmol/kg) or vehicle solution 5 min before dark onset. Food was returned at dark onset and food intake was recorded.

**Effect of IP GLP-1 on gastric emptying in the CART inhibition models**

One week prior to the experiment, rats were habituated to receiving test meals, to the restricted feeding schedule (test meal at dark onset, *ad libitum* food access from 3 to 8 h after dark onset, food deprivation otherwise) and to the restraint for tail vein blood sampling. On experimental days, all 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). Injections of IP GLP-1 and/or NTS CART antibody solution were done in identical conditions as during food intake measurements (described above). In both CART inhibition models, drug administration was randomized in a crossover design. Baseline tail vein blood was taken 30 min prior to test meal onset and post-meal blood was collected according to the scheduled timepoints. Blood was immediately placed on ice, centrifuged (10 min at 8370 g) and stored at -80°C for further processing. Paracetamol concentrations were measured with a commercial kit (Cambridge Life Sciences K8002) adapted for small plasma volumes and reading in a 96-well plate.

**Figures and statistical analysis**

Data normality was verified using the Shapiro-Wilk (when n ≥ 7) and the Kolmogorov-Smirnov (when 5 ≤ n ≤ 6) tests and homoscedasticity was checked by visualizing the distribution of residuals. Non-parametric tests were used otherwise. Tests used and P-values are given in the figure legends. Data are presented as means ± SEM. P-values < 0.05 were considered significant. All graphs were generated using GraphPad Prism (version 6.05 for Windows).
RESULTS

GLP-1 increased CART expression in primary vagal afferent neurons

In a primary culture of VAN, we first tested whether GLP-1 modulates the expression profile of known neuropeptides involved in eating regulation. Among the peptides screened, we found that only CART was increased by 2 h incubation with GLP-1 (10 nM), whereas others remained unchanged (Fig. 1A). Using immunocytochemistry, we measured an increase in the number of CART positive cells after GLP-1 incubation (Fig. 1C, D). We further tested whether this effect was dependent on the presence of GLP-1R. Pre-incubation of the primary VAN culture with the GLP-1R antagonist Ex-9 (100 nM) prevented the GLP-1-induced increase in CART gene expression (Fig. 1B) and number of CART-expressing neurons (Fig. 1C, D). Together, these results show that GLP-1 can induce CART mRNA and protein expression in a subpopulation of VAN via the GLP-1R.

Figure 1: GLP-1 (10 nM) induced CART expression in primary VAN via the GLP-1R
(A) Relative mRNA expression of the calcitonin (calc), neuropeptide y (npy), pro-melanin-concentrating hormone (pmch), substance P (subP) and CART (cart) genes in primary VA incubated with 10 nM GLP-1 or the vehicle solution for 1 h (n = 5; Student t-test, P < 0.05 for cart). (B) Relative mRNA expression of the cart gene in primary VAN incubated with vehicle, 100 nM of Ex-9, 10 nM
GLP-1 or co-incubated with GLP-1 and Ex-9 (n = 5; Two-way ANOVA GLP-1 x antagonist; GLP-1 effect P = 0.1, antagonist effect P = 0.1, interaction P < 0.05; * indicates significant difference from control in Bonferroni post hoc comparisons). (C) Representative pictures of cultured VA incubated with vehicle (top left), 100 nM of Ex-9 (top right), 10 nM GLP-1 (bottom left) or co-incubated with GLP-1 and Ex-9 (bottom right). (D) Percentage of CART positive cells among all cells in a primary culture of VA after incubation with vehicle, 100 nM of Ex-9, 10 nM GLP-1 or co-incubated with GLP-1 and Ex-9 (n = 5; Two-way ANOVA GLP-1 x antagonist; GLP-1 effect P < 0.05, antagonist effect P < 0.05, interaction P < 0.05; * indicates significant difference from control in Bonferroni post-hoc comparisons).

CART is expressed in GLP-1R-expressing vagal afferent neurons and is induced by IP administration of GLP-1

The data above suggest that CART expression is induced in the VAN expressing GLP-1R. Colocalization of CART and GLP-1R in VA is a pre-requisite for CART to mediate the effects of vagal GLP-1R activation. Thus, we next visualized CART protein expression in the GLP-1R expressing cells of the rat NG using double-immunostaining. CART was visible in a perinuclear localization in nearly half of the VA and GLP-1R were mainly expressed at the cell membrane (fed state, Fig. 2A, B, F and G). Due to the weak labeling of the GLP-1R antibody, we chose to determine whether the subpopulation of neurons clearly labeled as GLP-1R positive were also labeled as CART positive (CART+), and saw a strong overlap between the expression of the two proteins (Fig. 2A-D). CART expression was, however, not limited to GLP-1R expressing neurons. As previously demonstrated, CART expression depended on the feeding status, i.e., CART staining was not visible in the NG of fasted rats, but appeared when animals were refed ad libitum for 4 h (Fig. 2E and G). An IP injection of GLP-1 (10 nmol/kg) was sufficient to induce CART mRNA and protein expression in the NG (Fig. 2F, G). Together, these data raise the possibility that endogenous intestinal GLP-1 induces CART expression in the NG via activation of VAN GLP-1R. We consequently further tested the functional role of CART in the physiological effects of endogenous GLP-1 on food intake and gastric emptying.
Figure 2: CART and GLP-1R colocalized, and IP GLP-1 is sufficient to induce CART expression in the nodose ganglion of fasted rats
(A) GLP-1R staining, (B) CART staining and (C) merged picture of a rat nodose ganglion (ad libitum fed). (D) Percentage of CART-positive cells among GLP-1R-positive cells in a rat nodose ganglion (n = 4). (E) CART staining in the nodose ganglion of a rat fasted for 24 h and injected IP with a vehicle solution, (F) injected IP with GLP-1 (10 nmol/kg) or (G) refed ad libitum for 4 h. (H) Relative mRNA expression of the cart gene in the nodose ganglia (n = 3/5/2; Kruskal-Wallis test, P < 0.05; * indicates significant difference with the fasting condition after Dunn’s comparisons).

Full CART expression in vagal afferents is necessary for the IP GLP-1-induced reduction in food intake and gastric emptying

To test whether CART is a necessary player in the vagal mediation of GLP-1’s effects, we specifically knocked down the CART mRNA transcript in VAN. For this, we used our previously described lentivirus-based shRNA delivery via bilateral NG injection. Using this approach, CART expression was reduced by 35 % (Fig. 3A) in the NG of rats injected with LV-shCART compared to control injected rats. In this CART kd model, the eating-inhibition induced by the IP injection of GLP-1 (10 nmol/kg) was significantly attenuated (Fig. 3B). Moreover, we previously described that VAN GLP-1R activation is associated with a reduction in gastric emptying that parallels the eating inhibition. In VAN CART kd rats, meal-induced gastric emptying was accelerated, as shown by a faster appearance of paracetamol in the plasma. The gastric emptying inhibition induced by IP GLP-1, however, was abolished by the CART kd (Fig. 3C).
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Figure 3: CART kd in the NG attenuated the inhibition of eating and gastric emptying induced by IP GLP-1

(A) Relative mRNA expression of the cart gene in the nodose ganglia of rats injected with an anti-CART shRNA-carrying lentivirus, or a control lentivirus (n = 8/7; Student t-test, P < 0.05). (B) 30 min food intake (n = 8/8/7/7; Two-way mixed ANOVA knockdown x drug; Knockdown effect NS, drug effect P<0.05, interaction P=0.01; different letters indicate a significant difference by Bonferroni-adjusted post-hoc comparisons) and (C) plasma paracetamol concentrations after IP injection of GLP-1 (10 nmol/kg) or vehicle in control and CART KD rats (n = 8/8/7/7; Mixed MANOVA knockdown x drug x time; knockdown effect P<0.01, drug effect NS, time p<0.01, interaction knockdown * drug NS)

Together, these results demonstrate that the food intake reduction and the gastric emptying inhibition following IP GLP-1 injection require unaltered VAN CART expression, suggesting that CART is a downstream mediator of VAN GLP-1R signaling.

**NTS CART antibody attenuated IP GLP-1-induced reduction in food intake and gastric emptying**

A possible role for CART in the eating-inhibitory effect of GLP-1 may reside in the release of CART vesicles in the NTS. Thus, to test whether the presence of CART in the NTS is necessary for GLP-1 to inhibit eating and gastric emptying, we infused a CART antibody into the NTS prior to GLP-1 IP administration. The pre-injection of a CART antibody into the NTS blocked the effects of IP GLP-1 on food intake (Fig. 4A) and gastric emptying (Fig. 4B). These results are consistent with the idea that the release of CART in the NTS from VAN is necessary for the eating-inhibition induced by GLP-1.
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Figure 4: CART antibody in the NTS blocked the inhibition of eating and gastric emptying by IP GLP-1

(A) 30 min food intake (n = 10; Two-way ANOVA antibody x ip drug; antibody effect P < 0.05, IP drug effect P = 0.06, interaction P < 0.05; different letters indicate a significant difference among groups by Bonferroni-adjusted post-hoc comparisons) and (B) plasma paracetamol concentrations after IP injection of GLP-1 (10 nmol/kg), NTS pre-injection with the CART Ab and respective control vehicle injections (n = 5/6/9/8; MANOVA IP drug x NTS Ab x time; IP drug effect P < 0.05, NTS Ab effect P = 0.12, time effect P < 0.01, interaction IP drug * NTS Ab P = 0.1).

DISCUSSION

We here report that GLP-1 induced CART mRNA and protein expression via the GLP-1R in primary VAN. Moreover, in vivo, CART co-localized with GLP-1R, and IP GLP-1 was sufficient to induce CART expression in the NG of fasted rats. We then further tested whether CART mediates the eating-inhibitory effect of VAN GLP-1R activation. First, we knocked down CART expression in VAN using a lentiviral-mediated RNA interference approach. Second, we blocked CART signaling in the NTS by administering a CART antibody into the NTS. In both models, CART inhibition abolished IP GLP-1-induced satiation and gastric emptying. We conclude that GLP-1 induces CART synthesis in VAN and that CART released from VAN into the NTS is involved in the mediation of the physiological effects of GLP-1 on satiation and gastric emptying. Together, our data indicate that VAN CART is a necessary downstream mediator of endogenous GLP-1’s effects.

Based on our results, it is possible to speculate that VAN GLP-1R activation is involved in a/ an increase in CART transcription, and protein expression, and/or b/ the transport of the CART in vesicles to VAN terminals for their release into the NTS. This sequence of events is
further suggested by the study of CCK-induced CART release in VAN. CCK induces CART expression as early as after 10 min incubation in primary VAN cultures (23) but the highest expression is reached after 2 h, indicating that CCK action on CART gene transcription does not fully explain the short term (<30min) satiating effect of CCK. Moreover, the transport of CART from the soma to the VAN terminals is likely to be a rate-limiting step between CCK-induced CART expression and the eating inhibition. Indeed, CART vesicles have been reported in neurite terminals only after 85 min of incubation with CCK (in vitro) (20), and CART positive fibers appear in the NTS within 2 h of refeeding (26). Based on all these facts, it is likely that CCK promotes CART release by increasing VAN firing in addition to the transcriptional regulation. Similarly, considering the short-term GLP-1 effect on food intake, it seems more plausible that VAN GLP-1R activation simultaneously induces gene transcription and the release of pre-existing CART vesicles into the NTS. The activation of CART transcription may maintain and increase CART abundance for subsequent eating bouts, rather than exert an intra-meal effect. Thus, it stands to reason that a permanent downregulation of VAN CART (that reduces the amount of CART produced and, hence, released), and an acute neutralization of CART in the NTS via an antibody, have similar effects on GLP-1-induced inhibition of eating and gastric emptying. It is, however, surprising that a relatively mild downregulation of VAN CART expression leads to a substantial reduction in vagal GLP-1R-induced eating inhibition. This may indicate that reduced CART transcription leads to a rapid decrease in the amount of CART peptides available for release into the NTS, and therefore blocks the primary mechanism underlying GLP-1 induced eating inhibition.

Several studies indicate that CART can inhibit eating and gastric emptying via hindbrain mechanisms. Indeed, 4th ventricular injection of the CART peptide reduced food intake (19,27-30) and gastric emptying (29). While injection of CART in the lateral or 3rd ventricles produced similar effects to 4th ventricular injections (27,31), cerebral aqueduct occlusion attenuated the effects of 3rd ICV injection of CART on eating inhibition, further suggesting
that the inhibitory effect of CART on food intake originates within the hindbrain. In addition, 4\textsuperscript{th} ventricular injection of the CART peptide induced c-Fos in the NTS but the phenotype and projections of these CART-sensitive neurons are unknown (19).

At the neuronal and synaptic levels, the exact role and the mechanisms of CART action in the NTS remain speculative. Zheng and colleagues (19) hypothesized that CART could be co-released from VAN with glutamate in the NTS, thus providing a modulation of the glutamatergic satiation signal. Rather than having an immediate effect, CART could induce longer lasting pre- or post-synaptic changes in NTS neurons and, consequently, a longer-term influence on the control of food intake. Indeed, structural differences between classical neurotransmitters and neuropeptides suggest that neuropeptides can modulate synaptic transmission rather than being a fast-acting signal: glutamate is present in small synaptic vesicles that are released and recycled at the synapse while neuropeptides like CART are synthesized in the NG and transported in large vesicles to the axon terminals (32); the release of such large vesicles has been shown to occur outside of the synaptic terminals (33), indicating a modulatory role more than the ability to transmit a signal from neuron-to-neuron. CART has also been speculated to provide the early depolarizing event required for NMDA receptor activation in NTS neurons, thus increasing neuron specificity and reducing potential negative outcomes of glutamate spillover (8). Finally, meal-to-meal variations in the release of CART in the NTS based on feeding status could provide a certain plasticity to the vagal control of food intake that cannot be obtained with glutamatergic transmission alone (8).

Interestingly, several reports suggest that activation of central GLP-1R is necessary for the hypophagia induced by ICV CART. Indeed, pre-treatment (lateral ventricle) or co-infusions (4\textsuperscript{th} ventricle) of the GLP-1R antagonist attenuated the effects of ICV CART on food intake (30,34). This indicates that the effects of 4\textsuperscript{th} ICV CART require the central release of GLP-1.

We can thus speculate that one of the neuronal targets of vagal-derived CART may be the GLP-1 producing neurons in the NTS. This idea requires further testing but it is tempting to
think that the release of CART in the NTS by VAN relays the signal of intestinal GLP-1 secretion to the central GLP-1-producing neurons. The lack of an identified CART receptor (35,36), though, is a major obstacle to the identification of the central targets of CART.

Figure 4: Proposed mechanisms by which CART mediates the satiating effects of endogenous intestinal GLP-1 via vagal afferent neurons.
The activation of GLP-1R in the gut mucosa induces CART transcription (via an intracellular pathway to be identified) in VAN and activates the release of CART vesicles in the NTS. The role and neuronal targets of CART in the NTS are still unknown but it is probable that CART provides specificity and/or plasticity to the neuron-to-neuron glutamatergic transmission.

Despite the uncertain role of CART in the NTS, our data reiterate CART as an integrator of nutrient- and energy-related signals in VAN. Indeed, as mentioned earlier, CART has been shown to mediate the effects of CCK in VAN, and these effects are modulated by both leptin and ghrelin. Showing that CART is also a major downstream mediator of vagal GLP-1’s effects further enhances the potential importance of CART in the neural control of food
intake. Moreover, it naturally leads to the question whether CART integrates these different signals at the cellular level. Studies investigating the effects of various combinations of GLP-1, CCK, leptin, ghrelin or other vagally-sensed metabolic signals on CART expression and release are needed to understand how VAN CART may compute these diverse inputs to generate behavioral outcomes. Given its role as a mediating hub for several nutrient- and energy-related signals, VAN CART has also been hypothesized to contribute to the pathophysiology of obesity. Indeed, the eating-induced increase in CART levels is blocked in diet-induced obese rats (preliminary observations reported in (2)). Moreover, unpublished observations of our lab (Krieger, Lee, de Lartigue, 2015) indicate that the kd of CART in VAN is sufficient to induce hyperphagia and weight gain. Further studies are required to confirm a causal role for declining VAN CART levels in diet-induced obesity.

In sum, our data are the first demonstration to date that CART mediates the effects of VAN GLP-1R activation on food intake and gastric emptying. Our study is therefore a further step towards considering VAN CART as a major integrator of nutrient-derived signals along the gut-brain axis. The possibility that VAN CART plays a role in the pathophysiology of obesity makes it a potential therapeutic target.
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.

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

_De Lartigue G._ participated in the study conception and design, participated in the experiments/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.

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CHAPTER 5:

GLP-1 RECEPTORS IN VAGAL AFFERENT NEURONS MODULATE ENERGY EXPENDITURE VIA BROWN ADIPOSE TISSUE THERMOGENESIS

AUTHORS


Physiology and Behavior Laboratory, ETH Zürich, Switzerland

STATUS

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ABSTRACT

The glucagon-like peptide-1 (GLP-1) is a gut peptide involved in the control of energy intake and glucose metabolism. Whether intestinal GLP-1 also plays a role in the regulation of energy expenditure, is, however, unknown. Recent studies have shown that central GLP-1 receptor (GLP-1R) activation acutely increases energy expenditure via a stimulation of brown adipose tissue (BAT) thermogenesis, raising the possibility that peripheral GLP-1R activation may also be involved in the regulation of BAT thermogenesis. To test this hypothesis, we first investigated the effects of peripheral GLP-1R activation on energy expenditure in male Sprague-Dawley rats while controlling for potential confounding factors, i.e., food intake and gastric emptying. In contrast to central GLP-1R activation, peripheral GLP-1R activation by the GLP-1R agonist exendin-4 induced a transient decrease of whole-body energy expenditure, accompanied by a decrease in interscapular BAT (iBAT) temperature. The absence of GLP-1R in iBAT prompted us to search for a new pathway by which endogenous peripheral GLP-1 can modulate BAT activity. Using a retrograde viral tracing technique, we here present evidence that iBAT adipocytes are synaptically connected to vagal afferent neurons (VAN). To test the physiological relevance of this pathway, we knocked down GLP-1R in VAN. When exposed to a high-fat diet (HFD), GLP-1R kd rats showed an increase in energy expenditure and BAT activity during the dark phase compared to control rats, which alleviated HFD-induced obesity and glucose intolerance. Together, our data indicate that endogenous GLP-1 plays a role in energy expenditure and that a functional gut-BAT crosstalk via VAN is important for the pathophysiology of obesity.
INTRODUCTION

The development of obesity is indisputably influenced by fat- and carbohydrate-rich «western» diets, but the nutrient sensing mechanisms leading to the dysregulation of energy balance when exposed to such diets remains to be fully elucidated. The vagus nerve is a key neuronal element in the gut-brain communication: therefore, its role in nutrient sensing and, more broadly, in the regulation of energy metabolism has recently received increasing attention. In recent years, newly developed strategies allowed specific genetic manipulation targeting vagal afferent neurons (VAN) and provided important insights into the molecular mechanisms controlling eating and energy expenditure (1-5). Recently, using the bilateral injection of a viral vector in the nodose ganglion (NG), we demonstrated that VAN GLP-1R expression is necessary for the effects of endogenous intestinal glucagon-like peptide-1 (GLP-1) on eating and glycemia (5). GLP-1R knockdown (kd) in VAN increased meal size and slowed down gastric emptying without affecting 24 h food intake or body weight, and raised post-meal glucose levels by blunting insulin secretion.

The role of endogenous intestinal GLP-1 in the control of energy intake and glucose metabolism is well characterized, whereas the physiological effects of intestinal GLP-1 on energy expenditure and its components have not yet been systematically investigated. Central GLP-1R activation acutely increases whole-body energy expenditure (6-8) and stimulates brown adipose tissue (BAT) thermogenesis (7) in rodents, but whether endogenous intestinal GLP-1 can access central GLP-1R is unclear. Studies using peripheral administration of GLP-1 or GLP-1R agonists should better address the role of intestinal GLP-1 but led to conflicting reports on energy expenditure and its surrogate measures in mice, rats and humans (8-13). In addition, there is evidence indicating a role of the vagus nerve in controlling energy expenditure and BAT thermogenesis. Indeed, diverse techniques of vagus nerve stimulation (VNS) were linked with a modulation of whole-body energy expenditure and BAT activity in rodents and humans (14-17). Moreover, alterations of lipid sensing pathways in VAN (via downregulation of the nuclear receptors peroxisome proliferator-
activated receptor γ, PPARγ, and liver X receptors α and β, LXRα/β) alleviated high-fat diet (HFD)-induced obesity, mainly by increasing BAT thermogenesis (2,3). The exact role of VAN in the regulation of whole-body energy expenditure and BAT thermogenesis, however, is still elusive.

Considering the pivotal role of VAN in mediating the GLP-1 signal to the brain and the common feature of VAN and GLP-1 to modulate BAT activity, we hypothesized that a/ peripheral GLP-1R activation can modulate whole-body energy expenditure, b/ this modulation is dependent on VAN GLP-1R and c/ the effect of peripheral GLP-1 on energy expenditure is at least partly due to changes in BAT activity. In order to clarify the effect of endogenous intestinal GLP-1 on energy expenditure, we activated peripheral GLP-1R using an intraperitoneal (IP) injection of exendin-4 (Ex-4) in rats and measured whole-body energy expenditure and BAT thermogenesis. We further investigated the potential role of VAN for the modulation of BAT activity using retrograde tracing studies from the interscapular BAT (iBAT) pads. Finally, we tested the physiological relevance of this vagal pathway in rats with a reduced GLP-1R expression in VAN. Overall, our data propose a new role for endogenous GLP-1 to tune down energy expenditure after a meal via a previously undescribed gut-brain-BAT pathway. Furthermore, increased intestinal GLP-1 secretion during HFD feeding may reduce the dissipation of nutrients as heat, thus contributing to body weight gain and glucose intolerance.

**METHODS**

**Animals and housing**

Male Sprague-Dawley rats (Charles River) were individually housed in a temperature and humidity-controlled environment (21±1°C, 55±5% HR) with a 12/12 h dark/light cycle. Standard laboratory chow (3433, Klika Nafag, energy density: 3.13 kcal/g) was provided ad
Ad libitum unless otherwise noted. All procedures were approved by the Zurich Cantonal Veterinary Office.

**Acute effects of IP Ex-4 injection on energy expenditure, BAT temperature and locomotor activity**

Rats were individually housed and habituated for 6 days to the new environment and to IP injections of NaCl prior to the experiment. The routine daily health check was performed 2 h before the dark phase onset. On experimental days, rats (350-450 g) were allowed food access **ad libitum** until food was withdrawn 2 h into the dark phase. Two h later, rats received an IP injection of NaCl or Ex-4 (0.3 µg/kg) and were immediately brought back to their cages. This protocol was repeated four times to a/ measure whole-body energy expenditure and RER, b/ measure iBAT temperature with infrared imaging, c/ measure locomotor activity and d/ collect tissue for subsequent molecular analysis. Injections were randomized and a minimum of 4 days separated each experimental day.

**Indirect calorimetry**

Rats were individually housed in an open circuit indirect calorimetry Phenomaster system (TSE). Individual body weights were used to normalize energy expenditure. Data are presented as 1 h time bins.

**BAT Infrared imaging**

To increase the sensitivity of the BAT temperature measurements, the interscapular area was shaved. Infra-red (IR) pictures were taken with an E60 camera (FLIR), mounted vertically over the area of interest (distance 30 cm). Rats were lightly restrained in a stretched position under the camera and 3 snapshots/timepoint were taken for further analysis. Baseline IR pictures were taken 2 h after dark onset and a second series of IR pictures was taken 2 h after the IP injection. Final analysis of the IR pictures was done by defining a standard rectangular area (60 x 20 px) over the interscapular region and averaging the mean area temperatures of two pictures (FLIR Tool software for PC).
**Locomotor activity**

Ninety min after IP injection of Ex-4 (to overlap the phase during which EE was at the lowest), rats locomotor activity was monitored for 45 min during an open field test. The open field test was conducted in two identical arenas (80x80x50 cm), filmed by a digital camera and analyzed with a computer running the EthoVision tracking system (see (18) for a full description).

**Tissue collection**

Subcutaneous white adipose tissue (ScWAT) and brown adipose tissue (BAT) were immediately collected after decapitation of the animals 2 h after IP Ex-4 injection. Tissues were immediately frozen in liquid nitrogen and stored at -80°C until further processing.

**Retrograde labeling from the iBAT**

Rats (520-570 g on surgery day) were anesthetized with ketamin (Ketalar, 88 mg/kg IP, Zurich Cantonal Pharmacy) and xylazin (Rompun 2%, 5 mg/kg IP, Zurich Cantonal Pharmacy). An incision of 4 cm was made above the interscapular area to allow visualization of and access to the brown fat pads. One µL of a pseudorabies 263 virus (PRV-263, 1.4x10^9 pfu/mL, provided by Lynn Enquist, Princeton University, virus center grant p40RR018604) was injected into each iBAT pad. After injection, the incision was immediately stitched and rats received appropriate post-surgical care (20 mg/kg sulfadoxin, 5 m/kg trimethoprim, subcutaneously, SC and 5 mg/kg carprofenum, SC, Zurich Cantonal Pharmacy). Four days after PRV-263 injection, rats were euthanized with CO₂. Nodose ganglia were immediately collected and fixed in 4% PFA. Fixed nodose ganglia were cut at 10 µm in a cryostat (CM 1950, Leica) and fluorescence was visualized with an Axioscope.A1 microscope (Zeiss) equipped with an AxioCam MRC camera (Zeiss).
GLP-1R knockdown in vagal afferent neurons

**Lentiviral-mediated knockdown**

The production of lentiviral particles targeting the GLP-1R mRNA via a shRNA construct was carried out as previously described (5). Briefly, a U6 promoter-driven shRNA sequence targeting the rat GLP-1R mRNA was expressed in a pLKO.1-puro vector (Sigma-Aldrich). A non-target shRNA sequence (Sigma-Aldrich) expressed in a similar vector was used as a control. Each of these plasmids has been co-transfected with the pMD2.G and psPAX2 plasmids (Didier Trono, Addgene #12259 and 12260) in HEK 293T cells, and lentiviral particles were collected, concentrated to $10^{10}$ particles/mL using 8% PEG6000 (Millipore) in 0.3M NaCl and resuspended in PBS. Rats (270-330 g on surgery day) were anesthetized with ketamin (Ketalar, 88 mg/kg IP, Zurich Cantonal Pharmacy) and xylazin (Rompun 2%, 5 mg/kg IP, Zurich Cantonal Pharmacy). An incision in the anterior neck was made to provide successive access to the left and right cervical branches of the vagus nerve. 1.5 µL of the concentrated viral solution were injected into each nodose ganglion (NG) with a Picospritzer III injector (Parker Hannifin). The incision was sutured and post-surgery care was given according to the above-mentioned protocol. Before and during 20 days after surgery, all animals had access to standard chow. After the 20-day period left for the knockdown to fully establish, animals had *ad libitum* access to a HFD (D12492, Sniff, 60% energy from fat, energy density: 5.16 kcal/g).

**Measurement of food intake and body composition**

Daily food intake and meal patterns were measured by an automated system (see (19) for description) unless animals were placed in the indirect calorimetry system (see below). Analysis of body composition was conducted 10 weeks after introduction of the HFD using a computerized tomography system (La Theta LCT-100, Aloka) with a previously validated method (20). Briefly, rats were anesthetized with isoflurane and placed supine in a cylindric holder (120 mm inner diameter). An initial whole-body sagittal image was obtained to ensure proper placement of the animal. Scans were done from vertebrae L1 to L6 with a 2 mm pitch.
size. Volumes of adipose tissue (fat mass), bone, air, and the remainder (lean mass) are detected by the Aloka software based on their X-ray absorption. Fat mass and lean mass were computed using the density factors of 0.92 g/cm$^3$ and 1.10 g/cm$^3$, respectively. Fat ratio is defined as fat mass / (lean + fat mass).

**Continuous measurements of energy expenditure, locomotor activity, body (IP) and iBAT temperatures**

A telemetry sensor (TA-F40, DSI) was surgically implanted IP to monitor activity in freely-moving animals. After recovery, rats were placed in an indirect calorimetry system (Phenomaster, TSE) and habituated for 5 days to the environment prior to the data collection. Energy expenditure and activity were monitored in parallel in the indirect calorimetry chambers, and averaged over 3 days. Daily health check of the rats was performed 2 hours before the dark phase onset. Individual body weights were used to normalize energy expenditure. To monitor BAT temperature, a dual telemetry sensor (F40-TT, DSI) was surgically implanted (SC) in a separate rat cohort 10 weeks after introduction of a HFD. The two flexible temperature probes were placed in close apposition with the interscapular brown fat pads, or in the peritoneal cavity, respectively, and secured with sutures. After 10 days of recovery, BAT and IP temperatures were continuously measured in ad libitum HFD-fed animals (weeks 11 to 12, Dataquest ART v3.1, DSI).

**Oral glucose tolerance test (OGTT)**

An OGTT was performed 11 weeks after the introduction of the HFD. Fourteen h-food deprived rats adapted to gavage and restraining received an oral bolus of 40% glucose solution (2 g/kg). Tail vein blood samples for glucose and insulin were taken at baseline and 15, 30, 60, 90, and 120 min after the oral glucose bolus. Blood glucose was measured twice using Accucheck (Roche). Insulin was measured from plasma using an immunoassay (MesoScale Discovery single-spot for mouse/rat K152BZC).
**Intraperitoneal insulin sensitivity test (IPIST)**

An IPIST was performed 12 weeks after the introduction of the HFD. Fourteen h-food deprived rats adapted to injections and restraining received an intraperitoneal injection of 1 IU/kg of insulin (Actrapid HM, Novo Nordisk). Tail vein blood glucose was measured twice per timepoint using Accucheck (Roche) at baseline and 15, 30, 45, 60, 90, 120 and 180 min after injection.

**HFD test meal-induced GLP-1 levels**

10 weeks after introduction of the HFD, rats were habituated to test meals and restricted feeding schedule for a week (Test meal at dark phase onset, ad libitum food access from 3 to 8 h after dark phase onset, food deprivation otherwise). On experimental days, rats received a 3 g HFD test meal at dark phase onset. Blood was sampled from hepatic portal vein (HPV) catheters in unrestrained animals 30 min prior to (baseline) and according to the scheduled timepoints. At each time point, 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 was measured using an immunoassay (MesoScale Discovery multi-spot K15171C).

**Tissue collection of GLP-1R kd rats**

Animals received an IP injection of pentobarbital (100 mg/kg, Zurich Cantonal Pharmacy) and NG (ganglia from the same animal were pooled), brain, subcutaneous white adipose tissue (ScWAT) and iBAT were immediately collected. For gene and protein expression analysis, tissues were immediately frozen in liquid nitrogen and stored at -80°C until further processing. The nucleus tractus solitarii (NTS), arcuate nucleus (Arc) and paraventricular nucleus of the hypothalamus (PVH) were collected after brain cryosection and micropunching. For stainings with haematoxylin and eosin (H&E), adipose tissues were fixed in 4% PFA (Sigma-Aldrich) and processed in a STP 120 (Microm) according to the standard protocol. Paraffin-embedded samples were cut at 5 µm on a Hyrax M55 (Zeiss),
deparaffinized and stained on a Varistain 24-4 (Shandon). Pictures were obtained with an Axioscope A.1 microscope (Zeiss).

**Gene expression and protein analysis**

RNA and proteins were extracted using Trizol (Life Technologies). RT-qPCR analysis was performed using SybR Green on a OneStep Plus instrument (Applied Biosystems) and results were analyzed using the 2ddCt method. Western blots were performed to detect the GLP-1R protein (Rabbit antibody 39072, 1:400, Abcam) or the Uncoupling protein-1 (UCP1; Rabbit antibody PA1-24894 1:2000, ThermoFisher) using β-actin as reference (Mouse antibody AC-74, 1:3000, Sigma-Aldrich).

**Statistical analysis**

Data normality was verified using the Shapiro-Wilk (when \( n \geq 7 \)) and the Kolmogorov-Smirnov (when \( 5 \leq n \leq 6 \)) tests and homoscedasticity was checked by visualizing the distribution of residuals. Non-parametric tests were used otherwise. Tests used and P-values are given in the figure legends. Data are presented as means ± SEM. P-values < 0.05 were considered significant. All graphs were generated using GraphPad Prism (version 6.05 for Windows).

**RESULTS**

**Peripheral GLP-1R activation induced a transient decrease of energy expenditure**

To test the effects of peripheral GLP-1R activation on whole-body energy expenditure, we performed an IP injection of the GLP-1R agonist Ex-4 at a dose that previously inhibited food intake via the activation of VAN GLP-1R (0.3 µg/kg) (5). Moreover, to isolate the effects of GLP-1R activation on EE per se, we standardized food intake and gastric emptying using a restricted feeding schedule (no access to food 2h before and 4h after the IP Ex-4 injection—Fig. 1A). Our data demonstrate that IP injection of Ex-4 transiently reduced whole-body
Figure 1: IP injection of the GLP-1R agonist Ex-4 (0.3 µg/kg) transiently reduced energy expenditure, RER and iBAT thermogenesis

(A) Experimental protocol used to study the effects of IP Ex-4 on energy homeostasis independently of the effects of food intake and gastric emptying (B) Hourly heat production following IP Veh or Ex-4 injection (n = 7/9; Two-way ANOVA group x time; group effect NS, time effect P < 0.0001, interaction P = 0.1) (C) Cumulative energy expenditure during the 4 h following IP Veh or Ex-4 injection (n = 7/9; Student t-test; P < 0.05) (D) Hourly evolution of the respiratory exchange ratio following IP Veh or Ex-4 injection (n = 7/9; Two-way ANOVA group x time; group effect NS, time effect P < 0.0001, interaction P < 0.005) (E) Total distance moved during a 45-min open field test (n = 8/7; Student t-test; NS) (F) Average temperature of the interscapular area before and 2 h after IP Veh or Ex-4 injection, with representative pictures. White rectangles indicate the area considered to evaluate interscapular skin temperatures (n = 8; Two-way ANOVA group x time; group effect NS, time effect NS, interaction P < 0.01; * indicates a significant difference with Veh-injected rats 2 h post-injection - Bonferroni post-hoc test) (G) Relative mRNA expression of thermogenic markers in the iBAT; (n = 8/7; Student t-tests; P < 0.01 for B3AR; P < 0.1 for PGC1α and PPARγ; P < 0.2 for UCP1, Cidea, FGF21, HSL and MGL; P > 0.3 for ATGL and DIO2 (H) Relative UCP1 protein levels in the iBAT 2 h after IP Veh or Ex-4 injection, with representative bands (n = 7; Student t-test; NS)

Abbreviations: OF, open field; UCP1, Uncoupling protein-1; B3AR, β-3 adrenergic receptor; PGC1α, peroxisome proliferator-activated receptor γ coactivator α-1; PPARγ, peroxisome proliferator-activated receptor γ; DIO2, type 2 iodothyronine deiodinase; FGF21, fibroblast growth factor 21; ATGL, adipose triglyceride lipase; HSL, hormone-sensitive lipase; MGL, monoacylglycerol lipase
energy expenditure (Fig. 1B, C) and respiratory exchange ratio (RER) (Fig. 1D) in a timeframe of 4 h after injection. Because Ex-4 is known to induce visceral malaise (21), it may impact energy expenditure via a reduction in locomotor activity. To test this, we measured locomotor activity in a 45 min open field test after IP injection of Veh or Ex-4 and showed that Ex-4 at the dose effective for reducing energy expenditure did not reduce locomotor activity (Fig. 1E and Supp. Fig. 1). Together, our data indicate that peripheral GLP-1R activation reduces whole-body energy expenditure independent of a decrease in food intake and without reducing locomotor activity.

**Peripheral GLP-1R activation induced a transient decrease in iBAT temperature**

We further tested the idea that the reduction in energy expenditure induced by peripheral GLP-1R activation was at least partially due to the changes in BAT thermogenesis. Using a similar paradigm as above, we found that interscapular skin temperature was decreased 2 h after IP Ex-4 injection compared to vehicle injection (Fig. 1F), indicative for a reduced iBAT activity. Furthermore, at the same time point, the expression of thermogenic markers tended to be lower in the iBAT of Ex-4 injected rats compared to controls (Fig. 1G). We, however, failed to detect the decrease in the protein levels of UCP1 in iBAT of Ex-4 injected rats (Fig. 1H).

**Potential control of iBAT activity by VAN**

iBAT showed no detectable mRNA or protein expression of the GLP-1R, as measured by RT-PCR and Western blot of iBAT and NG extracts (Fig. 2A, B). This eliminates the possibility of a direct action of GLP-1 on brown adipocytes. To test if VAN are involved in the control of BAT activity, we first tested whether iBAT and NG are synaptically linked using a neuroanatomical tracing technique. For this, we injected the retrograde tracer PRV-263 expressing tdTomato bilaterally in the iBAT pads. 4 days after PRV injection, a subset of neurons in both NG were labeled with tdTomato (Fig. 2C). These data indicate a neuronal network linking the gut and the iBAT. Together with our previous results, it suggests that endogenous GLP-1 can modulate energy expenditure and BAT thermogenesis via VAN.
Figure 2: Absence of GLP-1R in the iBAT of the rat and synaptic connections between the iBAT and VA
(A) Products of a RT-PCR ran with GLP-1R primers only (lane 1 - “Neg”) or together with iBAT cDNAs (lanes 2 to 5 - “iBAT”) or with NG cDNAs (lanes 6 to 8 - “NG”); expected GLP-1R amplicon size: 79bp
(B) Relative expression of the GLP-1R protein in the iBAT and in the NG, with representative bands (n = 4/5; Mann-Whitney test; P < 0.05) (C) Representative fluorescent picture of a NG 4 days after injection of a PRV-263 virus into the iBAT. Arrows indicate labeled cells, top right frame shows labeling of fibers within the NG

GLP-1R kd in VAN reduced HFD-induced body weight gain and impairments of glucose homeostasis

To test the relevance of GLP-1R activation in VAN for the control of BAT activity, we used our established model of Sprague-Dawley rats with a specific kd of GLP-1R in VAN (5). Injection of lentiviral particles containing a GLP-1R targeting shRNA construct led to a significant reduction of GLP-1R mRNA (Fig. 3A) and protein (Fig. 3B) expression in the NG compared to control injected rats. In this model, body weight is equivalent to the one of control rats (5). When fed a 60% HFD, however, the body weight gain of GLP-1R kd rats diverged from controls, leading to a significantly lower body weight gain after week 9 in GLP-1R kd rats (Fig. 3C). As indicated by a trend towards a lower fat mass without any change in lean mass, the reduction in HFD-induced weight gain in GLP-1R kd rats may predominantly be explained by less fat accumulation (Fig. 3E). Surprisingly, although GLP-1R kd rats
Figure 3: GLP-1R kd in VAN alleviated HFD-induced body weight gain and glucose intolerance

(A) Relative GLP-1R mRNA expression in control and GLP-1R kd rats (n = 10/9; Student t-test; P < 0.001)

(B) Relative GLP-1R protein expression in control and GLP-1R kd rats, with representative bands (n = 7/6; Student t-test; P < 0.05)

(C) Body weight gain of control and GLP-1R kd rats after introduction of the HFD (n = 13/14; Two-way ANOVA, group x time; group effect P < 0.05, time effect P < 0.0001, interaction effect P < 0.01; * indicates a significant difference between control and GLP-1R kd rats - post-hoc Bonferroni comparisons)

(D) Daily intake of HFD by control and GLP-1R kd rats (n = 13/14; Two-way ANOVA, group x time; group effect NS, time effect P < 0.0001, interaction effect NS)

(E) Lean and fat mass of control and GLP-1R kd rats (n = 13/13; Student t-tests; NS)

(F) Blood glucose during an OGTT (2 g/kg) in control and GLP-1R kd rats (n = 13/14; Two-way ANOVA, group x time; group effect NS, time effect P < 0.0001, interaction effect NS; * indicates a main group effect)

(G) Area under curve of blood glucose during OGTT (n = 13/14; Student t-test; P < 0.01) in control and GLP-1R kd rats

(H) Plasma insulin during OGTT (n = 12/14; Two-way ANOVA group x time; group effect P < 0.05, time effect P < 0.0001, interaction effect NS; * indicates a main group effect) in control and GLP-1R kd rats

(I) Blood glucose during an IPIST in control and GLP-1R kd rats (n = 6/7; Two-way ANOVA group x time; group effect NS, time effect P < 0.0001, interaction effect P < 0.05; * indicates a significant difference between control and GLP-1R kd rats - post-hoc Bonferroni comparisons)
showed a larger meal size than controls before being switched to HFD, HFD exposure did not lead to alterations in either daily food intake or meal patterns (Fig. 3D, Supp. Fig. 3). We further tested whether the lower body weight gain in GLP-1R kd rats was associated with improved glucose intolerance and insulin sensitivity. GLP-1R kd rats showed a reduced glucose excursion compared to controls from 60 to 120 min after an oral glucose bolus (Fig. 3F), leading to an overall reduced glucose AUC (Fig. 3G). Consistent with glucose levels, plasma insulin levels were also lower in GLP-1R kd rats than in controls after the oral glucose bolus (Fig. 3H). An IP insulin sensitivity test (IPIST) also showed a more robust reduction in blood glucose 15 min after IP injection of insulin in GLP-1R kd rats than in controls (Fig. 3I). These results indicate that GLP-1R kd in VAN conferred a relative protection from HFD-induced weight gain, glucose intolerance and insulin resistance.

GLP-1R kd in VAN increased dark phase energy expenditure and iBAT temperature during HFD exposure

When chronically challenged with a 60% HFD, GLP-1R kd rats showed an increased heat production in the early dark phase compared to their controls (Fig. 4A, B). This increase in energy expenditure was not due to changes in locomotor activity (Supp. Fig. 2). Consistent with the absence of changes in food intake, RER was similar between the two groups (Fig. 4C). In another cohort, we implanted a dual temperature probe and measured in parallel IP and BAT temperatures in control and GLP-1R kd rats. iBAT temperature was constantly elevated during the dark phase in GLP-1R kd rats compared to controls, whereas it remained similar to controls during the light phase (Fig. 4E). IP temperature remained similar throughout the day between the two groups (Fig. 4D).

GLP-1R kd in VAN increased thermogenic capacity in both BAT and ScWAT during HFD exposure

iBAT H&E staining showed a smaller average cell size in the GLP-1R kd group, indicative for increased BAT thermogenesis in this group (Fig. 4G). In addition, the molecular analysis of the iBAT after 12 weeks on HFD revealed an increased UCP1 mRNA and protein expression
Figure 4: GLP-1R kd in VAN increased dark phase energy expenditure and BAT and ScWAT thermogenic capacity during HFD exposure

(A) Hourly evolution of heat production over 24 h in control and GLP-1R kd rats (n = 7/8; Two-way ANOVA; group x time; group effect P = 0.1, time effect P<0.0001, interaction effect NS) (B) Cumulative energy expenditure split in day quarters in control and GLP-1R kd rats (n = 7/8; Sidak-Bonferroni corrected t-test; P < 0.05 for 0 - 6 h, NS for 6 - 12 h and 12 - 18 h, P = 0.1 for 18 – 24 h) (C) Hourly evolution of respiratory exchange ratio (RER) over 24 h in control and GLP-1R kd rats (n = 7/8; Two-way ANOVA; group x time; group effect NS, time effect P<0.0001, interaction effect NS) (D) Real-time 24 h monitoring of IP temperatures (n = 6/6; Two-way ANOVA group x time; group effect NS, time effect NS, interaction effect NS) (E) Real-time 24 h monitoring of iBAT temperatures (n = 6/6; Two-way ANOVA group x time; group effect P = 0.06; time P < 0.0001; interaction P < 0.001) (F) Relative mRNA expression of thermogenic markers in the iBAT (n = 8/8; Student t-tests; P<0.05 for UCP1 and
and a trend towards an increase in β3-adrenergic receptor (B3AR) expression in the GLP-1R kd rats compared to controls (Fig. 4F). In addition, RT-qPCR indicated an increase in thermogenic markers (including UCP-1, PPARγ, and B3AR) and a trend towards a reduction in the expression of lipogenic enzymes (FAS, ACC) in the scWAT of GLP-1R kd rats compared to controls (Fig. 4H). A robust increase in UCP1 was also detected at the protein level (Fig. 4I). Together, these results indicate an increased BAT thermogenic phenotype and browning of the ScWAT via increased sympathetic inputs as a result of VAN GLP-1R kd.

**GLP-1R kd in VAN led to a maintenance of ppg expression in the NTS during HFD exposure**

We further investigated whether central regulators of energy balance and glucose homeostasis were modulated by the GLP-1R kd in VAN. In the arcuate nucleus of the hypothalamus (Arc), there was no difference in the expression of feeding related neuropeptides, consistent with the similar food intake between two groups (Fig. 5B). Similarly, in the paraventricular nucleus of the hypothalamus (PVN), there was no change in genes involved in the neuroendocrine control of energy metabolism (Supp. Fig. 4A). Preproglucagon (PPG) is a precursor for the GLP-1 and is produced in the NTS in the hindbrain. Interestingly, we found that HFD feeding decreased ppg mRNA expression in the NTS compared to normal chow feeding in control rats (Supp. Fig. 4B). GLP-1R kd prevented this HFD-induced decrease in PPG mRNA expression (Fig. 5A and Supp. Fig. 4B) whereas it did not affect the central GLP-1R expression (Fig. 5A, B and Supp. Fig. 4B). Because central GLP-1R activation modulates BAT functions, the protective effect of VAN GLP-1R kd on NTS
ppg expression suggests a potential central mechanism linking peripheral GLP-1R signaling to the adipose tissue function.

**Figure 5:** GLP-1R kd in VAN increased NTS ppg expression during HFD exposure

(A) Relative mRNA expression in the NTS (n = 6/7; Student t-test; P < 0.05 for ppg, NS for other genes)

(B) Relative mRNA expression in the Arc (n = 7/8; Student t-test; NS)

Abbreviations: AgRP, Agouti-Related Peptide; Arc, arcuate nucleus of the hypothalamus; DBH, dopamine β-hydroxylase; GLP-1R, glucagon-like peptide-1 receptor; NTS, nucleus tractus solitarii; POMC, proopiomelanocortin; PPG, preproglucagon; NPY, neuropeptide-Y

**DISCUSSION**

Recent studies have highlighted the newly emerging role of GLP-1 in the control of energy expenditure and, in particular, the stimulatory effects of central GLP-1R activation on BAT thermogenesis. This study is, to our knowledge, the first to unequivocally demonstrate the differential role of peripheral GLP-1R activation on EE and BAT thermogenesis.

First, our study shows that peripheral GLP-1R agonist treatment decreases whole-body energy expenditure and BAT thermogenesis. There were conflicting results in the literature regarding the effects of peripheral GLP-1 or GLP-1R agonist treatment on energy expenditure (8,9,11-13). These discrepancies may be explained by the use of different routes of administration (IP, IV, SC), doses (see chapter 1, table 1), agonists (GLP-1, Ex-4, liraglutide) or species (mice, rats, hamsters, humans). Furthermore, it is important to consider that the reduction in food intake and gastric emptying induced by GLP-1 administration can lead to adaptive thermogenesis. Therefore, it was crucial to dissect the direct effect of GLP-1R activation on energy expenditure by eliminating these parameters.
with a restricted feeding schedule. Our data clearly demonstrate that peripheral GLP-1R activation by a low dose of IP Ex-4 reduces RER, energy expenditure and BAT temperature in the absence of eating, suggesting a direct effect. It remains unclear how IP Ex-4 reduces iBAT temperature, given the relatively mild modulation of thermogenic markers and the absence of apparent change in UCP1 protein levels. Because we measured gene and protein expression 2 h after Ex-4 injection, when energy expenditure started to increase again, it remains therefore possible that the BAT thermogenic activity is already normalized to the baseline level.

Since endogenous GLP-1 requires normal GLP-1R expression in VAN for the control of food intake and glycemia, we next hypothesized that this gut-brain pathway may also be involved in the regulation of BAT thermogenesis. Injections of retrogradely transported viral tracers have been widely used to find the CNS origins of the sympathetic regulation of the BAT ((22,23) among others). Using a fluorescent-labeled transsynaptic PRV, we visualized VAN in the NG retrogradely labeled from the iBAT, suggesting a vagally-mediated control of BAT thermogenesis rather than a direct activation of GLP-1R in the BAT. We acknowledge that our tracing data identified synaptically connected neurons between VAN and iBAT but did not provide indisputable evidence that this pathway is relevant for the effects of peripheral GLP-1R activation on energy expenditure and BAT activity. Further studies are on the way to investigate whether these PRV-infected VAN express GLP-1R and whether the acute effects of IP Ex-4 on energy expenditure and BAT temperature are attenuated in GLP-1R kd rats. Nonetheless, we could speculate on the potential central pathways involved in this gut-to-BAT pathway. The premotor neurons in the rostral raphe pallidus (rRPa) were shown to be the key mediators of the sympathetic control of BAT thermogenesis (24,25), providing an excitatory drive to BAT sympathetic preganglionic neurons in the spinal intermediolateral nucleus. rRPa premotor neurons activity is modulated by hypothalamic and brainstem projections (25-27). Indeed, decerebrate rats showed higher BAT temperature than intact rats after injection of a melanocortin receptor agonist into the rRPa (28), indicating that the
caudal brainstem circuit is sufficient for tuning BAT thermogenesis. Moreover, disinhibition of NTS neurons via a GABAA receptor antagonist blocked the increase in BAT sympathetic nerve activity and BAT temperature induced by various stimuli (including cold, and direct activation of rRPa neurons) in an anesthetized rat model (26). Therefore, it is reasonable to speculate that rRPa neurons integrate vagally-mediated signals via inhibitory NTS projections to modulate BAT sympathetic outflow.

Our study further demonstrated the physiological relevance of a gut-BAT crosstalk involving gut-derived GLP-1 in the pathophysiology of obesity. Indeed, permanent downregulation of GLP-1R kd in VAN increased BAT thermogenesis and energy expenditure, which was associated with reduced HFD-induced body weight gain and markedly improved glucose homeostasis. Several recent studies have suggested the involvement of the vagus nerve in the regulation of HFD thermogenesis. VA express genes that are involved in lipid sensing mechanism and are thus relevant for the sympathetic regulation of adipose tissue and lipid metabolism. Indeed, the loss of nuclear receptor expression involved in lipid sensing, (PPARγ (2) and LXRα/β (3)) in VAN of mice increased mRNA levels of BAT thermogenic markers such as UCP1 and β3-adrenergic receptors (2), which were associated with an increase in energy expenditure, and with attenuated lipid accumulation during HFD exposure (3). Moreover, hepatic vagotomy restored the reduced BAT thermogenic capacity and rRPa c-Fos expression induced by a liver glucokinase overexpression in HFD-fed mice (29), further strengthening the idea that VAN could be the crucial link relaying the effects of peripheral signals to the CNS to modulate BAT activity.

The fact that, in the GLP-1R kd rat model, the disruption of a gut-BAT crosstalk translates into whole-body energy expenditure and body weight effects during exposure of HFD, but not chow, raises an important question. Recent data have shown that 3 to 13 wk HFD-feeding increases the GLP-1 response to a mixed-meal compared to low fat diet-fed rats (30). In our study, chronic HFD-fed rats had 3-fold higher meal-induced GLP-1 levels in the hepatic portal vein than the chow-fed rats after an isocaloric test meal (Supp. Fig. 3E). Hence, it is a
plausible speculation that an increased endogenous gut GLP-1 secretion in response to HFD, and the vagal GLP-1R activation that follows, may be the gut-brain mechanism that signals the high amount of fats in the diet and promote the central mechanisms favoring the storage of the excess fat. Therefore, blocking this signaling pathway by GLP-1R kd in VAN prevented the fat accumulation via an increase in BAT thermogenesis and browning of the ScWAT. As mentioned earlier, HFD exposure modulates the expression pattern of the nuclear receptors involved in lipid sensing in VAN (2). Because these nuclear receptors have been shown to regulate the thermogenic response to HFD exposure, we can also speculate that GLP-1 signal within the NG interacts with these nuclear receptors. Further investigation of the role of the lipid sensing in VAN and its interaction with GLP-1 signaling in regards to the control of energy expenditure is clearly warranted. Intriguingly, our data show a decrease in NTS *ppg* expression after chronic HFD exposure in control, but not in GLP-1R kd rats. Acute central injection of GLP-1 or GLP-1R agonists has been shown to transiently increase energy expenditure in rodents (6-8), via a mechanism involving hypothalamic GLP-1R, adenosine monophosphate-activated protein kinase (AMPK) (7) and increased sympathetic outflow to the BAT (31). This suggests that increased central GLP-1 production in GLP-1R kd rats during HFD exposure may be one of the mechanisms contributing to the increased BAT activity in this model. NTS *ppg*-expressing neurons respond to food-related cues, such as CCK, gastric distension, and leptin, and *ppg* kd results in hyperphagia and fat accumulation, suggesting the involvement of central GLP-1 system in the regulation of energy balance (32). Although GLP-1 does not directly activate *ppg* neurons (33), chronic peripheral treatment with the GLP-1R agonist liraglutide decreased central *ppg* gene expression compared to *ad libitum* fed and weight-matched control rats (34). The results from our GLP-1R kd study indicate that GLP-1R in VAN are necessary for the modulation of central *ppg* expression induced by HFD exposure. Together, this raises the possibility that the amplified gut secretion of GLP-1 during HFD exposure leads to a downregulation of the central GLP-1 system via VAN, which contributes to reduced BAT thermogenesis and fat accumulation.
In the light of our data, antagonizing elevated GLP-1 levels early in the development of obesity could be a promising strategy to alleviate the course of body weight gain and insulin resistance. This may seem contradictory to the growing body of evidence showing that GLP-1R agonists treatments are effective at treating obesity via a reduction in eating and a possible effect on energy expenditure. Several studies, however, have shown that the effects of the GLP-1R agonist liraglutide on body weight and food intake are mediated by central GLP-1R (4,34), i.e. receptors physiologically activated by the central GLP-1 system. The effects of GLP-1R agonists on eating and body weight may predominantly compensate for the alteration of the central GLP-1 system induced by the gut-brain mechanisms speculated here.

The existence of a pathway that tunes down BAT thermogenesis after nutrient-induced GLP-1 secretion implies that, at the whole organism level, heat dissipation of nutrients/fat is being limited, probably to favor the uptake of upcoming nutrients by peripheral organs. In other words, GLP-1 may elicit a counter-regulatory mechanism against meal-induced thermogenesis (the supplementary energy cost above basal metabolic rate for the use and storage of nutrient) by decreasing heat generation in the BAT. This idea supports recent findings indicating that diet-induced thermogenesis is limited in the BAT (see (35) for review). This “thrift” mechanism may better distribute the energy source to maintain the overall energy balance between the meals, which could be an evolutionary advantage in an environment where food is scarce. During exposure to a high-caloric diet rich in fat, this mechanism may however serve to promote the positive energy balance which contributes to the development of obesity.

In this study, we demonstrated a new role for endogenous intestinal GLP-1 in the control of energy expenditure via BAT thermogenesis. The clinical relevance of this mechanism in the development of obesity requires further investigation but our data raised the possibility that interfering with the GLP-1 gut-BAT crosstalk can alleviate the development of diet-induced obesity.
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Chapter 5: GLP-1R in vagal afferents, energy expenditure and brown adipose tissue


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SUPPLEMENTS

Supplementary figure 1: Locomotor activity during a 45-min open field test in rats injected with IP Veh or Ex-4 90 min before the start of the test (n = 8/7; Two-way ANOVA group x time; group effect NS, time effect P<0.0001, interaction NS)

Supplementary figure 2: 24 h locomotor activity in freely-moving ad libitum fed control and GLP-1R kd rats (n = 7/8; Two-way ANOVA group x time; group effect NS, time effect P < 0.0001, interaction NS)
Chapter 5: GLP-1R in vagal afferents, energy expenditure and brown adipose tissue

Supplementary figure 3: (A) Changes in 24 h energy intake, (B) average daily meal size, (C) average daily meal duration and (D) average daily meal numbers before and after introduction of HFD (n = 7/8; Sidak-Bonferroni corrected t-tests; * indicates a difference between control and GLP-1R kd rats) (E) HPV plasma levels of GLP-1 in rats maintained on chow after a chow meal or in rats maintained on HFD after an isocaloric HFD meal (n = 6/7/8/8; MANOVA group x meal x time; group effect NS; meal effect P < 0.0001; time effect P < 0.0001; group*diet NS); data of the chow group have been published elsewhere (5)

Supplementary figure 4: (A) Relative mRNA expression the paraventricular nucleus of the hypothalamus of HFD-fed control and GLP-1R kd rats (n = 8/8; Student t-test; NS) (B) Relative mRNA expression of ppg in the NTS of age-matched chow- or HFD-fed control and GLP-1R kd rats (n = 7/7/5/7; Two-way ANOVA group x diet; group effect NS, diet effect P < 0.05; interaction effect P < 0.05; * indicates a significant difference with chow-fed control rats with Bonferroni post-hoc test; HFD levels of ppg expression presented here are the same data presented in fig. 5G, to allow comparison with the age-matched chow-fed control and GLP-1R kd rats)

Abbreviations: AVP, arginine vasopressin; CRH, corticotropin releasing hormone; GLP-1R, Glucagon-like peptide-1; PPG, preproglucagon; TRH, thyrotropin releasing hormone
1 Overview of the main findings

In the manuscripts compiled in this thesis, we contributed to a better understanding of how the gut communicates with the brain to control energy intake and energy expenditure. We specifically investigated the role of the vagus nerve (VN), the main neuronal route of communication between the gut and the brain, in mediating the effects of the glucagon-like peptide-1 (GLP-1), a powerful incretin and satiating peptide.

An initial review of the available literature (CHAPTER 2) revealed several points that guided our subsequent experimental approach:

1. Lesions of the VN suggested a role for vagal afferent neurons (VAN) in mediating GLP-1’s effects on food intake and glycemia. The exact role of VAN GLP-1 receptors (GLP-1R) in mediating the effects of endogenous intestinal GLP-1 was unknown and required a new experimental approach. We set up a bilateral nodose ganglion (NG) injection technique to deliver a lentiviral vector and knock down GLP-1R in VAN via RNA interference. Using this approach, we showed that GLP-1R in VAN are necessary for the normal control of meal size, gastric emptying and post-meal glycemia (CHAPTER 3).

2. Several neuropeptides have been identified in the NG and some of them, i.e., the melanin-concentrating hormone (MCH) and the Cocaine- and Amphetamine-Regulated Transcript (CART) were already implicated in the eating-inhibition induced by cholecystokinin (CCK). Whether neuropeptides were involved in the vagally-mediated effects of GLP-1 was, however, unknown. We screened neuropeptides in the NG for their transcriptional response to GLP-1 in a primary VAN culture model and found that only CART was upregulated. We further showed that GLP-1R activation induces VAN CART expression in vivo. Moreover, using two models of loss of CART function, we demonstrated that a full CART expression in VAN and CART signaling in the NTS are necessary for the full eating and gastric emptying effects induced by intraperitoneal (IP) GLP-1. These results implicate for the first time CART as a neuropeptide involved in the brainstem processing of the GLP-1 signal (CHAPTER 4).
The neuronal targets and the functional consequences of CART release in the NTS by VAN remain to be further investigated.

3. The initial review of the literature did not comment on energy expenditure because the role of the VN in the control of energy expenditure has been unexplored in the field until recently. Moreover, whether peripheral GLP-1 can modulate energy expenditure also remained to be determined, notably because the effects of GLP-1 on eating and gastric emptying were confounding factors. We initially set up a protocol to investigate the effects of peripheral GLP-1R activation on energy expenditure in wild-type rats while controlling for food intake and gastric emptying. We found that peripheral GLP-1R activation induced a transient decrease of whole-body energy expenditure, accompanied by a decrease in interscapular brown adipose tissue (iBAT) temperature. We further tested whether a permanent downregulation of GLP-1R in VAN was associated with changes in energy expenditure. Indeed, during HFD feeding, when endogenous peripheral GLP-1 secretion is stimulated, VAN GLP-1R KD prevented the HFD-induced reduction in energy expenditure presumably by increasing BAT activity during the dark phase. This was sufficient to alleviate the HFD-induced body weight gain and glucose intolerance. Together with a retrograde tracing from the iBAT and central gene expression analysis, we proposed a new vagally-mediated pathway by which endogenous peripheral GLP-1 tunes down iBAT activity after a meal (CHAPTER 5).

Together, these results enhanced our understanding of the gut-to-brain GLP-1 signal. Many questions, derived directly or indirectly from our results, have emerged and remain to be answered. Some of the specific questions are already discussed separately in each chapter. Broader questions, however, will be discussed in the following sections.
2 Established and potential non-vagal targets of intestinal GLP-1 in the control energy balance and glyceremia

The results presented in this thesis clearly showed a role for VA in mediating the effects of endogenous intestinal GLP-1 on both food intake and energy expenditure, which are the key factors in maintaining energy balance. This, however, does not mean that the VN is the only or most important route for the GLP-1 signal. Indeed, GLP-1 acts in a paracrine fashion but also enters the systemic blood and lymph circulation (1). Thus, despite its degradation by DPP-IV in the liver and the systemic circulation, it can have direct effects via the activation of non-vagal peripheral GLP-1R. I will briefly discuss other non-vagal potential mechanisms of GLP-1 effects on parameters implicated in the energy homeostasis.

2.1 Potential satiating effects of circulating GLP-1 via central GLP-1R

It is possible that circulating GLP-1 can bind to central GLP-1R. Indeed, GLP-1 may have access to brain areas outside the blood-brain barrier (BBB) and may also diffuse through the BBB to get access to other brain areas (2). As mentioned in CHAPTER 2, several experiments aimed at mimicking the release of GLP-1 in the circulation (via injection into the hepatic portal vein - HPV) supported the idea that the effect of GLP-1 on food intake may also rely on a direct access to central GLP-1R (3), especially in the area postrema (AP) (4). In support of this, injection of \(^{125}\)I-labeled GLP-1 in the aorta of anesthetized mice was found in the subfornical organ and the AP (5). Pharmacological doses of the fluorescent-labeled GLP-1R agonist liraglutide were shown to access both circumventricular and hypothalamic areas (6). It is then possible that elevations of the GLP-1 levels in the HPV following a meal can exert an eating inhibition by binding directly to GLP-1R in the brain, independently of VAN. The direct activation of central GLP-1R, however, can also be an artifact induced by intravenous (IV) injections of GLP-1 or an exclusive effect of pharmacological doses of GLP-1R agonists and do not necessarily reflect the physiological mechanism by which endogenous intestinal GLP-1 exerts its satiating effect.
Several approaches can be suggested to address the question whether endogenous intestinal GLP-1 activates central GLP-1R in physiological conditions. First, based on quantitative data about meal-induced GLP-1 secretion, degradation by DPP-IV, and penetration through the BBB, a compartmental model can be established to estimate the concentration of endogenous GLP-1 that may reach central GLP-1R (a classic approach in nutrition or toxicity studies to estimate the concentration of a substance in an organ (7)).

More physiologically, GLP-1 can also be fluorescently labeled with a small fluorochrome (such as “VivoTag”) and injected via catheters in the HPV or in the superior mesenteric artery, at a dose and a rate that mimics the meal-induced release of GLP-1, before being visualized in the brain. This approach, however, may present several caveats. First, similarly to the study by Orskov and colleagues (5), it involves an IV injection, which might not replicate the spread of endogenous GLP-1 in other compartments, such as the mesenteric lymph, and raises the question of the adequate dose to simulate meal-induced GLP-1 levels. Moreover, the attachment of a tag to GLP-1 may modify its kinetics, including its penetration through the BBB. Alternatively, in mice with a CNS-specific deletion of ppg expression (that abolishes central GLP-1 production), detecting GLP-1 (or GLP-1/GLP-1R interaction) in the brain would give the indication that endogenous gut GLP-1 can access to central GLP-1R. Eventually, an intestinal-specific modification of the ppg gene can lead to the secretion by the gut of a GLP-1 molecule that differs from the central GLP-1 production (via the insertion/deletion of a few amino acids without affecting GLP-1 kinetics) and the subsequent identification of this modified GLP-1 molecule in the brain by proteomics.

2.2 Direct insulinotropic effects of GLP-1 in pancreatic β-cells

For its glycemic effects, GLP-1 is thought to be in high enough concentration to exert its insulinotropic effect on β-cells via a direct activation of the GLP-1R. The re-expression of the human GLP-1R in the pancreatic islets and ductal cells of Glp-1r -/- mice was sufficient to normalize the glucose and insulin responses to glucose tolerance tests (both oral and IP) (8). This result strongly indicates that GLP-1R on the pancreatic β-cells largely mediate the
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incretin effect of endogenous GLP-1. Our results showed a role for VA GLP-1R in amplifying the early insulin response to a meal. We propose to conceptualize the insulinotropic effect of endogenous intestinal GLP-1 as a collaboration between an early neuroincretin pathway involving a gut-brain-pancreas vago-vagal reflex and an endocrine effect of GLP-1 on pancreatic β-cells. Indeed, because the direct incretin effect of GLP-1 on β-cells is mild at euglycemic levels (9), we can speculate that a neuronal activation of islet cells participates to an early insulin production, before glycemia starts to rise significantly and before the direct incretin effect of GLP-1 on β-cells intensifies. Consequently, this early insulin release can be a sensitization signal for peripheral organs to activate the insulin-sensitive intracellular pathways and thus, efficiently anticipate the elevation in glycemia. Further elevation of blood glucose levels, then, may mainly recruit the direct incretin effect, as suggested by the above-mentioned experiments (8).

2.3 Potential other GLP-1 targets in the control of energy balance and glycemia

Several other GLP-1R populations may play a direct or indirect role in the control of energy balance. GLP-1R expression has been reported in the dorsal root ganglia (DRG) (10): this opens the possibility of another route for the GLP-1 signal to the brain involving spinal afferents. Available studies, however, indicate a functional relevance of the DRG GLP-1R in neuroprotection and pain sensitivity (11,12) rather than in the control of energy balance.

GLP-1R have also been detected in the enteric nervous system (ENS) of mice (10) and primates (13). The clear interaction between GLP-1 and the ENS has not been shown. Some reports, however, indicate that GLP-1 could play a local role on the regulation of gastric emptying by controlling smooth muscle activation via a nitric oxide-dependent pathway (14).

Additionally, GLP-1R are expressed in both subcutaenous and visceral white adipose tissue (ScWAT and vWAT) (15). GLP-1 has been shown to increase lipolytic processes in 3T3-L1 cells (15). Moreover, GLP-1R activation by GLP-1 or liraglutide increased the differentiation and reduced the apoptosis of pre-adipocytes both in vitro and in vivo (16), thus favoring
adipose tissue hyperplasia. Interestingly, morbidly obese women showing hyperplasia in ScWAT and vWAT showed a better glucose, insulin and lipid profiles compared to individuals with adipose hypertrophy (17). Thus, it is possible to speculate that endogenous gut GLP-1 access to GLP-1R on white adipocytes to induce lipolysis and induce the differentiation of new adipocytes, thus favoring adipose tissue hyperplasia. This idea, however, requires further research.

In brief, based on our results and the available literature, we can say that VAN are the key mediator of endogenous peripheral GLP-1’s effects on eating, gastric emptying, post-meal glycemia and energy expenditure. On the other hand, circulating GLP-1 also participates in the insulinotropic effects of GLP-1 in the pancreas and may access central GLP-1R to further control food intake. Our data from the HFD-induced obese rats suggest that this apparent role of VAN GLP-1R cannot be generalized and depends on the diet and the obesity status.

3 A role for vagal afferent neurons in the long-term control of energy balance: potential implication in the development of obesity

As pointed out in CHAPTER 2, lesions of VAN resulted in acute effects on meal size when animals were exposed to standard chow. Compensations in meal frequency, however, prevented chronic changes in daily food intake and body weight, leading to the view that VAN are only involved in the short-term control of food intake (18,19). As mentioned previously, lesions studies are very limited in their interpretation. Based on our and other recent findings, we ask the following questions: a/ How does the exposure to a HFD modulate the apparent function of VAN GLP-1R? b/ Aside from GLP-1R, is there evidence that VAN function is modulated during HFD exposure or obesity?
3.1 The chronic effect of VAN GLP-1R kd in the overall energy balance is revealed by HFD challenge

In **CHAPTER 3**, we found that GLP-1R in VAN are necessary for normal satiation. The daily energy intake, however, was similar between control and VAN GLP-1R kd rats, due to compensations in meal number. Overall, the energy balance and, thus, body weight, was not affected by the kd when rats were fed *ad libitum* with standard chow diet. We therefore concluded that VAN GLP-1R had a role in the meal-to-meal control of food intake rather than in the long-term control of the energy balance. In **CHAPTER 5**, however, we report that the introduction of a high-fat diet (HFD) to VAN GLP-1Rkd rats resulted in unexpected and interesting phenotypes. First, the previously described changes in meal pattern (increased meal size and duration) are no longer present after the introduction of a HFD. More noticeable is a clear disruption in the energy balance: the energy expenditure was increased in the dark phase in VAN GLP-1R kd rats with no changes in food intake, leading to a smaller body weight gain than control rats after 8-9 weeks of HFD exposure. Together, these results suggest that exposure to HFD changed the relevance of VAN GLP-1R effects in the chronic regulation of energy balance. Potential mechanistic explanations of this phenomenon are in part discussed in **CHAPTER 5** and will be further discussed below.

First, the introduction of a 60% HFD may mask the control of meal size by VAN GLP-1R. Indeed, there is a large body of evidence showing that the hedonic processes triggered by the consumption of palatable food can result in caloric intake largely exceeding energy requirements (see (20) for review). It is then plausible that the reward-related consumption of palatable food rapidly overrides the homeostatic effects on meal size induced by VAN GLP-1R activation, thus eliminating the differences in meal size between GLP-1R kd and control rats. In addition, unpublished data of our laboratory (Rouault and Krieger, 2015) suggest that VAN GLP-1R kd affect the consumption of palatable diet but this effect is short-lived. While the mechanisms explaining this observation remain unknown, it would be interesting to find the molecular switch counteracting the normal satiating process during exposure to a HFD.
Moreover, the introduction of HFD in a rodent model causes profound changes in the intestinal secretion of GLP-1. Indeed, we found a 3-fold increase in HPV postprandial levels of GLP-1 in rats exposed to HFD receiving a HFD meal compared to rats fed with chow receiving an isocaloric chow test meal. We can speculate that the high GLP-1 secretion during HFD exposure leads to a more pronounced activation of the VAN GLP-1R, thus amplifying the effects of the kd on energy expenditure and BAT thermogenesis. Moreover, in chow-fed rats, the exogenous administration of Ex-4 was sufficient to decrease energy expenditure and BAT thermogenesis, likely via a vagal mechanism. In summary, these findings support the idea that VAN GLP-1R mediate an effect of endogenous secretion of GLP-1 on energy expenditure and BAT activity that is revealed by a HFD challenge.

The modulatory effects of HFD on the central GLP-1 system may provide another interesting possibility regarding the chronic effects of VAN GLP-1R on energy balance. Indeed, the decrease in NTS *ppg* expression induced by HFD exposure was greatly attenuated in VAN GLP-1R kd rats. Because the central GLP-1 system has been shown to play a role in the HFD-induced hyperphagia and fat accumulation (21), the decreased NTS *ppg* expression would provide another possible explanation by which the VAN GLP-1R kd can exert a chronic influence on the energy balance during HFD exposure. HFD-induced downregulation of NTS *ppg* expression and its inverse relationship with body weight is, however, inconsistent in the literature. Indeed, NTS *ppg* expression was strongly upregulated in obese Zucker rats, independent of the diet (22), and was correlated with fat mass in HFD-exposed Long-Evans rats (21). Moreover, a 2-week exposure to a HFD was sufficient to increase NTS *ppg* expression in mice (23). Whereas NTS PPG neurons express the leptin receptor and *ppg* expression is regulated by leptin in mice, there is no evidence for leptin receptor expression and leptin regulation in rat PPG neurons, highlighting species differences in the central GLP-1 system between rats and mice (24).

In addition, because intestinal GLP-1 secretion is elevated in our model of HFD-fed, the HFD-induced downregulation of NTS *ppg* expression suggests an inverse relationship
between peripheral and central GLP-1 production and b/ that VAN GLP-1R are necessary mediators of the balance between peripheral and central GLP-1. Little is known about the interplay between the central and peripheral GLP-1. As explained in our general introduction (CHAPTER 1), the regulation of NTS PPG neurons by endogenous peripheral GLP-1 is unlikely to happen directly (because NTS PPG neurons do not express the GLP-1R (25)) or via a relay in the AP (because GLP-1R expressing AP neurons are catecholaminergic and NTS PPG neurons do not receive catecholaminergic inputs (26)). Alternatively, PPG neurons may be acutely modulated by VAN GLP-1R activation, because they receive direct monosynaptic glutamatergic inputs from VAN (25). An acute IP Ex-4 injection, however, did not induce c-Fos protein expression in the rat NTS GLP-1-expressing neurons (unpublished observations, Lutz and Vrang). There is, however, indication that pharmacological doses of IP Ex-4 (unpublished observations, Lee SJ, 2015) or the chronic administration of liraglutide reduces the expression of ppg in the NTS (6). These observations are consistent with the idea that a sustained activation of GLP-1R, including peripheral GLP-1R, may lead to a reduction in NTS ppg expression, although peripheral GLP-1 does not acutely activate NTS PPG neurons. A crosstalk between the high intestinal secretion of GLP-1 and the NTS ppg expression in the context of a HFD, as well as the potential mediators of this crosstalk, deserve further investigation. Indeed, interfering with this crosstalk during obesity may maintain NTS ppg expression and reduce hyperphagia and fat accumulation (21).

In addition, it is plausible that the apparent function of VAN changes in an obesogenic context following the modulation of lipid sensing pathways within VAN. Indeed, VAN express nuclear receptors (NR) involved in fat metabolism, and HFD exposure modulates the expression pattern of these NR in mice (27). The loss of these NR have been linked to an increase in the thermogenic response to HFD exposure (27,28). In other words, the exposure to HFD reduces the thermogenic power of VAN via NR signaling. How NR in the VAN detects the presence of fat in the gut and how this translates into metabolic effects is currently unknown. Based on our findings, it is interesting to speculate that VAN GLP-1R
activation may interact with this vagal lipid sensing and thus exerts its effect on energy expenditure. This would explain why only HFD-exposed, but not chow-fed, VAN GLP-1R kd rats showed changes in energy expenditure compared to their controls.

In conclusion, the role of VAN GLP-1R in the regulation of energy homeostasis is probably context-specific. In brief, we can say that, in a physiological context, VAN GLP-1R activation is short-lived and influences the acute post-meal control of eating and glycemia. In an obesogenic context, however, the introduction of palatable food may modulate the apparent role of VAN GLP-1R activation in the regulation of energy balance via the high peripheral GLP-1 secretion, the decreased central ppg expression and the interaction with lipid sensing mechanisms within VAN.

3.2 Evidence for a modulation of vagal afferent function in obesity

Beyond the GLP-1R signaling, the exposure to an obesogenic diet or obesity can modulate vagal afferent function. There is evidence that the satiating effects of gut nutrients and gut peptides are reduced in diet-induced obesity (DIO) (29-31). Moreover, maintenance on a HFD attenuated the NTS response to the intestinal infusion of oleate (32) and to the injection of CCK (33), as shown by a reduction in the number of neurons stained for the activity marker c-Fos. This suggests that the VAN molecular pathways governing satiation are altered during the development of diet-induced obesity. Indeed, maintenance on a HFD decreases mechanosensory properties of gastric VAN (34,35), which may be related to the modulatory effect of ghrelin on gastric VAN sensitivity to distension and contact. Moreover, there is overwhelming evidence that the sensitivity of VAN to CCK, as well as other gut peptides (e.g., bombesin) and serotonin, is reduced in diet-induced obese (DIO) rats and mice (32,36-38). The reduced sensitivity of VA to mechanical signals and intestinal satiating compounds coincides with a reduced excitability of the neuronal cell membrane (35), which may lead to an overall decrease in action potential frequency. Additionally, the development of leptin resistance in VAN has been proposed as a potential mechanism for the decreased sensitivity of VAN to satiating signals. Indeed, VAN develop leptin resistance early in the
onset of obesity, which coincides with the onset of hyperphagia and the reduction of VAN sensitivity to CCK (39). Moreover, conditional knockout of the leptin receptor in Nav1.8-expressing neurons led to an uncompensated increase in meal size, higher body weight and adiposity compared to control mice (40). This showed that the development of leptin resistance in VAN can induce hyperphagia and weight gain. This is of particular interest knowing the characterized interaction between the anorectic effects of leptin and GLP-1. Indeed, IP leptin in rats strongly enhanced the acute eating-inhibition and weight loss induced by IP GLP-1 or Ex-4 in rats (41,42). Moreover, reduced circulating leptin levels induced by fasting (41) or leptin resistance induced by the maintenance on a HFD (31) were both shown to reduce the anorectic effect of IP GLP-1. Hence, knowing the pivotal role of VAN in mediating the GLP-1R-induced satiation, it is possible to speculate that the HFD-induced leptin resistance in VAN drives a reduction of the anorectic effect of endogenous gut GLP-1, thus favoring HFD-induced hyperphagia. Moreover, because leptin potentiates the effects of CCK on CART abundance in VAN (43), it is likely that an interaction between VAN leptin and GLP-1R signaling also happens via the regulation of CART expression.

In addition, the normal plasticity of VAN is lost after chronic exposure to a HFD. Indeed, in physiological situations, the feeding status modulates the sensitivity of VAN to peripheral signals: this is achieved by the modulation of the expression of receptors and neuropeptides involved in satiation (see (44) for review). During chronic HFD exposure, the postprandial rise of expression of Y2 receptors and CART, as well as the downregulation of MCH, its receptor, and the cannabinoid 1 receptor, are blunted (39). Moreover, unpublished results from our lab in collaboration with G. de Lartigue (2015) indicate that knocking down one of the anorexigenic peptides, CART, in VAN, is sufficient to induce hyperphagia and an increase in body weight gain in chow-fed rats. This further strengthens the idea that VAN plasticity is necessary for the mediation of appropriate satiation signals to the brain and, thus, for the long-term control of energy intake.
In summary, these studies confirm that VAN are indeed the relays of short-term satiation signals, but they also indicate that a diet-induced alteration of VAN function can promote the overconsumption of food and the development of an obese phenotype. Together with our findings, this further suggests that VAN play a role in the regulation of energy balance that depends on the diet and the adiposity status.

4 Putative evolution of the endogenous GLP-1 system during the development of obesity and therapeutic implications

Our results (CHAPTER 3) highlighted the protective effects against HFD-induced metabolic impairments conferred by the VAN GLP-1R kd. At first, these results seemed counter-intuitive for us. Indeed, based on the known role of GLP-1 and our previous results (CHAPTERS 3 & 4), interfering with normal satiation during HFD exposure should exacerbate food intake and lead to an increase in body weight gain. Moreover, the pharmacological administration of GLP-1R agonists, although not targeted only at VAN GLP-1R, leads to a reduction in body weight and an improvement in glucose homeostasis in obese patients. Several reports, however, were discordant with this rather simplistic view: indeed, acute systemic blockade of the GLP-1R with Ex-9 decreased food intake in rats exposed to a HFD (31). Moreover, glucose tolerance was improved in mice fed a HFD injected for 4 weeks with Ex-9 (45), suggesting that decreased GLP-1R signaling could be beneficial in some conditions. We will try to reconcile these findings by exploring the idea that a high endogenous intestinal GLP-1 response to HFD exposure can be detrimental during the development of obesity.

4.1 Our model tested the role of VAN GLP-1R in response to an obesogenic diet, not in an established obese phenotype

First, in the experimental model used in CHAPTER 5, the VAN GLP-1R kd was established prior to HFD exposure. This brings an important point to consider: we interfered with the gut-
brain communication during the dynamic phase in which the whole-body metabolism is modulated by the introduction of a HFD rather than in a context of established obesity with several established metabolic impairments. Thus, our model helps us to investigate the role of peripheral endogenous GLP-1 acting on VAN in the development of HFD-induced obesity, but does not answer the question whether VAN GLP-1R can alleviate or worsen an already-established obese phenotype. Knocking down the GLP-1R in VAN of DIO rats would be an interesting experiment to further investigate this point.

4.2 Intestinal GLP-1 secretion is amplified during the development of HFD-induced obesity

It is often reported that fasting and postprandial plasma GLP-1 levels are reduced in obese animals (29,46) and humans (47,48) compared to their lean controls. We, however, have found much higher meal-induced GLP-1 levels in HFD-maintained rats compared to chow-maintained rats receiving an isocaloric amount of their respective diet as a test meal. This apparent discrepancy may be a key to understand the role of endogenous GLP-1 in the pathophysiology of obesity. First, we measured the meal-induced GLP-1 release after 10 weeks of exposure to a HFD. Others have found a similar upregulation of GLP-1 secretion in rodents fed an obesogenic diet for up to 13 weeks (49,50) or in humans briefly exposed to a high-fat diet (51). Together, these data suggest that HFD exposure results in an elevation of endogenous GLP-1 secretion, due to the increase in fat and energy intake for an initial period of at least 13 weeks in rat models (49,50). Fasting and meal-induced GLP-1 levels appear to diminish in obese animals and humans and may be due to the development of leptin resistance in L-cells (46).

Moreover, technical considerations may also have allowed us to highlight a clear difference in meal-induced GLP-1 levels between chow-fed and HFD-fed animals. Indeed, in contrary to other reports (46,49), we consistently sampled HPV blood rather than tail vein blood as a more dynamic readout of GLP-1 secretion, and repeatedly did so in freely-moving animals. In addition, we gave HFD-maintained rats a HFD test meal, i.e. a meal that obviously differs in
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nutrient composition to the chow meal given to chow-maintained rats (although isocaloric) rather than a similar test meal. We argue that this approach is more informative about the endogenous GLP-1 levels at which rats are exposed during HFD feeding.

To summarize, based on our data as well as the literature, it is undisputable that GLP-1 secretion is affected by the introduction of a high fat content in the diet and must have an important function in the development of obesity. The time course of the rise and fall of GLP-1 during the development of obesity, however, needs to be further systematically investigated in the future.

4.3 Putative effects of high intestinal GLP-1 levels on glucose and lipid metabolism

In our model, the repeated elevation of postprandial GLP-1 levels induced by hyperphagia and by the nutrient composition of HFD meals is associated with a/ the tuning down of BAT activity and whole-body energy expenditure and b/ the downregulation of the central GLP-1 system, which may contribute to hyperphagia and fat accumulation (52). In addition, we cannot rule out that an increased GLP-1 secretion during HFD exposure exacerbates the insulinotropic action of GLP-1 (including its neuroincretin component) and, therefore, may have a direct effect on the morphology and gene expression pattern of the adipose tissues. Indeed, the BATIRKO mouse model (BAT-specific deletion of the insulin receptor) showed a reduced BAT cell size, likely due to reduced lipid accretion, and an elevated level of UCP1 compared to controls (53). Together, these results suggest that the initial elevation of intestinal GLP-1 secretion upon HFD exposure can have detrimental consequences on components of energy balance and glucose homeostasis by generating hyperinsulinemia and altering insulin sensitivity.

To test this idea, a chronic, systemic, blockade of peripheral GLP-1R using GLP-1R antagonist can be performed during the onset of diet-induced obesity. To our knowledge, this experiment has not been specifically performed for the purpose of testing this hypothesis. We can, however, learn from other experimental paradigms. Indeed, the Glp-1r KO mice is
protected from HFD-induced insulin resistance and, to some extent, body weight gain (in females) (54). A similar phenotype has been observed in the double-incretin receptor KO (DIRKO) mice (55). Moreover, chronic infusions with exendin-9 (Ex-9) in wild-type mice exposed simultaneously to a HFD for one month block the HFD-increase in fasting glycemia and improve oral glucose tolerance (45). In the same experiment, body weights were unchanged in Ex-9-treated mice compared to their non-treated controls, but energy intake was increased, suggesting an effect of the peripheral GLP-1R activation in the control of energy expenditure. These results would support the hypothesis of a time window during which HFD-induced elevated GLP-1 levels foster a modulation of energy expenditure and glucose homeostasis (see Fig.1).

This idea remains, however, largely speculative and lacks further scientific support. Moreover, the pharmacological use of a GLP-1R antagonist as a way to temper or slow the development of an obese phenotype seems unrealistic. Indeed, obese animal models can be experimentally generated by a diet manipulation, which allows testing the effects of a pharmacological intervention in a controlled manner. In humans, identifying a period in which high GLP-1 levels should be neutralized seems a difficult therapeutic strategy. In this regard, the pharmacological use of GLP-1R agonists when obesity and diabetes are established is an effective strategy to enhance β-cell insulin production and reduce appetite via central GLP-1R (6). Nevertheless, measurements of endogenous GLP-1 levels could informative in the diagnosis of pre-diabetics states. Hence, it is important to understand how GLP-1 can be a contributing factor to fat accumulation in response to an obesogenic diet.

To summarize, our results presented in CHAPTER 5, which may appear counter-intuitive to the conventional GLP-1 functions, shed new light on the contribution of intestinal endogenous GLP-1 secretion to the development of an obese phenotype in a DIO model. These results may suggest a benefit of blocking peripheral GLP-1R during the development of obesity to slow weight gain and impairment of glucose homeostasis, but are by no means
contradictory to the beneficial effects of pharmacological GLP-1R agonists in established obesity.

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Figure 1: Proposed evolution of the secretion of endogenous GLP-1 (peripheral and central) during the development of diet-induced obesity and summary of the phenotypes of VAN GLP-1R kd rats

<table>
<thead>
<tr>
<th>Phenotype of VAN GLP-1R kd vs control</th>
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<tbody>
<tr>
<td>Satiation</td>
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<tr>
<td>Daily energy intake</td>
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<tr>
<td>Gastric emptying</td>
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<tr>
<td>Insulin secretion</td>
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<tr>
<td>BAT activity / Energy exp.</td>
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<td>Body weight</td>
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5 GLP-1 and the vagus nerve, the future of obesity treatment?

The manuscripts in this thesis are part of a broader literature suggesting a role for the VN in the control of energy balance. Hence, the VN can be seen as a therapeutic target against obesity and its comorbidities. In this final section, we will discuss potential therapeutic strategies that derive from our findings as well as current developing approaches targeting the VN.

5.1 New pharmacological ways to access GLP-1 receptors on vagal afferents

First, as mentioned earlier, the currently used GLP-1R agonists are thought to reduce body weight via a direct access to central GLP-1R. In other words, GLP-1R agonists do not require the vagal route to produce the beneficial effects on body weight and appetite reduction (6).
Based on our results showing a role for GLP-1R in VAN to mediate satiation, recruiting the vagal route may have an additional therapeutic advantage. Two possibilities (not exclusive) can explain that GLP-1R agonists do not require an intact vagal route: a/ long-acting GLP-1R gain access to peripheral and central GLP-1 but the effects of central GLP-1R activation is largely predominant or b/ agonists do not gain access to GLP-1R on VAN because they are injected subcutaneously for therapeutic usage. This second possibility leads to the idea of delivering GLP-1 or GLP-1R agonists by an oral route, which would provide them access to VAN in the intestinal mucosa. Orally administered GLP-1 is absorbed by the gut mucosa and significantly elevates circulating GLP-1 levels in humans (56). In addition, oral GLP-1 affects glucose homeostasis after an OGTT and the total meal energy intake in humans (57,58). Moreover, the oral GLP-1R agonist Semaglutide (Novo Nordisk) has recently moved to a phase 3a clinical trial (59), based on previous “encouraging results”. For oral delivery, GLP-1 and GLP-1R agonists must withstand the gastrointestinal milieu and were stabilized with specific proprietary agent (SNAC or Eligen Carrier Technology®). Nevertheless, the oral dose needed to achieve comparable effect to the once-daily injectable semaglutide is likely to be high (in the phase 3 trial, the injected doses to be tested fall between 0.5 to 1 mg, whereas oral doses range between 3 and 14 mg). The design of oral GLP-1R agonist was primarily motivated by the desire to find an alternative to injectable drugs. It will however be interesting to observe whether an oral delivery of GLP-1R agonists, that may recruit the vagal route, have similar effects on body weight and glucose homeostasis to the injectable versions or whether it can, indeed, enhance the effect.

A way to (partly) avoid enzymatic degradation is to have GLP-1 or an agonist secreted by a host directly in the intestine. Indeed, bacteria and yeast have been engineered to secrete GLP-1 and administered by gavage to rodent models of diabetes (60,61). In both cases, the authors reported acute improvements of glucose homeostasis. Hence, using a micro-organism as a vector to deliver GLP-1 in close proximity to their site of endogenous intestinal release may represent another viable therapeutic strategy. Intriguingly, the same strategy...
applied to a commensal bacterial strain engineered to secrete the non-insulinotropic form of GLP-1, GLP-1(1-37), has been shown to reprogram epithelial cells in the intestine to produce insulin (62). This unexpected source of insulin has been shown to reduce hyperglycemia in a rat model of type 1 diabetes. In both cases, however, the therapeutic potential of these approaches in humans might be legally limited by the fact that they involved the ingestion of genetically-modified micro-organisms. Alternatively, the use of certain prebiotics, including non-digestible fermentable carbohydrate, has been shown to reduce appetite and improve glucose tolerance in humans and correlate with increased post-prandial GLP-1 levels in humans (63,64). The mechanisms are partially understood and may rely on the production of short-chain fatty acids by colonic microbes and changes in the colonic microbial ecosystem.

In summary, delivering GLP-1 or an agonist orally or via an intestinal host may provide them access to GLP-1R on VA and have some therapeutic benefits. Whether the effects of these new therapeutic approaches are indeed mediated by VAN, and whether they will bring a distinct therapeutic benefit to the currently-used GLP-1R injectable agonists remains to be explored.

5.2 The promises of neuromodulation approaches

Alternatively, therapeutic approaches that target not only the GLP-1R signaling but the VN itself may be successful to alleviate the development of obesity. Initially, results of vagotomy or subdiaphragmatic deafferentation experiments suggested that blocking the VN signal could acutely impact satiation, but could not affect body weight over the long-term, due to an increase in meal number that compensated for the decrease in meal size. As we pointed out in CHAPTER 2, results of lesion studies are difficult to analyze because most of the lesions affect both VAN and VEN and may mask potential antagonist effects of different vagal fibers. Despite these results, there is recently growing interest in the therapeutic potential of another, rather unspecific, VN-targeting approach, i.e., VN neuromodulation. Neuromodulation techniques can be divided into two apparently antagonists approaches. First, VN stimulation (VNS) involves the implantation of electrodes on the VN to generate
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electrical pulses and, thus, initiate action potentials. Second, VN blockade (VNB) uses implantable electrodes in order to locally disturb the propagation of action potentials in subdiaphragmatic VN branches. Both techniques present several advantages: first, they provide the possibility to switch the device on or off based on the patient’s needs and to individualize the characteristics of the currents applied. Moreover, the implantation of the device is a relatively minor surgery compared to gastric bypass techniques. Finally, VNS is already FDA-approved for the treatment of refractory epilepsy. So, are these VN-targeting techniques the future of anti-obesity strategies, based on the available results in animals and humans?

Initially, diverse VNS techniques produced a reduction in body weight and food intake in dogs (65), pigs (66), rabbits (67,68) and rats (69,70) compared to their unstimulated controls. More interestingly, similar techniques were shown to attenuate the weight gain induced by an obesogenic diet in rats (71-73) and pigs (74). Retrospective studies in patients treated with VNS for epilepsy or depression showed mixed results: two studies indicated that a large proportion of the followed cohort showed weight loss or stable weight (75,76), whereas two other studies found no effects of VNS on body weight (77,78). Interestingly, studies in depressed patients with VNS indicated a change in the emotional response to sweets and reduction in food cravings (79,80). These behavioral changes may correlate with and/or explain the VNS-induced reduction in body weight measured in some studies. To summarize, there is pre-clinical evidence suggesting that VNS can be an interesting therapeutic approach to reduce obesity. The results of large clinical studies targeting obese patients are required to further substantiate this claim.

Interestingly, VNB showed positive results to reduce eating and body weight in advanced clinical trials. Indeed, in a first set of clinical study, VNB led to a sustained reduction in body weight in obese patients that had not achieved satisfactory weight loss with behavioral intervention or weight-loss drugs (81,82). Larger clinical trials confirmed these findings (83-86).
In summary, VN neuromodulation approaches, and especially VNB, have shown clinical potential to reduce body weight in obese patients. Mainly, a reduction in caloric intake has been reported and seems to account for the loss of body weight. An effect of VNS on energy expenditure may also contribute to the shift in energy balance (87). It is, however, difficult to conceptualize that VNB can lead to a reduction in body weight via a decrease in food intake. Indeed, our results and others have shown that VAN carry satiation signals and blocking them via VNB should result in an increase in meal size and hyperphagia. There is evidence, however, that some compounds that stimulate food intake, such as the fatty acid oxidation inhibitor mercaptoacetate, act via an activation of VAN (88,89). Moreover, VNB also blocks the electrical activity of VEN, which probably induces a reduction in gastric motility and secretion, thus reducing nutrient absorption. Eventually, the effects of VNB on food intake and body weights probably are the results of blocking both feeding stimulatory afferent signals as well as inhibiting the efferent control of GI secretion and motility.

Moreover, the reason why VNB, an intermittent blockade of vagal signals, showed a greater therapeutic potential than a permanent disruption of vagal signaling using subdiaphragmatic vagotomy or SDA, is unclear. The chronic and constant application of an electrical current to the VN may increase the risk of VN damage, as shown in dogs (90) and pigs (74), although the risk appears to be limited. Intermittent strategies, however, may be a superior option to prevent the emergence of stimulation-induced neuropathy and the compensatory mechanisms associated with the absence of normal autonomic and sensory nerve functions. Further research should clarify the mechanisms by which VN neuromodulation techniques induce weight loss.

In addition, these neuromodulation techniques brought us to further speculate the cellular and molecular mechanisms underlying the beneficial effects on body weight and appetite control. Our study in CHAPTER 4, among others (see (91) for review), highlighted the role of CART as a vagal neuropeptide required for the effects of GLP-1 on eating and gastric emptying. How neuromodulation techniques affect the expression and release of vagal

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neuropeptides is unknown. Moreover, in the case of diet-induced obesity, a reduced leptin signaling is thought to be responsible for a phenotypic switch of VAN to an orexigenic state (40, 44), with low CART and high MCH expression. If neuromodulation in obese patients restores a normal VAN function, *i.e.* normalizes neuropeptide expression in VAN, has not been investigated. Probably, neuromodulation techniques that primarily trigger or block action potentials in VN neurons may not fully recapitulate the satiation-related cellular and molecular events induced by endogenous vagal chemosensing. A deeper understanding of the role of the neuropeptides in the vagal control of energy balance may lead to the development of new therapeutic approaches or complement existing strategies.

### 5.3 The use of recent neuroscience tools to further understand the role of vagal afferents in the control of energy balance

The research on the VN functions can greatly benefit from newly developed neuroscience tools to manipulate neuronal activity in a cell-specific manner. Indeed, a controlled activation or inhibition of VAN is possible in animal models using optogenetics or DREADD (Designer Receptors Exclusively Activated by Designer Drugs). To our knowledge, optogenetics has recently been used in VAN to test the role of distinct neuronal populations in the control of breathing (with unilateral light activation of VAN in the NG of anesthetized rat) (92). Moreover, the use of the DREADD technique does not require a sophisticated optogenetics setup and may also be useful to investigate the effects of activating or inhibiting a specific VAN population. To my knowledge, so far no studies have used these established neuroscience tools to study VAN function in the context of eating behavior. The development of the bilateral NG injection technique during my thesis yields the possibility to inject viral vectors bilaterally and thus apply these new techniques to the study of VAN functions. Moreover, conditional (*i.e.*, cre-dependent) expression of DREADD-activated receptors can be used to specifically test the effect of a subgroup of VAN, such as those expressing a particular marker or the VAN coming from a particular vagal branch. More recently, these techniques were further utilized to specifically express light-activated receptors such as
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channelrhodopsin or DREADD in neurons previously activated by a drug or an environmental stimulus (using an immediate early gene-driven tagging by tetracycline and conditional expression of the appropriate receptor (93,94)). This allows for the later reactivation of these functionally-defined neurons by light (optogenetics) or CNO (DREADD). Such tools may be helpful to provide the signature pattern of VAN activation during a meal. By manipulating this signature pattern, we may reproduce or block nutrient-induced satiation and further study the contribution of VAN to eating behavior. In summary, many innovative and promising clinical approaches are targeting the GLP-1 system and/or the VN and are on the verge of becoming real therapeutic alternatives to current treatments. Moreover, constant improvements in neuronal mapping and in vivo control of neuronal activity allow to further investigate the potential of VAN in regulating the energy balance.

Figure 2: Overview of current innovative therapeutic and research strategies involving GLP-1 receptors on vagal afferents or neuromodulation of the vagus nerve
Clinically-tested strategies involve neuromodulation of the vagus nerve using stimulation or blockade and oral administration of GLP-1R agonists. Emerging approaches are based on the oral administration of prebiotics to increase the short-chain fatty acid-induced secretion of GLP-1 or the genetic engineering of commensal bacteria to secrete GLP-1. Current research may benefit from the use of cell-specific manipulation of VAN activity via DREADD or optogenetics. DREADD, Designer Receptors Exclusively Activated by Designer Drugs; GLP-1R: glucagon-like peptide-1 receptor; VA: vagal afferents; VN: vagus nerve
In conclusion, I can emphasize that the work presented in this dissertation is of particular interest in the context of the development of new strategies against diabetes and the associated metabolic diseases. Indeed, the development of treatments targeting the gut-brain communication itself (VNS, VNB, ...) requires a deeper understanding of the gut-brain signals both at the functional and molecular levels. By dissecting the role of the VN in mediating the effects of gut endogenous GLP-1 on eating, glycemia and energy expenditure, I believe that this thesis work extends the current knowledge about the role of the gut-brain axis in the regulation of the energy balance. Many important questions, however, arose from this work and remained unanswered. The experimental tools developed during my thesis, as well as the new approaches suggested in this dissertation, will allow to further improve our understanding of the role of VAN in regulating the energy balance.
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