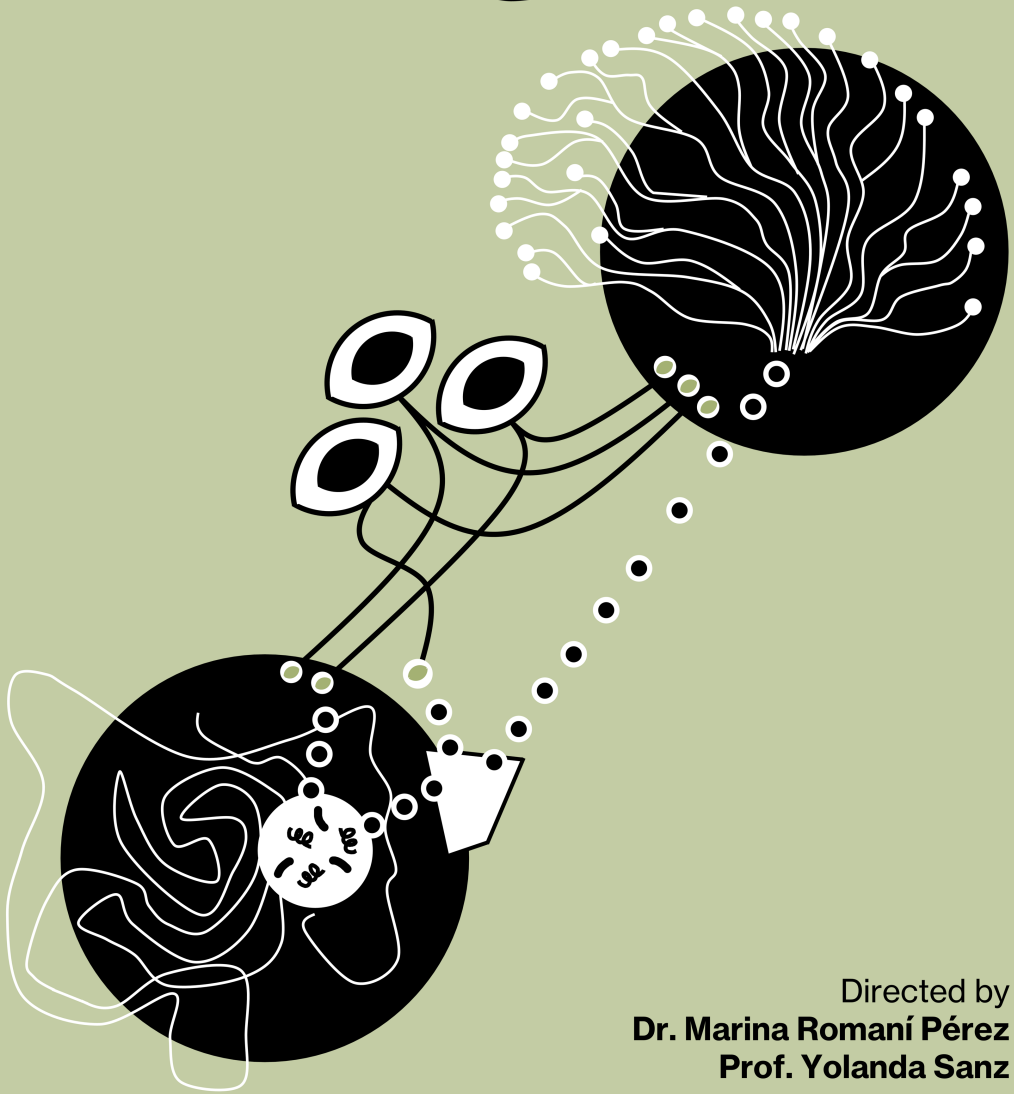


Influence of gut-to-brain neuroendocrine pathways and intestinal microbiota on energy homeostasis

PhD Thesis by

Clara Bullich Vilarrubias



Directed by
Dr. Marina Romaní Pérez
Prof. Yolanda Sanz

Valencia, April 2024



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Clara Bullich Vilarrubias

Co-directed by
Dr. Marina Romaní Pérez
Prof. Yolanda Sanz

Submitted to the Department of Biotechnology of the
Polytechnic University of Valencia (UPV)



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CERTIFICAN

que la Graduada en Biología, Clara Bullich Vilarrubias, ha realizado bajo su dirección el trabajo con título “Influence of gut-to-brain neuroendocrine pathways and intestinal microbiota on energy homeostasis”, y autorizan su presentación para optar al grado de Doctor.

Y para que así conste, expiden y firman el presente certificado en Valencia, en enero de 2024.

Dra. Marina Romaní Pérez

Prof. Yolanda Sanz Herranz



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Contents

Abstract	7
Resumen en castellano.....	9
Resum en valencià	11
Introduction	13
1. Obesity	15
2. Central control of energy balance	18
3. Endocrine and neural intestinal afferent signals controlling energy balance	20
4. Gut microbiota and obesity	31
5. Circadian control of energy homeostasis	37
6. Gut based therapies to combat obesity	41
Objectives	45
Results	49
Chapter 1.....	51
Chapter 2.....	87
Chapter 3.....	133
General Discussion	175
1. Novel functions of Nav1.8-expressing sensory neurons in the control of energy homeostasis.....	178
2. <i>P. faecium</i> DSM 32890 induces anti-obesity effects modulating gastrointestinal functions, such as enteroendocrine signaling, gut transit and lipid absorption	185
Conclusions	189
References.....	193
Abbreviations Table	221

Abstract

Obesity is a major global public health challenge that has reached epidemic proportions. In addition to its profound impact on health and well-being, this metabolic disorder represents a significant economic burden to society. Increasing trends in the prevalence of obesity are fueled by our "westernized" environment, where high-calorie foods are readily available and, along with a sedentary lifestyle, represent a major driver of the chronic imbalance between energy intake and energy expenditure that cause obesity. The limited effectiveness of behavioral interventions in managing long-term weight loss highlights the urgent need to develop more effective and minimally invasive approaches to prevent and treat obesity and its comorbidities. The development of gut-targeted therapeutic strategies to improve metabolic health requires a comprehensive understanding of the gut neuroendocrine signaling pathways that, in interaction with the gut microbiota, control feeding behavior to ultimately maintain energy balance. Therefore, the aim of this thesis has been to gain insight into gut-to-brain interactions, including those mediated by endocrine, neural and gut microbial components, involved in the control of energy homeostasis, with a focus on obesogenic diet-related dysfunctions that increase susceptibility to the development of obesity.

In Chapters 1 and 2, we have investigated novel functions of sensory afferent neurons expressing the sodium channel Nav1.8 in the control of energy homeostasis, taking into account sex-specificities, by generating a mouse model lacking Nav1.8-expressing neurons through a diphtheria toxin ablation strategy. In Chapter 1, we show that Nav1.8+ neurons are required to control neural and endocrine pathways involved in energy homeostasis in a sex-specific manner. Specifically, ablation of Nav1.8+ neurons in females improves postprandial glucose regulation by enhancing glucagon-like peptide-1 (GLP-1) enteroendocrine signalling and accelerating intestinal transit, whereas in males it induces resistance to weight gain in response to an obesogenic diet. To further explore the role of Nav1.8+ neurons in controlling food intake and pre- and post-prandial daily rhythms that influence metabolic phenotype, in Chapter 2 we show in males that ablation of Nav1.8+ sensory neurons impairs the coordinated control of food intake and body weight fluctuations throughout the day. The loss of these neurons also alters the physiological enteroendocrine signaling and daily gut microbiota oscillations in response to the nutritional status (fasting/refeeding cycles), and disrupts intestinal immune homeostasis.

In Chapter 3, we used a diet-induced obese mouse model to investigate the mechanisms by which *Phascolarctobacterium faecium* DSM 32890, a gut bacterial strain isolated from metabolically healthy humans, prevents obesity by modulating food intake. We show that administration of *P. faecium* reduces caloric intake by promoting hypersecretion of a satiating gastrointestinal hormone, the peptide YY (PYY). Independently of its anorexigenic effects, the bacterium exerts its metabolic benefits via complementary mechanisms, specifically by stimulating intestinal transit and reducing intestinal lipid absorption, thereby preventing body fat accumulation.

In conclusion, this doctoral thesis provides preclinical evidence for a better understanding of gut-to-brain neuroendocrine pathways and the role of gut microbiota in the regulation of food intake and energy expenditure. Specially, we highlight the importance of Nav1.8+ sensory afferent neurons in gut chemosensing for maintaining energy balance in both sexes, which prompts novel research lines and opportunities to design of sex-specific neuromodulation tools selectively targeting Nav1.8+ neurons for the prevention and treatment of diet-induced metabolic disorders. We also highlight that *P. faecium* is a promising next-generation probiotic candidate, as it modulates the host enteroendocrine system and prevents obesity in a preclinical model. Overall, our findings contribute to the development of gut-based therapeutic strategies to combat obesity and its associated comorbidities.

Resumen en castellano

La obesidad es un gran reto de salud pública que ha alcanzado proporciones epidémicas. Además del impacto que supone en la salud y el bienestar, este trastorno metabólico representa una importante carga económica para la sociedad. El aumento de la prevalencia de la obesidad se ve impulsada por el entorno "occidentalizado" en el que vivimos, caracterizado por la amplia accesibilidad a alimentos hipercalóricos, junto con un estilo de vida sedentario. Estos factores contribuyen al desequilibrio crónico entre la energía ingerida y el gasto energético que causan la obesidad. Las intervenciones conductuales diseñadas para la pérdida de peso tienen limitada efectividad a largo plazo, por lo que existe una urgente necesidad de desarrollar estrategias más eficaces y seguras para prevenir y tratar la obesidad y sus comorbilidades. El desarrollo de estrategias terapéuticas dirigidas al intestino para mejorar la salud metabólica requiere un conocimiento en profundidad de las vías de señalización neuroendocrina intestinal que regulan el la ingesta y el equilibrio energético, así como su interacción con la microbiota. Así pues, el objetivo de esta tesis ha sido profundizar en las interacciones intestino-cerebro implicadas en el control de la homeostasis energética, incluyendo los componentes endocrinos, neurales y la microbiota intestinal. La tesis se centra especialmente en las disfunciones que aumentan la susceptibilidad al desarrollo de la obesidad inducida por una dieta hipercalórica.

En los Capítulos 1 y 2, hemos explorado nuevas funciones de las neuronas sensoriales aferentes que expresan el canal de sodio Nav1.8 en el control de la homeostasis energética, teniendo en cuenta las diferencias entre sexos. Con ese fin, hemos generado un modelo de ratón que carece de las neuronas que expresan Nav1.8 mediante una estrategia de ablación basada en la toxina diftérica. En el Capítulo 1, hemos demostrado que las neuronas Nav1.8+ son indispensables para regular, de manera específica según el sexo, las vías neurales y endocrinas implicadas en la homeostasis energética. En concreto, la ablación de estas neuronas en hembras mejora la regulación de la glucosa postprandial potenciando la señalización enteroendocrina del péptido similar al glucagón-1 (GLP-1, por sus siglas en inglés) y acelera el tránsito intestinal, mientras que en machos induce resistencia al aumento de peso inducido por una dieta obesogénica. Para profundizar en el papel de las neuronas Nav1.8+ en el control de la ingesta de alimentos y los ritmos diarios pre- y postprandiales que influyen en el fenotipo metabólico, en el Capítulo 2 hemos demostrado que, en machos, la ablación de las neuronas Nav1.8+ altera el control coordinado de la ingesta de alimentos con las variaciones de peso corporal a lo largo del día. Además, la depleción de estas neuronas altera la señalización enteroendocrina y las oscilaciones diarias de la microbiota intestinal en respuesta al estado nutricional (ciclos de ayuno/ingesta), así como también perturba la homeostasis del sistema inmune intestinal.

En el capítulo 3, hemos usado un modelo de ratón con obesidad inducida por la dieta para investigar los mecanismos por los cuales *Phascolarctobacterium faecium* DSM 32890, una cepa bacteriana intestinal aislada de humanos metabólicamente sanos, previene la obesidad

modulando la ingesta de alimentos. Hemos demostrado que la administración de *P. faecium* reduce la ingesta calórica mediante la hipersecreción de una hormona gastrointestinal saciante, el péptido YY (PYY). Independientemente de sus efectos anorexigénicos, la bacteria ejerce sus beneficios metabólicos a través de mecanismos complementarios, concretamente estimulando el tránsito intestinal y reduciendo la absorción intestinal de lípidos, evitando así la acumulación de grasa corporal.

En conclusión, esta tesis doctoral proporciona evidencia preclínica que contribuye a una comprensión más precisa de las vías neuroendocrinas que comunican el intestino y el cerebro, y del papel que tiene la microbiota intestinal en la regulación de la ingesta de alimentos y el gasto energético. En especial, destacamos la importancia de las neuronas sensoriales aferentes Nav1.8+ en la detección de estímulos intestinales por quimiorreceptores (“chemosensing” en inglés) para regular el balance energético en ambos sexos, lo cual abre una nueva línea de investigación y oportunidades para diseñar herramientas basadas en la neuromodulación selectiva de las neuronas Nav1.8+ con el fin de prevenir y tratar los trastornos metabólicos inducidos por la dieta, de forma específica para cada sexo. También destacamos que *P. faecium* es una bacteria candidata como probiótico de nueva generación, ya que es capaz de modular el sistema enteroendocrino del hospedador y prevenir la obesidad en un modelo preclínico. En conjunto, estos hallazgos proporcionan una base para el desarrollo de estrategias terapéuticas basadas en el intestino dirigidas a combatir la obesidad y sus comorbilidades asociadas.

Resum en valencià

L'obesitat és un gran repte de salut pública que ha assolit proporcions epidèmiques. A més de l'impacte que suposa en la salut i el benestar, aquest trastorn metabòlic representa una important càrrega econòmica per a la societat. L'augment de la prevalença de l'obesitat està impulsat pel nostre entorn "occidentalitzat", caracteritzat per l'àmplia accessibilitat a aliments hipercalòrics, conjuntament amb un estil de vida sedentari. Aquests factors contribueixen al desequilibri crònic entre l'energia ingerida i el despesa energètica que causen l'obesitat. Les intervencions conductuals dissenyades per a la pèrdua de pes tenen una eficàcia limitada a llarg termini, per la qual cosa hi ha una necessitat urgent de desenvolupar estratègies més eficaces i segures per a prevenir i tractar l'obesitat i les seues comorbiditats. El desenvolupament d'estratègies terapèutiques dirigides a l'intestí per a millorar la salut metabòlica requereix un coneixement en profunditat de les vies de senyalització neuroendocrina intestinal que regulen la ingesta i l'equilibri energètic, així com la seua interacció amb la microbiota. Així doncs, l'objectiu d'aquesta tesi ha sigut aprofundir en les interaccions intestí-cervell implicades en el control de l'homeòstasi energètica, incloent els components endocrins, neurals i la microbiota intestinal. La tesi se centra especialment en les disfuncions que augmenten la susceptibilitat al desenvolupament de l'obesitat induïda per una dieta hipercalòrica.

En els Capítols 1 i 2, hem explorat noves funcions de les neurones sensorials aferents que expressen el canal de sodi Nav1.8 en el control de l'homeòstasi energètica, tenint en compte les diferències entre sexes. Amb aquesta finalitat, hem generat un model de ratolí que careix de les neurones que expressen Nav1.8 mitjançant una estratègia d'ablació basada en la toxina diftèrica. En el Capítol 1, hem demostrat que les neurones Nav1.8+ són indispensables per a regular, de manera específica segons el sexe, les vies neurals i endocrines implicades en l'homeòstasi energètica. En concret, l'ablació d'aquestes neurones en femelles millora la regulació de la glucosa postprandial potenciant la senyalització enteroendocrina del pèptid similar al glucagó-1 (GLP-1, per les seves inicials en anglès) i accelera el trànsit intestinal, mentre que en mascles induïx resistència a l'augment de pes induït per una dieta obesogènica. Per a aprofundir en el paper de les neurones Nav1.8+ en el control de la ingesta d'aliments i els ritmes diaris pre- i postprandials que influeixen en el fenotip metabòlic, en el Capítol 2 hem demostrat que, en mascles, l'ablació de les neurones Nav1.8+ altera el control coordinat de la ingesta d'aliments amb les variacions de pes corporal al llarg del dia. A més, la depleció d'aquestes neurones altera la senyalització enteroendocrina i les oscil·lacions diàries de la microbiota intestinal en resposta a l'estat nutricional (cicles de dejuni/ingesta), així com també pertorba l'homeòstasi del sistema immunitari intestinal.

En el capítol 3, hem utilitzat un model de ratolí amb obesitat induïda per la dieta per tal d'investigar els mecanismes pels quals *Phascolarctobacterium faecium* DSM 32890, una soca bacteriana intestinal aïllada d'humans metabòlicament sans, prevé l'obesitat modulant la ingesta d'aliments. Hem demostrat que l'administració de *P. faecium* redueix la ingesta calòrica mitjançant la hipersecreció d'una hormona gastrointestinal saciant, el pèptid YY (PYY). Independentment dels seus efectes anorexigènics, el bacteri exerceix els seus beneficis metabòlics a través de mecanismes complementaris, concretament estimulant el trànsit intestinal i reduint l'absorció intestinal de lípids, evitant així l'acumulació de greix corporal.

En conclusió, aquesta tesi doctoral proporciona evidència preclínica que contribueix a una comprensió més precisa de les vies neuroendocrines que comuniquen l'intestí i el cervell, i del paper que té la microbiota intestinal en la regulació de la ingesta d'aliments i la despesa energètica. Especialment, destaquem la importància de les neurones sensorials aferents Nav1.8+ en la detecció d'estímuls intestinals per quimioreceptors ("chemosensing" en anglès) per a regular l'equilibri energètic en ambdós sexes, que obri una nova línia d'investigació i oportunitats per a dissenyar ferramentes basades en la neuromodulació selectiva de les neurones Nav1.8+ amb la finalitat de prevenir i tractar els trastorns metabòlics induïts per la dieta, de forma específica per a cada sexe. També destaquem que *P. faecium* és un bacteri candidat com a probiòtic de nova generació, ja que és capaç de modular el sistema enteroendocrí de l'hoste i prevenir l'obesitat en un model preclínic. En conjunt, aquests troballes proporcionen una base per al desenvolupament d'estratègies terapèutiques basades en l'intestí dirigides a combatre l'obesitat i les seues comorbiditats associades.

Introduction

Introduction

1. Obesity

1.1. Definition of obesity, prevalence and etiology

Obesity represents a major health and societal challenge worldwide that, due to its high prevalence, has already reached epidemic proportions. The prevalence of overweight and obesity in the adult population has nearly tripled from 1975 to 2016 ¹. In 2016, 15% of women and 11% of men were obese, representing a 13% of the world's adult population ², and it is predicted to grow up to 17.5% in 2030, representing one billion people worldwide ³. In the countries belonging to the World Health Organization (WHO) European Region, obesity and overweight is estimated to affect 59% of adults, being more frequent in men (63%) than in women (54%), and the highest prevalence resides in Mediterranean and Eastern European countries ⁴. Specifically, in Spain, the prevalence of obesity in 2020 was near 16.5% and 15.5% in men and women, respectively ⁵.

According to the WHO, the definition of obesity relies on the body mass index (BMI, ≥ 30 kg/m²) criterium ². The public misconception that obesity is a lifestyle choice resulting from insufficient willpower has been gradually challenged over the past 25 years ⁶. In fact, obesity is a chronic disease characterized by a long-term imbalance between energy intake and energy expenditure leading to abnormal or excessive accumulation of adipose tissue and body weight gain ⁶⁻⁸. Obesity is associated with a range of associated non-communicable diseases ², such as type 2 diabetes mellitus, dyslipidemia, cardiovascular diseases, chronic respiratory diseases, cancer, hypertension, or depression. Many of these comorbidities occur more frequently with excess adiposity ⁹⁻¹⁸.

Obesity greatly impairs life quality and reduces life expectancy, highlighting the urgent need to halt this epidemic due to its burden for both the patient and the healthcare system. Measures to address the growing obesity epidemic are included in the ambitious goals of the 2030 Agenda as well as in the Health Cluster of the Horizon Europe Strategic Plan ^{19,20}.

The pathophysiology of obesity and the related metabolic alterations is largely mediated by the adipose tissue. In obesity, there is an aberrant secretion of adipocyte-derived endocrine signals involved in the long-term control of energy homeostasis, such as leptin, leading to leptin resistance. This occurs along with hyperlipidemia and release of pro-inflammatory cytokines from adipocytes and infiltrated macrophages, ultimately leading to chronic low-grade inflammation and insulin resistance ^{21–27}. The identification of leptin as a peripheral endocrine signal targeting the brain to control energy balance ²⁸ was a landmark discovery in endocrine research, prompting further exploration into the new endocrine and neural peripheral signals importantly involved in transmitting energy needs to the brain. Some of these pathways are reviewed in Section 3.

Concerning the etiology, obesity is a complex multifactorial disease involving genetic, epigenetic, and environmental factors, along with socioeconomic aspects determining access to healthcare and healthy nutritional habits. The consumption of high caloric foods stands out as a major environmental factor contributing to the high prevalence of obesity ^{29–33}.

Trends of human dietary patterns worldwide have drastically shifted in the past 50 years towards a Western dietary pattern. A Western diet is characterized by elevated intake of a hypercaloric foods, including ultra-processed and highly palatable foods with a high content in saturated fats and simple sugars, and low in fiber ^{34–36}. These types of foods are relatively cheap and accessible, favoring unhealthy dietary habits that significantly contribute to obesity ³⁵. Numerous investigations support that the Western diet pattern, along with a sedentary lifestyle reducing energy expenditure, is a major driver of the chronic imbalance between caloric intake and caloric expenditure causing obesity ^{2,34,37}.

1.2. Western diet and effects on metabolism

In terms of macronutrient composition, the Western diet is rich in fats and simple sugars, which are considered the main contributors to the metabolic impairments associated with obesity. The impact of these dietary components on metabolism are reviewed below.

Fats

The main long-chain fatty acids (LCFAs) in the Western diet are saturated fatty acids (SFAs), such as myristic, palmitic and stearic acid ³⁸. Palmitate is found in foods of animal origin and in palm oil used in ultra-processed foods ^{39–41}. Several studies report that diets enriched in palmitic acid are associated with increased circulating cholesterol levels, thereby raising the risk of cardiovascular disease ^{42–45}. Furthermore, high levels of dietary palmitate induce insulin resistance. For instance, *in vitro* experiments demonstrated that sustained exposure of cultured muscle cells to palmitate impaired the insulin intracellular cascade, specifically decreased protein kinase B (PKB, or AKT serine/threonine kinase) phosphorylation, resulting in deficient glucose uptake in muscle ⁴⁶. The negative effect of palmitate on insulin signaling has been functionally confirmed in humans. In

particular, the intake of palmitate impaired the postprandial secretion of insulin by pancreatic β -cells and the insulin sensitivity in response to oral glucose ⁴⁷.

Due to the SFAs-associated detrimental effects, the 2020 Dietary Guidelines for Americans recommend replacing dietary SFAs with polyunsaturated fatty acids (PUFAs) fats to reduce the risk of cardiovascular disease ⁴⁸. However, the proportions of each type of PUFA are not specified, which has been questioned given their differential effects on metabolism. PUFAs are classified in omega-3 (n-3) and omega-6 (n-6) PUFAs ⁴⁹. So far, the beneficial effects of n-3 PUFAs in diet-induced obesity are clear. For instance, preclinical studies in high-fat diet-fed mice show that enrichment with n-3 PUFAs prevents body weight gain, adiposity, insulin resistance, liver steatosis and lipogenesis ^{50,51}, and dampens the SFA-induced inflammation in the adipose tissue and the increased gut permeability ⁵⁰. However, increasing the content of dietary n-6 PUFAs maintains a functional intestinal barrier but fails to prevent excessive body weight gain and fat mass, the perigonadal adipose tissue inflammation and the metabolic benefits of n-3 PUFAs ^{50,51}.

In fact, the Western diet is characterized by an excess of n-6 PUFAs and a deficiency of n-3 PUFAs, which has been linked to an increased risk of obesity and metabolic syndrome ^{52–55}. Throughout evolution, the dietary ratio of n-6 to n-3 PUFAs has drastically changed, with modern diets exhibiting ratios as high as 20:1 or even 34:1 in cafeteria diets ^{53,56}, compared to the balanced 1:1 ratio corresponding to the Paleolithic diet ⁵³. This evidence highlights the importance of promoting the presence of n-3 PUFAs while reducing the intake of n-6 PUFAs to improve metabolic health.

Sugars

Excessive intake of simple sugars like fructose or sucrose disrupts both metabolism and brain circuits controlling feeding. Daily consumption of sweet and fatty snacks, enriched with added sugars, fuel a preference for high-fat, high-sugar foods, that leads to exacerbated caloric intake, weight gain and fat accumulation ^{57–61}. Interestingly, both preclinical and clinical research shows that, independently of an elevated caloric intake and body weight, high fructose consumption can directly contribute to metabolic syndrome by promoting hyperlipidemia, fat deposition in liver, skeletal muscle and visceral adipose tissue, pancreatic inflammation and insulin resistance ^{62–65}. Nevertheless, current evidence suggests that sugar may be detrimental for metabolic health when consumed as a surplus of calories, rather than being per se a direct cause of obesity ^{66–68}.

Therefore, in view of the current obesogenic environment, a better understanding of the molecular bases governing food intake and energy expenditure is crucial for identifying effective strategies to improve the energy balance and so, to reduce the prevalence of obesity. In this regard, how the central nervous system (CNS) perceives the whole-body energy needs has become a relevant focus of interest to clinical and preclinical research in obesity. Consequently, the key components involved in these signaling pathways contributing to the chronic positive energy balance, and how they are affected by the consumption of a Western diet and obesity will be detailed henceforth.

2. Central control of energy balance

In mammals, the homeostatic control of energy balance is maintained by adjusting energy intake and energy expenditure. A variety of signals from the periphery, including endocrine and neural signals originated in the adipose tissue, liver, pancreas, or intestine, among other organs, provide the CNS with information relative to fluctuations in the energy status. The CNS coordinates the detection and responses to these cues to ultimately maintain energy balance and body weight by modulating feeding behavior and energy metabolism in peripheral tissues ⁶⁹.

Acute energy indicators such as periprandial signals (before, during and after meals) mediate hunger, satiety and metabolic energy expenditure to regulate short-term energy balance including feeding behavior, energy metabolism, and energy expenditure ^{70–72}. In contrast, chronic signals from hormones positively correlated with body fat content, such as leptin from adipose tissue and insulin from pancreas, transmit a tonic afferent signal to the brain to control energy homeostasis at the long term ^{69,73–75}.

2.1. The homeostatic control of feeding behavior

Feeding behavior results from the brain integration of metabolic, nutritional, hormonal, and neural peripheral signals. In the CNS, two main regions are involved in the homeostatic control of feeding behaviour and body weight: the hypothalamus, that gathers hormonal and nutritional signals from systemic circulation through the blood-brain barrier (BBB), and the caudal brainstem, which also receives afferent neuronal inputs from the periphery, in addition to hormonal and nutritional signals ^{75,76}. The crosstalk between the hypothalamus and brainstem enables assessment of the body energy status and induces appropriate responses according to the energy needs. Moreover, these brain areas interact with reward and motivation pathways influencing food seeking behavior ⁷⁷, which will not be detailed in this Doctoral Thesis.

The homeostatic brain circuits that modulate feeding behavior respond exclusively to cover body's energy needs, so that food intake is stimulated or inhibited when energy stores are depleted or full, respectively ^{78,79}. In the hypothalamus, the arcuate nucleus (ARC) is a key homeostatic area for its location, near the median eminence, where a capillary basement membrane and glial cells, called tanycytes, as part of the BBB, allow dynamic diffusion of peripheral hormones, nutrients and other metabolic signals from circulation ^{76,80,81}. The ARC contains functionally counteracting neuronal populations that, together with brainstem neurons, constitute the melanocortin system, an important neuronal pathway involved in the control of feeding behavior, energy expenditure and autonomic control of energy metabolism ^{70,75}. On one hand, ARC neurons expressing the neuropeptides proopiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) mediate the catabolic pathways of the melanocortin system in response to energy sufficiency and, subsequently, are responsible for suppressing food intake and increasing energy expenditure, resulting in body weight loss. On the other hand, the anabolic arm of this system is governed by neurons expressing neuropeptide Y (NPY) and agouti-related peptide (AgRP) that are activated by energy insufficiency to induce food intake and reduce energy expenditure, ultimately promoting body weight gain ⁷⁰. Within the ARC, the activity of POMC neurons is inhibited

by projections from NPY/AgRP neurons, that in addition to neuropeptides, also release gamma-aminobutyric acid (GABA) ⁷⁵.

Within the hypothalamus, ARC neurons project to the paraventricular nucleus (PVN), where the α -melanocyte stimulating hormone (α MSH), derived from POMC, binds to the melanocortin-4-receptor (MC4R) to inhibit food intake ^{70,75}. Complementary, food intake is stimulated when AgRP, an antagonist of MC4R, binds to this receptor in the PVN ^{70,75}. Besides the projections to the PVN, POMC and AgRP neurons also project to other secondary areas involved in satiety, such as the ventromedial hypothalamus (VMH) and the parabrachial nucleus; or in hunger, such as the lateral hypothalamus (LH) ^{70,82–84}.

In the caudal brainstem, the nucleus of the solitary tract (NTS) and the area postrema, which also display incomplete BBB ⁷⁵, are nuclei where sensory information is integrated to induce meal termination ^{85,86}. The NTS integrates neural (vagal afferents), hormonal and nutritional inputs from periphery via melanocortin system since this area also contains POMC neurons and express MC4Rs ⁸⁵. Both, the hypothalamus and NTS are bidirectionally connected establishing a complex neural circuit to control food intake ^{75,85}.

2.2. Autonomic control of energy metabolism

In addition to control food intake, the hypothalamus and the brainstem integrate energy-related peripheral signals to control energy metabolism through the autonomic nervous system (ANS) ^{87,88}, that comprises the sympathetic and parasympathetic nervous systems innervating the liver, skeletal muscle, pancreas, and adipose tissue ⁸⁷, among others. The sympathetic branch of the ANS stimulates energy expenditure and thermogenesis in brown adipose tissue, which is regulated by ARC and PVN neurons ^{89,90}, and lipolysis in white adipose tissue, which is controlled by the ARC, LH and VMH ^{87,91}. Glucose homeostasis is regulated through ANS innervation of the liver and pancreas. The ARC, VMH and PVN control hepatic glucose production through sympathetic and parasympathetic nerves ^{92–94}, while the ARC, VMH and dorsal vagal complex of the brainstem regulate insulin production in the pancreas through vagal parasympathetic efferent neurons ^{87,95}. Furthermore, gastrointestinal (GI) motility is also governed by ANS, in such a way that the sympathetic nervous system inhibits gut motility, whereas the parasympathetic innervations exerts both inhibitory and excitatory effects on the intestinal muscle ⁹⁶.

Brain circuits involved in the homeostatic control of feeding behavior and in the autonomic control of energy metabolism are affected by the Western diet. For example, within one day after high-fat diet feeding, mice already show reduced levels of hypothalamic POMC, which is maintained in chronic high-fat feeding ⁹⁷. The Western diet disrupts the mechanisms whereby peripheral signals transmit information about energy needs to the CNS ⁷⁸. Indeed, chronic high-fat diet feeding in mice induces a long-term desensitization of AgRP to dietary fat or hormones mediating hunger and satiety, effects that cannot be reversed by weight loss ⁹⁸. Excessive intake of Western diet impairs the afferent sensory signaling from the GI tract to the brain transmitting nutrient sensing, which contributes to the altered feeding behavior and energy metabolism in obesity.

From a clinical perspective, strategies that restore how brain perceives nutrient sensory information from the GI tract contribute to improving the metabolic phenotype. Indeed, the GI tract

has become a target organ for the development of anti-obesity strategies ^{99,100} due to its huge range of afferent pathways transmitting short-term energy signals ^{70–72}, altogether modulated by the interactions of dietary molecules with gut microorganisms ¹⁰¹. The following sections will provide details on the main components of the gut-to-brain axis involved in energy homeostasis, including endocrine and neural pathways, along with key nutritional and gut microbial molecules influencing these pathways, with insights into their implications in obesity.

3. Endocrine and neural intestinal afferent signals controlling energy balance

The GI tract is a huge sensory organ housing enteroendocrine cells (EECs) that, together with the enteric nervous system (ENS) and vagal afferent neurons, constitute an intricate sensory network distributed throughout the GI tract, also called gut connectome ¹⁰². In essence, the gut connectome conveys sensory information to the brain about the ingested food inducing a feedback regulation in the CNS to control food intake and energy homeostasis ^{103–105}.

Scattered along the GI tract, EECs are specialized secretory cells embedded in the GI epithelium that sense the fluctuating composition of the luminal content, nutrient absorption, and epithelial barrier integrity to ultimately regulate food intake, gut motility, digestion, and systemic metabolism ^{103,104}. Gut hormones secreted by EECs can directly act on the brain by entering the systemic circulation (endocrine signaling) or indirectly by contacting sensory neurons in the lamina propria, including the intrinsic neurons of the ENS and the extrinsic neurons of the vagal afferents innervating the GI tract (paracrine signaling) ^{106,107}. Enteric neurons can modulate EECs secretion and vagal afferent neuron activity, besides sensing gut hormones, absorbed nutrients or metabolites ^{108,109}. Interestingly, Kaelberer et al. have identified synaptic connections in EECs, termed neuropod cells, establishing synapses with vagal afferent neurons implying a rapid and direct connection between the intestine and the brainstem ¹⁰⁷.

Importantly, EECs, vagal afferent endings, and enteric neurons show a wide range of chemosensors, commonly membrane G-protein coupled receptors (GPCRs), that bind to nutrients or other ligands such as bile acids (BAs) and gut microbiota-derived molecules such as metabolites and bacterial structural components ¹¹⁰. The gut connectome signaling is crucial to mediate brain sensing of energy-related signals. The molecular aspects involved in this signaling (**Figure 1**) and how it is affected in obesity will be discussed in the following sections of this Introduction.

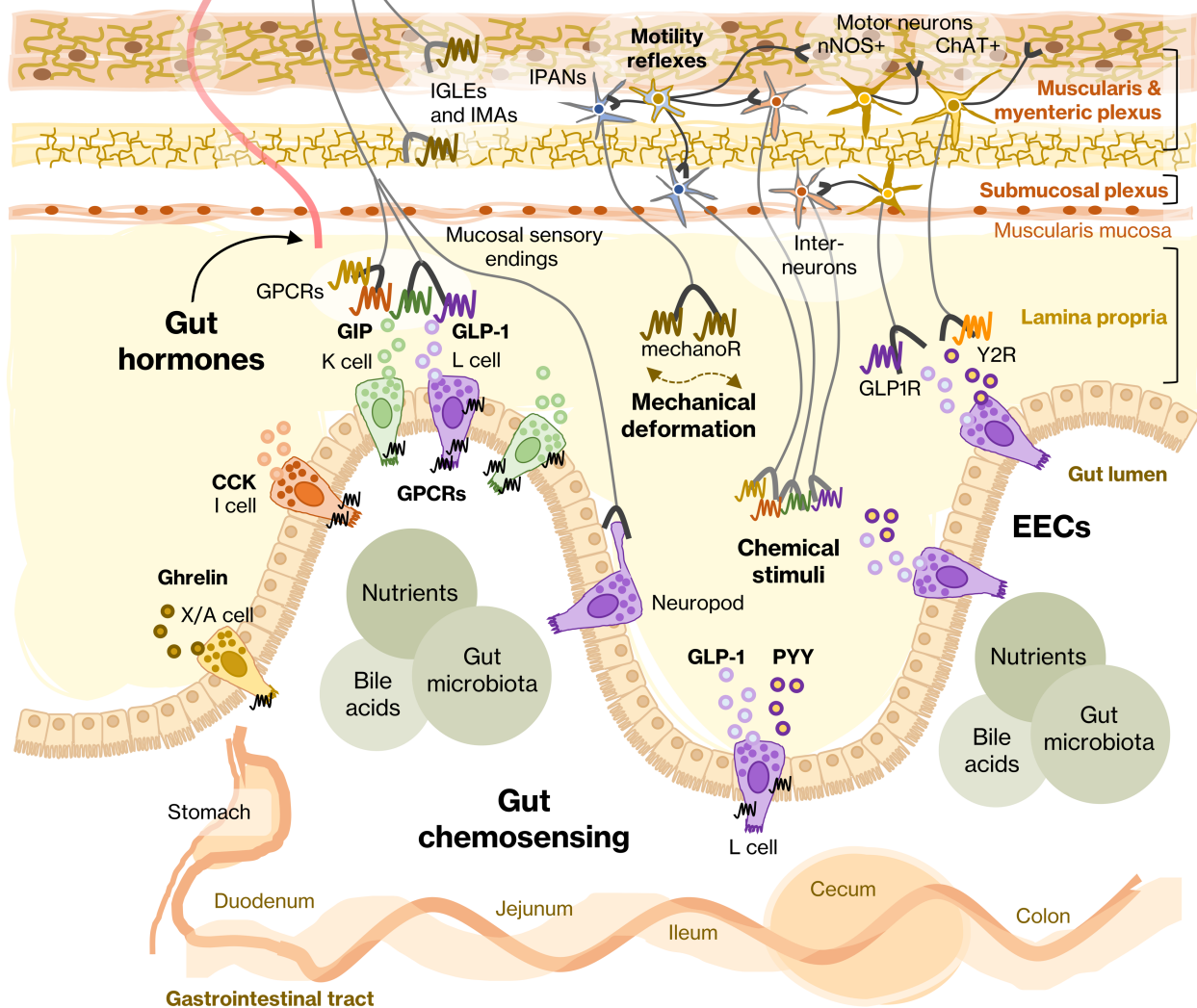
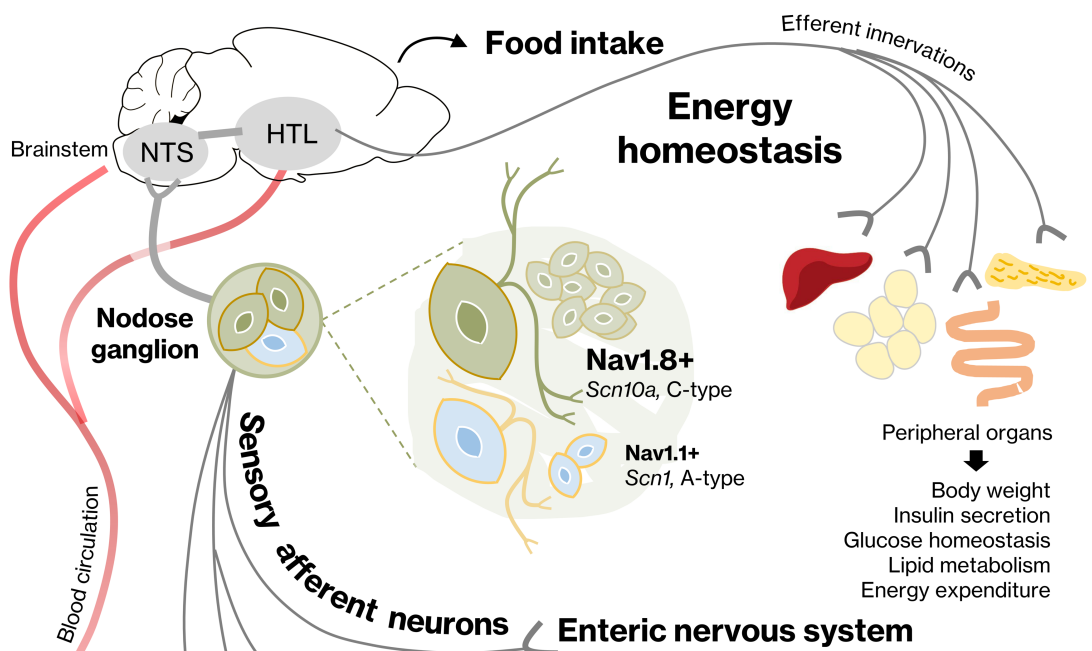


Figure 1. The gut connectome and energy homeostasis. In the gut, the enteroendocrine cells (EECs), sensory afferent neurons and the enteric nervous system form the gut connectome, which transmits sensory information about the ingested nutrients via humoral and neural pathways to the brain, and ultimately controls food intake and energy balance via efferent innervations in peripheral organs. EECs, mucosal sensory endings, and enteric neurons such as interneurons and secretomotor neurons, express chemosensors, commonly GPCRs, that bind to nutrients, to other ligands such as bile acids and gut microbiota-derived molecules, or to GI hormones to collect signals from chemical stimuli in the gut. Sensory afferent neuron terminals from IGLEs and IMAs, and enteric neurons such as IPANs, interneurons and motor neurons, are embedded in the muscularis, myenteric and submucosal plexus, and express mechanosensors to detect mechanical stimuli like gastric stretch and distension. Once activated, these receptors lead to hormone secretion and modulation of neural activity that reach the NTS in the brainstem and the hypothalamus to modulate food intake and energy balance.

Abbreviations: CCK: cholecystokinin; ChAT: choline acetyltransferase; EECs: enteroendocrine cells; GI: gastrointestinal; GIP: glucose-dependent insulintropic polypeptide; GLP-1: glucagon-like peptide-1; GLP1R: GLP-1 receptor; GPCRs: membrane G-protein coupled receptors; HTL: hypothalamus; IGLEs: intraganglionic laminar endings; IMAs: intramuscular arrays; mechanoR: mechanoreceptors; nNOS: nitric oxide synthase; NTS: nucleus of the solitary tract; PYY: peptide tyrosine tyrosine; Y2R: Neuropeptide Y receptor type 2. Figure created with Microsoft Power Point.

3.1. Endocrine components of the gut connectome

Although EECs constitute a minor proportion, around 1%, of the total epithelial cell population, they are considered the primary chemosensory cells in the intestine secreting gut hormones, collectively representing the largest endocrine organ in the body ^{108,111,112}.

At least 12 distinct EECs subtypes producing around 20 different hormones have been identified. Among this diversity, the most studied EECs are those producing ghrelin, cholecystokinin (CCK), peptide tyrosine tyrosine (PYY), or the incretin hormones glucose-dependent insulintropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1). EECs are spatially distributed following a gradient along the proximal-distal GI tract. For instance, X/A cells secreting ghrelin are mainly located in the stomach, while I and K cells secreting CCK and GIP, respectively, are predominantly found in the proximal small intestine. L cells are mainly present in the distal small intestine (ileum) and colon, and release GLP-1 and PYY ^{113,114}.

These gut peptides play a crucial role in the short-term regulation of feeding. During fasting (preprandial state), efferent sympathetic signaling from the brainstem induce the secretion of ghrelin ^{115,116}, a potent orexigenic hormone, producing feelings of hunger inducing food intake. After a meal (postprandial state), ghrelin levels fall rapidly, and the presence of nutrients in the intestine stimulates the release of anorexigenic hormones such as CCK, GIP, GLP-1 and PYY, which promote satiety. These hormones coordinate food digestion and absorption, as well as ensure that absorbed nutrients are adequately assimilated and stored in peripheral tissues ¹⁰⁴.

Soon after food intake, GIP is released mainly in the duodenum to promote fat storage in adipocytes ¹¹⁷. Together with GLP-1, which is also produced in the proximal small intestine by L cells, these two gut peptides respond to intestinal glucose absorption to promote insulin secretion, known as incretin effect, to ultimately reduce glycemia after a meal ^{118,119}. CCK is secreted in the upper GI tract, especially in response to fat intake to control meal size ^{105,120–122}. Besides stimulating gallbladder contraction and facilitating pancreatic enzyme secretion ¹²³, CCK mediates satiety signaling through vagal afferent neurons innervating the upper GI tract ^{124,125}.

L cells in the distal GI tract acutely secrete GLP-1 and PYY in response to nutrients ¹²⁶. The postprandial increase of GLP-1 and PYY occurs rapidly after initiation of feeding, within 15-30 min ^{127,128}, much before nutrients reach the luminal L cell surface in distal regions of the intestine, probably due to a neuroendocrine reflex regulation. For instance, GLP-1 release from L cells in the distal intestine is stimulated by parasympathetic vagal efferents induced by GLP-1 signaling arising from the proximal intestine ¹²⁹. GLP-1 secretion from distal intestinal regions can also be stimulated by GIP and CCK actions on L cells ^{130,131}, and PYY secretion can be induced by CCK-mediated mechanisms ^{132,133}. GLP-1 and PYY are also released tonically at basal levels during fasting, since L cells in the colon are constantly exposed to non-absorbed nutrients and microbial derived products after feeding ¹³⁴.

GLP-1 not only has a potent glucoregulatory role by inducing glucose-dependent insulin secretion and by reducing glucagon secretion, but also slows gastric emptying and reduces food intake ¹³⁵. GLP-1 acts on GLP-1 receptor (GLP1R), predominantly expressed in pancreatic β cells, where it exerts its insulinotropic effects. GLP1R is also present in the hypothalamus ¹³⁶, where it induces satiety and regulates body weight, as demonstrated experiments in rodents receiving an intracerebroventricular injection of the GLP1R agonist exendin-4 ^{137,138} or the GLP1R antagonist exendin-(9-39) ¹³⁹.

Upon food intake PYY is secreted to suppress food intake and to promotes weight loss through its interactions with Neuropeptide Y receptor type 2 (Y2R) expressed in the ARC ^{140–143} and through activation of vagal afferent neurons by binding to Y2R expressed in the nodose ganglion neurons ¹⁴⁴. Besides its potent anorexigenic effects, PYY decelerates gastric emptying and reduces GI transit ¹³².

3.2. Neural components of the gut connectome

The neural components of the gut connectome include extrinsic afferent innervations and intrinsic neural plexuses of the ENS. In communication with the CNS and/or autonomously, they modulate processes involved in energy balance, such as nutrient digestion and absorption, hunger and satiety, or GI motility and secretion ^{96,145}.

Extrinsic afferent innervations of the GI tract: the afferent arm of the vagal nerve

Anatomy of the vagal nerve

The vagal nerve (X cranial nerve) is critical for the autonomic control of many physiological processes ¹⁴⁶. Vagal efferents consist of parasympathetic neurons originating from the brainstem ^{147,148} that innervate organs like the GI tract to control intestinal contractility, secretion, and immune functions ^{149–152}. Vagal afferents, specialized in sensing and transmitting sensory information, are pseudobipolar neurons with cell bodies in the nodose and jugular ganglia ¹⁵³ that send bidirectional projections to the CNS and the periphery ¹⁵³. Towards the CNS, vagal afferents send inputs to the brainstem and hypothalamus to coordinate gut functions ¹⁵³. Towards the GI tract, they sense mechanical and chemical cues in response to food intake. Notably, vagal afferent fibres outnumber efferent fibres by approximately 9:1 ¹⁵⁴.

Methodologies to dissect the function of intestinal vagal afferent neurons

Research in vagal afferents, that densely innervate the gut, primarily in the upper intestine and gradually decreasing along the GI tract¹⁵⁵, has focused on understanding the role of these neurons in feeding behavior. Progress has been made by using surgical, chemical and molecular methods to selectively target different subtypes of vagal afferents and elucidate their functions¹⁵⁶.

For decades, vagotomy has been used in rats to define their importance in mediating CCK-induced satiety^{157,158} and glucose homeostasis^{159–162}, although this methodology does not distinguish between afferent and efferent nerves, leading to confounding effects. Furthermore, vagal afferent desensitization with capsaicin lacks specificity for vagal afferent subtypes^{163,164}. Recent molecular tools offer improved cellular specificity, such as neural tracing with viral-based tools for vagal afferent projections mapping^{156,165}. Cre-LoxP-based strategies, a genetic engineering strategy based on the use of the Cre recombinase to manipulate DNA in living organisms, have enabled both neural tracing and *in vivo* modulation of neural activity through optogenetic and chemogenetic tools^{156,165–170}.

Classification and function of vagal afferents

Based on their electrophysiological properties, 20% of the nodose ganglia neurons are A-type neurons with myelinated axons, which transmit strong and short electrical impulses for rapid neurotransmission, while the 80% are C-type neurons with unmyelinated axons, which conduct weaker but sustained action potentials^{153,171,172}.

Based on their gut-related sensing properties, GI vagal afferents are typically classified into mechanosensitive and chemosensitive types¹⁵⁵. During food intake, intraganglionic laminar endings (IGLEs) and intramuscular arrays (IMAs), that are located in the myenteric plexus, are specialized in mechanosensitive processes and sense gut distention from the stomach and the proximal intestine to induce meal termination^{153,155,173–175}. Mucosal afferent endings, embedded in the lamina propria near the basal side of EECs in duodenum and jejunum, mediate chemosensitive processes and respond to nutrient-induced hormone release through receptors (R) like ghrelin receptor (GHSR), CCK receptor (CCK1R), GLP1R and NPY2R^{112,147,153,175–179}.

Intestinal vagal afferent neurons are key mediators of the gut-to-brain communication to regulate feeding and energy balance at the short and long term¹⁴⁵. The role of vagal afferents in short-term satiety signaling, particularly in mediating the anorexigenic effects of CCK, has been studied for decades. Abdominal and subdiaphragmatic vagotomy in rats reduced the satiating effects of intraperitoneally administered CCK^{157,158}. Moreover, bilateral injection of CCK conjugated with the neurotoxin saporin into the nodose ganglia to selectively target CCKR-expressing afferents¹⁸⁰ increased meal frequency and blunted meal termination following lipid intake¹⁸¹.

The effects of endogenous PYY in appetite suppression and meal patterning through vagal afferent Y2R signaling have been recently demonstrated by molecular-based tools. Y2R knockdown mice were generated by bilateral injection of an adeno-associated viral construct expressing Cre into the nodose ganglia of mice with floxed Y2R¹⁷⁸. Specifically, these mice showed faster, smaller, and more frequent meals, along with accelerated gastric emptying¹⁷⁸. Similarly, the vagal afferent-mediated effects of GLP-1 signaling have been shown in a GLP1R knockdown animal model, generated by injecting in the nodose ganglia of wild-type rats a lentiviral

vector expressing a short hairpin (sh)RNA to silence the GLP1R gene ¹⁶². These rats showed increased meal size, accelerated gastric emptying, and upregulated postprandial glycemia along with blunted insulin secretion ¹⁶².

Although less studied, the vagal afferents mediate orexigenic signaling. By using chemogenetic and optogenetic tools, Chen et al. have recently described an orexigenic vagal-NTS pathway that stimulates feeding through transient activation of afferent nodose ganglion neurons and NTS neurons coexpressing epinephrine and NPY ¹⁶⁶.

The role of vagal afferents in long-term control of energy homeostasis has been shown through a vagal afferent-specific knockdown of leptin receptor (LepR) in mice, which showed disrupted postprandial afferent signaling ultimately leading to hyperphagia, increased body weight and adiposity ¹⁸².

Recent advances in the molecular phenotyping of vagal sensory neurons: a focus on Nav1.8 expressing neurons

The use of genetic tools has evidenced certain inaccuracy in the traditional classification of vagal afferents based on the presence of mechanoreceptors or chemoreceptors. Recently, Kupari et al. have generated a molecular atlas of vagal sensory neurons in the mouse using single-cell RNA sequencing, demonstrating that most subtypes of vagal afferents express both types of receptors ¹⁸³. These findings reveal the complex molecular heterogeneity of vagal afferents and provide a new perspective on the intricate afferent sensory processing. Specifically, the authors reported a diversity of 24 vagal afferent neuron subtypes of which 6 subtypes, identified in the jugular ganglion, were somatosensory and nociceptive neurons similar to the dorsal root ganglion (DRG) spinal neurons, and 18 subtypes, detected in the nodose ganglion, showing a different molecular profile ¹⁸³.

Further molecular classification of afferent neuron subtypes in the nodose ganglion was based on myelinated or unmyelinated neurons, enriched with the voltage-gated sodium channels genes *Scn1* or *Scn10a*, respectively ¹⁸³. In the GI tract, the authors highlight that the neuron subtype enriched in *Scn10a*, coding for the sodium channel Nav1.8, innervated the mucosa and the muscularis layer. Contrary to the traditional view, they identified mucosa subtypes enriched with receptors responding to noxious stimuli, while muscularis subtypes showed gut hormone receptors ^{183,184}.

Typically, the tetrodotoxin-resistant sodium channel Nav1.8 has been described in spinal sensory neurons of the DRG involved in pain sensing ¹⁸⁵. Specific deletion of the Nav1.8 gene in these neurons was initially achieved by driving the expression of Cre recombinase, which catalyzes a site-specific recombination of DNA flanked by loxP (locus of crossover x P1) sites, under the control of the Nav1.8 promoter, generating a heterozygous Nav1.8-Cre mouse line ¹⁶⁹. By crossing these mice with homozygous mice carrying floxed diphtheria toxin fragment A (DTA), Abrahamsen et al. ablated all Nav1.8-expressing neurons and demonstrated the essential role of these sensory neurons in pain sensing to mechanical, cold, and inflammatory stimuli ¹⁸⁶. This strategy based on Cre-mediated expression of DTA for *in vivo* genetic ablation has been described in detail by Ivanova et al. in other cell types ¹⁸⁷. In any case, this mouse breeding approach to specifically

study the function of Nav1.8 sensory neurons has been used in other studies including this thesis, and it is described below and represented in **Figure 2**.

With years, the versatility of Cre-LoxP-based strategies has enabled the tracing neurons expressing Nav1.8 in peripheral organs, demonstrating their vagal, as well as spinal, nature and suggesting their involvement in transmitting visceral information. In this regard, Serlin et al. used Nav1.8-Cre mice crossed with tdTomato reporter mice to restrict expression of this fluorescent protein in all Nav1.8+ afferent neurons and demonstrated by abdominal vagotomy in these mice that the afferent neurons innervating the GI mucosa were vagal in origin ¹⁸⁸. Using the same mouse model, Gautron et al. visualized the innervations Nav1.8 neurons expressing tdTomato in several viscera. Results revealed expression in the gallbladder, liver and pancreas, highlighting a dense Nav1.8+ innervation in the intestinal mucosa and myenteric plexus, including IGLs ¹⁶⁵. The fact that mechanoreceptors in IGLs can suppress food intake by inhibiting orexigenic AgRP neurons in the hypothalamus ¹⁸⁹ suggest that Nav1.8+ GI afferents may play a role in the regulation of feeding. In addition, vagal Nav1.8-expressing afferents express GPCRs involved in nutrient sensing. Specifically, through quantitative PCR, RNA sequencing and *in situ* hybridization in the nodose ganglia of control versus Nav1.8-ablated mice, Egerod et al. demonstrated the expression of gut hormone receptors in the mucosa, such as the neurotensin receptor NTS1R, and in the muscularis layer, such as NPY2R, CCK1R and GLP1R ¹⁹⁰. In addition, lipid immunomodulatory receptors such as the cannabinoid receptor 1 (CB1R), cysteinyl leukotriene receptor 2 (CYSLTR2) and prostaglandin E receptor 4 (PTGER4), that bind to the endocannabinoids anandamide (AEA) and 2-arachidonoylglycerol (2-AG), leukotrienes, and prostaglandin E2, respectively, were also identified in vagal Nav1.8-expressing afferents in both mucosa and muscularis ¹⁹⁰.

The exploration of the functions of Nav1.8+ neurons on energy homeostasis using animal models reveals contradictory results. For instance, Alonso et al. evidenced that mice with selective deletion of Y2R in Nav1.8 sensory neurons showed blunted food intake suppression upon the exogenous administration of PYY₃₋₃₆, at dose similar to the physiological PYY release, demonstrating that PYY anorexigenic signaling requires Nav1.8 sensory neurons ¹⁷⁸. Moreover, a study using mice lacking the lipid sensing nuclear receptor LXR α/β (liver X receptor alpha and beta) in Nav1.8+ neurons demonstrated that these neurons are required for physiological adaptations to high-fat diets, by limiting energy expenditure and weight loss ¹⁹¹. In contrast to these results, a study using the Cre/DTA-induced Nav1.8+ ablated mouse model indicates a modest function of these neurons in the control food intake, body weight or energy expenditure ¹⁹². Rather than in the control of energy balance, this study identifies a key role of these neurons in limiting the acute inflammation triggered by the intake of high-fat diet ¹⁹². Spinal and vagal Nav1.8+ neurons innervating the intestine crosstalk with mucosal immune cells populations and gut microbiota to mediate protective responses against pathogens ¹⁹³. Moreover, the role of Nav1.8-expressing neurons in mediating immune functions has been suggested in other tissues. For example, in the lung, Talbot et al. showed that Nav1.8+ nociceptive neurons enhance adaptive immune responses during allergic inflammation ¹⁹⁴. Using Nav1.8-Cre/DTA mice, they showed that IL-5 from activated effector immune cells acts directly on Nav1.8 neurons innervating the airways, leading to VIP release. Subsequently, VIP stimulates group 2 innate lymphoid cells (ILC2)

and T helper 2 (Th2), thus amplifying a proinflammatory signaling loop that contributes to allergic inflammatory hyperresponsiveness in the lung ¹⁹⁴.

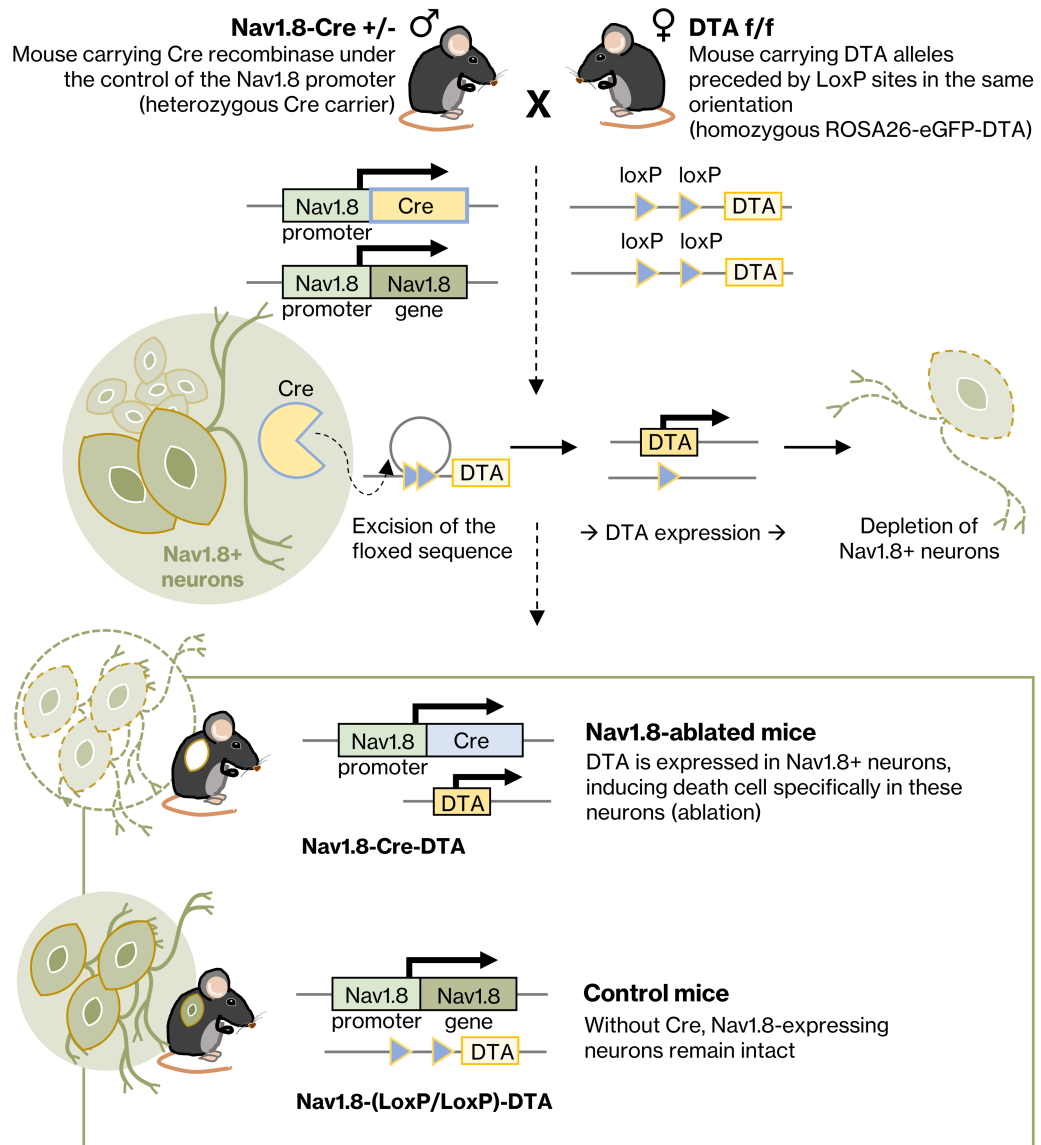


Figure 2. Generation of mice lacking Nav1.8-expressing neurons using a diphtheria toxin ablation strategy. Transgenic heterozygous male mice carrying Cre recombinase under the control of the Nav1.8 promoter are mated with homozygous female mice carrying the DTA gene at the ROSA26 locus, where DTA expression is prevented by a stop cassette (LoxP sites). This mating scheme results in an offspring that expresses Cre recombinase, which excises the floxed region flanking DTA, allowing for the expression of the toxin that ultimately induces cell death. This results in the specific ablation of Nav1.8+ neurons. Offspring that inherit the wild-type allele lack the Cre recombinase gene and thus retain Nav1.8-expressing neurons and serve as control mice. Abbreviations: DTA: diphtheria toxin fragment A; loxP: locus of crossover x P1. Figure created with Microsoft PowerPoint.

Overall, the identification of the role of Nav1.8-expressing sensory neurons in the control of energy balance is still an emerging field of research and its potential as a therapeutic target for obesity remains to be elucidated.

Intrinsic innervations of the GI tract: the enteric nervous system

The ENS is a complex local neural network embedded in the intestinal wall which, independently of the CNS, regulates local digestive processes such as gut motility and secretion¹⁰³. The ENS is composed by intrinsic primary afferent neurons (IPANs), motor neurons, and interneurons¹⁹⁵. IPANs are sensory neurons located in the myenteric and submucosal plexuses, forming interconnected networks that detect mechanical deformation and chemical stimuli from the musculature and intestinal lumen. Motor neurons innervate the muscle surrounding the intestine and EECs and are divided into muscle motor and secretomotor neurons. These neurons are excitatory or inhibitory, the first are mainly cholinergic, expressing choline acetyltransferase (ChAT), and the latter are mainly nitrergic, expressing nitric oxide synthase (nNOS), which produce acetylcholine and nitric oxide (NO) as neurotransmitters, respectively. Last, interneurons, which are located in the myenteric plexus, are involved in local motility reflexes through cholinergic and NO neurotransmission. They establish connections with IPANs and motor neurons^{112,148,195}.

Besides its local effects on the gut, the ENS interacts with vagal efferent and afferent neurons^{149,196}, allowing its contribution to the central control of energy homeostasis. For instance, preclinical studies suggest a mechanism whereby duodenal contractility is sensed by the CNS via vagal afferents to ultimately control glucose utilization by peripheral tissues^{197,198}. Specifically, it is suggested that after a meal, nNOS-expressing neurons reduce duodenal contractility allowing NO hypothalamic signaling, which in turn facilitates glucose uptake by muscle, liver and adipose tissue^{197,198}. This signaling has clinical relevance since, as experiments in mice have demonstrated, diabetes is associated with duodenal hypermotility, which exacerbates intestinal glucose absorption, hyperglycemia and insulin resistance¹⁹⁹. Thus, molecules inducing duodenal relaxation could help designing anti-diabetic therapeutic approaches.

Under healthy conditions, enteric neuronal cell bodies are also able to respond to gut hormones. For example, enteric neurons positive for nNOS colocalize with intestinal expression of GLP1R^{200,201}. At a functional level, enteric neurons from transgenic mice expressing fluorescent *Glp1r* driven by Cre-recombinase show enhanced neural activity upon exposition to GLP-1²⁰⁰. Moreover, intraperitoneal injection of PYY inhibits colonic motor function through Y2R, which expression was detected in nerve terminals around enteric neurons suggesting PYY-mediated inhibition of cholinergic neurotransmission in myenteric neurons²⁰². Altogether, these findings suggest a direct action of GLP-1 and PYY on the ENS to modulate gut motility.

3.3. Gut connectome in obesity

Enteroendocrine cell function in obesity

Dietary changes have a rapid effect on EECs functionality. Within one day of switching to a high fat diet, EECs, like other intestinal cell types, experience cellular stress²⁰³. Further on, the number of secretory cells is reduced, as stem and progenitor cell populations switch from a secretory to an absorptive transcriptomic program in response to the increased fat content²⁰³. The secretory

profile of EECs is subsequently affected, impairing the short-term meal-related gut hormone signaling, which, together with disturbed long-term peripheral energy indicators, such as leptin resistance, contributes to energy imbalance ²⁰⁴. For instance, in humans with obesity, ghrelin levels are lower ^{205–207}, probably as a compensatory mechanism to the positive energy balance. In response to lipids, diet-induced obese rats show decreased expression of CCK and PYY in response to intragastric lipid infusion compared to diet-induced obese resistant rats. Similarly, obese humans and mice display attenuated secretion of endogenous PYY after a lipid load or after a meal ^{142,208}. In response to glucose, diabetic mice show impaired glucose sensing by EECs, as evidenced by decreased secretion of GLP-1 and GIP coupled with attenuated activation of vagal afferent neurons ²⁰⁹. However, other studies in rodents and humans with obesity and/or diabetes show inconsistent results in postprandial GLP-1 secretory responses ^{208,210–213}, and warrant further investigation both at the level of EEC secretory function and its associated neural sensory signaling. Taken together, these findings highlight an impaired peripheral satiety signaling in obesity based on altered hormone secretion, ultimately leading to hyperphagia ²¹⁴.

Sensory afferent-mediated signaling in obesity

Nutrient sensing is attenuated in vagal afferents upon chronic consumption of a high-fat diet, as duodenal infusion of oleate in diet-induced obese rats reduced the neuronal activation marker cFos in the NTS and in the small intestine ²¹⁵. Moreover, studies in rodents confirm that chronic high-fat diet feeding induces loss of plasticity of pre- and postprandial vagal afferent signaling, leading to hyperphagia, body weight gain and obesity ^{145,216}. In fact, the expression profile of vagal afferents experiences a shift towards an orexigenic signaling profile under high fat diet. For instance, in the nodose ganglion of obesity-prone, but not obesity-resistant rats, high-fat diet upregulates expression of orexigenic-related receptors, such as GHSR ²¹⁷. In addition, mice under a high-fat diet show reduced CCK1R expression levels in the nodose ganglion and decreased CCK sensitivity, translated into impaired postprandial responses to intragastric macronutrient loads ²¹⁸.

Chronic high calorie feeding affects vagal afferent signaling not only in the short-term but also in the long-term control of feeding behavior and body weight management. Through its receptor, leptin modulates the sensitivity of vagal afferents to meal-related signals by potentiating CCK-induced stimulation of these neurons to induce satiety ¹⁸². However, deletion of LepR in mice under high-fat diet feeding show disrupted postprandial afferent signaling, leading to hyperphagia and obesity ¹⁸².

Enteric nervous system in obesity

To date, limited research has examined the effects of nutrients and metabolites on the ENS. However, emerging evidence suggests that the ENS, along with EECs and vagal afferents, plays a critical role in coordinating responses to gut-related signals ^{219–221}. Obesity is often associated with motility disorders such as constipation, diarrhea and dysphagia ^{222–224}. In obesity, altered GI motility affects different parts of the GI tract, either accelerated in the small intestine, or delayed, typically in the colon ^{222,225,226}.

As the ENS senses chemical stimuli of the gut lumen and responds with adaptive changes to maintain intestinal homeostasis, a phenomenon known as enteric neuroplasticity ²²⁰, the effects

of the Western diet on enteric neurons are increasingly being studied. In male and female mice, feeding a high-fat diet slows gut motility associated with loss of nNOS myenteric neurons and NO neurotransmission²²⁷, as in nNOS knockout mice the propulsion velocity of boluses in the intestine is slowed down^{228,229}. Specifically, in female mice intestinal dysmotility preceded the development of glucose intolerance, insulin resistance and obesity²²⁷, which also occurs in humans, where enteric neuropathy and motility disorders have been shown to precede diabetes²³⁰. However, the underlying mechanisms by which the ENS is affected by the Western diet deserve further investigation. To date, based on the growing recognition of the ENS in metabolic disorders, Knauf et al. have recently coined the term “enterosynes” to define intestinal bioactive molecules of variable nature, including neurotransmitters and hormones, nutrients, microbial compounds, and immune factors, that can modulate the function of specific enteric neuron subtypes with potential therapeutic functions in obesity and related metabolic disorders^{219,231}.

3.4. Nutrient chemosensing

Intestinal nutrient sensing is the ability of intestinal cells to detect and respond to nutrient fluctuations²³². The endocrine and extrinsic neural components of the gut connectome sense molecules derived from the macronutrient digestion in the intestinal lumen (nutrient stimuli) through chemosensors or nutrient sensors, which are mainly membrane GPCRs. Upon binding to specific nutrient ligands, GPCRs trigger chemosensation (intestinal nutrient sensing) that controls gut-to-brain neuroendocrine signaling involved in food intake and energy balance. Proteins, carbohydrates, lipids, and their derived metabolites induce hormone secretion, thereby triggering physiological responses from the gut.

Lipids. Fat ingestion has a strong satiety effect and inhibits gastric emptying by triggering secretion of the postprandial hormones CCK, GLP-1, GIP and PYY^{108,233–236}. The main GPCRs responding to free fatty acids are GPR40 (FFAR1) and GPR120, which are stimulated by medium- and long-chain fatty acids (MCFA and LCFA, respectively)^{237,238}, and are enriched in EECs expressing ghrelin, GIP, CCK, GLP-1, and PYY^{239–242}. Functionally, PUFAs improve glucose metabolism through GLP-1 secretion and prevents obesity in high-fat fed mice²⁴³. In addition, LCFA and MCFA can be directly sensed by vagal afferent neurons via GPR40²⁴⁴.

Carbohydrates. As mentioned above, carbohydrate ingestion induces a strong incretin effect through the release of GIP and GLP-1 from K and L cells, respectively¹⁰³. Carbohydrate sensing is based on several glucose sensing mechanisms, the best known of which is the sodium-glucose cotransporter type 1 (SGLT1). After a meal rich in carbohydrates or free sugars, SGLT1 expressed on K and L cells induces membrane depolarization with the subsequent secretion of GIP or GLP-1, respectively^{245–247}. Glucose can also be sensed by the sweet taste receptors TAS1R2-TAS1R3, although the mechanisms of action stimulating GLP-1 secretion remain to be established^{134,248}.

Proteins. Protein ingestion is sensed by GPCRs such as the calcium-sensing receptor (CaSR), GPRC6A, metabotropic glutamate receptors (mGluR1 and mGluR4), GPR142 and umami taste receptor T1R1/T1R3^{134,249,250}. CaSR is expressed in I and L cells, among other EECs, triggering CCK and GLP-1 secretion, respectively^{134,251,252}.

Bile acids. Although bile acids (BAs) are not nutrients, their secretion is paired with food intake since they facilitate digestion and absorption of dietary fats ²⁵³. Luminal BAs modulate GLP-1 and PYY release from L cells ^{254–256} by signaling through the nuclear receptor farnesoid X (FXR) receptors and the basolateral G-protein-coupled bile acid receptor 1, also called Takeda G-protein-coupled receptor 5 (TGR5) ^{257,258}. TGR5 is also expressed in inhibitory motor neurons of the myenteric plexus probably inducing a deceleration of gastric emptying ²⁵⁹.

4. Gut microbiota and obesity

The human GI tract harbors trillions of microorganisms which constitute a complex and dynamic ecosystem ²⁶⁰. The gut microbiota provides protection against pathogens and contributes to maintaining host metabolic and immune homeostasis ²⁶¹. By modulating gene expression involved in host nutrient metabolic pathways, the gut microbiota influences energy metabolism, including adiposity and glucose homeostasis ^{262–264}. Importantly, diet is recognized as a critical factor in shaping the microbial communities ²⁶⁵. The effects of diet on the gut microbiota composition and function are reviewed in this section and summarized in **Figure 3**.

4.1. Gut microbial signatures associated with Western diet and obesity

Intestinal dysbiosis, defined as dysfunctional changes in gut microbiota ²⁶⁶, may disrupt the intestinal barrier and the gut-associated lymphoid tissue ²⁶⁷, compromising protection against pathogens and other harmful agents ²⁶⁸. In obesity, dysbiosis exacerbates the chronic low-grade inflammation, leading to insulin resistance ^{269–271}, and affects intestinal lipid absorption and metabolism, and the production and secretion of GI peptides involved in food intake control, altogether leading to increased adiposity ^{272,273}. Research has long sought to identify specific microbial signatures associated with metabolic disorders. Obesity has been associated with a higher Bacillota/Bacteroidota (formerly named Firmicutes/Bacteroidetes) ratio compared to normal weight humans, reduced diversity and/or increased abundances of Proteobacteria and reduced of *Bifidobacterium*, among others ^{262,274–276}. Although there is no consensus on which microbiota signatures are unequivocally associated with obesity, it is clear that the Western diet induces substantial changes in gut microbial communities, but these can vary between individuals due to the high interindividual variability of their microbiota and other variables such as the diet. For example, the diversity of fatty acids of the diet that reach the gut could differently affect the gut microbiota and host metabolism differently between individuals ²⁷⁴.

The gut microbiota adapts to changes in dietary lipid intake, adjusting to contexts of under- or over-nutrition by modulating lipid digestion and absorption ²⁷⁷. In mice, switching to a high-fat high-sugar diet (HFHSD) leads to significant changes in the gut microbiota structure, metabolic pathways, and gene expression within one day ²⁷⁸. Fecal microbiota transplantation from humans on a Western diet transmits the obese phenotype in mice ²⁷⁸. Similarly, cultured gut microbiota from adult twins discordant for obesity transmit the body weight, adiposity, and other metabolic traits to mice ²⁷⁹, overall revealing prompt effects of diet-microbiota interactions. In this regard, germ-free mice, which are resistant to diet-induced obesity, show increased lipid excretion in feces

²⁸⁰, and antibiotic-treated rats show reduced lipid absorption ²⁸¹, highlighting the impact of the gut microbiota on intestinal lipid processing. Obese rats fed a cafeteria diet show increased levels of circulating saturated fatty acids due to gut dysbiosis ⁵⁶, which is influenced by the amount and type of fatty acids consumed ²⁷⁴. For example, a palmitate-enriched diet reduces microbial diversity and richness in mice, which is associated with increased permeability, endotoxemia and inflammation in the colon and liver ²⁸². Conversely, dietary supplementation with n-3 PUFAs reverses the dysbiosis associated with type 2 diabetes or obesity, and reduces inflammation in rodents ^{283,284}.

For these reasons, the gut microbiome is increasingly recognized as a critical modulator of energy homeostasis. Recently, Corbin et al. tested a microbiome enhancer diet, designed to deliver nutrient substrates such as fiber and minimally processed products to the gut microbiome to quantify the contribution of the gut microbiome to human's energy balance ²⁸⁵. Compared to a Western diet, the microbiome enhancer diet led to decreased adiposity and increased energy output through feces, resulting in less metabolizable energy for the host. In addition, the microbiome enhancer diet increased microbial biomass, beta diversity, fermentation products and GLP-1 secretion, indicating its impact on the enteroendocrine system ²⁸⁵.

4.2. Chemosensing of gut microbiota stimuli by the gut connectome

The gut microbiota generates a vast array of metabolites from carbohydrates, lipids, and proteins from diet ²⁸⁶. Besides nutrient-related molecules, microbial ligands also activate chemosensing in EECs, vagal afferents, and enteric neurons by binding to specific receptors, mainly GPCRs, to modulate neuroendocrine gut-to-brain pathways thus affecting energy balance ^{110,286}. In addition, via humoral pathways, microbial ligands reach other organs, including the brain. These microbial ligands include metabolites such as short chain fatty acids (SCFAs), microbial-derived LCFAs, secondary BAs, amino acid-derived metabolites, tryptophan metabolites such as indole, and bacterial components such as lipopolysaccharide (LPS), muramyl dipeptide (MDP), and caseinolytic peptidase B (ClpB), among others ¹¹⁰. Some of these bacterial ligands and their interaction with the gut connectome are described henceforth.

Bacterial ligands sensed by chemosensors of the gut connectome

Short-chain fatty acids

SCFAs, such as acetate, propionate and butyrate as the major ones, are produced by anaerobic bacterial fermentation of non-digestible fibers in the colon, having beneficial effects on energy metabolism by binding to GPR41 (FFAR3) and GPR43 (FFAR2) ^{279,287,288}. By increasing GLP-1 and PYY secretion, SCFAs lead to improved glucose homeostasis and insulin sensitivity, appetite suppression, reduced body weight gain, and increased fat oxidation and energy expenditure in humans and rodents with obesity ^{289–296}. Acetate is produced by the genera *Prevotella*, *Ruminococcus*, *Bifidobacterium*, *Bacteroides*, *Clostridium* and *Akkermansia*, among others ²⁹⁷. *Prevotella*, *Bacteroides*, *Akkermansia muciniphila* and *Coproccoccus* are propionate producers ^{298–300}, and species from *Roseburia*, *Eubacterium* and *Coproccoccus* are butyrate producers ²⁹⁹.

Figure 3. The interaction of gut microbiota with the gut connectome in health and obesity.

Under a healthy diet, the gut microbiota interacts with the gut connectome in response to the presence of nutrients in the gut lumen by producing metabolites, such as BCAAs, SCFAs, secondary BAs, LCFAs, or directly through their subcellular components, such as LPS and MDP. These microbial products interact with GPCRs or microbial-associated molecular pattern receptors of the immune system expressed in EECs and/or vagal afferent neurons and enteric neurons, inducing hormonal and neural signaling to the brain and modulating intestinal motility by interacting with nNOS- and ChAT-expressing neurons, all contributing to the whole-body energy balance. Consumption of a high-calorie, low-fibre diet induces dysfunctional changes in the gut microbiota (gut dysbiosis), which compromises the integrity of the intestinal epithelial barrier, which becomes permeable to LPS (leaky gut), inducing inflammation and metabolic endotoxemia. Overgrowth of certain bacteria promotes ER stress in EECs, leading to EEC silencing, and oxidative stress in nNOS-expressing neurons, leading to dysmotility. All of these changes affect lipid absorption and the secretion of GI peptides involved in the control of food intake, glucose homeostasis and lipid metabolism, resulting in impaired energy balance.

Abbreviations: BCAA: branched-chain amino acid; CCK: cholecystokinin; ChAT: choline acetyltransferase; ClpB: caseinolytic peptidase B; EEC: enteroendocrine cell; ER: endoplasmatic reticulum; GI: gastrointestinal; GLP-1: glucagon-like peptide-1; GLP1R: GLP-1 receptor; HTL: hypothalamus; LCFA: long-chain fatty acid; LPS: lipopolysaccharide; LTA: lipoteichoic acid; MDP: muramyl dipeptide; nNOS: nitric oxide synthase; NO: nitric oxide; NOD2: nucleotide-binding oligomerization domain-containing protein 2; NTS: nucleus of the solitary tract; ox: oxidative; PYY: peptide tyrosine tyrosine; sBA: secondary bile acid; SCFA: short-chain fatty acid; T1R1/T1R3: umami taste receptors; TLR4: Toll-like receptor 4. Figure created with Microsoft Power Point.

Long-chain fatty acids and endocannabinoid-like fatty acids

Although less studied, microbial-derived LCFAs are increasingly described for their major impact on the host's energy balance through the lipid sensors GPR40 and GPR120. The microbial production of LCFAs is essential for membrane biosynthesis and utilized as bacterial energy source³⁰¹, as well as for quorum sensing and as anti-virulence agents against enteric pathogens^{302,303}. For instance, *Lactobacillus plantarum* converts linoleic acid, an essential n-6 PUFA, to 10-hydroxy-cis-12-octadecenoic acid³⁰⁴, which prevents diet-induced obesity in mice through GPR40- and GPR120-mediated GLP-1 secretion³⁰⁵. It has been postulated that gut bacteria produce endocannabinoid-like lipid metabolites such as oleoylethanolamide (OEA) from oleic acid³⁰⁶, which binds to the fat sensor GPR119 in L cells^{307–309} and triggers GLP-1 and PYY secretion to regulate glucose homeostasis and food intake^{310–313}.

On a Western diet, the gut microbiota adjusts its lipid metabolism to handle the excess of lipids in the colon. In mice fed a high-fat diet, the commensal bacterium *Fusimonas intestini* overexpresses the gene *fadR*, which favors fatty acid biosynthesis, and genes involved in the production of the trans-fatty acid elaidate, thus contributing to impaired energy balance³¹⁴. Species of the genera *Prevotella*, *Lactobacillus* and *Alistipes* express *Fab* genes, indispensable for producing saturated LCFAs, and are positively correlated with the abundance of heptadecanoic and stearic acids in the rat colon, causing gut dysmotility³¹⁵.

Secondary bile acids

The gut microbiota contributes to the modulation of host lipid metabolism by deconjugating primary BAs, such as cholic acid (CA) and chenodeoxycholic acid (CDCA), to the secondary BAs deoxycholic acid (DCA) and lithocholic acid (LCA), respectively, using bacterial bile salt hydrolases (BSH)³¹⁶. By binding to FXR and TGR5, the resulting BA pool modulates energy balance^{317–319}. In mice, gut dysbiosis induced by a high-fat diet feeding alters the BA composition and affects energy metabolism³²⁰. For example, high-fat diet-induced dysbiosis in mice caused a reduction in CDCA, LCA and ursodeoxycholate (UDCA), which was positively correlated with the abundance

of *Clostridium scindens*, and a downregulated expression of GLP-1 in ileum and the thermogenic marker UCP1 in brown adipose tissue, all contributing to obesity³²¹. In contrast, absorbed BAs enhance TGR5-mediated GLP-1 and PYY secretion, improving the metabolic phenotype and protecting against obesity^{254,322,323}.

Branched-chain amino acids

Valine, leucine and isoleucine are dietary branched-chain amino acids (BCAAs) that can also be produced de novo from glucose by bacteria such as *Akkermansia muciniphila*^{324–326}. The role of BCAAs in metabolism is still controversial, with some studies associating increased BCAA levels with insulin resistance and obesity^{325,327–329}, while others with beneficial effects in diet-induced obese mice^{330–332}. Although the satiating effects of BCAAs remain to be investigated, Genton et al. reported that BCAA supplementation increased circulating PYY levels in patients with chronic kidney disease³³³, a common complication of type 2 diabetes³³⁴. *In vitro*, BCAAs stimulate CCK secretion through activation of T1R1/T1R3 in a porcine jejunum model, suggesting an anorexigenic effect of BCAAs³³⁵.

Bacterial subcellular components

Structural components of bacterial cell walls, such as LPS of Gram-negative bacteria and MDP of both Gram-positive and Gram-negative bacteria, reduce food intake via sickness-associated immune pathways^{336,337}. LPS and MDP bind to two microbial-associated molecular pattern receptors of the immune system, the toll-like receptor 4 (TLR4) and the nucleotide-binding oligomerization domain-containing protein 2 (NOD2), respectively, and modulate GLP-1 signaling^{338–340}. In addition, the bacterial heat shock protein ClpB, an αMSH mimetic protein produced by *Escherichia coli* and *Hafnia alvei*, promotes satiety, possibly by enhancing PYY secretion^{341–346}.

Interaction between the gut microbiota and the gut connectome components

Crosstalk with enteroendocrine cells

A growing body of evidence reveals the molecular mechanisms by which the gut microbiota influences the functionality of EECs, thereby affecting host metabolism^{101,110,347}. For instance, SCFAs elicit EECs generation from stem cells in mouse and human intestinal organoids, contributing to increased L cell abundance³⁴⁸. BAs can also induce L cell differentiation through interaction with the TGR5, expressed in the basolateral membrane of EECs³⁴⁹. Other studies demonstrate the pivotal role of gut bacteria in the gene expression, functionality, and region-specificity of EECs throughout the intestine³⁵⁰. Specifically, in germ-free mice transplanted with the gut microbiota of conventionally raised mice, the transcriptome kinetics of ileal L-cells is rapidly altered, associated with increased intracellular GLP-1 content³⁵¹. Furthermore, conventionalization with the commensal gut bacterium *Bacteriodes thetaiotaomicron* helps normalize EEC numbers and intestinal localization specificity, as well as circulating GLP-1 levels^{352,353}.

High-fat diet-induced alterations in the gut microbiota disrupt nutrient sensing in EECs, known as “EEC silencing”³⁵⁴. In this study, Ye et al. showed that feeding zebrafish with a high-fat diet induced intestinal overgrowth of *Acinetobacter* sp. This may explain the endoplasmic reticulum stress they observed in EECs, which was associated to the retraction of EEC apical processes and ultimately to a reduced sensitivity to nutrients³⁵⁴. Collectively, these findings suggest

mechanisms by which the gut microbiota contributes to the secretory component of the gut connectome, subsequently affecting the chemosensing to modulate energy homeostasis.

Crosstalk with sensory afferent neurons

A recent study conducted in mice demonstrated the expression of GPR41, GPR119, GPR35, and CaSR in vagal Nav1.8-expressing afferents innervating the GI tract ¹⁹⁰. Preclinical experiments demonstrate that propionate elicits anorexigenic effects through a mechanism dependent on GPR41 expressed in vagal afferent neurons ³⁵⁵. Likewise, activation of GPR119 by OEA is associated with satiating effects mediated through peripheral sensory afferents ³⁵⁶. Activation of GPR35, a receptor for kynurenic acid and other amino acidic metabolites enriched in Nav1.8 afferent neurons ¹⁹⁰, increases satiety signaling and reduces food intake ³⁵⁷. Interestingly, administration of the commensal bacterial strain *Holdemanella biformis* CECT 9752 to diet-induced obese mice improved glucose homeostasis and increased GLP-1 secretion coupled to upregulated expression of intestinal GLP1R ³⁵⁸, which has been detected in vagal afferents ¹⁹⁰. Electrophysiological recordings in cultured nodose ganglion neurons showed that, upon incubation with *H. biformis*, these neurons increased GLP-1 sensitivity ³⁵⁸, suggesting a sensory afferent-mediated mechanism for the glucoregulatory effects of this bacterial strain.

Crosstalk with the enteric nervous system

The gut microbiota regulates the ENS and gut motility. Administration of *Lactobacillus rhamnosus* GG increases ChAT expression in myenteric neurons through bacterial adhesion and redox mechanisms, enhancing GI transit ³⁵⁹. Similarly, conventionalization of SPF mice with *Bacteroides thetaiotaomicron* restores ChAT and nNOS expression in enteric neurons, promoting colonic motility, possibly mediated by LPS binding to TLR2 expressed in enteric cells ³⁶⁰. Through the ENS, the gut microbiota also influences the central control of glucose homeostasis. Experiments in mice show that LPS and MDP, via TLR4, NOD2 and CD14 (acting as TLR co-receptor), increase the sensitivity to GLP-1 in nNOS enteric neurons. Thus, in addition to the subsequent inhibition of gastric emptying, this signaling pathway favors glucose regulation. However, dysbiosis induced by a high-fat diet disrupts these pathways, resulting in GLP-1 resistance and contributing to the development of diabetes ³⁴⁰.

Indeed, diet-induced dysbiosis leads to dysregulation of the GI transit ³⁶¹. In mice, delayed intestinal motility and the loss of nNOS myenteric neurons induced by high-fat diet feeding have been correlated with decreased abundance of *Bifidobacterium*, *Lactobacillus* and *Clostridium* ²²⁷, and increased abundance of Gram-positive bacteria ³⁶². In fact, Nyavor et al. have shown that lipoteichoic acid from Gram-positive bacteria, known to induce oxidative stress in neurons ³⁶³, reduce the number of nitrergic myenteric neurons ³⁶². Increased intestinal LPS also induces delayed motility and enteric neuropathy in mice fed a high-fat diet ^{362,364,365} by activating pro-inflammatory pathways via TLR4, as TLR4 knockout mice fed a high-fat diet show normal GI transit and myenteric neuron density ^{364,365}. Nevertheless, the mechanisms underlying the crosstalk of gut microbiota and the ENS deserve further exploration.

5. Circadian control of energy homeostasis

Circadian rhythms are 24-hour patterns of many physiological processes regulated by cyclic environmental cues that act as entrainment signals, known as “zeitgebers” (from German, “time givers”) ³⁶⁶. Light is the principal stimulus that synchronizes the suprachiasmatic nucleus (SCN) in the hypothalamus, the central pacemaker of the circadian timing system ^{367,368}. Besides, peripheral metabolic tissues express autonomous clocks, which are entrained by food intake to maintain the diurnal metabolic homeostasis. Mistimed eating can reset circadian rhythms in peripheral organs and brain, but not in the SCN, meaning that food intake, independently of light, and so, of SCN, governs circadian patterns complementary to the central clock ³⁶⁹.

The secondary food clock synchronizes food intake in phase with environmental cycles. Therefore, the time of most food availability (light phase in humans and dark phase in rodents) corresponds to the active phase when most feeding occurs. Likewise, the circadian phase corresponding to fasting or reduced feeding states is the resting phase (dark and light phase in humans and rodents, respectively) ^{369,370}. This temporal organization coordinates feeding patterns with related metabolic routes (anabolic or catabolic) governed by anorexigenic signals after meals during the active phase and orexigenic signals at the end of the resting phase. Given that meal timing is a potent zeitgeber for peripheral organs ³⁷¹ and the ingested food first contacts the GI tract, the intestine is recognized as a crucial peripheral clock for the circadian control of metabolism ³⁷². The regulation of energy homeostasis in response to circadian rhythms of gut related signals is represented in **Figure 4**.

5.1. Circadian rhythms in enteroendocrine secretion

Gut hormones follow circadian patterns to anticipate feeding or fasting phases to optimize the effects of enteroendocrine signaling on energy metabolism ³⁶⁶. For instance, in rats, the levels of ghrelin in circulation peak rapidly before the onset of the dark phase and drop immediately after, indicating the start of the feeding phase ³⁷³. Postprandial GLP-1 and insulin secretion patterns also follow a circadian rhythm. In rats, just before the onset of the feeding phase, GLP-1 peaks to stimulate insulin secretion ³⁷⁴ and, independent of dark/light cues, the response of these hormones to oral glucose is greater at the end of the fasting phase, probably to increase pancreatic β -cell sensitivity to feeding-induced GLP-1 ³⁷⁴. Similarly, in humans, the faster postprandial secretion of the incretins GLP-1 and GIP in the morning is coupled to the rapid insulin responses ^{375,376}. In mice, basal levels of PYY also vary throughout the day, reaching the lowest point at the beginning of the dark phase ³⁷⁷. Maroni et al. showed that, in light-phase fasted mice, exogenous PYY administration was much more effective in suppressing food intake compared to dark-phase fasted mice ³⁷⁷.

In light of this evidence, the presence of independent peripheral clocks in EECs has been indicated in rodents ³⁷⁴. Although a recent study in *Drosophila* reported that EECs may be clock-deficient ³⁷⁸, it remains to be elucidated whether these results extend to other animal models.

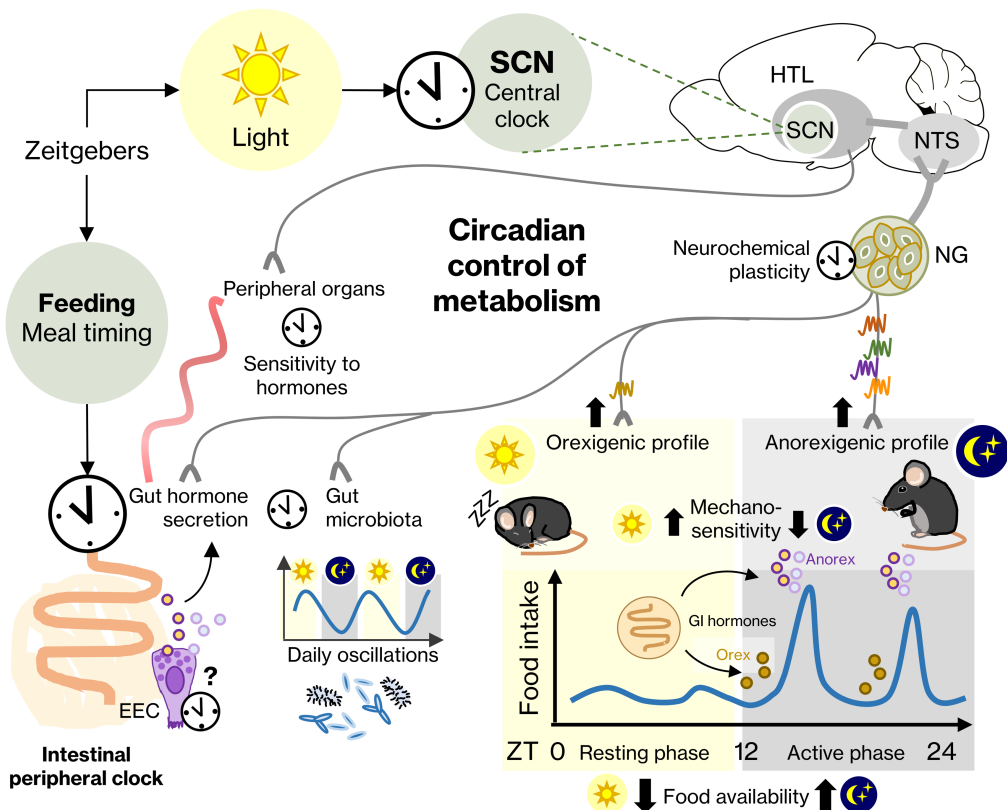


Figure 4. Circadian control of energy homeostasis. Circadian rhythms are regulated by “zeitgebers” to maintain the diurnal metabolic homeostasis: light synchronizes the SCN, the master pacemaker located in the hypothalamus, and food intake synchronizes the gut, which acts as an essential peripheral clock to regulate energy balance in accordance with the circadian food availability. In turn, the gut communicates with peripheral organs that express autonomous clocks entrained by food intake. Fasting and feeding cycles induce oscillations in gut hormone secretion and gut microbiota composition and function, anticipating changes in nutrient availability and influencing postprandial gut hormone release. In addition, vagal afferents convey nutrient-related information to the brain by favoring the expression of anorexigenic and orexigenic receptors during the active and dark phases, respectively, according to food availability. These changes are coupled with reduced vagal mechanosensitivity during the active phase to favor the intake of larger amounts of food. In mice, pre- and post-prandial gut-derived signals govern the two feeding peaks in the dark phase, to communicate the nutritional status to the hypothalamus and ultimately contribute to the circadian control of metabolism.

Abbreviations: anorex: anorexigenic; EEC: enteroendocrine cell; HTL: hypothalamus; NG: nodose ganglion; NTS: nucleus of the solitary tract; orex: orexigenic; SCN: suprachiasmatic nucleus; ZT: zeitgeber time. Figure created with Microsoft PowerPoint.

5.2. Circadian rhythms in vagal afferent-mediated signaling

Vagal afferent signaling in response to nutrient-related stimuli fluctuates throughout the day with circadian rhythmicity to induce satiety³⁷⁹. In response to gastric stretch, mouse vagal afferents exhibit profound circadian variations in mechanosensitivity that are inversely correlated with meal size³⁸⁰. Indeed, mechanosensitivity in rodents is reduced during the dark phase, when most feeding occurs³⁸¹, allowing larger meal sizes to be consumed before satiation is reached³⁸⁰.

These findings suggest a coordination of vagal afferent mechanosensitive signaling in response to the energy requirements of each phase, likely governed by the circadian expression of molecular clock genes observed in the nodose ganglia ³⁸⁰.

Furthermore, the neurochemical profile of vagal afferents also shows plasticity in the pre- and postprandial periods. Specifically, after a meal, CCK stimulates the expression of NPY2R and CART, thus promoting an anorexigenic expression profile to facilitate meal termination. Conversely, during fasting, the reduced plasma CCK levels allow the expression of melanin-concentrating hormone (MCH)-1 receptors and the orexigenic neuropeptide MCH, as well as the upregulation of GHSR expression ^{216,382,383}. However, whether the diurnal plasticity of vagal afferent chemosensitivity is controlled by peripheral clocks in the nodose ganglia similar to vagal mechanosensitivity remains to be further determined.

5.3. Disrupted circadian rhythms under a high fat diet

Disrupted or desynchronized circadian rhythms, especially those related to feeding behavior, drive to impairments in energy homeostasis, increasing the risk of obesity and type 2 diabetes ^{384–386}. Adherence to a Western diet and mistimed eating patterns both contribute to circadian disruption. In mice, the daily feeding rhythms are immediately altered by consumption of a high-fat diet ³⁸⁷, which has been associated to blunted circadian fluctuations of gastric vagal afferent mechanosensitivity, leading to larger meal sizes and higher meal frequency during the light phase ³⁸⁸. These alterations could also be explained by a direct action of palmitate on hypothalamic clock genes that alters the rhythmicity of NPY expression, as reported in cultured murine hypothalamic neurons ^{389,390}.

In humans, abnormal meal timing, such as shift work schedules, skipping breakfast or late-night snacking, leads to increased adiposity and impaired regulation of postprandial glycemia favoring diabetes and obesity ^{386,391–393}. In mice, high fat feeding restricted to the light phase leads to reduced energy expenditure and subsequent weight gain ³⁹⁴. In contrast, restricting food access within the active phase (time-restricted eating/feeding) displays metabolic benefits ^{395–397}. For instance, in mice fed a high-fat diet, food availability limited to the dark phase enhances thermogenesis in adipocytes ³⁹⁴, improves insulin sensitivity and prevents body weight gain ³⁹⁸. Importantly, the restriction of feeding to 9-10 hours in the active phase induces metabolic benefits even under clock genes-impaired conditions, as demonstrated in a study in clock-deficient mice in which feeding restriction to the dark phase prevents the hyperlipidemia, the altered glucose homeostasis and obesity in response to high-fat diet ³⁹⁹. The metabolic benefits of time-restricted feeding have also been attributed to improved chemosensing ⁴⁰⁰. For example, in rats, restricted food access resets the expression of nutrient sensing-related genes in peripheral organs, thus restoring peripheral clocks and lipid metabolism mainly in the liver ⁴⁰¹.

In line with this scientific evidence, chrononutrition is a specialized field involving nutritional strategies to synchronize eating times with peripheral clocks. Thus, chrononutrition enforces the rhythms of nutrient utilization to induce a rhythmic activation of clock genes, to amplify nutrient sensing pathways and ultimately optimize energy metabolism and promote a healthy metabolic phenotype ³⁹⁹. Recently, a human randomized controlled trial in obese patients showed that 4-

and 6-hours restricted eating for two months reduced body weight, insulin resistance and oxidative stress compared to having no restrictions in meal timing ⁴⁰².

5.4. Daily oscillations of gut microbiota for metabolic homeostasis

The gut microbiota composition and function show circadian oscillations mainly modulated by the host feeding rhythms and dietary composition. Studies in *Per1/2*-deficient mice, lacking key clock genes synchronizing feeding time and energy metabolism ⁴⁰³, show dysbiosis and aberrant gut microbiota daily fluctuations ⁴⁰⁴. Thaïss et al. demonstrated that abundant gut bacteria such as from the orders Lactobacillales, Bacteroidales and Clostridiales, as well as some metabolic pathways involved in carbohydrate and amino acid metabolism, showed 24 hour-circadian fluctuations, suggesting the gut microbiota's ability to anticipate changes in nutrient availability ⁴⁰⁴. Notably, they confirmed that feeding time, rather than light-dark cycles, controls gut microbiota oscillations, as subjecting *Per1/2*-deficient mice to light- or dark-restricted feeding to normalize feeding patterns fully restored the gut microbiota rhythmicity ⁴⁰⁴. Thus, independent of light, the gut microbiota oscillates in response to food availability in the gut lumen.

Moreover, the gut microbiota affects the circadian rhythms of postprandial gut hormone secretion. It has been postulated that the dynamics of gut bacterial growth phases induced by nutrients influx align with the dynamics of postprandial secretion of PYY and GLP-1 ⁴⁰⁵. Other evidence shows that the circadian GLP-1 release is dependent on both the diet and the daily oscillations of the gut microbiota composition to maintain the diurnal rhythms in metabolic homeostasis ^{372,406}.

Studies in mice reveal that diet-induced obesity blunts the circadian feeding patterns and disrupts the daily oscillations in gut microbiota structure and function ^{397,407}. The gut microbiota induces a circadian rhythmic expression of the epigenetic regulator histone deacetylase 3 (HDAC3) in the mouse small intestine, resulting in fluctuations in the expression of host genes related to lipid absorption and metabolism, and nutrient transport. However, under a high-fat diet, this rhythmicity is disrupted, potentially contributing to the metabolic outcomes associated with obesity ⁴⁰⁸.

The fact that the diurnal variations of the gut microbiota depend on food-entrainable cues is relevant to design chrononutritional strategies. In mice fed a high-fat diet, time-restricted feeding partially normalizes gut microbiota oscillations and the ileal host transcriptome related to GLP-1 secretion and bile acid signaling, probably mediating the metabolic benefits observed ³⁹⁸. In addition, time-restricted feeding resynchronizes gut microbiota oscillations of genera such as *Lactobacillus*, ameliorating obesity and non-alcoholic steatohepatitis ⁴⁰⁹. Interestingly, only the microbiota isolated from the feeding phase, but not the fasting phase, restored microbial rhythmicity and protected from nonalcoholic steatohepatitis ⁴⁰⁹. Collectively, these findings highlight the importance of developing approaches to modulate diurnal gut microbiota fluctuations in interaction with the gut connectome to enhance metabolic benefits to combat obesity.

6. Gut based therapies to combat obesity

Traditionally, obesity and diabetes have been treated by targeting the end metabolic organs, such as the pancreas, adipose tissue, muscle, or brain ¹⁰⁰. However, the gut is now considered as an important target for the management of energy homeostasis in obesity and associated metabolic disorders.

To date, bariatric surgery is the most effective gut-based treatment for severe obesity (BMI > 40), resulting in significant sustained weight loss and improved glucose homeostasis ^{410,411}. By reducing the stomach size and/or rearranging the digestive tract, it increases nutrient availability and gut microbiota-derived metabolites in the distal regions of the GI tract, thereby enhancing chemosensitivity in the gut connectome and ultimately contributing to the benefits of bariatric surgery ^{99,411}. However, given the side effects and risks of this irreversible intervention ^{410,412–415}, research is increasingly focused on developing less invasive approaches to mimic the amplified gut-to-brain neuroendocrine signaling associated with bariatric surgery ^{99,100}.

6.1. Gut peptide-based pharmacotherapy

Pharmacotherapy targeting gut peptide signaling offers a less invasive alternative to bariatric surgery ^{7,100}. For example, GLP1R agonists, such as liraglutide and semaglutide, effectively improve glycemic control in obese and diabetic patients by improving insulin sensitivity while inducing mild or strong body weight loss ^{416,417}. Moreover, long-acting GIP receptor (GIPR) agonists promote body weight loss and reduce food intake, probably through hypothalamic signaling ^{418,419}. Polyagonists, such as GLP-1/GIP hybrid peptides with dual GIP/GLP-1 receptor agonist activity, also known as “twincretins”, have benefits in glycemia ^{99,420,421}. Specifically, GIPR agonism appears to enhance GLP1R-mediated weight loss, ameliorate hyperglycemia and protect against ectopic lipid deposition ^{99,420} and reduces the GLP1R agonists-induced nausea ^{422,423}. Furthermore, PYY analogs have also been developed recently for weight loss based on the anorexigenic properties of this hormone ^{7,100,424}, and are currently being tested for obesity treatment alone, or in combination with semaglutide for diabetes ⁷. However, gut-peptide agonists such as GLP1R agonists have been associated with nausea or pancreatitis ^{422,425}, so other gut-based approaches are currently under exploration to avoid unwanted side effects of pharmacotherapy.

6.2. Vagal nerve stimulation

Peripheral sensory fibers are increasingly recognized for their role in mediating metabolic functions, leading to the development of device-assisted vagal neuromodulation strategies such as vagal nerve stimulation (VNS) and vagal blocking for obesity control (VBLOC) ^{216,426}. Both are based on the application of electrical currents to the vagal nerve, at low frequency for stimulation in the case of VNS therapy and at high frequency to inhibit the vagal nerve in the case of VBLOC ²¹⁶. Specifically, the VBLOC device, that has been approved for weight loss in obese patients ^{69,427}, involves the subdiaphragmatic implantation of electrodes that deliver high-frequency, low-energy electrical pulses to intermittently block the transmission of the vagal nerve ^{427(p20),428–430}, to inhibit the aberrant vagal orexigenic signaling in obesity from the stomach to the brain ^{216,431}.

Preclinical and clinical evidence demonstrates the efficacy of VNS and VBLOC therapy, in achieving sustained weight loss and rapid satiation ^{429,431–440}, along with potential benefits in energy expenditure ²¹⁶, glucose homeostasis, and cardiovascular health ⁴⁴⁰. Nevertheless, some studies in patients with severe obesity show no significant changes in body weight ^{441,442} and report side effects such as intestinal discomfort ⁴³⁸, besides eventual perioperative complications ⁴³⁷. In addition, VNS cannot distinguish between afferent and efferent vagal nerve fibers ^{216,443}. To address these disadvantages and certain inconsistencies, larger clinical trials are needed to provide more conclusive results ²¹⁶.

6.3. Gut microbiome-based therapies

Gut microbiome-based therapies rely on gut commensal bacteria and their derived components and metabolites, offering an achievable therapeutic option to promote the presence of luminal ligands that modulate the gut connectome. These approaches provide a safer profile and are increasingly being tested as for obesity mitigation. Fecal microbiota transplantation (FMT), prebiotics, probiotics, and postbiotics are coadjuvant therapies based on the modulation of the gut microbiome composition or function to restore metabolic health.

Fecal microbiota transplantation

In humans, FMT for obesity, which consists in the infusion of the fecal microbiota from a metabolically healthy donor into the GI tract of an obese recipient to restore the composition of the gut microbiota ⁴⁴⁴, is still at an early stage, with only a few studies conducted to date. Two clinical trials showed that duodenal infusion of microbiota obtained from small intestine biopsies of lean donors increased insulin sensitivity in male patients with metabolic syndrome ^{445,446}. However, administration of oral capsules containing fecal microbiota from lean donors did not result in body weight benefits ^{447,448}, which warrants further investigation addressing the delivery technique with larger and diverse cohorts regarding age and sex.

Prebiotics, Probiotics and Postbiotics

Prebiotics, such as inulin, fructooligosaccharides and human milk oligosaccharides, are substrates selectively utilized by host microorganisms conferring a health benefit ⁴⁴⁹. Some of the trials specially with inulin-derived prebiotics have shown beneficial effects in obesity ⁴⁴⁹.

Probiotics are “live microorganisms which when administered in adequate amounts confer a health benefit on the host”, as defined by the International Scientific Association for Probiotics and Prebiotics (ISAAP) ⁴⁵⁰. Bacterial strains of *Bifidobacterium* and *Lactobacillus* are traditionally used as probiotics in humans ⁴⁵¹, but new commensal gut bacterial species, mostly isolated from metabolically healthy individuals, are being identified as potential next-generation probiotics to combat obesity ^{101,452}. Indeed, mainly preclinical studies show that the oral administration of bacterial strains such as *Bacteroides uniformis* CECT 7771 ^{453,454}, *Eubacterium hallii* L2-7 ⁴⁵⁵, *Akkermansia muciniphila* ⁴⁵⁶, *Holdemanella bififormis* CECT9752 ³⁵⁸, *Christensenella minuta* DSM33407 ⁴⁵⁷, *Blautia wexlerae* ⁴⁵⁸, and *Parabacteroides distasonis* AS93 and PF-BaE11 ⁴⁵⁹ exert metabolic benefits in obesity models. These include improved insulin sensitivity, glucose tolerance and lipid metabolism, as well as reduced body weight gain and adiposity. Overall, the modes of action of these strains are mainly related to their anti-inflammatory and gut barrier protective

effects and to enteroendocrine effects. However, very few explore the precise cellular and molecular mechanisms by which, for example, they enhance enteroendocrine or enteric/afferent neural signaling to improve energy homeostasis. For example, strains of *H. biformis* induce increased GLP-1 levels, possibly through enhancement of vagal GLP1R signaling ^{358,459}.

Finally, postbiotics are defined as “preparation of inanimate microorganisms and/or their components that confers a health benefit on the host” ⁴⁶⁰. For example, SCFAs, vitamins, peptides, teichoic acids, and enzymes may show postbiotic properties for obesity since, via GLP-1 and PPY, suppress food intake and increase energy expenditure ^{347,461–463}. However, the effects may differ depending, for example, on the GI region where the SCFAs are released, and *in vivo* production may not exert the same effects as the oral supplementation of the metabolites. More recently, the heat-shock protein ClpB from *E. coli* and *Hafnia alvei* has shown to exert beneficial effects in energy homeostasis through its interaction with the components of the gut connectome. Specifically, ClpB is associated to reduced food intake and body weight in mice and humans ^{342,344,345}, probably by increasing PYY secretion ^{344,345}, which mediates endocrine or neural signaling, ultimately increasing hypothalamic activity and the expression of anorexigenic neuronal populations ^{341,344,464}.

Currently, microbiome research partly focuses on the identification of keystone intestinal bacteria and their derived metabolites and structural components with potential to modulate the gut-to-brain neuroendocrine signaling to restore energy balance in obesity. The ultimate goal is to progress in the development of next-generation probiotics and postbiotics, perceived as biotherapeutics agents ⁴⁶⁵, that provide non-invasive and safe alternatives for obesity, in combination with pharmacotherapy and/or healthy lifestyle habits (diet and exercise).

Objectives

Objectives

Currently, we live in a “Westernized” environment where high caloric and palatable foods are right at our fingertips which, combined with a sedentary lifestyle, fuel the increasing obesity prevalence worldwide. The lack of effectiveness of behavioural interventions in managing long-term weight loss⁷ highlights the urgent need to develop novel, efficient and safe approaches for prevention and treatment of obesity and its associated comorbidities. Complementary to the existing treatments, the development of innovative gut-targeted therapeutic strategies to improve metabolic health is a promising research area. This requires a comprehensive exploration of the intestinal neuroendocrine signaling pathways governing feeding behavior to ultimately maintain energy balance, as well as the complex interactions between these neuroendocrine components with the gut microbiota.

The **global objective** of this Doctoral Thesis has been to gain insight in the gut-to-brain interactions, mediated by endocrine, neural and microbial components, involved in the control of energy homeostasis, with a particular focus on obesogenic diet-related dysfunctions that increase the vulnerability to develop obesity.

To attain this general objective, two major aims have been addressed, as follows:

Objective 1

To investigate **novel functions of afferent neurons expressing the sodium channel Nav1.8** in the control of **energy homeostasis**, considering **sex-specificities**, with a particular emphasis on their role in governing **food intake** and **pre- and postprandial daily rhythms** that influence the metabolic phenotype, such as **body weight variations**, **gut hormone secretion**, and **gut microbiota composition**.

Objective 2

To investigate the **mechanisms** through which *Phascolarctobacterium faecium* DSM 32890, a human intestinal commensal bacterial strain, exerts **anti-obesity effects**, with a particular focus on **enteroendocrine pathways** involved in the control of **food intake**.

To address **Objective 1**, a murine model lacking Nav1.8-expressing neurons was generated through a diphtheria toxin ablation strategy. **Chapter 1**, relative to Objective 1, reports that Nav1.8+ neurons are needed to control energy homeostasis in a sex-dependent manner. Specifically, these neurons are involved in postprandial glucose regulation in females, while in males are required to gain weight in response to an obesogenic diet. **Chapter 2**, relative to Objective 1, demonstrates that Nav1.8+ neurons are critical to maintain food intake and the pre- and postprandial variations of body weight, gut hormones, and gut microbiota.

Chapter 3, relative to **Objective 2**, demonstrates that *Phascolarctobacterium faecium* DSM 32890 mediates its metabolic benefits in diet-induced obese mice by reducing food intake via PYY oversecretion and through modulation of gut transit and intestinal lipid absorption, overall preventing fat accumulation.

Results

Chapter 1

Chapter 1

The Ablation of Sensory Neurons Expressing the Nav1.8 Sodium Channel Improves Glucose Homeostasis and Amplifies the GLP-1 Signaling in Obese Female Mice

Marina Romaní-Pérez^{1*}, Clara Bullich-Vilarrubias^{1*}, Inmaculada López-Almela^{1,2}, Yolanda Sanz¹

¹ Microbiome, Nutrition and Health Unit, Institute of Agrochemistry and Food Technology, Spanish National Research Council (IATA-CSIC), Valencia, Spain.

² Present address: Research Group Intracellular Pathogens: Biology and Infection, Department of Animal Production and Health, Veterinary Public Health and Food Science and Technology, Faculty of Veterinary Medicine, Cardenal Herrera-CEU University, Valencia, Spain.

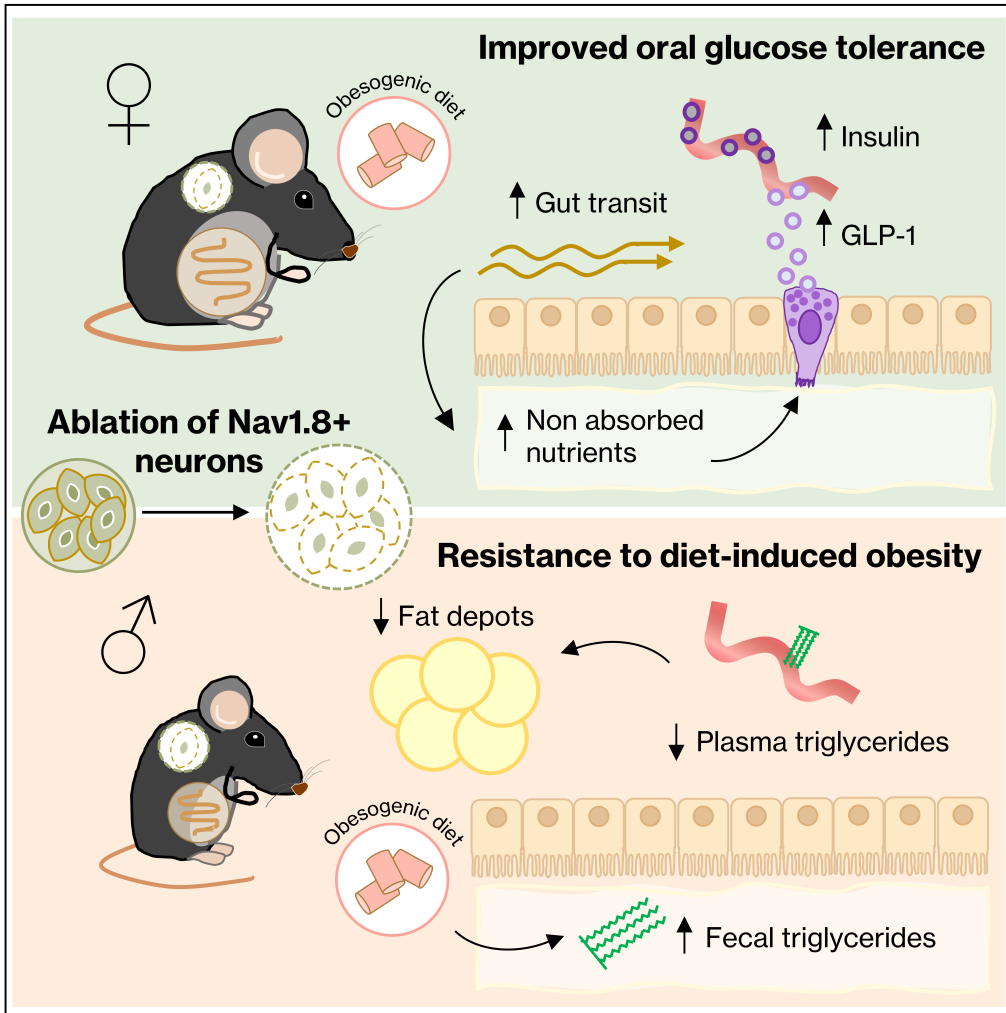
*Equal contribution

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My contribution to this research article was: Study design, *In vivo* experiments, Sampling procedures, Data analysis, Manuscript revision.

Chapter 1

Graphical Abstract



The Ablation of Sensory Neurons Expressing the Nav1.8 Sodium Channel Improves Glucose Homeostasis and Amplifies the GLP-1 Signaling in Obese Female Mice

Abstract

Sensory neurons expressing the sodium channel Nav1.8 contain a repertoire of receptors for nutrient, hormonal and inflammatory ligands. However, their function in key regulators of energy homeostasis control, is not well understood and is completely unexplored in females. We generated mice lacking Nav1.8-expressing neurons (Nav1.8-cre/DTA), using a diphtheria toxin ablation strategy, to investigate whether these neurons modulate body weight, food intake, gut hormone secretion, gastrointestinal transit and glucose tolerance in response to nutrient challenges in a sex-dependent manner. Male Nav1.8-cre/DTA mice show resistance to gain weight in response to high-fat high-sugar diet (HFHSD), whereas females lacking Nav1.8+ neurons have improved oral glucose tolerance accompanied by higher insulin levels and attenuated glucagon secretion after an oral glucose load. Female Nav1.8-cre/DTA mice also show higher fasting and postprandial GLP-1 levels with an increased number of GLP-1-positive cells. Finally, ablation of Nav1.8-expressing neurons accelerated the gastrointestinal transit in female mice under HFHSD. Our data demonstrate sex-dependent differences in the Nav1.8-mediated regulation of energy metabolism, and provide new insights that might help in the design of sex-specific neuromodulation therapies for metabolic disorders induced by diets rich in fats and simple sugars.

1. Introduction

Obesity has reached epidemic proportions globally, and has myriad associated comorbidities, such as type 2 diabetes (T2D), which increase morbidity and mortality. Despite the great strides made to understand the key factors controlling energy homeostasis, efforts are still needed to reduce the prevalence of obesity, including new therapies.

The gut is now considered a key target in the development of therapies for obesity. GI endocrine and neural sensory pathways play an important role in nutrient sensing to further control energy homeostasis. The major players of these pathways are enteroendocrine cells (EECs) and vagal afferents. Nutrient sensing G protein-coupled receptors (GPCRs) on EECs are activated by dietary molecules and trigger the secretion of gut hormones that control many physiological functions via endocrine paths such as gut motility, appetite, and glucose metabolism, among others. This activation also stimulates vagal afferents to rapidly transmit nutrient sensing to the brain ^{1,2}.

Bariatric surgery, the most effective treatment for weight loss and glycemic control ^{3,4}, induces its benefits by enhancing the postprandial secretion of gut hormones in the distal gut, owing to the higher availability of unabsorbed nutrients in this region ⁵. Bariatric surgery mimetic approaches for T2D include non-invasive gut-peptide strategies or those that favor gut hormone secretion ^{6,7}. Analogues of the gut hormone glucagon-like peptide-1 (GLP-1) are established glucose-lowering agents for T2D ⁸, and the clinical efficacy of agonists of the chemoreceptors that induce GLP-1 secretion are under intense investigation ⁶.

In a different approach, neuromodulation therapies including either stimulation (vagus nerve stimulation therapy, VNS) or inhibition (vagal nerve blocking therapy, VBLOC) of the vagal nerve by low- or high-frequency currents, respectively, induce weight loss and improve obesity-related metabolic parameters ^{9–11}. Nevertheless, the mechanisms responsible for these benefits, and whether afferent or efferent tone are preferentially involved, are enigmatic ¹².

Currently, the therapeutic potential of the modulation of sensory neurons from the peripheral nervous system remains understudied, as its diverse molecular specialization makes it difficult to investigate the role of specific neuronal populations ¹³.

Genetic tools to target neurons expressing specific molecular markers are now reshaping our understanding of the functions of afferent neurons on energy homeostasis control. For instance, Cre-LoxP-based experiments conducted in mice point out new functions of sensory neurons expressing the sodium channel Nav1.8, hitherto characterized as spinal neurons mediating pain sensation ¹⁴. More specifically, the vagal origin of Nav1.8+ neurons innervating the GI tract has been demonstrated ^{15,16} highlighting their function driving visceral information to the brain and so, controlling energy balance. Investigations on this topic demonstrated that these neurons are dispensable for the control of body weight, food intake or energy expenditure ¹⁷. In contrast, another study revealed that, although Nav1.8+ neurons are not involved in weight gain due to high-fat dietary intake, they are necessary for maintaining fat stores while contributing to metabolic balance ¹⁸. In addition, characterization of GPCR gene expression profiles points to a role for these neurons in mediating gut-to-brain communication triggered by gut-derived molecules such as hormones or immunomodulatory lipids ¹⁹. In this regard, it is not yet known whether Nav1.8+

neurons are needed for GI functions that influence postprandial control of energy metabolism and whether they exhibit sex-dependent effects. This research would help advance more precise neuromodulation-based strategies for obesity by targeting a specific neuronal population in the afferent arm of the peripheral nervous system. Using a murine ablation model lacking Nav1.8-expressing neurons we have identified sex-dependent metabolic functions of these neurons. Specifically, in females, we have unraveled intestinal factors through which Nav1.8+ neurons regulate energy homeostasis.

2. Materials and Methods

Mice

We crossed heterozygous Nav1.8 knock-in Cre-recombinase male mice (EM:04582, EMMA/Infrafrontier repository, Munich, Germany) with homozygous ROSA26-eGFP-DTA female mice (stock #006331; The Jackson Laboratory, Bar Harbor, ME) to obtain 1:1 litters of Nav1.8-deficient mice (with Cre and DTA alleles: Nav1.8-cre/DTA or Cre⁺ in Figures) and control mice (without Cre: control littermates or Cre⁻ in Figures). Genotyping was conducted using the Phusion High-Fidelity PCR Kit (Thermo Fisher Scientific, Waltham, MA) (**Table S1**). RNA isolation and reverse-transcription were performed with the Ambion® RiboPure Kit (Invitrogen, Carlsbad, CA) and SuperScript™ VILO™ Master Mix (Thermo Fisher Scientific), respectively, in randomly selected nodose ganglia. The TaqMan® PreAmp Master Mix (Applied Biosystems, Thermo Fisher Scientific) was used for cDNA pre-amplification, and TaqMan® Gene Expression Assays (FAM-MGB; Applied Biosystems) using TaqMan® Fast Advanced Master Mix (Applied Biosystems, Thermo Fisher Scientific) for gene expression analysis of *Scn10a* (coding for NAV1.8) and the housekeeping gene *Rpl19* (**Table S1**). Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method to represent data as fold-change relative to control littermates.

Experimental design and dietary information

Three cohorts were generated for the experimental protocol detailed in **Figure S1A**. Collectively housed mice were maintained under constant conditions of humidity and temperature ($23 \pm 2^\circ\text{C}$) and 12-h light/dark cycle with *ad libitum* access to food (and water) unless otherwise stated.

Female and male control and Nav1.8-cre/DTA mice (6 weeks old) were randomly allocated in groups fed either a control diet (CD: 10% of energy from fat without sucrose) or a high-fat high-sugar diet (HFHSD: 45% of energy from fat and 21% from sucrose) (D12450K or D12451, respectively, Research Diets) for 11 weeks. The composition of the diets is shown in **Table S2**.

Sample size (**Figure S1A**) was calculated using G*Power software based on a previously estimated effect size. Body weight was monitored weekly. Food efficiency, GI transit time and glucose tolerance were conducted in females and males between week 6 and 11. To estimate food efficiency (weight gain/food ingested during refeeding), mice were 4 h fasted in the dark phase followed by 2 h of free access to food. GI transit time was measured in overnight fasted mice receiving an oral gavage of 6% non-absorbable carmine red in 0.9% saline with 0.5% methylcellulose²⁰. For 6 h, mice had free access to food and the color of feces was checked every 30 min to determine the time from the oral gavage to the first appearance of carmine red in feces.

Feces after the overnight fasting and feces after the GI transit time (6 h of refeeding) were collected to measure triglyceride levels. For glucose tolerance tests (GTTs), glycemia was determined at 0, 15, 30, 60 and 120 min after an oral (OGTT) or intraperitoneal (ipGTT) glucose challenge. At week 7, overnight-fasted male mice were ad libitum fed for 2 h to measure postprandial triglycerides in plasma.

Between week 7 and 11, intestinal glucose absorption and the glucose-induced hormone secretion (GLP-1, GIP, insulin and glucagon) was explored in females. Intestinal glucose absorption was estimated by calculating the slope of the glycemia between 0 and 5 min after a glucose oral challenge²¹, and hormone secretion by measuring hormones in plasma at 0 and 15 min after an oral glucose load.

For GTTs, intestinal glucose absorption and hormone secretion, mice were fasted for 4 h before glucose challenge (2 g/kg). For the GTTs and glucose absorption, blood was drawn from the saphenous vein, and for hormone secretion tests, from the submandibular vein into Microvette® 500 K3E tubes containing DPPIV inhibitors (Sigma), and immediately centrifuged to obtain plasma. Glycemia was determined with Contour XT glucometer (Bayer, Leverkusen, Germany).

At the end of the experiment, 4 h fasted mice were orally administered the mixed nutrient solution Ensure® (12.4 kcal/kg) to assess GLP-1 levels at 0 and 15 or 90 min after Ensure® challenge. In one group of females, blood was collected 15 min after Ensure®, and mice were then sacrificed by cervical dislocation to isolate duodenum, ileum, colon and brain, which were immediately frozen until processed. The remaining small intestine was embedded in cold FACS buffer for further isolation of immune cells. Another group of female and male mice were anaesthetized with an intraperitoneal injection of pentobarbital (50 mg/kg) 90-min following Ensure® administration, and were then transcardially perfused with PBS followed by 10% neutral buffered formalin. The gonadal and inguinal white adipose tissue (gWAT and iWAT, respectively) of males and females and ileum and colon of females were isolated for processing.

Animal welfare was assessed on a daily basis. Signs of sickness or harm observed were immediately reported to the veterinarian in charge of the animal facility for further evaluation. Assessments were focused on excessive body weight loss, spine posture, dullness of mice coat and injury such as ulcerative dermatitis induced by self-mutilation. All the experimental procedures using animals, as well as evaluation of signs of sickness, were in accordance with European Union 2010/63/UE and Spanish RD53/2013 guidelines, and were approved by the ethics committee of the University of Valencia (SCSIE, UV, Spain) and authorized by Dirección General de Agricultura, Ganadería y Pesca (Generalitat Valenciana) (approval ID 2019/VSC/PEA/0020 and 2020/VSC/PEA/0022).

Hormone levels in plasma

GLP-1 (total), GIP, insulin, and glucagon in plasma were measured using a MMHE-44K-04 Milliplex Kit (Merck, Darmstadt, Germany) in a Luminex MAGPIX® System (Luminex, Austin, TX) or an ELISA kit for total GLP-1 (Merck).

Triglycerides in plasma and feces

Only in male mice, triglycerides levels were measured in feces after 12 h of overnight fasting and 6 h of refeeding, and in plasma after 2 h of refeeding. Lipids were isolated from feces based on a method adapted from ^{22,23} as previously described ²⁴. Triglycerides were measured using a Triglyceride Colorimetric Assay Kit (Elabscience Biotechnology Co. Ltd., Bethesda, MD).

Histology and GLP-1 immunostaining

Hematoxylin-eosin staining was performed in gWAT and GLP-1 immunostaining in intestinal samples according to standardized protocols by Patologika Laboratorio S.L. (Valencia, Spain). Images were taken using an Eclipse 90i Nikon wide-field microscope (Nikon Corp., Tokyo, Japan) with a CFI Plan Fluor DIC M/N2 (MRH00200) Nikon dry-air objective (20× or 4×) combined with an optical zoom factor of 0.8×. Fiji software (ImageJ 1.49q Software, National Institutes of Health) and Nis elements BR 3.2 software (Nikon Corp.) were used for quantification according to standardized protocols of the imaging service from IATA-CSIC.

RT-qPCR

RNA was isolated from the hypothalamus, duodenum, ileum and colon using TRIsure™ (Bioline Reagent, London, UK). Reverso-transcription and qPCR were performed as described ²⁵. Relative change of cDNA abundance was calculated according to the $2^{-(\Delta\Delta Ct)}$ method and represented as fold-change expression relative to the control group. **Table S3** shows the specific primer pairs.

Immune phenotyping by flow cytometry

Splenic suspensions, obtained by crushing and washing with FACS buffer (PBS-10% fetal bovine serum, Thermo Scientific), were filtered (70 µm nylon cell strainers, Biologix) and centrifuged (450 g, 5 min, 4°C). Intestines were incubated with Ca²⁺/Mg²⁺-supplemented Hank's balanced salt solution (HBSS) (Thermo Fisher Scientific) with 5 mM EDTA, 1 mM DTT and antibiotics (100 µg/mL streptomycin-100 U/mL penicillin, Merck) for 30 min at 37°C, filtered (100-µm nylon cell strainers, Biologix) and incubated again with HBSS containing antibiotics and 0.5 mg/mL collagenase D, 1 mg/mL DNase I (Roche), and 3 mg/mL dispase II (Sigma) for 30 min at 37°C. Lamina propria cells were isolated by filtration (70-µm nylon cell strainers, Biologix) and centrifugation (450 g, 5 min, 4°C) of cell suspensions.

A combination of flow cytometry antibodies against surface and intracellular markers (**Table S4**) was used for immunolabeling FACS preserved cells (30 min at 4°C, protected from light). Cells were first permeabilized and fixed using a fixation/permeabilization solution (BD Bioscience) for intracellular markers. Data acquisition and analysis were performed using a BD LSRFortessa flow cytometer operated with FACS Diva software v.7.0 (BD Biosciences).

Statistics

GraphPad Prism 9 was used for statistics. Normality and equality of variances were assessed with Shapiro-Wilk and Bartlett's tests, respectively. Two-way ANOVAs were used for main effects and interactions between genotype and diet, genotype and sex, or genotype and prandial conditions. Multiple comparisons using Tukey's *Post-hoc* test were performed when interactions were detected. One-way ANOVA followed by Tukey's *Post-hoc* test or Kruskal-Wallis followed by Dunn's *Post-hoc* test were conducted for the analyses restricted to control mice fed either CD or HFHSD and Nav1.8-cre/DTA mice fed HFHSD. Matched pairs data were analyzed with paired t-

tests and Student's t test was used to explore differences between two independent groups. Correlations between two variables were explored with Bravais-Pearson correlation coefficient. Survival was analyzed using Log-rank (Mantel-Cox) Chi square test.

3. Results

Lack of Nav1.8-expressing neurons has a sex-dependent effect on body weight gain, fasting induced refeeding and glucose tolerance in mice fed a high-fat high-sugar diet

Since vagal afferents, rather than spinal neurons, are involved in nutrient sensing to further control energy homeostasis, Nav1.8 gene expression was explored in nodose ganglia. Mice expressing Cre, hereinafter referred to as mice lacking Nav1.8⁺ neurons or Nav1.8-cre/DTA mice, had almost undetectable *Scn10a* mRNA levels (coding for NAV1.8), indicating effective ablation of Nav1.8⁺ neurons in the offspring (**Figure S1B**).

We examined the vulnerability to gain weight in response to HFHSD-feeding in Nav1.8-cre/DTA mice and potential interactions between sex and the genotype. The effects on body weight were examined after 5 weeks of HFHSD-feeding, before conducting other functional tests (between week 6 and 11) to avoid the influence of the stress associated with these tests.

An interaction between the group and the week of CD/HFHSD-feeding was identified in the body weight of both, females and males (**Figure 1A**), indicating that this parameter varies differentially over 5 weeks of dietary intervention. As expected, at least from week 4, HFHSD-feeding increased the body weight of female and male control mice compared with controls fed CD (**Figure 1A**). Furthermore, under HFHSD and only in males, Nav1.8-cre/DTA mice showed reduced body weight than their control littermates (**Figure 1A**). Overall, 5 weeks of HFHSD-feeding increased the body weight gain of both, female and males since a main diet effect was identified in both sexes (**Figure 1B**). A genotype effect was also detected, but only on the body weight gain of male mice, supporting that loss of Nav1.8-expressing neurons induces obesity resistance in males (**Figure 1B**).

Ad libitum food intake was not markedly affected by the genotype in female and male mice (**Figure S2A**). By contrast, analysis of 2-h food intake after fasting revealed a diet effect only in females irrespective the genotype, with reduced food intake in the refeeding period in mice fed HFHSD (**Figure 1C**, left graph). An interaction between diet and the genotype was identified in males (**Figure 1C**, right graph). *Post-hoc* analysis revealed that HFHSD-fed Nav1.8-cre/DTA male mice had a higher food intake after fasting than controls fed the obesogenic diet or Nav1.8-cre/DTA mice fed CD (**Figure 1C**, right graph). Body weight gain after the refeeding was lower in HFHSD fed female and male mice irrespective of the genotype compared with CD-fed groups (**Figure 1D**). Food efficiency was also affected by the diet and the genotype in female and male mice (**Figure 1E**). Specifically, we found an interaction between diet and genotype in females, whereby food efficiency was lower in HFHSD-fed control mice than in CD-fed mice, with no effects in Nav1.8-cre/DTA mice (**Figure 1E**, left graph). In males, the diet and the genotype impacted food efficiency, and both variables showed a trend for interaction ($p=0.08$) (**Figure 1E**, right graph). Analysis restricted to HFHSD-fed mice indicated that food efficiency showed a trend to be lower in Nav1.8-

cre/DTA mice than in control littermates (Student's t test, Cre⁻ HFHSD *versus* Cre⁺ HFHSD, $p = 0.09$).

In response to an obesogenic diet, we explored whether the loss of Nav1.8+ neurons affected weight gain and food efficiency differently in males or females by identifying sex and genotype interactions on these parameters (datasets from figures 1B and 1E). First, we observed high inter-individual variability in the body weight gain of Nav1.8-Cre/DTA male mice fed HFHSD (see extending whiskers in **Figure 1B** indicating variability outside the upper and lower quartiles), supporting that loss of Nav1.8+ neurons may induce diverse responses to HFHSD-feeding, as already evidenced by others¹⁷. Using a protocol similar to that used in previous rodent studies²⁶⁻²⁸, we employed quartile distribution to identify individual vulnerability to diet induced obesity allowing a more accurate exploration of sex-genotype interactions taking into account the variability of response to an obesogenic diet. Thus, each HFHSD-fed group was subdivided into weight-gaining or obesity-prone mice, whose weight gain belonged to the first quartile (Q1); non-weight-gaining or obesity resistant mice, belonging to Q2 and Q3; and weight-losing or cachectic mice, belonging to Q4 (**Figure 1F**).

No sex-associated differences in the quartile distribution of weight gain data were found irrespective of the genotype (males: $\chi^2 = 0.564$, $p = 0.754$ or females: $\chi^2 = 0.012$, $p = 0.994$). We found an interaction between genotype and sex in the weight gains distributed in Q2/Q3 and in Q4 (**Figure 1F**). Specifically, in response to 5 weeks of HFHSD-feeding, male Nav1.8-Cre/DTA mice, but not females, gained less weight than their control littermates, occasionally showing weight loss in the case of male mice distributed in Q4 (**Figure 1F**). No interaction was found in mice from Q1 (**Figure 1F**). In addition, a combinatorial effect between sex and genotype was found in food efficiency, with a tendency to show reduced food efficiency in male mice lacking Nav1.8+ neurons compared to control littermates ($p = 0.09$), not shown in females (**Figure 1G**).

Genotype- and sex-related effects on fat depots were also examined. First, we examined fat distribution, calculated as the percentage of individual fat pad mass (gWAT or iWAT) relative to total fat pad mass (iWAT plus gWAT). As indicated by a main effect of fat type, there was a greater contribution of gWAT to total fat depots in both female and male mice (**Figure S2B**). In addition, the analysis of the combinatory effects of genotype and sex revealed that fat distribution was affected by sex but not by the genotype (**Figure S2C**). Indeed, regardless the genotype, a main effect of sex was identified on the distribution of gWAT and iWAT, indicating higher gonadal fat pads in male than in female and higher iWAT in female than in male (**Figure S2C**).

Despite fat distribution remained unaffected by the genotype, we identified that, only in males, the loss of Nav1.8-expressing neurons had an impact on gWAT weight. Indeed, after 11 weeks of HFHSD-feeding, Nav1.8-cre/DTA female mice showed the same gWAT weight as controls (controls: 0.67 ± 0.12 g *versus* Nav1.8-cre/DTA: 0.55 ± 0.20 g, $p = 0.60$), while Nav1.8-Cre/DTA male mice (from Q2 and Q3) had reduced gWAT (controls: 1.70 ± 0.20 g *versus* Nav1.8-cre/DTA: 0.69 ± 0.27 g, $p = 0.04$). The lower gWAT in male mice lacking Nav1.8+ neurons was further supported by the smaller adipocyte size compared with HFHSD-fed controls, reaching values similar to CD-fed control mice (**Figure 2A**), suggesting a reduced storage of lipids in adipocytes.

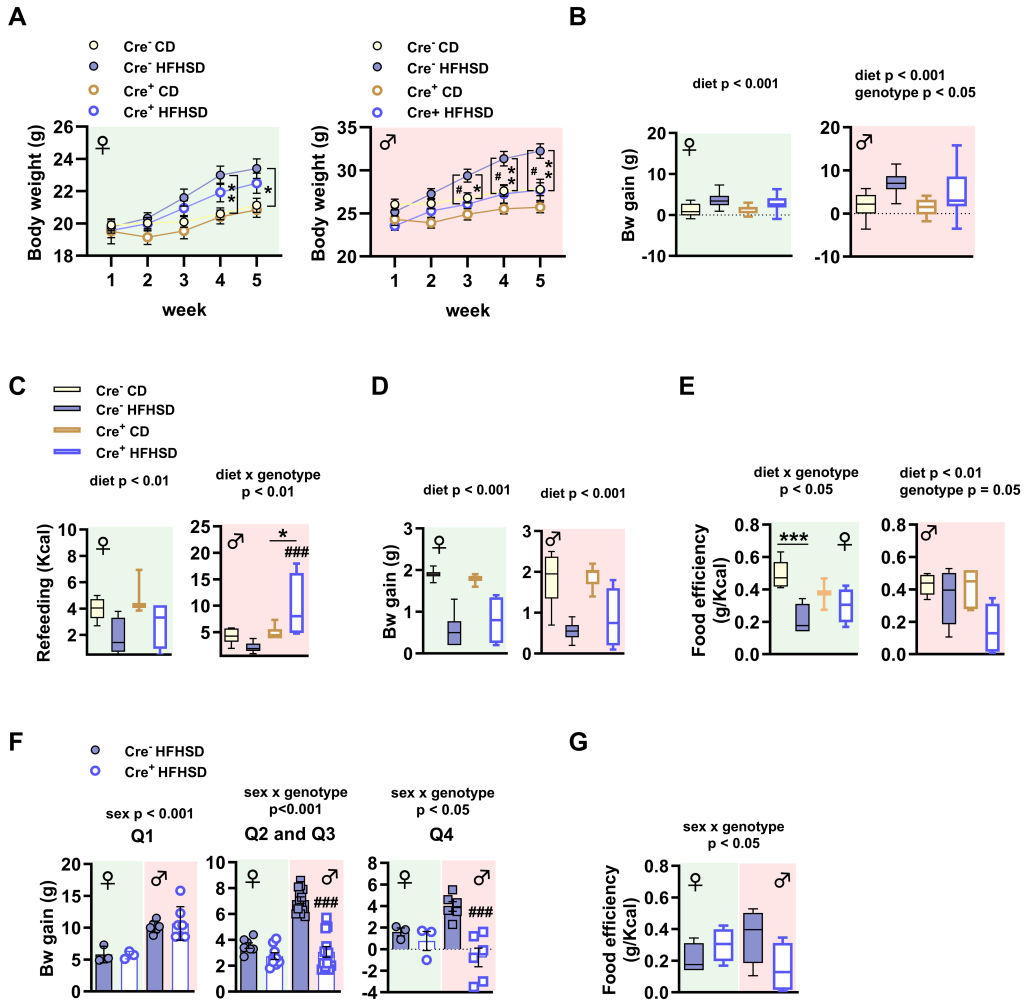


Figure 1. Ablation of Nav1.8-expressing neurons has sex-dependent effects on body weight under an obesogenic diet.

In female and male control and Nav1.8-cre/DTA mice (Cre^{-/-}/Cre^{+/+}) fed either control or high-fat high-sugar diet (CD/HFHSD), we measured: **(A-B)** Body weight (Bw) follow-up and Bw gain of females and males in response to 5 weeks of CD or HFHSD. **(C)** Food intake (kcal) during refeeding after fasting in the dark phase. **(D)** Bw gain after refeeding. **(E)** Food efficiency. **(F)** Comparisons of the Bw gain separated into quartiles (Q) of female and male mice fed HFHSD. **(G)** Comparison of the food efficiency of female or male mice fed HFHSD. Data show the mean $\pm$ SEM or the median, the interquartile range and the minimum and maximum values (whiskers). (A, B and F): Data from 3 cohorts, no significant cohort effect was observed. Female Cre^{-/-} CD/HFHSD, n = 20; female Cre^{+/+} CD, n = 8; female Cre^{+/+} HFHSD, n = 15; male Cre^{-/-} CD/HFHSD, n = 23/25; male Cre^{+/+} CD, n = 13, and Cre^{+/+} HFHSD, n = 27. (C-E and G): Data from 1 cohort. Female Cre^{-/-} CD/HFHSD, n = 6; female Cre^{+/+} CD/HFHSD, n = 4; male Cre^{-/-} CD/HFHSD, and n = 8 and male Cre^{+/+} CD/HFHSD, n = 7/5. HFHSD-data of (B) are also represented in (F). HFHSD-data of (E) are also represented in (G). Two-way ANOVA followed by Tukey's *Post-hoc* test when significant interactions were detected. Main effects and interactions are indicated on top of the graphs. *p<0.05 and ***p<0.001 vs CD-fed mice with the same genotype. ###p<0.001 vs HFHSD-fed mice with different genotypes.

To unravel how loss of Nav1.8-expressing neurons limits fat depots in males, we examined in these mice the influence of the genotype on dietary lipids excretion and absorption by measuring triglycerides in feces and plasma after a meal and by exploring GI transit time. A main effect of the prandial condition (fasting/refeeding) on fecal triglycerides levels was identified, with higher triglycerides levels in feces after 6 h of refeeding compared with 12 h of fasting (**Figure 2B**). After fasting, fecal triglycerides levels did not show significant differences between groups, but total excreted triglycerides during 6 h of refeeding were lower in HFHSD-fed control male mice than in equivalent Nav1.8-Cre/DTA mice, which had levels similar to those of CD-fed control mice (**Figure 2B**). Furthermore, under HFHSD, Nav1.8-cre/DTA male mice showed reduced postprandial triglycerides levels in plasma than controls (**Figure 2C**). The GI transit time, which may impact on the intestinal excretion and absorption of lipids, was not significantly affected by the diet or the genotype (**Figure 2D**).

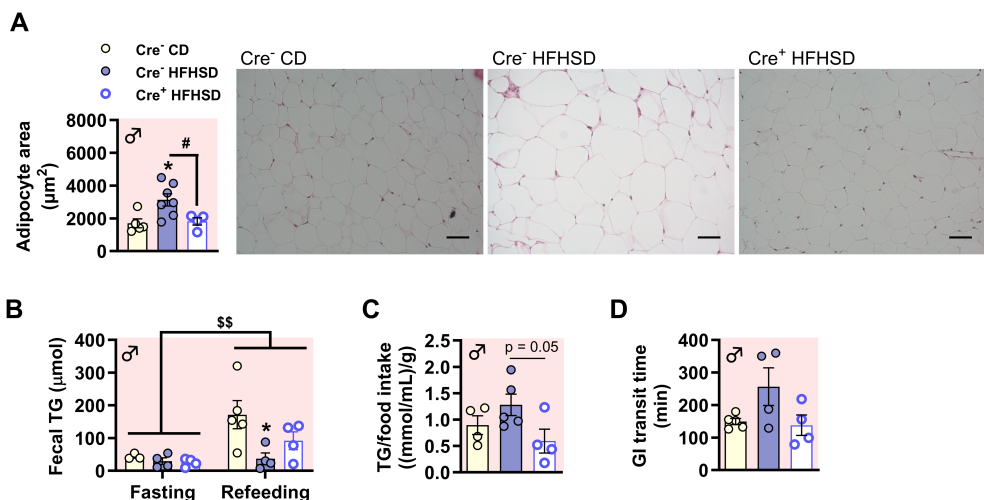


Figure 2. Loss of Nav1.8+ neurons limits fat storage in the gonadal adipose tissue in male mice fed high-fat high-sugar diet

In male controls (Cre⁻) fed control diet (CD) or high-fat high-sugar diet (HFHSD) and mice lacking Nav1.8+ neurons fed HFHSD, we measured: **(A)** Adipocytes area in the gonadal white adipose tissue at the end of the experiment. **(B)** Total triglyceride (TG) levels excreted in feces after 12 h of overnight fasting, preceding the GI transit time trial and during the trial (6 h of refeeding) after 8 weeks of CD or HFHSD-feeding. **(C)** TG in plasma after 2 h of refeeding at week 7 of CD or HFHSD-feeding. **(D)** Time required for orally administered carmine red to appear in feces (GI transit time) after 8 weeks of CD or HFHSD-feeding. Data are shown as the mean ± SEM. Cre⁻ CD, n = 5, Cre⁻ HFHSD, n = 7-5, and Cre⁺ HFHSD, n = 4. (A, C and D): One-way ANOVA followed by Tukey's *Post-hoc* test. (B): Two-way ANOVA. *p<0.05 vs CD-fed control mice (Cre⁻ CD). #p<0.05 vs HFHSD-fed mice with different genotypes. \$\$p<0.01 main effect of prandial condition (fasting vs refeeding).

We then explored the impact of Nav1.8+ neurons ablation on the control of glucose homeostasis. In female mice, an interaction between the genotype and the diet was identified in fasting glycemia (**Figure 3A**). Further *post-hoc* analysis indicated that HFHSD increased the basal glucose levels compared with CD in Nav1.8-cre/DTA female mice (**Figure 3A**, left graph). In male mice, neither main effects nor interactions between the genotype and the diet were detected (**Figure 3A**, right

graph). The analysis of the OGTT indicated a main effect of the genotype in female mice (**Figure 3B**) but not in males (**Figure 3C**) indicating that the whole-body glucose clearance was more efficient in female Nav1.8-Cre/DTA mice than in their control littermates after an oral glucose load under either CD or HFHSD (**Figure 3B**). When glucose was intraperitoneally (ip) administered to mice, the whole-body glucose clearance was improved in male Nav1.8-Cre/DTA mice (**Figure 3E**) but not in females (**Figure 3D**). A diet effect was found in the area under the curve (AUC) of the OGTT and ipGTT in female and male mice (**Figures 3B-3E**), confirming that, irrespective the genotype, HFHSD impairs glucose tolerance. We found an interaction between sex and genotype for the AUC of the OGTT in mice fed HFHSD with more robust glycemia reduction in female Nav1.8-Cre/DTA mice than in males as indicated the *post-hoc* test (**Figure 3F**, left graph), although no combinatory effects were observed for the AUC of the ipGTT (**Figure 3F**, right graph).

Taken together, our findings support sex-dependent metabolic functions of Nav1.8+ neurons in which, in response to an obesogenic diet, Nav1.8+ silencing attenuates weight gain and adiposity in males but not in females and improves oral glucose tolerance in females but not in males.

Loss of Nav1.8-expressing neurons increases the plasma levels of total GLP-1 and insulin in female mice fed a high-fat high-sugar diet

Longer term analysis of health status of mice (9 weeks of HFHSD-feeding) revealed that female Nav1.8-cre/DTA mice showed optimal health and welfare in response to HFHSD-feeding, whereas male Nav1.8-cre/DTA showed sickness behavioral responses to HFHSD. Indeed, in response to 9 weeks of HFHSD-feeding, female mice lacking Nav1.8+ neurons gained the same weight as controls (**Figure S2D** and **S2E**). In contrast, some Nav1.8-cre/DTA male mice showed body weight loss through the HFHSD intervention (**Figure S2F**, right graph) occasionally reaching almost 8% weight loss from their initial body weight (**Figure S2G**, right graph). Those Nav1.8-cre/DTA male mice that lost weight under HFHSD showed ulcerative dermatitis (data not shown) and finally died. Indeed, only the 50% of Nav1.8-cre/DTA male mice survived after 9 weeks of HFHSD-feeding (**Figure S2H**, right graph). By contrast, the obesogenic diet did not affect the survival of Nav1.8-cre/DTA female mice (**Figure S2H**, left graph). To note, the experiments in male mice (**Figure 2** and **Figure 3**) were conducted in mice that did not show sickness behaviour (mice which body weight gain belonged to Q2 and Q3).

As Nav1.8+ neurons ablation did not affect female health, the exploration of the effects of long-term exposure to HFHSD were restricted to female mice.

On the basis that Nav1.8+ neurons mediate neuroimmune pathways in mucosa layers ^{29,30}, we began to explore the intestinal mechanisms underlying the glucoregulatory benefits of Nav1.8+ neurons in diet-induced obese female mice by first examining intestinal immunity, which imbalance may disturb glucose homeostasis ³¹.

In the lamina propria of the small intestine, Nav1.8-cre/DTA mice had higher levels of type 1 and type 2 macrophages (M1 and M2) and regulatory T cells (Treg) (**Figure S3A-S3C**). In addition, the absence of Nav1.8+ neurons conferred protection against the spleen enlargement showed in HFHSD-fed control mice (**Figure S3D**), increased the splenic levels of memory CD4+ T cells (**Figure S3E**) and tended to reduce the levels of effector CD4+ T cells (**Figure S3F**).

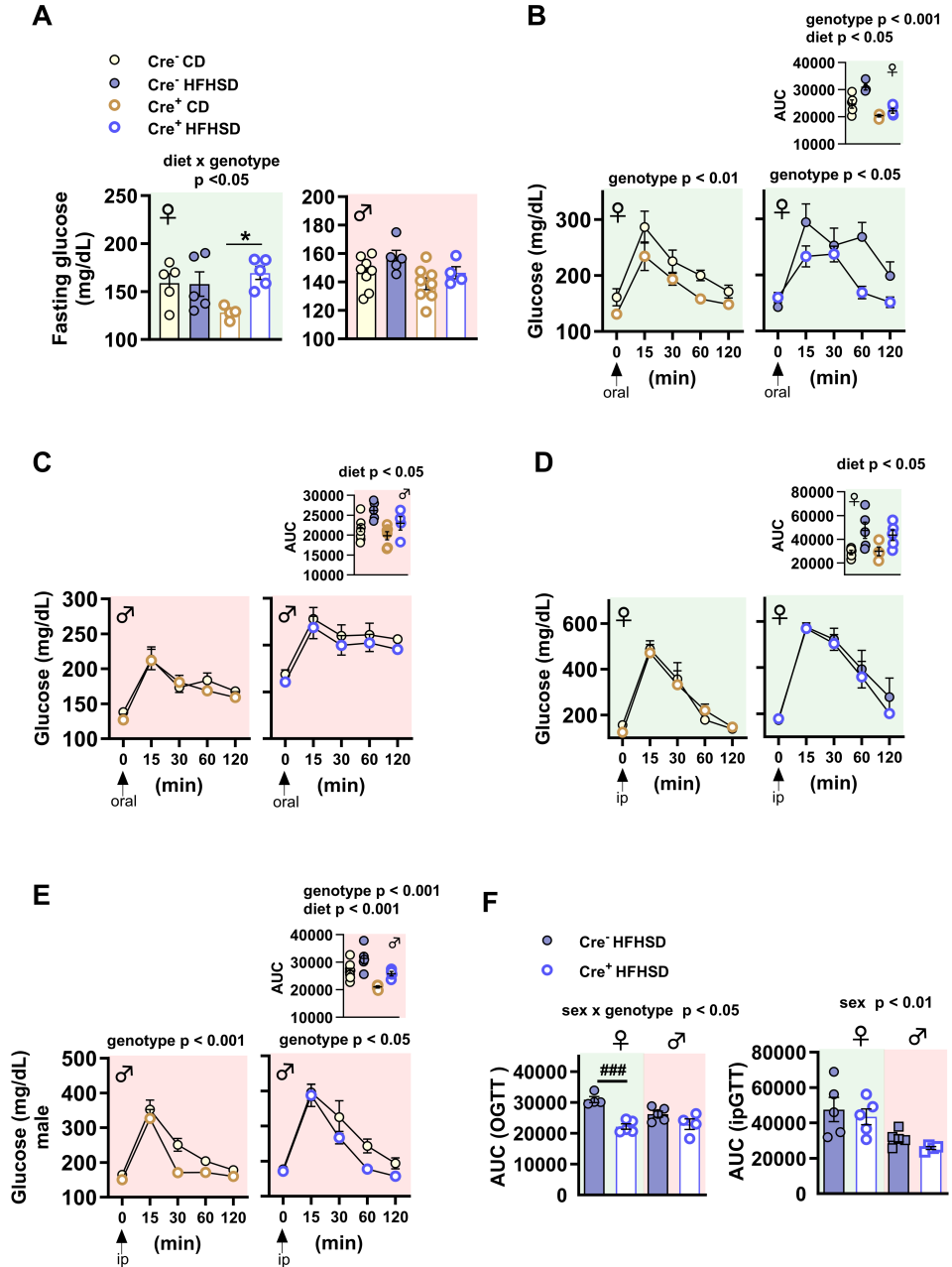


Figure 3. Deficiency of Nav1.8+ neurons improves oral glucose tolerance in females and intraperitoneal glucose tolerance in males.

In female or male controls and Nav1.8-cre/DTA mice (Cre⁻/Cre⁺) fed either control or high-fat high-sugar diet (CD/HFHSD) for 10 weeks, we measured:

(A) Fasting glucose levels in plasma of female and male mice. (B and C) OGTT and the area under the curve (AUC). (D and E) Intraperitoneal glucose tolerance test (ipGTT) and AUC. (F) Comparison of the AUC of the OGTT between females and males (Cre⁻/Cre⁺) fed HFHSD (left) and comparison of the AUC of the ipGTT

between females and males (Cre⁺/Cre⁺) fed HFHSD (right). Data are shown as the mean $\pm$ SEM. Female mice, n = 5 per group and male mice, n = 5-8 per group. HFHSD-data of AUC of (B-E) are also represented in (F). Two-way ANOVA followed by Tukey's *Post-hoc* test when significant interactions were detected. Main effects and interactions are indicated on top of the graphs. *p<0.05 Cre⁺ fed CD vs HFHSD. ####p<0.001 vs HFHSD-fed mice with different genotypes.

As alternative intestinal mechanisms potentially involved in the glucoregulatory benefits of the Nav1.8+ neurons depletion, we also investigated glucose absorption and incretin hormone (GLP-1 and GIP) secretion together with the hormones regulated by those incretins (insulin and glucagon) in response to an oral glucose challenge. Intestinal glucose uptake was faster in Nav1.8-cre/DTA mice than in control mice, as indicated by the enhanced early appearance of glucose in the bloodstream immediately after an oral glucose challenge (5 min post-glucose) (**Figure 4A**), despite their lower glycemia in the OGTT from minute 15 onwards, which refers to glucose clearance (**Figure 3A**). No changes were identified in the gene expression of the glucose transporters *Slc2a2* (coding for GLUT2) (**Figure S3G**) and *Slc5a1* (coding for SGLT1) (**Figure S3H**) in the proximal small intestine, or in the small intestine length (**Figure S3I**).

Compared with CD-fed controls, HFHSD-fed control mice had lower total GLP-1 levels in plasma but higher GIP during fasting, and showed increased GIP and glucagon levels after an oral glucose load (**Figure 4B**). Nav1.8-cre/DTA mice had higher insulin levels than CD-fed mice both during fasting and after glucose challenge, with similar levels of total GLP-1, GIP and glucagon (**Figure 4B**). Combinatorial effects of the genotype and the prandial condition on hormone levels were investigated in HFHSD-groups. We found an interaction between genotype and prandial condition for total GLP-1 (**Figure 4B**), with *post-hoc* analysis indicating that total GLP-1 levels were higher in Nav1.8-cre/DTA mice than in control mice during fasting but not after the oral glucose load (**Figure 4B**). Furthermore, independently of the prandial condition, the absence of Nav1.8-expressing neurons prevented the increase in GIP levels induced by HFHSD-feeding (**Figure 4B**). In addition, a main effect of the genotype was also detected on insulin indicating that Nav1.8-cre/DTA mice showed increased levels of this hormone in both conditions (fasting and after an oral glucose load) compared with HFHSD-fed control mice (**Figure 4B**). At the end of the experiment, total GLP-1 levels in plasma were measured in response to an oral load of the mixed-nutrient solution, Ensure®. No changes were observed 15 min after Ensure® (**Figure 4C**), but after 90 min, a main effect of the diet and the genotype were identified on the total GLP-1 levels in plasma (**Figure 4D**). These main effects indicated that HFHSD-feeding reduced total GLP-1 whatever the genotype, and that the ablation of Nav1.8+ neurons increased its levels compared with control littermates irrespective of the diet (**Figure 4D**).

The loss of Nav1.8+ neurons might impact gut-to-brain nutrient sensing and, consequently, the central control of glucose homeostasis. Analysis of the expression of key hypothalamic genes for energy homeostasis control evidenced no major changes associated with the genotype. *Pomc* (proopiomelanocortin) and *Cart* (cocaine- and amphetamine-regulated transcript) were unchanged whatever the group, whereas *Agrp* (agouti-related peptide) and *Npy* (neuropeptide Y) transcripts were lower in control and Nav1.8-cre/DTA mice fed HFHSD than in CD-fed control mice (**Figure S3J**), reflecting that Nav1.8-cre/DTA mice downregulated orexigenic neuropeptides to counteract the excess of high-energy dense food as did HFHSD-fed control mice.

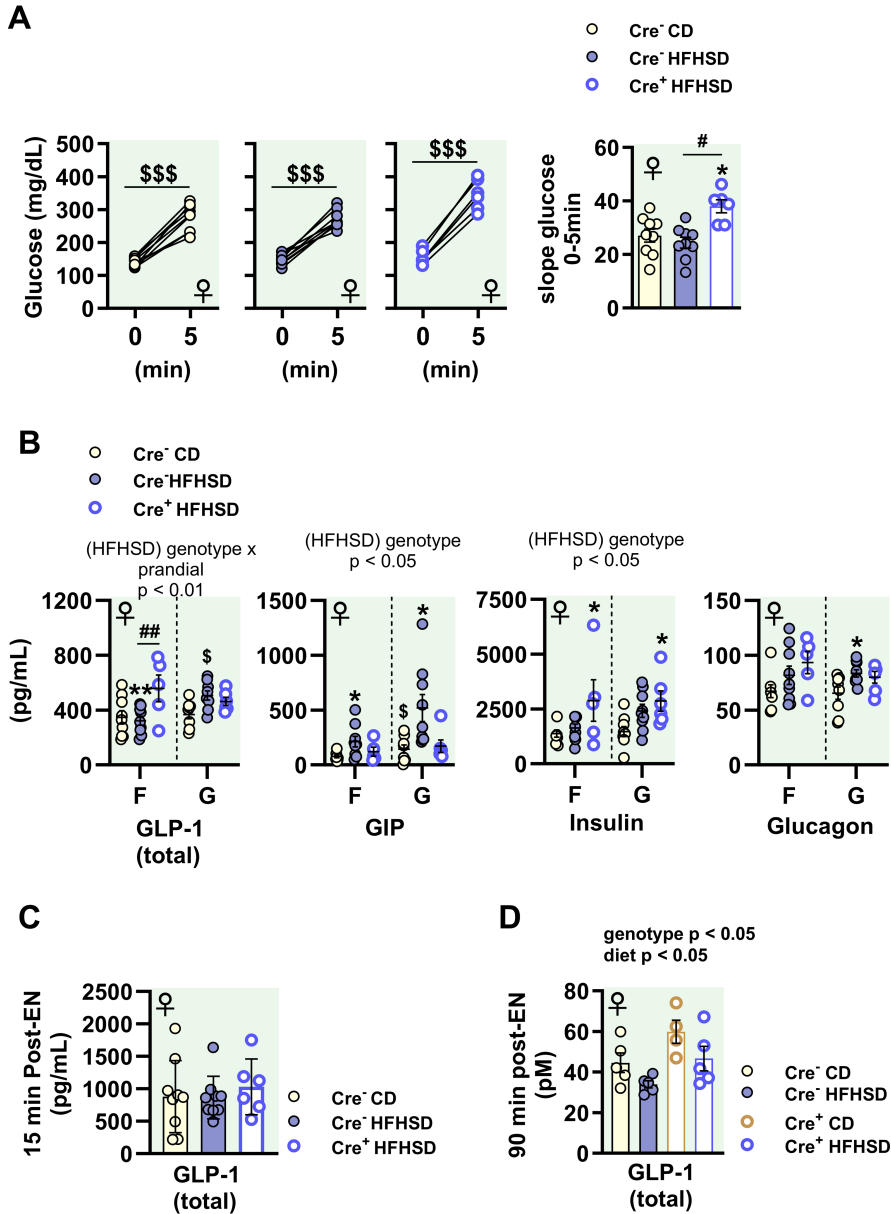


Figure 4. Deficiency of Nav1.8⁺ neurons increases plasma levels of total GLP-1 and insulin in female mice fed high-fat high-sugar diet.

In female controls (Cre⁻) fed control or high-fat high-sugar diet (CD or HFHSD, respectively) and mice lacking Nav1.8⁺ neurons (Cre⁺) fed HFHSD, we measured: **(A)** Fasting glycemia and 5 min after an oral glucose load and the slope between these two points, indicating glucose intestinal uptake, after 7 weeks of CD- or HFHSD-feeding. **(B)** Plasma levels of total GLP-1, GIP, insulin and glucagon, in fasting (F) and 15 min after an oral glucose load (G), after 7 weeks of CD- or HFHSD-feeding. **(C)** Plasma levels of total GLP-1 15 min after the oral administration Ensure® (EN). **(D)** Plasma total GLP-1 levels 90 min after administering Ensure®. Data are shown as the mean $\pm$ SEM or paired individual values. Cre⁻ CD/HFHSD, $n = 5-9$, Cre⁺ CD = 4 and Cre⁺ HFHSD,

n = 5–6. (A): Paired t-test; (slope data in A and C): One-way ANOVA followed by Tukey's *post-hoc* test. B (HFHSD data) and D: Two-way ANOVA followed by Tukey's *post-hoc* test when interactions were detected. Main effects and interactions are indicated on top of the graphs. (B): One-way ANOVA followed by Tukey's *post-hoc* test for comparisons with Cre⁻ CD group. *p<0.05 and ***p<0.001 vs Cre⁻ CD-fed mice. #p<0.05, ##p<0.01 comparisons between Cre⁻ and Cre⁺ fed HFHSD. \$p<0.05 and \$\$\$p<0.001 F vs G.

Loss of Nav1.8+ neurons increases the density of intestinal cells expressing GLP-1 in female mice fed a high-fat high-sugar diet

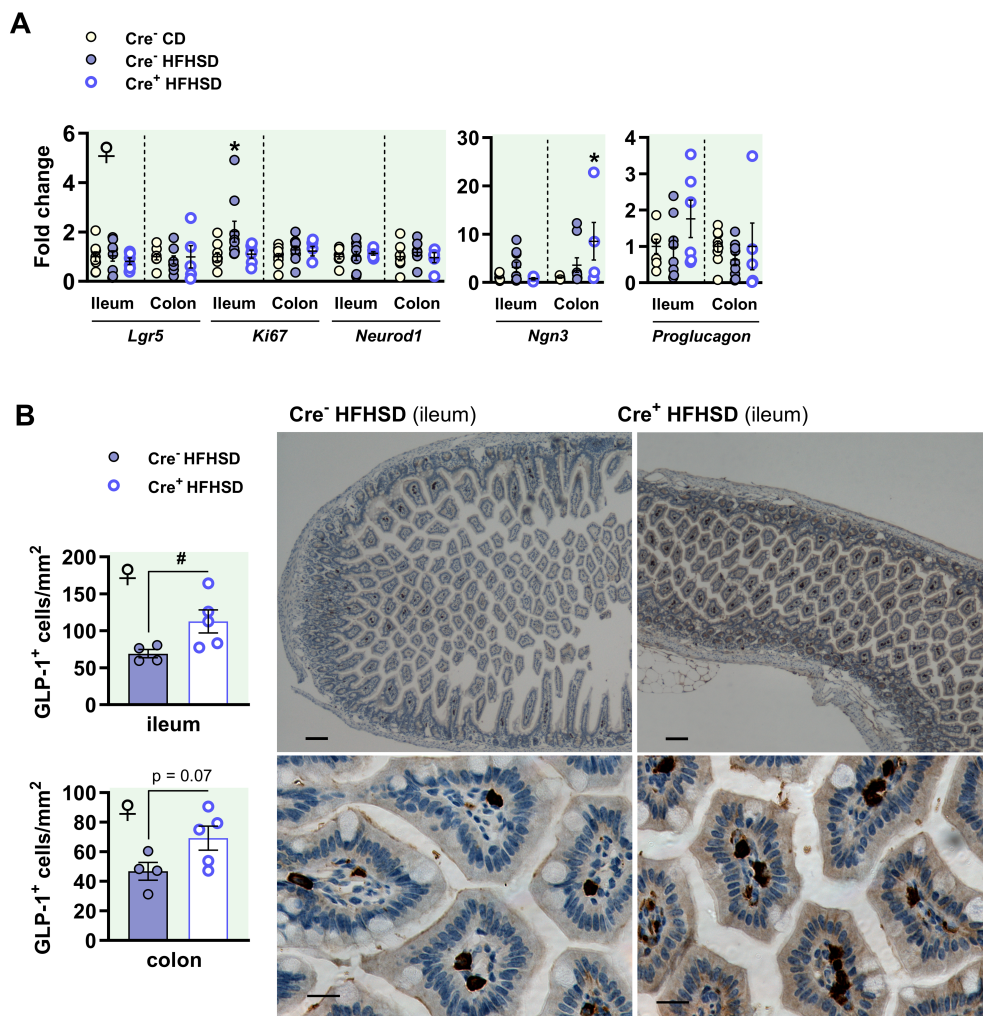
Gene expression analysis of markers of stem cells, proliferation and L-cell differentiation after oral Ensure® challenge revealed that the loss of Nav1.8+ neurons prevented the higher ileal expression of *Ki67* induced by HFHSD-feeding and enhanced the colonic expression of the neuroendocrine cell differentiation marker *Ngn3* as compared with control mice fed CD (**Figure 5A**) suggesting the attenuation of cellular proliferation under HFHSD-feeding and the enhancement of neuroendocrine cell differentiation. Quantification of cells expressing GLP-1 in the distal gut in HFHSD-fed mice revealed higher cellular density in the ileum of Nav1.8-cre/DTA mice than in control mice and a trend for a higher density in colon (p = 0.07) (**Figure 5B**).

Lack of Nav1.8-expressing neurons leads to faster gastrointestinal transit in high-fat high-sugar diet-fed female mice

We reasoned that the absence of Nav1.8+ neurons might also affect the intestinal efferent tone and/or the ENS, in turn modulating gut transit and the pool of dietary GLP-1 secretagogues in the distal gut.

Gene expression analysis of neural markers involved in gut motility (relaxation or contraction) revealed lower ileal levels of the noradrenaline and VIP receptors (*Adrb2* and *Vipr2*, respectively), involved in relaxation processes, in Nav1.8-cre/DTA mice than in control mice on HFHSD (**Figure 6A**), and higher expression of *Vipr2* in HFHSD-fed controls than in CD-fed controls. Transcripts of the glial cell marker *Gfap* were increased in HFHSD-fed groups irrespective of the genotype (**Figure 6A**). In colon, HFHSD-fed Nav1.8-cre/DTA mice had higher *Adrb2* mRNA levels than both control groups (CD and HFHSD), and increased gene expression of the glutamate receptor (*Grm5*), involved in contraction processes, compared with CD-fed controls (**Figure 6B**).

Functional exploration of gut transit indicated that, compared with CD-fed control mice, HFHSD-feeding in controls, but not in Nav1.8-cre/DTA mice, increased the GI transit time (**Figure 6C**) indicating a partial protection of the Nav1.8+ neurons ablation against the effect induced by the obesogenic diet. In addition, under HFHSD, the ablation of Nav1.8-expressing neurons increased the production of feces normalized by food intake (**Figure 6D** and **Figure S4A** and **S4B**), likely as a consequence of the partial restoration of the GI transit time, as both variables showed a negative correlation (**Figure 6E**).



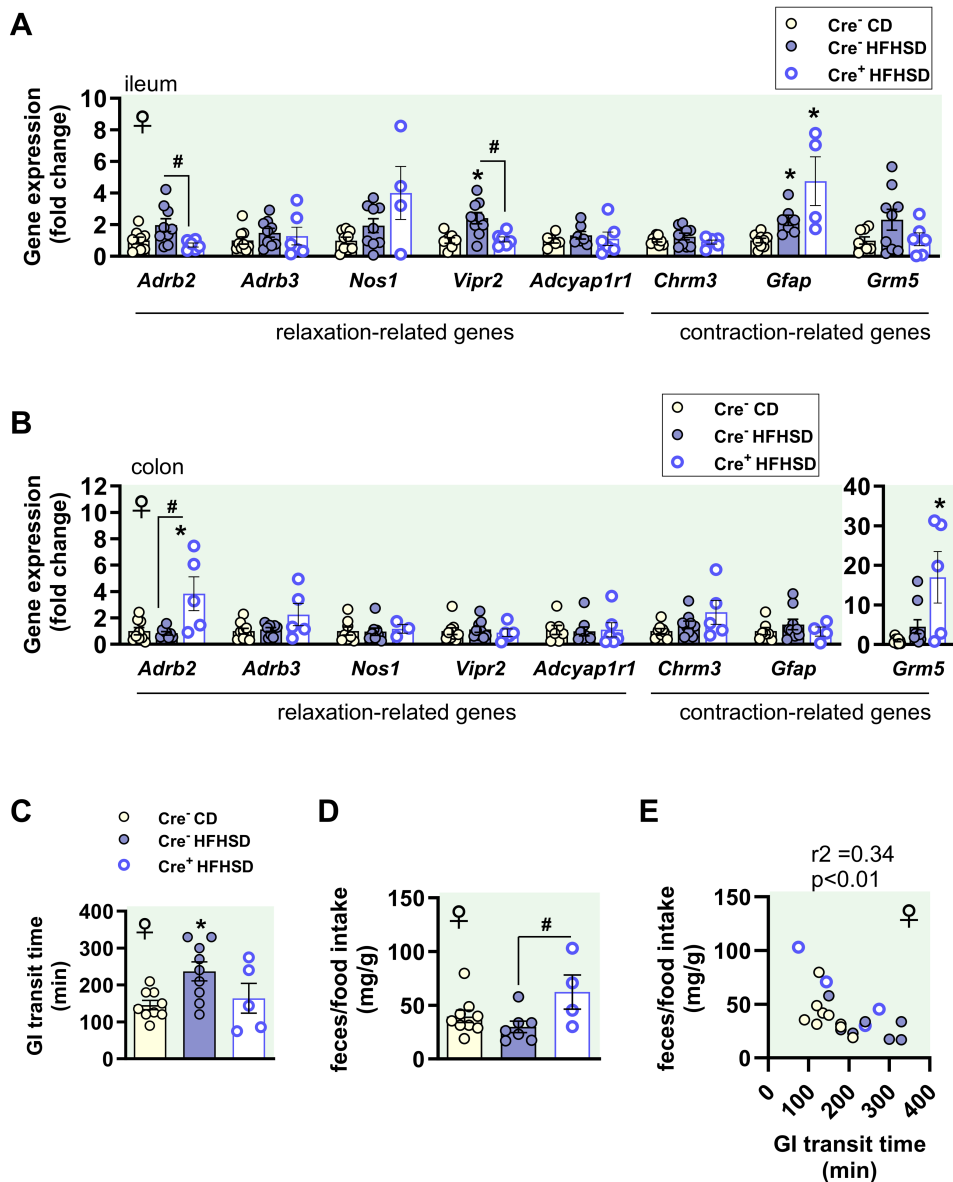


Figure 6. Deficiency of Nav1.8⁺ neurons alters the gene expression of gut motility-related neural markers and accelerates the gastrointestinal transit in female mice fed high-fat high-sugar diet.

In female controls (Cre⁻) fed control diet (CD) or high-fat high-sugar diet (HFHSD) and females lacking Nav1.8⁺ neurons (Cre⁺) fed HFHSD, we measured: **(A and B)** At the end of the experiment, the ileal and colonic mRNA levels of adrenergic receptors (*Adrb2* and *Adrb3*), nitric oxide synthase (*Nos1*), the receptor of VIP and PACAP (*Vipr2* and *Adcyap1r1*), the cholinergic receptor muscarinic 3 (*Chrm3*), the glial marker (*Gfap*) and the glutamate metabotropic receptor (*Grm5*). **(C)** Time required for orally administered carmine red to appear in feces (GI transit time) after 8 weeks of CD- or HFHSD-feeding. **(D)** The production of feces in the carmine red trial normalized by the food intake. **(E)** Correlation between the normalized feces production and the GI transit

time. Data are represented as the mean $\pm$ SEM. Cre⁻ CD/HFHS, n = 8–9; Cre⁺ HFHS, n = 4–6. One-way ANOVA followed by Tukey's *post-hoc* test or the Kruskal-Wallis test followed by Dunn's *Post-hoc* test. * $p < 0.05$ vs Cre⁻ CD-fed mice. # $p < 0.05$ comparisons between Cre⁻ and Cre⁺ fed HFHS.

4. Discussion

Our data support a role for Nav1.8+ sensory neurons on the control of glucose homeostasis in female mice through a mechanism originating in the gut that involves the upregulation of GLP-1 action.

Energy metabolism is differently regulated in females and males. For instance, fat depots show a sex-dependent distribution, with a preferential accumulation in visceral WAT in males, and in subcutaneous WAT in females³², as indicated by preclinical studies. This fat distribution confers protection against metabolic disturbances in females and increases the susceptibility in males, in line with the higher T2D prevalence in men³³. Nevertheless, women, especially with menopause, are more prone to accumulate fat, leading to a higher prevalence of visceral obesity and metabolic syndrome than in men³⁴. Despite these gender/sex-specificities, the development of strategies to improve metabolic health remains underexplored in females, even in preclinical models.

To provide mechanistic explanations of sex-related metabolic differences, we investigated further the recently identified function of Nav1.8+ neurons on energy balance¹⁵⁻¹⁹ with an especial focus on GI tract elements affecting energy metabolism.

Our study demonstrated sex-related influence of these neurons on body weight gain and glucose intolerance in mice on an obesogenic diet.

The relevance of Nav1.8+ neurons on the metabolic phenotype was already demonstrated in a recent work¹⁸. More specifically, the constitutive mTOR activation in Nav1.8+ neurons due to tuberous sclerosis complex 2 gene (*Tsc2*) knocking out in these neurons (Nav1.8-TSC2KO mice) modifies the distribution of adipose tissues in both female and male mice, promoting an obese phenotype, but prevents high fat diet-induced weight gain¹⁸. Our findings further support the role of Nav1.8-expressing neurons on energy metabolism. In particular, using Nav1.8+ neurons-deficient mice, we found that these neurons are dispensable for diet-induced obesity in females, but their absence induces resistance to gain weight in males. We identified that Nav1.8-cre/DTA male mice show diverse responses to HFHS-feeding. The analysis of the body weight gain data in separated quartiles revealed that Nav1.8-cre/DTA male mice from the first quartile gained the same weight than their control littermates in response to HFHS-feeding, which is in line with previous investigations¹⁷. In contrast, male mice from second, third and fourth quartiles gained less weight than their control littermates or even showed body weight loss relative to their initial weight. We also found that, while gonadal fat depots of Nav1.8-cre/DTA female mice were not affected, male mice lacking Nav1.8+ neurons that did not lose weight but showed resistance to obesity (Q2 and Q3) exhibited reduced gWAT with smaller adipocytes. Food intake does not seem to contribute to the resistance to obesity in Nav1.8-cre/DTA male mice. Indeed, loss of Nav1.8+ neurons did not affect *ad libitum* food intake and surprisingly exacerbated HFHS refeeding after fasting, but with minimal weight gain. In this regard, the reduced food efficiency of Nav1.8-cre/DTA male mice fed HFHS supports a low contribution of food as a source for weight gain in these

mice. Defective cholesterol and fatty acid sensing in Nav1.8+ neurons exacerbates energy expenditure³⁵ which could also be the case of the total ablation of Nav1.8-expressing neurons, similarly contributing to attenuate fat storage under HFHSD. Complementary, our findings support that loss of Nav1.8+ neurons diminished lipid absorption after a meal in male mice, which could lead to reduce hypothalamic lipid sensing after a meal, explaining the exacerbated refeeding we have identified.

Importantly, the occasional weight loss we observed in Nav1.8-cre/DTA male mice fed HFHSD was associated with ulcerative dermatitis with skin lesions similar to those shown by Nav1.8-TSC2KO mice¹⁸. Although we did not perform behavioral analyses in Nav1.8-cre/DTA male mice, these skin lesions could be a consequence of an itch-related behavior as exhibited by Nav1.8-TSC2KO mice¹⁸. This sickness-related phenotype was only detected in males fed an obesogenic diet and finally caused death in our model. In agreement with other findings, these observations in Nav1.8-cre/DTA mice could reflect their inability to resolve the lesion-related inflammation due to defective immune response especially under high fat diet, as previously suggested¹⁷.

Since visceral fat is a main contributor to insulin resistance in obesity³², the reduced visceral (gonadal) fat observed in Nav1.8-cre/DTA male mice might favor glucose tolerance. We found sex-dependent effects of Nav1.8+ neurons on this variable. The improved glucose tolerance in male Nav1.8-cre/DTA mice was seen in response to intraperitoneal challenge with glucose, whereas oral glucose tolerance was enhanced in female Nav1.8-cre/DTA mice, suggesting a contribution of the intestinal Nav1.8+ afferents in the control of the postprandial whole-body glucose clearance in females but not in males.

There is evidence that supports improvements in glycemic control in people with obesity and T2D subjected to neuromodulation strategies such as VBLOC therapy^{36,37}. Mechanistically, the inhibition of vagal afferents prevents the PPAR α -mediated glucose intolerance induced by glucocorticoids³⁸, although neither oral glucose tolerance nor sex-associated effects were explored in the aforementioned study. Our present findings demonstrate: (1) a postprandial glucoregulatory role of Nav1.8+ sensory neurons, occurring preferentially in females, (2) particularly those Nav1.8+ neurons innervating the gut, and (3) independently of confounding effects due to body weight variations.

Different explanations for the glucoregulatory effects of Nav1.8+ neurons in obese females are possible. Focusing on GI mechanisms, on the one hand, the absence of Nav1.8+ neurons seemed to provide protection against systemic inflammation, as suggested by the effect of the neuronal ablation preventing splenomegaly, which could be a consequence of the anti-inflammatory tone of the intestine in Nav1.8-cre/DTA mice. Indeed, using the same mouse model, Talbot and colleagues demonstrated that the absence of Nav1.8+ neurons reduces the antigen-induced airway inflammation and bronchial hyperresponsiveness³⁹. Nevertheless, further investigations are required to explore potential neuroimmune interactions between Nav1.8+ neurons and Treg and macrophages in the gut.

We also revealed changes in the enteroendocrine system which via endocrine routes can directly modulate glucose homeostasis in peripheral organs. The ablation of Nav1.8+ neurons impeded GIP oversecretion induced by HFHSD-feeding, which in turn may contribute to preventing the

higher glucagon levels that accounts for the development of T2D ^{40,41}. The attenuated GIP oversecretion under an obesogenic diet in this mouse model may be a consequence of the accelerated GI transit that in turn could reduce the availability of GIP secretagogues in the upper gut ⁴².

On the other hand, the faster intestinal glucose uptake in Nav1.8-cre/DTA mice could lead to an earlier glucose sensing in pancreatic β -cells to induce insulin secretion. In addition, the depletion of Nav1.8+ neurons prevented the reduction of total GLP-1 levels induced by HFHSD-feeding seen in control mice, which could in turn improve pancreatic β -cell function and peripheral insulin sensitivity ⁴³. Altogether, these mechanisms may lead to the oversecretion of insulin observed in Nav1.8-cre/DTA mice independently of the prandial condition, allowing for a more efficient postprandial glucose clearance. Nonetheless, it cannot be ruled out that the insulin oversecretion without meal-related stimuli (i.e., fasting) might finally lead to long-term insulin resistance.

Analysis of the GLP-1 system in Nav1.8-cre/DTA mice revealed that the lack of Nav1.8+ neurons did not improve total GLP-1 secretion after 15 min of oral glucose or Ensure®, which corresponds to the early hormone release regulated by nutrient-mediated neural pathways ⁴⁴. By contrast, whatever the diet, postprandial total GLP-1 was higher in mice lacking Nav1.8+ neurons at 90 min after the oral Ensure®, corresponding to the later GLP-1 release directly driven by nutrients not absorbed in upper regions and reaching the distal gut ⁴⁴. In line with this, the accelerated GI transit shown by female mice lacking Nav1.8+ neurons could contribute to increasing the availability of unabsorbed dietary GLP-1 secretagogues in distal intestinal regions, in turn favoring GLP-1 secretion and L-cell proliferation.

Since GLP-1 reduces gut motility and so increases GI transit time ⁴⁵⁻⁴⁷, we explored alternative mechanisms allowing a transit time reduction in female mice lacking Nav1.8+ neurons. The gene expression analysis of neural markers of the extrinsic and intrinsic nervous system involved in muscle contraction and relaxation in the intestine ⁴⁸⁻⁵² revealed that the depletion of Nav1.8+ neurons upregulated muscle contraction markers and downregulated muscle relaxation markers, in line with the accelerated transit. Complementary, ENS changes due to the depletion of Nav1.8+ neurons may also have an impact on GLP-1, as the neuropeptides secreted by these neurons locally control GLP-1 release ².

Overall, the Nav1.8-cre/DTA phenotype in females resembles some of the metabolic benefits of bariatric surgery, including accelerated GI transit, elevated levels in plasma of GLP-1 and insulin, and improved oral glucose tolerance ⁵. These similarities suggest that, at least in part, the metabolic benefits of bariatric surgery could be a consequence of the loss of Nav1.8+ neurons. Indeed, it has been suggested that these benefits could be caused by the paradoxical increase of sensory inputs resulting from the unintentional vagotomy occurring during bariatric surgery ⁵³, in turn altering efferent visceral outflow and so, the physiology of the innervated organs.

Altogether, our study reveals sex-related differences in the function of Nav1.8+ afferent neurons, which might illuminate a path to personalized neuromodulation therapies that, through specific inhibition, could limit fat depots in overweight/obese men or improve postprandial glycemia in women with T2D. Notably, we describe that the improved postprandial glucose clearance in females due to ablation of Nav1.8+ afferents is aligned with intestinal adaptations leading to an

amplified GLP-1 secretion and so, insulin secretion. These results open new possibilities for T2D treatment specifically in women based on non-invasive strategies by silencing Nav1.8-expressing neurons that mimic bariatric surgery effects. Further studies are required to determine the spinal or vagal nature of the Nav1.8+ afferent neurons controlling energy homeostasis in a sex-dependent manner.

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Author Contributions

MRP, CVB and YS designed the study. MRP and CBV conducted the *in vivo* experiments and MRP supervised the research. CBV conducted the sampling procedures. MRP and CBV analyzed the data. MRP prepared figures and drafted the manuscript. YS edited the manuscript and supervised the project.

Data Availability Statement

Data available on request from the corresponding author.

Conflict of interests

The authors declare no competing interests.

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6. Supplementary material

Supplemental Tables

Table S1. Primer pairs and sequences used to detect Nav1.8 knock-in Cre and floxed DTA alleles and their respective wild-type fragments

Genotype	Primer's pairs	Name of primer's pairs	Amplicon length
Wild-type or Nav1.8 knock-in Cre-recombinase	Common forward / wild-type reverse	mSNS13s/mSNS12a	438bp
	Common forward / Nav1.8 knock-in Cre reverse	mSNS13s/Cre5a	461bp
	Nav1.8 knock-in Cre forward / Nav1.8 knock-in Cre reverse	Cre2s/Cre5a	249bp
Wild-type or floxed DTA	Common forward / DTA reverse	ROSA26 F/floxed DTA	340bp
	Common forward / common reverse	ROSA26 F/ROSA 26 R	650bp
Primer name	5'-3' sequence		
mSNS13s	TGT AGA TGG ACT GCA GAG GAT GGA		
mSNS12a	TTA CCC GGT GTG TGC TGT AGA AAG		
Cre5a	AAA TGT TGC TGG ATA GTT TTT ACT GCC		
Cre2s	CTG CAT TAC CGG TCG ATG CAA CGA		
ROSA26 F	AAA GTC GCT CTG AGT TGT TAT		
floxed DTA	GCG AAG AGT TTG TCC TCA ACC		
ROSA 26 R	GGA GCG GGA GAA ATG GAT ATG		
PCR conditions			
Primer pair	Initial denaturation	Denaturation-annealing-extension	Final extension
mSNS13s/mSNS12a	94°C (2 min)	94°C (30 s)-63°C (45 s)-72°C (1 min) x 34 cycles	72°C (10 min)
mSNS13s/Cre5a and Cre2s/Cre5a	94°C (2 min)	94°C (30 s)-62°C (30 s)-72°C (1 min) x 29 cycles	72°C (10 min)
ROSA26 F/floxed DTA and ROSA26 F/ROSA 26 R	98°C (3 min)	95°C (20 s)-60°C (45 s)-72°C (20 s) x 30 cycles	72°C (5 min)
TaqMan® Gene Expression Assays used for validation of the Nav1.8 genetic ablations			
Target name	Assay reference	Assay ID	Amplicon length
Scn10a (Nav1.8)	4448892	Mm00501467_m1	82bp
Rpl19	4331182	Mm02601633_g1	69bp
Pre-amplification conditions: 95°C (10 min) – [95°C (15 s) - 60°C(4 min)] x 10 cycles – 99°C (10 min). Eppendorf thermocycler qPCR: 50°C (2 min) - 95°C (20 s) - [95°C (3 s) - 60 °C (30 s)] x 40 cycles. LightCycler® 480 Instrument (Roche).			

Table S2. Composition of the diet

COMPOSITION (%)		
	Control diet (CD) D12450K	High-fat High-sugar diet (HFHSD) D12451
Protein: Casein, L-Cystein	19.24	23.66
Carbohydrate: Sucrose, Lodex 10, starch, Corn	66.73	40.73
Fiber: Solka Floc, FCC200	4.74	5.83
Fat: Lard, Soybean oil	4.26	26.51
Fatty acids: C12:0	0.01	0.05
Fatty acids: C14:0	0.04	0.29
Fatty acids: C16:0	0.69	5.33
Fatty acids: C18:0	0.31	2.92
Fatty acids: C20:0	0.02	0.07
Fatty acids: C16:1	0.05	0.62
Fatty acids: C18:1	1.30	9.42
Fatty acids: C18:2	1.43	3.46
Fatty acids: C18:3	0.15	0.37
Minerals	4.74	5.83
Vitamin premix	0.28	0.35
ENERGY CONTENT (% KJ)		
	Control diet (CD) D12450K	High-fat High-sugar diet (HFHSD) D12451
Protein	20	20
Carbohydrate	70	35
Fat	10	45

Table S3. Genes, primer pair sequences and conditions of the qPCR

Gene name	Abbreviation	Sequence 5'-3'
Adenylate cyclase activating polypeptide 1 receptor 1	<i>Adcyap1r1</i>	Forward: TATTACTACCTGTCGGTGAAG Reverse: ATGACTGCTGTCCTGCTC
Adrenergic receptor, beta 2	<i>Adrb2</i>	Forward: CAAGTTCGAGCGACTACAAA Reverse: CTTGAAGGGCGATGTGATAG
Adrenergic receptor, beta 2	<i>Adrb3</i>	Forward: TAGGGAAAAGAGAGCACCCC Reverse: TGGTCCAAGATGGTGCTTAG
Agouti-related neuropeptide	<i>Agrp</i>	Forward: AGGTCTAAGTCTGAATGGC Reverse: CGGTTCTGTGGATCTAGC
Cholinergic receptor muscarinic 3	<i>Chrm3</i>	Forward: ACCTGTTACACGACCTACATCA Reverse: AGTGAGTGGCCTGGTAATAGAAA
Cocaine- and amphetamine-regulated transcript	<i>Cart</i>	Forward: AAGAAGTCCTGAAGAAGCTC Reverse: CAAGCACTTCAAGAGGAAAG

Gene name	Abbreviation	Sequence 5'-3'
Farnesoid X-activated receptor	<i>Fxr</i>	Forward: TGTGAGGGCTGCAAAGTTT Reverse: ACATCCCCATCTCTCTGCAC
Free fatty acid receptor 1	<i>Ffar1</i>	Forward: TTTCATAAACCCGGACCTAGGA Reverse: CCAGTGACCAGTGGGTTGAGT
Free fatty acid receptor 4	<i>Ffar4</i>	Forward: ATCTTGATCCAAAACCTCCG Reverse: GTAAAAATGGCTCCCTTCTC
G protein-coupled bile acid receptor 1	<i>Gpbar1</i>	Forward: GCTCCTGTCAGTCTTGGCCTAT Reverse: TTCCTCGAAGCACTCGTAGACA
Glial fibrillary acidic protein	<i>Gfap</i>	Forward: TCCTGGAACAGCAAAACAAG Reverse: CAGCCTCAGGTTGGTTTCAT
Glutamate receptor, metabotropic 5	<i>Grm5</i>	Forward: ACGAAGACCAACCGTATTGC Reverse: AGACTTCTCGGATGCTTGA
Leucine rich repeat containing G protein-coupled receptor 5	<i>Lgr5</i>	Forward: CCTACTCGAAGACTTACCCAGT Reverse: GCATTGGGGTGAATGATAGCA
marker of proliferation Ki-67	<i>Ki67</i>	Forward: CAGACTTGCTCTGGCCTACC Reverse: GGTGGCGTTTCTCTCTTT
Neurogenic differentiation 1	<i>Neurod1</i>	Forward: AGGAATTCGCCACGCAGAAG Reverse: CTCCTCTGCATTTCATGGCTTCAAG
Neurogenin 3	<i>Ngn3</i>	Forward: ACTCAGCAAACAGCGAAGAAG Reverse: CAGTGCCCAGATGTAGTTGTG
Neuropeptide y	<i>Npy</i>	Forward: AATCTCATCACCAGACAGAG Reverse: CTTTCCTTCATTAAGAGGTCTG
Nitric oxide synthase 1	<i>Nos1</i>	Forward: CCTGTGCGAGATCTTCAAGC Reverse: GAGACGCTGTTGAATCGGAC
Porglucagon	<i>Proglucagon</i>	Forward: CAAACCAAGATCACTGACAAGAAAT Reverse: GGGTTACACAATGCTAGAGGGA
pro-opiomelanocortin-alpha	<i>Pomc</i>	Forward: AAAAGAGGTTAAGAGCAGTG Reverse: ACATCTATGGAGGTCTGAAG
ribosomal protein L19 (housekeeping)	<i>Rpl19</i>	Forward: CCTTGTCTGCCTTCAGCTTGT Reverse: GAAGGTCAAAGGGAATGTGTTCA
solute carrier family 2 member 2	<i>Slc2a2</i>	Forward: TTGTGCTGCTGGATAAATTC Reverse: AAATTCAGCAACCATGAACC
solute carrier family 5 member 1	<i>Slc5a1</i>	Forward: GCCCTTTATACAATCACAGG Reverse: AAGGCTTCATATCCTCTAC
Vasoactive intestinal peptide receptor 2	<i>Vipr2</i>	Forward: CTCCTGGTAGCCATCCTT Reverse: ATGCTGTGGTCGTTTGTG
Conditions of the qPCR reactions - denaturation: 95°C (10 min); 45 amplification cycles: 95°C (10 s), 60°C (30 s), and 72°C (5 s); final melting stage: 95°C (5 s), and 65°C (1 min) All primer pairs were supplied by Isogen Life Science		

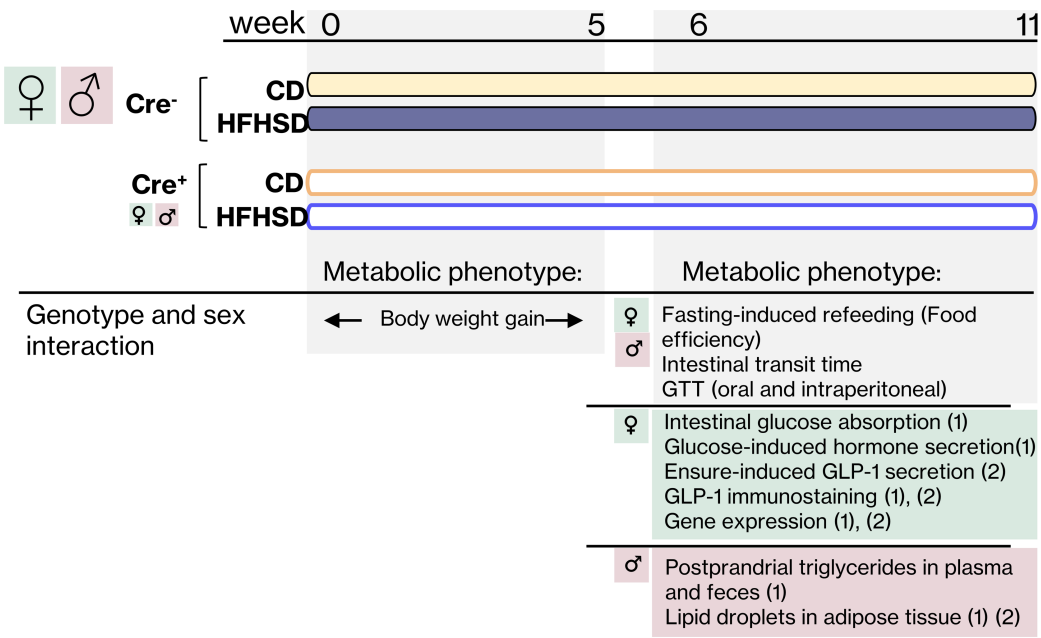
Table S4. Anti-mouse antibodies against immune markers to quantify immune cells in the lamina of the small intestine propria by flow cytometry

Immune cells	markers	antibodies	Supplier	References
Macrophages	M1: F4/80+ CD80+ iNOS+	FITC-conjugated anti-F4/80+	Miltenyi Biotec	130-102-327
	M2: F4/80+ CD206+ Arg1+	Pe-Vio770-conjugated anti-CD80	Miltenyi Biotec	130-102-372
		APC-conjugated anti-iNOS*	Thermo Fisher Scientific	17-5920-80
		PerCPCy5.5-conjugated anti-CD206	Biolegend	141716
		PE-conjugated anti-Arg1*	R&D Systems	IC5868P
CD4+ T cells	Treg: CD4+ CD25+ Foxp3+	BV711-conjugated anti-CD4	BD-Bioscience	563050
		APCCy7-conjugated anti-CD25	BD-Bioscience	557658
		PE-conjugated anti-Foxp3*	Miltenyi Biotec	130-111-678
CD4+ T cells	Memory T cells: CD4+ CD44 ^{high} CD62 ^{low}	BV711-conjugated anti-CD4	BD Bioscience	563050
		PerCP-Cy™5.5-conjugated anti-CD44	BD-Bioscience	560570
	Effector T cells: CD4+ CD44- CD62-	APC-conjugated anti-CD62	BD-Bioscience	561919

**antibodies against intracellular markers*

Supplemental Figures

A



- (1) Cre⁺ female and male mice-fed CD groups were not included in the assay
(2) Post-mortem analysis (w11)

Sample size	♀				♂			
	Cre ⁻		Cre ⁺		Cre ⁻		Cre ⁺	
Cohort	CD	HFHSD	CD	HFHSD	CD	HFHSD	CD	HFHSD
1	6	6	4	4	8	8	7	5
2	5	5	4	5	10	10	6	7
3	9	9		6	5	7		15

B

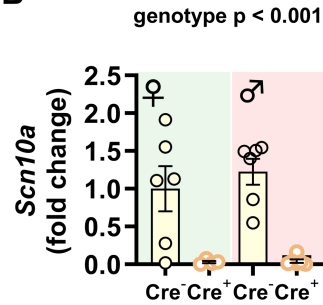


Figure S1. (A) Graphical representation of the experimental procedure: Mice lacking Nav1.8+ neurons (Cre^+) and their control littermates (Cre^-) were randomly allocated to control diet (CD) or high-fat high-sugar diet (HFHSD) groups (Cre^- CD/HFHSD and Cre^+ CD/HFHSD). Mice had free access to CD or HFHSD for 11 weeks. Body weight (Bw) was monitored weekly and different functional tests were conducted between week 6 and 11, including fasting-induced refeeding, intestinal gastric time assessment, glucose tolerance tests (GTT), intestinal glucose absorption and glucose-induced hormone secretion. At the end of the experiment, we also analyzed the Ensure®-induced GLP-1 secretion, intestinal cells expressing GLP-1, postprandial triglycerides in feces and plasma, adipocyte size, and the expression of key genes in energy homeostasis control. **(B)** Validation of the ablation of Nav1.8-expressing neurons in the offspring by gene expression analysis of *Scn10a* coding for NAV1.8 in nodose ganglia samples from Cre^-/Cre^+ female/male mice.

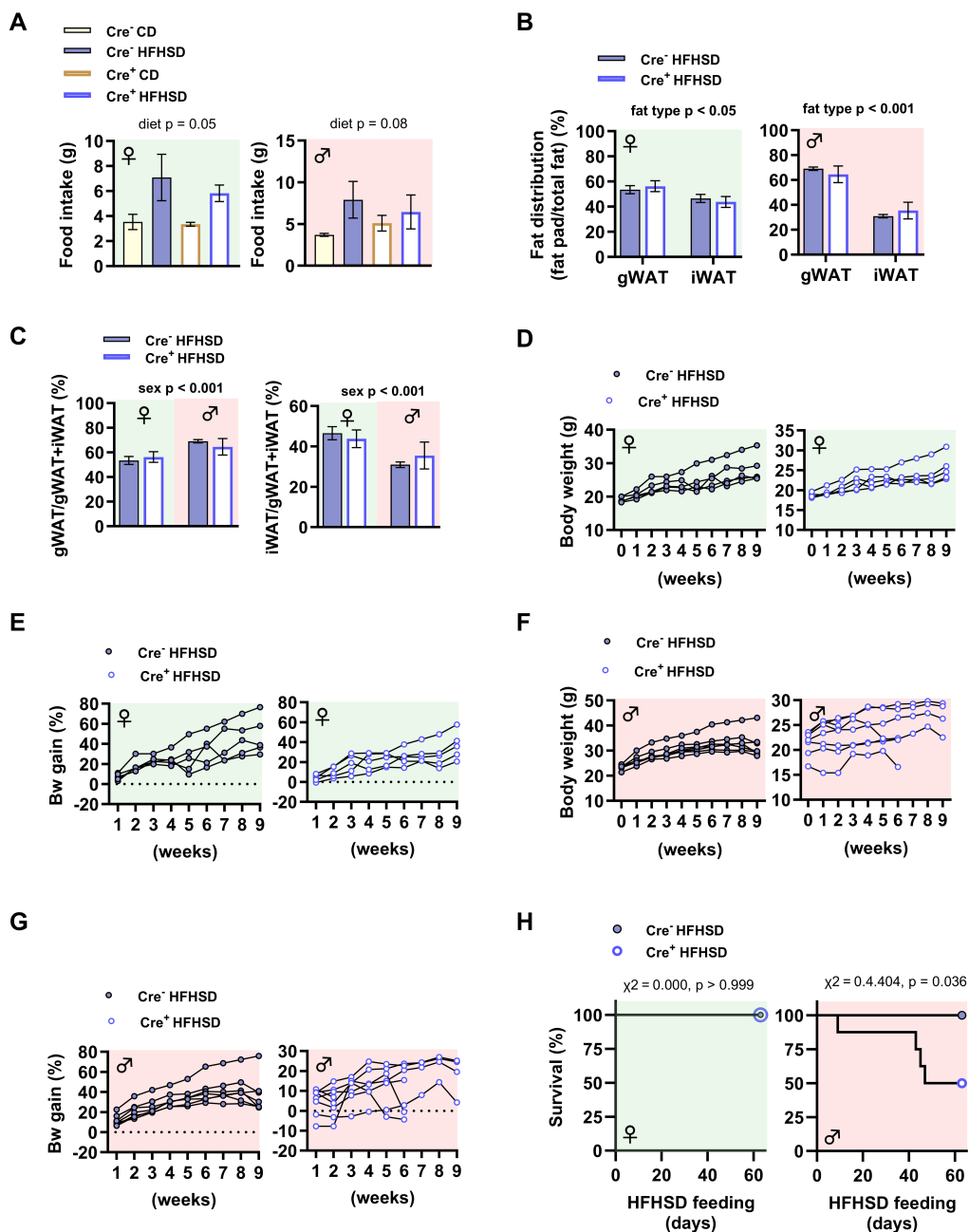


Figure S2. In male and female control mice (Cre⁻) or male and female mice lacking Nav1.8+ neurons (Cre⁺), we measured: **(A)** *Ad libitum* food intake under control diet (CD) and high fat high sugar diet (HFHSD). **(B and C)** Fat distribution of HFHSD-fed mice measured as the percentage of individual fat pad mass relative to gWAT plus inguinal WAT (iWAT) (total fat pad mass). **(D-G)** Effects of long-term exposure (9 weeks) to HFHSD on weekly body weight in grams (D and F) and percentage of body weight variations (increase or decrease)

relative to the initial body weight (E and G). (H) Survival of Cre- and Cre+ female (left graph) and male (right graph) mice over 9 weeks of HFHSD-feeding. (A-C): Data are shown as the mean $\pm$ SEM. (D-G): connected plots indicated individual values; (H): Groups (Cre- or Cre+) are plotted using survival curves in which each drop of the staircase pattern represents a death in a time-point (X axis) and the corresponding percentage of survival (Y axis). n = 9-6 per group. (A-C): Two-way ANOVA with main effects indicated on top of the graphs. (H): Survival was analyzed using Log-rank (Mantel-Cox) Chi square test.

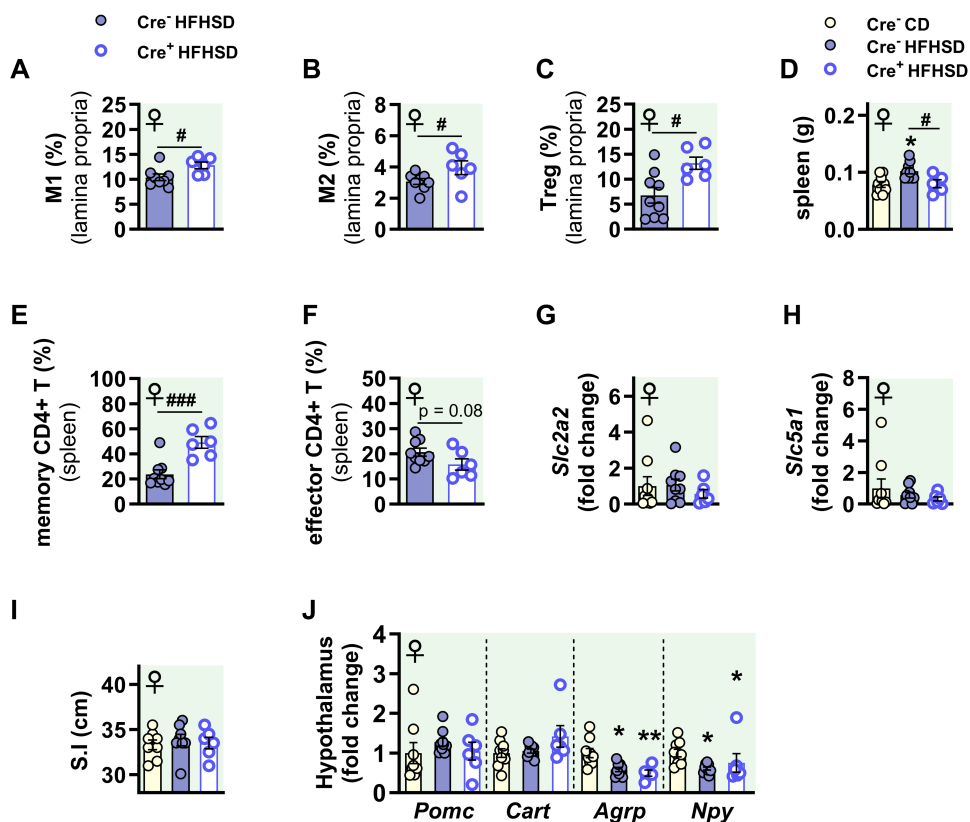


Figure S3. In female control mice (Cre-) fed control or high-fat high-sugar diet (CD or HFHSD) or female mice lacking Nav1.8+ neurons (Cre+) fed HFHSD, we measured: (A-C) Levels of type 1 and type 2 macrophages and regulatory T cells (M1, M2, Treg) in the lamina propria. (D) Weight of the spleen. (E and F) Levels of memory and effector CD4+ T cells in spleens. (G) Gene expression of *Slc2a2* coding for GLUT2 in the duodenum. (H) Gene expression of *Slc5a1* coding for SGLT1 in the duodenum. (I) Length of the small intestine (S.I.). (J) Gene expression of hypothalamic neuropeptides *Pomc*, *Cart*, *Agrp*, and *Npy*. Data are shown as the mean $\pm$ SEM Cre- CD/HFHSD, n = 8-9 and Cre+ HFHSD, n = 4-6. (D, H and I): One way ANOVA followed by Tukey's *post hoc* test; (A-C, and E and F): Student's t test. #p<0.05 comparisons between Cre- and Cre+ fed HFHSD and ***p<0.001 vs Cre- CD-fed mice.

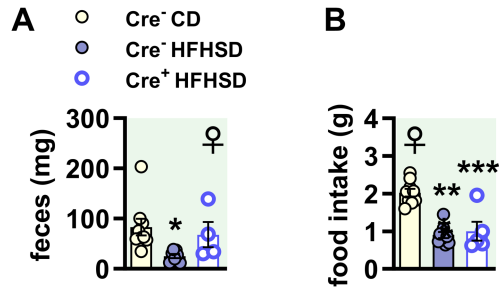


Figure S4. In female control mice (Cre⁻) fed control or high-fat high-sugar diet (CD or HFHSD) or female mice lacking Nav1.8+ neurons (Cre⁺) fed HFHSD, we measured: **(A)** Produced feces during the assessment of the gastric time. **(B)** Food intake during the assessment of the GI time. Data are shown as the mean $\pm$ SEM Cre⁻ CD/HFHSD, n = 8-9 and Cre⁺ HFHSD, n = 4-6. One way ANOVA followed by Tukey's *post hoc* test; *p< 0.05 and ***p<0.001 vs Cre⁻ CD-fed mice.

Chapter 2

Chapter 2

Nav1.8-expressing neurons control daily oscillations of food intake, body weight and gut microbiota in mice

Clara Bullich-Vilarrubias^{1*}, Marina Romaní-Pérez^{1*}, Inmaculada López-Almela^{1,3}, Teresa Rubio¹, Carlos García Hernández-Gil², Francisco A. Tomás-Barberán², Yolanda Sanz¹

¹ Microbiome, Nutrition and Health Unit, Institute of Agrochemistry and Food Technology, Spanish National Research Council (IATA-CSIC), Valencia, Spain.

² Quality, Safety and Bioactivity of Plant Foods, CEBAS-CSIC, Murcia, Spain.

³ Present address: Research Group Intracellular Pathogens: Biology and Infection, Department of Animal Production and Health, Veterinary Public Health and Food Science and Technology, Faculty of Veterinary Medicine, Cardenal Herrera-CEU University, Valencia, Spain.

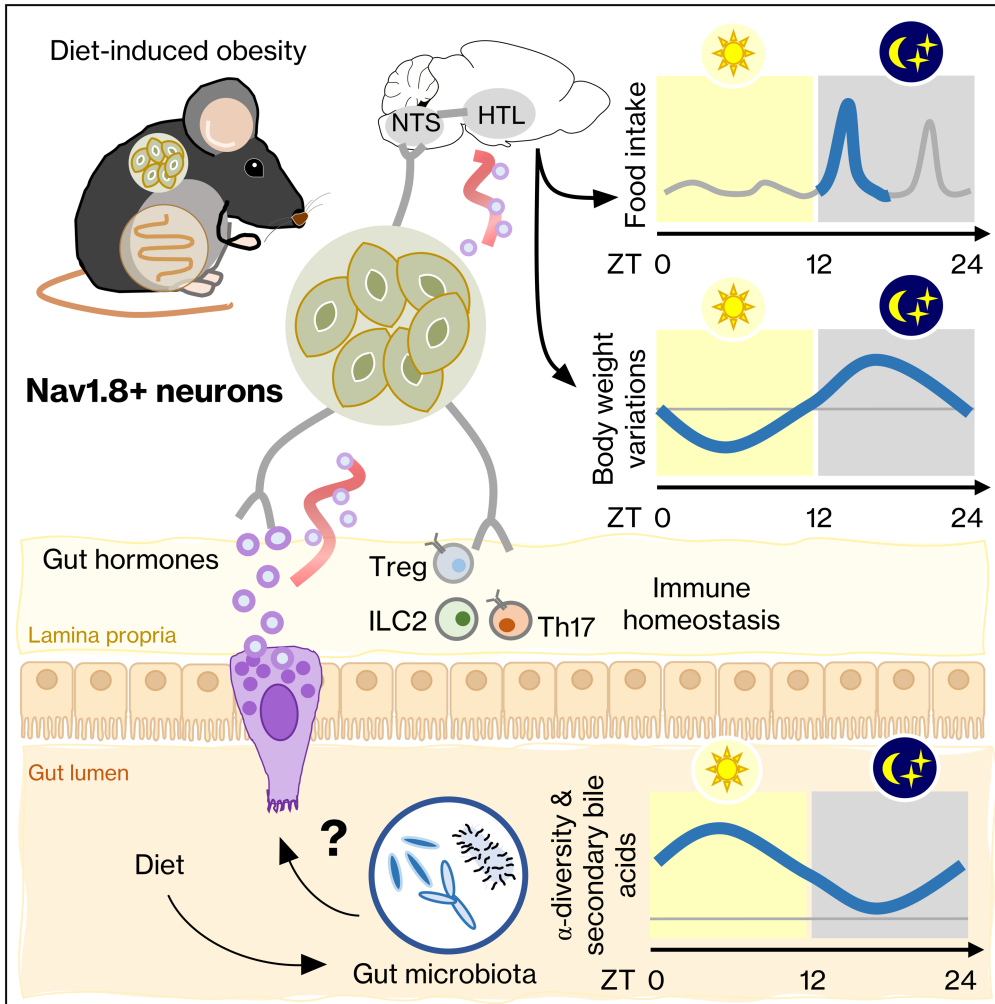
*Equal contribution

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My contribution to this research article was: Investigation and Methodology, Data curation, Manuscript Review.

Chapter 2

Graphical Abstract



Nav1.8-expressing neurons control daily oscillations of food intake, body weight and gut microbiota in mice

Abstract

Recent evidence suggests a role of sensory neurons expressing the sodium channel Nav1.8 on the energy homeostasis control. Using a murine diphtheria toxin ablation strategy and *ad libitum* and time-restricted feeding regimens of control or high-fat high-sugar diets, here we further explore the function of these neurons on food intake and on the regulation of gastrointestinal elements transmitting immune and nutrient sensing. The Nav1.8+ neuron ablation increases food intake in *ad libitum* and time-restricted feeding, and exacerbates daily body weight variations. Mice lacking Nav1.8+ neurons show impaired prandial regulation of gut hormone secretion and gut microbiota composition, and altered intestinal immunity. Our study demonstrates that Nav1.8+ neurons are required to control food intake and daily body weight changes, as well as to maintain physiological enteroendocrine and immune responses and the rhythmicity of the gut microbiota, which highlights the potential of Nav1.8+ neurons to restore energy balance in metabolic disorders.

1. Introduction

The peripheral nervous system (PNS) allows the communication between the central nervous system (CNS) and the peripheral tissues. The afferent or sensory arm of PNS sends information from the periphery to the CNS while the efferent innervations transmit effector signals from the CNS to the peripheral tissues to maintain homeostasis.

Sensory ganglia contain the cell bodies of pseudo-bipolar sensory neurons. Spinal neurons of the dorsal root ganglia (DRG) and vagal sensory neurons of the nodose ganglia transmit somatosensory and visceral information, respectively, to the CNS. The majority of the spinal and vagal sensory neurons express the sodium channel Nav1.8^{1,2}. Spinal sensory neurons expressing Nav1.8 are involved in nociception and pain^{3,4}, while Nav1.8+ vagal sensory neurons in the nodose ganglia mediate the viscerosensory transmission from lung and GI tract as suggest prediction-based studies⁵. Targeted expression of dTomato in Nav1.8+ neurons in mice through Cre-LoxP-based methods reveals that Nav1.8-expressing afferents innervate the intestinal mucosa and the intraganglionic laminar endings (IGLEs) of the myenteric plexus^{1,6}. Complementary vagotomy experiments confirm the vagal origin of these afferents^{1,7}.

Moreover, Nav1.8+ nodose ganglion neurons express G protein coupled receptors (GPCRs) for gut hormones, immunomodulatory lipids, microbial-derived metabolites and receptors involved in neurotransmitters signaling⁸, suggesting a relevant interaction of these neurons with key GI paths whereby energy and immune homeostasis are controlled. Indeed, spinal and vagal Nav1.8+ neurons are reported to protect against invasion, colonization and dissemination of enteric pathogens, by modulating intestinal mucosal immune cells⁹. All these evidences suggest that, beyond nociception and pain, intestinal spinal and/or vagal Nav1.8+ neurons might be important to mediate gastric distension, and nutrient and immune sensing from the intestine to the brain, suggesting their relevance in maintaining energy homeostasis in response to fat-enriched diets. In this regard, under an obesogenic diet, Nav1.8-expressing neurons are required to limit acute inflammation due to dietary lipids and have a modest role on meal patterns, since mice lacking these neurons do not show the reduced meal size and the increased feed rate detected in control littermates in response to high fat diet¹⁰. In addition, recent publications indicate that stimulation of IGLEs mechanoreceptors, highly expressing Nav1.8^{1,6}, reduces food intake after a meal by transmitting gastric distension to the brain for further inhibiting AgRP-orexigenic neurons⁶. Preclinical studies that characterize genetic markers of sensory neurons combined with targeted neuronal manipulation identified specific functions of sensory neurons of the PNS^{6,11,12}. This evidence would help to establish the basis for the design of new strategies to restore the disturbed neural control of the energy homeostasis in obesity and associated metabolic disorders. In this regard, a better understanding of how Nav1.8+ neurons maintain the energy homeostasis pre- and postprandially, particularly in response to high energy-dense foods and to interventions for weight loss, such as time-restricted feeding, might be key to identify novel strategies for restoring energy balance.

Here, we used Cre-LoxP technology to selectively ablate Nav1.8+ neurons in mice, allowing us to examine their role in energy balance. We show that Nav1.8+ neurons are required to control food intake and body weight fluctuations, as well as to maintain prandial regulation of gut hormone

secretion, daily oscillations of gut microbiota and bile acid (BA) composition, and immunity under different dietary regimens, including *ad libitum* and time-restricted feeding under a control diet (CD) or a high-fat, high-sugar diet (HFHSD). Our results highlight the potential of Nav1.8+ neurons to restore energy homeostasis in metabolic disorders.

2. Materials and Methods

Generation and validation of Nav1.8-cre/DTA mice

Heterozygous Nav1.8 knock-in Cre-recombinase male mice (EM:04582, EMMA-infracore repository, Munich, Germany) were crossed with homozygous ROSA26-eGFP-DTA female mice (stock #006331; The Jackson Laboratory) to obtain 1:1 litters of Nav1.8-deficient mice (mice expressing Cre and DTA alleles: Nav1.8-cre/DTA, indicated as Cre⁺ in Figures) and control mice (mice with DTA but without Cre allele: control littermates, indicated as Cre⁻ in Figures). Mice were bred in barrier facility using ventilated racks and cages at the animal facility of the University of Valencia (Animal Production Section, SCSIE, University of Valencia, Spain). Only male offspring were employed in the present study, females were employed for other investigations. Genomic DNA from ear punches of male mice was used for PCR; wild-type or Nav1.8-Cre fragments and wild-type and floxed DTA fragments were amplified in an Eppendorf thermocycler using the Phusion High-Fidelity PCR Kit (Thermo Fisher Scientific) and primer sequences detailed in **Table S1**. For the mSNS13s/mSNS12a primer pair PCR, DNA was incubated at 94°C for 2 min followed by 34 cycles of denaturation at 94°C for 30 s, annealing at 63°C for 45 s and extension at 72°C for 1 min, with a final step at 72°C for 10 min. For the mSNS13s/Cre5a and Cre2s/Cre5a primer pair PCR, DNA was incubated at 94°C for 2 min followed by 29 cycles of denaturation at 94°C for 30 s, annealing at 62°C for 30 s and extension at 72°C for 1 min, with a final step at 72°C for 10 min. For the ROSA26 F/floxed DTA and ROSA26 F/ROSA 26 R primer pair PCR, DNA was incubated at 98°C for 3 min followed by 30 cycles of denaturation at 95°C for 20 s, annealing at 60°C for 45 s and extension at 72°C for 20 s, with a final step at 72°C for 5 min. The amplified fragments were separated by electrophoresis.

The effectiveness of the Nav1.8 genetic ablation was explored as described previously¹⁰. In brief, RNA from randomly selected nodose ganglia samples was isolated and reverse transcribed with the Ambion® RiboPure Kit (Invitrogen) and SuperScript™ VILO™ Master Mix (Thermo Fisher Scientific), respectively. To analyze the expression of *Scn10a* (coding for Nav1.8), cDNA was first pre-amplified in triplicate with the TaqMan® PreAmp Master Mix (Applied Biosystems, Thermo Fisher Scientific) and incubated at 95°C for 10 min followed by 10 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 4 min, with a final step for enzyme inactivation at 99°C for 10 min in an Eppendorf thermocycler. Pre-amplified cDNA was further amplified by qPCR reactions using TaqMan® Gene Expression Assays (FAM-MGB; Applied Biosystems, Thermo Fisher Scientific; references provided in **Table S1**) and incubated with TaqMan® Fast Advanced Master Mix (Applied Biosystems, Thermo Fisher Scientific) at 50°C for 2 min and at 95°C for 20 s, followed by 40 cycles of denaturation at 95°C for 3 s and annealing/extension at 60°C for 30 s in a LightCycler® 480 Instrument (Roche). *Rpl19* was used as a housekeeping gene. The $2^{-(\Delta\Delta Ct)}$

method was employed to calculate relative gene expression, represented as fold change relative to Cre⁻ mice for each gene.

Mouse experimental design and metabolic phenotyping

Two cohort of male mice were generated to conduct the experiments described in this study.

Figure S1 details the experimental procedure. For all experiments, six-week-old male mice were individually housed and maintained under constant conditions of humidity and temperature ($23 \pm 2^{\circ}\text{C}$) and a regular 12-h light-dark cycle, in which zeitgeber time (ZT)0 corresponds to lights on and ZT12, to lights off.

For experiments using cohort 1 (**Figure S1A**), Nav1.8-ablated mice ($n = 4$) and their control littermates ($n = 6$) were fed *ad libitum* with HFHSD (D12451; 45% of energy from fat and 21% from sucrose, Research Diets) for 8 weeks. Body weight was weekly determined and 24 h-food intake was measured at the end of the experiment. Mice were anaesthetized with an intraperitoneal injection of pentobarbital (50 mg/kg) and then transcardially perfused with PBS followed by 10% neutral buffered formalin. BAT was isolated for immunohistochemistry.

For experiments using cohort 2 (**Figure S1B**), Nav1.8-ablated mice ($n = 13$) and their control littermates ($n = 20$) were fed either CD (D12450K containing 10% of energy from fat and without sucrose, Research Diets) or HFHSD for 14 weeks.

Throughout this period, animals were submitted to the following feeding regimes: 5 weeks of free access to food: *ad libitum* feeding (week 0-5); followed by 3 weeks of free access to food only during the light and then the dark phase sequentially: light- (week 6-8) and dark- (week 9-11) restricted feeding, respectively (LFR and DRF). During the period of *ad libitum* feeding, body weight was monitored weekly. In both LRF and DRF, body weight loss and body weight gain were calculated daily after 12 h of fasting and 12 h of refeeding, respectively. Under these feeding schedules, food intake was also determined at 6 h and at 12 h of the refeeding period for 2 consecutive days, and the feeding response to an oral load (nutritional challenge) of a mixed nutrient solution (Ensure®) was explored. In parallel, feces were collected after 12 h of fasting, and 6 h and 12 h of refeeding. After the food restriction period, mice were switched again to an *ad libitum* feeding schedule for 3 weeks (week 12-14). In the last 24 h of this period, food intake and body weight variations were determined every 6 h and fecal samples were collected. At the end of the experiment (14 weeks), the secretion of energy metabolism-related hormones in response to the oral administration of Ensure® (detailed in subsection 2.4) was determined. Mice were sacrificed by cervical dislocation for sample collection. Adipose tissues (eWAT, iWAT and BAT) and spleens were weighed. Brain, adipose tissue and the first and the last 2-cm of the upper and distal small intestine (duodenum and ileum, respectively) and colon were immediately frozen and stored at -80°C . The remaining small intestines and the spleens were embedded in cold FACS buffer for further isolation of immune cells (detailed in subsections 2.6 and 2.7).

We have complied with all relevant ethical regulations for animal use. All experimental procedures using animals were in accordance with European Union 2010/63/UE and Spanish RD53/2013 guidelines and approved by the ethics committee of the University of Valencia (Animal Production Section, SCSIE, University of Valencia, Spain) and authorized by Dirección General de

Agricultura, Ganadería y Pesca (Generalitat Valenciana) (approval ID 2019/VSC/PEA/ 0020 and 2020/VSC/PEA/0022).

Feeding response to oral administration of Ensure®

12-h fasted mice received by oral gavage saline solution or Ensure® (nutritional challenge) (12.4 kcal/kg body weight Ensure®, Abbot, or the corresponding volume of saline solution) at ZT0, in the LRF schedule or ZT12, in DRF. After 10 min, mice had free access to food to determine food intake after 120 min from when food was provided. Saline trials were conducted before the Ensure® trials for four consecutive days, which was the time required to obtain a regular feeding response. A similar number of trials were performed with Ensure®. Ensure®-induced food intake suppression was calculated as the percentage of food intake suppression after an oral gavage of Ensure® relative to food intake after oral gavage of saline.

In vivo hormone secretion assay

Blood from the submandibular vein was collected after 4 h of fasting and 15 min after an oral gavage of Ensure® (12.4 kcal/kg of body weight) in Microvette® 500 K3E tubes (Sarstedt) containing inhibitors of DPPIV and serine proteases (Pefabloc) (Sigma). Blood was immediately centrifuged and plasma was stored at -80°C until hormone measurement.

Glycemia and hormone measurements

Before blood centrifugation, glycemia was measured using glucose test strips and a glucometer (Contour® Next meter). The levels of glucagon, active ghrelin, insulin, leptin, total PYY, total GIP and total GLP-1 were measured using a MILLIPLEX Mouse Metabolic Hormone Expanded Panel-7 Plex on a Luminex® MAGPIX System (Milliplex, Merck Group). CCK and α MSH were quantified in plasma using Mouse Cholecystokinin 8 (CCK8) ELISA kit (MyBioSource, Quimigen) and Mouse α MSH (Alpha-Melanocyte Stimulating Hormone) ELISA Kit (Elabscience, Quimigen), respectively, on a CLARIOstar Microplate reader (BMG Labtech).

Immunohistochemistry

UCP1 immunostaining was conducted in BAT samples according to standardized protocols by Patologika Laboratorio S.L. (Valencia, Spain). Images were obtained using an Eclipse 90i Nikon wide-field microscope (Nikon Corp., Tokyo, Japan) with a CFI Plan Fluor DIC M/N2 (MRH00200) Nikon dry-air objective (20× or 4×) combined with an optical zoom factor of 0.8×. Fiji software (ImageJ 1.49q Software, National Institutes of Health) and Nis elements BR 3.2 software (Nikon Corp.) were used for quantification according to standardized protocols of the imaging service from IATA-CSIC.

Immune cell isolation from spleen and the small intestinal epithelium and lamina propria

Spleens were gently crushed using the plunger of a syringe and washed with FACS buffer (PBS-10% fetal bovine serum, Thermo Scientific). The splenic suspensions were filtered (70 μ m nylon cell strainers, Biologix) and centrifuged (450 g, 5 min, 4°C). Isolated cells were preserved in cold FACS buffer until immunolabeling.

Small pieces of longitudinally-opened small intestines were incubated twice with calcium/magnesium-supplemented Hank's balanced salt solution (HBSS) (Thermo Fisher Scientific) with 5 mM EDTA, 1 mM DTT and antibiotics (100 μ g/mL streptomycin-100 U/mL penicillin, Merck) for 30 min at 37°C. After each incubation, intestinal solutions were filtered (100-

µm nylon cell strainers, Biologix) and centrifuged to collect epithelial cells. Cells from the lamina propria were isolated by incubating the remaining intestinal tissues twice with HBSS containing antibiotics and 0.5 mg/mL collagenase D, 1 mg/mL DNase I (Roche), and 3 mg/mL dispase II (Sigma) for 30 min at 37°C. Cell suspensions were filtered (70-µm nylon cell strainers, Biologix) and centrifuged (450 g, 5 min, 4°C) to collect lamina propria cells. Cells from both intestinal compartments were preserved in FACS solution until immunolabeling.

Immune phenotyping by flow cytometry

The isolated cells from spleen, intestinal epithelium and intestinal lamina propria were incubated with a combination of flow cytometry antibodies against surface and intracellular immune markers for 30 min at 4°C (protected from light). For intracellular marker immunolabeling, cells were first permeabilized and fixed using a fixation/permeabilization solution (BD Bioscience). The combinations of anti-mouse flow cytometry antibodies for labeling specific immune cell populations are indicated in **Table S2** and **Figure S7**. Spleens were tested for macrophages (type 1 and type 2, M1 and M2, respectively) and CD4⁺ T cells, including memory and effector CD4⁺ T cells. The intestinal epithelium was tested for type 1 innate lymphoid cells (ILC), including natural killer (NK) cells and ILC1. The lamina propria was tested for macrophages (M1 and M2), type 2 and type 3 ILCs (ILC2 and ILC3), regulatory T (Treg) cells and T helper 17 (Th17) cells.

Data acquisition and analysis were performed using a BD LSRFortessa flow cytometer operated with FACS Diva software v.7.0 (BD Biosciences).

RNA isolation and RT-qPCR

Total RNA from hypothalamus, epididymal white adipose tissue, duodenum, ileum and colon was isolated using TRIsure™ reagent (Bioline). 1-2 µg of total RNA was retrotranscribed with high-capacity cDNA reverse transcription kit (Applied Biosystems, Thermo Fisher Scientific) incubated at 25°C (10 min), 37°C (120 min) 85°C (5 min) in an Eppendorf thermocycler. Gene amplification was conducted using 300 nM of gene-specific primer pair sequences (**Table S3**) with a cDNA dilution previously validated in our lab and the LightCycler 480 SYBR Green I Master Mix (Roche) on a LightCycler® 480 Instrument (Roche). Changes of gene expression were calculated based on $2^{-\Delta\Delta C_t}$ method and expressed as fold-change expression relative to the control group. Conditions of the qPCR reactions are indicated in **Table S3**.

Quantification of intestinal acetylcholine, noradrenaline and vasoactive intestinal peptide

For the analyses of acetylcholine (Ach) and noradrenaline (NE), fractions from the upper and distal small intestine (duodenum and ileum, respectively) and colon (~10 mg) were homogenized with 80% acetonitrile with a Tissue Lyser (Qiagen) and centrifuged (9,400g, 10 min, 4°C) to collect supernatants. NE and Ach in the collected supernatants were measured using a linear ion trap quadrupole LC/MS/MS mass spectrometer (QTRAP® 6500 LC-MS/MS System, Sciex) according to standardized protocols by SCSIE (University of Valencia). Analytes were separated with a Kinetex C8 column (1.7 µm, 2.1 × 100 mm, Phenomenex) using formic acid (0.1%) and acetonitrile as mobile phases with a 0.35 mL/min flow rate and a gradient elution program. VIP content in the duodenum and ileum was determined using a mouse vasoactive intestinal peptide (VIP) ELISA Kit (Elabscience).

Bile acid analysis in feces

Chemicals. Acetonitrile and water 0.1% (v/v) formic acid were purchased from J.T. Baker (Deventer), and formic acid was obtained from Panreac. Authentic standards of 3,7-Dihydroxy-5-cholan-24-oic Acid (chenodeoxycholic acid) and 3-Hydroxy-11-oxo-5-cholan-24-oic Acid (3-oxo-chenodeoxycholic acid) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). The N-(3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24-oyl)-glycine (glycocholic acid), N-(3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24-oyl)-taurine (taurocholic), 3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24-oic acid (cholic acid), 3 α ,7 β ,12 α -Trihydroxy-5 β -cholan-24-oic acid (ursocholic), 3 α ,6 β -Dihydroxy-5 β -cholan-24-oic acid (murideoxycholic acid), 3 α ,6 α -Dihydroxy-5 β -cholan-24-oic acid (hyodeoxycholic acid), 3 α ,7 β -Dihydroxy-5 β -cholan-24-oic acid (ursodeoxycholic acid), 3 α ,12 α -Dihydroxy-5 β -cholan-24-oic acid (deoxycholic acid), 3 α -Hydroxy-5 β -cholan-24-oic acid (lithocholic) and 3 β -Hydroxy-5 β -cholan-24-oic acid (isolithocholic) were purchased from Cayman Chemical.

Extraction of bile acids. Bile acids extraction was performed according to the protocol previously described ¹³ with modifications: 50 μ L of 1:10 dilution stool samples were suspended in 450 μ L of 50 mM cold sodium acetate buffer (pH 5.6) and were then mixed with 1.5 ml of ethanol and under stirring during 20 min at 25°C. After centrifugation, the supernatant was diluted four times with water and eluted through C18 cartridge (500 mg/3 ml; Hypersep Spe, Thermo Scientific). The cartridge was washed with ethanol (3 mL) and bile acids were eluted with ethanol (3 mL). Then, the solvent was evaporated and the residue dissolved in 500 μ L of methanol. Finally, the solution was filtered by a 0.22 μ m PVDF filter (Merck Millipore) prior to the UPLC-ESI-QTOF-MS analysis.

UPLC-ESI-QTOF-MS analysis. The metabolomics analysis was performed on a U-HPLC (Infinity 1290; Agilent) coupled to a high-resolution mass spectrometer with a quadrupole time-of-flight mass analyser (6550 iFunnel Q-TOF LC/MS; Agilent) with an Agilent Jet Stream (AJS) electrospray (ESI) source. The mass analyzer was operated in negative mode under the following conditions: gas temperature 150°C, drying gas 14 L/min, nebulizer pressure 40 psig, sheath gas temperature 350°C, sheath gas flow 11 L/min, capillary voltage 3500 V, fragmentor voltage 100 V, and octapole radiofrequency voltage 750 V. Data were acquired over the m/z range of 50–1700 at the rate of 2 spectra/s. The m/z range was autocorrected on reference masses 112.9855 and 1033.9881. The MS/MS target product ion spectra were acquired at m/z 100–1100 using a retention time window of 1 min, collision energy of 30 eV and an acquisition rate of 10 spectra/s. The chromatographic analysis was performed with a reversed-phase C18 column (Poroshell 120, 3 $\times$ 100 mm, 2.7 μ m pore size) at 30°C, using water + 0.1% formic acid (Phase A) and acetonitrile + 0.1% formic acid (Phase B) as mobile phases with a flow rate of 0.4 mL/min. The gradient started with 50% B, increased in 4 min to 90% B, in 3 min to 99% B, held for 3 min and decreased to the initial conditions during 1 min. The injection volume for all samples was 2 μ L. Raw data were processed by Profinder 10.0 software (Version B.10.0, Agilent software metabolomics, Agilent Technologies) through the application of a batch targeted featured extraction based on an in-house database built for bile acids. After data processing, the bile acids identified were analyzed by Mass Hunter Qualitative 10.0 qualitative (Version B.10.0, Agilent software metabolomics, Agilent Technologies).

Metataxonomic analysis of microbiota

At the end of the last *ad libitum* regimen, fecal samples were collected every 6 h for 24 h (ZT6, ZT12, ZT18 and ZT24); in the last 24 h of the dark-restricted feeding period, samples were collected after 12 h of fasting (ZT12), and after 6 and 12 h of refeeding (ZT18 and ZT24, respectively) (**Figure S1B**). Samples were immediately snap-frozen in liquid nitrogen and stored at -80°C. Before DNA extraction, ~30 mg of feces was incubated for 1 hour at 37°C with lysozyme and mutanolysin (Sigma-Aldrich) for Gram-positive bacterial cell wall lysis. The QIAamp® PowerFecal® Pro DNA kit (Qiagen) was used for isolation of microbial DNA, but instead of using the vortex adapter described in the protocol, samples were homogenized in the provided bead tubes using a Mini-BeadBeater-8 (BioSpec Products) at medium-high speed 3 times for 1 min, with 1 min incubation on ice between bead-beatings. DNA was eluted in 30 µL of nuclease-free water and yields were quantified by fluorimetry with Qubit® (Invitrogen) using the dsDNA HS Assay reagent kit. Concentration of DNA samples ranged from 1 to 50 ng/µL. For metataxonomic analysis, library preparation was performed using Nextera XT v2 Index (Illumina) targeting the V3-V4 region of the 16S rRNA gene and sequenced on an Illumina® MiSeq platform (2×300 bp paired-end reads) at the Genomics Unit of Institute of Parasitology and Biomedicine “López-Neyra” (IPBLN, National Research Council, Granada, Spain).

Quantification of amplicon sequence variants (ASVs) was performed with the DADA2 v.1.24 R package¹⁴. Raw reads were truncated after 280 bp for forward and 250 bp for reverse reads, and 30 nucleotides were additionally removed at the start of both paired-end reads. Reads were filtered for quality assurance; clean pairs of reads were merged into contig sequences and chimeric sequences were discarded. Taxonomy was assigned by comparing sequences with the SILVA v.138 database¹⁵. Taxa with 0 reads in 30% of the samples, in at least 3 experimental groups, were removed from subsequent analyses. The alpha diversity was calculated through the estimation of the observed ASVs, and the Shannon and inverse Simpson indices using the Phyloseq v.1.40 R package¹⁶.

Statistics and Reproducibility

Graphs were plotted with GraphPad Prism 9. Statistical analyses were also performed using GraphPad Prism 9, with the exception of the microbiota-related data, which will be detailed later in this section. When data met assumption of normality and equality of variances, determined by conducting Shapiro-Wilk and Bartlett's tests, respectively, different parametric tests were conducted. A two-way ANOVA was used to analyse the main effects and interactions between genotype (controls: Cre⁻ and Nav1.8-cre/DTA: Cre⁺) and diet (CD or HFHSD), genotype and prandial condition (fasting/Ensure®), or genotype and time (zeitgeber time [ZT]) as between-subject factors. Multiple comparisons using Bonferroni's *post hoc* test were performed when interactions were detected. The unpaired Student's t test or the Mann-Whitney U test was used to analyze differences between two independent variables for normally and non-normally distributed data, respectively. The paired t test was used to identify differences in paired samples. The Bravais-Pearson correlation coefficient was used to test correlations between two variables.

The data of microbial alpha diversity showed normality and equality of variances and were, therefore, analyzed by two-way ANOVA to assess main effects and interactions between genotype (controls: Cre⁻ and Nav1.8-cre/DTA: Cre⁺) and zeitgeber time (ZT) as between-subject factors in

mice fed the same diet. Multiple comparisons using Bonferroni's *post hoc* test were performed when interactions were detected.

Regarding the DRF dataset, the evaluation of the community structure across groups was performed through principal coordinates analysis (PCoA) (phyloseq::ordinate function and "bray" distance). For differential abundance analysis of ASVs across groups, the edgeR v.1.38 R package¹⁷ was applied using trimmed mean of M values (TMM) data normalization, quasi-likelihood F-tests and Bonferroni *post hoc* correction. Pairwise comparisons between the experimental groups were undertaken to identify the main effects and interactions, making contrasts between genotype (Cre⁻ and Cre⁺) and prandial condition (ZT12, fasting; ZT18, 6 h of refeeding, and ZT24, 12 h of refeeding) for CD- or HFHSD-fed mice.

Identification of oscillating signals in the microbiota of the *ad libitum* dataset was assessed with the web application DiscoRhythm (<https://disco.camh.ca>). The dataset was divided into two independent groups (Cre⁻ CD and Cre⁺ CD) and the JTK approach was selected for oscillation detection with Benjamini and Hochberg multiple testing correction.

The univariate t-test was applied to study, individually, the variations in bile acids according to genotype (Cre⁻ and Cre⁺), diet (CD or HFHSD) and prandial condition.

Sample sizes and number of replicates are provided in the figure legends for each analysis.

3. Results

Nav1.8-expressing neurons are required for weight gain and for food intake control after fasting in mice fed a high-fat high-sugar diet

To evaluate the role of neurons expressing the ion channel Nav1.8 in key elements controlling energy homeostasis, we selectively depleted these neurons by a diphtheria toxin ablation strategy (as described in the Methods section) to obtain Nav1.8-deficient mice and control mice.

Since vagal afferents are involved in the energy homeostasis control, we examined whether the expression of Nav1.8 was cancelled in the nodose ganglion of the offspring. Compared with controls, mice expressing Cre, hereinafter referred to as Nav1.8-cre/DTA mice or mice lacking Nav1.8⁺ neurons (**Figure S1A-B** and **S2A** Cre⁻ or Cre⁺, for controls or Nav1.8-cre/DTA mice, respectively) showed a 28-fold decrease in *Scn10a* (coding for Nav1.8) mRNA levels in the nodose ganglion (**Figure S2B**), demonstrating the efficient genetic ablation of Nav1.8⁺ neurons in the vagal nerve.

The role of Nav1.8⁺ neurons in the control of body weight and food intake under an obesogenic diet was investigated (**Figure S1A**). Specifically, control and ablated mice fed on chow until 6 weeks of age were switched to HFHSD feeding during 8 weeks. After this period, Nav1.8-cre/DTA mice showed less body weight gain than their control littermates (**Figure 1A**). The weight gain resistance to diet-induced obesity of mice lacking Nav1.8⁺ neurons was not due to lower food intake; in fact, contrary to what was expected from their lean phenotype, Nav1.8-cre/DTA tended to eat more than controls (**Figure 1B**). Alternatively, we explored whether thermogenesis was involved in the genotype-related body weight differences. The quantification of cells expressing

the thermogenesis marker uncoupling protein 1 (UCP1) in the brown adipose tissue (BAT) revealed that mice lacking Nav1.8-expressing neurons tended to have increased number of UCP1+ cells in BAT compared with controls ($p = 0.08$) (**Figure 1C**).

Since the loss of Nav1.8-expressing neurons tended to dysregulate food intake and metabolic routes of fat utilization, we submitted mice to daily nutritional challenges to further explore the role of these neurons in the short-term control of food intake and body weight. Accordingly, we generated another cohort of mice (controls or Nav1.8-cre/DTA) that were first fed *ad libitum* with either control diet (CD) or HFHSD, and then switched to 12 h of fasting followed by refeeding (12 h of restricted feeding) first in the light phase (LRF) for 3 weeks and then in the dark phase (DRF) for 3 weeks to explore the influence of feeding time or light/dark cues on feeding behavior and body weight variations (**Figure S1B**).

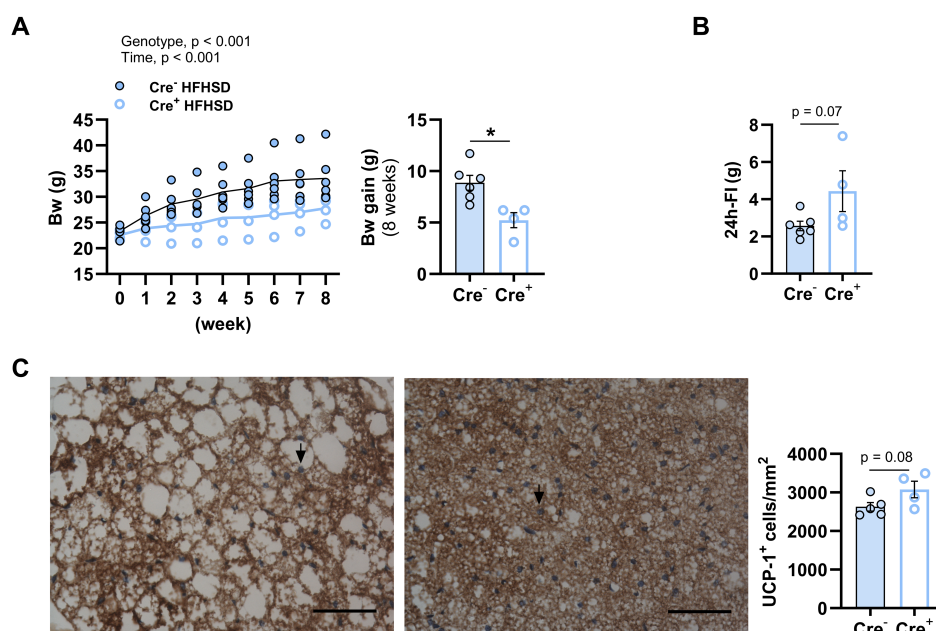


Figure 1. Effects of the ablation of Nav1.8+ neurons on body weight, food intake and thermogenesis in mice fed high-fat high-sugar diet *ad libitum*

In mice lacking Nav1.8+ neurons (Cre⁺) and their control littermates (Cre⁻) fed high-fat high-sugar diet (HFHSD) for 8 weeks, we measured: **(A)** Weekly body weight (Bw) and Bw gain; **(B)** 24 h- food intake (FI); and **(C)** Number of cells expressing the uncoupling coupled protein 1 (UCP-1) (graph) and immunostaining of UCP-1⁺ cells in brown adipose tissue (BAT), scale bar = 50 μ m, black arrows indicate UCP1+ immunolabeling (in blue). Data are represented as scatter plots indicating individual values and the mean follow-up curve or scatter plots for individual values and bars with mean $\pm$ SEM (Cre⁻ HFHSD, $n = 6-5$ mice, and Cre⁺ HFHSD, $n = 4$ mice). Individual mice fed HFHSD are depicted by solid blue (Cre⁻) or empty blue (Cre⁺) circles; mean values in the follow-up curves in **A** are represented by black (Cre⁻) or bold blue (Cre⁺) lines. **(A)** Two-way ANOVA with genotype (Cre⁻ or Cre⁺) and week as between-subject factors; **(B, C)**: unpaired Student's t test. * $p < 0.05$.

The analysis of body weight changes in response to 5 weeks of *ad libitum* feeding with CD or HFHSD revealed that Nav1.8-cre/DTA mice showed reduced body weight gain especially under

HFHSD. Indeed, an interaction between genotype and diet was identified on body weight gain (**Figure S3A**). *Post hoc* analysis indicated that Nav1.8-cre/DTA mice fed HFHSD, but not CD, had lower weight gain than equivalent controls (**Figure S3A**).

Daily food intake and body weight variations were assessed during restricted feeding. As feeding patterns of rodents show peaks at dawn and dusk ¹⁸, caloric intake was measured in two consecutive 6-h periods in the last 2 days of the LRF [zeitgeber time (ZT)0-ZT6 and ZT6-ZT12] and the DRF (ZT12-ZT18 and ZT18-ZT24). We observed that HFHSD-fed control mice ate less during the dusk peak (ZT12-ZT18) than equivalent CD-fed mice subjected to DRF, but not to LRF (**Figure 2A**). In the dusk peak, we also identified an interaction between genotype and diet (**Figure 2A**) showing that Nav1.8-cre/DTA mice blunted the HFHSD-induced food intake reduction under DRF and ate more than controls (**Figure 2A**). We replicated these results in the first 2 h of free access to food under LRF or DRF, when feeding behavior is governed by hunger signals after a period of fasting. In particular, a main diet effect indicated that HFHSD reduced food intake in control mice whatever the food-restricted regimen (LRF or DRF) (**Figure 2B**), while the interaction between diet and genotype under DRF denoted that Nav1.8-cre/DTA mice fed HFHSD ate more than equivalent control mice (**Figure 2B**).

We also explored food intake suppression in response to an oral load of Ensure® administered after 12 h of fasting by measuring 2 h of food intake at the onset of the refeeding period. Under DRF, but not in LRF, we identified a main effect of the genotype in food intake suppression (**Figure 2C**). The analysis restricted to either CD or HFHSD indicated that the absence of Nav1.8+ especially inhibited the Ensure®-induced food intake suppression under HFHSD rather than under CD (**Figure 2C**).

We also analyzed daily fasting-refeeding body weight loss and gain. Under LRF and compared with CD-fed mice, HFHSD-feeding induced resistance to weight loss or weight gain after 12 h of fasting or 12 h of refeeding, respectively, irrespective of the genotype (**Figure 2D and Figure S3B and 3c**). Under DRF, in addition to the HFHSD effect on limiting the fasting-refeeding body weight variations, a genotype effect was also evident (**Figure 2E and Fig S3D and S3E**), indicating that daily body weight loss and gain was higher in Nav1.8-cre/DTA mice than in control mice irrespective of the diet.

Analysis of the global effect of time-restricted feeding on body weight revealed that control mice fed HFHSD, but not fed CD, lost weight after 6 weeks of 12-h-restricted feeding (LRF followed by DRF) as compared with their weight under *ad libitum* feeding (**Figure 2F**). By contrast, time-restricted feeding had no influence on the body weight of Nav1.8-cre/DTA mice fed either CD or HFHSD (**Figure 2F**).

In summary, we demonstrated that the loss of Nav1.8+ neurons exacerbates food intake at dusk and the body weight loss after fasting, and attenuates the food intake suppression after a meal, especially under HFHSD feeding. Taking together, our findings suggest that these neurons mitigate hunger at the beginning of the active phase through mechanisms facilitating satiety and/or limiting the loss of energy stores during fasting. To note, the role of Nav1.8+ neurons on the short-term control of food intake seems to be governed by light/dark cues rather than feeding time since these effects were observed when food was provided in the dark phase, but not in the light phase.

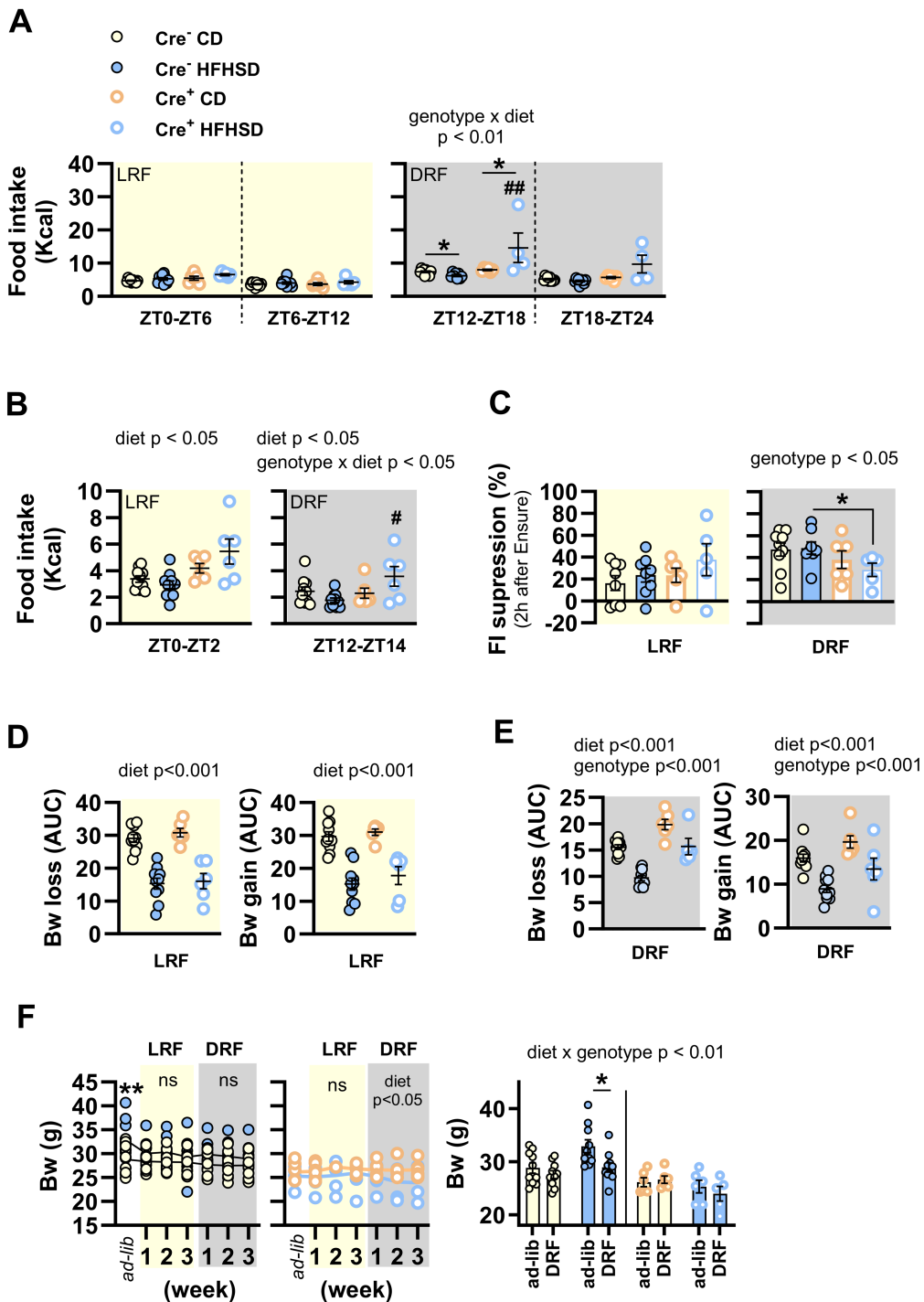


Figure 2. Influence of the ablation of Nav1.8+ neurons in the control of food intake and body weight and of mice under time-restricted feeding.

In mice lacking Nav1.8+ neurons (Cre⁺) and their control littermates (Cre⁻), fed either control or high-fat high-sugar diet (CD and HFHSD, respectively), we determined: **(A)** Food intake during the zeitgeber time (ZT)0 to ZT6 and ZT6-ZT12 in light restricted feeding (LRF) and during the ZT12-ZT18 and ZT18-ZT24 in dark restricted feeding (DRF). Data are the mean of 2 consecutive days at the end of each feeding restriction schedule; **(B)** Food intake during ZT0-ZT2 in the LRF and during ZT12-ZT24 in the DRF. Data are the mean of 3 consecutive days in the third week of the LRF or DRF; **(C)** Food intake (FI) suppression in the LRF or DRF represented as percentage of food intake after an oral gavage of Ensure® relative to FI after an oral gavage of saline (FI for either saline or Ensure® conditions was calculated using the mean of 3 consecutive days in the third week of the LRF or DRF); **(D)** Areas under the curve (AUC) of the Bw loss after 12 h of fasting and Bw gain after 12 h of refeeding during 3 weeks of LRF; **(E)** AUC of the Bw loss after 12 h of fasting and Bw gain after 12 h of refeeding during 3 weeks of DRF; **(F)** Bw follow-up in different feeding regimens: *ad libitum* (*ad-lib*), LRF and DRF; and impact of the time-restricted feeding on Bw (Bw is shown after *ad-lib* and after DRF).

Data are represented as scatter plots indicating individual values and bars with mean $\pm$ SEM or scatter plots for individual values and the mean follow-up curve (Cre⁻ CD and Cre⁻ HFHSD, $n = 10$ mice; Cre⁺ CD, $n = 6$ mice; Cre⁺ HFHSD, $n = 5-7$ mice). Mice fed CD are depicted by solid yellow (Cre⁻) or empty orange (Cre⁺) circles, mice fed HFHSD are depicted by solid blue (Cre⁻) or empty blue (Cre⁺) circles; mean values in the follow-up curves in **(F)** are represented by black (Cre⁻ CD, Cre⁻ HFHSD), bold orange (Cre⁺ CD) or bold blue (Cre⁺ HFHSD) lines; shadings in light yellow and grey represent LRF and DRF regimes, respectively; dashed lines separate the 6-hour ZT timeframe. **(A-F)**: Two-way ANOVA with genotype (Cre⁻ or Cre⁺) and diet (CD or HFHSD) as between-subject factors. Right graph of **(F)**: Three-way ANOVA with genotype (Cre⁻ or Cre⁺), diet (CD or HFHSD) and regimen (*ad libitum* or DRF) as between-subject factors. Main effects or interactions are indicated in the top of the graphs. When interactions were identified, differences between groups were assessed with Bonferroni's *post hoc* test. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs CD groups either Cre⁻ or Cre⁺ **(A, F)**, vs *ad libitum* feeding **(F)**, or vs saline **(C)**. # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$ vs Cre⁻ fed the same diet (HFHSD); ns: non-significant.

Nav1.8+ neurons modulate the intestinal immune system and affect gut microbiota by influencing its daily rhythms and pre- and postprandial oscillations

The gut microbiota shows food-entrainable diurnal oscillations¹⁹ and, through its interaction with the diet, modulates feeding patterns by governing enteroendocrine signaling of the gut-to-brain axis²⁰⁻²². In addition, innervations of the GI tract establish a complex connection with the gut microbiota²³. On these bases, we explored whether the ablation of Nav1.8+ neurons affects the gut microbiota composition with a particular emphasis in its pre- and postprandial oscillations during DRF, which in turn could affect feeding behavior.

The analysis of the influence of genotype and diet on the gut microbiota after fasting (ZT12) and refeeding (ZT18 and ZT24) showed that Nav1.8+ neurons were necessary to shape oscillations of alpha diversity, measured as Shannon index and InvSimpson index, as Nav1.8-cre/DTA mice fed either CD or HFHSD did not show significant prandial variations as controls (**Figure 3A and 3b**). In CD-fed control mice, the Shannon and InvSimpson indices were reduced from fasting (ZT12) to 6 h of refeeding (ZT18) (**Figure 3A**), whereas in HFHSD-fed controls, the Shannon index was lower in both postprandial periods (ZT18 and ZT24) than in the fasting period (ZT12) (**Figure 3B**).

When exploring beta diversity under CD, the prandial condition rather than the genotype shaped two different microbiota communities in fasted mice and 6-h refed mice (**Figure 3C**, circles for ZT12 and triangles for ZT18). In both genotypes, gut microbiota did not appear to shape a specific cluster after 12 h of refeeding (ZT24), but it was distributed across ZT12 and ZT18 clusters (**Figure 3C**, smaller square dots). Nevertheless, HFHSD shaped different clusters depending on the

genotype and the prandial condition. Indeed, in contrast to control mice, Nav1.8-cre/DTA mice did not exhibit different sample clustering in fasting and in 6 h of refeeding (**Figure 3D**).

We also analyzed the main effects of genotype and zeitgeber time on amplicon sequence variants (ASVs) for each diet (CD and HFHSD), to identify bacterial taxa whose abundance varied across fasting and refeeding depending on the genotype. The genotype altered the abundance of some ASVs between refeeding periods (from ZT18 to ZT24) in CD-fed mice. The absence of Nav1.8+ neurons impeded the increase of the fecal abundance of *Bilophila* spp. and of ASVs from the families Ruminococcaceae, Desulfovibrionaceae and Peptococcaceae from 6 h to 12 h of refeeding (**Figure 3E**). Also, Nav1.8-cre/DTA mice showed postprandial variations in *Stenotrophomonas* spp., *Acinetobacter* spp., and *Dietzia* spp., with abundances higher in both postprandial periods than during fasting (ZT12 vs ZT18 or vs ZT24) (**Figure 3E**). On HFHSD, Nav1.8+ ablation affected the prandial-related oscillations of an ASV from the family Desulfovibrionaceae and from *Alistipes* spp. Indeed, in contrast to control mice, the loss of Nav1.8+ neurons impeded the 12-h-postprandial increase of Desulfovibrionaceae compared with fasting (ZT12 vs ZT24). In addition, Nav1.8-cre/DTA mice did not show the decrease of *Alistipes* spp. after 6 h of refeeding (ZT12 vs ZT24) or an increase in its abundance after 12 h of refeeding (ZT18 vs ZT24) observed in controls (**Figure 3E**).

We next questioned whether Nav1.8+ neurons impact the prandial variations of microbiota-derived metabolites. We focused on secondary bile acids (SBAs), which are biotransformed by the microbiota from primary bile acids (PBAs) synthesized by the host from dietary cholesterol²⁴ and secreted to the intestinal lumen to digest ingested food. Under CD, the prandial-related variations in BAs were not significantly affected by genotype, except for an increase of cholic acid in fasted Nav1.8-cre/DTA mice compared with fasted mouse controls (**Figure 4A**). By contrast, under HFHSD the fasting levels of SBAs were higher in control mice than in equivalent Nav1.8-cre/DTA mice, particularly deoxycholic, hyodeoxycholic, murideoxycholic, 3-oxo-chenodeoxycholic and ursodeoxycholic acids, followed by a progressive decrease in their levels in the postprandial period (**Figure 4B**). The results indicate that, in controls but not in Nav1.8-cre/DTA mice, microbial BA metabolism was active, particularly in fasting conditions once PBAs generated in the prior refeeding period reach the distal gut.

We then assessed whether Nav1.8-expressing neurons are also involved in the daily fluctuations of the gut microbiota when mice have 24-hour free access to food. Thus, after time-restricted feeding, mice were again switched to *ad libitum* regimen during 3 weeks.

The free access to HFHSD throughout the whole day again increased the body weight of control mice but not of Nav1.8-cre/DTA mice (**Figure S4A** and **S4B**). Importantly, under a HFHSD, the switch from restricted to *ad libitum* feeding reduced the survival of Nav1.8-cre/DTA mice (5 dead mice out of 7) and so, gut microbiota under *ad libitum* feeding conditions was only investigated in CD-fed mice.

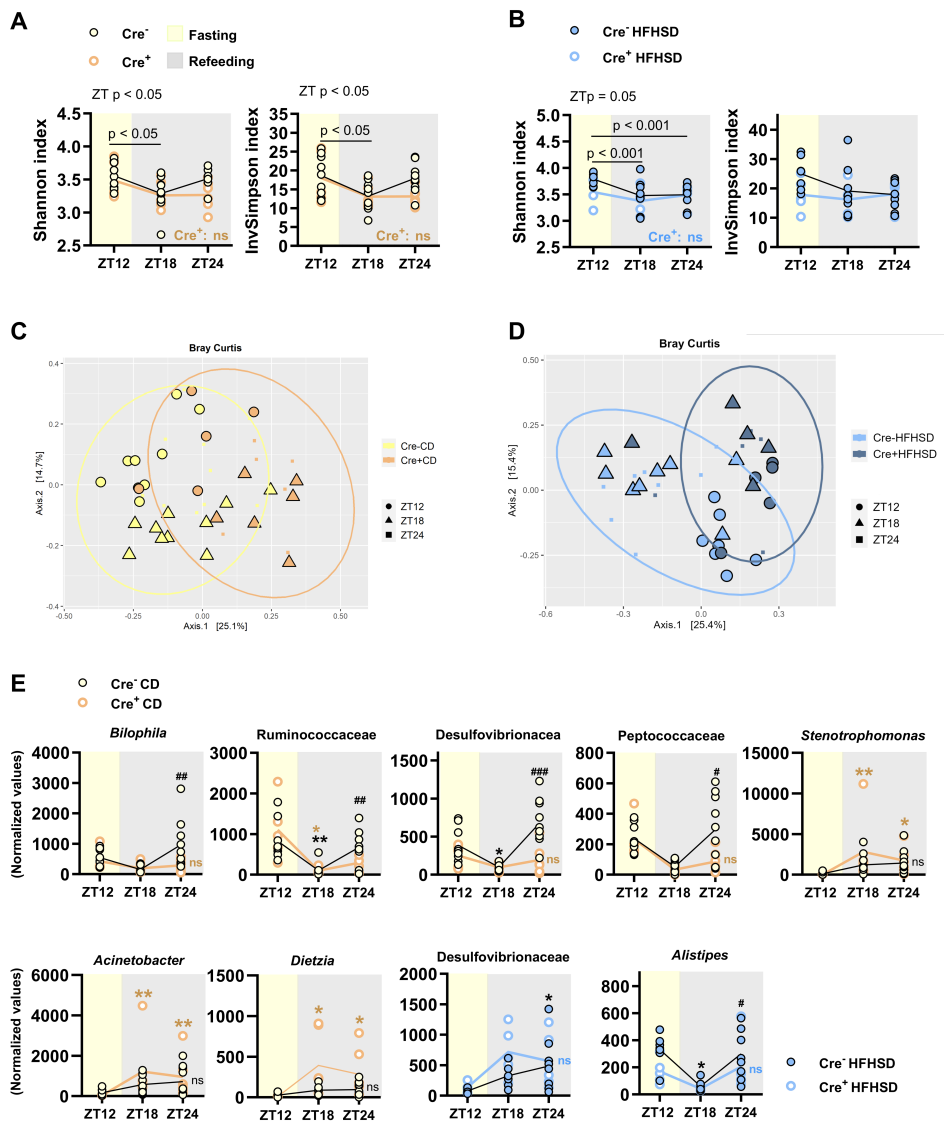


Figure 3. Effects of the ablation of Nav1.8+ neurons on gut microbiota composition during dark-restricted feeding.

At the end of the dark restricted feeding, in mice lacking Nav1.8+ neurons (Cre⁺) and their control littermates (Cre⁻), fed either control or high-fat high-sugar diet (CD and HFHSD, respectively), we determined: **(A), (B)** Alpha diversity after 12 h of fasting (ZT12) and 6 and 12 h of refeeding (ZT18 and ZT24) measured with the Shannon and Inverse Simpson indices; **(C), (D)** Beta diversity after 12 h of fasting (ZT12) and 6 and 12 h of refeeding (ZT18 and ZT24), determined by principal coordinate analysis (PCoA) (phyloseq::ordinate function and “bray” distance); **(E)** Normalized data using trimmed mean of M values (TMM) of the abundance of amplicon sequence variants (ASVs) after 12 h of fasting (ZT12) and 6 and 12 h of refeeding (ZT18 and ZT24). Data are represented as scatter plots indicating individual values and the mean follow-up curve (Cre⁻ CD and Cre⁻ HFHSD, n = 9 mice; Cre⁺ CD, n = 5-6 mice; Cre⁺ HFHSD, n = 5 mice). Mice fed CD are depicted by solid yellow (Cre⁻) or empty orange (Cre⁺) circles, mice fed HFHSD are depicted by solid blue (Cre⁻) or empty blue (Cre⁺) circles; mean values in the follow-up curves in **A, B** and **E** are represented by black (Cre⁻ CD, Cre⁻ HFHSD), bold orange (Cre⁺ CD) or bold blue (Cre⁺ HFHSD) lines; shadings in light yellow and grey represent

fasting (light phase) and refeeding (dark phase) periods, respectively; in (C) and (D), circles, triangles and squares represent ZT12, ZT18 and ZT24, respectively, mice on CD feeding are represented in solid yellow (Cre⁻) or solid orange (Cre⁺), mice on HFHSD feeding are represented in solid light blue (Cre⁻) or solid dark blue (Cre⁺). (A, B): Two-way ANOVA with genotype (Cre⁻ or Cre⁺) and zeitgeber time (ZT) as between-subject factors followed by Bonferroni's *post hoc* test. $p < 0.05$, $p < 0.01$ and $p < 0.001$ indicate significant differences between ZT within the same experimental group; (E) quasi-likelihood F-tests and Bonferroni *post hoc* correction. # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$ vs ZT18. * $p < 0.05$ and ** $p < 0.01$ vs ZT12 (black for Cre⁻ and orange for Cre⁺). ns: non-significant differences.

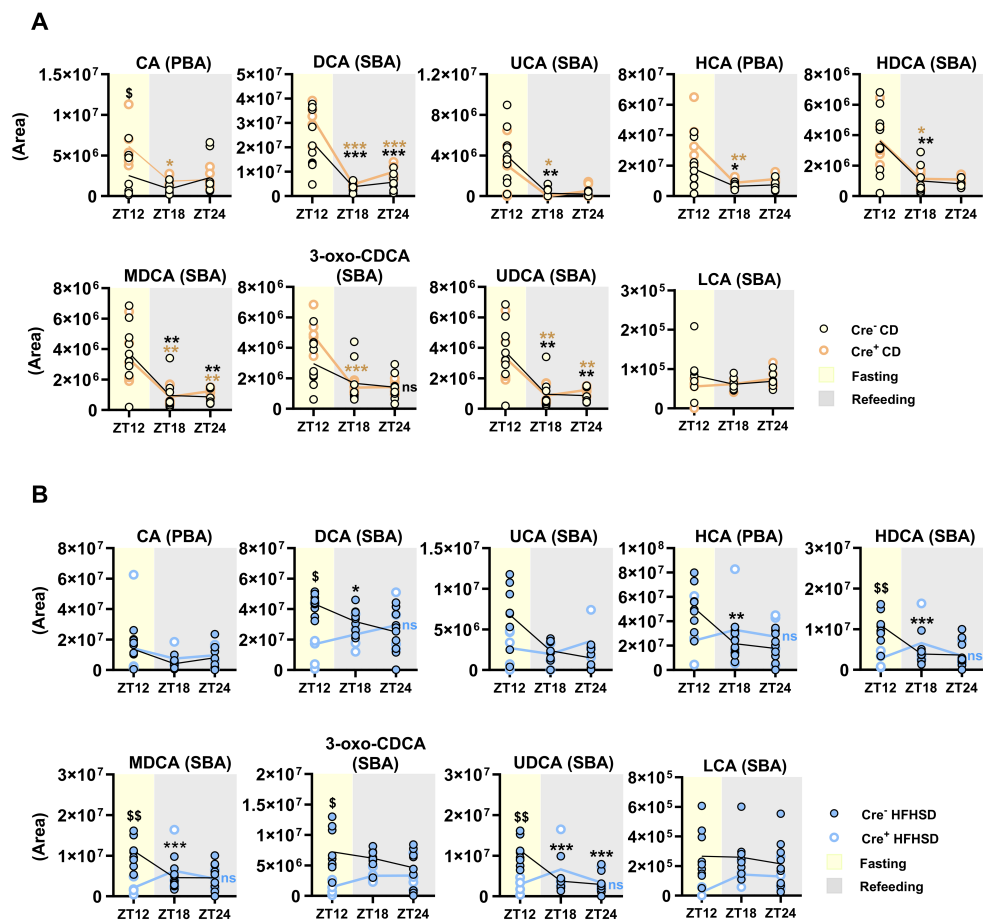


Figure 4. Influence of the ablation of Nav1.8+ neurons on fecal bile acids levels during dark-restricted feeding.

(A) From left to right: Fecal levels of the primary bile acid (PBA) cholic acid (CA), and its derived secondary bile acids (SBAs) deoxycholic acid (DCA) and ursodeoxycholic acid (UCA); Fecal levels of the PBA hyocholic acid (HCA), and its derived SBAs hyodeoxycholic acid (HDCA) and murideoxycholic acid (MDCA); Fecal levels of the SBAs 3-oxo-chenodeoxycholic acid (3-oxo-CDCA), ursodeoxycholic acid (UDCA) and lithocholic acid (LCA), derived from chenodeoxycholic acid (the latter not shown) in control and Nav1.8-cre/DTA mice at the end of the dark-restricted feeding of control diet (CD) after 12 h of fasting (zeitgeber time, ZT12), and 6 h and 12 h of refeeding (ZT18 and ZT24, respectively); (B) The metabolites mentioned in (A) measured in the same ZTs in control and Nav1.8-cre/DTA mice fed high-fat high-sugar diet (HFHSD) at the end of the dark-restricted feeding.

Data are represented as scatter plots indicating individual values and the mean follow-up curve shown as chromatographic peak area. (Cre⁻ CD/HFHS, n = 9-10 mice and Cre⁺ CD/HFHS, n = 4-6 mice). Mice fed CD are depicted by solid yellow (Cre⁻) or empty orange (Cre⁺) circles, mice fed HFHS are depicted by solid blue (Cre⁻) or empty blue (Cre⁺) circles; mean values in the follow-up curves are represented by black (Cre⁻ CD, Cre⁻ HFHS), bold orange (Cre⁺ CD) or bold blue (Cre⁺ HFHS) lines; shadings in light yellow and grey represent fasting (light phase) and refeeding (dark phase) periods, respectively. The univariate t-test was applied to study separately the variations of bile acids according to the genotype (Cre⁻ and Cre⁺) and prandial condition (ZT). [§] p < 0.05 and ^{§§} p < 0.01 Cre⁻ HFHS vs Cre⁺ HFHS or Cre⁻ CD vs Cre⁺ CD for a specific ZT; * p < 0.05, ** p < 0.01 and *** p < 0.001 vs ZT12.

We explored the role of Nav1.8+ neurons on the daily variations of alpha diversity in gut microbiota every 6 h, as well as on the rhythmicity of bacterial taxa. Alpha diversity, measured as observed ASVs, Shannon index and InvSimpson index, was affected by the zeitgeber time but not by the genotype, with a reduction from the light phase to the beginning of the dark phase, followed by an increase at the end of this phase (**Figure 5A**). Using the JTK_cycle algorithm, we found that Nav1.8+ neurons were critical to maintain the daily rhythmicity of some ASVs (**Figure 5B**). Specifically, in controls, but not in Nav1.8-cre/DTA mice, we detected five rhythmic ASVs that were higher in abundance in the dark phase, including those belonging to the order Gastranaerophilales or species from the genera *Alistipes* or *Colidextribacter* (**Figure 5C**). ASVs from the families Muribaculaceae and Oscillospiraceae also showed daily rhythms in control mice (**Figure 5C**).

Since gut microbiota and intestinal immunity are closely related ²⁵, we then examined whether these microbiota changes were associated with alterations in the gene expression of gut barrier markers and in the abundance of immune cells in the intestinal mucosa. The gene expression of several markers of intestinal barrier defence and integrity were lower in Nav1.8-cre/DTA mice than in control mice, including the ileal levels of *Tcf4*, involved in Paneth cell development, and the colonic levels of the tight junction proteins *Ocln* and *Cldn3* (**Figure 5D**). Gene expression of antimicrobial peptides (*DefA*, *Lyz1*, *Reg3g*) or proliferation (*Ki67*) markers in ileum and colon was unaffected by the genotype (**Figure S5A**). The abundance of natural killer cells (NK) and type 1 innate lymphoid cells (ILC1) in the small intestinal epithelium was unaffected by the genotype (**Figure S5B**). Contrastingly, analysis of the lamina propria revealed a reduction in the abundance of type 2 ILC (ILC2), involved in type 2-like responses and tissue repair (**Figure 5E**), and regulatory T cells (Treg) (**Figure 5F**), critical to control inflammation and to develop immune tolerance, in Nav1.8-cre/DTA mice, and an increase in T helper 17 cells (Th17) (**Figure 5G**), suggesting disrupted intestinal immune homeostasis. Other immune cells such as type 3 ILC (ILC3) or type 1 and type 2 macrophages (M1 and M2) were unaffected by the absence of Nav1.8+ neurons (**Figure S5B**). Notably, spleen weight was greater in Nav1.8-cre/DTA mice than in control mice (**Figure S5C**), suggesting systemic inflammation, although no changes were observed in the splenic abundance of memory or effector T cells or macrophages between genotypes, except for a trend for a decrease in M2 in Nav1.8-cre/DTA mice (**Figure S5D**).

We next addressed how the loss of Nav1.8+ neurons could affect intestinal inflammation. Thus, on the basis that Nav1.8+ neurons express VIP ²⁶, a neural-produced peptide with immunomodulatory properties, we next examined the duodenal and ileal levels of this neuropeptide. VIP protein was undetectable in the duodenum. In the ileum, we found a trend for decreased VIP levels in Nav1.8-cre/DTA mice; indeed, while most of the control mice showed detectable levels of VIP most Nav1.8-cre/DTA mice had undetectable levels (**Figure S5E**). We

reasoned that the absence of Nav1.8+ neurons might impact brain-to-gut communication through the sympathetic and parasympathetic efferent tone, influencing the intestinal inflammatory profile. The sympathetic tone in the duodenum, but not in the distal regions, was enhanced in Nav1.8-cre/DTA mice, as indicated by the higher levels of noradrenaline (NE) (Figure 5H). By contrast, the levels of acetylcholine (ACh) were unaffected (Figure 5H).

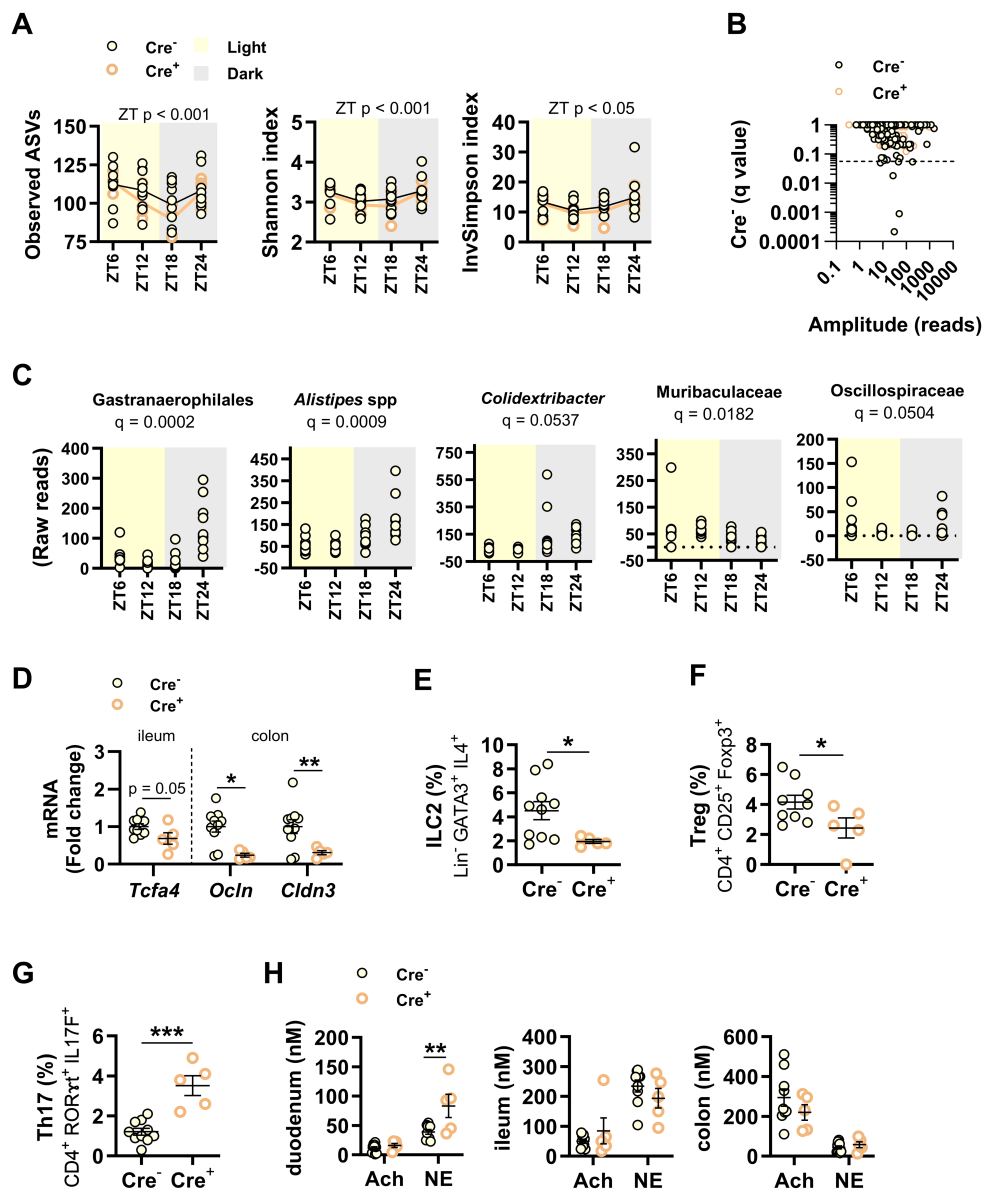


Figure 5. Effects of Nav1.8+ neuron ablation on the daily variations of gut microbiota and on gut barrier integrity and defence in mice fed control diet *ad libitum*.

At the end of the experiment, in Nav1.8-cre/DTA mice (Cre⁺) and their control littermates (Cre⁻) fed control diet (CD), we measured: **(A)** Alpha diversity of fecal microbiota every 6 h for 24 h, measured as observed amplicon

sequence variants (ASVs) and the Shannon and Inverse Simpson indices; **(B)** Amplitude of ASVs daily oscillations. The dashed line indicates a significant cut-off of $p \leq 0.05$; **(C)** Representation of the abundance of oscillating ASVs throughout the day; **(D)** mRNA levels of transcription factor 4 (*Tcf4*), occludin (*Ocln*) and claudin 3 (*Cldn3*) in ileum and colon; **(E)-(G)** Percentage of type 2 innate lymphoid cells (ILC2) **(E)**, regulatory T cells (Treg) **(F)** and T helper 17 cells (Th17) **(G)** in the lamina propria of the small intestine; **(H)** Acetylcholine (ACh) and noradrenaline (NE) levels in duodenum, ileum and colon.

Data are represented as scatter plots for individual values with mean follow-up curve or as scatter plots indicating individual values $\pm$ SEM (Cre⁻ n = 9-10 mice and Cre⁺ n = 5 mice). Mice fed CD are depicted by solid yellow (Cre⁻) or empty orange (Cre⁺) circles; mean values in the follow-up curves in **(A)** are represented by black (Cre⁻) or bold orange (Cre⁺) lines; shadings in light yellow and grey represent light and dark phases, respectively. **(A)**: Two-way ANOVA with genotype (Cre⁻ or Cre⁺) and zeitgeber time (ZT) as between-subject factors followed by Bonferroni's *post hoc* test; **(B)**: JTK approach with Benjamini and Hochberg multiple testing correction (DiscoRhythm); **(D-H)**: unpaired Student's t test; * $p < 0.05$ and ** $p < 0.01$.

Altogether, these analyses establish that Nav1.8⁺ neurons are relevant to shape daily oscillations of the gut microbiota. Especially under DRF of HFHSD, Nav1.8⁺ neurons govern the postprandial decrease of the alpha diversity and SBAs production at the beginning of the active phase. The function of Nav1.8⁺ neurons regulating gut microbiota could be attributed to their effects as modulators of immune cells in the intestine, which could be in turn modulated by a balanced efferent tone.

Nav1.8⁺ neurons are required for the secretion of gut hormones involved in hunger and satiety signaling

Next, we assessed whether the altered daily rhythms of the gut microbiota of Nav1.8-cre/DTA mice were aligned with altered secretion of gut hormones as primary drivers of short-term control of food intake.

At the end of the experiment, we measured gut hormones during fasting and after an oral load of Ensure® (**Figure 6A-f**) in mice fed CD *ad libitum*, since, as mentioned above, most Nav1.8-cre/DTA mice did not survive a final period of *ad libitum* HFHSD feeding.

We identified interactions between genotype and prandial condition (fasting and post-Ensure® exposure) on gut hormones, predominantly those secreted in the upper small intestine such as ghrelin and GIP (**Figure 6A** and **6b**). In particular, the fasting levels of ghrelin were lower in Nav1.8-cre/DTA mice than in control mice, with no effect in response to Ensure® (**Figure 6A**). Contrastingly, the Ensure®-induced secretion of GIP was boosted in Nav1.8-cre/DTA mice, whereas fasted levels remained unaffected by the genotype (**Figure 6B**). CCK levels remained unchanged irrespective of the genotype or the prandial condition (**Figure 6C**). The distal gut anorexigenic hormones PYY and GLP-1 were also affected by the genotype (**Figure 6D** and **6e**). Whereas PYY levels were higher in Nav1.8-cre/DTA mice whatever the prandial condition (**Figure 6D**), the genotype and the prandial condition showed a trend for interaction on GLP-1 (**Figure 6E**). Analysis of GLP-1 restricted to either fasting or oral Ensure® revealed that fasting GLP-1 levels were higher in Nav1.8-cre/DTA mice than in controls (**Figure 6E**). Comparable results were found for neuropeptide α MSH (**Figure 6F**), with a significant interaction between genotype and prandial condition (**Figure 6F**). Analysis of the slope between the fasting and Ensure® states for GLP-1 and α MSH levels (**Figure 6G**) supported the defective Ensure®-induced increase of these peptides in Nav1.8-cre/DTA mice (**Figure 6E** and **6F**), as the slopes of both peptides were lower than those of controls (**Figure 6G**).

The ileal and colonic transcript levels of PYY and GLP-1 (*Pyy* and *Proglucagon*) were unchanged between genotype (**Figure S6A**); however, the expression of the transcriptional activator *Neurod1* was lower in ileum and *Ngn3* mRNA levels remained unchanged in duodenum, ileum and colon (**Figure 6H**), suggesting downregulated *Neurod1*-mediated ileal L-cell differentiation.

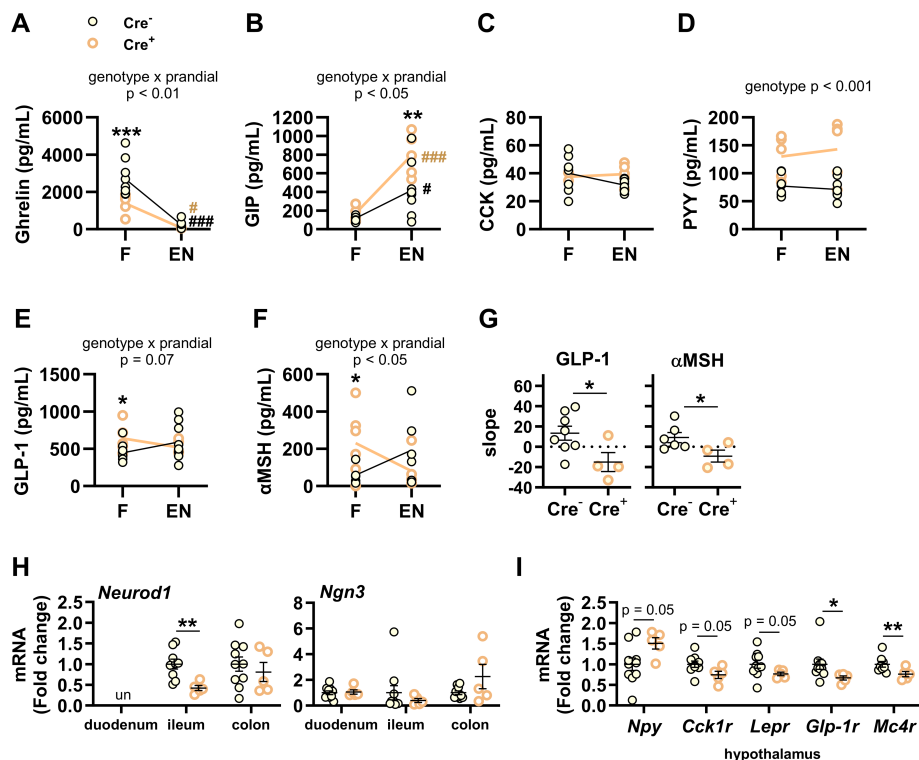


Figure 6. Effects of the ablation of Nav1.8⁺ neurons on gut-to-brain orexigenic and anorexigenic signals in mice fed a control diet *ad libitum*.

At the end of the experiment in Nav1.8-cre/DTA mice (Cre⁺) and their control littermates (Cre⁻) fed control diet (CD), we measured the following: **(A-F)** In 4-h-fasted mice (F) and 15 min after an oral load of Ensure® (EN), plasma levels of **(A)** Ghrelin, **(B)** GIP, **(C)** CCK, **(D)** PYY, **(E)** GLP-1 and **(F)** αMSH; **(G)** Slope calculated between fasting and post-Ensure® levels of GLP-1 and αMSH; **(H)** mRNA levels of *NeuroD1* and *Ngn3* in duodenum, ileum and colon; **(I)** mRNA levels of *Npy*, *Cck1r*, *Lepr*, *Glp1r* and *Mc4r* in the hypothalamus.

Data are represented as scatter plots for individual values with mean follow-up curve or as scatter plots indicating individual values ± SEM (Cre⁻ n = 9-10 mice and Cre⁺ n = 5 mice). Mice fed CD are depicted by solid yellow (Cre⁻) or empty orange (Cre⁺) circles; mean values in **A-F** are represented by black (Cre⁻) or bold orange (Cre⁺) lines. **(A-F)**: Two-way ANOVA with genotype (Cre⁻ or Cre⁺) and prandial condition (F/EN) as between-subject factors (main effects and interactions are indicated in the top of the graphs) followed by Bonferroni's *post hoc* test **(A-C)** or Student's t test **(E, F)**; **(G, H, I)**: unpaired Student's t test (for *Glp1r* in **I**) Mann-Whitney U test was conducted). * p < 0.05, ** p < 0.01, *** p < 0.001 vs Cre⁻ (when applicable in a specified prandial condition) and # p < 0.05, ### p < 0.001 vs fasting condition for a specified genotype (black for Cre⁻ and orange for Cre⁺).

In mice lacking Nav1.8⁺ neurons, we also found disturbances in the gene expression of hypothalamic markers involved in food intake control. Specifically, the gene expression of the orexigenic neuropeptide *Npy* was higher in Nav1.8-cre/DTA mice than in controls, whereas the

transcripts of receptors involved in transmitting anorexigenic signals such as CCK, leptin, GLP-1 and αMSH receptors (*Cck1r*, *Lepr*, *Glp1r* and *Mc4r*) were lower (**Figure 6I**). Other hypothalamic neuropeptides such as *Agrp*, *Pomc* or *Cart* remained unaffected (**Figure S6A**).

Food intake and body weight variations throughout the day were also analysed in these mice. Analyses restricted to each phase indicated that, in the dark phase (ZT12-ZT24), Nav1.8-cre/DTA mice had higher caloric intake than their control littermates, with no change in the light phase (ZT0-ZT12) (**Figure 7A**), although 24 h-food intake (ZT0-ZT24) was not affected by the ablation of Nav1.8+ neurons (**Figure 7A**). We also measured food intake and body weight every 6 h during 24 h in line with the bimodal pattern of nocturnal food intake in rodents. Food intake was higher in Nav1.8-cre/DTA mice than in controls at dusk (ZT12-ZT18) (**Figure 7B**), and this was accompanied by a higher body weight loss in the first 6 h of the light period (ZT0-ZT6) (**Figure 7C**). In addition, the positive correlation between 24-h body weight variation and 24-h food intake in control mice was lost in Nav1.8-cre/DTA mice (**Figure 7D**), suggesting that Nav1.8+ neurons are required to properly control body weight in response to food intake and *vice versa*. Supporting this, the weight of epididymal white adipose tissue (eWAT), but not that of inguinal WAT (iWAT) or BAT, was lower in Nav1.8-cre/DTA mice than in control mice (**Figure 7E**), as were the plasma levels of leptin, irrespective of the prandial state (fasting or after an oral load of the liquid mixed nutrient solution, [Ensure®]) (**Figure 7F**). Gene expression analysis of eWAT revealed a trend for higher expression of the beta 3 adrenergic receptor, *Adrb3*, which stimulates the noradrenergic-mediated fat mobilisation, in Nav1.8-cre/DTA mice than in control mice, whereas *Adrb2* expression was unchanged (**Figure 7G**). In line with the apparent energy deficient status, Nav1.8-cre/DTA mice had reduced fasting glycemia (**Figure 7H**) independently of changes in plasma insulin and glucagon levels, which were unaffected in Nav1.8-cre/DTA mice (**Figure S6B and 6C**).

Taken together, these findings indicate that even when mice are fed CD *ad libitum*, Nav1.8-expressing neurons are required for controlling food intake at the beginning of the active phase and for body weight loss in the resting phase. Nav1.8+ neurons could govern feeding patterns by influencing gut-to-brain transmission of gut hormone-mediated orexigenic and anorexigenic signaling.

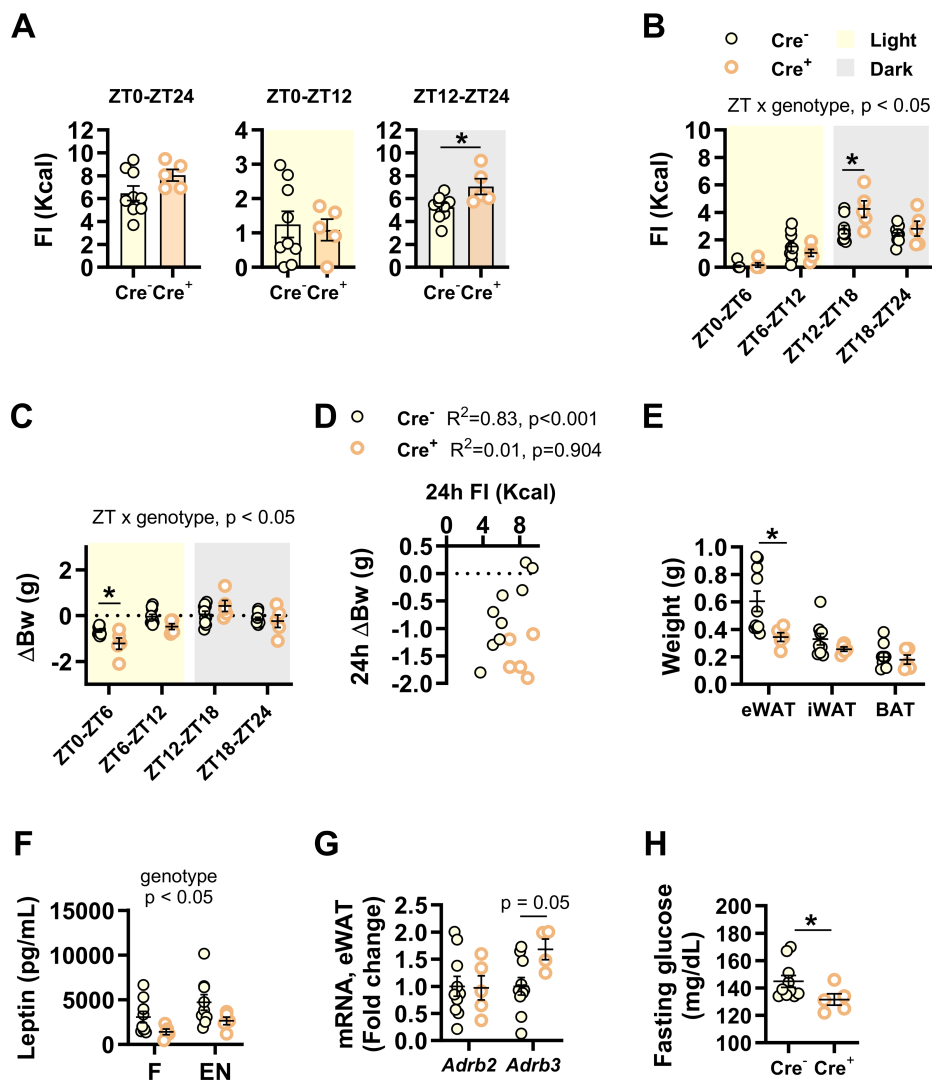


Figure 7. Impact of the ablation of Nav1.8+ neurons on daily food intake and body weight in mice fed control diet *ad libitum*.

At the end of the experiment we measured: **(A)** *Ad libitum* food intake (FI) of control diet (CD) in Nav1.8-cre/DTA mice (Cre⁺) and their control littermates (Cre⁻) during 24h (zeitgeber time, ZT0-ZT24) and in the light and dark phase (ZT0-ZT12 and ZT12-ZT24, respectively); **(B)** *Ad libitum* FI of CD during ZT0 to ZT6, ZT6 to ZT12, ZT12 to ZT18, and ZT18 to ZT24; **(C)** Body weight variations (ΔBw) in the same ZT periods as in **(B)**; **(D)** Correlation between 24-h *ad libitum* FI of CD and ΔBw; **(E)** Weight of different fractions of the adipose tissue: epididymal and inguinal white adipose tissue (eWAT and iWAT, respectively), and brown adipose tissue (BAT); **(F)** Plasma levels of leptin after 4 h of fasting (F) and after oral administration of Ensure® (EN); **(G)** Relative gene expression of beta 2 and 3 adrenergic beta receptor genes (*Adrb2* and *Adrb3*) in eWAT; **(H)** Fasting glycemia.

Data are represented by scatter plots indicating individual values with mean ± SEM, (Cre⁻ n = 9-10 mice and Cre⁺ n = 5-6 mice). Mice fed CD are depicted by solid yellow (Cre⁻) or empty orange (Cre⁺) circles; shadings in light yellow and grey represent light and dark phases, respectively. **(B, C, F)**: Two-way ANOVA with

genotype (Cre⁻ or Cre⁺) and ZT or genotype and prandial condition (F/EN) as between-subject factors (main effects and interactions are indicated in the top of the graphs) followed by Bonferroni's *post hoc* test; (D): Pearson correlation; (A, E, G): Student's t test; and (H): Mann-Whitney U test. * $p < 0.05$.

4. Discussion

Using *ad libitum* feeding and food restriction regimens, we identify a role for sensory neurons expressing the Nav1.8 sodium channel in the control of body weight and food intake. We also show that these neurons are relevant for maintaining the gut-to-brain endocrine pathways transmitting hunger and satiety signals, and in shaping the pre- and postprandial oscillations of the gut microbiota. At the long-term, these neurons are required to show normal weight gain in response to HFHSD feeding probably by modulating metabolic routes of fat utilisation (**Figure 8**).

The ablation of Nav1.8⁺ neurons dysregulated the control of body weight and food intake. Specifically, under *ad libitum* regimen, Nav1.8-cre/DTA mice were resistant to weight gain in response to HFHSD, and showed higher body weight loss in the resting phase when fed CD. Under food restriction regime, Nav1.8-cre/DTA also showed a disturbed body weight control irrespective of the type of diet with exacerbated body weight loss and gain in fasting-refeeding cycles only under DRF (the active phase of mice).

On the other hand, the absence of these sensory neurons exacerbated food intake in *ad libitum* feeding and DRF, which could result from the depletion of IGLEs since they express Nav1.8⁺ and are involved in mechanical-induced meal-termination^{6,8}. Specifically, this effect was observed at the beginning of the dark phase, when food intake is mainly governed by hunger. For instance, in the ZT12-ZT18 period, DRF caused hypophagia after fasting in HFHSD-fed control mice but induced hyperphagia in mice lacking Nav1.8⁺ neurons pointing out a critical role of these neurons mediating hunger signaling. In addition, Nav1.8⁺ neurons appear to be involved in nutrient-induced satiation since Nav1.8-cre/DTA mice showed defective Ensure®-induced food intake suppression. The absence of effects under LRF suggests that the role of Nav1.8⁺ neurons in regulating energy homeostasis after fasting is dependent on light-entrainable cues rather than food-entrainable cues. Our findings contrast with the study of Udit et al. that describes a minor role for Nav1.8⁺ neurons in energy intake in a mouse model of primary afferent neuron ablation¹⁰. Discrepancies between studies require further investigations, but methodological differences such as the ageing of the animals and/or fasting-refeeding cycles during the food restriction regimen could be involved.

Our data also support an uncoordinated regulation of weight gain and feeding behavior induced by the ablation of Nav1.8⁺ neurons, since Nav1.8-cre/DTA mice chronically fed with HFHSD showed attenuated weight gain despite their tendency toward increased food intake. Exploration of possible mechanisms involved in this uncoordinated control suggested that ablation of Nav1.8⁺ neurons might overstimulate energy-dissipating metabolic pathways, which in turn would contribute to limit fat depots and exacerbate food intake, as a mechanism to compensate for the energy deficient state. In fact, we showed that Nav1.8-cre/DTA mice fed *ad libitum* HFHSD tended to have higher UCP1 expression in BAT, suggesting increased thermogenic potential helping to limit their fat depots. In line with this, previous investigations revealed that in response to Western diet feeding, mice without the lipid sensing nuclear receptor LXRα/β (liver X receptor alpha and

beta) in Nav1.8-expressing neurons also showed increased thermogenesis activity in BAT coupled with reduced body weight gain²⁷. Further supporting an increased energy loss in Nav1.8-cre/DTA mice, our study also identified reduced eWAT depots in these mice fed *ad libitum* CD accompanied by the overexpression of the β 3 adrenergic receptor, suggesting higher sympathetic-mediated fatty acid release for oxidation²⁸. In line with this, it was recently demonstrated that the ablation of Nav1.8+ neurons in mice reduces food efficiency and lipid absorption after a meal²⁹ suggesting a limited contribution of HFHSD to weight gain in this animal model.

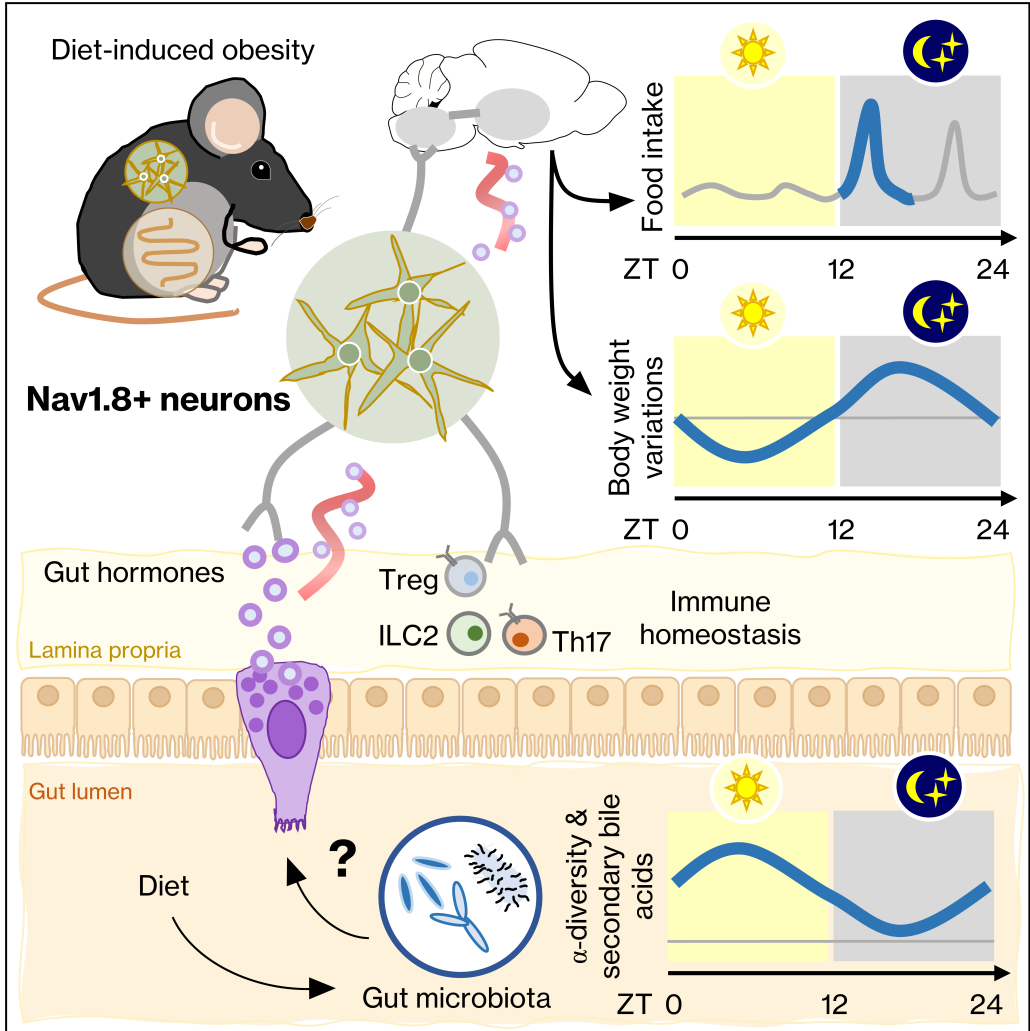


Figure 8. Graphical summary of the main roles of Nav1.8+ neurons on daily control of food intake, body weight and intestinal endocrine, immune and microbial signals.

In mice, Nav1.8+ neurons are involved in the daily control of food intake showing a relevant role in regulating food intake at the beginning of the active phase (dusk peak). Body weight variations along the day are also affected by Nav1.8+ neurons since limit body weight loss in the resting phase and after 12 h of fasting. At intestinal level, these neurons maintain the homeostasis of immune system, shape pre- and postprandial gut microbiota oscillations, and preserve the secretion of gut hormones that transmit hunger and satiety. At long-term, Nav1.8+ neurons are required to develop diet-induced obesity. ZT, zeitgeber time.

On the other hand, we observed that Nav1.8-cre/DTA mice did not survive longer periods of HFHSD consumption (14 weeks in cohort 2-related experiments). Nav1.8+ sensory neurons have been shown to protect host from endotoxic death³⁰ and to be necessary for immune homeostasis in response to an excess of dietary lipids¹⁰. Accordingly, chronic HFHSD feeding could trigger inflammatory processes in Nav1.8-cre/DTA mice, underlying the enhanced mobilization of lipids that is seen in cachexia³¹ and ultimately causing death due to their inability to tolerate the exacerbated lipid-induced inflammation.

Thus, due to the inability of Nav1.8-cre/DTA to survive 14 weeks of HFHSD feeding, the impact of the ablation of Nav1.8+ neurons in the gut-to-brain circuits controlling food intake was explored only in mice fed with CD. In line with the increased food intake, we identified an upregulated and downregulated orexigenic and anorexigenic signaling in the hypothalamus, respectively. Nav1.8+ neurons appear to control food intake by maintaining the secretion of orexigenic and anorexigenic gut hormones, especially ghrelin, GLP-1 and PYY, during the pre- and postprandial periods. Intestinal sympathetic and parasympathetic innervations control ghrelin and GLP-1 secretion, respectively³²⁻³⁴. Accordingly, changes in efferent tone resulting from the absence of Nav1.8+ afferents could potentially affect gut hormone secretion. In this line, duodenal noradrenaline levels were increased in Nav1.8-cre/DTA mice, and thus sympathetic-mediated stimulation of cells producing ghrelin could initially increase ghrelin levels followed by long-term aberrant ghrelin secretion due the chronic overstimulation, resulting in reduced fasting levels of this hormone. The diminished levels of ghrelin in fasting Nav1.8-cre/DTA mice were not sufficient to normalize food intake, as the postprandial enteroendocrine signaling inducing meal termination was also dysregulated. For instance, the ablation of Nav1.8+ neurons induced the oversecretion of GLP-1 during fasting and a lack of response after a meal. Studies in vagotomized rats propose a proximal-distal neuroendocrine loop underlying the early postprandial secretion of GLP-1. This signaling is initiated in the proximal small intestine by nutrient-dependent stimulation of vagal afferents to indirectly induce GLP-1 secretion in the distal gut through parasympathetic innervations³³. Our study suggests that Nav1.8+ neurons might target this loop, explaining that loss of these neurons could contribute to disturbed L-cell functionality, affecting also PYY secretion. On the other hand, Nav1.8+ neurons are required to maintain plasma α MSH levels, which diffuse from the central nervous system to circulation³⁵. Mice lacking Nav1.8+ neurons showed increased fasting levels of α MSH, which could overstimulate PYY and GLP-1 secretion through MC4R in L-cells³⁶.

We also observed an enhanced proinflammatory tone in the intestinal mucosa of mice lacking Nav1.8+ neurons, which could contribute to the observed long-term dysregulation of energy homeostasis. The proportions of ILC2 and Treg cells were reduced in the lamina propria of the small intestine in Nav1.8-cre/DTA mice, coupled to an increase in Th17 cell number. The loss of Nav1.8+ neurons appears to alter intestinal immunity by disrupting neuro-immune communication mediated by Nav1.8+ neurons and/or the efferent sympathetic and parasympathetic innervations in the gut. In the context of neuro-immune communication, the immunomodulatory neuropeptide VIP was almost undetectable in the absence of Nav1.8+ neurons, which produce this neuropeptide²⁶. Thus, loss of Nav1.8+ neurons would interrupt VIP-ILC2 communication, dysregulating the intestinal type 2 inflammatory response as occurs in the pulmonary mucosa²⁶. Correspondingly, as Nav1.8+ neurons express immune-related receptors (i.e., for prostaglandins and leukotrienes)

⁸, their absence would change the anti-inflammatory vagovagal reflex, and/or the intestinal sympathetic tone. The sympathetic tone acts through the β 2-adrenergic receptor to maintain ILC2 cell functionality, allowing acute protective type 2 responses but preventing pathological ILC2 overstimulation ³⁷. In this regard, the increased noradrenaline levels in the upper gut of Nav1.8-cre/DTA mice suggest that an upregulated sympathetic tone could contribute to reducing the abundance of ILC2 cells. Indeed, in bacterially-infected mice, β 2-adrenergic receptor agonists inhibit intestinal ILC2 cell proliferation, resulting in higher worm burdens due to the attenuated type 2 response ³⁷. On the other hand, the reduced abundance of Treg cells in Nav1.8-cre/DTA mice could be driven by the higher intestinal noradrenaline content, related to a sympathetic-mediated apoptotic effect on these cells ^{38,39}. Therefore, the disrupted intestinal immune homeostasis associated with the loss of Nav1.8+ neurons could prompt systemic inflammation, suggested by the observed splenomegaly. This could, in turn, contribute to dysregulating energy balance, leading to reduced energy depots despite the enhanced food intake. Indeed, Nav1.8-cre/DTA mice showed changes in plasma metabolic markers suggestive of energy deficiency (reduced leptin and glucose levels).

The gut microbiota and its interactions with diet, which ultimately modulate gut-to brain neuroendocrine signaling, could also be affected by the greater vulnerability to inflammatory dietary signals through the loss of Nav1.8+ neurons.

The alpha diversity of fecal gut microbiota was not significantly affected by the loss of Nav1.8+ neurons under CD feeding *ad libitum*, in line with other investigations ⁹. Nevertheless, their loss impeded the daily rhythms of *Alistipes* spp. and *Colidextribacter* spp., whose amplitudes in control mice were increased during feeding periods. This suggests that nutrient supply in the intestinal lumen contributes to the increased postprandial abundance of these genera. Indeed, *Alistipes finegoldii* assembles its membrane lipids from dietary fatty acids ⁴⁰, which are more available after feeding.

Under DRF, we could more acutely identify the pre- and postprandial variations of the gut microbiota and its associated metabolites dependent on Nav1.8+ neurons. The gut microbiota structure and derived BAs were particularly affected by the loss of Nav1.8+ neurons under HFHSD. Indeed, their loss impeded the refeeding-induced decline in gut microbiota diversity. The postprandial reduction of alpha diversity in control mice suggests an increase in the abundance of some microbial taxa and a reduction in others in response to fasting-induced refeeding. Although we could not detect major interactions between the genotype and the zeitgeber time on specific ASVs, we found an impaired fasting-to-refeeding decrease in *Alistipes* spp. Tentatively, based on previous investigations ⁹, greater prandial-related ASV variations dependent on the genotype could be detected in the ileal intestinal content.

The loss of Nav1.8+ neurons also blunted the reduction in fecal SBA levels induced by refeeding due to their reduced levels under fasting. This could be the result of lower hepatic PBA production, lower SBA production (owing to compositional microbial changes) or to higher intestinal absorption. An imbalance in the production of BAs by hepatocytes under HFHSD is a likely possibility, being continuous and coordinated in control mice in response to lipid intake but discontinuous and uncoordinated in Nav1.8-cre/DTA mice, reflecting their dysregulated feeding

patterns. Correspondingly, the results also indicate that the gut microbiota of control mice can convert unused PBAs into SBAs upon reaching the colon in the fasting period instead of being absorbed. Contrastingly, the reduced production of SBAs of fasted mice lacking Nav1.8+ neurons could be explained by the absence of PBAs reaching the colon, as they were completely used in the upper gut through the digestion of a higher amount of lipids because of the evident hyperphagia. Also, the changes of the fasted gut microbiota composition of Nav1.8-cre/DTA mice might underlie their inability to produce SBAs from PBAs.

Recent studies have revealed that BAs modulate feeding behavior and energy metabolism by favoring the secretion of gut hormones ⁴¹, intestinal lipid sensing ⁴², and hypothalamic TGR5 signaling, to modulate the sympathetic efferent tone of the adipose tissue ⁴³. Assuming that the altered profile of BA in feces induced by the loss of Nav1.8+ neurons produces similar alterations in the distal intestinal content, the reduction in BAs during fasting could contribute to disrupting enteroendocrine signaling in Nav1.8-cre/DTA mice and, consequently, their daily control of food intake and body weight variations.

In summary, we have identified a role of Nav1.8-expressing neurons in conveying peripheral signals to the brain to govern daily food intake and body weight variations. We propose that these neurons contribute to energy homeostasis not only because they are part of the neural signaling network transmitting peripheral sensory information to the brain, but also because they are necessary to maintain (1) prandial regulation of the enteroendocrine system, (2) fasting body weight loss, and (3) prandial gut microbiota configurations and BA signaling in the distal gut. Accordingly, the loss of Nav1.8+ neurons could account for the overconsumption, inflammation and dysbiosis in obesity, and for the exacerbated weight loss and inflammation in cachexia ⁴⁴. Further studies are needed to determine the spinal or vagal nature of the Nav1.8+ neurons controlling food intake, body weight and endocrine, immune and microbial intestinal signals, as well as the possible causal role of the microbial changes identified on the gut-to-brain axis for controlling food intake and energy homeostasis.

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Author contributions

Conceptualization: MRP and YS; Data curation: CBV; Formal analysis: MRP, TR, CGH-G; Funding acquisition: MRP and YS; Investigation and Methodology: MRP, CBV, and ILA; Supervision: MRP and YS; Writing - original draft: MRP and CGH-G; Writing - review & editing: YS and FTB; Project administration: MRP and YS

Data availability

Raw data from 16S rRNA amplicon sequencing has been deposited at European Nucleotide Archive (ENA). Accession number: PRJEB58911. The procedures for obtaining source data can be found in the general guide on ENA data retrieval. Source data for the graphs and charts are provided as Supplementary Data 1. Any remaining information can be obtained from the corresponding author upon reasonable request.

Conflict of Interest

The authors declare no competing interests.

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6. Supplementary material

Supplemental Tables

Table S1. Primer pairs and sequences used to detect Nav1.8 knock-in *Cre* and floxed *DTA* alleles and their respective wild-type fragments.

Genotype	Primer pairs	Name of primer's pairs	Amplicon length
Wild-type or Nav1.8 knock-in Cre-recombinase	Common forward / wild-type reverse	mSNS13s/mSNS12a	438bp
	Common forward / Nav1.8 knock-in Cre reverse	mSNS13s/Cre5a	461bp
	Nav1.8 knock-in Cre forward / Nav1.8 knock-in Cre reverse	Cre2s/Cre5a	249bp
Wild type or floxed DTA	Common forward / DTA reverse	ROSA26 F/floxed DTA	340bp
	Common forward / common reverse	ROSA26 F/ROSA 26 R	650bp
Primer name	5'-3' sequence		
mSNS13s	TGT AGA TGG ACT GCA GAG GAT GGA		
mSNS12a	TTA CCC GGT GTG TGC TGT AGA AAG		
Cre5a	AAA TGT TGC TGG ATA GTT TTT ACT GCC		
Cre2s	CTG CAT TAC CGG TCG ATG CAA CGA		
ROSA26 F	AAA GTC GCT CTG AGT TGT TAT		
floxed DTA	GCG AAG AGT TTG TCC TCA ACC		
ROSA 26 R	GGA GCG GGA GAA ATG GAT ATG		
TaqMan® Gene Expression Assays used for validation of the Nav1.8 genetic ablations			
Target name	Assay reference	Assay ID	Amplicon length
<i>Scn10a (Nav1.8)</i>	4448892	Mm00501467_m1	82bp
<i>Rpl19</i>	4331182	Mm02601633_g1	69bp

Table S2. Anti-mouse antibodies against immune markers to quantify immune cells in the spleen and intestinal epithelium and lamina propria by flow cytometry.

Immune cells	markers	antibodies	Supplier	Reference
ILC1 group	ILC1: Lin- Tbet+ IFN γ + CD49a+	PerCP-Cy™5.5-conjugated anti-Lineage antibody cocktail (LIN)	BD Bioscience	561317
	NK: Lin- Tbet+ IFN γ + CD49a-	PE-Vio615-conjugated anti-Tbet*	Miltenyi Biotec	130-107-611
		APC-conjugated anti-IFN γ *	Miltenyi Biotec	130-109-723
		PE-vio 770- conjugated anti-CD49a	Miltenyi Biotec	130-107-590

Immune cells	markers	antibodies	Supplier	Reference
ILC2	Lin- GATA3+ IL4+	PerCP-Cy™5.5-conjugated anti-Lineage antibody cocktail (LIN)	BD Bioscience	561317
		BUV395- conjugated anti-GATA3*	BD Bioscience	565448
		Pe-Cy7- conjugated anti-IL4*	Biolegend	504118
ILC3	Lin- ROR γ t+ IL22+	PerCP-Cy™5.5-conjugated anti-Lineage antibody cocktail (LIN)	BD Bioscience	561317
		BV421- conjugated anti- ROR γ t*	BD Bioscience	562894
		PE- conjugated anti-IL22*	Biolegend	516404
Macrophages	M1: F4/80+ CD80+ iNOS+	FITC-conjugated anti-F4/80	Miltenyi Biotec	130-102-327
			Miltenyi Biotec	130-102-372
	M2: F4/80+ CD206+ Arg1+	Pe-Vio770-conjugated anti-CD80		
		APC-conjugated anti-iNOS*	Thermo Fisher Scientific	17-5920-80
		PerCPCy5.5-conjugated anti-CD206	Biolegend	141716
CD4+ T cells	Treg: CD4+ CD25+ Foxp3+	PE-conjugated anti-Arg1*	R&D Systems	IC5868P
	Th17: CD4+ ROR γ t+ IL17F+	BV711-conjugated anti-CD4	BD-Bioscience	563050
			BD-Bioscience	557658
		APCCy7-conjugated anti-CD25		
D4+ T cells	Memory T cells: CD4+ CD44 ^{high} CD62 ^{low}	PE-conjugated anti-Foxp3*	Miltenyi Biotec	130-111-678
		BV421- conjugated anti- ROR γ t*	BD Bioscience	562894
		PerCP-Cy5.5 conjugated anti-IL17F*	BD Bioscience	562194
	Effector T cells: CD4+ CD44- CD62-	BV711-conjugated anti-CD4	BD Bioscience	563050
			BD-Bioscience	560570
		PerCP-Cy™5.5-conjugated anti-CD44		
		APC-conjugated anti-CD62	BD-Bioscience	561919

***antibodies against intracellular markers**

Table S3. Genes, primer pair sequences and conditions of the qPCR reactions

Gene name	Abbreviation	Sequence 5' - 3'	tissue
Neurogenic differentiation 1	Neurod1	Forward: AGGAATTCGCCCACGCAGAAG Reverse: CTCCTCTGCATTCATGGCTTCAAG	duodenum, ileum and colon
Neurogenin 3	Ngn3	Forward: ACTCAGCAAACAGCGAAGAAG Reverse: CAGTGCCCAGATGTAGTTGTG	duodenum, ileum and colon
Neuropeptide Y	Npy	Forward: AATCTCATCACCAGACAGAG Reverse: CTTTCCTTCATTAAGAGGTCTG	hypothalamus
Melanocortin 4 receptor	Mc4r	Forward: GCGTTTCGAATGGGTTCGAAACCA Reverse: CCGCAATGGAAAGCAGGCTGCAA	hypothalamus
Cholecystokinin A receptor	Cck1r	Forward: GACAGCCTTCTTATGAATGGGAG Reverse: GCTGAGGTTGATCCAGGCAG	hypothalamus
Glucagon-like peptide 1 receptor	Glp1r	Forward: GGCGTCAACTTTCTTATCTTC Reverse: CAAAGATGACTTCATGTGTCC	hypothalamus
Leptin receptor	Lepr	Forward: GGACACAGGTGGGACACTCT Reverse: CCCACAGCACATTTTTCTT	hypothalamus
Pro-opiomelanocortin-alpha	Pomc	Forward: AAAAGAGGTTAAGAGCAGTG Reverse: ACATCTATGGAGGTCTGAAG	hypothalamus
Cocaine- and amphetamine-regulated transcript	Cart	Forward: AAGAAGTCCTGAAGAAGCTC Reverse: CAAGCACTTCAAGAGGAAAG	hypothalamus
Agouti related neuropeptide	Agrp	Forward: AGGTCTAAGTCTGAATGGC Reverse: CGGTTCTGTGGATCTAGC	hypothalamus
Neuropeptide Y receptor Y2	Npy2r	Forward: CATCTTCACCGTGTTCCACA Reverse: AAGGCCGAGAGGAAAGCTTT	hypothalamus
Growth hormone secretagogue receptor	Ghsr	Forward: CTCAGGGACCAGAACCACAAAC Reverse: ACAAAGGACACCAGGTTGCAG	hypothalamus
Lysozyme 1	Lyz1	Forward: GCCAAGGTCTACAATCGTTGTGAGTT Reverse: CAGTCAGCCAGCTTGACACCACG	ileum and colon
Defensin A	DefA	Forward: GGTGATCATCAGACCCAGCATCAGT Reverse: AAGAGACTAAACTGAGGAGCAGC	ileum and colon

Gene name	Abbreviation	Sequence 5' - 3'	tissue
Regenerating islet-derived protein 3 gamma	<i>Reg3g</i>	Forward: TTCCTGTCCTCCATGATCAAA Reverse: CATCCACCTCTGTTGGGTTC	ileum and colon
Marker of proliferation Ki-67	<i>Ki67</i>	Forward: CAGACTTGCTCTGGCCTACC Reverse: GGTTGGCGTTTCTCCTCTTT	ileum and colon
Occludin	<i>Ocln</i>	Forward: ATGTCCGGCCGATGCTCTC Reverse: TTTGGCTGCTCTTGGGTCTGTAT	ileum and colon
Claudin 3	<i>Cldn3</i>	Forward: TCATCGGCAGCAGCATCATCAC Reverse: ACGATGGTGATCTTGGCCTTGG	ileum and colon
Adrenergic receptor, beta 2	<i>Adrb2</i>	Forward: CAAGTTCGAGCGACTACAAA Reverse: CTTGAAGGGCGATGTGATAG	white adipose tissue (epididymal)
Adrenergic receptor, beta 2	<i>Adrb3</i>	Forward: TAGGGAAAAGAGAGCACCCC Reverse: TGGTCCAAGATGGTGCTTAG	white adipose tissue (epididymal)
Ribosomal protein L19	<i>Rpl19</i>	Forward: CCTTGTCTGCCTTCAGCTTGT Reverse: GAAGGTCAAAGGGAATGTGTTCA	housekeeping
Conditions of the qPCR reactions - denaturation: 95°C (10 min); 45 amplification cycles: 95°C (10 s), 60°C (30 s), and 72°C (5 s); final melting stage: 95°C (5 s), and 65°C (1 min) All primer pairs were supplied by Isogen Life Science except primers for <i>Glp1r</i> amplification, which were supplied by Sigma.			

Supplemental Figures

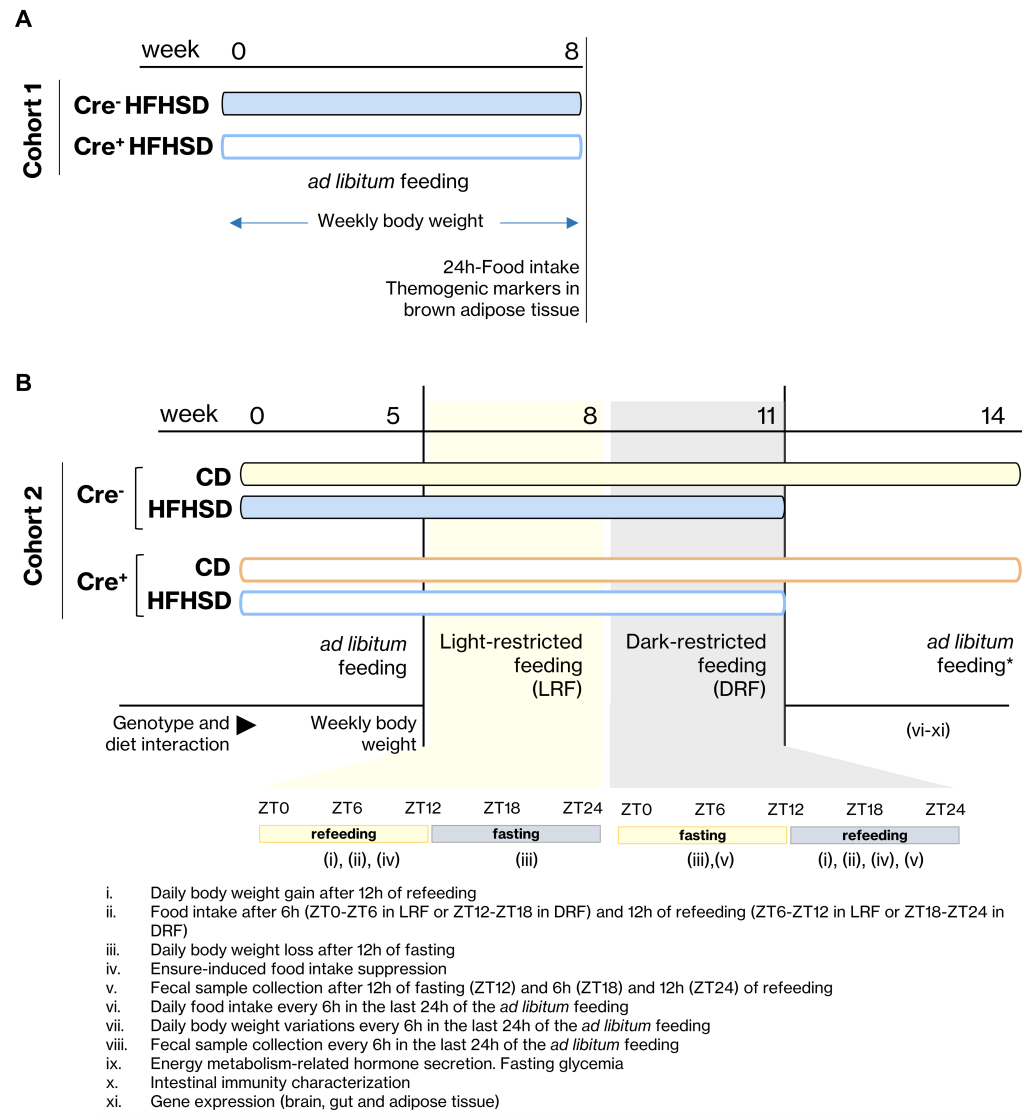


Figure S1. Graphical representation of the experimental procedure.

(A) In cohort 1, mice lacking Nav1.8+ neurons (Cre⁺) and their control littermates (Cre⁻) were fed a high-fat high-sugar diet (HFHSD) for 8 weeks (Cre⁻ HFHSD: n = 6 and Cre⁺ HFHSD: n = 4 mice). **(B)** In cohort 2, Cre⁺ and Cre⁻ mice were randomly allocated to control diet (CD) or HFHSD groups (Cre⁻ CD/HFHSD: n = 10 per group; and Cre⁺ CD/HFHSD: n = 6 and n = 7 mice, respectively). Mice were submitted to different feeding schedules along 14 weeks: *ad libitum* feeding (5 weeks), followed by light- and dark-restricted feeding (LRF/DRF) (3 weeks each) and *ad libitum* feeding (3 weeks). Roman numerals indicate functional assessments and molecular/cellular analyses performed at each feeding schedule at specified zeitgeber time (ZT), with ZT0-ZT12 and ZT12-ZT24 corresponding to the ZTs of the light and dark phases, respectively. Measurements of the last *ad libitum* feeding are only shown for CD-fed mice (Cre⁻/Cre⁺) as HFHSD-*ad libitum* feeding profoundly reduced the survival of Cre⁺ mice.

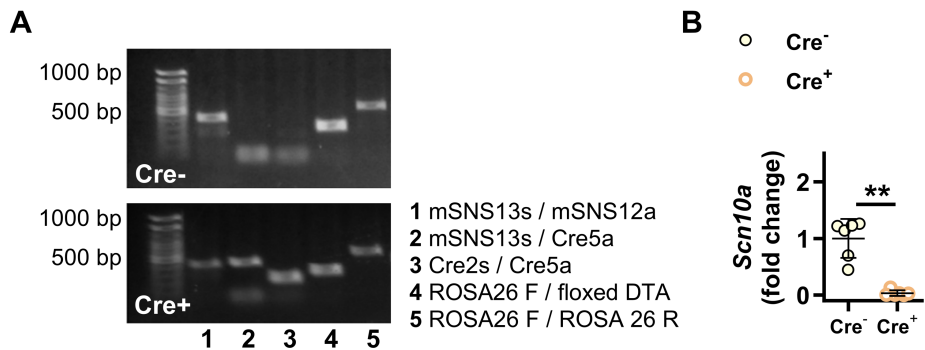


Figure S2. Validation of the ablation of Nav1.8+ cells in the offspring by diphtheria toxin A (DTA)-targeted expression based on the Cre-LoxP system.

(A) Electrophoretic separation of amplified DNA fragments (base pairs, bp) of DTA and Cre recombinase (presented in approximately half of the mice). (B) Silenced gene expression of *Nav1.8* in nodose ganglia of Cre⁺ mice (Nav1.8-cre/DTA) relative to that observed in control littermates (Cre⁻). Results are represented as scatter plots indicating individual values with mean $\pm$ SEM ($n = 5$ mice). Mice fed CD are depicted by solid yellow (Cre⁻) or empty orange (Cre⁻) circles.

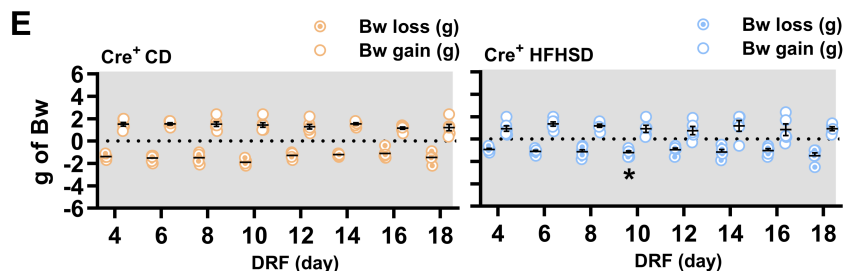
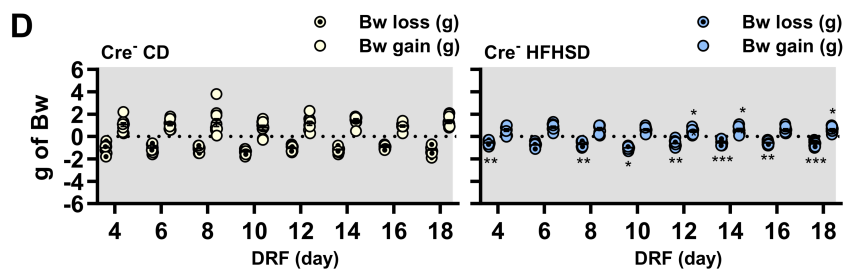
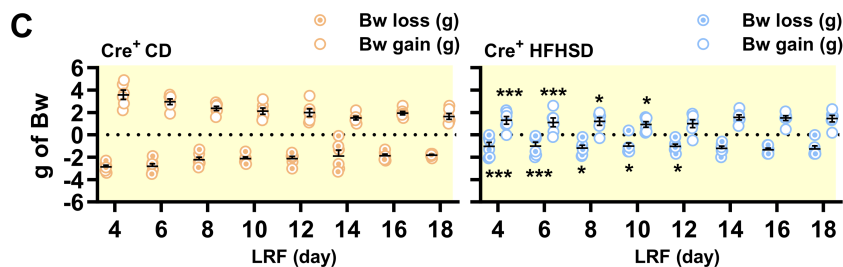
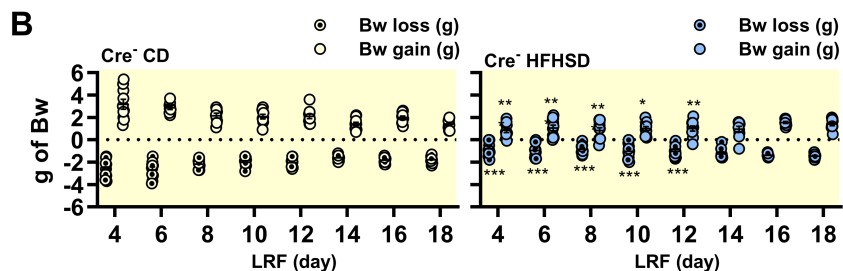
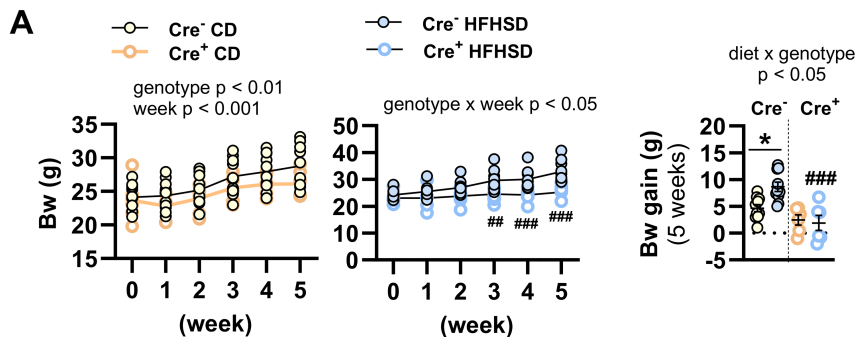


Figure S3. Related to Figure 2. (A) Body weight (Bw) follow-up and Bw gain of control mice (Cre⁻) and Nav1.8-cre/DTA mice (Cre⁺) fed *ad libitum* with either control diet (CD) or high fat high sugar diet (HFHSD) for 5 weeks. 12h-body weight gain (positive values) and body weight loss (negative values) every 2 days in: (B) Control mice fed CD or HFHSD during the light restricted feeding (LRF). (C) Nav1.8-cre/DTA mice fed CD or HFHSD during LRF. (D) Control mice fed CD or HFHSD during DRF. (E) Nav1.8-cre/DTA mice fed CD or HFHSD along DRF. Data are represented as scatter plots for individual values with mean follow-up curve or as scatter plots indicating individual values $\pm$ SEM (Cre⁻ CD and Cre⁻ HFHSD, n = 10 mice; Cre⁺ CD, n = 6 mice; Cre⁺ HFHSD, n = 5-7 mice. Mice fed CD are depicted by solid yellow (Cre⁻) or empty orange (Cre⁺) circles, mice fed HFHSD are depicted by solid blue (Cre⁻) or empty blue (Cre⁺) circles; shadings in light yellow and grey represent LRF and DRF regimes, respectively. (A): Two-way ANOVA with genotype (Cre⁻ or Cre⁺) and diet (CD/HFHSD) as between-subject factor; (B-E): Two-way ANOVA with diet (CD or HFHSD) and day of the LRF or DRF as between-subject factors. Bonferroni's *post hoc* test. a ## p < 0.01 and ### p < 0.001 vs Cre⁻ HFHSD and * p < 0.05 vs Cre⁻ CD; (b-e) * p < 0.05, ** p < 0.01 and *** p < 0.001 vs either Cre⁻ CD or Cre⁺ CD.

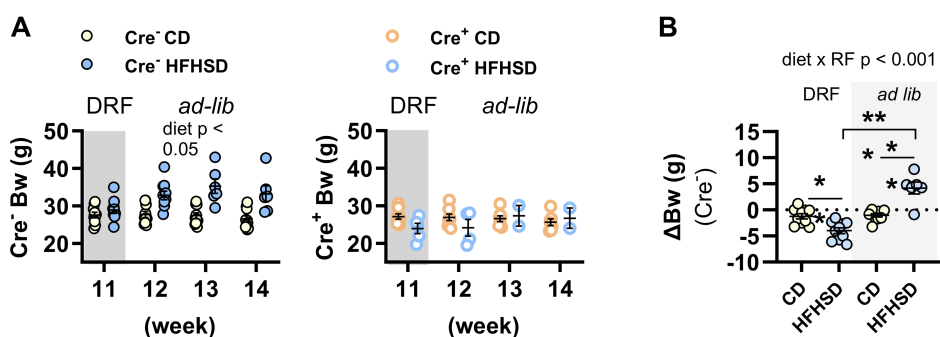


Figure S4. Body weight changes under 24-hour free access to food conditions during 3 weeks.

(A) Body weight (Bw) follow-up along the last 3 weeks of the experiment when control mice (Cre⁻) and mice lacking Nav1.8+ neurons (Cre⁺) on either control diet (CD) or high-fat high-sugar diet (HFHSD) were switched from dark-restricted feeding (DRF) to *ad libitum* feeding (*ad-lib*). (B) Impact of the *ad libitum* feeding reverting the effects of DRF on Bw: calculated Bw differences from the end of the *ad-lib* feeding and the DRF (ΔBw). Results are represented as scatter plots indicating individual values with mean $\pm$ SEM. (Cre⁻ CD and Cre⁻ HFHSD, n = 5–10 mice; Cre⁺ CD, n = 5–6 mice; Cre⁺ HFHSD, n = 2–5 mice). Mice fed CD are depicted by solid yellow (Cre⁻) or empty orange (Cre⁺) circles, mice fed HFHSD are depicted by solid blue (Cre⁻) or empty blue (Cre⁺) circles; shading in grey represents DRF regime. Two-way ANOVA with genotype (Cre⁻ or Cre⁺) and diet (CD or HFHSD) as between-subject factors followed by Bonferroni's *post hoc* test.

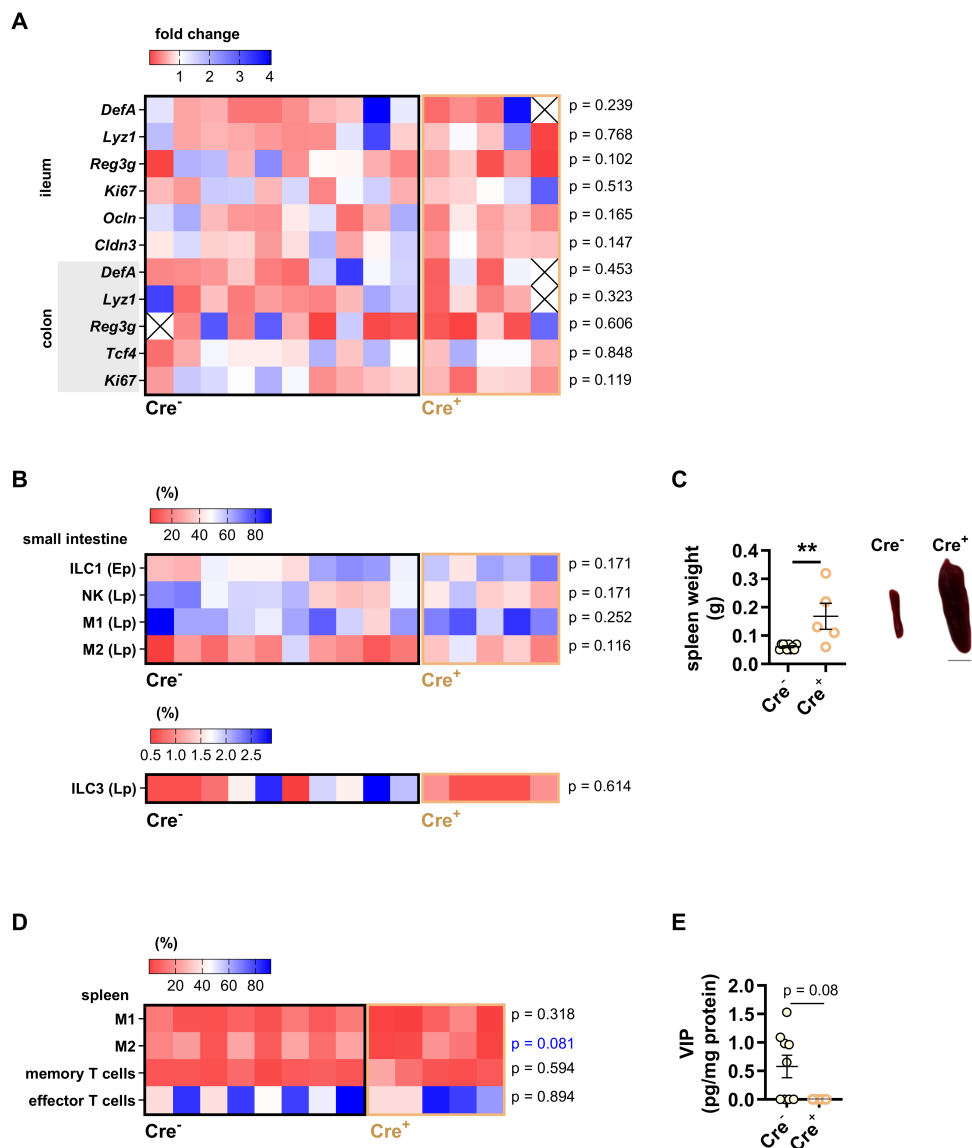
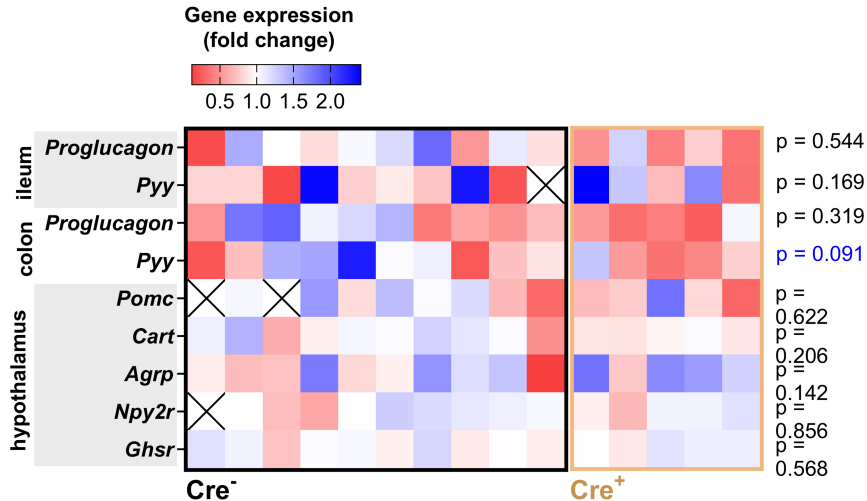


Figure S5. Related to Figure 5. Analysis of immune markers at the end of the experiments in mice fed control diet.

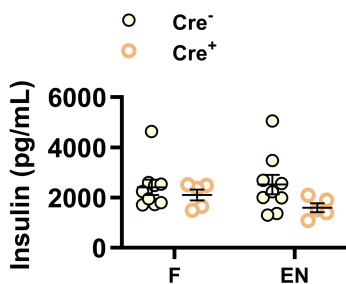
(A) Gene expression of antimicrobial peptides (*DefA*, *Lyz1*, and *Reg3g*), tight junction proteins (*Ocln* and *Cldn3*) and markers of proliferation (*Ki67*) and differentiation (*Tcf4*) in ileum and colon, represented as fold-change of the gene expression relative to control littermates. (B) Percentage of type 1 innate lymphoid cells (ILC1), natural killer cells (NK), and type 1 and type 2 macrophages (M1 and M2, respectively) in the lamina propria of the small intestine. (C) Weight of the spleen, scale bar: 0.5 cm. (D) Percentage of M1, M2, and memory and effector T cells in the spleen. (E) Vasoactive intestinal peptide (VIP) concentration in ileum. Results are shown in a heatmap in which color intensity represents the relative gene expression (A) or the relative abundance of immune cells expressed as a percentage (B, D), and missing values are indicated as "X" (A, B, D); or shown as scatter plots indicating individual values with mean $\pm$ SEM (C, E) (Cre⁻ $n = 9$ –10 mice and Cre⁺ $n = 5$ mice). Mice fed CD are depicted by solid yellow (Cre⁻) or empty orange (Cre⁺) circles. All

measurements were analyzed using Student's t test except for (C), where mRNA levels of *DefA*, *Lyz1* and *Ki67* in ileum and *DefA* and *Reg3g* in colon were analyzed with the Mann-Whitney U test. **p < 0.01.

A



B



C

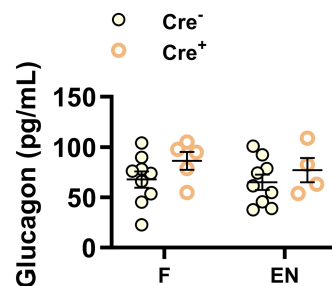
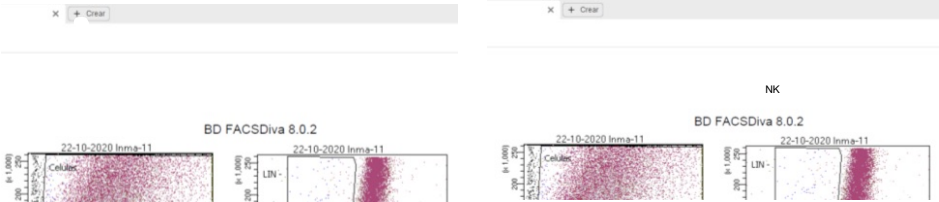


Figure S6. Related to Figure 6 and 7: Gene expression of hypothalamic markers involved in food intake control and plasma levels of hormones involved in regulating glycemia.

In mice lacking Nav1.8+ neurons (Cre⁺) and their control littermates (Cre⁻) fed control diet (CD) we analyzed at the end of the experiment: (A) mRNA levels of different genes including the precursor of GLP-1 (*Proglucagon*) and peptide tyrosine tyrosine (*Pyy*) in ileum and colon, and food intake-related peptides and receptors in the hypothalamus (including *Pomc*, *Cart*, *Agrp*, *Npy2r*, and *Ghsr*). The heatmap shows the gene expression variations, represented by fold-change relative to control littermates; color intensity depicts the relative gene expression, and missing values are indicated as "X". (B, C) Plasma insulin and glucagon levels in 4-h fasted f mice and 15 min after an oral gavage of the nutrient mixed solution (ensure, EN). Results are represented as scatter plots indicating individual values with mean $\pm$ SEM. (Cre⁻ n = 6–10 mice and Cre⁺ n = 5 mice). Mice fed CD are depicted by solid yellow (Cre⁻) or empty orange (Cre⁻) circles. (A) Student's t test. (B, C): Two-way ANOVA with genotype (Cre⁻ or Cre⁺) and prandial condition (fasting/ensure, F/EN) as between-subject factor.

a ILC1 group (intestinal epithelium)



b ILC2 and ILC3 group (intestinal lamina propria)



c Macrophages (intestinal lamina propria)



d Treg and Th17 (intestinal lamina propria)



e Macrophages (spleen)



f Memory and effector T cells (spleen)

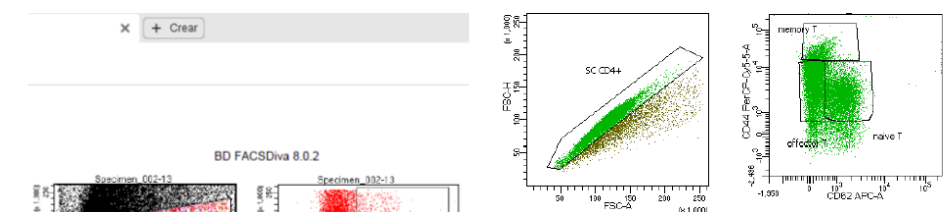


Figure S7. Gating strategy for flow cytometry analysis of immune cells in mice fed control diet.

(A) Gating strategy for type 1 innate lymphoid cells (ILC1) and natural killer cells (NK) in the intestinal epithelium. Total cells were selected from a FSC-A vs SSC-A dot plot, and lineage negative (LIN)⁻ cells were selected using a mouse lineage antibody cocktail. Subsequently, ILC1 group was characterized as Tbet⁺ IFN γ ⁺, which was subsequently selected for CD49a expression as ILC1, while CD49a⁻ cells were characterized as NK. **(B)** Gating strategy for type 2 and type 3 innate lymphoid cells (ILC2 and ILC3, respectively) in the intestinal lamina propria. LIN⁻ cells were selected as for ILC1 group **(A)**. ILC2 were then characterized as GATA3⁺ IL4⁺, and ILC3 were identified as ROR γ t⁺ IL22⁺. **(C)** Gating strategy for macrophages in the intestinal lamina propria. F4/80⁺ cells were selected from FSC-A vs SSC-A dot plot. M1 and M2 macrophages were characterized as iNOS⁺CD80⁺ and Arg1⁺CD206⁺, respectively. **(D)** Gating strategy for Treg and Th17 in the intestinal lamina propria. CD4⁺ cells were selected from FSC-A vs SSC-A dot plot. CD4⁺ cells were selected to subsequently identify IL17F⁺ ROR γ t⁺ population (Th17) and CD25⁺ FoxP3⁺ cells (Treg). **(E)** Gating strategy for macrophages in the spleen. F4/80⁺ cells were selected from FSC-A vs SSC-A dot plot. M1 and M2 macrophages were characterized as iNOS⁺CD80⁺ and Arg1⁺CD206⁺, respectively. **(F)** Gating strategy for memory and effector T cells in the spleen. CD4⁺ cells were selected from a FSC-A vs SSC-A dot plot. CD4⁺ cells were selected to subsequently identify CD44^{high}CD62^{low} as memory T cells and CD44⁺CD62⁻ as effector T cells.

Chapter 3

Chapter 3

***Phascolarctobacterium faecium* induces resistance to diet-induced obesity in mice by inducing PYY anorexigenic effects and by modulating gastrointestinal functions**

Clara Bullich-Vilarrubias^{1*}, Marina Romaní-Pérez^{1*}, Inmaculada López-Almela^{1,2}, Alfonso Benítez-Páez¹, Verónica Tolosa-Enguís¹, Yolanda Sanz¹

¹ Microbiome, Nutrition and Health Unit, Institute of Agrochemistry and Food Technology, Spanish National Research Council (IATA-CSIC), Valencia, Spain.

² Present address: Research Group Intracellular Pathogens: Biology and Infection, Department of Animal Production and Health, Veterinary Public Health and Food Science and Technology, Faculty of Veterinary Medicine, Cardenal Herrera-CEU University, Valencia, Spain.

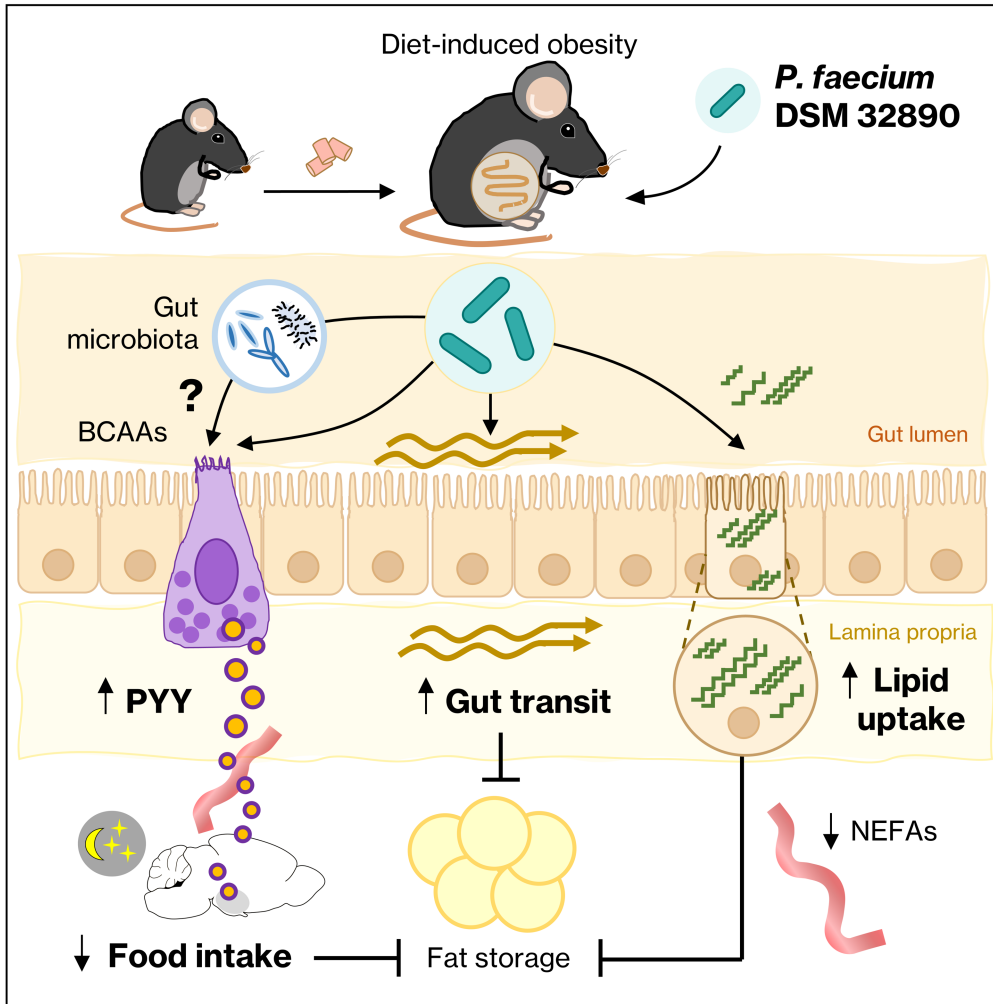
*Equal contribution

Manuscript under preparation

My contribution to this research article was: Conceptualization and Study Design, Methodology, Formal analysis, Investigation, Writing - Original draft.

Chapter 3

Graphical Abstract



***Phascolarctobacterium faecium* induces resistance to diet-induced obesity in mice by inducing PYY anorexigenic effects and by modulating gastrointestinal functions**

Abstract

Disturbed feeding behaviour contributes to adiposity in obesity. Here, we investigated whether the human intestinal bacterium *Phascolarctobacterium faecium* DSM 32890 prevents obesity through changes in food intake in a diet-induced obesity mouse model. *P. faecium* administration reduced caloric intake through the induction of PYY oversecretion since early stages of obesity development. We also showed that the bacterium itself and the branched chain amino acids (valine, leucine and isoleucine), increased in the cecum after *P. faecium* administration, act as PYY secretagogues on neuroendocrine cells cultures. A pair-feeding experiment demonstrated that, the anorexigenic effect of *P. faecium* is a major contributor to its metabolic benefits, but other pathways are also involved. The bacterium accelerated gut transit and attenuated intestinal lipid absorption, limiting adiposity independently of caloric intake. This study identifies the modes of action of a new human intestinal bacterium against obesity, which includes the restoration of eating behaviour and intestinal transit.

1. Introduction

Obesity has already reached epidemic proportions ^{1,2}. The multifactorial etiology of this disorder hampers the development of effective therapies to curb its increased prevalence over the years. Consumption of hypercaloric diets, rich in saturated fats and simple sugars, disturbs the energy balance, especially by altering the hypothalamic brain circuits controlling food intake in obesity ^{3–9}.

The gut plays an important role in the control of food intake and energy homeostasis by transmitting information from nutrients in the intestine to the brain (nutrient sensing). Nutrient sensing is mediated by enteroendocrine cells (EECs) ^{10,11} specialized in producing gut hormones ^{12,13} that target the brain through endocrine routes after reaching blood circulation, or through paracrine routes by activating vagal afferent neurons innervating the intestinal mucosa ^{14,15}. Obesity is associated with altered secretion of GI anorexigenic hormones, such as glucagon-like peptide 1 (GLP-1) or peptide YY (PYY) ^{16–19}. On this basis, obesity treatments are being designed, including gut peptide-based strategies for mimicking the bariatric surgical enteroendocrine responses ²⁰ and agonists of G protein coupled receptors (GPCRs) inducing gut hormone secretion, known as secretagogues ²¹.

A large body of evidence also demonstrates that intake of Western diets induces alterations in gut microbiota composition ^{22–25} that contribute to obesity by increasing the energy harvest from diet ²⁶. The intestinal bacteria can also influence host feeding behavior by modulating satiety and reward pathways ^{27,28}. Bacterial structural components and microbial-derived metabolites resulting from food digestion and fermentation can activate EECs through toll-like receptors (TLRs) and GPCRs, respectively, to induce production and release of appetite-regulating hormones ^{29–32}. For instance, nutrient-induced growth of *Escherichia coli* promotes the production of the bacterial protein ClpB contributing to meal termination by increasing PYY secretion and stimulating hypothalamic POMC neurons ³³.

Despite the potential of human intestinal bacteria caloric intake in obesity, evidence is scarce. An observational study in humans revealed that the abundance of the genus and species *Phascolarctoacterium faecium* was decreased in children showing excessive body weight gain compare to those showing normal one during a four-year follow-up ³⁴. Moreover, this genus has been associated with the antidiabetic effects of metformin or berberine treatment in obese rats ³⁵.

To progress from associations to causation, we have investigated whether the bacterial strain *P. faecium* DSM 32890, isolated from feces of a metabolically healthy individual, could modulate the enteroendocrine system and energy intake, a mechanism that remains largely unexplored, and thus exert anti-obesogenic effects in diet-induced obesity (DIO), combining *in vivo* (hormone blocking and paired-fed models) and *in vitro* experimental models. Specifically, we demonstrated that the anorexigenic effects of *P. faecium* DSM 32890 through enhancement of PYY secretion is key in the control of body weight, and that both the bacterium and the enhanced metabolites (branched-chain amino acids) act as PYY secretagogues. We also prove that, independently of the reduced energy intake, this bacterial strain accelerates gut transit time and reduces lipid absorption, which also contribute to prevent obesity.

2. Materials and Methods

Phascolarctobacterium faecium isolation and culture conditions

P. faecium DSM 32890 was isolated from feces of healthy volunteers as previously described ³⁶. For *in vivo* experiments with mice, *P. faecium* was grown in modified PYG medium supplemented with sodium succinate (8 g/L) instead of glucose at 37°C under anaerobiosis for 48 h. Cells were centrifuged (10000g, 10 min, 4°C) followed by two phosphate-buffered saline (PBS) washes and re-suspended in PBS (0.05% cysteine, 20% glycerol). Quantification of the number of viable bacteria (colony forming units, cfu/mL) was performed by plate counting using the aforementioned media and incubated at 37°C under anaerobic conditions for 48 h.

Mice, diets and experimental design

C57BL/6 male mice (6-8 weeks old) (Charles River Laboratories) were individually housed under controlled conditions of humidity and temperature (23±2°C), and maintained under a 12-h light/dark cycle (lights on/off: 6:00/18:00, in which zeitgeber time (ZT)0 corresponds to lights on and ZT12, to lights off).

Animals were fed *ad libitum*, unless otherwise stated, with CD (D12450K), containing 10% of kcal from fat and without sucrose, or HFHSD (D12451), containing 45% of kcal from fat and 21% from sucrose (Research Diets). Sample size, detailed in **Figure S6**, was calculated using G*Power software based on a previously estimated effect size. Mice were randomly allocated in each experimental group. All experimental procedures using animals were performed in accordance with European Union 2010/63/UE and Spanish RD53/2013 guidelines, and were approved by the ethics committee of the University of Valencia (SCSIE, UV, Spain) and authorized by Dirección General de Agricultura, Ganadería y Pesca (Generalitat Valenciana; approval ID 2017/VSC/PEA/00015, 2019/VSC/PEA/0020 and 2020/VSC/PEA/0022).

The effects of the oral supplementation of *P. faecium* on DIO in mice was explored by conducting the experiments detailed below and graphically represented in **Figure S6**.

Experiment 1 (Figure S6A): HFHSD-fed mice received daily an oral gavage of *P. faecium* (1-5x10⁹ cfu/mouse in PBS 0.05% cysteine, 20% glycerol) or vehicle (PBS 0.05% cysteine, 20% glycerol), whereas CD-fed mice received only vehicle. Body weight was monitored weekly and 24-h individual caloric intake was measured at 3, 4 and 12 weeks of HFHSD-feeding. At week 4, body weight loss after overnight fasting was estimated in HFHSD-fed mice receiving vehicle or *P. faecium*. After 4 and 12 weeks of HFHSD-feeding, overnight fasted mice were anaesthetized with isoflurane for blood collection by cardiac puncture. After cervical dislocation, brains and cecal content were isolated and immediately frozen until processing.

Experiment 2 (Figure S6B): For the pair-feeding experiment, mice were fed HFHSD for 12 weeks and received an oral daily dose of vehicle or *P. faecium* as in experiment 1. An additional group also fed HFHSD was pair-fed over a 24 h-period to the average food consumed the previous day by the *P. faecium*-treated group. The pair-fed group was orally administered with vehicle. Body weight was monitored weekly and caloric intake was measured daily at ZT12.

Different functional assessments were performed throughout the intervention including: intestinal absorption of lipids, PYY secretion and glycemia in response to an oral nutritional challenge, intestinal transit time, and oral glucose tolerance test (OGTT).

-Intestinal absorption of lipids, PYY secretion assays and postprandial glycemia: At weeks 4 and 12, 4-h fasted mice received an oral load of intralipid 20% (300 μ L per mouse, Sigma), as previously reported ³⁷ at ZT12, and blood was collected from the saphenous vein at 0 (fasting), 20 min, 2 and 4 h after intralipid administration. Triglycerides and NEFAs were determined at 0, 120 and 240 min, whereas PYY were measured in samples collected at 0 and 20 min. For PYY analysis, intralipid administration and blood sampling were also performed at ZT0 (light phase) to determine the phase in which the gut hormone secretion is more efficient. Glycemia was determined in blood from the saphenous vein at fasting and 20 min after intralipid administration using glucose test strips and a Contour XT glucometer (Bayer).

-GI transit time: At week 7, intestinal transit time was determined as previously reported ³⁸. Briefly, mice with free access to food were administered an oral gavage of carmine red dissolved in 0.5% of methylcellulose in 0.9% PBS 1X at ZT12. The color of fecal pellets of each mouse was examined every 30 min. Intestinal transit time was measured as the time between oral gavage and first appearance of carmine dye in feces.

-OGTT: At week 10, an OGTT was carried out as previously described ³⁶.

At week 12, overnight fasted mice were intraperitoneally injected with insulin (1.5 U/kg, NovoRapid FlexPen®, Novo Nordisk, Denmark). After 15 min, mice were sacrificed by cervical dislocation. Blood glucose from saphenous vein was measured at fasting and after insulin administration. eWAT, subWAT, and BAT were isolated and weighted. Fragments of eWAT, subWAT and colon were extracted and immersed in 10% neutral buffered formaldehyde for fixation for hematoxylin-eosin staining and immunohistochemistry. In addition, fragments of subWAT were immediately frozen until gene expression processing.

Experiment 3 (Figure S6C): The experiments were conducted in mice fed HFHSD for 4 weeks. First, we conducted two dose testing experiments to validate the immunoneutralization of PYY through PYY-antibody administration. Then we performed a PYY-blocking experiment to explore whether or not *P. faecium* required PYY for inducing anorexigenic effects.

In the first testing experiment, we assessed the capacity of the administration of an anti-PYY antibody (Phoenix Pharmaceuticals) to reduce energy intake. Two concentrations of the anti-PYY antibody were tested: a dose 10 and 100 times higher than the PYY plasma levels in *P. faecium*-treated mice (i.e: 1500 pg/mouse or 15000 pg/mouse, respectively). Mice were intraperitoneally injected with saline, 10x dose or 100x dose of anti-PYY antibody, 1 h before the onset of the dark phase (ZT11). *Ad libitum* caloric intake was measured at ZT24 for 2 consecutive days.

In the second testing experiment, we determined whether the lower effective dose of the anti-PYY antibody was able to block the anorexigenic effect of PYY, exogenously administered. For periods of two consecutive days, mice were intraperitoneally injected: saline at ZT11 and at ZT12; saline followed by 5 μ g/kg of PYY₃₋₃₆ (Tocris™ Bioscience) at ZT11 and ZT12, respectively; and a 10x anti-PYY antibody dose followed by PYY₃₋₃₆ at ZT11 and ZT12, respectively. *Ad libitum* caloric

intake was measured 4 h after the saline/saline, saline/PYY and anti-PYY antibody/PYY₃₋₃₆ administrations (ZT16) for 2 consecutive days.

For the PYY-blocking experiment, mice daily received an oral gavage of *P. faecium* at the same dose as in the experiment 1 or vehicle during 4 weeks. At the end of the intervention, at ZT11, mice were injected intraperitoneally with saline and then with 10x anti-PYY antibody, for 2 consecutive days each. *Ad libitum* caloric intake was determined after 24 h.

For all experiments, blood samples were collected in Microvette® 500 K3E tubes (Sarstedt). For GLP-1 analysis, blood tubes contained dipeptidyl-peptidase (DPP)-4 inhibitor (Sigma). Plasma was obtained by immediate centrifugation and stored at -80°C until analysis.

Hormone measurements

Plasma levels of total PYY, total GLP-1 and insulin were quantified using a MILLIPLEX Mouse Metabolic Hormone Expanded Panel on a Luminex® MAGPIX System (Milliplex, Merck). CCK plasma levels were measured using a Mouse Cholecystokinin 8 (CCK8) ELISA kit (MyBioSource, Quimigen) and leptin levels were determined with Mouse Leptin ELISA (Merck), according to manufacturer's instructions on a CLARIOstar Microplate Reader (BMG Labtech). Mouse Insulin ELISA (Mercodia) was used to measure insulin at 0 and 15 min of OGTT.

Triglycerides, cholesterol and citrate synthase measurements

Triglycerides, cholesterol, and NEFAs were measured in plasma using Triglyceride Colorimetric Assay Kit (Elabscience) or Triglyceride Quantification Colorimetric Assay Kit (Abcam), Cholesterol Liquid Kit (Química Clínica Aplicada SA), and Free Fatty Acid Colorimetric Assay Kit (Abcam). Citrate synthase activity was measured in subWAT using the Citrate Synthase Assay Kit (Abcam).

Acute PYY secretion assay in STC-1 cell line

Enteroendocrine murine STC-1 cells (ATCC®) were grown in Dulbecco's Modified Eagle's Medium (DMEM; ATCC®) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco™), 1% penicillin/streptomycin (Sigma) and 0.1% amphotericin B (Corning®) in 75 cm² cell culture flasks (SPL Life Sciences) and maintained in an incubator at 37°C in a 5% CO₂ atmosphere. Cells were then seeded in 24-well cell culture plates (SPL Life Sciences) at a density of 1 x 10⁵ cells per well and incubated at 37°C for 48 h until reaching 80% confluence. Cells were washed with PBS 1X and incubated with *P. faecium* for 16 h (cell-to-bacteria 1:10 ratio) for being further incubated (1 h) or not with either valine, leucine or isoleucine (100 µM each BCAA). Wells incubated only with PBS were used as negative control. After incubations, supernatants and cells were harvested and processed as follows. Protein from supernatants was concentrated using Amicon® ultrafiltration tubes (0.5 mL) (Merck Millipore). Cells were removed by adding 1X Trypsin-EDTA Solution (ATCC®, Virginia, USA) and lysed with RIPA buffer (Sigma) with protease inhibitor (Roche). Isolated proteins were stored at -80°C prior to PYY determinations using the Mouse PYY EIA kit (RayBiotech).

Lipid secretion assays in Caco-2 cells

Human colonic Caco-2 cells (ATCC®) were grown in Eagle's Minimum Essential Medium (EMEM; ATCC®) supplemented with 10% heat-inactivated FBS (Gibco™), 1% penicillin/streptomycin (Sigma) and 0.1% amphotericin B (Corning®) and maintained in a 5% CO₂ humidified atmosphere at 37°C. Cultured Caco-2 cells were seeded in 6-well Corning® Transwell® plates with

polycarbonate membrane inserts (12 mm diameter, 1.12 cm², 0.4 µm pores; Corning®) at a density of 1 x 10⁵ cells per insert. A lipid secretion assay was conducted when cell monolayers reached a trans-epithelial electrical resistance (TEER) > 600 Ω x cm², measured with a Millicell-ERS-2 voltohmmeter (Merck-Millipore).

P. faecium (cell-to-bacteria 1:10 ratio) or PBS, as control condition, were added to cells of the apical compartment and incubated for 16 h. After one wash with PBS, medium containing lipid micelles (2 mM sodium taurocholate, 0.05 mM cholesterol, 0.6 mM oleic acid, 0.2 mM 2-mono-oleoylglycerol, 0.2 mM L-α-lysophosphatidylcholine [Sigma] and 0.02 mM of the fluorescent fatty acid BODIPY™ FL C₁₆ [Thermofisher]) was added to the apical compartment. After a 15 min incubation, medium containing micelles was replaced by fresh medium. BODIPY™ FL C₁₆ secretion was determined by measuring fluorescence of both apical and basal compartments at 0, 2 and 4 h after removal of micelles using a CLARIOstar Microplate Reader (BMG Labtech). At 4 h, cells were collected using 1X Trypsin-EDTA Solution (ATCC®) and lysed with 0.1% Triton™ X-100 solution and sonicated in an ultrasonic bath for 15 minutes. Lysed cells were centrifuged at 16000 g for 3 min at 4°C and supernatants were collected for immediate fluorescence measurements.

Metabolomic analyses in cecal content

Bile acids (BAs), short chain fatty acids (SCFAs) and branched amino acids (BCAAs) were analysed in the cecal content of mice from *Experiment 1* according to standardized protocols of LEITAT Technological Center (Valencia, Spain). In brief, samples were extracted in a PreCellys system for BAACs quantification by UPLC following AccQ-Tag chemical derivatization. SCFA were analyzed by gas chromatography-mass spectrometry (GC-MS) and concentration of BAs was determined by ultra-performance liquid chromatography (UPLC).

Histology and immunohistochemistry

Fixed eWAT, subWAT and colon tissues were paraffin-embedded. Following standardized protocols by Patologica Laboratorios S.L. (Valencia, Spain), WAT samples were stained with hematoxylin and eosin, whereas intestinal samples underwent immunohistochemical staining for PYY-positive cells quantification using a 1:9000-dilution of anti-PYY antibody (Abcam) and a mix of goat anti-rabbit and goat anti-mouse as secondary antibody. Bright-field digital images were captured by Eclipse 90i Nikon wide-field microscope (Nikon Corporation) coupled to a CFI Plan Fluor DIC M/N2 (MRH00200) Nikon dry-air objective of 20X combined with an optical zoom factor of 0.8X for adipose tissue samples and 4X for intestinal samples. Individual adipocyte fat area was selected using Fiji software (ImageJ 1.49q Software, National Institutes of Health, USA) and quantified by Nis elements BR 3.2 software (Nikon Corporation). Data are based on measurements of 70-300 adipocytes from 4-6 sections per mouse. For intestinal samples, PYY-positive cells and mucosal area were selected and quantified using Nis elements BR 3.2 software, and the number of PYY-positive cells was calculated per unit of mucosal area.

RNA extraction and RT-qPCR analysis

For total RNA extraction of hypothalamus and subWAT, TRIsure™ reagent (Bioline) was used according to manufacturer's instructions. For reverse transcription, between 1-2 µg of total RNA were incubated at 25°C for 10 min, 37°C for 120 min and 85°C for 5 min with the High-Capacity

cDNA Reverse Transcription Kit (Thermo Fisher Scientific) in an Eppendorf thermocycler. Real time qPCR was carried out incubating previously validated cDNA dilutions with 300nM of gene-specific primer pair sequences (**Table S1**, Isogen) and LightCycler 480 SYBR Green I Master Mix (Roche) in a LightCycler® 480 Instrument (Roche). Relative mRNA levels were calculated for each gene according to the $2^{-(\Delta\Delta C_t)}$ method. The amount of target gene, normalized to the housekeeping gene ribosomal protein L19 (*Rpl19*), is represented as mRNA fold-change expression relative to the control group.

Fecal microbiota analysis

Feces were obtained from almost all animals (N=29) at the end-point of the experiment and ~100 mg were used to isolate microbial DNA using the QIAamp PowerFecal DNA kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The DNA concentration was measured by UV methods (Nanodrop, Thermo Scientific, Wilmington, USA). The V3-V4 hypervariable regions of the 16S ribosomal ribonucleic acid (rRNA) gene were amplified using 1 μ L aliquot DNA (~20 ng) and 25 PCR cycles consisting of the following steps: 95 °C for 20 sec., 55 °C for 20 sec. and 72 °C for 20 sec. Phusion High-Fidelity Taq Polymerase (Thermo Scientific, Wilmington, USA) and the 6-mer barcoded primers, S-D-Bact-0341-b-S-17 (TAGCCTACGGGNGGCWGCAG) and S-D-Bact-0785-a-A-21 (ACTGACTACHVGGGTATCTAATCC) that target a wide repertoire of bacterial 16S rRNA genes³⁹ were used for PCR. Dual barcoded PCR products, consisting of ~500 bp, were purified from triplicate reactions with the Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, UK) and quantified through Qubit 3.0 and the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). The samples were multiplexed in one sequencing run by combining equimolar quantities of amplicon DNA (~50 ng per sample) and sequenced in one lane of the Illumina MiSeq platform with 2x300 PE configuration (Eurofins Genomics GmbH, Germany). Raw data was delivered in fastq files and pair ends with quality filtering were assembled using Flash v1.2.11⁴⁰. Sample de-multiplexing was carried out using sequence information from forward/reverse primers, the respective DNA barcodes, and the SeqKit v0.12.1 suite of analysis⁴¹. After demultiplexing, the barcodes/primers were removed and sequences were processed for chimera removal using UCHIME algorithm⁴² and the SILVA reference set of 16S sequences (Release 132)⁴³. Operational Taxonomic Unit (OTU) information was retrieved by using a rarefied subset of 10,000 sequences per sample, randomly selected after multiple shuffling (10,000X) from of the original dataset, and the UCLUST algorithm implemented in USEARCH v8.0.1623⁴⁴. Common alpha diversity descriptors including the Chao's index, Reciprocal Simpson's index, Simpson's evenness, dominance, and phylogenetic distance (PD) were computed using QIIME v1.9.1⁴⁵. For phylogenetic-based metrics the OTUs sequences were aligned using the PyNAST algorithm implemented in QIIME and the SILVA aligned reference database, whereas the tree topology reconstruction was completed with the FastTree algorithm⁴⁶ using the generalized time-reversible (GTR) model and gamma-based likelihood. The evaluation of the community structure across the sample groups was performed with the vegan R package v2.5-6 through interpretative multivariate and constrained appraisal based on the distance-based redundancy analysis (dbRDA) (vegan::dbRDA function and "bray" method). For taxonomy assessment, we used the transformation of compositional microbiota-derived data by applying the centered log-ratio (clr) algorithm implemented in *CoDaSeq::codaseq.clr* with prior execution of *zCompositions::cmultRepl* R function supporting a Bayesian-Multiplicative replacement of count zeros. Taxonomy

identification for OTUs of interest was assisted by utilization of SINA aligner⁴⁷ and SILVA database (Release 138).

Statistical analysis

Statistics were performed with GraphPad Prism version 9.5.1 for Windows (GraphPad Software). Normality and equality of variances of data were assessed through the D'Agostino & Pearson test and Bartlett's test, respectively. One-way analysis of variance (ANOVA) followed by Tukey post hoc test was conducted to compare three independent groups, either CD-Veh, HFHSD-Veh and HFHSD-*P. faecium* or HFHSD-Veh, HFHSD-*P. faecium* and HFHSD-Veh paired group. Kruskal-Wallis test followed by Dunn's test for multiple comparisons was used for nonparametric data. Two or three-way ANOVAs were run to assess main effects and interactions between variables. When interactions were identified, Tukey's post hoc tests were conducted. Paired t-tests were applied to compare two matched data. Differences were considered significant when p-values were $p < 0.05$. Graphs were plotted using GraphPad Prism version 9.5.1. Statistical analyses on microbiota data were conducted in R v4.1.2 applying non-parametric methods such as Kruskal-Wallis and pairwise Wilcoxon Rank Sum tests (for unpaired samples) with Benjamini-Hochberg post hoc correction for multiple group comparison across the alpha diversity descriptors. Statistical robustness for differences of the microbial community structure assessed by interpretative approaches was completed using the permutation-based *vegan::adonis* function. Differential abundance of OTUs across groups was assisted by application of Kruskal-Wallis test with Benjamini-Hochberg post hoc correction. Highly divergent OTUs in terms of abundance were selected when Kruskal-Wallis corrected p-value ≤ 0.01 . Correlations were determined using Kendall rank correlations. Graphs and plots were drawn using GraphPad Prism version 9.5.1, and *ggplot2* and *grid* R v4.1.2 packages. Heatmap hierarchical clustering of OTUs (scaled DNA read counts) was achieved using Euclidean distance and the complete clustering method.

3. Results

P. faecium DSM 32890 reduces caloric intake by amplifying PYY production and secretion in diet-induced obese mice

The oral supplementation of *P. faecium* to high-fat-high sugar-diet (HFHSD)-fed mice (or DIO mice) for 12 weeks reduced body weight, reducing the area under the curve (AUC) of the body weight follow-up (**Figure S1A**). Body weight gain (**Figure 1A**), as well as the weights of eWAT and subWAT were also reduced in DIO mice treated with *P. faecium* (**Figure 1B**). Compared to control diet (CD)-fed mice, untreated DIO mice showed increased 24 h-caloric intake, but this was reduced by *P. faecium* in HFHSD-fed mice, reaching similar values as CD-fed mice after the 12-week intervention (**Figure 1C**). To evaluate the time-point of HFHSD feeding when *P. faecium* induced a food intake suppressing effect, caloric intake was measured weekly in another experiment. *P. faecium* treatment prevented the hyperphagia of HFHSD-fed mice at 4 weeks, but not before at 3 weeks (**Figure 1D**). Specifically, the food intake suppression was detected during the dark but not in the light phase after 12 h of *ad libitum* feeding (**Figure 1D**).

We also investigated whether the anorexigenic effects of *P. faecium* at early stages of DIO were already associated with metabolic benefits. We observed that the *P. faecium* treatment reduced

body weight throughout the 4 weeks of administration, reducing the AUC of the body weight follow-up (**Figure 1E**). Compared to untreated HFHSD-fed mice, the weight of subcutaneous white adipose tissue (subWAT) tended to be reduced in *P. faecium*-treated mice, while no differences were detected in epididymal white adipose tissue (eWAT) (**Figure 1F**). Compared with untreated HFHSD-fed mice, plasma triglycerides and cholesterol levels were reduced in *P. faecium*-treated HFHSD-fed mice after 4 weeks of intervention (**Figure 1G** and **1H**). Moreover, *P. faecium* prompted a greater body weight loss in response to 12 h of fasting (**Figure 1I**), suggesting an enhanced fasting-induced utilization of fat depots due to the bacterium intervention at early stages of DIO.

We next analyzed the gene expression profile of hypothalamic neuropeptides involved in the central control of food intake at weeks 4 and 12 of HFHSD feeding. The analyses of the mRNA levels of anorexigenic neuropeptides revealed that, only at the end of the intervention (week 12), *Pomc* gene expression was enhanced in untreated HFHSD-fed mice compared to CD-fed mice, which was normalized by *P. faecium* (**Figure 2A**), while *Cart* expression remained unaffected by HFHSD feeding or the treatment (**Figure 2A**). At both 4 and 12 weeks of HFHSD feeding, the mRNA levels of the orexigenic neuropeptide *Npy* were decreased in untreated HFHSD-fed mice compared to control mice (**Figure 2A**). The *Npy* downregulation was also shown after 12 weeks but not after 4 weeks of *P. faecium* treatment (**Figure 2A**). The transcript levels of the orexigenic neuropeptide *Agrp* mirrored the *Npy* gene expression profile showing reductions only after 12 weeks of HFHSD feeding treated or untreated with *P. faecium* (**Figure 2A**).

To examine the putative mechanisms underlying the anorexigenic effect of *P. faecium* in DIO mice, plasma levels of insulin and leptin, both involved in the long-term control of energy intake, were measured after 4 weeks of HFHSD feeding. Insulin and leptin remained unaffected by HFHSD and the *P. faecium* treatment (**Figure 2B** and **2C**). Plasma levels of the anorexigenic hormones CCK, GLP-1 and PYY, which are postprandially secreted by the enteroendocrine cells, were measured at short and long term of HFHSD feeding (at weeks 4 and 12). At week 12, CCK was increased in *P. faecium*-treated DIO mice compared to untreated mice fed HFHSD (**Figure 2D**), being undetectable in CD-fed mice as well as in all groups at week 4 of HFHSD feeding. GLP-1 and PYY were differently affected by *P. faecium*. GLP-1 remained unchanged in either untreated or treated DIO mice at any HFHSD feeding time point (**Figure 2E**). *P. faecium* increased the plasma levels of PYY at both time points of HFHSD feeding (weeks 4 and 12) while the levels did not differ in untreated HFHSD-fed mice compared to CD-fed mice (**Figure 2F**).

Thus, these data indicate that administration of *P. faecium* induced an anorexigenic effect from early stages of DIO, potentially through a PYY hypersecretion, that might precede the metabolic benefits over body weight and adiposity observed in the long-term.

- CD-Veh
- HFHSD-Veh
- HFHSD-*P.fae*

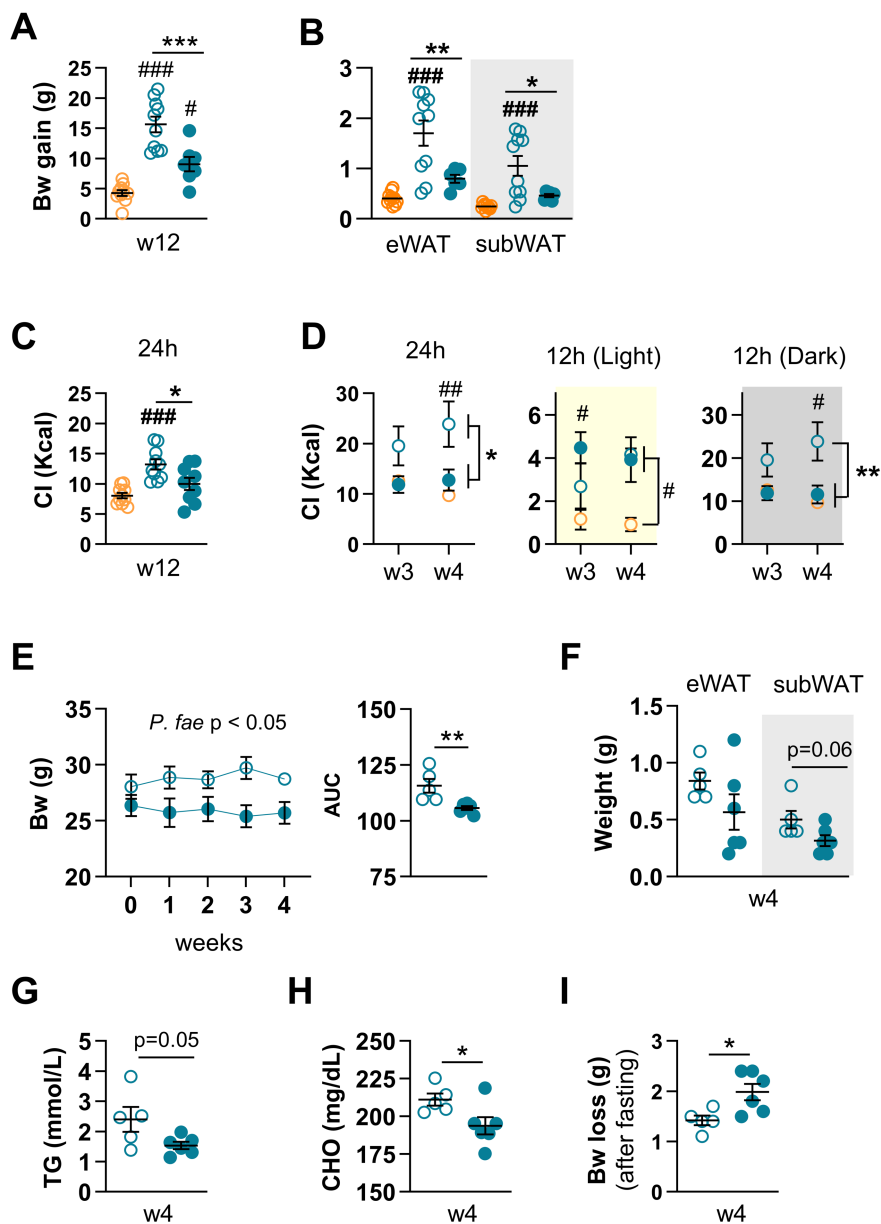


Figure 1. *Phascolarctobacterium faecium* improves the metabolic phenotype of diet-induced obese mice and reduces caloric intake.

In mice fed CD or HFHSD treated with Veh or with *P. fae*, we measured: **(A)** Bw gain, **(B)** weights of eWAT and subWAT, and **(C)** 24 h-FI after 12 weeks of intervention. **(D)** 24 h- and 12 h- (light and dark phase) FI after 3 and 4 weeks of intervention. **(E)** Bw evolution and AUC after 4 weeks of intervention. **(F)** Weights of eWAT and subWAT after 4 weeks of intervention. **(G)** TG and **(H)** CHO plasma levels after 4 weeks of intervention. **(I)** Body weight loss after overnight fasting after 4 weeks of intervention. Abbreviations: AUC: area under the curve; Bw: body weight; CD: control diet; CHO: cholesterol; CI: Caloric intake; eWAT: epididymal white adipose tissue; HFHSD: high fat high sugar diet; *P. fae*: *Phascolarctobacterium faecium*; subWAT: subcutaneous white adipose tissue; TG: triglycerides; Veh: vehicle; w: week. 12-weeks intervention: n=9-10 per group; 4-weeks intervention: n= 5-6 per group. Data shown as mean $\pm$ SEM. One-way ANOVA followed by Tukey's *post-hoc* test (**A**, **B** and **C**); two-way ANOVA followed by Tukey *post-hoc* test (**D** and **E** [body weight evolution]), main effects are shown in the graph; Student's t-test (**E** [AUC] and **F-I**). *p<0.05, ##p<0.01 and ###p<0.001 vs. CD-Veh group; *p<0.05, **p<0.01 and ***p<0.001.

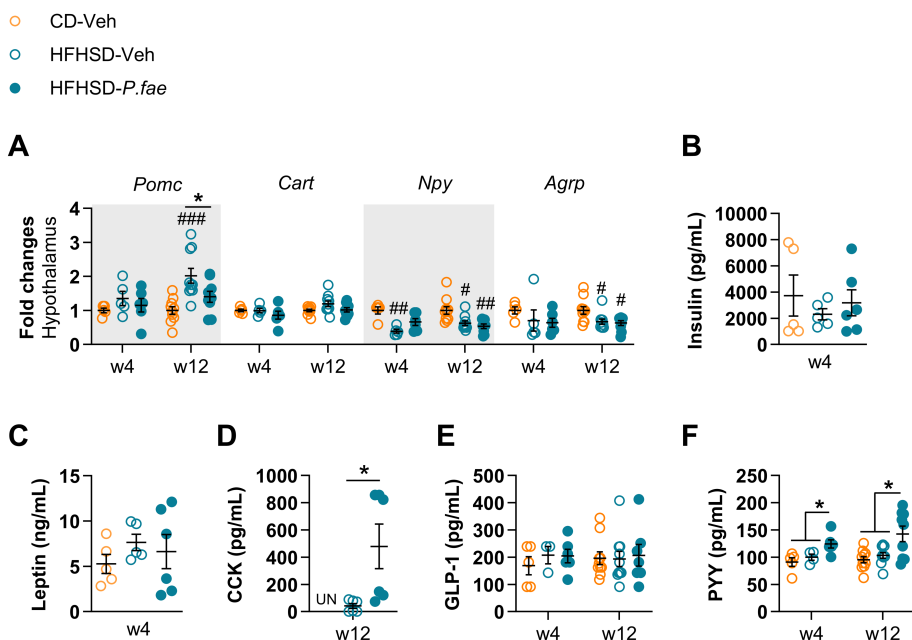


Figure 2. *Phascolarctobacterium faecium* increases the plasma levels of the anorexigenic hormones CCK and PYY in diet-induced obese mice.

In mice fed CD or HFHSD treated with Veh or with *P. fae*, we measured: **(A)** Gene expression of anorexigenic (*Pomc* and *Cart*) and orexigenic (*Npy* and *Agrp*) neuropeptides in the hypothalamus after 4 and 12 weeks of intervention. **(B)** Plasma levels of insulin and **(C)** leptin after 4 weeks of intervention. **(D)** Plasma levels of CCK after 12 weeks of intervention. **(E)** Plasma levels of GLP-1 and **(F)** PYY after 4 and 12 weeks of intervention. Abbreviations: CD: control diet; HFHSD: high fat high sugar diet; *P. fae*: *Phascolarctobacterium faecium*; UN: undetectable; Veh: vehicle; w: week. 12-weeks intervention: n=9-10 per group; 4-weeks intervention: n= 5-6 per group. Data shown as mean $\pm$ SEM. One-way ANOVA followed by Tukey's *post-hoc* test (**A-C**, **E** and **F**); Student's t-test (**D**). #p<0.05, ##p<0.01 and ###p<0.001 vs. CD-Veh group; *p<0.05.

To further explore the effects of *P. faecium* as PYY secretagogue, we functionally assessed PYY secretion in response to lipid intake, using a 20% lipid emulsion (intralipid) as a nutritional challenge. First, we determined in which phase of the light-dark cycle PYY was more efficiently secreted in response to the intralipid stimulus in untreated and *P. faecium*-treated HFHSD-fed mice. We observed that the treatment (vehicle / *P. faecium*), the prandial condition (fasting / intralipid) and the daily phase (light / dark) influenced the PYY plasma levels. The highest PYY

levels were detected in *P. faecium*-treated mice, after intralipid administration, and in the dark phase (**Figure S2A**). Moreover, *post-hoc* analyses specifically indicated that the postprandial levels of PYY in *P. faecium*-treated mice were higher in the dark than in the light phase (**Figure S2A**). Accordingly, the secretion assay was performed in the dark phase after 4 and 12 weeks of HFHSD feeding. At both, 4 and 12 weeks of DIO *P. faecium* increased the postprandial levels of PYY after oral intralipid exposure compared with fasting, which was not observed in untreated HFHSD-fed mice (**Figure 3A** and **3B**).

Since these data further support a main effect of *P. faecium* controlling PYY secretion at short and long term of DIO, we investigated whether blocking PYY reverts the effects of *P. faecium* suppressing food intake. First, we tested the efficacy of the PYY immunoneutralization on blocking the food intake suppression by administering intraperitoneally an IgG antibody against this hormone. In an experiment testing the antibody dose we demonstrated that HFHSD-fed mice pretreated with a concentration of 10x anti-PYY antibody, but not with a concentration of 100x, increased 12 h-food intake in the dark phase compared to control mice injected a saline solution (**Figure S2B**). Moreover, in a consecutive 4-week preliminary experiment to further assess the antibody efficacy, we demonstrated that the administration of a 10x dose of the anti-PYY antibody blunted the suppression of food intake induced by exogenous administration of PYY in HFHSD-fed mice (**Figure S2C**). Based on this evidence, food intake was measured after 4 weeks of HFHSD feeding in untreated and *P. faecium*-treated mice receiving a 10x dose of the anti-PYY antibody. In line with previous results (**Figure 1C**), *P. faecium* reduced food intake in HFHSD-fed mice receiving saline compared with untreated HFHSD-fed mice, but this effect was not observed when mice were administered the PYY antibody (**Figure 3C**).

On the other hand, expression of PYY was examined by immunohistochemical analysis in the colon, revealing an increased number of PYY-positive cells per unit of mucosal area in *P. faecium*-treated mice compared to untreated mice fed HFHSD (**Figure 3D**). Therefore, *P. faecium* increases both, the number of PYY-producing enteroendocrine cells and PYY secretion, and this PYY hypersecretion is required for reducing food intake in DIO mice.

***P. faecium* DSM 32890 drives changes in gut microbiota composition and derived metabolites**

In cecal contents, we measured PYY secretagogues such as bile acids (BAs), short chain fatty acids (SCFAs), including their branched forms (BCFA) and their precursor branched-chain amino acids (BCAAs) associated with protein-induced anorexigenic hormone secretion ^{48,49}. Among all BAs analyzed (**Figure 4A** and **S3A**), concentrations of the secondary BA ω MCA and its taurine-conjugated form τ MCA, as well as the primary BA α MCA and β MCA, were increased in the cecum content of untreated HFHSD-fed mice compared to CD-fed mice (**Figure 4A**). While the levels of τ MCA and ω MCA were completely restored by administration of *P. faecium*, the levels of α MCA and β MCA resulted partially normalized by *P. faecium* without reaching statistical differences compared to untreated HFHSD-fed mice (**Figure 4A**).

○ HFHSD-Veh
● HFHSD-*P.fae*

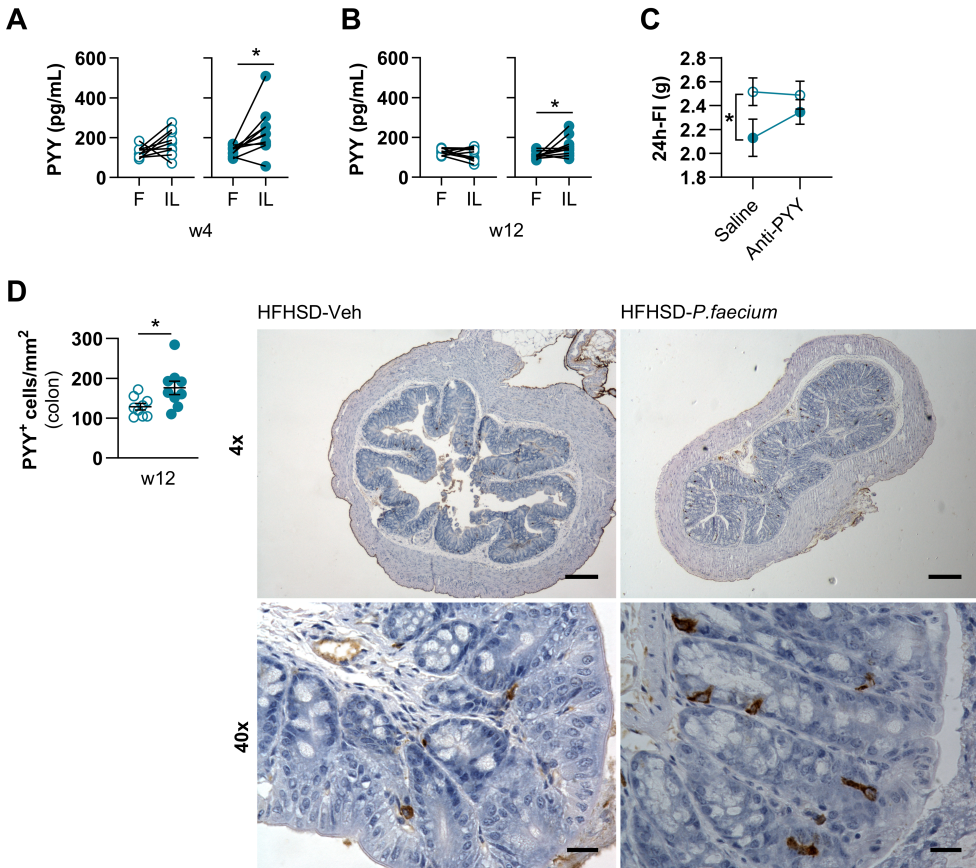


Figure 3. *Phascolarctobacterium faecium* increases the PYY levels after intralipid oral load and the number of PYY-producing cells, and requires PYY to suppress caloric intake in diet-induced obese mice.

In mice fed HFHSD treated with Veh or with *P. fae*, we measured: **(A)** PYY levels in plasma at fasting and 20 min after an oral load of Intralipid 20% at the beginning of dark phase after 4 weeks and **(B)** 12 weeks of the intervention. **(C)** 24 h-FI 1 h after intraperitoneal administration of PYY antibody after 4 weeks of intervention. **(D)** Quantification of PYY-positive cells in colon and representative bright field images of PYY immunohistochemical staining at 4X magnification (top images, scale bar = 200 μm) and 40X magnification (bottom images, scale bar = 20 μm). Abbreviations: CD: control diet; CI: Caloric intake; F: fasting; HFHSD: high fat high sugar diet; IL: 20 min after intralipid; *P. fae*: *Phascolarctobacterium faecium*; Veh: vehicle; w: week. n=8-10 per group. Data shown as mean ± SEM. paired Student's t-test (**A** and **B**); Student's t-test (**C** and **D**). n.s.: non-significant; *p<0.05.

The analysis of SCFAs (**Figure 4B** and **S3B**), revealed an increase of the BCFA- isovalerate in *P. faecium*-treated HFHSD-fed mice compared to untreated mice fed either HFHSD or CD (**Figure 4B**). Similarly, acetate was increased in *P. faecium*-treated mice compared to CD-fed mice, and only when the analysis was restricted to HFHSD-fed groups, *P. faecium*-treated mice showed higher acetate concentrations compared to untreated mice (Student's t-test, p = 0.04) (**Figure 4B**).

Heptanoate levels were reduced in untreated HFHSD-fed mice compared to CD-fed mice, and this effect was not observed in mice treated with the bacterium (**Figure 4B**). Assessment of BCAAs demonstrated that, compared to untreated mice fed either HFHSD or CD, *P. faecium*-treated mice had increased cecal concentrations of valine, leucine and isoleucine (**Figure 4C**).

We next examined whether BCAAs could mediate the effects of *P. faecium* on PYY secretion. The incubation of murine enteroendocrine cell line STC-1 with the BCAAs valine, leucine or isoleucine increased PYY secretion compared to control conditions (**Figure 4D**). Besides, *P. faecium* enhanced PYY release by STC-1 cells compared to control (**Figure 4D**).

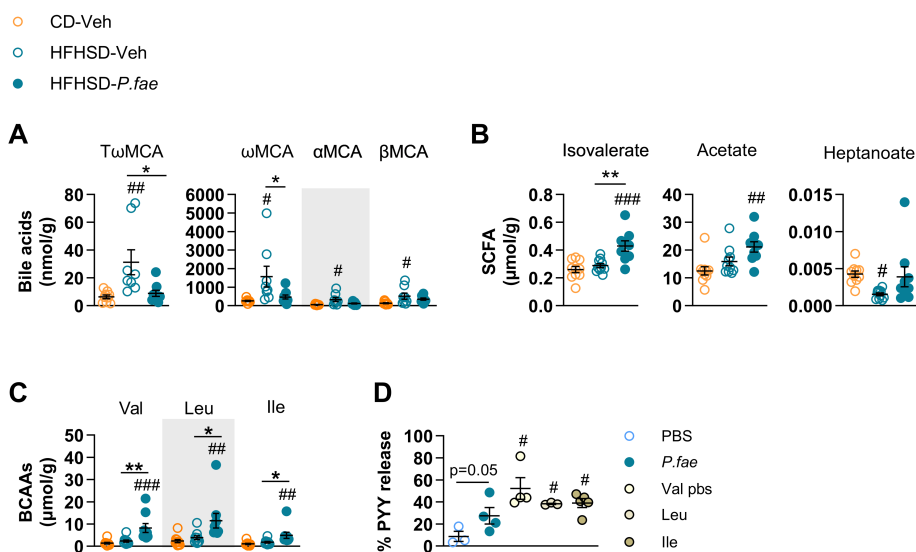


Figure 4. *Phascolarctobacterium faecium* increases the cecal levels of branched-chain aminoacids which act as PYY secretagogues in SCT-1 neuroendocrine cells.

(A) Levels of bile acids, (B) SCFAs and (C) BCAAs in the caecum content of mice fed CD or HFHSD treated with Veh or with *P. fae*, after 12 weeks of intervention. (D) Percentage of PYY secretion *in vitro* by STC-1 cells incubated with PBS (control conditions), *P. fae* and BCAAs pre-incubated with PBS or *P. fae*. Abbreviations: αMCA: α-muricholic acid; βMCA: β-muricholic acid, ωMCA: ω-muricholic acid; BCAAs: branched-chain aminoacids; CD: control diet; HFHSD: high fat high sugar diet; Ile: isoleucine; Leu: leucine; Val: valine; *P. fae*: *Phascolarctobacterium faecium*; SCFAs: short-chain fatty acids; TωMCA: tauro-ω-muricholic acid; Veh: vehicle. n=9-10 per group in (A-C) and n= 5 per experimental condition in (D). Data shown as mean ± SEM. One-way ANOVA followed by Tukey's post-hoc test (A-C); Student's t-test and two-way ANOVA followed by Tukey's post-hoc test (D) n.s.: non-significant main effects between pre-incubation with PBS or *P. faecium*. *p<0.05, **p<0.01 and ***p<0.001 and #p<0.05, ##p<0.01 and ###p<0.001 vs. CD-Veh group in (A-C); #p<0.05 vs. PBS in (D).

The impact of *P. faecium* treatment on the gut microbiota was assessed using typical alpha and beta diversity approaches on the presence and abundance of the 696 OTUs detected. We did not detect drastic differences in richness supported by the differential distribution of total OTUs in HFHSD mice (Chao index, KW = 4.09, p < 0.390), which suggests no loss of bacterial species (**Figure 5A**). However, descriptors such as the reciprocal Simpson's index, dominance and evenness were significantly affected by the administration of *P. faecium*, suggesting a change of proportions in particular OTUs (balance among species), thus partially reversing the phenotype

observed in HFHSD mice and beyond the CD group values (**Figure 5A**). The study of the microbial community structure as a whole by multivariate analysis, based on Bray-Curtis dissimilarity index among samples and redundancy analysis (RDA), permitted to discern a radical shift in the gut microbial community as a consequence of the HFHSD feeding (Adonis = 9.66, $p < 0.001$) (**Figure 5B**). The HFHSD-induced gut microbiota alteration was not reversed by *P. faecium* to the CD shape, but it generated a distinctive microbiota structure compared to untreated HFHSD-fed mice (**Figure 5B**). When we analyzed the effects of the treatment of individual bacterial taxa, we found that approximately one third of the OTUs evaluated showed differential abundance across all experimental groups (N = 209). HFHSD feeding induced a depletion of several OTUs belonging to the Muribaculaceae family, which are predominant in murids (top-half heatmap at **Figure 5C**). This loss of commensal gut bacteria was not completely restored by *P. faecium* administration. Nevertheless, we found that *P. faecium* reduced to CD levels the Lachnospiraceae-enriched OTUs, (also including few species of the families Ruminococcaceae, Oscillospiraceae, Eggerthellaceae, and Erysipelotrichaceae), which were increased in untreated HFHSD-fed mice (bottom-half heatmap at **Figure 5C**). Finally, compared to untreated CD- and HFHSD-fed mice, *P. faecium*-HFHSD-treated mice showed a significant increase of *Lactobacillus* spp. (OTU43, OTU104 OTU517, $p = 0.031$) and *Akkermansia muciniphila* (OTU197, $p = 0.017$) (**Figure 5D**). Both *Lactobacillus* and *Akkermansia* species positively correlated with cecal BCAA levels, specifically OTU104, OTU517, and OTU197 with Val (**Figure S4A**), Leu (**Figure S4B**), and Ile (**Figure S4C**). Also, undefined species from the family Erysipelotrichaceae such as OTU3, OTU94, and OTU595, were significantly increased by *P. faecium* administration (KW > 15.5, $p < 0.005$), and exhibited positive correlations with BCAA cecal levels (Kendall's tau > 0.45, $p < 0.001$) (data not shown).

Altogether, our findings support underlying mechanisms by which *P. faecium* supplementation elicits PYY secretion, including direct pathways via *P. faecium* structural components and indirect pathways via changes in the gut microbiota composition linked to BCAAs-mediated signaling.

The anorexigenic effect of *P. faecium* DSM 32890 contributes to prevent diet-induced obesity in mice

To determine the contribution of the anorexigenic effect of *P. faecium* on its associated metabolic benefits at long term DIO (**Figure 1**), we conducted an experiment in HFHSD-fed mice, including an untreated and *P. faecium*-treated *ad libitum*-fed groups and an untreated pair-fed group food-restricted to the same HFHSD amount consumed by *P. faecium*-treated mice.

Compared to untreated *ad libitum*-fed mice, *P. faecium*, but not pair-feeding, reduced the body weight gain after 12 weeks of intervention (**Figure 6A**). We next explored whether treatment with *P. faecium* influenced postprandial glucose homeostasis. Both, *P. faecium* and pair-feeding slightly improved the oral glucose tolerance by reducing glycemia 15 min after the oral glucose load (**Figure 6B**). Nonetheless, the AUC of the OGTT remained unaffected by *P. faecium* or pair-feeding (**Figure 6B**), probably due to the high inter-individual variability displayed by pair-fed mice. In this regard, the analysis restricted to untreated (*ad libitum*-fed mice) and *P. faecium*-treated mice revealed that the supplementation with the bacterium reduced the AUC (Student's t-test, $p = 0.05$), which was not observed in the analysis restricted to *ad libitum* and pair-fed untreated groups (Student's t-test, $p = 0.81$). Besides, no differences were detected in insulin levels in fasting or

after 15 min of oral glucose administration (**Figure S5A**) and glycemia was reduced after 15 min of insulin intraperitoneal injection irrespective of the group (**Figure S5B**) indicating that the slight improvements in glucose clearance observed were independent of insulin. Postprandial glycemia was also examined after the oral administration of the 20% intralipid, since fat is a main component of HFHSD. The prandial condition (fasting or intralipid) and groups tended to interact ($p = 0.06$) and the *post-hoc* analysis indicated that, compared with fasting levels, glycemia was increased 20 min after the oral intralipid load in untreated mice (*ad libitum* and pair-fed) but not in *P. faecium*-treated mice (**Figure 6C**). In addition, glycemia after intralipid administration was lower in *P. faecium*-treated mice compared to *ad libitum* untreated mice (**Figure 6C**).

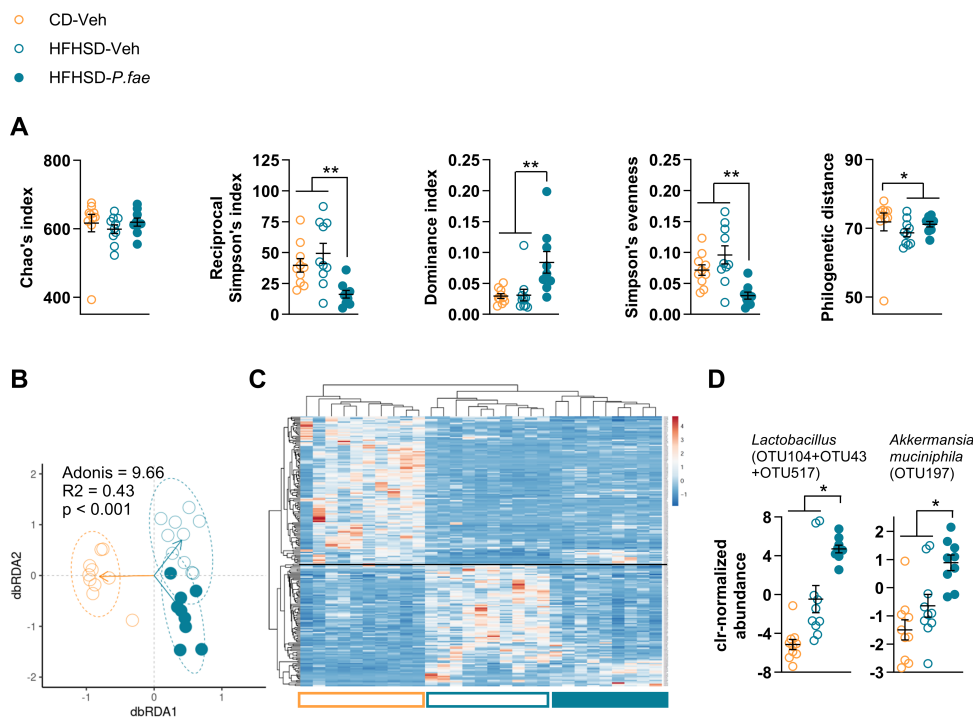


Figure 5. *Phascolarctobacterium faecium* drives changes in gut microbiota composition.

In mice fed CD or HFHSD treated with Veh or with *P. fae*, we assessed in feces: **(A)** The alpha diversity including study of the observed OTUs, Simpson's reciprocal index, Simpson's evenness index, dominance index, and phylogenetic distance descriptors. **(B)** Beta diversity evaluation of microbial community structure using distance-based redundancy analysis (dbRDA). The two gradients of dataset dispersion in ordination space explaining more variation of this constrained approach are shown in a scatter-plot fashion. **(C)** Scaled read counts for top differentially abundant OTUs ($n=209$, chi-squared test ≥ 11.5 , corrected $p \leq 0.01$) across groups are shown as a heatmap. Clustering of OTUs was carried out by using euclidean distance and "complete" clustering method. The OTUs from major clusters were identified using SINA aligner. Heat scale is based on Z-scores resulting from rarefied read counts per OTU (raw scaling). A horizontal black line divide OTUs clustering into two major groups supporting effect of HFHSD (top half) and the reversing phenotype of *P. faecium* (bottom half). **(D)** Distribution of clr-normalized DNA read counts for OTUs identified as *Lactobacillus* (OTU104, OTU43, OTU517) and *Akkermansia muciniphila* (OTU197). $n=9-10$ per group. Kruskal-Wallis (KW) test and pairwise comparisons with respective adjusted (FDR) p -values are indicated in each graph **(A)**; Adonis test with centroids of data dispersion indicated with arrows' heads **(B)**; pairwise comparisons between groups and adjusted (FDR) p -values **(D)**. Abbreviations: CD: control diet; clr: centered log-ratio; HFHSD: high fat high sugar diet; *P. fae*: *Phascolarctobacterium faecium*; Veh: vehicle.

Compared with untreated *ad libitum*-fed mice, the weights of eWAT and subWAT were reduced in *P. faecium*-treated mice but not in pair-fed mice (**Figure 6D**). The weight of brown adipose tissue (BAT) remained unaffected after *P. faecium* or pair-feeding intervention (**Figure 6D**). The analysis of adipocytes in hematoxylin-eosin stained eWATs indicated that the frequency of adipocyte diameters and groups tended to interact ($p = 0.08$) (**Figure 6E**). Compared with untreated *ad libitum*-fed mice, *P. faecium*, but not pair-feeding, increased the frequency of smaller adipocytes ($\leq 50 \mu\text{m}$) (**Figure 6E**). Moreover, *P. faecium*-treated mice, but not pair-fed mice, tended to have lower adipocyte size than untreated *ad libitum*-fed mice ($p=0.09$, **Figure 6F**). Neither the frequency of adipocyte diameters nor the adipocyte size were affected in subWAT (**Figure S5C and S5D**). In addition, the gene expression analysis of markers of lipogenesis, lipolysis, β -oxidation and adipogenesis in subWAT remained unchanged by both, *P. faecium* and pair-feeding (**Figure S5E**).

Overall, these findings support that the anorexigenic effect of *P. faecium* contributes to improving the energy metabolism but it is not the only mechanism involved, as pair-fed mice show an intermediate phenotype between untreated and *P. faecium*-treated mice fed *ad libitum*.

***P. faecium* DSM 32890 accelerates gastrointestinal transit in diet-induced obese mice and reduces lipid absorption in cultured colonocytes**

Since the effect of reducing caloric intake appeared not to be the exclusive mechanism through which *P. faecium* prevented the increase of body weight gain and adiposity in response to HFHSD-feeding, we sought to investigate complementary pathways that influence energy homeostasis such as thermogenesis and intestinal functions including GI transit and lipid absorption.

Although transcripts of thermogenesis markers in subWAT remained unchanged by the bacterium and pair-feeding (**Figure S5E**), the citrate synthase activity, a marker of mitochondrial density which helps assess thermogenesis^{50,51} (**Figure 7A**), was increased by both, *P. faecium* and pair-feeding. This suggests that the anorexigenic effect of the bacterium potentiates thermogenesis which partially contributes to reduce adiposity (**Figure 6D**).

On the other hand, obesity is associated to prolonged GI transit time, allowing a higher intestinal lipid absorption with the subsequent weight gain⁵². Thus, we measured GI transit time after 7 weeks of HFHSD-feeding. Compared to untreated mice fed a HFHSD *ad libitum* or pair-fed, *P. faecium*-treated HFHSD-fed mice displayed a faster transit time (**Figure 7B**), indicating that, independently of caloric intake, the bacterium accelerates gut transit which might affect intestinal lipid absorption.

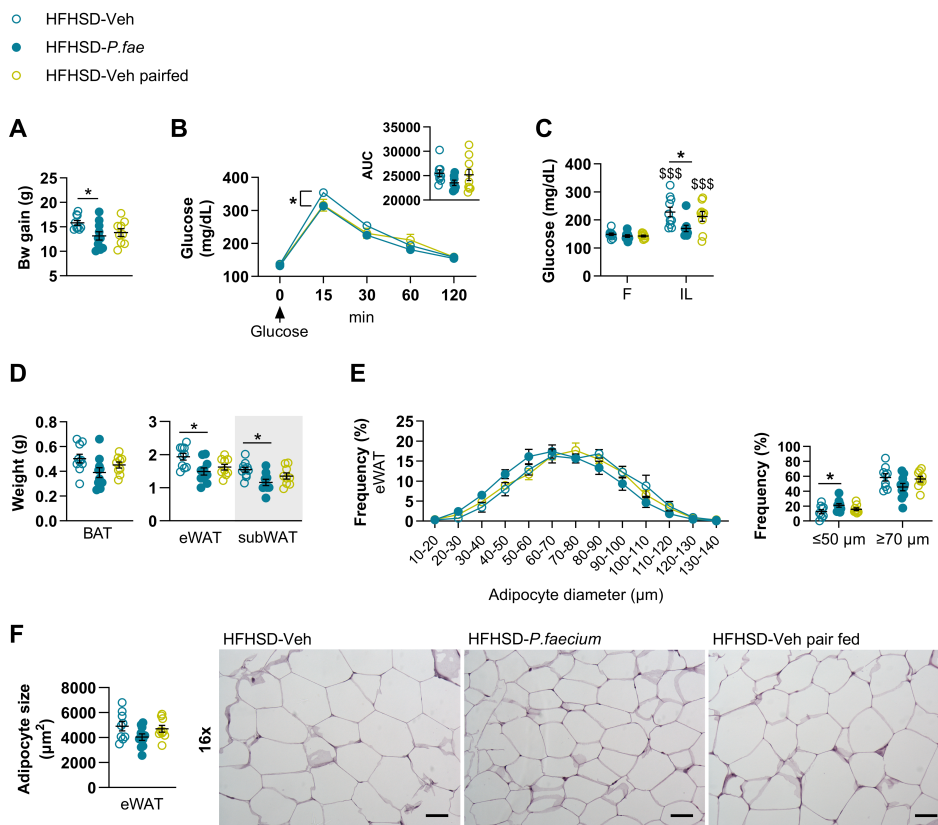


Figure 6. *Phascolarctobacterium faecium* partially prevents body weight gain, postprandial hyperglycemia and adiposity in diet-induced obese mice by reducing caloric intake.

In mice fed HFHSD treated with Veh (fed *ad-libitum* or pair-fed to *P. fae*-treated group) or treated with *P. fae*, we measured: (A) Bw gain. (B) OGTT: glycemia after 0, 15, 30, 60, and 120 min of an oral load of glucose (2 g/kg) after 4 h of fasting, and AUC at week 10 of the intervention. (C) Blood glucose levels in fasting and 20 min of an oral load of Intralipid 20% at week 10 of the intervention. (D) Weights of BAT, eWAT and subWAT after 12 weeks of intervention. (E) Frequency of adipocytes of different diameters in eWAT after 12 weeks of intervention. (F) Quantification of individual adipocyte fat area in eWAT and representative bright field images of hematoxylin and eosin staining (scale bar = 50 μm, 16X magnification). Abbreviations: CD: control diet; HFHSD: high fat high sugar diet; Veh: vehicle; *P. fae*: *Phascolarctobacterium faecium*; Bw: Body weight; OGTT: oral glucose tolerance test; AUC: area under the curve; BAT: brown adipose tissue; eWAT: epididymal white adipose tissue; subWAT: subcutaneous white adipose tissue; F: fasting; IL: 20 min after intralipid. n=9-10 per group. Data shown as mean ± SEM. One-way ANOVA followed by Tukey's *post-hoc* test (A, B [AUC], D, E [upper graph] and F); two-way ANOVA followed by Tuckey's *post-hoc* test (B, C and E). $^{***}p < 0.001$ vs fasting conditions; * $p < 0.05$.

To assess the implication of *P. faecium* in intestinal lipid absorption, we measured plasma levels of non-esterified fatty acids (NEFAs) and triglycerides along time after an intralipid oral load in mice fed HFHSD for 12 weeks. Compared with fasting levels (0 h), all groups showed an increase of NEFAs levels in plasma 2 h after intralipid administration, and only in *P. faecium*-treated mice NEFAs decreased after 4 h compared with their levels after 2 h (Figure 7C). Moreover, an interaction between time and groups was detected ($p < 0.01$), and *post-hoc* analysis revealed that mice treated with *P. faecium* exhibited reduced NEFA levels 4 h after the intralipid oral load

compared to untreated fed *ad libitum* or pair-fed groups (**Figure 7C**), suggesting that treatment with the bacterium was able to induce a more rapid clearance of circulating postprandial NEFAs into adipose tissue independently of food intake.

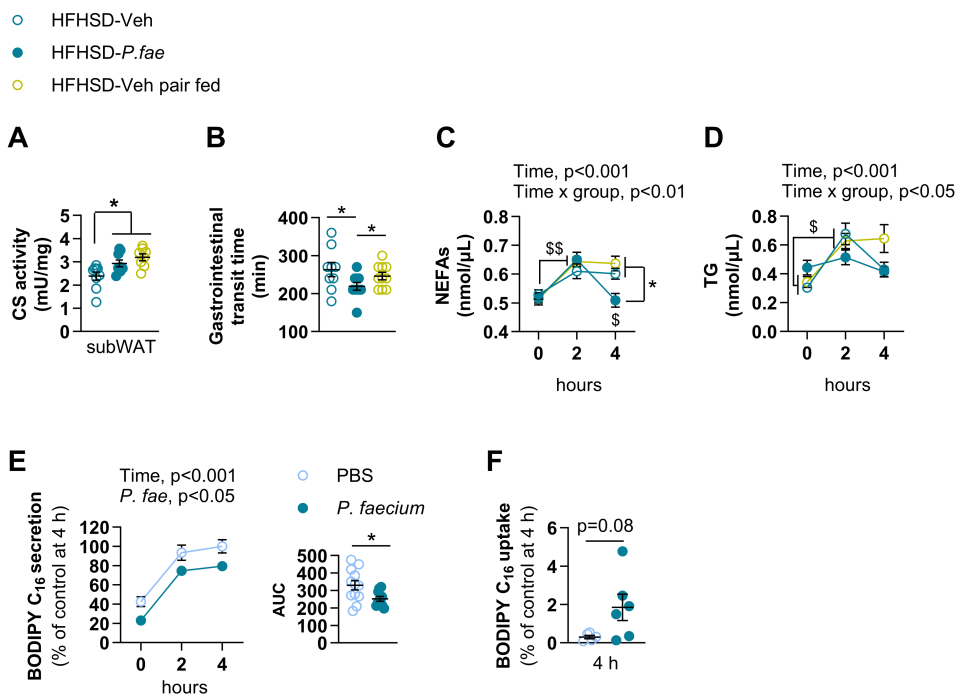


Figure 7. *Phascolarctobacterium faecium* induces faster GI transit and clearance of non-esterified fatty acids in blood and *in vitro* limits intestinal lipid absorption.

In mice fed HFHSD treated with Veh (fed *ad-libitum* or pair-fed to *P. fae*-treated group) or treated with *P. fae*, we measured: (A) Quantification of citrate synthase activity in subWAT. (B) GI transit time: time between oral gavage and first appearance of dye in feces in mice fed *ad libitum*. (C) Plasma NEFAs and (D) plasma TG levels after an oral load of intralipid 20%. (E) BODIPY C₁₆ secreted in the basolateral compartment by Caco-2 cells at 4 h after addition of lipid micelles, and AUC. (F) BODIPY C₁₆ uptake by Caco-2 cells at 4 hours after addition of lipid micelles. Amount of BODIPY C₁₆ is expressed as percentage (%) of fluorescence relative to control conditions (PBS). Abbreviations: CD: control diet; HFHSD: high fat high sugar diet; CS: citrate synthase; NEFAs: non-esterified fatty acids; *P. fae*: *Phascolarctobacterium faecium*; TG: triglycerides; Veh: vehicle. n=5-10 per group in (A-D), n=12 per experimental condition in (E) and n=5-6 per experimental condition in (F). Data shown as mean ± SEM. One-way ANOVA followed by Tukey's *post-hoc* test (A,B); two-way ANOVA followed by Bonferroni's *post-hoc* test (C-E), significant main effects are shown in the graph; unpaired Student's t-test (E [AUC] and F). \$p<0.05 vs. previous time point. *p<0.05 and ***p<0.001.

Plasma triglyceride levels were increased in untreated mice fed a HFHSD *ad libitum* and pair-fed 2 h after intralipid administration compared with fasting levels, while no changes were detected in *P. faecium*-treated mice along time (**Figure 7C**). NEFAs and triglycerides plasma levels in response to intralipid were also measured after 4 weeks of HFHSD-feeding but no significant differences were found between groups (**Figure S5F**).

We next explored the effects of *P. faecium* in lipid absorption in the distal gut by performing an *in vitro* experiment using human colonic Caco-2 cells grown in transwell plates and incubated with

fluorescent fatty acid BODIPY™ FL C₁₆. Compared to control conditions, pre-treatment with *P. faecium* induced a decreased secretion of BODIPY™ FL C₁₆ to the basolateral compartment (**Figure 7E**), while the amount of non-absorbed BODIPY™ FL C₁₆ in the apical compartment remained unaffected by the bacterium (not shown). BODIPY™ FL C₁₆ cellular uptake showed a non-significant increasing trend at 4 h in *P. faecium*-treated Caco-2 cells compared to control conditions (Student's t-test, $p=0.08$) (**Figure 7F**).

Thus, our data suggest that, independently of the effect reducing caloric intake, *P. faecium* may prevent DIO by inducing faster GI transit and reduced lipid absorption, both limiting fat storage.

4. Discussion

Here, we demonstrate that *Phascolarctobacterium faecium* DSM 32890, an intestinal bacterial strain isolated from metabolically healthy humans, exerts metabolic benefits in a rodent model of DIO through its appetite-suppressant action via PYY hypersecretion from early stages of obesity progression. *P. faecium* elicits PYY release by potentiating the presence of BCAAs in the distal gut as PYY secretagogues. Along with its anorexigenic outcomes, *P. faecium* also influences key aspects of the intestinal function ameliorating obesity, including an accelerated gut motility and a limited intestinal lipid absorption accompanied by a reinforced postprandial lipid clearance from bloodstream.

Our study unequivocally identifies *P. faecium* as a gut bacterium with appetite suppressant effects and potentially useful in obesity treatment. Indeed, we showed that, *P. faecium* prevented the hyperphagia displayed by untreated DIO mice, which was coupled with elevated levels of the anorexigenic hormone PYY in plasma and increased number of PYY positive cells in colon. Recent preclinical and clinical investigations also highlight the function of another commensal intestinal bacterium, *Hafnia alvei* 4597, as probiotic for obesity due to its anorexigenic effects induced by the shock protein ClpB, an antigen-mimetic of the anorexigenic α -MSH^{53,54}. Of note is that very few studies have identified intestinal bacteria with PYY secretagogue capacity in obesity⁵⁵⁻⁵⁷. For instance, Liang et al. described the anti-obesity effects of *Ligilactobacillus salivarius* LCK11 in mice, through the enhancement of intestinal and plasma PYY after 8 weeks of oral supplementation⁵⁷, but the causal role of this effect in obesity was not proven.

In our study, we also observed that the oral administration of *P. faecium* increased the plasma levels of CCK but not those of GLP-1, the two additional anorexigenic hormones investigated. A primary role of PYY on the effect of *P. faecium* modulating caloric is suggested by its increased levels in plasma already from early stages of treatment to the end of the intervention. To what extent CCK is needed for the anorexigenic effect of *P. faecium* deserves further investigations.

In line with its effects preventing hyperphagia, *P. faecium* seemed to restore a functional hypothalamic control of food intake, since treated mice did not overexpress the anorexigenic neuropeptide POMC as a compensatory mechanism in response to excessive energy intake, as untreated obese mice did. Although how hypothalamic and PYY paths are related in *P. faecium*-treated mice remains unknown, our study demonstrated that PYY oversecretion from early stages of treatment underlies the anorexigenic effects of *P. faecium*, since the PYY immunoneutralization

in mice after 4 weeks of treatment blunted the effect of the bacterium reducing food intake. In addition, *P. faecium* specifically suppressed appetite during the dark but not during the light phase. Consistently with this, we further demonstrated that *P. faecium* induced PYY secretion in response to lipid intake only in the dark phase. Concerning how *P. faecium* potentiates PYY secretion, it could be hypothesized that the elevated CCK levels in plasma due to the bacterium administration might be involved in the long-term effects, as it is known that CCK stimulates PYY release from L cells ^{58,59}. Besides, the fact that the anorexigenic effect of *P. faecium* was only detected in the dark phase, the active phase in rodents, suggests that its anorexigenic effect probably requires active feeding increasing the intestinal abundance of dietary and/or microbial molecules able to activate gut-to-brain nutrient sensing, in line with bacterial growth dynamic-based model of appetite control ^{33,60}. Although we did not specifically explore whether intestinal metabolites showed light/dark variations, we identified increased levels of valine, leucine and isoleucine in the cecum of *P. faecium*-treated mice. This could be attributed to the ability of *P. faecium* to shape a gut microbiota, reducing the abundance of bacterial species with capability for BCAA uptake and degradation ⁶¹⁻⁶³, and increasing those with capability for *de novo* BCAA biosynthesis ^{61,62}. Indeed, we found that *P. faecium*-treated mice showed higher abundance of *Akkermansia muciniphila*, a species with capacity of synthesizing BCAAs from glucose ⁶⁴, and that was positively correlated with BCAAs levels in the cecum in our study.

BCAAs have been associated with insulin resistance, glucose intolerance and obesity ^{62,65-70}. In contrast, other investigations have shown metabolic benefits of BCAAs. For instance, isoleucine reduced postprandial glycemia in rats ^{71,72} and dietary supplementation with leucine and isoleucine reduced body weight gain and fat mass in DIO mice ⁷³⁻⁷⁵. Our findings from mechanistic experiments with STC-1 neuroendocrine cells support that the enhanced PYY release may be induced by a BCAA-dependent pathway acting as PYY secretagogues. In line with our results, Genton et al. have reported that a 4-month oral supplementation with valine, leucine, and isoleucine increased plasma levels of PYY in patients with chronic kidney disease ⁴⁹, a common complication of type 2 diabetes ⁷⁶.

P. faecium could also exert enteroendocrine effects through changes in BAs, which are described as PYY secretagogues ⁷⁷⁻⁷⁹. In our study, *P. faecium* normalized the levels of the primary BAs α MCA and β MCA, and the secondary BAs ω MCA and τ MCA in the cecum, suggesting higher BAs reabsorption in the distal gut, which in turn could contribute to increase PYY secretion by activating the basolateral TGR5 in L cells ^{78,80}. Alternatively, the *P. faecium*-induced caloric intake reduction could secondarily result in the lower BAs levels detected in the cecum.

Complementary, we also proved that the bacterium itself exerts direct effects on SCT-1 cells increasing PYY secretion. Similarly, other *in vitro* data with this cell line have demonstrated the PYY secretagogue properties of *Ligilactobacillus salivarius* LCK11, mediated by the ability of its peptidoglycan to activate the Toll-like receptor 2 (TLR2)/NF- κ B signaling pathway ⁵⁷. Based on other studies demonstrating that enteroendocrine cells express TLRs ^{30,31,57}, and their role in promoting *Pyy* gene expression in L cells ⁸¹, it is likely that TLR ligands could mediated the *P. faecium* effects on the enteroendocrine system to boost PYY release.

Importantly, *P. faecium* prevented the exacerbated energy intake since early stages of HFHSD feeding in DIO, indicating that the modulation of feeding behaviour is a key mechanism through which the bacterium improved the metabolic phenotype. Indeed, body weight evolution and plasma triglyceride and cholesterol levels were already improved after 4 weeks of treatment. The relevance of the ability of *P. faecium* to control caloric intake for preventing obesity was explored by conducting a pair-feeding protocol. Pair-fed mice and *P. faecium*-treated mice showed similar reduction of body weight, adiposity and postprandial glycemia, indicating that the metabolic benefits induced by the bacterium are influenced by caloric intake. Nevertheless, pair feeding did not completely replicate the improved metabolic phenotype since, contrary to *P. faecium* treatment, there were no significant differences between pair-fed and untreated *ad libitum* fed mice. Therefore, we hypothesized that *P. faecium* also triggers food intake-independent mechanisms that altogether are responsible for its metabolic benefits.

Accordingly, we investigated additional mechanism that could mediate the metabolic improvement, including effects on the GI transit and intestinal lipid absorption. In obesity, GI motility is often characterized by delayed transit times and reduced peristalsis, thus facilitating a longer nutrient absorptive timeframe leading to body weight gain ^{52,82-84}. The pair-feeding protocol indicated that, independently of the lower food intake, *P. faecium* accelerates GI transit which may speed the nutrient flux throughout the intestine increasing the amount of unabsorbed dietary proteins products reaching the colon. This could be a source of BCAAs that, together with *de novo* synthesized BCAAs by gut bacteria, contribute to the enhanced BCAAs pool, which will subsequent stimulate PYY secretion. Ferrier et al also showed that the PYY could inhibit intestinal transit; however, this effect was due to PYY₃₋₃₆, and not to PYY₁₋₃₆, the full-length isoform, which increases muscle contractions and motility ^{85,86}. Nonetheless, this possible relationship between the transit time and the different PYY forms, could not be established in our study since we only measure total PYY.

Irrespective of caloric intake, the ability of *P. faecium* to accelerate GI transit could limit intestinal lipid absorption helping to prevent the increased fat storage under HFHSD feeding. Indeed, both 4 and 12 weeks of *P. faecium* supplementation reduce the weight of subWAT, in parallel with lower tissue weight and higher frequency of smaller adipocytes in eWAT elicited by 12-week *P. faecium* supplementation. Indeed, *P. faecium* modestly prevented the 2 h- postprandial triglycerides peak shown by untreated DIO after the oral administration of intralipid, suggesting lower intestinal lipid absorption. In addition, *P. faecium* induced an earlier clearance of postprandial NEFAs from blood 4 h after intralipid administration, indicating faster storage of NEFAs into the adipose tissue. Considering that the weight of fat depots was lower in *P. faecium*-treated mice, the faster whole-body NEFAs clearance seem to prevent the deleterious effects of HFHSD-induced dyslipidemia, instead of contributing to adiposity. In addition, the lower fat storage due to *P. faecium* supplementation could result from boosted catabolic routes, as 4 weeks of treatment with the bacterium increased body weight loss after food restriction, which is related to higher energy expenditure ⁸⁷. Supporting these findings, 12 weeks of treatment with *P. faecium* increased mitochondrial activity in subWAT, an adipose tissue fraction that is more metabolically active than eWAT, suggesting that, through its anorexigenic effects, the bacterium may enhance catabolism in subcutaneous fat depots through mitochondrial biogenesis. This could lead to enhanced

thermogenesis and energy expenditure, similar to studies in DIO mice treated with potential probiotics or prebiotics showing higher mitochondrial density associated to increased thermogenic activity and energy expenditure ^{88,89}. However, at the transcriptional level, *P. faecium* did not induce changes in lipid metabolism and thermogenesis-related genes in subWAT. Evidence shows that elevated BCAA plasma levels associated to the metabolic complications previously mentioned are exacerbated by insufficient BCAA catabolism in adipose tissue ^{90–92}. Thus, based on the higher metabolic activity observed in subWAT of *P. faecium*-treated mice, we hypothesize that *P. faecium* could enhance BCAA catabolism in adipose depots, thereby preventing the metabolic complications attributed to high BCAA circulating levels.

Based on the assumption that accelerated GI transit would increase the amount of unabsorbed lipids reaching the colon in *P. faecium*-treated mice, we complementarily investigated the influence of the bacterium on lipid absorption in distal gut. Through *in vitro* experiments, we proved that *P. faecium* enhanced cellular lipid uptake and utilization by colonocytes, as it prevented crossing of lipids from the apical to the basolateral side of Caco-2 cells, thus limiting intestinal lipid absorption and helping to prevent the increased adiposity under HFHSD feeding. Interestingly, it is reported that some gut bacteria are able to promote lipid storage in enterocytes, thereby restricting chylomicron secretion ⁹³.

Overall, our study demonstrates the causal role of the *P. faecium* DSM 32890 anorexigenic effects, mediated by PYY oversecretion, in the control of body weight under an obesogenic diet. It also proves that the bacterium accelerates GI transit time and reduces fat uptake additionally contributing to improving the metabolic phenotype through complementary mechanisms. Altogether, our findings could help to advance therapies targeting specially food intake and eating behavior, a key obesity driver.

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Author contributions

Conceptualization, CBV, MRP and YS; Methodology, CBV, MRP, ABP, ILA and VTE.; Formal analysis, CBV, ABP and MRP.; Investigation, CBV and MRP.; Writing – Original Draft, CVB.; Writing – Review & Editing, MRP and YS.; Funding Acquisition, YS.; Supervision, MRP and YS.

Data availability

The raw sequencing data derived from microbiota analysis on mice cecal content is publicly available at the European Nucleotide Archive (ENA) via accessing the bioproject PRJEB66189.

Conflict of Interest

YS, MR-P, ABP and IL-A are authors of a patent on *Phascolarctobacterium faecium* DSM 32890. The remaining authors declare no competing interests.

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6. Supplementary material

Key Resources Table

REAGENT OR RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-mouse PYY antibody (for PYY immunostaining)	Abcam	Cat. #ab22663
Anti-mouse PYY purified IgG Antibody (for PYY immunoneutralization experiments <i>in vivo</i>)	Phoenix Pharmaceuticals	Cat. #G-059-03
Bacterial and Virus Strains		
<i>Phascolarctobacterium faecium</i> DSM 32890	This thesis	N/A
Chemicals, peptides, and recombinant proteins		
PYY (3-36)	Tocris Bioscience	Cat. #1618
2-mono-oleoylglycerol	Sigma-Aldrich	Cat. #870451O
BODIPY™ FL C16	Thermo Fisher Scientific	Cat. #D3821
Carmine red	Sigma-Aldrich	Cat. #C1022-5G
Cholesterol	Sigma-Aldrich	Cat. #C8667
Insulin NovoRapid FlexPen	Novo Nordisk	EAN13: 8470006567746; CN: 656774
LightCycler® 480 SYBR Green I Master	Roche	Cat. #4887352001
L-Isoleucine	Sigma-Aldrich	Cat. #I7403-10MG
L-Leucine	Thermo Fisher Scientific	Cat. #J62824-22
L-Valine	Sigma-Aldrich	Cat. #V0500-25G
L- α -Lysophosphatidylcholine	Sigma-Aldrich	Cat. #L1381
Oleic acid	Sigma-Aldrich	Cat. #01008-1g
Sodium taurocholate	EDQM	Cat. #S0900000
TRIsure™	Bioline	Cat. #BIO-38033
Critical commercial assays		
Cholesterol Liquid Kit	Química Aplicada	Cat. #995282
Free Fatty Acid Colorimetric Assay Kit	Abcam	Cat. #ab282927
High-Capacity cDNA Reverse Transcription Kit	Thermo Fisher Scientific	Cat. #4368814
Illustra™ GFX™ PCR DNA and Gel Band Purification Kit	GE Healthcare	Cat. #GE28-9034-66
MILLIPLEX Mouse Metabolic Hormone Expanded Panel	Merck Group	Cat. #MMHE-44K

Mouse Cholecystokinin 8 (CCK8) ELISA kit	MyBioSource	Cat. #MBS263980
Mouse Insulin ELISA	Mercodia	Cat. #10-1247-01
Mouse Leptin ELISA	Merck Group	Cat. #EZML-82K
Mouse PYY EIA Kit	RayBiotech	Cat. #EIAM-PYY-1
Phusion High-Fidelity PCR Kit	Thermo Fisher Scientific	Cat. #F553L
QIAamp PowerFecal DNA Kit	Qiagen	Cat. #12830-50
Qubit dsDNA HS Assay Kit	Thermo Fisher Scientific	Cat. #Q32851
Triglyceride Colorimetric Assay Kit	Elabscience	Cat. #E-BC-K261-S
Triglyceride Quantification Colorimetric Assay Kit	Abcam	Cat. #ab282924

Deposited data

16S V3-V4 amplicon data	ENA	PRJEB66189
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Experimental models: Cell lines

Human Caco-2 cells	ATCC	Cat. #HTB-37
Mouse STC-1 neuroendocrine cells	ATCC	Cat. #CRL-3254

Experimental models: Organisms/strains

C57BL/6 mice	Charles River Laboratories	N/A
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Oligonucleotides

Primer pairs for gene expression	Isogen Life Science and Sigma-Aldrich	All primer pairs are referenced in Supplemental Table S1
Primer pairs 16S V3-V4 amplicon	Klindworth et al. 2013	doi: 10.1093/nar/gks808

Software and algorithms

ImageJ V1.49q (Fiji software)	NIH, USA	https://imagej.nih.gov/ij/
Flash v1.2.11	Magoc et al. 2011	doi: 10.1093/bioinformatics/btr507
GraphPad PRISM V9.5.1	GraphPad Software	https://www.graphpad.com/
Illumina MiSeq platform	Centro Nacional de Análisis Genómico – CNAG, Barcelona, Spain.	N/A
Nis elements BR 3.2 software	Nikon Corporation	https://www.nissoftware.net/NISsoftware/
SeqKit v0.12.1	Shen et al. 2016.	doi: 10.1371/journal.pone.0163962
UCHIME v	Edgar et al. 2011	doi: 10.1093/bioinformatics/btr381
USEARCH v8.0.1623	Edgar, RC. 2010	doi: 10.1093/bioinformatics/btq461
QIIME v 1.9.1	Caporaso et al. 2010	doi: 10.1038/nmeth.f.303

FastTree	Price et al. 2009	doi: 10.1093/molbev/msp077
SILVA database v138	Quast et al. 2013	doi: 10.1093/nar/gks1219
SINA aligner	Pruesse et al. 2012	doi: 10.1093/bioinformatics/bts252
R v4.1.2	The R Foundation	https://cran.r-project.org/

Other

Amicon Ultra-0.5 Centrifugal Filter Units	Millipore	Cat. #UFC501096
Control diet chow	Research Diets	Cat. # D12450K
High-fat high-sugar diet chow	Research Diets	Cat. # D12451
Intralipid, 20% emulsion	Sigma-Aldrich	Cat. #I141-100ML

Supplemental Tables

Table S1. Genes, primer pair sequences and qPCR conditions

Gene name	Abbreviation	Primers sequence 5'3'	
Hypothalamus			
Agouti-related neuropeptide	Agrp	Forward	AGGTCTAAGTCTGAATGGC
		Reverse	CGGTTCTGTGGATCTAGC
Cocaine- and amphetamine-regulated transcript	Cart	Forward	AAGAAGTCCTGAAGAAGCTC
		Reverse	CAAGCACTTCAAGAGGAAAG
Neuropeptide Y	Npy	Forward	AATCTCATCACCAGACAGAG
		Reverse	CTTTCCTTCATTAAGAGGTCTG
Pro-opiomelanocortin-alpha	Pomc	Forward	AAAAGAGGTTAAGAGCAGTG
		Reverse	ACATCTATGGAGGTCTGAAG
Adipose tissue			
Acetyl-Coenzyme A carboxylase alpha	Acaca	Forward	TAATGGGCTGCTTCTGTGACTC
		Reverse	CTCAATATCGCCATCAGTCTTG
Cell death-inducing DNA fragmentation factor alpha-like effector A	Cidea	Forward	TGCTCTTCTGTATCGCCCAGT
		Reverse	GCCGTGTTAAGGAATCTGCTG
Carnitine palmitoyltransferase 1a	Cpt1a	Forward	TTTGAATCGGCTCCTAATGG
		Reverse	CCCAAGTATCCACAGGGTCA
Carnitine palmitoyltransferase 1b	Cpt1b	Forward	CAACTCCTGGAAGAAACGCC
		Reverse	TCCACCTTGCAGTAGTTGGA
Fas cell surface death receptor	Fas	Forward	GGAGGTGGTGATAGCCGGTAT
		Reverse	TGGGTAATCCATAGAGCCCAG
Lipase, hormone sensitive	Lipe	Forward	ATGCCACTCACCTCTGATCC
		Reverse	CTGTCCTGTCCTTCCCGTAG
Lipoprotein lipase	Lpl	Forward	TGAAAGCCGGAGAGACTCAG
		Reverse	AGTGTGAGCCAGACTTCTTCAG
Uncoupling protein 1	Ucp1	Forward	ACTGCCACACCTCCAGTCATT
		Reverse	CTTTGCCTCACTCAGGATTGG
Peroxisome proliferator-activated receptor alpha	Ppara	Forward	GATGTCACACAATGCAATTC
		Reverse	CAGTTTCCGAATCTTTCAGG
Peroxisome proliferator-activated receptor gamma	Pparg	Forward	GCATGGTGCCTTCGCTGA
		Reverse	TGGCATCTCTGTGTCAACCATG
Peroxisome proliferative-activated receptor gamma coactivator 1 alpha	Ppargc1a	Forward	AGCCGTGACCACTGACAACGAG
		Reverse	GCTGCATGGTTCTGAGTGCTAAG
qPCR conditions: preincubation at 95°C for 10 min followed by 45 denaturation cycles at 94°C for 10 s, annealing at 60°C for 30 s and extension at 72°C for 5 sec.			

Supplemental Figures

- CD-veh
- HFHSD-Veh
- HFHSD-*P.fae*

A

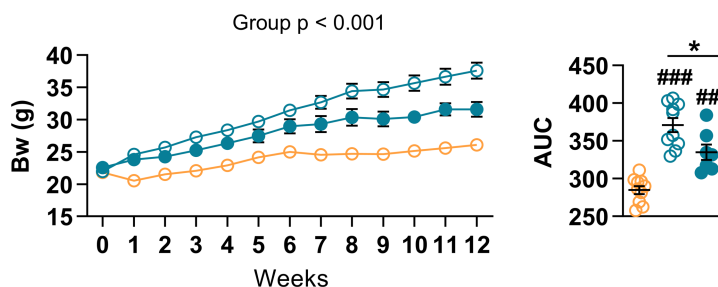
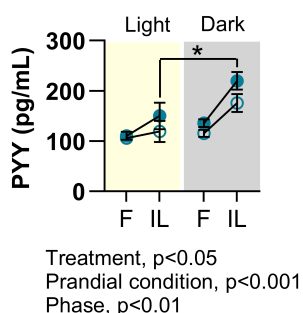


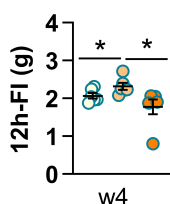
Figure S1 (relative to Figure 1). (A) Bw evolution and AUC after 12 weeks of intervention. Data shown as mean $\pm$ SEM, (n=7-10 per group). Abbreviations: AUC: area under the curve; CD: control diet; HFHSD: high fat high sugar diet; *P. fae*: *Phascolarctobacterium faecium*; Veh: vehicle. Two-way ANOVA followed by Tukey post-hoc test (A [body weight evolution]), main effects are shown in the graph; one-way ANOVA followed by Tukey's post-hoc test (A [AUC]). ## p<0.01 and ### p<0.001 vs. CD-Veh group; *p<0.05.

- HFHSD-Veh
- HFHSD-*P.fae*
- Saline
- 10x anti-PYY
- 100x anti-PYY

A



B



C

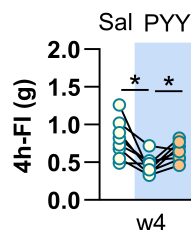


Figure S2 (relative to Figure 3). (A) Plasma levels of PYY at fasting and 20 min after an oral load of Intralipid 20% at the onset of the light and dark phase in 4 h-fasted mice fed HFHSD for 4 weeks (n=7-8). (B) 12 h-food intake of HFHSD-fed mice intraperitoneally injected with saline, 10x (1500 pg/mouse) or 100x (15000 pg/mouse) anti-PYY antibody 1 hour before the onset of the dark phase (n=6). (C) 4 h-food intake of HFHSD-fed mice receiving saline or 10x anti-PYY antibody 1 hour before the onset of the dark phase and injected with

exogenous PYY3-36 (5 µg/kg body weight) Data shown as mean ± SEM, (n=8 per group). Abbreviations: F: fasting; FI: food intake; IL: 20 min after oral load of intralipid; Sal: saline; w: week. Three-way ANOVA followed by Tukey's post-hoc test (A); one-way ANOVA followed by Tukey's post-hoc test (B); one-way repeated measures ANOVA followed by Tukey's post-hoc test (C); significant main effects are shown in the graph. *p<0.05, **p<0.01 and ***p<0.001.

- CD-Veh
- HFHSD-Veh
- HFHSD-*P.fae*

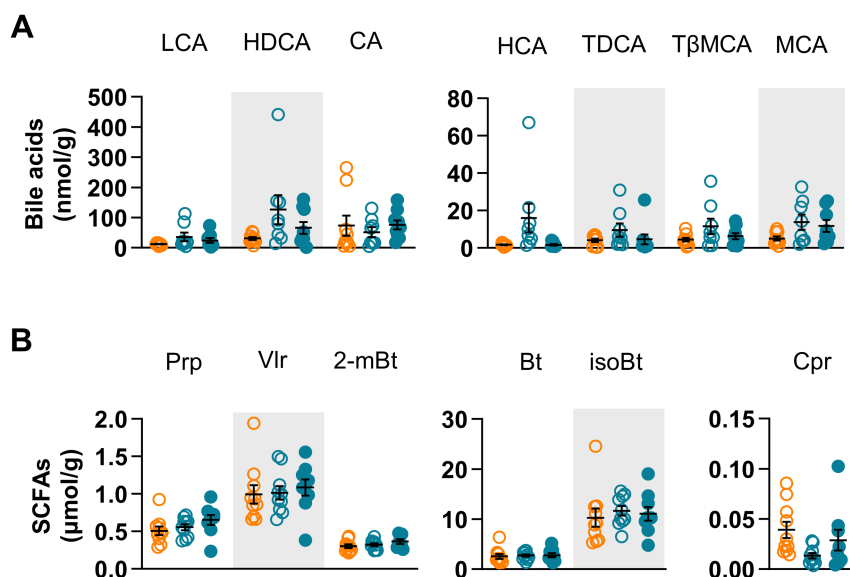


Figure S3 (relative to Figure 4). (A) Levels of bile acids and (B) short-chain fatty acids in caecum content of mice fed a CD or HFHSD treated with vehicle or with *P. faecium* at 12 weeks of intervention. Data shown as mean ± SEM, (n=9-10 per group). Abbreviations: 2-mBt: 2-methylbutyrate; Bt: butyrate; CA: cholic acid; CD: control diet; Cpr: caproate; LCA: lithocholic acid; HDCA: hyodeoxycholic acid; HCA: hyocholic acid; HFHSD: high fat high sugar diet; isoBt: isobutyrate; MCA: muricholic acid; *P. fae*: *Phascolarctobacterium faecium*; Prp: propionate; TDCA: taurodeoxycholic acid; TβMCA: tauro-β-muricholic acid; Veh: vehicle; Vlr: valerate. One-way ANOVA followed by Tukey's post-hoc test.

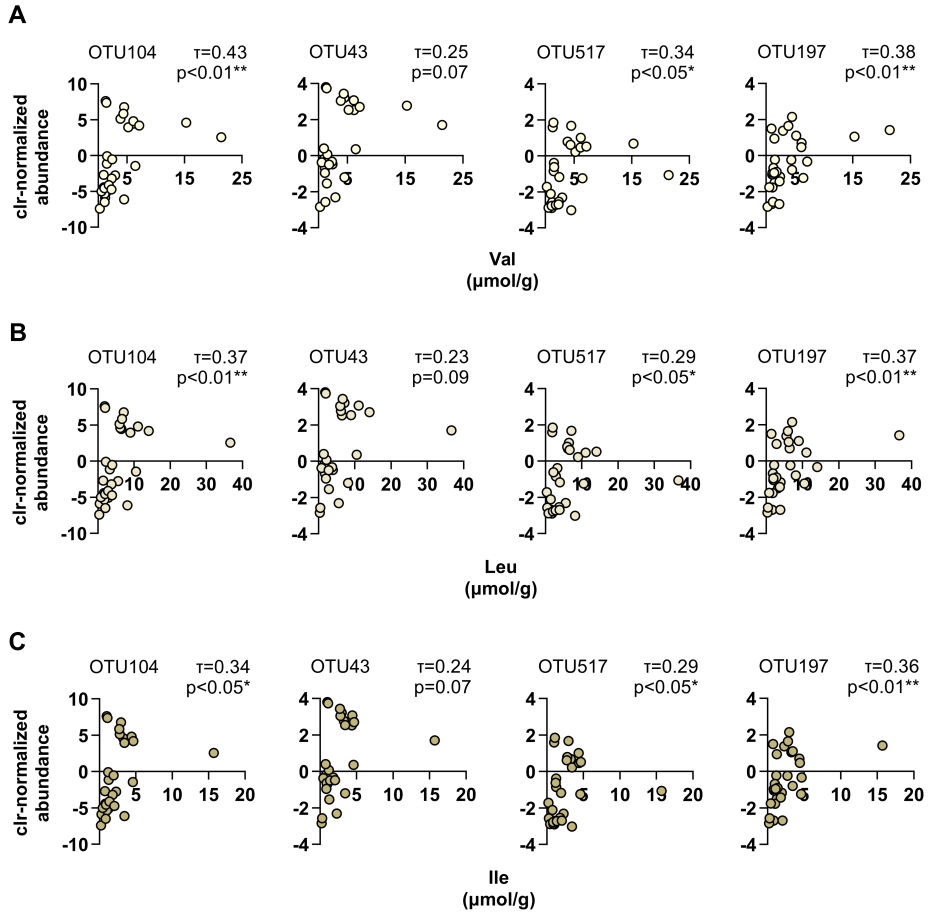


Figure S4 (relative to Figure 5). Correlations between abundance of *Lactobacillus* species (OTU104, OTU43, OTU517) or *Akkermansia muciniphila* (OTU197) and the BCAAs **(A)** Val, **(B)** Leu, and **(C)** Ile in cecum content of mice at 12 weeks of intervention. Abbreviations: clr: centered log-ratio, Ile: isoleucine; Leu: leucine; Val: valine; τ : Kendall's tau. Kendall rank correlations. * $p<0.05$ and ** $p<0.01$.

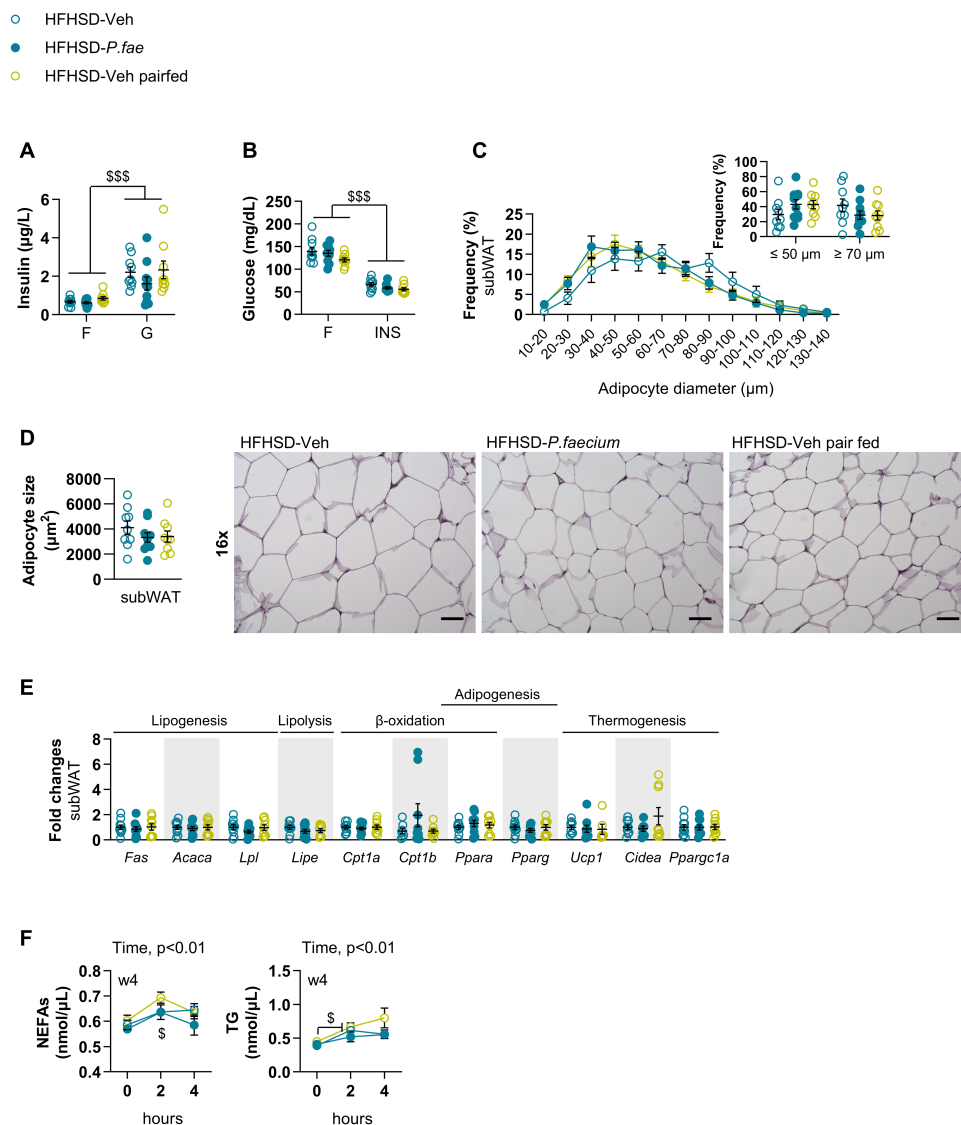


Figure S5 (relative to Figure 6 and Figure 7). (A) Plasma insulin levels in fasting and 15 min after an oral glucose load (2 g/kg). (B) Blood glucose levels after in fasting and 15 min after an i.p. injection of insulin (1.5 U/kg). (C) Frequency of adipocytes of different diameters in subWAT. (D) Quantification of individual adipocyte fat area in subWAT and representative bright field images of hematoxylin and eosin staining (scale bar = 50 μm , 16X magnification). (E) Gene expression analyses of markers of lipogenesis, lipolysis, β -oxidation, adipogenesis and thermogenesis in subWAT. (F) Plasma non-esterified fatty acids and triglycerides in fasting (0 h) and 2 and 4 h after an oral load of Intralipid 20%. Abbreviations: F: fasting; G: 15 min after glucose load; INS: 15 min after insulin injection; NEFAs: non-esterified fatty acids; w: week. Data shown as mean $\pm$ SEM, (n=5-10 per group). Two-way ANOVA followed by Tukey's post-hoc test (A, B, C and F); One-way ANOVA followed by Tukey's post-hoc test (C: upper graph, and D). \$ $p < 0.05$, and \$\$\$ $p < 0.001$.

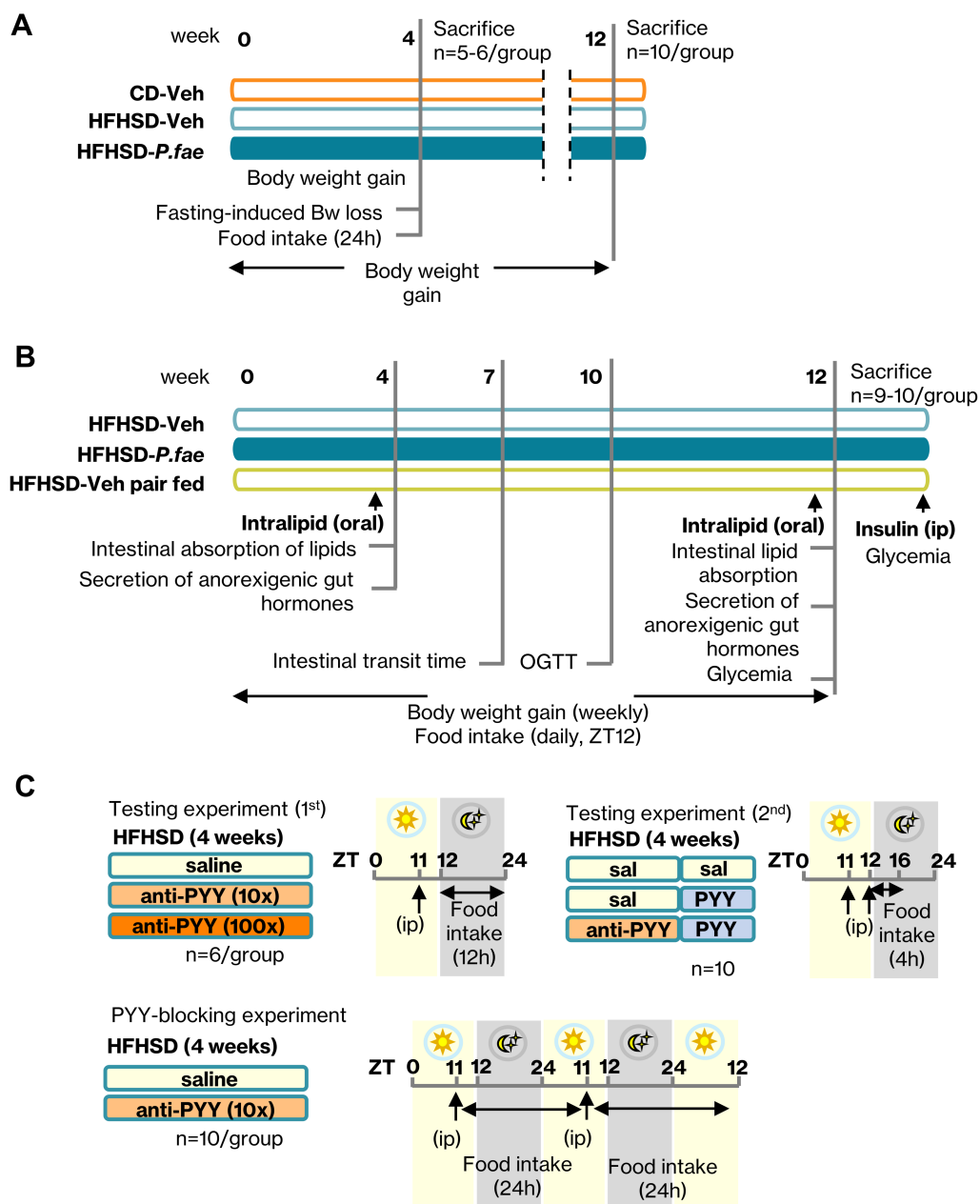


Figure S6. Graphical representation of the experimental designs. (A) Experiment 1 (effects of *P. faecium* in diet-induced obese mice): 4- and 12-week interventions with oral supplementation of *P. faecium* to diet-induced obese mice. (B) Experiment 2 (pair-feeding experiment): 12-week intervention with oral supplementation of *P. faecium* to diet-induced obese mice including a group pair-fed with the *P. faecium*-treated group. (C) Experiment 3 (PYY-blocking experiments in HFHSD-fed mice). Validation of PYY immunoneutralization with PYY-antibody administration (1st: PYY-antibody dose testing experiment; 2nd: PYY-antibody efficacy testing experiment with exogenous PYY). PYY-blocking experiment in *P. faecium*-treated mice. Abbreviations: Bw: body weight; CD-Veh: control diet-vehicle, HFHSD-Veh: high fat high sugar diet-vehicle, HFHSD-*P. fae*: high fat high sugar diet-*P. faecium*; ip: intraperitoneal.

General Discussion

General Discussion

Globally, overweight and obesity have reached epidemic proportions which, in addition to their impact on health and well-being, impose a significant economic burden to society ^{2,466}. Tackling this metabolic disorder is therefore recognised as a priority for achieving the Sustainable Development Goals (SDGs) for the 2030 Agenda, particularly SDG3, which includes reducing mortality from noncommunicable diseases through prevention and treatment ^{2,19}.

In our modern westernised environment, characterized by easy access to high calorie foods and a sedentary lifestyle, reducing the prevalence of obesity remains a major challenge. In this Doctoral Thesis we have explored the gut-to-brain neuroendocrine interactions that influence energy balance, including the role of intestinal microbiota, to progress in their understanding and potential role as therapeutic targets for obesity management.

The present section summarizes the main preclinical evidence resulting from this Doctoral Thesis and discusses the potential implications of these findings on energy homeostasis for the prevention and treatment of obesity and associated metabolic alterations.

1. Novel functions of Nav1.8-expressing sensory neurons in the control of energy homeostasis

The impact of Nav1.8+ neurons in the regulation of energy balance has been raised in recent studies ^{165,190,192}. In Chapters 1 and 2 of this Doctoral Thesis, we have successfully generated a mouse model lacking neurons expressing the sodium channel Nav1.8 through a diphtheria toxin ablation strategy, as in previous studies ^{186,192}, to elucidate the impact of these neurons on energy homeostasis in both males and females. This depletion approach has enabled us to assess how the absence of Nav1.8+ neurons affects neural and endocrine signaling pathways that contribute to the adverse effects on energy balance associated with diet-induced obesity from a sex-specific perspective for the first time.

Role of Nav1.8+ neurons in glucose homeostasis in females

Research in metabolic disorders considering sex differences is still scarce, even in preclinical studies. For instance, relevant works addressing the role of vagal afferent neurons in obesity were exclusively conducted in male rodents ²¹⁶. The underrepresentation of females in obesity-related basic research hampers the development of effective anti-obesity therapies, since both sexes are equally represented in the population world-wide ⁴⁶⁷, and current research is mainly designed for males. Importantly, in Chapter 1 we reveal that Nav1.8+ neurons are required to regulate energy homeostasis in a sex-dependent manner. Briefly, we show that under an obesogenic diet intact Nav1.8+ sensory neurons are required to induce oral glucose intolerance in females, while in males, they are necessary for body weight gain and fat storage.

Sex differences in the control of glucose homeostasis, such as glycemia, postprandial glucose absorption rates and insulin sensitivity, have been sparingly and gradually reported in humans ^{468–471}. For instance, compared to individuals with normal glucose tolerance, elderly women with T2D display reduced GLP-1 responses to an oral glucose challenge, which seem to precede T2D and obesity development ²¹⁰. In this line, in Chapter 1 we demonstrated that Nav1.8+ sensory neurons underlie sex differences in the energy homeostasis control. Specifically, we show that the depletion of Nav1.8+ neurons in female mice improves glucose clearance after an oral glucose challenge, while in males this occurs when glucose is injected intraperitoneally. Moreover, the loss of Nav1.8+ neurons in females is accompanied by elevated circulating levels of GLP-1 and insulin, which could facilitate glucose clearance. This suggests that in females, but not in males, postprandial glucose may be regulated by intestinal mechanisms mediated by Nav1.8+ afferents innervating the gut, through modulation of GLP-1 release.

In humans, enhanced GLP-1 release upon bariatric surgery has a strong and beneficial impact in the control of postprandial glycemia, and this enhanced GLP-1 has been associated with weight loss and diabetes remission independently of the eating-inhibitory effects of the surgery ^{472–475}. Similarly, our results in diet-induced obese female mice show a relevant role of GLP-1 on postprandial glucose homeostasis without affecting caloric intake. Still, the depletion of Nav1.8+ neurons does not affect body weight as bariatric surgery do, so it would be of interest to explore whether the sustained levels of GLP-1 could eventually lead to body weight loss in females in the longer term.

We also show that the depletion of Nav1.8+ neurons in females restores gut transit which, as expected, was reduced by the consumption of a high calorie diet. Moreover, the normalized gut transit does not appear to affect fat weight or distribution. Thus, as discussed in Chapter 1, rather than reducing the time frame for intestinal fat absorption, the accelerated gut motility induced by the loss of Nav1.8+ neurons may help to drive nutrients to the distal parts of the intestine, facilitating the presence of GLP-1 secretagogues that ultimately contribute to glucose homeostasis. The ENS and sympathetic and parasympathetic efferent innervations are key players in modulating GI transit^{96,476} and may therefore be affected by the absence of Nav1.8+ neurons, although characterisation of these neural adaptations that facilitate gut motility needs to be addressed. Furthermore, reversible Nav1.8+ silencing strategies could allow identification of the optimal time frames in which modulation of postprandial gut motility would optimize the availability of dietary GLP-1 secretagogues in the distal gut to amplify GLP-1 release, and thus more precisely regulate postprandial glucose.

Based on our results, silencing the activity of Nav1.8+ neurons in females could contribute to the prevention of glycemic disturbances after a meal induced by a high-calorie diet through enhancement of GLP-1 endocrine signaling.

Role of Nav1.8+ neurons in body weight, food intake and energy expenditure in males

Strong evidence in male rodents reveal that chronic consumption of high calorie foods reduces vagal afferent neuron sensitivity to peripheral signals, ultimately leading to obesity²¹⁶. These neurons show a wide variety of molecular phenotypes¹⁸³, and the identification of metabolic functions governed by specific vagal neurons subtypes could help advance in the understanding of obesity pathophysiology and so, in the development of more effective therapies. Thus, in our study with mice, the depletion of Nav1.8+ sensory neurons, unmyelinated neurons classically associated with spinal mediating signaling, in combination with the characterization of the metabolic phenotype, allowed us to disentangle new functions of these neurons.

In contrast to females, in Chapters 1 and 2 we show that in male mice Nav1.8+ neurons considerably affect the control of body weight and food intake, especially under HFHSD feeding. In Chapter 1, close examination of the high variability detected in body weight gain in response to HFHSD feeding allowed us to identify different obese phenotypes induced by depletion of Nav1.8+ neurons, in line with a previous study using the same mouse model¹⁹². Specifically, the loss of Nav1.8+ neurons prevents weight gain in response to HFHSD in the majority of mice, which is also reported in Chapter 2, while has no effect in others, or even induces weight loss. Moreover, we report that the absence of these neurons induces reductions in food efficiency, intestinal fat absorption, and adiposity (Chapter 1), and possibly increases thermogenic activity (Chapter 2), supporting that the resistance to HFHSD-induced weight gain is possibly the result of a defective assimilation of ingested nutrients in combination with exacerbated energy expenditure. In view of these results, we suggest that Nav1.8+ neurons impact body weight by influencing efferent outputs to peripheral organs with a relevant role on the energy homeostasis control, such as the GI tract and the adipose tissue. To note, the impact of Nav1.8+ neurons on the sympathetic efferent tone

targeting organs to modulate energy balance should be further elucidated to ultimately assess the role of Nav1.8+ neurons on energy expenditure.

Based on these first results in males, we further disentangle the role of Nav1.8+ neurons in the control of feeding and body weight in pre- and post-prandial states. In this regard, Chapters 1 and 2 highlight the critical role of Nav1.8+ neurons in male mice in regulating hunger after food deprivation and enhancing satiety signaling after a meal. Specifically, we show that male mice lacking Nav1.8+ neurons under obesogenic feeding exhibit an exacerbated body weight loss during fasting, followed by intensified food intake, possibly comparable to binge eating. Concretely, this overfeeding occurs at dusk, as shown when mice are food-restricted to the dark. Under these conditions we also show that, after an acute nutritional stimulus, mice lacking Nav1.8+ neurons reduce satiety when re-exposed to food. To note, when food is available only during the light phase, none of these effects are observed, that is why we suggest that Nav1.8+ neuron modulation on feeding is more responsive to light/dark cues rather than feeding time.

At first glance, these results seem to suggest that silencing Nav1.8+ neurons activity in males could counteract the effects of overeating by preventing weight gain. However, the hyperphagia observed in Nav1.8-ablated mice seems to be an attempt to compensate for the abnormally reduced body weight gain, which could be exacerbated somehow by the consumption of a high-calorie diet. Interestingly, we report that diet-induced obese mice (control genotype) are unable to display compensatory overfeeding upon food restriction, as previously shown ⁹⁸, possibly due to a diet-induced desensitization of the gut-to-brain signaling involving Nav1.8+ afferent neurons. At the end of the time-restricted feeding protocol, diet-induced obese control mice show reduced body weight, which does not occur when Nav1.8+ neurons are depleted. Thus, our findings suggest that Nav1.8+ neurons could be implicated in mediating body weight loss under time-restricted feeding, probably by “reactivating” their ability to sense nutritional states.

Therefore, stimulating the activity of Nav1.8+ neurons in diet-induced obese male mice to sensitize gut-to-brain afferent sensory signaling may be an efficient approach to improve the regulation of body weight variations in consonance with food intake, ultimately ameliorating the energy imbalance in the short and long term. This approach would also ease the benefits of intermittent fasting regimens to reduce body weight in overweight and obesity. To note, Nav1.8+ neuron sensitivity might be differently affected depending on the individual degree of obesity or other related factors, which should be considered to personalize such therapeutical approaches.

Role of Nav1.8+ neurons in the enteroendocrine gut-to-brain signaling in males

Complementary to the attempt to compensate for abnormal energy deficits, discussed above, we reasoned that the altered feeding behavior showed by mice lacking Nav1.8+ neurons could be also a consequence of an altered enteroendocrine signaling driving enhanced hunger and reduced satiety signals to the hypothalamus.

Mechanistic enteroendocrine events underlying the role of Nav1.8+ neurons controlling food intake were only explored in male mice lacking Nav1.8+ neurons fed CD ad-libitum since, under a high calorie diet, the depletion of Nav1.8+ neurons had detrimental health effects in mice along the

course of the experiment. Overall, we show in these mice impaired enteroendocrine and hypothalamic hunger signaling resulting in exacerbated food intake at the beginning of the active phase. Specifically, in the gut, we demonstrate that Nav1.8+ neurons in males are necessary to maintain the levels of the orexigenic hormone ghrelin elevated in fasting and the levels of the anorexigenic gut hormones GLP-1 and PYY reduced. Particularly for GLP-1, Nav1.8+ neurons are required for a proper hormonal secretion after a meal. Furthermore, our findings support that Nav1.8+ neurons are critical to maintain a responsive hypothalamus to the hormonal signals arising from the intestine to ultimately control food intake.

How Nav1.8+ neurons maintain the functionality of EECs cells and whether this is critical for their role controlling food intake requires further investigations, especially in the context of diet-induced obesity. In this regard, it should be explored whether Nav1.8+ neurons directly influence ECCs or whether these neurons impact on the ENS and/or the intestinal efferent tone to indirectly affect EECs functionality ^{153,477–479}.

In the context of obesity, as already mentioned, we show in Chapters 1 and 2 that the loss of Nav1.8+ neurons under obesogenic diet induces overfeeding following a fasting period, even after and acute nutritional stimulus (Ensure®), resulting in uncoupled weight gain. This suggests dysfunctional nutrient sensing, at intestinal and hypothalamic levels, unable to adequately control body weight. To unravel the relevance of Nav1.8+ neurons in the control of food intake in obesity it is still needed to decipher whether these neurons are desensitized by hypercaloric diets leading to defective nutrient sensing, including defective enteroendocrine signaling, that affects food intake. This could help to figure out whether stimulation of Nav1.8+ neurons in obesity could activate functional nutrient sensing transmission from the gut to the hypothalamus to ultimately ensure proper synchronization of feeding behavior and body weight.

In contrast to females, where silencing of Nav1.8+ neurons appears to drive glucose-related benefits in obesity likely due to increased GLP-1, stimulation of Nav1.8+ neuron activity in males may help to normalise pre- and postprandial gut hormone secretion and so feeding behavior. This again underscores the sex-specific impact of Nav1.8+ neuron function on energy homeostasis and highlights the need to test sex-specific and personalised neuromodulation strategies in obesity.

Role of Nav1.8+ neurons in the daily oscillations of the gut microbiota and intestinal barrier components in males

Previous findings show the relevance of diurnal rhythmicity of the gut microbiota composition in response to circadian feeding stimuli ⁴⁸⁰. In this Doctoral Thesis we demonstrate in male mice that the daily fluctuations of gut microbiota diversity are shaped by Nav1.8+ neurons in response to feeding states. Differences in the composition of the diet nuance how Nav1.8+ neurons mediate these oscillations. For instance, under an obesogenic diet, Nav1.8+ neurons control changes in microbial BA metabolism in response to the prandial state, probably explained by the consumption of a fat-enriched diet inducing a subsequent rise in BA secretion for lipid digestion and absorption. This may enhance the BA pool available for intestinal bacterial transformation into secondary BAs in consonance with the microbiota variations observed.

Overall, we find that these neurons are required to drive reductions of the gut microbiota diversity and BAs after the first meal of the active phase, demonstrating a relationship between gut microbiota and Nav1.8+ neurons as a function of feeding behavior. The direction of this relationship should be further studied by exploring whether the prandial-related fluctuations of the gut microbiota govern the function of Nav1.8+ neurons in food intake; or whether the role of Nav1.8+ neurons controlling food intake governs the gut microbiota prandial-related oscillations. This could help to design non-invasive microbiome-based strategies for restoring feeding patterns via Nav1.8+ neurons.

Parallel to these findings, we show that under metabolically healthy conditions Nav1.8+ neurons are necessary to preserve a functional gut barrier by reinforcing the tight junctions within the intestinal epithelium and by maintaining the levels of protective immune cell populations. Thus, as discussed in Chapter 2, Nav1.8+ neurons innervating the intestine might enhance a balanced efferent tone which, by modulating intestinal immune cells, in turn modulates variations on gut microbiota ¹⁹³.

Thus, intermittent activation of Nav1.8+ neurons in coordination with established meal timings in the active phase would enable daily fluctuations of beneficial microbial communities reinforced by an anti-inflammatory intestinal immune tone. To note, it would be of interest to study the contribution of silencing Nav1.8+ neurons in the composition of the gut microbiota in the active phase of females, to assess to which extent this may contribute to the enhanced GLP-1 secretion.

Role of Nav1.8+ neurons in immune health in males and females

In Chapters 1 and 2 we show in males that the consequences of depleting Nav1.8+ neurons are considerably detrimental when mice are challenged with a high-fat-diet. Moreover, we report low survival rates and immune system alterations, which have already been discussed in the corresponding Chapters, that are in line with previous findings demonstrating that Nav1.8+ neurons are required to maintain the immune homeostasis to limit the acute inflammation induced by excessive dietary lipid intake ¹⁹². Notably, in contrast to males, the health of females under HFHSD feeding is not compromised by the depletion of Nav1.8+ neurons, which rather promotes an intestinal anti-inflammatory cell population profile coupled with the prevention of splenomegaly.

Thus, Nav1.8+ neuron silencing approaches in obese females could potentially entail the mentioned benefits in immunity which, in turn, may underlie or feedback the improvements observed in glucose homeostasis, which is directly linked with the immune system ²⁷⁰. Stimulation of Nav1.8+ neurons in males deserves further investigation to effectively prevent the adverse effects of a high fat diet on immunity.

Prospects and applications of modulating Nav1.8+ neuronal activity

By establishing a preclinical model of genetic ablation of Nav1.8+ neurons to study energy homeostasis, Chapters 1 and 2 of this Doctoral Thesis elaborate the first hypotheses about the potential of modulating the activity of Nav1.8+ sensory neurons to restore energy balance in female and male population with obesity.

On the one hand, our findings in females suggest that silencing Nav1.8+ neurons could serve as a tool to control glucose homeostasis in diet-induced obese and/or diabetic women, including for type 2 diabetes prevention in menopausal women, in whom glucose problems appear to increase with age ²¹⁰. As the chronic depletion of Nav1.8+ neurons in our female mouse model did not cause immune health problems, either sustained or intermittent neuron silencing can be considered to achieve optimal benefits in glucose homeostasis when designing neuromodulation strategies for obese and/or diabetic female population. In this regard, VBLOC targeting Nav1.8+ neurons could help to precisely assess the effects of neuron silencing in females.

VBLOC is a neuromodulation-based therapy used in patients with obesity and diabetes to inhibit the vagal signaling resulting in successful weight loss ^{429,431,438–440} and improved glucose homeostasis and cardiovascular health ⁴⁴⁰. Interestingly, in clinical trials testing 18 or 24 months of VBLOC therapy, adverse events such as abdominal pain, heartburn or dysphagia were rare, demonstrating the safety of the treatment over 2 years ^{438,440}. In addition, electrical stimulation could be adjusted in amplitude and in number of hours per day (12 or 14 hours) to minimize adverse sensations derived from the therapy while optimizing weight loss ^{438,440}, adding flexibility in personalizing VBLOC therapy.

On the other hand, our findings in males suggest that strategies to modulate the activity of Nav1.8+ neurons to prevent or treat obesity should focus on acute and intermittent, rather than sustained stimulation. Indeed, synchronizing the activation of Nav1.8+ neurons with the active phase of mice (nighttime) or humans (daytime) would allow an optimal and healthy regulation of body weight fluctuations in coordination with meal timing, in turn modulating gut microbiota oscillations and ensuring a functional enteroendocrine signaling to enhance energy homeostasis. Given the strong association of these neurons with immunity reported in this Doctoral Thesis, the effects of Nav1.8+ neuron activation on the immune system in males on a high-calorie diet should be carefully evaluated. In addition, we reported a variable effect of Nav1.8+ neurons on the obese phenotype in male mice. This highlights the importance of inter-individual differences in parameters that influence body weight to achieve a personalized approach in the development of neuromodulation strategies targeting obesity in males. Therefore, daily energy expenditure (e.g., physical activity or resting metabolic rate), age, dietary composition or eating habits should be considered in Nav1.8+ neuromodulation studies as factors affecting body mass.

VNS therapy has also been used to increase the vagal tone in order to enhance a postprandial sensation of fullness ⁴³⁶, with clinical and preclinical studies showing reduced caloric intake and sustained body weight loss ^{432–437}. Thus, intermittent VNS in obese males following the patterns here suggested might help to increase the anorexigenic signaling and restore a proper vagal regulation of feeding. However, since in males Nav1.8 neurons seem to have a complex role in energy homeostasis, as presented in this Doctoral Thesis, specifically targeting afferent Nav1.8+ neurons by other techniques could contribute to improving the regulation of energy balance.

However, to the best of our knowledge, VNS and VBLOC trials lasting longer than 2 years have not yet been tested to assess whether the benefits in obesity are sustained in the longer term. Importantly, although VNS/VBLOC therapies appear to be effective, their underlying mechanisms warrant further investigation, as these techniques involve vagal afferent and efferent populations

indistinctly, and the complete inhibition or stimulation of the electrical current in these neurons is not yet clear ^{216,443}. In this context, a recent study in rats achieved directional activation of vagal nerve fibers by anodal block (ABL), which elicited a greater afferent response to VNS when stimulating the left vagal branch ⁴⁸¹. Although this technique could partially avoid the off-target effects of VNS on the adverse outcomes mentioned above, ABL studies are scarce and have shown inconsistent results. For these reasons, the potential applicability of selective modulation of vagal afferents, such as Nav1.8+ neurons, to alleviate diet-induced obesity in females and males is promising and merits further investigation.

Collectively, our findings suggest that the role of Nav1.8+ neurons in energy homeostasis underlies gut-related mechanisms. Furthermore, given the predominant vagal origin of Nav1.8+ neurons innervating the gut ¹⁸⁸, we highlight their importance as key mediators in gut chemosensing. This reinforces the need for preclinical research aimed at developing tools that allow specific control of the Nav1.8+ afferent neuron population innervating the gut, with the possibility of accurately enhance timed stimulation or silencing of these neurons.

Limitations and future perspectives

Based on the contrasting results between males and females, which underscore the importance of developing sex-specific approaches to address obesity, the molecular mechanisms underlying these differences deserve future attention. In this context, estrogen receptors expressed in vagal afferent neurons, including Nav1.8+ neurons, play a role in regulating food intake, body weight and enteroendocrine signaling ^{482,483}. Therefore, the depletion of Nav1.8+ neurons in our model may impact the potential synergistic effects of estrogens on gut-derived postprandial signals.

Our work provides a robust basis for the application of optogenetic or chemogenetic approaches. Given that the Cre-LoxP-based methodology has been successfully used for genetic tracing of Nav1.8+ vagal and spinal afferents ^{165,188} and for genetic ablation of these neurons *in vivo* ^{186,192} as we did in this Doctoral Thesis, it provides with a practical strategy to selectively target Nav1.8+ neurons and modulate them with optogenetic or chemogenetic tools. The use of Cre-expressing transgenic mouse strains combined with retrograde tracing with viral vectors carrying Cre-dependent light-sensitive channels or engineered receptors (DREADDs) would allow transient stimulation and inhibition of Nav1.8+ neurons, helping to refine the study of their metabolic functions. However, selectively targeting the intestinal Nav1.8+ afferent neuronal population of vagal origin remains a challenge, as vagal afferent endings innervate the intestine in different neural substructures (IGLEs, IMAs and mucosal afferents) and regions along the GI tract ^{153,173}. In addition, Nav1.8 is also a marker of spinal neurons. Although these have been traditionally related to sensing of noxious stimuli, the separate contribution of Nav1.8-expressing spinal or vagal sensory afferent neurons on energy homeostasis remains to be elucidated.

Overall, this preclinical model would allow acute and intermittent stimulation or inhibition of intestinal afferent Nav1.8+ neurons, ultimately broadening the understanding of the function of these neurons in energy homeostasis in both females and males. In the future, the efficacy of this strategy could be validated in combination with other anti-obesity interventions, for instance time-

restricted feeding or fibre-enriched diets, to ultimately assess its potential use in the design of sex-specific anti-obesity/diabetes therapeutic strategies.

2. *P. faecium* DSM 32890 induces anti-obesity effects modulating gastrointestinal functions, such as enteroendocrine signaling, gut transit and lipid absorption

New perspectives for developing anti-obesity therapies are oriented to tackle the altered enteroendocrine responses in this metabolic disorder^{99,100}. Considering the ability of the gut microbiota to modulate EECs, the use of microbial ligands represents a promising strategy to target the EECs and, thus, achieve gut hormone secretion outcomes that mimic the enteroendocrine changes upon bariatric surgery leading to weight loss¹⁰⁴. In Chapter 3, we demonstrate that oral administration of *P. faecium* DSM 32890 prevents fat accumulation by enhancing food intake suppression through PYY hypersecretion and by promoting gut motility, as well as limiting intestinal lipid absorption. Importantly, our study contributes to identifying gut bacteria that modulate the enteroendocrine system, an emerging area of research.

Role of PYY in mediating the satiating effects of *P. faecium*

PYY is a gut hormone that regulates body weight not only by reducing caloric intake, but also by increasing energy expenditure via stimulation of fat oxidation⁴⁸⁴. Under metabolically healthy conditions, PYY responds primarily to dietary fats, also depending on the fatty acid composition and on the fatty acid type⁴⁸⁵, rather than to protein or glucose^{472,486,487}. It is also well known that a fat-enriched diet is associated with increased energy intake and adiposity⁴⁸⁸, which is consistent with the diet-induced obese mouse model used in our study. In Chapter 3 we show that *P. faecium* increases the number of colonic PYY-secreting cells and enhances the PYY secretory responses to an oral lipidic stimulus at early and late stages of treatment in diet-induced obese mice. Moreover, incubation with the bacterium increases PYY release in cultured EECs. Therefore, *P. faecium* appears to contribute to the sensitization of the enteroendocrine system to fat, by enhancing PYY-induced satiety in diet-induced obese mice. In search of the underlying mechanisms of enhanced PYY secretion, we initially suggest that *P. faecium*, directly or indirectly through changes in the microbial ecosystem, might contribute to mitigating the diet-induced EEC alterations affecting hormonal secretory responses, as occurs in diet-induced EEC silencing^{354,489}. Based on this hypothesis, *P. faecium* could modulate EEC sensing by enhancing *Pyy* transcription and PYY vesicular storage and exocytosis, although further research would clarify the bacterium/cell-to-cell interactions involving the intracellular transcription machinery.

Interestingly, we show that after 4 weeks of HFHSD feeding, when metabolic hormone profiles have not yet been disturbed by the diet in mice, the *P. faecium*-induced PYY over-secretion is associated with the prevention of hyperphagia in the dark phase. In this regard, the absence of satiety effects of *P. faecium* in another cohort of mice with immunoneutralized PYY and receiving the bacterium confirms the causal role of PYY in the anorexigenic effect of *P. faecium*. Thus, *P. faecium* appears to exert its metabolic benefits by initially targeting control of food intake at early

stages of obesity development, and we show that these PYY-mediated anorexigenic effects are maintained in the long term. Importantly, through pair-feeding protocol we also demonstrate that the effect of *P. faecium* modulating food intake partially drive its metabolic benefits. Therefore, as the onset and exacerbation of obesity are particularly influenced by excessive caloric intake, *P. faecium* may be an effective strategy for the early prevention of this metabolic disorder.

Modulation of gastrointestinal functions contributing to the anti-obesity effects of *P. faecium*

In Chapter 3 we show that, in the long term, *P. faecium* also exerts beneficial metabolic effects in obesity through other mechanisms that are independent of food intake (anorexigenic mechanisms). Specifically, *P. faecium* enhances GI transit and leads to rapid clearance of NEFAs from the systemic circulation. Our results suggest accelerated gut motility, which seems somewhat paradoxical given the traditionally ascribed role of PYY in slowing intestinal transit ^{202,490,491}. However, as discussed in Chapter 3, the differential contribution of PYY isoforms to gut motility ^{492,493} should be quantified. In addition, functional testing of gut motility earlier in time, that is, when PYY-mediated anorexigenic effects occur, would provide additional information on the contribution of PYY to gut transit in addition to the anorectic properties.

Notably, the effects on lipid clearance occur in the long, but not in the short term of *P. faecium* treatment, reinforcing the importance of the PYY-induced anorexigenic effects as an initial trigger for *P. faecium*-induced metabolic benefits. In addition, we report that a structural component of *P. faecium* reduces intestinal lipid absorption by enhancing cellular lipid uptake *in vitro*. In this regard, bacterial-derived metabolites rather than bacterial structural components have been investigated for being able to modulate the use of dietary lipids by enterocytes. For instance, enterocyte lipid metabolism is directly influenced by *Lactobacillus paracasei* or *Escherichia coli* fermentation products, which promote cellular lipid storage and oxidation, respectively ⁴⁹⁴. In this line, *P. faecium* produces propionate ⁴⁹⁵, another fermentation metabolite that induces lipolysis gene expression in enterocytes via the AMPK/LSD1 activation pathway ⁴⁹⁶, suggesting a still unexplored mechanism of interaction between *P. faecium* and enterocytes.

Role of *P. faecium* in modulating gut microbiota and intestinal BCAA as PYY secretagogues

Regarding the effects of *P. faecium* on the intestinal bacterial community, we show that the intervention increases the abundance of *Akkermansia muciniphila*, a commensal bacterium beneficial for the host's metabolic homeostasis, which may contribute to the maintenance of the intestinal barrier integrity through the production of immunomodulatory lipids ^{326,497–499}. Using glucose as a substrate, *A. muciniphila* synthesizes BCAAs ³²⁶, which strengthen tight junctions in the intestinal epithelial barrier ⁵⁰⁰. As *A. muciniphila* is positively associated with BCAAs levels in our study, the microbial interaction between *P. faecium* and *A. muciniphila* should be further investigated. In this respect, it could be possible that succinate supports the cross-feeding between these two bacterial strains since *A. muciniphila* is a succinate producer, while *P. faecium* is a succinate consumer ⁴⁹⁹. Moreover, *P. faecium* produces shikimic acid, an intermediate of the

shikimate pathway, the central metabolic pathway for the biosynthesis of aromatic compounds⁵⁰¹. *A. muciniphila*, on the other hand, has been found to be the main contributor to shikimate pathway in the gut microbiota, as it possesses all the transcriptionally active genes required for this pathway⁵⁰².

Given the importance of PYY in the anorexigenic effects of *P. faecium*, we also show that this bacterial strain may interact directly with EECs to induce PYY over-secretion, or indirectly through microbial-derived BCAA production due to modulation of the gut bacterial community. Most importantly, we identified increased cecal levels of the BCAAs as potential PYY secretagogues in *P. faecium*-treated DIO mice. BCAAs can have quite opposite effects depending on the nutritional state (pre- or post-prandial), which determines catabolic and anabolic signaling in the organism⁵⁰³. Since *P. faecium* facilitates fasting-induced body weight loss in the early stages of treatment, accompanied by reduced body weight gain and fat deposition at the end of the intervention, we consider the possibility that the bacterium could target the restoration of a functional BCAA catabolism over anabolism in adipose tissue, thus limiting fat storage, which has previously been reported as a potential strategy to alleviate metabolic disorders^{504,505}.

In the search for *P. faecium*-derived molecules with PYY secretory capacity, we suggest that bacterial structural components could be putative candidate ligands. TLRs are increasingly recognized as essential gut sensors in the microbiome-host crosstalk, stimulating the release of gut hormones when activated by bacterial molecules^{338,339,506–509}. As *P. faecium* is a Gram-negative bacterium⁵¹⁰, its outer membrane consists of LPS, a TLR4 ligand of L cells^{338,508,511}. The bacterium could directly access the basolateral TLRs, as the integrity of the intestinal barrier is partially compromised by HFHSD³³⁸. However, we cannot rule out the possibility that the *P. faecium*-induced changes in the microbial community contribute to a pool of bacterial components that potentially bind to TLRs and induce PYY secretion.

We hypothesize that the *P. faecium*-increased gut motility may also facilitate the accumulation of BCAAs in the cecum, either from unabsorbed dietary proteins or from gut microbial *de novo* BCAA synthesis. In addition, the accelerated intestinal transit may lead to higher potential pathogens shedding⁵⁰⁹, thus leaving a nutritional niche for metabolically beneficial bacteria, such as *Akkermansia* and *Lactobacillus*^{512–516}. Interestingly, commensal bacteria contribute to GI motility by modulating TLR4^{365,517,518}. Thus, *P. faecium* could interact with this receptor directly or indirectly through microbial modifications, ultimately mitigating the high-fat diet-induced effects on delayed intestinal transit, without triggering exacerbated LPS/TLR4-mediated pro-inflammatory responses adversely affecting enteric neurons^{364,365}.

From a whole-organism perspective, studying the effects of *P. faecium* administration on the gut microbiota and derived metabolites, such as BCAA levels in the gut and circulation at different time points, could shed more light on the contribution of the bacterium itself, the microbial community, and derived metabolites in maintaining the high PYY secretion observed throughout the intervention. In addition, the isolation of *P. faecium* structural components, together with TLR signaling screening *in vitro* and *in vivo*, would be a further step in the identification of specific ligands and receptors that stimulate PYY release. In addition, the characterization of *P. faecium*-derived metabolites through metabolomic approaches in cultures of the bacterium alone or in

consortium with other key intestinal bacteria would shed light into how *P. faecium* modulates the BCAA metabolism and allow the identification of other potential metabolites with PYY secretagogue properties.

Limitations and future perspectives

Although *P. faecium* DSM 32890 was obtained from a healthy human source, our results in mice provide strong evidence for the metabolic benefits of this bacterial strain, suggesting that its efficacy as a potential probiotic is likely to be transferable to humans. Therefore, clinical trials are needed to translate our preclinical findings into therapeutic approaches for humans. In this context, investigating whether the preventive properties of *P. faecium* in obesity are also applicable to treatment strategies would also complement our findings.

Overall, our findings in Chapter 3 suggest that *P. faecium* could serve as a next-generation probiotic that could prevent and ameliorate overweight and obesity through specific mechanisms that modulate the host enteroendocrine system to limit fat storage. Specifically, *P. faecium* may be able to target altered feeding patterns such as hyperphagia, thereby restoring a functional homeostatic control of food intake to restrict fat accumulation.

In addition to the enteroendocrine system, other mechanisms by which *P. faecium* mediates the metabolic benefits in diet-induced obesity include the modulation of gut motility. Targeting the ENS, a key component in the control of gut motility, would provide new insights into the mode of action of *P. faecium*. Therefore, mechanistic preclinical studies are essential to advance the use of *P. faecium* as a biotherapeutic agent with both PYY secretagogue and enterosyne properties. In addition, the combination of this bacterial strain with current gut hormone-based anti-obesity drugs could potentially increase their efficacy by allowing lower doses, thereby improving tolerability and reducing side effects in patients.

Conclusions

Conclusions

1. Depletion of **sensory neurons expressing the sodium channel Nav1.8** in mice **affects** neural and endocrine pathways involved in **energy homeostasis** in a **sex-specific** manner.
2. In **diet-induced obese female** mice, **ablation of Nav1.8+ sensory neurons improves postprandial glucose homeostasis** by enhancing GLP-1 enteroendocrine signaling, whereas in **male** mice, depletion of these neurons induces **resistance to diet-induced obesity** by reducing intestinal triglyceride absorption and adiposity.
3. In **male** mice, **ablation of Nav1.8+ sensory neurons impairs** the coordinated control of **food intake** and **body weight** fluctuations throughout the day. At the intestinal level, ablation of Nav1.8+ sensory neurons alters the physiological **enteroendocrine responses** and the **daily oscillations of the gut microbiota** in response to nutritional status (fasting/refeeding cycles) and disrupts **intestinal immune homeostasis**.
4. *Phascolarctobacterium faecium* **DSM 32890** exerts anti-obesity effects by inducing **PYY-mediated satiation, accelerating intestinal transit** and **reducing intestinal lipid absorption**, together with enhanced lipid clearance in the circulation.

References

**General Introduction and
Discussion sections**

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General Introduction and Discussion

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Abbreviations Table

Ach	Acetylcholine
AGRP	Aguti-related peptide
ARC	Arcuate nucleus of the hypothalamus
ASV	Amplicon sequence variants
BA	Bile acid
BAT	Brown adipose tissue
BCAA	Branched-chain amino acid
Bw	Body weight
CART	Cocaine- and amphetamine-regulated transcript
CCK	Cholecystokinin
CCK1R	Cholecystokinin receptor
CD	Control diet
ChAT	Choline acetyltransferase
CHO	Cholesterol
ClpB	Caseinolytic peptidase B
CNS	Central nervous system
DIO	Diet-induced obesity
DRF	Dark-restricted feeding
DRG	Dorsal root ganglion
DTA	Diphtheria toxin fragment A
EEC	Enteroendocrine cell
ENS	Enteric nervous system
ER	Endoplasmatic reticulum
eWAT	Epididymal white adipose tissue
FXR	Farnesoid X receptor
GHSR	Growth hormone secretagogue receptor (ghrelin receptor)
GI	Gastrointestinal
GIP	Glucose-dependent insulintropic polypeptide
GIPR	Glucose-dependent insulintropic polypeptide receptor
GLP-1	Glucagon like peptide-1
GLP1R	Glucagon like peptide-1 receptor
TGR5	Takeda G-protein-coupled receptor 5
GPCR	G protein-coupled receptor
gWAT	Gonadal white adipose tissue
HFHSD	High-fat high-sugar diet
HTL	Hypothalamus
IGLEs	Intraganglionic laminar endings
IL	Intralipid
ILC2	Group 2 innate lymphoid cells
Ile	Isoleucine
IMAs	Intramuscular arrays
IPANs	Intrinsic primary afferent neurons
iPGTT	Intraperitoneal glucose tolerance test
iWAT	Inguinal white adipose tissue

LCFA	Long-chain fatty acid
Leu	Leucine
LoxP	Locus of crossover x P1
LPS	Lipopolysaccharide
LRF	Light-restricted feeding
M1, M2	Type 1-, type 2- macrophages
MC4R	Melanocortin-4-receptor
MDP	Muramyl dipeptide
Nav1.8	Tetrodotoxin-resistant sodium channel Nav1.8
NE	Noradrenaline
NEFAs	Non esterified fatty acids
NG	Nodose ganglion
nNOS	Nitric oxide synthase
NO	Nitric oxide
NPY	Neuropeptide Y
NPY2R (or Y2R)	Neuropeptide Y receptor type 2
NTS	Nucleus of the solitary tract
OGTT	Oral glucose tolerance test
OTU	Operational taxonomic unit
<i>P. faecium</i>	<i>Phascolarctobacterium faecium</i>
POMC	Proopiomelanocortin
PUFA	Polyunsaturated fatty acid
PYY	peptide tyrosine tyrosine (YY)
SBA	Secondary bile acid
SCFA	Short-chain fatty acid
SCN	Suprachiasmatic nucleus of the hypothalamus
Scn10a	Voltage-gated sodium channel Nav1.8 gene
SFA	Saturated fatty acid
SGLT1	Sodium-glucose cotransporter type 1
subWAT	Subcutaneous white adipose tissue
TG	Triglycerides
Th17	T helper 17 cells
Th2	T helper 2
TLR	Toll-like receptor
Treg	T regulatory cells
Val	Valine
VBLOC	vagal blocking for obesity control
VIP	Vasoactive intestinal peptide
VNS	Vagus nerve stimulation therapy
Y2R (or NPY2R)	Neuropeptide Y receptor type 2
ZT	Zeitgeber time
αMSH	α -melanocyte stimulating hormone

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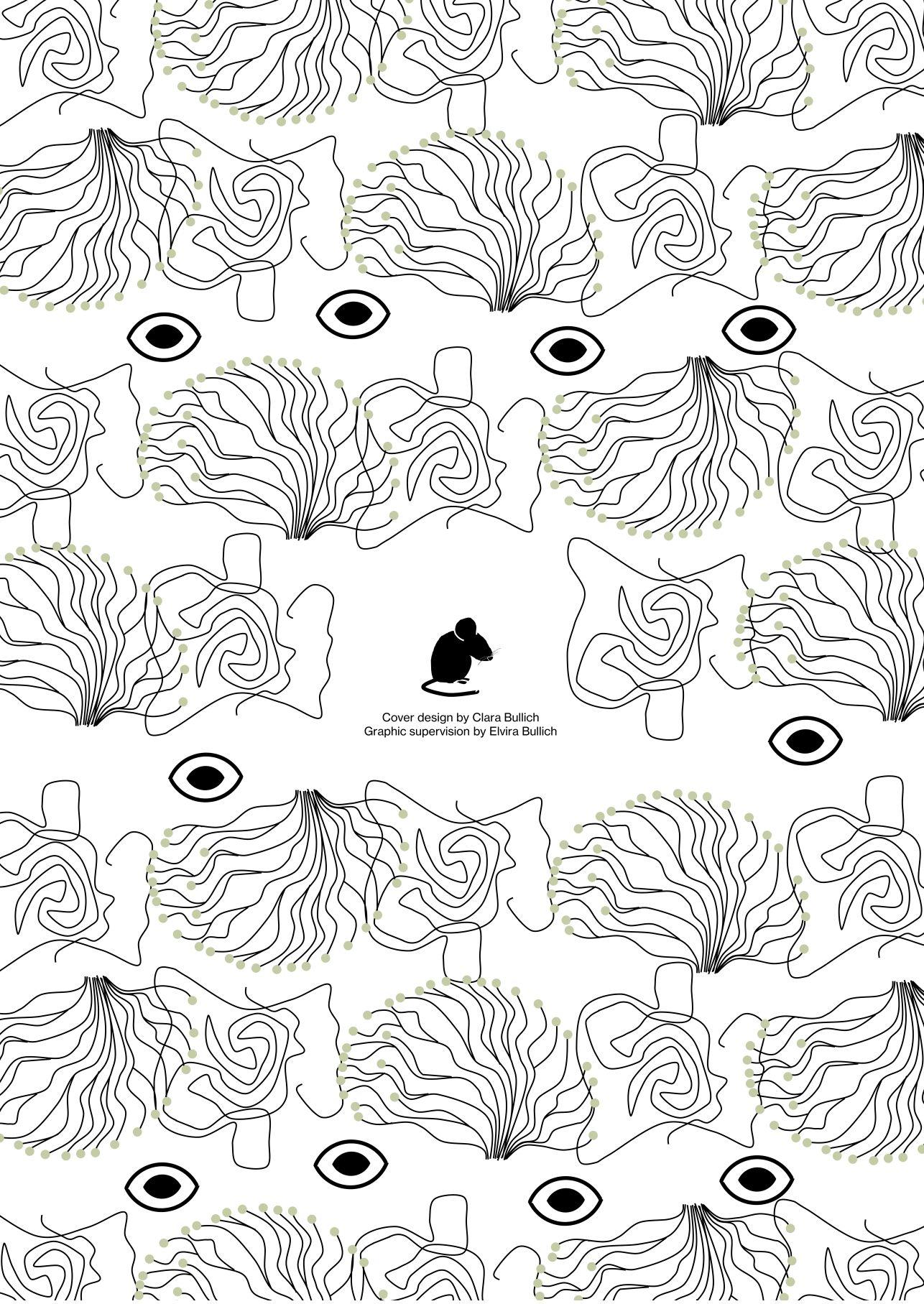
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