SATIATING PROPERTIES OF A GRAPE SEED PROANTHPCYANIDIN EXTRACT

Joan Serrano López

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Satiating properties of a grape seed proanthocyanidin extract

DOCTORAL THESIS
Supervised by Dr. Anna Ardévol Grau

Biochemistry and Biotechnology Department
MoBioFood Research Group

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FAIG CONSTAR que aquest treball, titulat "Satiating properties of a grape seed proanthocyanidin extract", que presenta Joan Serrano López per a l'obtenció del títol de Doctor, ha estat realitzat sota la meva direcció al Departament Bioquímica i Biotecnologia d'aquesta universitat.

I STATE that the present study, entitled "Satiating properties of a grape seed proanthocyanidin extract", presented by Joan Serrano López for the award of the degree of Doctor, has been carried out under my supervision at the Department Bioquímica i Biotecnologia of this university.

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El/s director/s de la tesi doctoral
Doctoral Thesis Supervisor/s

Anna Ardévol Grau

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RESUM

A causa de problemes de salut associats amb el sobrepès i l'obesitat, part de la recerca actual s'ha centrat en el desenvolupament de teràpies que redueixen la ingesta a través de la modulació de certes hormones gastrointestinals amb efectes sobre l'apetit. Prèviarment, el nostre grup de recerca va observar que l'administració d'un extracte de proantocianidines de pinyol de raïm (GSPE) augmenta la producció de l'hormona GLP-1 en rates, la qual s'associa amb una disminució de l'apetit. Aquest fet ens va portar a postular que l'administració d'aquest extracte en rates influiria en la secreció de les principals hormones gastrointestinals, influint així en la ingesta d'aliments.

Els nostres estudis aguts amb rates femella han confirmat un efecte saciant del GSPE amb la mateixa dosi que s'havia descrit anteriorment fer augmentar GLP-1 (1 g/kg). Dosis menors mostren efectes saciants des de 0.35 g/kg, administrant-se abans del primer àpat dels animals. Alhora, hem observat resultats similars en rates mascle i rates sotmeses a una dieta hipercalòrica, altament apetitiva. En tractaments subcrònics de 8 dies consecutius, hem observat que tant la dosi d'1 g/kg com de 0.5 g/kg GSPE mantenen un efecte saciant similar durant tot l'experiment, reduïnt de manera similar el pes corporal i l'absorció de nutrients. En un segon tractament subcrònic la dosi de 0.5 g/kg va mantenir el seu efecte saciant i la reducció de pes corporal, mentre que la dosi d'1 g/kg no va produir cap efecte en absolut. A més, el grup tractat amb 0.5 g/kg GSPE catabolitza més greix i energia que el grup control, un efecte no observat en el grup d'1 g/kg.

De manera aguda, hem observat que l'administració de GSPE incrementa els nivells de GLP-1, així com disminueix els nivells de CCK i augmenta els nivells de ghrelina, una hormona inductora de l'apetit. Contràriament, en estudis subcrònics amb 0.5 o 1 g/kg GSPE la ghrelina es reduí 24 hores després de l'última dosi, tant a nivell plasmàtic com d'expressió gènica. Donada la importància d'aquesta hormona examinàrem els efectes de diferents compostos de GSPE en en un línia cel·lular productora de ghrelina. En aquest model els flavanols monomèrics estimulen la secreció de ghrelina través de la interacció amb receptors de sabor amarg, un fet que podria explicar l'augment de ghrelina en plasma després dels tractaments aguts així com també a curt termini després d'un tractament subcrònic amb GSPE. Per altra banda, el GSPE, les proantocianidines i l'àcid gàl·lic inhibeixen la secreció de ghrelina. Per al GSPE, observem efectes similars al treballar amb segments de teixit intestinal. L'àcid gàl·lic inhibeix la secreció de ghrelina en segments de teixit intestinal i en el plasma de rates immediatament després de l'última dosi d'un estudi de 8 dies. Malgrat aquest fet, els estudis subcrònics d'ingesta amb aquest compost mostren una adaptació gradual i una pèrdua total d'efecte, accentuant la importància de la resta de compostos del GSPE.

Estudiant el paper d'aquestes hormones en l'efecte saciant, hem trobat que els efectes aguts del GSPE i l'àcid gàl·lic són bloquejats per l'administració prèvia d'un antagonista del receptor de GLP-1. Subcrònicament, hem observat que la disminució de la ghrelina i l'augment de la senyalització de GLP-1 a l'hipotàlem estan relacionats amb la disminució d'ingesta en el grup de 0.5 grup g/kg, així com amb l'augment de l'activitat lipolítica en el teixit adipós subcutani. D'altra banda, aquests resultats suggereixen una contra-regulació de les neurones hipotalàmiques anorexigèniques cap a l'excessiva senyalització de GLP-1, el que explicaria la resistència al tractament en el grup d'1 g/kg.

En conclusió, els estudis que presentem en aquesta tesi assenyalen que el GSPE té un efecte saciant en rates sota una dosi adequada a través dels seus efectes sobre les hormones ghrelina i GLP-1.

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ABBREVIATIONS

5HT 5-hydroxytriptamine

ABCB1 ATP-binding cassette B1

AgRP agouti-related protein

Apo apolipoprotein

ARC arcuate nucleus

AVP arginine vasopressin

B0AT1 B0 neutral aminoacid transporter

BAT brown adipose tissue

BDNF brain-derived neurotrophic factor

BMI body mass index

BW body weight

CART cocaine- and amphetamine-regulated transcript

CaSR calcium-sensing receptor

CCK cholecystokinin

CCK1 cholecystokinin A receptor

CCK2 cholecystokinin B receptor

CNS central nervous System

COMT catechol-O-methyltransferase

CPT1 carnitine palmitoyltransferase 1

CRH corticotropin-releasing hormone

Db/db leptin receptor deficient

DMC dorsal motor complex

DMN dorsal motor nucleus

DPP4 diaminopeptidyl peptidase-4

EGCG epigallocatechin-gallate

ENS enteric nervous System

FDA United States Food and Drug administration

FFAR free fatty acid receptor

FOXO1 forkhead box protein O1

GABA gamma-aminobutyric acid

GAL galanin

GH growth hormone

GHRH growth hormone releasing hormone

GHSR growth hormone secretagogue receptor

GLP-1 glucagon-like peptide-1

GLP1R glucagon-like peptide-1 receptor

GOAT ghrelin O-acyltransferase

GPCR G protein-coupled receptor

GRPP glicentin-related pancreatic polypeptide

GSPE grape-seed proanthocyanidin extract

h hours

IP intervening peptide

KATP ATP-sensitive potassium channels

LH lateral hypothalamus

LPAR lysophosphatidic acid receptor

MC1R to MC4R melanocortin receptors

MCH melanin-concentrating hormone

min minutes

MMC migrating motor complex

MPGF major proglucagon fragment

MSH melanocyte-stimulating hormone

NAc nucleus acumbens

NOS nitric oxide synthase

NPY neuriopeptide Y

NT neurotensin

NTS nucleus of the solitary tract

Ob/ob leptin deficient

Ob-Rb long form of the leptin receptor

OLETF Otsuka Long-Evans Tokushima Fatty

OR olfactory receptor

OX orexin

OXT oxytocin

PAM peptidylglycine alpha-amidating monooxygenase

PBN parabrachial nucleus

PC prohormone convertase

PEPT1 peptide transporter 1

POMC proopiomelanocortin

PP pancreatic polypeptide

PVN paraventricular nucleus

PYY peptide YY

RYGB Roux-en-Y gastric bypass

SCFA short chain fatty acid

SGLT1 sodium-glucose linked transporter 1

SIM1 single-minded 1

SREBP2 sterol regulatory element-binding protein 2

STAT3 Signal transducer and activator of transcription 3

T3 triiodothyronine

T4 thyroxine

TASR taste receptors

TRH tyrotropin-releasing hormone

TRP transient receptor potencial channel

UCP uncoupling protein

VTA ventral tegmental area

Y1 to Y6 neuropeptide Y receptors

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INTRODUCTION

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1. OPENING

1.1 A global concern for body weight management

Physical wellbeing relies on the preservation of the body's internal environment despite the changes in the external conditions, a process sustained by a dynamic equilibrium defined as homeostasis by the American physiologist Walter Cannon [1]. Homeostasis is a necessary condition for life, acting at all levels of the living systems by a complex interplay of biochemical and physiological regulatory actions to sustain health [2]. The homeostatic regulation of body weight is a balance between energy income and outcome by controlling food intake and energy expenditure. Thus, imbalances in this system results in a body weight below or above its optimum value, compromising the physical wellbeing [3]. An optimum value of body weight is found between a Body Mass Index (BMI, body weight related to body height) of 20-25kg/m2. Values below and above these boundaries are defined as underweight and overweight and obesity is defined for BMI values above 30kg/m2 [4]. Overweight and obesity are a major risk of health problems, such as cardiovascular diseases (hypertension, heart disease and stroke) type 2 diabetes, osteoarthritis and some cancers such as endometrial, breast and colon cancers [5].

During the last decades, the prevalence of overweight and obesity has dramatically increased in both the developing and developed societies, raising from a 25% prevalence of overweight on 1980 to the actual 35% of world's population [6,7]. This increase coincides perfectly with the prevalence estimations made on 2005, which predicted that by 2030 a 38% of world's population will be overweight and a 20% will be obese [8]. By this means, overweight is actually the 5th leading risk of global deaths, plus being directly related to other major risks such as high blood glucose and cholesterol and high blood pressure [9]. Despite it has been identified a large heritability of obesity, a number as low as only 5% of the morbid obese present a known monogenic cause of obesity [10,11]. It is generally accepted that the main causes of overweight are a high calorie consumption and a low physical activity, two conditions found simultaneously in the industrialized countries due to the low costs of food production and the use of labor-saving technological devices [12,13]. From a strictly biological point of view several hypotheses had been stated to explain why body weight is not properly corrected by the body regulatory mechanisms (i.e. increasing satiety and energy expenditure), as for example it is hypothesized that humans have evolved within multiple ecological and feeding stresses, so that the energy homeostatic system is biased toward weight gain in order to store energy and ensure our survival [3,14,15]. Either way, it is also accepted that overweight is a complex multifactorial problem that includes not just biological aspects but socio-economical, cultural and psychological aspects that may bypass the own body regulatory mechanisms [16–19].

1.2 Focusing on appetite suppression to intervene in body weight

Given that body weight represents the equilibrium between energy intake and energy expenditure, the main advices given to individuals to manage a high body weight are to engage in regular physical activity and to limit the overconsumption of energy-dense food [5]. For those individuals who succeed in weight loss, maintaining the optimum body weight is even a greater challenge [20].

Therefore, complementary strategies are needed to aid weight loss and promote a sustained weight management. These strategies include the blockade of intestinal lipid absorption [21], the increase in the lipid metabolism [22] or the appetite suppression [23]. The main proposed pharmacological and natural treatments to increase energy expenditure involve the activation of the sympathoadrenal system, a strategy that may cause adverse effects such as anemia, inhibition of peristalsis and thrombus formation, but most importantly heart affections such as tachycardia and ischemia [24,25]. Despites this approach cannot be totally ruled out, complementary factors should also be taken into consideration to propose strategies for weight loss. In this sense, it should be an important matter of concern the global rates of undernourishment [26], which ethically compels to develop appetite suppression strategies ahead of nutrient blockade or thermogenic strategies.

1.3 The satiety cascade

The cyclic food intake patterns of humans are regulated by a wide interplay of several factors, involving physiological, psychological and behavioral processes. The sum of this processes, named appetite, reflects the resulting desire to eat food. A main factor influencing appetite is hunger, the conscious sensation that reflex a mental urge to eat, directly determining when and how much to consume. By its side, the satiating power of food is the food capacity to suppress hunger by means of several processes, roughly classified as cephalic, sensorial, cognitive, post-ingestive and post-absorptive. Collectively, the operation of this processes is referred as the satiety cascade. Technically, the conscious sensation opposed to hunger is named satiation during the course of the meal and is named satiety during the inter-meal periods. Thus, satiation brings an eating period to its termination, while satiety determines how long will the inter-meal period take [27].

Before the meal starts, at the cephalic phase, the thought of food and the anticipation of food reward influences appetite and triggers bodily reactions such as salivation, gastric acid secretion or insulin release to prepare the body to the incoming meal. These cephalic events continue in the sensory phase by the sight, smell and taste of food. Thus, the specific responses of the cephalic phase are a result of the previous experiences that occurred in previous meals along all its phases, in a cognitive cycle of liking, learning and wanting. This cognitive processes helps us to choose the type and amount of food that we need and prepare the body to initiate food-specific digestions [28–30].

Appetite is also determined by sensations at a gastrointestinal level. Before a meal, when stomach and intestines are empty, a specific 4-phases contraction pattern emerges in the gastrointestinal tract termed Migrating Motor Complex (MMC). The 3rd phase of MMC is characterized by a short burst of regular high-amplitude contractions that originates either in the stomach or duodenum. These phase-III contractions, believed to clean the stomach from debris, bacteria and secretions, are perceived as a rumbling often referred as hunger pangs, which directly influences the sensation of hunger. By the other side, the increase in the stomach volume during a meal generates the sensation of fullness through the activation of gastric mechanosensitive receptors, being a major determinant in the regulation of satiation [31].

The emptying of the stomach content after a meal plays also a main role in the control of appetite, as a bottleneck event regulating the intestinal exposure of the ingested nutrients and its subsequent

absorption [31]. Once the chime has reached the duodenum, the information originated in the gastrointestinal tract is the major contributor of the satiety cascade. In this post-ingestive phase, the physical characteristics of food, its caloric content and its nutrient composition are detected by intestinal chemo- and mechanosensitive receptors. During this phase, intestinal endocrine cells secrete peptide hormones that contribute to satiation and satiety either entering the circulation to reach the central nervous system (CNS) or acting at the vagus nerve. After this phase, the nutrients absorbed, processed and stored play the central role in the control of appetite. Satiety in the early post-absorptive phase is defined by the plasma increase of the absorbed nutrients and its consequent peripheral and cerebral metabolization, with a main role of the oxidative processes in the liver. By its turn, the amount of energy stored is the main satiety signal once the nutrient availability diminishes, in the late post-absorptive phase, in form of leptin and other adiposity signals [32–35].

1.4 Structure of the introduction

Given the global need for body weight management and the importance of the gut-brain axis in the control of appetite, we hereafter introduce the physiological background controlling appetite at a central and gastrointestinal level and review the developed and developing treatments to influence in this system, emphasizing in the effects of food ingredients and specially in the effects of plant-derived phenolic compounds, a yet understudied field of research.

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2. PHYSIOLOGICAL BACKGROUND OF NEURAL MECHANISMS CONTROLLING APPETITE AND ENERGY BALANCE

The short-term fluctuations in food intake and physical activity compel a tight regulation of energy balance. This regulation controlled by the CNS by adjusting food intake, energy expenditure and energy storage [1]. In the CNS, the hypothalamus is the central core of the homeostatic regulation, controlling food intake and energy expenditure, along with other vital physiological processes such as the control of body temperature, sleep and wakefulness and stress responses [2]. Placed in a privileged position behind the median eminence, where the walls of the blood-brain barrier are defenestrated, the hypothalamus directly senses circulating nutrients, metabolites and hormones reflecting a myriad of parameters such as the energetic status of the body [3,4]. The hypothalamus is the center of a complex neuronal network involving other regions of the brain, such as the cortical areas, the amygdala and other regions involved in emotion, behavior, motivation or memory [5]. Besides the hypothalamus, the brainstem plays a prominent role in homeostatic regulation, mediating the communication between the CNS and the nervous system in the gastrointestinal tract [6].

2.1 Mediation of satiety signaling by the nervous system in the gastrointestinal tract

As introduced before, the mechanisms underlying the shift from the sensation of hunger to the sensation of fullness after a meal encompass changes in gastric distension and gut motility, the detection of absorbed nutrients and the secretion of gut hormones. The peripheral nervous system plays a key role in the transmission of this information, acting as a direct channel of communication between the gastrointestinal system and the CNS.

Despite some sympathetic and parasympathetic neurons directly innervate the gastrointestinal system, many CNS actions in the gastrointestinal tract are mediated through its intrinsic nervous system, named Enteric Nervous System (ENS) [7,8]. The neurons of the ENS constitute the major neural network in the gastrointestinal tract and, besides the cross-talk with the CNS, the ENS is also capable to control the main digestion processes through direct reflexes [9,10]. The nerve cell bodies of the ENS neurons are grouped in aggregates called enteric ganglia, forming neural networks between the longitudinal and circular muscle layers (myenteric plexus) and beyond the muscularis mucosae (submucosal plexus) [11]. The intrinsic sensory neurons of the ENS project their axons from the plexuses to the gastrointestinal mucosa, encoding stretch-sensitive receptors or chemical-sensitive receptors to detect stimuli from the lumen [12,13]. The ENS effector neurons presents a vast number of different excitatory and inhibitory neurons acting on circular muscle, longitudinal muscle, muscularis mucosae, blood vessels and endocrine cells [14–18]. The ganglionated plexuses of the upper small intestine also interconnect with plexuses of ENS neurons in the gallbladder, cystic duct, biliar duct and pancreas, presumably to coordinate its functions with the tubular gastrointestinal tract [19–21]. In sum, by direct reflexes or cross-talk with the CNS, the ENS control enzyme and hormonal secretion and motility functions such as gastric accommodation, peristalsis and the MMC [22–26]. These processes affect the energy balance through nutrient absorption and the food intake cycles by its contributions to the satiety cascade.

Sympathetic afferents of the CNS in the gastrointestinal tract are spinal nerves projecting to the thoracolumbar segments of the spinal cord through prevertebral and paravertebral ganglia, displaying sensitivity to chemical, heat and mechanical stimuli [27,28]. High-threshold afferents are responsible of nociception in the gastrointestinal tract through mechanoreceptors, which sensitivity is influenced by a wide range of chemical mediators of injury and inflammation [29]. When present, these stimuli could activate both conscious and unconscious brain responses through the sympathetic projections of the dorsal horn of spinal cord to the brainstem, thalamus, amygdala and cortex, to associate the aversive stimulus with negative emotional connotations [30,31]. The sympathetic efferent pathways come into action when protective reflexes are activated, which could pass via the CNS or conducted from one part of the gastrointestinal tract to another through prevertebral ganglia. This postanglionar noradrenergic pathway reduces blood flow and intestinal motility directly or through interaction with ENS inhibitory neurons [32]. Then, the sensory and effector actions of the sympathetic nervous system in the gastrointestinal tract are related to noxious events and exert little influence in the regulation of food intake and energy balance in normal conditions. Nevertheless, the ENS intestinofugal neurons use sympathetic pathways to reduce the gastrointestinal motility of a proximal region of the gastrointestinal tract triggered by events taken in a more distal position. By this means, gastric emptying is inhibited when acidic or hyperosmotic conditions affect the duodenal mucosa and intestinal transit is inhibited when undigested nutrients reach the distal small intestine (i.e. ileal brake), both events with a strong influence in the satiety cascade [33–35].

The parasympathetic pathways in the gastrointestinal tract comprise the pelvic and the vagus nerves. The pelvic neurons innervate the distal colon and rectum to control the motility and and initiate defecatory reflexes by stimulating excitatory ENS neurons [36,37]. On the other hand, the vagus nerve innervate the upper gastrointestinal system until the proximal two-thirds of the transverse colon and play major contributions on the satiety cascade by indicating the volume and composition of the ingested food [38,39]. The vagus nerve is a cranial nerve originated in the dorsal motor nucleus of the vagus (DMN) in the brainstem [40]. The cell bodies of the vagal sensory neurons are located in the nodose ganglion and project to the nucleus of the solitary tract in the brainstem (NTS) [40]. Vagal sensory afferents innervate the stomach, liver, pancreas, small intestine and, in less number, the proximal colon [38]. Vagal afferent endings in gastrointestinal ganglia and smooth muscle encode tension and stretch mechanoreceptors that become activated in the physiological range of distension to a bolus of food [41]. The vagal sensory endings beneath the intestinal epithelium react to the chemistry of the lumen indirectly through the mediation of peptides and transmitters secreted by enteroendocrine cells [42,43].

Several gastrointestinal peptides influencing meal size are signaled to the NTS through the vagus nerve, including cholecystokinin (CCK), glucagon-like peptide-1 (GLP-1), apolipoprotein (Apo) A-IV, peptide YY (PYY) and ghrelin [44]. Direct glucose and peptide sensing of vagal afferents in the portal vein and the liver may also detect the amount of glucose and protein absorbed after a meal [45,46]. Together, the stimuli resulting from the ingestion of food activate vagovagal reflexes that regulate the activity of the stomach, intestines, pancreas and gallbladder [47–50]. As reviewed by Chang et al., vagal efferent stimuli are both stimulatory and inhibitory [51]. The vagal excitatory stimuli promote the closure of the pylorus and the MMC in the early prandial phase and the activity

of exocrine and endocrine secretory cells. The vagal inhibitory stimuli promote the gastric accommodation of food in the beginning of the prandial phase, and relax the pyloric sphincter in the late prandial phase. In the intestine, the vagal inhibitory activity slows the progression of the MMC and hence the propulsion of the bolus [51]. Thus, vagal afferents transmit predominantly satiety signals from the gastrointestinal system to the brain and influence this same satiety signals by controlling gastric accommodation, gastric emptying and intestinal motility, ultimately determining the absorption rate of nutrients and the release of pancreatic and gastrointestinal hormones involved in satiation and hunger.

2.2 Main neuronal and chemical factors of the arcuate nuclei of the hypothalamus

Food intake and energy balance are controlled in the CNS via interconnections of several neural clusters in communication through the release of different neurotransmitters. Melanocortins are a family of peptides derived from the common precursor protein pro-opiomelanocortin (POMC) by proteolytic cleavage. POMC is expressed in a subset of neurons of the CNS, giving several peptidic products such as α, β and γ-melanocyte-stimulating hormone (MSH) and adrenocorticotropic hormone (ACTH). In mammals, melanocortins bind in different affinities to five subclasses of melanocortin receptors (MC1R to MC5R), being MC3R and MC4R the most expressed in the CNS. On the other hand, the agouti-related protein (AgRP) is an inverse agonist neuropeptide of MC3R and MC4R, which is expressed by a subset of neurons that typically coexpress neuropeptide Y (NPY) and γ-aminobutyric acid (GABA) [52].

A highly dense cluster of POMC and AgRP-producing neurons is found in the arcuate nucleus (ARC), a ventral zone of the hypothalamus exposed to the bloodstream via the median eminence [3,53]. In this position, the AgRP and POMC neuronal cell bodies sense the homeostatic signals carried by the bloodstream and produce an adequate response that will be delivered to second order neurons to adjust food intake and energy expenditure. The axons of this cells converge in several brain nuclei inside and outside the hypothalamus where MC3R and MC4R are expressed, hence counteracting the action one type of cell with the other [53]. In addition to blocking the action of α-MSH and other melanocortins released to second-order neurons, the AgRP neurons also inhibit POMC neurons directly via GABA release [54,55].

POMC neurons respond to energy abundance by firing α-MSH. Central injection of α-MSH reduces food intake and increases energy expenditure and the genetic ablation of POMC results in an obese phenotype [56,57]. The knockout of MC4R in mice leads to hyperphagia and lower energy expenditure, resulting in an obese phenotype [58]. By its side, knockout of MC3R in mice leads to increased fat mass despite hypophagia due to higher feed efficiency [59]. The central injection of the α-MSH agonist MTII increases the metabolic rate and decreases energy expenditure in wild type mice but is ineffective in MC4R, suggesting a limited role for MC3R [60]. In humans, MC4R mutations are considered the most frequent genetic cause of obesity up to date [61]. POMC neurons in the ARC co-express cocaine- and amphetamine-regulated transcript (CART), which is also expressed in other regions of the hypothalamus [62–64]. Similarly to α-MSH, CART central injections reduce food intake and increase lipid oxidation in rats [65]. This CART-leaded increase in energy expenditure is most probably mediated by the activation of sympathetic preglanglionic

neurons in the spinal cord [66]. Some evidences also suggest different roles of CART in different brain areas, where comes into attention a converse orexigenic effect of CART in discrete brain nuclei [67,68]. Broadly, other effects of CART include a role in reward and cognitive processes [69,70], conditioned place preference or taste aversion [71,72], the mediation of the orexigenic effects of nocioceptin and cannabinoids [73,74], the regulation of the pituitary function [75], and in stress and anxiety behavior [76]. Worth to mention is the fact that CART receptors had not been disclosed yet, which probably hampers the study of CART physiology.

On the contrary to POMC neurons, AgRP neurons respond to energy-deficient situations by firing AgRP and NPY, promoting food intake and reducing energy expenditure. Central injection of AgRP increase food intake and reduce energy expenditure [77]. The ablation of AgRP neurons in adult animals, but not in neonates, lead to hypophagia and weight loss in mice, suggesting that other pathways could compensate for this loss during development [78]. Similarly to AgRP, central injection of NPY also increases food intake and reduces energy expenditure [79,80]. NPY acts on five different receptors (Y1, Y2, Y4, Y5 and Y6) [81]. The administration of Y5 agonists, increases food intake and energy expenditure in mice, while both Y1 and Y5 agonists increases body weight [82,83].

2.3 Homeostatic integration in the arcuate nuclei

A first sensing mechanism of ARC cells is fuel-sensing via its cellular metabolism. In abundance of circulating nutrients, such as glucose, cell metabolism increase resulting in high cytosolic ATP concentrations. In POMC neurons, the rise in the ATP/ADP ratio inactivates the ATP-sensitive potassium (KATP) channels, leading to membrane depolarization and an increased firing of melanocortins [84]. On the other hand, the firing rate of AgRP neurons increases in energy deficient situations, when the cytosolic ATP/AMP ratio decrease. In this situation, the rise in AMP leads to the activation of AMP-activated protein kinase (AMPK), membrane depolarization and subsequent firing, probably via opening P/Q type calcium channels [85]. Since glycolysis and β-oxidation are reciprocally inhibited, glucose concentrations could also promote opposite effects on the activation of both types of cells at a metabolic level. In conditions of energy sufficiency, the levels of malonyl-CoA increase and saves the use of fatty acids as fuel by inhibiting its transport to the mitochondrion via carnitine palmitoyltransferase 1 (CPT1). The increase in ARC malonyl-CoA increases POMC and decreases AgRP neuron activity in mice, decreasing food intake and energy expenditure [86–88].

In addition to circulating nutrients, ARC neurons integrate signals of energy storage via leptin and insulin receptors. Leptin is a peptidic hormone synthetized mainly in the adipose tissue and released in the circulation proportionally to the amount of stored fat and the activity of the adipocytes [89,90]. Circulating leptin is able to cross the blood brain barrier, conveying information of energy storage to the hypothalamus [91]. The administration of leptin to rodents leads to a reduction in food intake and an increase in energy expenditure those results in reduced weight gain [92–94]. Mice lacking the leptin gene (ob/ob) become hyperphagic and obese, but a normal phenotype is restored by leptin supplementation [95,96]. In the ARC, leptin interacts via the long form of leptin receptor (Ob-Rb) to activate POMC cells and inhibit AgRP cells [97]. The knockout of leptin

receptor (db/db) in mice leads to a similar phenotype those ob/ob mice, presenting hyperphagia and a decreased energy expenditure [98].

Insulin is mainly secreted after a meal to promote the absorption of glucose in peripheral tissues and increase its use for energy supply and storage. Despite its pancreatic origin, insulin is also considered an adiposity signal, since its basal and glucose-stimulated levels directly correlate with body adiposity [99]. In accordance to this role, the central administration of insulin reduces food intake in rodents, while brain-specific knock-outs of the insulin receptor induces obesity in mice [100,101].

In the ARC neurons, the activation of insulin and leptin receptors triggers similar effects. On the short term, the activation of leptin receptor leads to a decreased firing of AgRP cells via a membrane hyperpolarization process by kv2.1 channels, and increases firing of POMC cells via membrane depolarizaton by transient receptor potential cation 5 channel (TrpC5) [102–104]. On the long term, insulin and leptin promote changes in the gene expression of neuropeptides via control of its main transcription factors forkhead box protein O1 (FOXO1) and the signal transducer and activator of transcription 3 (STAT3), which produce converse effects in AgRP/NPY and POMC transcriptions. Upon activation of the receptors, the increase in the transcription factor STAT3 supresses AgRP gene expression and activates POMC gene expression, meanwhile the basal FOXO1 activity is reduced to stop the tonic transcription of AgRP and stop the tonic suppression of POMC gene expression [105,106].

2.4 Mediation of anorexigenic signals in the brainstem

Besides of the above-mentioned mechanical and hormone-triggered vago-vagal reflexes influencing gastrointestinal tone and motility, part of the brainstem-mediated actions on food intake are influenced by the hypothalamus and the melanocortin system. In addition to the ARC, POMC-expressing neurons are also found in the NTS, which in mice extend reciprocal connections, mainly via other brainstem centers as raphe magnus and raphe obscurus [107]. By its side, MC4R is found in the NTS, vagal afferents and the DMV [108,109]. A population of ARC POMC neurons also project to the DMV, which contains the higher MC4R density throughout the brain [110,111]. Vagal efferents containing MC4R neurons project its axons to liver, stomach, duodenum and pancreas [112,113].

Some evidences suggest that the activation of the hypothalamic-NTS circuit reduces food intake by amplifying the satiety signals driven by the vagus nerve. POMC cells in the NTS are activated by feeding or intraperitoneal CCK injection in mice, which could be blocked by MC4R antagonists [114]. Microinjection of α-MSH in the NTS reduced the amplitude of phasic contractions and gastric tone, pointing to the reduction of gastric emptying as a main target in the brainstem melanocortinergic system [115]. Furthermore, suppression of food intake produced by leptin administration in the ARC and activation of ARC POMC neurons is blocked by the administration of an MC4R antagonist in the NTS [116].

In addition to vagal efferent actions, the activation of the NTS also drives to satiety by stimulation of other brain centers. The parabrachial nucleus of the pons (PBN) receives information from the NTS

and sends it to the limbic system in amygdala. Both centers are activated in fasting-refeeding experiments in rodents and its stimulation produces satiety in optogenetic and pharmacogenetic experiments, implying neurons of the lateral PBN expressing calcitonin gene-related peptide and neurons of the amygdala central nucleus expressing protein kinase C-δ [117–119]. Since the limbic system is responsible of behavioural and emotional processes, behavioural studies were also performed in mice after stimulation of the amygdala central nucleus, showing no symptoms of discomfort, unpleasantness or malaise, but rather an anxiolytic effect [120].

2.5 The paraventricular nucleus of the hypothalamus as an anorexigenic center

The paraventricular nucleus (PVN) of the hypothalamus expresses MC4R and receives an important number of AgRP and POMC neural projections from the ARC [53,107,121]. Lesioning of PVN or the mechanical disruption of the ARC-PVN circuitry leads to increased food intake and produces obesity in rats [122,123]. Multiple peptidergic neurons coexist in the PVN, containing oxytocin (OXT), arginine vasopressin (AVP), corticotropin-releasing hormone (CRH), thyrotropin-releasing hormone (TRH) or brain-derived neurotrophic factor (BDNF) [124,125]. This heterogeneous neural population expresses the transcription factor single-minded 1 (SIM1), which regulates PVN development [126]. The haploinsufficency of Sim1 results in hyperphagic obesity in mice and humans, and its ablation in adult mice also leads to reduced activity and energy expenditure [127,128]. Altogether, these evidences highlight a role of the PVN as a satiety center in the hypothalamus.

The exact neuroanatomy involving each cell population in the PVN satiety mechanisms remains to be completely solved. Neurons expressing AVP are activated by the MC4R agonist melanotan-II, and its chemiogenetic activation reduces food intake in mice [129]. It was also reported that glutamatergic MC4R-expressing neurons in the PVN that do not express OXT, CRH or AVP are synaptically connected to the PBN and increase food intake after MC4R ablation [130,131]. Similarly, a subset of PVN neurons expressing nitric oxide synthase (Nos1) but not OXT projects to the NTS and PBN and reduce food intake in a comparable extent than Sim1 neurons when activated in mice [132]. BDNF neurons had also been postulated to play a role in the PVN satiety signaling, since its specific ablation in PVN SIM1 cells produces hyperphagia and obesity in mice [125].

Together with other hypothalamic regions downstream of the ARC, mainly the ventromedial hypothalamus, the PVN also modifies energy expenditure by neuro-endocrine and autonomic mechanisms. A subgroup of TRH neurons projects their axons to the median eminence, where the TRH released reaches the pituitary gland to stimulate the synthesis and release of tyroid-stimulating hormone, which in turn will stimulate the synthesis and release of thyroxine (T4) and triiodothyronine (T3). These thyroid hormones increase the metabolic activity of peripheral tissues, notably increasing the sympathetic tone and the expression of uncoupling proteins (UCP) in muscle and brown adipose tissue (BAT), increasing thermogenesis [133]. In the PVN, the gene expression of TRH decreases during fasting and increases after feeding by the regulation of NPY, AgRP, α-MSH and leptin [134]. Similarly to the TRH neurons, CRH neurons of the PVN decrease with fasting and increase with leptin, influencing BAT thermogenesis and adiposity [135–137]. In adult

mice, Sim1 ablation leads to lower BAT temperature, physical activity and energy expenditure before the onset of obesity [128]. BDNF ablation produced similar metabolic effects, which are attributed to act via sympathetic pathway [125].

2.6 The lateral hypothalamus as an orexigenic center

AgRP and POMC neurons of the ARC project to the lateral hypothalamus (LH), often referred as the brain's hunger or feeding center [107]. In rats, the bilateral ablation of these nuclei results in a complete absence of feeding, meanwhile its electrical stimulation promotes overeating [138]. Gross electrical stimulation of this region also produces a reinforced lever-pressing behavior in rats to gain additional stimulation, suggesting that the LH is also involved in reinforcement processes [139]. Several cell types coexist in the LH, characterized by distinct glutamate or GABA neurotransmission, in addition of several neuropeptides, including orexin A (OX), melanin-concentrating hormone (MCH), neurotensin (NT) and galanin (GAL) [140–144]. One characteristic of the LH is its dense connectivity with several brain regions besides the ARC, including the brainstem, the spinal cord, the prefrontal cortex, the mesolimbic system and other hypothalamic regions [145,146]. One of these efferent regions, the ventral tegmental area (VTA), is a region of mesolimbic system interconnected with the nucleus acumbens (NAc), which together are implied in reward and feeding reward [147–149].

MC4R is expressed both OX and MCH neurons [150]. AgRP-increased food intake has been associated with a higher OX neurons activity, with no effect on MCH neurons [151]. Conversely, AgRP administration increases MCH but not OX gene expressions [152]. On the other hand, NPY injections increase OX activity in rats [153]. OX injections or chemogenetic activation of OX neurons increase food intake in rodents [154,155]. OX infusions in the VTA activate dopaminergic neurons projecting to the NAc and promote reward-seeking of food or drugs [156,157]. In the NAc, OX infusions also promote food intake, enhancing wanting of palatable food or hedonic liking [158]. OX also presents notable effects on arousal and energy expenditure. OX neural stimulation increases arousal, physical activity energy expenditure [155]. Consistently, OX deficiency in rodents or humans leads to decreased food intake and energy expenditure and produce narcolepsia [159–162].

Similarly, infusions of MCH increases taste-independent food intake in rodents, but reduce energy expenditure presumably by inhibiting the sympathetic output of the brainstem [163–165]. MCH neurons project predominantly to the NAc, where its administration increases food intake even in sated rats [166]. In contrast to OX, activation of MCH cells promotes REM sleep [167]. A recent review comparing the complementary effects of MCH and OX argued that MCH and OX promote different feeding strategies depending on the wakefulness state of the animal [168]. Altogether, these evidences highlight a role of the LH as a hunger center in the hypothalamus.

2.7 Refferences

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3. PHYSIOLOGICAL BACKGROUND OF ENTEROENDOCRINE MECHANISMS CONTROLLING APPETITE

3.1 Enteroendocrine cells and chemosensation

Throughout the gut epithelium, from the stomach to the rectum, a scattered population of endocrine cells is found [1,2]. This cells, named enteroendocrine cells, mainly respond to meal-related stimuli secreting hormones with several meal-related effects, from the control of gastrointestinal motility and secretions to the control of food intake [3–5]. The distribution of the enteroendocrine cells is very sparse, accounting for approximately 1% of the gut epithelial population, but still is considered that the enteroendocrine system is the largest endocrine system of the body [2,3].

Such as other intestinal epithelial cells, the enteroendocrine cells originate from common pluripotent stem cells and maturate from the intestinal crypts to the villi with a 3-4 days turnover in the small intestine [6]. Enteroendocrine cells are characterized by the presence of secretory vesicles of both large dense-core type and small synaptic-like type [7]. From the secretory vesicles, the stored gut hormones are secreted upon stimulation by exocytosis, which is usually described as a basolateral process into the interstitial space beyond the basolateral membrane [3]. In addition, the morphology of most of the enteroendocrine cells responds to a lumen-sensing function due to the presence of a dense tuft of microvilli projecting to the lumen in its apical side [8]. Beside this lumen-projecting cells, named open-type enteroendocrine cells, a number of closed-type enteroendocrine cells not reaching the lumen also coexist in the gastrointestinal epithelia [3].

Table 1 - Chemosensing machinery in enteroendocrine cells

Receptor/transporter/channel	Acronym	Ligands	Refs.
Umami taste receptor	TAS1R1+TAS1R3	L-amino acids (enhanced by purine nucleotides)	[11–15]
Bitter taste receptors	TAS2R (>20 receptors)	Bitter phytochemicals	[16–20]
Sweet taste receptor	TAS1R2+TAS1R3	Sweet	[11,13,21–23]
Sodium/glucose cotransporter 1	SGLT1	Sodium and glucose	[24–26]
Calcium-sensing receptor	CaSR	Calcium, aromatic aminoacids	[27–30]
G protein-coupled receptor C6A	GPRC6A	Basic aminoacids	[31,32]
Peptide transporter 1	PEPT1	Di/tripeptides	[33–36]
B0 neutral aminoacid transporter	B0AT1	Neutral aminoacids	[37,38]
Lysophosphatidic acid receptor 5	LPAR5 (GPR92/93)	Lysophosphatidic acid and peptone	[39–42]
Glucose-dependent insulinotropic receptor	GPR119	2-oleolylglycerol, oleoylethanolamide	[43–45]
Free fatty acid receptor 1	FFAR1 (GPR40)	LCFA (palmitic, oleic)	[46–48]
Free fatty acid receptor 4	FFAR4 (GPR120)	LCFA (α-linolenic acid)	[49–52]
Free fatty acid receptor 2	FFAR2 (GPR43)	SCFA (butyric acid)	[53–56]
Free fatty acid receptor 3	FFAR3 (GPR41)	SCFA (propanoic acid)	[53,57,58]
Olfactory receptor 51E2	OR51E2	SCFA (propanoic acid)	[59–61]
Olfactory receptor 51E1	OR51E1 (GPR164)	Valeric acids	[62,63]
Transient receptor potential channel A1	TRPA1	Menthol, cinnamaldehyde	[68–70]

The open-type enteroendocrine cells directly detect the luminal nutrient concentrations due to the expression of several chemosensing machinery, similar to the taste-signalling machinery expresed in the taste buds of the tongue. This chemosensing machinery includes taste receptors (TASR), free fatty acid receptors (FFAR) and other G protein-coupled receptors (GPCR), plus transporters and channels (**table 1**) coupled to typical taste transduction mechanisms such as the G protein α-gustducin and the transient receptor potential channel type M5 (TRPM5) [9]. This machinery allows to detect the luminal concentrations of macronutrients, but also gut-derived products or phytochemicals, in order to initiate an adequate hormonal response. For example, glucose-sensing by sodium-glucose linked transporter 1 (SGLT1) triggers in the enteroendocrine cells of the intestine the release the hormone GLP-1, which enhances pancreatic insulin secretion to prepare the body for the incoming glucose load [10].

3.2 Enteroendocrine products and satiety

The study of the gut hormones emerged as early as 1901 with the work of Bayliss and Starling, which discovered that a duodenal chemical messenger, named secretin, stimulated pancreatic secretion via bloodstream and independently of neural mechanisms [71,72]. This concept of a chemical messenger, coined with the term "hormone" by its authors, directly clashed with the state-of-the-art neuronal reflex theory of Pavlov but contributed notably to the dawn of endocrinology [73,74]. Since the discovery of secretin, a large number of gut hormones with different target tissues and effects had been discovered (**table 2**), highlighting the importance of the enteroendocrine system not only by its size, but by its complexity.

Most of the hormones produced by the enteroendocrine cells are small peptides synthetized from precursor proteins named preprohormones. Inactive prohormones are synthetized in the endoplasmatic reticulum by cleavage of their signal peptide and are further processed along their transition in the Golgi apparatus until packed in secretory vesicles as mature hormones. The common post-translational modifications of gut prohormones include cleavage by endopeptidases such as prohormone convertases (PCs), cleavage by exopeptidases such as carboxypeptidases at the carboxyl terminus and amidation at the carboxyl terminus by peptidylglycine α-amidating monooxygenase (PAM) [75]. Usually, different peptides are often released from a single preprohormone, which may present different bioactivities. For example, gastrin peptides of different lengths (gastrin-17, gastrin-34, gastrin-71, gastrin-14 and gastrin-6) are secreted from G-cells, which in turn may be found amidated or not and sulfated or not, while only sulfated gastrin-17 is a considerable agonist of the CCK2 receptor [76,77].

Other group of hormones produced by the enteroendocrine cells are monoamines such as 5-hydroxytriptamine (5HT, serotonin), which is mainly produced by enterocromaffin cells (EC) [78]. In addition to endocrine signaling, autocrine and paracrine signaling has also been proposed for both groups of gut hormones. For example, an autocrine loop has been proposed for histamine production in EC-like cells [79], as well as somatostatin act in a paracrine manner upon parietal cells and G-cells to reduce gastric acid and gastrin secretion [80].

Table 2 - Location and main products of enteroendocrine cells

Hormone	Cells	Main location	Main effects	Refs.
Gastrin	G cell	Stomach	Stimulation of histamine and gastric acid secretion	[101–104]
Histamine	EC-like cell	Stomach	Stimulation of gastric acid secretion	[105–107]
Somatostatin	D cell	Stomach	Inhibition of gastrin release	[108–110]
Ghrelin	P/D1	Stomach	Stimulation of growth hormone release, intestinal motility and appetite	[111–114]
Nesfatin-1	P/D1	Stomach	Satiety factor	[115,116]
Secretin	S cells	Duodenum	pH regulation through bicarbonate secretion	[117–119]
GIP	K cells	Proximal small intestine	Stimulation of glucose-triggered insulin secretion	[120–122]
CCK	I cell	Proximal small intestine	Stimulation of gallbladder contraction and pancreatic secretion, inhibition of gastric emptying and food intake	[123–127]
Motilin	M cell	Small intestine	Initiates the migrating motor complex	[128–131]
5-HT	EC cell	Along the GI tract	Activates peristalsis and intestinal secretions, contributes to satiety	[89,132–135]
Substance P	EC cell	Distal small intestine and colon	Neurotransmitter involved in emetic and nocioceptive processes	[136–142]
Urocortin III	EC cell	Colon	Reduces gastric emptying and food intake	[143–147]
GLP-1	L-cell	Distal small intestine and colon	Stimulates glucose-triggered insulin secretion, reduces gastric emptying and food intake	[148–153]
GLP-2	L-cell	Distal small intestine and colon	Stimulates intestinal epithelial growth	[154–156]
Oxyntomodulin	L-cell	Distal small intestine and colon	Reduces gastric emptying and food intake	[157–161]
PYY	L-cell	Distal small intestine and colon	Reduces gastric emptying and food intake	[149,162–165]
Neurotensin	L-cell (N-cell)	Distal small intestine	Increases colonic motility	[92,166–169]
Insulin-like peptide 5	L-cell	Colon	Stimulates appetite and hepatic glucose production	[170–173]
Guanylin	Paneth, goblet, colonocytes	Distal small intestine and colon	Decreases intestinal fluid absorption, induces natriuresis and lipolysis	[174–180]
Uroguanylin	Tuft cell	Upper small intestine	Decreases intestinal fluid absorption, induces natriuresis and lipolysis, reduces food intake	[176–182]
b-endorphin and Met-enkephalin	Tuft cell	Duodenum	Increase intestinal fluid absorption and reduces gastrointestinal motility	[95–98]
Leptin	Chief and P cells	Stomach	Short-term postprandial satiety	[99,183–186]
Apo A-IV	Enterocytes	Small intestine	Inhibits gastric motility and secretion and reduces food intake	[100,187–189]

Besides the number of endocrine functions of gut hormones, the proximity of the enteroendocrine cells to the gut nervous system also suggested a neuroendocrine action of gut hormones [81], as was also suggested by other physiological evidences. For example, the low plasma concentration and short plasma half-life of GLP-1 has called into question until which extent the GLP-1 effects could be driven in an endocrine blood-mediated manner [82]. Usually, the possible neuroendocrine effects of gut hormones had been studied through capsaicin denervation or by vagotomy, pointing to a role of the vagus nerve in many cases [83]. More recently, the development of new imaging technologies has allowed to unravel an intimate anatomical relationship between the enteroendocrine cells and the gut nervous system and glia via a basolateral axon-like process named neuropod [84–86]. Almost 75% of the hormone-containing vesicles in PYY-producing cells are found in its neuropods, highlighting the importance of the neuroendocrine signaling in the enteroendocrine system [85]. This neuropods comprehend several neuron-like structures, such as neurofilaments, presynaptic and postsynaptic markers and synaptic vesicles, which can be added to other neuron-like processes of the enteroendocrine cells, such as the electrical excitability of the cells and the presence of voltage-gated channels [84–87]. The conjugation of the chemosensing mechanisms of the enteroendocrine cells with the intimate relationship with gut neurons and glia accentuates the complexity of the enteroendocrine system, a concept that has recently been emphasized with the new phrase “gut connectome” [88].

An extra level of complexity can also be stated in reference to the hormone production in enteroendocrine cells. As noted in **table 2**, enteroendocrine cells had traditionally been classified with a single letter acronym in function of the hormone they produced, such as G cells for gastrin production or S cells for secretin production. In this paradigm, some types of enteroendocrine cells are found in specific locations, such as the I cells in the duodenum, while other cells such as the EC cells are found throughout the gut. However, it has become clear that there is a considerable coexpression of hormones within individual cells. For example, EC cells also produce different peptide hormones depending on its location in the gastrointestinal tract, such as gastrin in the stomach, CCK in the duodenum or PYY in the colon [8]. In this sense, it has been recently proposed that the function of chromogranin A, which is coexpressed in all the enteroendocrine cells, act as a monoamine binding protein to facilitate the monoamine secretion in not only the EC cells but all the enteroendocrine cells, which would stress out the phenotypic overlaps between enteroendocrine cells [90]. Other examples include the expression of GIP, glucagon, secretin and neurotensin in I cells, the expression of glucagon and CCK in K cells or the expression of GIP, CCK, secretin and neurotensin in L cells [91–93]. In addition, it has recently been shown using super resolution microscopy that GLP-1 and PYY are stored in different storage vesicles in L cells, which may indicate the existence of different response mechanisms for each stimuli in each type of cell [94].

In addition to the hormonal products of the enteroendocrine cells, some products of other gut epithelial cells are also collected in **table 2** for its endocrine characteristics. For example, tuft cells have not been usually classified as enteroendocrine cells for its absence of secretory granules, but express chemosensory machinery and produce chemical mediators such as POMC-derived opioid peptides which exert remarkable activities such as the inhibition of gastrointestinal motility [95–98]. It is also noticeable the synthesis of leptin in the gastric exocrine chief cells or the synthesis of Apo A-IV in the enterocytes, with both being important satiety factors [99,100].

Satiety is, indeed, the most widespread target between the distinct effects of the gastrointestinal hormones, as can be seen in **table 2**, pointing to a complex fine-tuning mechanism to regulate food intake. From this vast number of hormones, four of them (CCK, GLP-1, PYY and ghrelin) are introduced hereafter for its importance in food intake regulation.

3.3 Cholecystokinin

3.3.1 Disclosure and main functions of CCK

In the 1900s several physiologists started to study the mechanisms underlying bile formation and gallbladder emptying after a meal, finding that duodenal extracts containing the hormone secretin stimulated bile release in some, but not all the experiments [190]. Further study of this matter leaded Ivy and Oldberg to conclude that a yet unknown intestinal hormone was present in some of the previous secretin extracts used, which promoted bile secretion via gallbladder contraction in cats and dogs, naming it cholecystokinin [191]. Similar discrepancies were also found when duodenal extracts containing secretin were used to study the pancreatic secretion of enzymes, finally leading to the conclusion that a yet unknown hormone, named pancreozymin, was the responsible of the pancreatic secretion of digestive enzymes [192]. Despite as early as in 1902 it was suggested that the postprandial secretion of bile and enzymes may be triggered by the same signal, it was not until the late 1960s that became apparent that CCK and pancreozymin were, indeed, the same hormone [72,193]. Together with these digestive functions, CCK contributes to satiation via vagal stimulation and delayed gastric emptying [194–196]. In the CNS CCK acts as a neurotransmitter, which is involved in other actions such as panic and anxiety behaviors [197,198].

3.3.2 Biosynthesis and structure of CCK

Specific immunohistochemistry analyses showed that CCK is produced in enteroendocrine I-type cells of the upper intestinal tract, mainly duodenum, in humans and other mammals [199]. CCK is produced from a 115 amino acids prepropeptide to a series of peptides of different lengths, all sharing the same carboxyl-terminal domain. This domain is characterized by a sulfated tyrosine seven positions before the carboxyl terminus and a carboxyl-terminal amide, which is highly conserved between mammals and partially shared with the gastric hormone gastrin [200,201]. The different length CCK peptides are produced after the proteolytic cleavage of its signal peptide and the tyrosine sulfatation [202,203]. The most prominent CCK forms are CCK58, CCK33, CCK22 and CCK8, which are specifically cleavaged from the propeptide by PC1/3, PC2 or PC5/6 in different amino-terminal positions [204–206]. To produce mature CCK, peptides of any lengths are cleavaged and amidated at its carboxyl terminus [207,208]. The production of different CCK lengths varies between tissues. Besides the intestinal mucosa, CCK is highly expressed in the CNS, which mainly produces short forms as CCK8 [204,209]. In contrast, larger CCK forms as CCK58 are mainly produced in the intestine and so found in the bloodstream [204,210].

When comparing the effects of CCK8 and CCK58 on isolated murine acinar cells, equimolar concentrations elicited similar increases on amylase secretion, whereas in rats only CCK58 presents a sustained stimulation of acinar cells [211,212]. Studies on CCK pharmacokinetics in dogs show that CCK8 presents a shorter half-live than CCK33 or CCK58 [213,214]. It has been argued that the longer half-live of CCK58 over CCK8 may cause the long-lasting stimulation of intestinal afferent nerves observed in rats, despite different pharmacodynamics were not discarded [215]. Regarding food intake, it has been observed that CCK58 and CCK8 are equally potent during the first 30min after the administration, but CCK58 inhibited cumulative food intake for a longer period [216]. Both forms also produced different effects on meal microstructure, with larger first meal latency, intermeal interval and satiety ratio for CCK58 [217,218].

3.3.3 CCK release

CCK is released after meal initiation in a continuous manner, as long as nutrients are present in the duodenal lumen [124]. The ingestion of equal amounts of each macronutrient produce different effects on CCK release. Fat and protein produce similar increases in circulating CCK, while carbohydrates produce no effect [219].

Dietary fat needs to be previously hydrolyzed to produce its CCK-releasing effect, suggesting that an early pancreatic enzyme secretion feedbacks on the subsequent pancreatic exocrine release through CCK [220]. The fatty acid chain length is also important in the stimulation of CCK release, which require fatty acids longer than 10 carbons [221]. In agreement, the LCFA receptors are expressed in I-cells [47,51,222]. In mice, immunohistochemical costaining has also shown the presence of the short chain fatty acid (SCFA) receptor FFAR3 on duodenal CCK-producing cells, which has been proposed to act as a basolateral sensor of the SCFA absorbed in the colon [223].

Unlike dietary fat, dietary protein may not require a previous hydrolization step to produce CCK release, as it was recently observed in the murine enteroendocrine cell line STC-1, human intestinal tissue segments and humans [224–226]. Both in STC-1 cells, rat mucosal cells and humans, different dietary protein hydrolysates are also potent stimulators of CCK release [227–229]. A direct role of the oligopeptide transporter PepT1 was discarded in STC-1 cells and mice I-cells for CCK secretion [34,35]. In STC-1 cells, the lysophosphatidic acid receptor LPA has been shown to play a role in peptone-triggered CCK release [230,231]. Also in STC-1 cells and mice intestinal tissue explants, the individual aminoacids phenylalanine, leucine and glutamic acid stimulate CCK release via the umami receptor T1R1-T1R3 [15]. The aromatic aminoacids phenylalanine and tryptophan are also potent stimulators of CCK release by activating CaSR in I-cells [232–234].

CCK is also secreted in response non-caloric dietary compounds. In STC-1 cells, the bitter tastants denatonium benzoate and phenylthiocarbamide elicit CCK release via activation of T2R [235]. T2R are up-regulated in STC-1 cells and mice by sterol regulatory element-binding protein 2 (SREBP2) to enhance the CCK release under low sterol conditions such as vegetable-rich diets. This leaded to postulate that CCK may act to protect the individual by potentially toxic bitter substances via delayed gastric emptying and absorption [236]. In mice, the CCK increase produced by phenylthiocarbamide increase the activity of ATP-binding cassette B1 (ABCB1) in the enterocytes,

which limits the absorption of toxic substances by increasing its efflux to the intestinal lumen [20]. In humans, intraduodenal quinine administration increases CCK and decreases food intake and gastric emptying, despite it was also observed in rodents that the satiating effects of bitter tastants may be CCK-independent [237,238].

3.3.4 Mechanisms of action of CCK

CCK binds to two G-protein coupled receptors, named CCK1 and CCK2. CCK1 binds sulfated CCK with 500-1000 times more affinity than sulfated gastrin or non-sulfated CCK, whereas CCK2 binds with similar affinity to CCK and gastrin and do not discriminate between sulfated and non-sulfated forms [239,240]. CCK1 is predominantly expressed in gastrointestinal tissues and the vagus nerve, meanwhile CCK2 is predominantly expressed in the CNS, despite also found in the gastrointestinal system [241,242].

In the stomach, CCK1 is found in D-cells to increase somatostatin secretion, while CCK2 is found in parietal cells and enterochromaffin-like cells to regulate acid secretion [243–246]. In the pyloric sphincter and duodenum, CCK1 is expressed in the smooth muscle and its activation leads to reduce gastric emptying and duodenal motility [243,247,248]. In pancreas, hormonal and enzyme secretion are mediated by CCK1 in endocrine cells and CCK2 in exocrine pancreas [241,249–251]. In addition, pancreatic δ-cells also express CCK2 to promote the release of somatostatin [252,253]. The cholecystokinetic effects of CCK may be directly linked to the expression of CCK1 in the gallbladder muscle [254,255].

The expression of both receptors has been observed in the vagus nerve and nodose ganglia, with higher abundance of CCK1 [256–259]. Since vagal afferents project into the duodenal mucosa in close proximity to CCK-producing cells, it has been argued that the CCK stimulation of the vagus nerve plays an important role mediating the effects of CCK [260]. In isolated human acinar cells neither CCK nor gastrin trigger the secretion of amylase, and CCK-stimulated amylase release appears to be dependent on vagal cholinergic stimulation of exocrine pancreas [261–263].

The inhibition in gastric motility evoked by CCK is also dependent of vagal stimulation in rats [264–266]. In agreement with the higher abundance of CCK1 in vagal afferents, the administration of CCK1 antagonists suppress the satiating effects of CCK or intestinal nutrients, while CCK2 antagonists does not [267,268]. This CCK1-dependent activation of vagal afferents produces satiation by acting on neurons of the NTS [269–271]. It is noteworthy that Otsuka Long-Evans Tokushima Fatty (OLETF) rats, which lack CCK1, are hyperphagic and obese and lack a normal satiating response to intestinal nutrients [272,273]. In addition, both vagotomy and capsaicin deafferentation abolish the satiating effect of CCK in rats, despite a slight inhibition of food intake could be observed for higher CCK doses [274–276]. This fact may suggest that the satiating effects of CCK are not solely mediated by the vagus nerve.

CCK1 and CCK2 are expressed widely in the CNS, where CCK8 is also produced postprandially, and infusions of CCK in certain CNS areas reduce food intake in rats [242,277–281]. The peripheral administration of CCK1 antagonists in vagotomized rats do not affect food intake if the antagonist cannot cross the blood-brain barrier, but increase food intake per se if is able to reach the CNS

[282,283]. It has been also found that CCK1 is also expressed in varicosities of the vagus nerve and increase intracellular calcium upon activation, presumably increasing the firing rate to the NTS [284]. This mechanism may also play a role in the above-mentioned studies, since it has been shown that the vagal afferent cell bodies survive to vagotomy [285]. In mice, a group of CCK-expressing cells has been found in NTS, which is activated after food intake and triggers meal termination via axonal projections to the PVN [286]. Other studies in mice show that a NTS population of CCK-expressing cells project to CGRP neurons of the PBN, reducing food intake once stimulated [287]. Whether CCK neurons in the NTS specifically respond to CCK stimulation of vagal afferents or to broader vagal stimulation has not been specifically studied.

3.4 Glucagon-like peptide 1

3.4.1 Disclosure and main functions of GLP-1

The existence of glucagon-like peptides has been largely suspected due to the glucagon-like hyperglycemic activity of certain intestinal extracts and the glucagon-like immunoreactivity of peptides larger than glucagon in both pancreas and intestine [288–293]. After these studies, the identification of the preproglucagon transcript in the anglerfish allowed its molecular cloning in several mammals, including humans, which showed that the same glucagon transcript is expressed in both pancreas and intestine in the form of a polyprotein precursor containing the sequences of glucagon and two similar peptides, named GLP-1 and GLP-2 [294–301]. The similarity of the GLP-1 and GIP sequences raised the speculation that GLP-1 and GIP may also have similar biological activities [296]. GIP was previously known to be secreted from the duodenum after an oral glucose load in order to stimulate insulin secretion, an action known as the incretin effect [120,302–305]. The possibility that GIP may not be the only incretin hormone arose after the finding that GIP depletion did not completely abolish the incretin effect [306–308]. As well, in patients with intestinal resections, the ileum, but not the duodenum, showed essential for the incretin effect, highlighting the importance of other incretin hormones acting in concert with GIP [309]. In agreement with these precedents, the bioactive forms of GLP-1 were shown to be potent insulinotropic agents in pancreas of rats and pigs and finally in humans [310–312]. This feature of GLP-1 as an incretin hormone has allowed the development of current drugs to treat type 2 diabetes mellitus, which comprise GLP-1 mimetics and inhibitors of the degradation of endogenous GLP-1 [313–316]. Beside this function, GLP-1 is also an inhibitor of gastric emptying and a satiety factor, which has been argued to be the major effects of GLP-1 under physiological conditions [152,317–319].

3.4.2 Biosynthesis and structure of GLP-1

Besides the pancreatic a-cells, proglucagon is also expressed in the L-cells of the intestine and certain areas of the CNS, especially the brainstem. This precursor is differently processed in each tissue to produce different sets of hormones [320–322]. In the pancreas, the 160 residues preproglucagon is cleaved in three lysine-arginine sites by PC2, giving rise to the glicentin-related

pancreatic peptide (GRPP), glucagon, the intervening peptide (IP) 1 and the major proglucagon fragment (MPGF), which is formed by the sequences of GLP-1, IP2 and GLP-2 [323,324]. On the other hand, in the L-cells and the CNS preproglucagon is cleaved in single or double arginine sites by PC1/3, giving rise to glicentin, GLP-1, IP2 and GLP-2. The glicentin protein is formed by the sequences of GRPP, glucagon and IP1, which could also be cleaved to form GRPP and an extended glucagon fragment with the sequence of IP1, named oxyntomodulin [325,326].

The resulting 37 residues GLP-1, GLP-1(1-37), is further cleaved in a single arginine site to give GLP-1(7-37) [311,325]. Both forms of GLP-1 may be amidated in its carboxyl terminus, where a substitution at the terminal glycine gives GLP-1(1-36)amide and GLP-1(7-36)amide [327,328]. In turn, these peptides are further cleaved in its amino terminus once released by the enzyme diaminopeptidyl peptidase-4 (DPP-4), forming GLP-1(9-37) and GLP-1(9-36) amide [329–331]. These GLP-1 forms are not equally released or found in plasma. GLP-1 is mostly processed to GLP-1(7-36)amide, which represents the major secreted form in humans [327]. Even though, this molecule have a plasma half-life of less than 2 minutes due to the action of DPP-4, being its product GLP-1(9-36)amide the major form found in plasma [332–334].

It is generally accepted that the longer and shorter forms of GLP-1 are not biologically active and that GLP-1(7-37) and GLP-1(7-36) amide are the most bioactive forms. When studying the insulinotropic actions of GLP-1, GLP-1(1-37) failed to modify insulin or glucose plasmatic levels in fasting rabbits, while GLP-1(1-36) amide showed a mild insulinotropic effect in rat pancreatic islets [335,336]. Further studies showed that the amino-terminal cleavage to GLP-1(7-37) is required to stimulate insulin release [310,337]. The amidated form GLP-1(7-36) amide presents the same insulinotropic activity than GLP-1(7-37), but a higher stability in human plasma [338]. Like the extended forms of GLP-1, the truncated products resulting of DPP-4 activity lack any insulinotropic activity [339].

In line with the insulinotropic activity, GLP-1(7-37) and GLP-1(7-36 amide are equally bioactive reducing gastrointestinal motility in humans [340]. These effects were mostly studied with GLP-1(7-36) amide, which produce a dose-response inhibition of the antro-pyloro-duodenal motility and gastric emptying, influencing postprandial glycaemia by reducing the rate of nutrients reaching the intestine [341–343]. It is also well stablished that GLP-1(7-36)amide is an active GLP-1 form reducing food intake, both in lean, overweight or diabetic humans [344–348].

On the other hand, cumulative evidences point out that the shorter DPP-4-degraded forms of GLP-1 may exert an insulin-like activity, reducing hepatic glucose production and producing protective effects in vascular and cardiac tissues [349–353]. Recent evidences also point out that even shorter degradation products of GLP-1, such as GLP-1(28-36)amide or GLP-1(32-36)amide, directly target the mitochondria and reduce ROS levels, which could be linked to the observed reduction of hepatic glucose production and increase in β-cell mass and proliferation observed for this molecules [354–359].

3.4.3 GLP-1 release

GLP-1 is rather rapidly released after nutrient ingestion, with a first major peak of secretion 15-30min after the meal, often followed by a plateau or a second minor peak occurring 60-120min thereafter [360–364].

Glucose is a main factor stimulating GLP-1 release. In immortalized L-cells and L-cell primary cultures, glucose is sensed via its internalization through SGLT-1, leading to changes in membrane potential and the exocytosis of GLP-1-containing vesicles due to the co-internalization of sodium ions [365,366]. In murine models, the depletion of sodium chloride in the lumen or the co-administration of a SGLT-1 inhibitor with glucose blocks the glucose induction of GLP-1 secretion [367,368].

Fructose, which is not a substrate of SGLT-1, also promotes GLP-1 secretion in immortalized L-cells, murine models and humans by an increase in ATP and the closure of ATP-sensitive channels, pointing that the carbohydrate catabolism in the L-cells may also play a role in GLP-1 release [365,369]. However, comparative studies point out that glucose transport through SGLT-1 is more important than facilitative glucose transport in the carbohydrate-mediated GLP-1 release [370,371]. The role of sweet taste receptors in GLP-1 release is more controversial. In healthy volunteers, glucose-induced GLP-1 release is impaired by the administration of the T1R2/T1R3 antagonist lactisole, but artificial sweeteners failed to induce GLP-1 release in humans, rats or mice [367,372–374]. On the other hand, the glucose-induced GLP-1 release is impaired in α-gustducin or T1R3 knockout mice [23,375]. This inconsistency may be explained by the finding that SGLT-1 expression is up-regulated by the sweet taste receptor activation [13].

Fatty acids are also important factors stimulating GLP-1 release. GPR40 agonists stimulate GLP-1 secretion in rodents and human primary L-cells, which has been recently found to be a basolateral, but not luminal mechanism in the rat intestine [65,376–379]. GLP-1 secretion is also stimulated by GPR120 agonists in STC-1 cells and rodents [49,380]. The activation of GPR119 also produce GLP-1 release in L-cell models and rats, and the administration of GPR119 agonists has been shown to effectively increase GLP-1 levels in humans [45,381,382]. The SCFA produced by the microbiota also stimulate GLP-1 secretion via activation of GPR41 and GPR43 and the administration of propionate leads to increased plasma GLP-1 in mice, rats and humans [383–385].

In isolated L-cells and mice, oligopeptides also stimulate GLP-1 release due to the activation of CaR and PEPT1, in line with evidences of increased GLP-1 in humans under a high-protein diet [41,386,387]. Several individual aminoacids are strong activators of GLP-1 release murine L-cells, highlighting glutamine, which promotes a stronger GLP-1 release than glucose and effective increases in plasma GLP-1 when administered to humans [388–392].

Non-caloric dietary molecules may also stimulate GLP-1 release. Several bitter tastants such as PTC, momordicoside, quinine, DB, berberine, 10-phenanthroline or ginsenosides promote GLP-1 secretion in immortalized L-cells or mice [393–399]. Biliary acids also stimulate GLP-1 release in cellular and murine models via TGR5, which has been shown to be a basolateral mechanism in the rat perfused intestine [66,400–402]. Both deoxycholic and taurocholate rectal infusions effectively increase plasma GLP-1 in humans [403,404].

The fact that GLP-1 producing cells are mostly located in the terminal ileum and colon has brought into question whether a direct interaction of the above-mentioned dietary molecules or digestion products may be responsible of the rapid rise in plasma GLP-1 after a meal. It was observed in rats that a GIP plasma peak precedes the increase in plasma GLP-1 after a duodenal fat treatment and that an administration of the same GIP concentration leads to an increase in plasma GLP-1 [405]. The effects of GIP on GLP-1 were confirmed in rats and primary canine L-cells, but physiological doses of GIP failed to increase GLP-1 in healthy or diabetic humans [406–408]. The infusion of fat in a ligated or transected duodenal region in rats leads to an increase in GLP-1 which is completely abolished in vagotomized rats. Vagotomy also abolishes the GIP-induced increase of GLP-1 in rats [409]. Also in rats, the duodenal or ileal administration of a protein hydrolysate increases GLP-1 secretion and capsaicin treatment of the vagus abolished the effects of the duodenal, but not ileal administration [410].

Muscarinic agonists have also been shown to stimulate GLP-1 secretion in STC-1 cells and rats [406,411]. In pigs, in which as in humans GIP produces no effect on GLP-1 release, GLP-1 is increased by muscarinic stimulation and not by vagal stimulation, pointing out that the ENS circuitry may be involved in the stimulation of the L-cells [412]. In addition to this, it should be remarked that a small number of GLP-1-producing cells is also found in the upper intestine of mice and humans, which could also play a role in the rapid rise of GLP-1 after a meal [91,413,414].

3.4.4 Mechanisms of action of GLP-1

The biological activity of GLP-1(7-37) and GLP-1(7-36) amide is explained by its interaction with the GLP-1 receptor (GLP1R). The longer forms of GLP-1, GLP-1(1-37) and GLP-1(1-36) amide are only partial agonists of GLP1R, while GLP-1(7-37) and GLP-1(7-36) amide are potent full agonists of GLP1R [415]. After the cleavage by DPP-4, the remaining GLP-1(9-36) amide loses almost all its binding affinity to GLP1R, becoming a weak partial agonist of GLP1R [416].

GLP1R is expressed in pancreatic b-cells coupled to adenylate cyclase and its activation leads to increased cAMP, closure of KATP channels and a subsequent membrane depolarization, calcium influx and exocytosis of insulin vesicles [417–419]. Furthermore, the activation of GLP1R in b-cells is also followed by the activation of other transduction pathways that lead to the regulation of gene transcription, cell growth and differentiation [420–424].

GLP1R is also expressed in vagus afferents in rats. The administration of GLP-1 stimulates the afferent activity of the vagus, which could be blocked by the co-administration of the GLP-1R antagonist exendin9 [425]. In rats treated with exendin9 or with vagus deafferentiation, such as in vagotomized humans, the administration of GLP-1 do not produce the expected reduction in gastric emptying, highlighting the role of the vagus-expressed GLP1R mediating this effect [426,427].

In the dorsal motor complex (DMC), vagal afferents activate a group of GLP-1-expressing neurons which project to the hypothalamus and the spinal and vagal motor nuclei, areas where GLP-1R is also expressed [428,429]. In rats, the administration of GLP-1 or the GLP-1R agonist exendin4 in the DMC activates NOS-positive vagal motor fibers innervating the stomach diminishing gastric tone, an effect which could be blocked by the co-administration of exendin9 [430].

The intraperitoneal administration of exendin4 in rats also activates neurons in the DMC, together with myenteric and submucosal ENS neurons in the upper intestine, an effect which is also blocked by exendin9 [431]. It has been recently found in mice that ENS neurons expressing GLP-1R and functionally responding to GLP-1 densely innervate the duodenum and stomach myenteric plexuses, especially in the pyloric region, being mostly NOS-positive neurons [432]. Treatment of mice duodenal muscle strips with GLP-1 affected circular, but not longitudinal, contractility in a NOS-dependent manner which could be also blocked by exendin-9, pointing to the ENS as a mediator of the effects of GLP-1 [433,434].

In rodents' CNS, GLP-1 is mainly expressed in the NTS, while GLP-1R is broadly expressed in regions including the hypothalamus, limbic system, cortex and brainstem regions such as the spinal and vagal motor neurons [432,435,436]. Intracerebrovesicular injections of GLP-1 diminish food intake in sated rats, an effect blocked by the co-administration of exendin9, pointing out that GLP-1 signaling in the CNS is an important factor in the central regulation of food intake [437]. The specific injection of GLP-1 in the ARC do not modify food intake, but regulate aspects of glucose metabolism such as an effective reduction of hepatic gluconeogenesis in rats [438]. Specific injections of GLP-1 have shown that GLP-1 signaling reduces food intake when acting in other brain areas such as the PVN the VMH and the NAc [439–441]. Some GLP-1-expressing neurons in the NTS also project directly to the PBN, where activate CGRP neurons to reduce food intake [442,443].

The short plasma half-life of active GLP-1 has brought into study how the L-cell products may stimulate this brain centers. In vagotomized humans, GLP-1 administration does not produce the expected reduction of food intake, pointing that this effect may be mediated from the L-cells to the hypothalamus via vagal afferents [427]. Intraperitoneal GLP-1 also fails to diminish food intake in vagotomized rats, but not in sham rats or rats with selective ablation of the common hepatic branch of the vagus [444]. On the contrary, GLP-1 administration reduces food intake on the subsequent meal when administered in the portal vein in vagotomized rats, suggesting a central effect under venous administration [445]. Studies with exendin4 or the long-lasting GLP-1R agonist liraglutide in vagotomized rats show that the vagus nerve is essential for the short-term satiating effects of GLP-1R activation, but not in the long-term, where the CNS GLP-1R may take part [446]. Site-specific knockouts of GLP-1R in mice also shown that GLP-1R expression in the vagus is essential for the short-term effects on food intake, while GLP-1R expression in the CNS is essential for the long-term satiating effects of liraglutide [447]. Altogether, the evidences suggest that in physiological conditions GLP-1 stimulates vagal afferents, which would pass the information through the CNS via glucagon-expressing neurons among other pathways from the NTS. In this sense, both transection of the vagus nerve or the transection of GLP-1 expressing neurons from the brainstem to the forebrain effectively prevents the expected reduction of food intake produced by the administration of intraperitoneal GLP-1 in rats [448].

3.5 Peptide YY

3.5.1 Disclosure and main functions of PYY

PYY was first discovered in porcine secretin preparations in the search of unidentified gut peptides containing the common C-terminal amide modification [449,450]. Posterior immunohistochemistry studies identified PYY in the distal intestine of several species, including humans, where is co-expressed with GLP-1 [451–454].

Preceding hindgut peptide preparations from cat, rat and pig mucosa exhibited an inhibitory activity of pancreatic exocrine secretion, in accordance with the effects of a hindgut humoral factor released by intraileal administration of oleate [455–460]. The biological activity of PYY resembled to the effects of this preceding hindgut factor, named pancreotone or anti-colecystokinin, inhibiting the pancreatic exocrine secretion in cats, dogs and rats [450,461–463].

In other studies, PYY reduced the intestinal motility in cats and the interdigestive stomach contractions in conscious dogs [451,464]. In agreement with these effects, PYY administration reduces gastric emptying and mouth-to-caecum transit time in humans [163,465].

PYY administration also reduces food intake in rodents and in lean or obese humans [466–469]. Despite it has been brought into question whether this satiating effect of PYY is clearly physiological, later studies has shown that effective and non-effective doses of GLP-1 and PYY act in an additive manner to reduce food intake in rodents and humans, which is interpreted as a physiological satiating effect of PYY [470–472].

3.5.2 Biosynthesis and structure of PYY

The structure of PYY is related to pancreatic polypeptide (PP) and NPY, with the same 36 amino-acids length and a significant sequence homology, which produce a similar hairpin structure known as the PP-fold [450,473–475].

PYY is synthetized as a large precursor molecule in the endoplasmatic reticulum and it has been proposed that its processing involves a dibasic prohormone convertase, a carboxypeptidase and an amidating enzyme to produce the mature PYY(1-36)amide, similarly to other intestinal hormones or its homolog NPY [476,477]. Like GLP-1, the secreted PYY is also digested by DPP-4, producing PYY(3-36)amide [478,479]. In human plasma, both PYY forms are similarly abundant, with PYY1-36 accounting for a 63% of total PYY in the fasted state and for a 46% in the fed state [480]. This two forms of PYY present different biological activities. In isolated perfused rat panceas, only PYY1-36 inhibits exocrine pancreatic secretion [481]. Also in rats, both forms reduce gastric emptying, but PYY3-36 is an order of magnitude more potent than PYY1-36 [482]. Similarly, in humans, both forms of PYY reduce gastric emptying but PYY3-36 is the most active form [483].

Regarding food intake, the two forms of PYY also produce converse effects. The precursor molecule PYY1-36 acts as an orexigenic molecule when its centrally administered in rats [484,485]. In rats, rabbits and humans, PYY3-36 acts as an anorexigenic molecule, hence being activated by DPP4 conversely to GLP-1 [466,468,471,486].

3.5.3 PYY release

PYY is also released in response to nutrient ingestion. In humans, plasma PYY steadily rises from approximately 30min after the meal to reach a plateau 120min thereafter, which concentration stands for several hours [453]. Few PYY-expressing cells are expressed in the upper intestine. In mice, only a fifth of the GLP-1-expressing cells in the duodenum co-express PYY, which could explain why PYY plasma levels rise later than GLP-1 [91].

In human colonic primary cultures, no GLP-1-specific or PYY-specific cells are found and both peptides are released from the same secretory vesicles in response to the same stimuli [65]. Hence, like GLP-1, PYY is released in response to glucose, in humans [372,487]; SCFA, in rats [488]; LCFA, in humans [489]; FFAR4 agonists, in cells [490]; intracolonic administration of aminoacids, in dogs [491]; bitter agonists, in cells and mice colonic cripts [395,492]; and bile acids, in cells [493].

Similar to GLP-1 release, foregut signaling also plays a role in PYY release. In dogs the complete interruption of the duodenal chime does not prevent PYY release, but the administration of the CCK antagonist devazepide block PYY release [494–496]. Studies in rats intestines and dogs has shown that direct vagal stimulation and cholinergic agonists stimulate PYY release, while atropine blocks the PYY-stimulating effect of intraduodenal fat, pointing that neural stimuli from the foregut also plays a role in early PYY release [497–499].

3.5.4 Mechanisms of action of PYY

The structural relationship between PYY with PP and NPY links the biological activity of PYY to Y receptors. Y1 and Y5 are specific receptors for NPY and PYY 1-36, presenting much lower affinities for PP and C-terminal peptides such as PYY3-36 [500–503]. Y2, on the other hand, binds to the C-terminus of these peptides, with similar affinities to NPY, PYY1-36 and PYY3-36. Hence, PYY3-36 is considered to be selective to Y2, in relation to Y1 [503–505]. On the other hand, PP is selective to Y4, which presents lower affinities to the other peptides [506–508].

These affinities and the specific expression of Y receptors on each tissue determine the biological activities of PYY1-36 and PYY3-36. In preliminary studies performed directly on isolated perfused rat pancreas, only PYY1-36 inhibited CCK-induced exocrine secretion, discarding PYY3-36 and Y2 as mediators of this effect [481]. However, later studies in conscious rats showed converse results, which was interpreted as a possible brainstem-mediated effect via Y2 [509].

In rats and humans, both peptides inhibit gastric emptying, but with a more potent action of PYY3-36. Since both peptides equally bind to Y2, these results may imply that PYY1-36 partially counteracts its effects on gastric emptying by converse effects via Y1 or Y5 receptors [482,483]. In this sense, it has been shown that circulating PYY binds to the DMC in rats, where Y2 activation inhibits gastric emptying and Y1 activation stimulates gastric emptying [510,511]. The role of DMC and descending motor vagus in the PYY-driven effects on gastric emptying were also confirmed by vagotomy experiments in rats [512].

The different effects of PYY1-36 and PYY3-36 on food intake are also explained by its different actions on Y receptors. In rats, the central administration of PYY1-36 produces potent orexigenic

effects [484,485]. These effects seem to be mediated by Y1, since in mice even a weak Y1 agonist as PYY3-36 may induce food intake after a central injection, but this effect is abolished in Y1 knockout mice [513]. Further studies with electrophysiological slice preparations has also shown that PYY3-36 potently inhibit POMC neurons via Y2, adding a level of complexity to hypothetical ARC-mediated anorexigenic effects of PYY3-36 [514]. Other studies had previously shown that centrally injected PYY3-36 do inhibit food intake in fasted rats, which could be influenced by the fasting condition [466,514].

Either way, the effects of peripheral PYY3-36 in rodents are clearly anorexigenic and Y2-dependent, as it has been shown by using Y2 knockout mice and Y2 antagonists [466,515]. Y2 is expressed in vagal afferents of rats, where is upregulated by CCK, and the effects of peripheral PYY3-36 on food intake may also imply vagal afferents [516]. In rats, vagotomy completely blocks the anorexigenic effects of PYY3-36 [517]. Also in rats, the administration of PYY3-36 activates neurons of the NTS and APY via vagal afferents, besides an activation of neurons in the ARC [518]. It has also been shown in rats that PYY is also synthetized in the nodose ganglia and that PYY-induced ARC activation is abolished after the transection of ascending fibers of the NTS, suggesting that part of the ARC activation and the anorexic effects of PYY are directly drived by vagal stimulation [517].

3.6 Ghrelin

3.6.1 Disclosure and main functions of ghrelin

Ghrelin was discovered in extracts of rat stomach when looking for endogenous ligands of the growth hormone (GH) secretagogue receptor (GHSR), for whom non-endogenous ligands were known at that time [519]. In pituitary cells and rats, ghrelin specifically increases growth hormone secretion with no effects on other pituitary hormones [519,520]. The human orthologue of this hormone only differs in two residues with rat ghrelin and also increase GH in humans after venous administration [519,521,522].

The fact that ghrelin acts on the pituitary from a distant synthesis on the stomach in place of from the nearby hypothalamus leaded to investigate a possible role of ghrelin in food intake. It was thereby found that, in rodents, ghrelin serum levels increase after fasting and decrease after refeeding and that central administration of ghrelin increases food intake in a dose-dependent manner. Besides, peripheral ghrelin administration increase body weight gain due to decreased energy expenditure in a GH-independent fashion, pointing to ghrelin as a signal to increase metabolic efficiency in energy-deficient situations [523–527]. Similarly, in humans, ghrelin plasma levels rise before a standard meal or after fasting and decrease after a standard meal or after refeeding, suggesting a role in meal initiation [528–530].

In parallel to its discovery as a GHSR ligand, ghrelin was also reported as a new gastric peptide with sequence similarity to motilin, hence named motilin-related peptide [531]. Similarly to motilin, ghrelin also affects gastrointestinal motility enhancing gastric emptying in rodents and humans [532–535]. By the same token, high fasting plasma ghrelin levels is correlated with a higher gastric emptying rate in healthy humans [536].

3.6.2 Biosynthesis and structure of ghrelin

Ghrelin is mainly synthetized in enteroendocrine cells of the oxyntic mucosa, named P or D1 cells in humans and X or A-like cells in other mammals [111,519,537,538]. This type of cells represents about a 20% of the enteroendocrine cell population in the human or rat oxyntic mucosa [537,538]. In addition, ghrelin is also synthetized in enteroendocrine cells along the intestinal mucosa, from the duodenum to the colon, with a decreasing cell density towards the rectum [537–539]. Morphologically, ghrelin-producing cells are closed-type cells in the stomach, but an increasing cell density of open-type cells is found towards the rectum, with up to 80% of colonic ghrelin cells being open-type cells [539]. Ghrelin synthesis is also extended in other locations beyond the gastrointestinal tract. In the pancreas ghrelin is synthetized in islet epsilon cells [540,541], while in the hypothalamus ghrelin is expressed in neurons of the ARC and in a group of neurons adjacent to the third ventricle [542–544].

Such as other peptide hormones, mature ghrelin is generated from a larger prepropeptide via posttranslational modifications. Preproghrelin is a 117 amino-acid peptide in humans and rodents, and the excision of the signal peptide in the endoplasmic reticulum generates a 94 amino acid proghrelin to enter the Golgi apparatus [519]. Ghrelin is generated from the first 28 amino-terminal amino acids by the action of PC1/3, despite PC2 and furin may also process proghrelin to the 28 amino acids ghrelin [545,546]. The most intriguing structural feature of mature ghrelin is its acylation in the third serine residue with an octanoyl group, becoming the only known octanoylated peptide up to date [519]. The enzyme behind such modification was found to be a membrane-bound acyltransferase named ghrelin O-acyltransferase (GOAT), which is located in the membrane of the endoplasmic reticulum [547,548]. The source of the octanoyl group is presumably octanoyl-CoA, which would be translocated from the cytoplasm to the endoplasmatic reticulum by GOAT to acylate the ghrelin propeptide [547,549]. Other medium chain fatty acids may also be used as GOAT substrates, so other ghrelin forms such as hexanoyl and decanoyl-ghrelin have been described in vitro [550]. Still, the major ghrelin forms found in stomach and blood are octanoyl-ghrelin and the non-acylated des-acylghrelin [551,552].

A 27 amino-acids ghrelin form has also been described in rodents due to the alternative splicing of the ghrelin primary RNA transcript, but despite accounting for about 20% of ghrelin in the stomach of rats, it is only found in minor amounts in humans [552,553]. In addition, it was proposed that from the 66-amino acid carboxy-terminal fragment of proghrelin another hormone is formed, named obestatin for its allegedly anorexigenic effects [554]. However, other groups failed to reproduce these findings and the possible actions of obestatin are still controversial [555–557].

3.6.3 Ghrelin release

As early mentioned, ghrelin levels rise during fasting and before meals and decrease after eating [529,558]. This secretory behavior is dependent of the feeding habits of each subject. For example, rats shift their peak in ghrelin secretion towards meal initiation when accustomed to a restricted feeding schedule [559]. In a similar manner, this meal anticipatory secretion is also observed in 24-hour fasting humans, where plasma ghrelin is increased before the regular breakfast, lunch and dinner times of each subject [560]. A first interpretation is that ghrelin secretion is stimulated by

top-down anticipatory cephalic mechanisms. The fact that in fasting the sympathetic drive is lower in heart, liver and pancreas but higher in the white adipose tissue, compelled to study whether ghrelin secretion could also be regulated by a fasting-related sympathetic drive, finding that ghrelin secretion increases in rats after stimulation of sympathetic nerves or infusion of adrenergic compounds into the gastric mucosa [561,562]. In this sense, it was also found that the adrenergic B1 receptor is highly expressed in ghrelin-producing cells, which are also highly stimulated by adrenergic compounds [563–567]. The role of this sympathetic drive during fasting was confirmed in fasting mice, which has been suggested to be triggered by hypoglycemia [565,568].

In addition to top-down regulatory mechanisms, it has also been shown that ghrelin secretion is dependent on chronobiological mechanisms from the same ghrelin-producing cells, which are entrainable per se by food-related cues [569]. Indeed, beyond neuronal and endocrine-sensing machinery, the ghrelin-producing cells express an array of chemosensory receptors, stressing out a regulatory role of food-related stimulus in ghrelin production even in closed-type cells [563].

In accordance to the notion of a food-related inhibition of ghrelin secretion, some receptors such as FFAR2, FFAR4 and the lactate receptor (GPR81) are mainly linked to inhibitory G proteins, so SCFA, LCFA or lactate inhibit ghrelin secretion [563,564,570,571]. For other nutrients, though, a counterintuitive increase in ghrelin release is observed in in vitro experiments, despite such effects are not always observed in vivo. CaSR has been related to inhibitory G proteins in mouse gastric isolated ghrelin cells, where calcium stimuli inhibit ghrelin release, but in contrast, peptone stimulates ghrelin release in a mouse ghrelin cell line and in stomach tissue segments [563,572]. However, peptone decreased plasma ghrelin in mice [572]. The aromatic amino acid receptor GPR142 has been also found in ghrelin cells, where a tryptophan stimuli increases ghrelin secretion [563,564], but intraduodenal tryptophan administration do not affect plasma ghrelin in humans [573]. In addition, on the contrary to glucose and carbohydrates, the artificial sweetener sucralose also increases ghrelin release in ghrelin producing cells and gastrointestinal tissue segments in a taste-dependent manner, but sucralose administration neither increase ghrelin release in mice [574]. Some bitter tastants, on the other hand, stimulate ghrelin release by a taste-dependent mechanism in both ghrelin producing cells and mice. Interestingly, after a short-term increase in ghrelin and food intake in vivo, bitter tastants reduced long-term gastric emptying and food intake [238]. In addition to this nutrient-sensing machinery, ghrelin secretion is also regulated by the products of other enteroendocrine cells, with GIP and secretin as stimulators and somatostatin and insulin as inhibitor of ghrelin release [562,563,575,576].

3.6.4 Mechanisms of action of ghrelin

The full form of GHSR, GHSR1a, is the putative mediator of ghrelin activity [519,577]. The acylation with the octanoyl group constitutes an important motif for GHSR1a activation and similar substitutes such as hexanoate and decanoate produce similar activities [578,579]. Therefore, the amino-terminal segment of ghrelin is essential for GHSR1a activation but also sufficient, so the first 4-5 residues sequence reproduces the full ghrelin activity [578,579]. By the same token, the activity of the 27-residue length ghrelin formed by alternative splicing is comparable to the activity of ghrelin [579]. On the other hand, des-acyl ghrelin is a very week agonist of GHSR1a, with a binding

efficiency 1000 times lowers than ghrelin [578,580]. Hence, taking as an example the archetypical bioactivity of ghrelin, it has been observed that ghrelin but not des-acyl ghrelin administration increases GH secretion in humans [581,582]. As expected, such a bioactivity is mediated by GHSR1a, which is expressed in the pituicytes but also in hypothalamic neurons expressing GH releasing hormone (GHRH) [583,584].

It has been shown that the octanoyl moiety is also necessary to allow the transport of ghrelin across the blood-brain barrier, where gastrointestinal ghrelin may act on GHRH neurons or other neurons [585,586]. Using fluorescently labelled ghrelin it has been shown that peripheral ghrelin crosses the blood-brain barrier to bind AgRP/NPY neurons [587]. Binding to GHSR1a, ghrelin depolarizes AgRP/NPY neurons and promotes AgRP/NPY transcription via AMPK activation while indirectly hyperpolarizes POMC/CART neurons via GABA release, hence triggering a neuronal orexigenic pathway to stimulate food intake [543,588–594]. Central ghrelin infusion in rats also increases the expression of adipose fat-storage promoting enzymes, reduces lipid oxidation and increases body weight, while this central adipogenic effects are prevented by the administration of a GHSR1a antagonist [595,596]. The stimulatory effects of ghrelin in gastric motility are also mediated by the expression of GHSR1a in the nerve cells of the gastric muscle [597,598].

Similarly to the POMC/CART neuron hyperpolarization produced in the hypothalamus, ghrelin may also disengage the satiety signaling at a vagal level. Ghrelin treatments blocks a CCK-induced CART expression of vagal afferents, as well as it has also been suggested that ghrelin restricts the location of GLP-1 receptors to the cytoplasm of vagal afferent neurons to disengage GLP-1 signalling [599,600]. On the other way around, ghrelin does not stimulate food intake in rats or humans subjected to a transection vagotomy, despite it has also been argued that the transection of vagal motor neurons may have influenced such results since in vagal deafferentiated rats ghrelin administration still promotes food intake [601–603]. Either way, GHSR1a is expressed in the DMC, where ghrelin promotes neurogenesis, triggers a noradrenergic signaling to the ARC and stimulates feeding in rodents [604–606]. In addition to the ARC and DMC, ghrelin has also been shown to stimulate feeding when microinjected in the LH, PVN, NAc and VTA [607–611].

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4. LITERATURE REVIEW OF THERAPIES INFLUENCING APPETITE: A FOCUS ON FOOD INGREDIENTS

The first line treatment to combat overweight and obesity consists in the promotion of lifestyle changes, so the relationship between energy intake and energy expenditure drives to an adequate body weight for each subject. These interventions are usually based in recommendations to an equilibrate diet, to a limited daily caloric intake, to a limited daily fatty acid intake and to an increase of daily physical exercise [1–3]. Even so, dietary lifestyle interventions reducing body weight may be poorly effectives at the long term [4]. Alternative treatments such as pharmacologic treatments could support lifestyle interventions to reduce and sustain body weight. The 2015 Endocrine Society Clinical Practice Guideline recommended pharmacotherapy as an adjunct to lifestyle modification to promote weight loss in obese people [5]. Considering the known physiological mechanisms regulating food intake, two main strategies could be design to pharmacologically control food intake: to directly influence in the central mechanisms controlling food intake or to indirectly influence in them via gastrointestinal signals.

4.1 Treatments to reduce food intake at the CNS level

Drugs intended to directly act in the CNS imply driving the drug to the brain and avoid any side-efect due to actions on similar targets or other brain regions where the same target may be involved in an action different from food intake control. The fact that the receptors involved in the central food intake regulation are mainly non-aminergic GPCR involve the extra difficulty that the size, lipophilicity and other physico-chemical properties of its potential ligands are against its absorption by oral administration, its distribution to the brain and its clearance [6]. Even so, several pharmacological designs have focused on central-acting mechanisms, such as MC4R or Y5 signaling.

The effects of several MC4R agonists had been studied in rodents, which caused an effective reduction in food intake when administered centrally [7–14] or peripherally [15–20], but the application of MC4R agonists in humans is less clear. A first concern regarding the application of MC4R agonists in humans was the increased blood pressure produced by the highly selective MC4R agonist LY2112688 after its administration to obese subjects, together with other melanocortinergic effects such as muscle stiffness, yawning and penile erections and other undesirable side effects such as headache, asthenia, nausea or diarrhea [21].

RM-493, also named MK-0493 or BIM-22493, is other MC4R agonist that do not produce cardiovascular effects in a non-human primate model nor produces penile erection in healthy humans, but produced nausea and vomiting over a given dose in preclinical tests in humans [22–24]. A high dose of RM-493 in obese humans produced a non-significant reduction of food intake in a preclinical study [25]. In a larger study in humans, the larger safe dose of RM-493 produced a significant but marginal reduction of food intake in obese subjects, but failed to reduce body weight after 18 weeks of treatment. These effects discarded the use of RM-493 as an effective weight-reducing agent in healthy obese humans [26].

Currently, RM-493 is under phase-II trials in MC4R haploinsufficient (Prader-Willy syndrome) and POMC-null obese subjects (NCT02311673 and NCT02507492 respectively; [27,28]), where is expected to reduce food intake to become an effective treatment in these special cases of obesity.

Similarly to MC4R agonists, a number of Y5 antagonists reduce food intake and body weight in murine models [29–36], but the applicability of a Y5-based therapy in humans is less clear.

MK-0557 is an orally-delivered, highly-selective Y5 antagonist. In a first proof-of-concept study, obese patients underwent a diet and exercise intervention for 6 weeks, following 12 weeks of treatment with several doses of MK-0557. All the treated groups with MK-0557 marginally, but significantly reduced its body weight as compared to placebo, but produced no changes in obesity-related comorbidity endpoints, such as fasting glucose or cholesterol. In a second larger study, MK-0557 significantly reduced body weight after 52 weeks of treatment, but produced no changes in obesity-related comorbidity endpoints or the number of patients with metabolic syndrome [37]. In a last study, MK-0557 was tested in obese patients after a very-low-calorie diet intervention. After 52 weeks of treatment, MK-0557 marginally, but significantly reduced body weight, but produced no changes in secondary endpoints such as blood pressure, waist circumference or lipid profile. It was hence concluded that the effects of MK-0557 are not clinically meaningful and its research was discontinued [38].

The Y5 antagonist S-2367 has been tested in phase-II trials in obese humans subjected to low or high caloric restrictions over 60 weeks. Patients underwent either of both diets benefited from S-2367 treatments, which significantly lowered body weight as compared to placebo [39]. Right after these trials, the effects of S-2367 were tested alone or in combination with the lipid absorption blocker Orlistat in 486 subjects under a reduced calorie diet (NCT01126970; [40]). Up to date, no results had been reported for this study and the research on S-2367 seems to be discontinued.

Besides these efforts in targeting the hypothalamic core-mechanisms of food intake control, other CNS-acting drugs have been shown useful to reduce food intake by other mechanisms. Two of these CNS-acting drugs had formerly been used as long-term treatments for obesity, but were later withdrawn for its unacceptable side effects: Sibutramine and Rimonabant. Sibutramine was a centrally-acting monoamine-uptake inhibitor, originally developed as antidepressant, which reduced body weight and effectively sustained the weight reduction in long-term treatments [41]. After some reported non-desirable cardiovascular side-effects, the European Medicines Agency concluded that the benefit did not out-weighted de cardiovascular risks and withdrawn the drug from the market [42,43]. Two years after, the United States Food and Drug Administration also forced the withdrawal of sibutramine from the American market for the same concern [44,45]. By its side, Rimonabant was an endocannabinoid antagonist that reduced food intake by decreasing the motivation to eat [46]. Soon after its approval in Europe, it was pointed its psychiatric side effects, which included anxiety, depression and suicidal ideation and was withdrawn from the European markets [47,48]. Due to the same concern, Rimonabant was never approved in the United States [49]. Two other central-acting drugs, the 5-HT receptor agonist lorcaserin, and the combination of a sympathomimetic phentermine and the anticonvulsant topiramate are approved only in the United States, despite the warnings of psychological and physiological adverse side effects [50].

4.2 Treatments to reduce food intake at the gastrointestinal level

Given the problems observed for these central-acting drugs, an alternative pharmacological strategy consists in targeting energy balance mechanisms at a gastrointestinal level. Nowadays, the only available drug for long-term therapies in Europe is the lipase inhibitor Orlistat, which blocks the absorption of lipids in the intestine [51]. Taking into account the importance of the gastrointestinal signals in the control of food intake, other strategies focus in enteroendocrine signaling to reduce food intake.

A first clinical evidence of the usefulness of the enteroendocrine signals in food intake and body weight reduction is observed in bariatric surgeries, where the reduction in gastric capacity and digestibility are intended to reduce body weight in obese people. Roux-en-Y gastric bypass (RYGB) is the most common bariatric procedure and produces a rapid and sustained reduction in body weight [52,53]. The procedure consists in resecting the stomach in two to create a small gastric pouch where the lower jejunum is anastomosed, following by a jejuno-jejunal anastomosis to empty the secretions of the gastric remnant, pancreas and gallbladder in the middle jejunum [54]. Besides the obvious reduction in gastric capacity, RYGB causes an exaggerated secretion of GLP-1 and PYY [55–60], which is sustained even 10 years after the RYGB intervention [61]. This increase in GLP-1 and PYY after RYBG may involve direct stimulation of L-cells by nutrients or biliary acids, but also an increase in the number of L cells, as observed in rats [62,63]. In any way, it has been recently suggested that the reduced food intake in patients subjected to RYGB is influenced by the actions of GLP-1 and PYY, since the blockade of both hormones increased food intake after RYGB [64]. As a less invasive procedure, a duodeno-jejunal bypass liner may be implanted up to 12 months in obese patients. The implantation of this device, which drives the ingested food directly to the ileum, increases PYY and GLP-1, produces early and prolonged satiety and reduces food intake [65–67]. These effects may resemble those of duodeno-jejunal bypass surgery, were it has been suggested that the delivery of nutrients and undiluted bile acids into the ileum play an important physiological role [68].

A second clinical evidence of the usefulness of the enteroendocrine signals in food intake and body weight reduction is observed in incretin therapies. Incretin therapies based on GLP-1 analogs or DPP-4 inhibitors enhance endogenous insulin secretion in a glucose-dependent manner and are current treatments for type-2 diabetes [69]. It was observed that incretin therapies using GLP-1 analogs produce the plus point of weight loss, which may be related to the observed decrease in gastric emptying, appetite and food intake [70]. These positive effects on satiety signaling leaded the long-lasting GLP-1 analog Liraglutide to be approved as a treatment for overweight and obesity in the United States and the European Union [71,72]. Other GLP-1 analogs, such as G3215, are currently under clinical trial (NCT02692040; [73]). By the same token and taking into account that the effects of GLP-1 and PYY on food intake are additive [74], PYY analogs are also of clinical interest. The long-lasting PYY analogue Y242 has shown to reduce food intake in rodents, but its administration to humans is yet in phase-I trials and its effects are not known (NCT01515319; [75]). A stronger PYY analog with a slower onset, Y3394, is currently pending to start a phase-I trial in humans (MR/L013088/1; [76]). CCK analogues has also been tested in rodents, alone or in combination with GLP-1 analogues [77,78], but no studies in humans have been performed yet in our knowledge. Preliminary studies in rodents with diaminopyrimidine and quinazolinone

derivatives have shown that the ghrelin receptor antagonism also effectively reduce food intake [79,80], but no studies in humans have been performed yet in our knowledge.

4.3 Studies on food ingredients to target enteroendocrine signaling and food intake

The above-mentioned evidences point out that the therapies focused on the enteroendocrine signals could be an effective strategy for food intake and body weight management. The fact that enteroendocrine cells naturally respond to food ingredients leads to screening on food components to discover new therapies to manage food intake and body weight. Besides its pharmacological application as drugs, food ingredients provide the extra bonus of a wider applicability to risk populations via dietary recommendations, functional foods or dietary supplements, which may prevent the development of overweight and obesity-related problems from early preclinical stages.

It is worth to mention that herbs and food supplements are consumed whether or not its effects are clear or its physiological mechanisms are known, so by the same token, the effects of these supplements on enterohormones are not always taken into consideration in human interventions. Hence, there is also large body of human interventional studies using dietary compounds to reduce body weight that have not assessed its effects on any gastrointestinal hormonee, such as studies on dietary fibers [81–87], conjugated linoleic acid [88–94], extracts of Garcinia cambogia [95–99], effedrine and caffeine [100–104] or diverse plant extracts [105–116].

Hereafter, we compile a series of studies found in PubMed in December 2016 after the search "(hormone) AND (food compound OR bioactive compound OR bioactive ingredient OR natural ingredient OR plant ingredient OR plant molecule OR bitter OR extract OR hydrolyzed OR hydrolysed OR supplement OR functional food)".

4.3.1 CCK modulation by food ingredients

Different types of fiber are recurring ingredients in weight-reducing diets. In humans, a significant correlation had been found between fasting CCK levels and the colonic transit time related to the intake of guar fiber [117]. Posterior studies confirmed that the administration of guar fiber to obese patients lead to increased CCK [118]. In non-obese human volunteers, the administration of a high fiber meal based on dry beans also increases CCK by twice when compared to a low fiber meal [119].

Other bean and vegetable-derived extracts have been used to increase CCK due to its content in amylase or protease inhibitors, which trigger CCK release. In rats, the administration of a Phaseolus vulgaris extract containing amylase inhibitors increased CCK and decreased food intake [120]. Potato extracts containing protease inhibitors also increase CCK in rats, but its application failed to succeed in humans [121–123].

Intact proteins and hydrolysates are also potent stimulators of CCK release [124–126]. The intraduodenal administration of whey protein hydrolysates to obese patients increased CCK and tended to decrease food intake [127]. In a recent study in overweight patients, the administration of a fish protein hydrolysate during 90 days increased CCK and effectively decreased body weight

[128]. In lean and obese subjects, the intraduodenal administration of pea intact protein also increased CCK and leads to reduced food intake [129].

As noted before, bitter tastants from plant origin stimulate enteroendocrine cells to release CCK and activate SNC satiety centers, a process enhanced by vegetable-rich diets via SREBP [130–132]. An extract from Hoodia gordonii, an African plant of known satiating effects, containing the steroid glicoside H.g.-12, increased CCK secretion in a cellular model and in rat's intestine via activation of TAS2R14 [133]. In a study in humans, the intraduodenal administration of quinine stimulates CCK release and produces and significantly reduces calorie intake after a standardized meal by approximately 15% [134]. In a similar study, intraduodenal quinine did not affect CCK levels [135].

Some flavonoids, a family of plant-derived phenolic compounds, had also been shown to increase CCK in STC-1 cells. This included apigenin, quercetin and kaempherol [136], but the effects of these compounds on human CCK levels and food intake have not yet been studied in our knowledge. In other studies in humans, the administration of plant extracts containing a mix of phenolic compounds has been shown to improve body weight and food composition, but CCK levels were not measured [137,138].

4.3.2 GLP-1 modulation by food ingredients

Dietary fiber can also be found in the literature as a GLP-1 secretagogue in cellular or murine models [139,140]. In vivo, these effects may be mediated by the SCFA released during fiber fermentation, since in both rats and humans the number of FFA2-positive L-cells increase due to dietary indigestible fermentable carbohydrates [141]. However, the effects of dietary fiber on human GLP-1 secretion differ from study to study.

In healthy subjects, a meal containing wheat bran oligosaccharides or brown beans did not affect GLP-1 levels, so did the administration of a beverage with barley b-glucans despite reduced food intake [142–144]. In a trial with overweight and obese individuals, the administration of oligofructose during 12 weeks neither produced a change in GLP-1 levels, despite produced a reduction in body weight [145]. In other studies with healthy subjects, GLP-1 was even diminished after the administration of psyllium fiber or resistant starch [146–148].

In other study, guar gum increased GLP-1 and satiety in woman, but not men [149]. In overweight women, the administration of different arabinoxylans increased GLP-1, but not affected appetite or energy intake [150]. In a 10-patients study with healthy subjects, the administration of fructans from chicory roots increased GLP-1 and lowered hunger rates by 3-fold [151]. Finally, the administration of high-wheat to hiperinsulinemic subjects during 12 months increased GLP-1 but did not change body weight [152].

With the same aim of increase SCFA, probiotics have also been shown to increase GLP-1 and decrease body weight in mice [153]. In humans, the administration of Lactobacillus reuteri during 4 weeks also significantly increased GLP-1 levels [154].

Dietary supplementations with green-plant thylakoids has also been shown to influence GLP-1 levels. In overweight women, the administration of green-plant thylakoids during 12 weeks

increased postprandial GLP-1 and decreased body weight compared to placebo [155]. In a similar study it was discussed that the increased satiety and decreased hunger and cravings for snacks after a single thylakoid administration in overweight woman may be related to an increase in GLP-1, but no measures of GLP-1 were actually presented [156].

The administration of protein hydrolysates also increases GLP-1 in rodents [157–159]. A number of studies with different hydrolysates or proteins also produce a coherent GLP-1 increase in humans. In lean healthy men, intraduodenal whey hydrolysate increased GLP-1 levels and decreased energy intake in a standardized meal [160]. In a study conducted in lean and overweight men, the intraduodenal administration of a whey hydrolysate equally increased GLP-1 independently of body weight [127]. Similarly, a hydrolyzed gelatin meal also increased GLP-1 in both lean and overweight subjects [161]. In a study with overweight people, the administration during 90 days of a fish protein hydrolysate increased GLP-1 levels and improved several parameters of body composition, including body weight [128].

Plant-derived extracts and plant-derived compounds comprise the larger bibliographic body of studies regarding the application of food ingredients to stimulate GLP-1 release, many of them due to the screening of antidiabetic molecules from previously known antidiabetic herbal medicines. A number of antidiabetic plants presented DPP-4 inhibitory activities when studied in vitro [162–166], resulting in an effective GLP-1 increase for some plant extracts studied in rats [167]. Cellular or murine studies reporting a direct GLP-1 secretagogue activity include the use of plant extracts of mate tea [168], hachimi-jio-gan [169], pomelo [170], beet [171], wild angelica [172], yam [173], sweet potato leaf [174], coffee phenolic compounds [175] or grape-seed phenolic compounds [176,177], together with plant-derived pure compounds such as pregnane glicosides [178] or chlorogenic acid [179].

Despite the high number of these studies, the translation to human studies is more modest and mainly involves the use of plant-derived phenolic compounds. In healthy adults, the administration of both an apple phenolic extract and a coffee phenolic extract also increased GLP-1 levels, but its effects on satiety were not assessed [180,181]. In a study in overweight patients, a single combination of the phenolic compound epigallocatechin gallate together with capsaicinoids, piperin and carnitine increased GLP-1 and satiety levels [182]. Other plant-derived compounds, such as Korean pine nut oil, also increase GLP-1 and satiety levels after a single dosage in overweight women [183].

Some plant extracts and plant-derived compounds have been shown to stimulate GLP-1 release due to its bitter-tasting proprieties in cellular or rodent models [184–188], but intraduodenal quinine did not affect GLP-1 levels in a study in humans [135].

4.3.3 PYY modulation by food ingredients

The number of studies reporting the use of food ingredients to stimulate PYY release is humbler. In rat models, studies with pea protein and resistant starch report increased PYY levels [140,189,190].

In overweight women, a single arabinoxylan fiber-rich breakfast increases circulating PYY without changing postprandial appetite [150]. In healthy youg adults, an evening meal with brown beans increased PYY and decreased hunger sensations in a standardized breakfast [143]. In overweight adults, a 6-week dietary supplementation with oligofructose decreased the motivation to eat and tended to increase PYY [191], while a 12-week intervention reduced body weight and increased PYY [145]. Neither alginate nor hydrolyzed gelatin or intact pea protein produced any effect on PYY concentrations in three independent studies in overweight patients [129,161,192].

4.3.4 Ghrelin modulation by food ingredients

As for other enterohormones, ghrelin levels were also measured in some studies on different kinds of dietary fiber. In healthy men, the administration of a bean extract reduced ghrelin secretion during in a larger time-window than placebo, together with a reduced desire to eat [193]. In other study with healthy men, an evening brown beans meal reduced ghrelin by a 14% and hunger sensations by a 15%, as compared to a white-bread isocaloric fed group [143]. Also in healthy adults, cereal-based bread enriched in fiber and wheat proteins reduced less postprandial ghrelin than control bread, despite increased satiety [194]. In other study, the administration of corn soluble or resistant starch did not change ghrelin levels, alone or in combination with pullulan, as neither changed satiety nor energy intake [195]. In subjects with impaired glucose tolerance, the administration of arabinoxylan reduced total, but not acylated ghrelin, together with serum glucose and insulin [196]. In obese individuals, a 10-day treatment with alginate neither change ghrelin levels, satiation scores or calorie intake [192]. The administration of alginate also failed to change ghelin levels in a larger 12-week study in obese patients, but effectively reduced body weight as compared to placebo [197]. Also in obese adults, the administration of oligofructose during 12 weeks reduced ghrelin, together with body weight and self-reported food intake [145].

Ghrelin levels had also been studied in some dietary interventions based on protein supplementations. In obese patients, neither oral nor intraduodenal administration of pea protein changed ghrelin levels, despite diminished food intake [129]. A single gelatin hydrolizate meal neither changed ghrelin levels in normal or obese subjects [161]. In a 6-month intervention in obese women, ghrelin levels were not changed by ghrelin supplementation as either was body weight, which equally diminished in the control and the treated group [198]. In a larger study with 90 obese patients, a dietary supplementation of whey protein during 23 weeks diminished both ghrelin and body weight, as compared to a isocaloric carbohydrate-supplemented group, while soy protein produced no effect [199].

A number of plant-derived extracts and compounds have also been studied for ghrelin secretion. Some plant extracts have been shown to increase ghrelin secretion, which could have an application to treat anorexia, cachexia or other pathologies [200–202]. Besides, most of the literature is focused

in the screening of ghrelin-reducing effects. In mice, the terpenes ursolic acid from Sambucus australis and betulinic acid from Clusia nemorosa has shown to increase circulating ghrelin and improve the lipid profile of mice under a high fat diet [203,204]. In rats, steroids from the guggulsterone family extracted from Commiphora mukul reduced ghrelin, food intake and body weight during a 15-day ptratment period [205]. The compound cinnamaldehyde extracted from cinnamon decrease ghrelin secretion in MGN3-1 cells and reduce food intake and body weight after its administration to mice [206]. Also in mice and rats, the administration of phenolic extracts from potato or pomelo reduce ghrelin levels [170,207].

In obese humans, a mixed extract from Dolichos biflorus and Piper betle, two Asian tropical plants, decreased ghrelin levels and body weight when administered thrice a day during eight weeks [208]. It is noteworthy that most of the resting studies with plant extracts or compouds in humans show no influence on ghrelin levels. From plant alkaloids, neither an acute dose of quinine in healthy subjects nor a 9-month supplementation of ephedra and caffeine in obese women affected ghrelin levels, despite the ephedra and caffeine supplementation effectively reduced body weight [134,209]. In a study with overweight and obese subjects, the administration of a Coleus forskohlii extract containing the terpene forskolin for 12 weeks neither influence the levels of ghrelin, nor body weight [210]. In obese woman, a supplementation with eicosapentaenoic acid and α-lipoic acid during 10 weeks reduced body weight without affecting ghrelin levels [211].

A last group of human studies is focused in the effect of flavanol structures, a family of phenolic compounds. Hsu et al. examined the effects of a green tea extract supplementation containing epigallocatechin-gallate in two different studies. In obese women, the green tea extract supplementation during 12 weeks did not affect body weight and it could only be disclosed that ghrelin levels only increased along the study in the placebo group [212]. Very similar results were found when the green tea extract was administered to obese type-2 diabetics during 16 weeks, with no effects of the treatment on body weight and ghrelin levels along the study only increasing in the placebo group [213]. Independently, Dostal et al. realized other two experiments to assess the effects of a green tea extract supplementation to overweight woman, showing no effects on ghrelin levels and body weight after 12 months of treatment [214,215].

Taking into account these prior studies on green tea extracts, Chen et al. performed a study in obese women with thrice the dosage of phenolic compounds that used by Hsu et al. After 12 weeks of treatment, the green tea extract diminished body weight, presented more favorable effects on anthropometric measurements and significantly decreased ghrelin levels [216].

4.3.5 Summary of interventions using food ingredients

The effective studies in humans presented above are summarized in **table 3**. The effective acute studies involve either increases in anorexigenic enterohormones or decreases in ghrelin, with no obvious differences between types of ingredients. On the other hand, it is noteworthy that the effective chronic inteventions are mostly associated with reported decreases in circulating ghrelin, which also presented the only reported significant correlation between food intake and hormonal levels.

Table 3 - Human studies on food ingredients reporting positive effects on enterohormones and food intake or body weight

Treatment			Subjects			Outcome						
Compound	Dose	Length	Gender	Condition	n	CCK	GLP-1	PYY	GHS	Food intake	BW	Study
Pea protein	0.25g/kg BW	Acute	Man	Healthy lean or obese	10	↑	↑	↑	=	Increased fullness and decreased food intake in an ad libitum meal	-	[129]
Quinine	18mg	Acute	Both	Healthy lean	20	↑	-	-	=	Reduced intake in the ad libitum meal	-	[134]
Whey hydrolisate	2, 24 or 48 g	Acute	Men	Healthy lean	16	-	↑	-	-	Dose-response decrease	-	[160]
Dietary fiber and resistant starch	31g of mixed dietary fiber and 6.5g of resistant starch	Acute	Both	Healthy lean	16	-	=	↑	↓	Lower hunger in the early postprandial period (45min)	-	[143]
β-glucan	3g of barley β-glucans	Acute	Both	Healthy lean	14	-	=	=	↓	Lower hunger and food intake in the subsequent 24h	-	[144]
α-amylase inhibitor	100mg bean extract containing α-amilase inhibitor	Acute	Both	Healthy lean	12	-	-	-	↓	Increased satiety from 2-3h after the meal	-	[193]
Fish hydrolisate	1.4g or 2.8g Slimpro/day	90 days	Both	Overweight	37-39	↑	↑	-	-	-	↓	[128]
Thyllakoids	5g/day green plant membranes extract	12 weeks	Women	Overweight	38	-	↑	-	=	Lower hunger cravings	↓	[155]
Oligofructose	21g/day	12 weeks	Both	Overweight and obese	18-21	-	=	↑	↓	Lower self-reported energy intake along the study	↓	[145]
Milk prot/calcium	568ml milk containing 1g calcium and 20g protein	6 month	Women	Overweight and obese	12-13	-	-	-	↓	Increased fasting fullness, in correlation with ghrelin	↓	[198]
Whey protein or soy protein	56g/day whey or soy protein	23 weeks	Both	Overweight and obese	23-25	-	-	-	↓	No changes in food intake or subjective satiety	↓	[199]
Dolichos biflorus and *Piper betle* extract	900mg/day extract	8 weeks	Both	Obese	20	-	-	-	↓	-	↓	[208]
EGCG	1.5g green tea extract with 856.8mg EGCG/day	12 weeks	Women	Obese	38-39	-	-	-	↓	-	↓	[216]

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5. LITERATURE REVIEW OF THE FLAVANOL EFFECTS ON THE ENTEROENDOCRINE SYSTEM AND FOOD INTAKE

5.1 Focusing on flavanols

Phenolic compounds are a large group of phytochemicals widespread in the plant kingdom, where are produced as secondary metabolites with a several functions, such as pigments, signaling compounds, ultraviolet sunscreens and chemical defenses against pests or pathogens [1, 2]. The main core of phenolic compounds is formed by at least one phenol ring, but usually in plants these compounds contain more phenolic rings, and thus they are called polyphenols [3]. Among the many families of polyphenols, flavanols are a subgroup of polyphenols which is pervasive in human diets [4, 5]. Such as for other dietary compounds, a large number of studies report the effects of flavanol compounds on body weight, mostly focusing in tea supplementation and not assessing the levels of gut hormones. A meta-analysis published in 2009 showed a modest positive effect of green tea supplementation on weight loss and manteinance [6]. Similar results were reported in a second meta-analysis published in 2010 [7], but later studies have not been included yet in an overall analysis. As mentioned in previously, a recent study has shown that flavanol supplementation reduce circulating ghrelin in obese women, but food intake was not assessed in the study [8]. It is therefore interesting to sum up the known effects of flavanols on enterohormone secretion beyond the clinical studies in humans. Hereafter we review the effects of flavanols on food intake and enterohormone release in humans, but also in rodents and cellular models from published studies at the beginning of this thesis in 2013.

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5.3 Effects of flavanols on the enteroendocrine system: repercussions on food intake

By Monserrat Pinent, Mayte Blay, Joan Serrano & Anna Ardévol

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Abstract

Flavanols are plant-derived bioactive compounds for which several beneficial effects have been described. When ingested, they reach the gastrointestinal tract, where they can interact with the enteroendocrine cells. In this paper, we consider the possibility that flavanols modulate enterohormone secretion. Because the regulation of food intake is among the principal functions of the hormones that are secreted in the gastrointestinal tract, we also compile the literature that covers how the effects of flavanols on food intake are measured. Although there are some papers showing the effects of flavanols on the regulation of enterohormones, there are very few papers that have addressed the specific effects at the food intake level. Instead, most of the findings are secondary to the study of the action of flavanols on body weight, which makes it difficult to reach a clear conclusion regarding the effects of flavanols on food intake.

Keywords

Catechins, proanthocyanidins, enterohormones, hunger, satiety, body weight

Introduction

Plant-derived foods have many minor components that have the capacity to alter enzymatic and chemical reactions, which exert biological responses in mammalian systems and, therefore, impact health both positively and negatively. One of the largest groups of these bioactive components is the flavonoids, a subclass of polyphenols (Beecher, 1999, 2003). In turn, flavonoids are divided into 6 subclasses, with flavanols (also known as flavan-3-ols) being the most structurally complex subclass of the flavonoids. Flavanols can scavenge free radicals, complex with metal ions, and interact with proteins, which leads to the modulation of protein function interactions with signaling cascades and modifications in gene expression. Through these mechanisms, flavanols can exert protective effects against cardiovascular diseases (reviewed in (Rasmussen et al., 2005)) and act as antioxidant (Puiggros et al., 2005), anti-inflammatory (Terra et al., 2009), and anti-carcinogenic (Nandakumar et al., 2008) molecules. Additionally, they can improve lipid homeostasis (Bladé et al., 2010) and modulate glucose homeostasis (Pinent et al., 2012). Despite all of these well-described effects, the bioavailability of these compounds remains a controversial point. Most of them are detected in several tissues (Manach et al., 2004) inside the organism. However, the enormous diversity of chemical structures that can arise after their metabolization makes it difficult most of the time to

identify the compound(s) that are responsible for the described effect. In contrast, it is very clear that flavonoids reach the gastrointestinal tract, where they can directly interact with the enteroendocrine system, which controls several digestive and metabolic processes as well as food intake.

The enteroendocrine system is one of the highest endocrine systems of the organism (Janssen & Depoortere, 2012). On the intestinal surface, there are absorptive enterocytes, bactericidal Paneth cells, mucus-producing goblet cells and hormone-secreting enteroendocrine cells. These last cells are fully differentiated cells that, together with the goblet and Paneth cells, constitute the secretory lineages in the intestine, composing 10% of the epithelium (Moran-Ramos et al., 2012). The different enteroendocrine cell types have been classified according to their epithelial localization: first, the "closed cells" that do not reach the gut lumen, and second, the "open cells" that project a tuft of apical microvilli into the intestinal lumen and extend to the basal lamina (lamina propria) (Sternini et al., 2008). The open type cells are considered to be primary chemoreceptors, responding to the luminal nutrients by releasing their secretory products, which activate neuronal pathways, nearby cells or distant targets. Closed cells can be regulated by luminal content indirectly through neural and humoral mechanisms (Sternini et al., 2008). Enteroendocrine cells have also been classified into at least 10 types based on their morphology, principal hormone product(s) and distribution along the intestinal tract (Janssen & Depoortere, 2012) (summarized in Table 1). The most studied enteroendocrine cells are I-, L-, and K-cells due to their secreted products, which are cholecystokinin (CCK), glucagon-like peptides, and GIP, respectively (Moran-Ramos et al., 2012).

Food intake is controlled by the brain, which receives hormonal, neural and metabolic signals that reflect the energetic status; the brain then responds to these inputs by coordinating adaptive alterations of energy intake and expenditure. There are several signals that emanate from the gastrointestinal system, such as pancreatic and intestinal satiation peptides. Panickar (2012) recently reviewed the effects of dietary polyphenols on neuroregulatory pathways that modulate food intake. They concluded that some polyphenols clearly appear to have the potential to modulate neuropeptides that are involved in food intake and satiety, but they remark that the studies that allow such conclusions are scarce (Panickar, 2012).

We hypothesize that flavonoid effects on food intake could be mediated by its interaction with "open cells" in the gastrointestinal epithelium. To further analyze this hypothesis, we next review the described effects of flavonoids on gastrointestinal signals that regulate food intake and their described bioactivity that is related to food intake.

Flavanol structure and metabolism

Flavanols range from simple monomers, (+)-catechin and its isomer (-)-epicatechin, to complex structures that include the oligomeric and polymeric proanthocyanidins, which are also known as condensed tannins. The monomeric forms can be hydroxylated to form gallocatechins, and monomers can also undergo esterification with gallic acid (Crozier et al., 2009). Catechins are found in many fruits, but the richest sources of catechins are green tea, chocolate, and red wine (D'Archivio et al., 2007). Catechin and epicatechin are the main flavanols in fruit, whereas gallocatechin (GC), epigallocatechin (EGC), and epigallocatechin gallate (EGCG) are found in

certain seeds of legumes, in grapes, and in tea. In contrast to other classes of flavonoids, flavanols are not glycosylated in food (Manach et al., 2004). Although the exact flavanol content is difficult to determine due to the wide range of structures, they are major components in the human diet due their widespread presence in fruits, berries, nuts, beans, some spices, cocoa-based products, wine, and beer (Gu et al., 2004).

The biological properties of polyphenols depend on their bioavailability. The chemical structure of polyphenols determines their rate, the extent of intestinal absorption and the nature of the metabolites that circulate in the plasma (reviewed in (Scalbert & Williamson, 2000; Aron & Kennedy, 2008)). Moreover, the degree of polymerization and galloylation of flavan-3-ols are factors that affect their bioavailability [19]. Monomeric flavan-3-ols are absorbed in the small intestine, where they are extensively metabolized into glucuronide conjugates. Flavan-3-ols can also enter the liver, where they are mainly sulfated and methylated (Monagas et al., 2010). Then, conjugated flavanols enter into systemic circulation or can be returned to the intestinal lumen via bile (entero-hepatic circulation) (Aura, 2008). Approximately 90-95% of the total consumed polyphenols cannot be absorbed by the small intestine; as a result, they pass to the colon and, in addition to the compounds returned by the entero-hepatic circulation, they are metabolized by the colonic microflora (Clifford, 2004). The microbial metabolites are absorbed by the colonocytes and arrive at the liver, where they are subjected to glucuronidation, methylation and sulphatation. Then, they enter into systemic circulation or to the kidneys, where they are excreted in the urine (Monagas et al., 2010). Flavonoids have been detected in a wide range of tissues in mice and rats, including the brain, endothelial cells, heart, kidney, spleen, pancreas, prostate, uterus, ovary, mammary gland, testes, bladder, bone, and skin (Manach et al., 2004). Numerous studies in animals and humans have shown that polymeric proanthocyanidins are not absorbed. The majority of them pass unaltered through the small intestine and are then metabolized by the colonic microflora to yield a number of simple phenolic acids (Déprez et al., 2000). However, procyanidin dimers and trimers have been detected in rat urine (Tsang et al., 2007) and plasma (Serra et al., 2010) following administration of grape seed procyanidins. Additionally, oligomers (up to pentamer size) were detected in rat plasma following administration of a procyanidin extract from apples (Shoji et al., 2006). Some *in vitro* assays that mimic gastrointestinal conditions have demonstrated the degradation of procyanidin oligomers to yield bioavailable monomers (Spencer et al., 2000). However, *in vivo* studies rejected the notion that procyanidins contribute to the pool of circulating flavanols via their breakdown into monomers in rats (Tsang et al., 2007) and humans (Ottaviani et al., 2012). The detection of dimeric procyanidins in human plasma has been reported in some studies (Sano et al., 2003; Holt et al., 2002; Urpi-Sarda et al., 2009).

Effects of flavonoids on enterohormone release

GLP-1 is secreted by L-cells of the intestine and participates in the regulation of food intake, although its main role is as incretin, a hormone that promotes insulin secretion. The modulation of incretins by procyanidins has been partly evaluated. In healthy rats, an acute oral dose of grape seed extract (1 g/kg bw) has been shown to increase GLP-1 levels after an oral glucose load. The mechanisms that exert this effect could arise from their capacity to inhibit DPP-4, their ability to

modulate GLP-1 secretion from L-cells, as shown in the enteroendocrine cell line STC-1, and/or by altering the number of enteroendocrine cells in the intestine (submitted results). A preventive dose of 25 mg/kg bw of the same extract, for 12 weeks, prevented the cafeteria-induced decrease in colon GLP-1 producing cells (submitted results). Similarly, a dose of 10 μg/kg bw of the procyanidin tetramer cinnamtannin A2 also increases plasma active GLP-1 when it is acutely administered to fasted mice (Yamashita et al., 2013). Furthermore, Torronen et al, working with healthy humans, showed that a single administration of a berry purée (800 mg polyphenols including anthocyanins, flavonols, phenolic acids, proanthocyanidins, and ellagitannins) administered together with sucrose tended to increase GLP-1 (Törrönen et al., 2011).

Other monomeric polyphenols have also been shown to modulate GLP-1 levels. Chlorogenic acid, which is a major phenol found in coffee, was shown to improve plasma GLP-1 levels (Johnston et al., 2003) and increase GLP-1 secretion and production in STC-1 cells, a murine enteroendocrine cell line (Rafferty et al., 2011). Berberine, which is a major active constituent of *Rhizoma coptidis*, has been reported to increase portal active GLP-1 levels in healthy and streptozotocin-induced diabetic rats (STZ) and to enhance GLP-1 secretion and biosynthesis in NCI-H716 cells, a human enteroendocrine cell line (Lu et al., 2009; Yu et al., 2010). Genistein and daidzein isoflavonoids, which are derived from soybean fermentation, have been reported to increase GLP-1 secretion from NCI-H716 cells (Kwon et al., 2011); glyceollins and phytoalexins that are derived from daidzein in soybean with a fungi infection showed the same effect *in vitro* (Park et al., 2010). Resveratrol, a polyphenolic compound produced by fruits such as red grapes or berries, was found to increase portal active GLP-1 levels and intestinal biosynthesis in high-fat diet-fed rats (HFD) (Dao et al., 2011). Finally, a recent paper has reported that curcumin, a phenolic compound that is isolated from the rhizomes of *Curcuma longa L.*, can increase GLP-1 secretion in the murine enteroendocrine cell line, GLUTag (Takikawa et al., 2013).

Ghrelin is produced by X-cells of the stomach, and its main role is highly related to the regulation of food intake. Ghrelin's modulation by isoflavones has been evaluated, while there is less information regarding other types of flavonoids. Soy isoflavones decreased plasma ghrelin, increased CCK, and increased, although not significantly, PYY when administered to ovariectomized rats that were fed a high-fat diet for 4 weeks. These changes were found at 3 doses of isoflavones: low (26 mg/kg bw), medium (74 mg/kg bw), and high (206 mg/kg bw). However, the body weight was increased at the lower dose and was reduced at the other two doses. At the higher doses, the energy intake was reduced (Zhang et al., 2009). A similar effect was found in female mice by Ryokkynen. In this case, 8 mg /kg bw/day of the isoflavone genistein administered to mice for 8 weeks reduced plasmatic ghrelin in females, while it had no effect in males. In these mice, food consumption was reduced at weeks 1 and 5 but not at the end of the experiment because these animals had pups that were in the lactation period (Ryökkynen et al., 2006). Thus, animal studies suggest that some isoflavones can modify the levels of ghrelin, and it appears that such a modification precedes changes in body weight. Similar studies in humans do not clearly show this effect, but these results could be due to the different doses that were administered. In healthy postmenopausal women, 80 or 120 mg (i.e., 1.19 or 1.79 mg/kg bw) of soy isoflavones for 12 months did not modify the fasting levels of appetitive hormones (ghrelin, insulin, leptin, and adiponectin). In the same sense, the body composition was not affected by soy isoflavones. Food intake was not assessed (Matvienko et al.,

2010). Similarly, in another study in healthy postmenopausal women, 50 mg/day of isoflavones neither affected preprandial ghrelin plasma levels, nor insulin, glucose body weight or energy intake. Instead, PYY was increased by isoflavones, and the authors concluded that this hormone level is not a major factor in the regulation of body weight (Weickert et al., 2006). In a smaller study (Nikander et al., 2004), isolated isoflavonoids (114 mg/day) for three months inhibited the age-dependent rise of fasting plasma ghrelin in postmenopausal women with a history of breast cancer, although this finding was not accompanied by modifications in the lipid profile or insulin sensitivity, and the body composition and food intake were not assessed.

An extract of *Citrus grandis* that is rich in naringenin was administered for 12 weeks at different concentrations (300, 600, 1200 mg/kg bw) in Zucker fatty rats that were fed a high fat/high cholesterol diet and did not induce significant changes in the body weight nor in the food intake, although the authors suggested that there was a tendency to reduce the body weight accompanied by an increased energy intake. The hormones were analyzed, and the extract appeared to counteract the HFD-induced decrease in ghrelin. The extract also decreased the plasma GLP-1 (which was not affected by the diet), while it did not change the insulin, PYY, leptin nor amylin (Raasmaja et al., 2013). In type 2 diabetic humans, a decaffeinated green tea extract (11.12 mg EGCG/Kg bw) for 16 weeks did not show any difference in plasma ghrelin or leptin compared to the placebo group. Treatment also did not modify the body weight or plasmatic parameters (insulin, glucose, HOMA-IR) (Hsu et al., 2011). Finally, in healthy humans, carob pulp reduced the acylated but not the total ghrelin. The effects on ghrelin might account for the observed reduction in NEFA and TAG and the change in the substrate utilization toward lipid oxidation (decrease in RQ) (Gruendel et al., 2006). The effects on plasma acylated ghrelin and fat oxidation after a meal were maintained 24-hour after carob pulp intake (Gruendel et al., 2007). Because the treatment was acute, no report on hunger or energy intake was made. Moreover, carob pulp is rich in insoluble dietary fiber and polyphenols, mainly gallic acid, gallotannins and flavonol glycosides, and from these experiments, it cannot be deciphered whether the effects were due to the fiber or the polyphenols (Gruendel et al., 2006).

There are very few studies that evaluate flavonoid effects on PYY; these studies were cited above. Raasmaja et al. (2013) showed no effects of an extract that was rich in naringenin gived simultaneously with a HFD in Zucker fatty rats. Zhang et al. (2009) reported that an isoflavone treatment of ovarectomitzed rats increased PYY. Finally, Weicker et al. (2006) described that soy isoflavone supplementation for eight weeks did not significantly reduce the energy intake or body weight, even though plasma PYY increased during the isoflavone treatment. The relation of CCK with flavonoids is indeed less analyzed. The scarce existing studies are already indicated above.

Taking these studies together, flavonoids have been shown to modulate GLP-1 levels. Other polyphenols also modulate GLP-1 and ghrelin. There is very little data that concerns the effects on other enterohormones. In all of these studies, whether such modulation involves effects on food intake has not been assessed.

Effects of flavanols on food intake

There are few studies that evaluate the effect of flavonoids on food intake, and most of them evaluate it secondarily in studies that were designed to analyze the effects on body weight and energy balance.

Tea catechins

Among the most studied flavanols are the tea catechins, of which EGCG is the most abundant polyphenol. A few animal studies showed the inhibition of food intake by tea catechins (summarized in Table 2a). Kao et al. (2000) showed that, in Sprague Dawley rats, intraperitoneal EGCG injection (~85 mg/kg bw) reduced food intake within 2-7 days of treatment. Instead, other catechins (catechin, epigallocatechin, epicatechin gallate) did not modify food intake. Such effects were also reproduced in Zucker fatty rats, which suggests that the inhibition of food intake by EGCG is leptin-receptor independent. Treated animals also showed a significant reduction in body weight, which the authors attributed mainly to the reduced food intake. EGCG when orally administered led to a lower reduction in food intake compared to the intraperitoneally treated rats, and no effects on the body weight were found (Kao, 2000). Long-term studies in mice also showed a reduction in the food intake. Female mice that were fed a diet that contains 4% green tea powder for 16 weeks showed suppressed body weight gain and food intake (Sayama et al., 2000). Additionally, Murase et al. showed that in C57Bl/6J mice that were fed 0.5% (estimated intake: 592 mg EGCG/kg bw) tea catechins with a high-fat diet for 11 months, the energy intake was reduced. However, the authors suggested that these effects are due to the reduction in body weight that was induced by this catechin dose, because a decreased body weight was observed prior (12 weeks) to the decreased energy intake. Lower (0.1% and 0.2%) catechin doses had no effects on the food intake, although they did reduce the body weight (Murase et al., 2002).

Human studies also show the effect of inhibiting the food intake. In overweight men, an intake of 1500 mg (1500/ 84.2= 17.8 mg/bw) of green tea extract reduced energy consumption in a 4-hour test. However, at the same time, green tea extract enhanced the desire to eat something sweet and something fatty (Belza et al., 2007)[53]. Intake inhibitory effects have been observed when a combination of catechins and other substances was tested; in these cases, it is difficult to conclude whether the effects are due to the individual components or due to additive/synergic effects. In overweight humans, a study of a preload of a beverage that contained fiber ± caffeine and EGCG followed by a test lunch and a recording of motivational ratings and food consumption showed that the beverage and caffeine was more satiating and led to a lower calorie intake at lunch than the beverage that had only fiber (Carter & Drewnowski, 2012). Additionally, in overweight subjects, an oily complex of EGCG (50 mg/capsule) and 85 mg N-oleyl-phosphatidilethanolamine (NOPE), a naturally occurring phospholipid, administered for 2 months together with a restricted energy intake, promoted diet compliance and an increased feeling of fullness and satiety and reduced feelings of hunger compared to the placebo group. Weight change, which was reduced by the low calorie diet, was not different between the treated group and the placebo (Rondanelli et al., 2009). Finally, in healthy humans who were under a three-week positive-balance intake, the ingestion of green tea plus capsaicin (a total daily intake of catechins: 1795.5 mg) for five separate days

significantly reduced their energy intake. These components individually showed a tendency toward reduction, but it was not significant. When individuals were subjected to a negative energy balance, the treatments did not significantly alter their energy intake. Hunger, the desire to eat and fullness were reduced, and satiety was increased, due to the combination of ingredients, in both positive and negative energy-balance experiments (Reinbach et al., 2009).

However, several other studies in animals, acute tests in humans or longer feeding studies in humans did not find changes in food intake due to tea catechins, although in many of these studies, a reduction in body weight was observed. Concerning animal studies, in female mice feeding on a diet with 2% (estimated intake: 2368 mg EGCG/kg bw) green tea powder for 16 weeks, there was a reduced body weight gain without any effects on energy intake. A combination of 0.3% catechins + 0.05% caffeine showed similar effects, and the catechins alone modified neither the energy intake nor the body weight (Zheng et al., 2004). Often, antiobesity effects are observed when there is an impedance of body weight gain rather than a reduction in the body weight. In obesity-prone NZB mice, TEAVIGO (green tea extract with an estimated daily dose of EGCG of 1.3 mg/kg bw and less than 0.1% caffeine) consumption for 29 days dependently reduced the increase in body weight observed after the feeding of an HFD, which was exclusively due to a reduction in body fat and without any effects on food intake (Klaus et al., 2005). Male Sprague –Dawley rats that were fed a high-fat diet together with green tea extract (estimated dose: 2300 mg/kg bw) showed a reduction in body fat gain without having any effects on the energy intake (Choo, 2003). In C57BL/6J mice that were fed a high-fat diet (60% energy as fat), supplementation with dietary EGCG treatment (3.2 g/kg diet; estimated dose: 425.6 mg/kg bw) for 16 wk reduced the body weight gain and the percent body fat. Additionally, 3-mo-old high-fat–induced obese mice that received short-term EGCG treatment (3.2 g/kg diet, 4 wk) had decreased mesenteric fat weight and tended to have a lower body weight. However, any of these experiments showed a modified energy intake that was due to EGCG treatment (Bose et al., 2008). No effect on the food intake was found in male C57BL/6 mice that were fed a high-fat/Western-style diet together with a 3.2 g EGCG/Kg diet (~ 10 cups/day of green tea) for 17 weeks. Although after 9 weeks of treatment, a significantly lower body weight gain in EGCG-treated rats was observed, at the end of the experiment, these animals weighed 9% less than the non-treated mice (Chen et al., 2011).

Concerning human studies, Gregersen et al. designed a study to analyze the effect of tea catechins ± caffeine on energy expenditure and fat oxidation, which included testing subjective appetite sensation by visual analogue scales. The study, a one-day test that was conducted on normal-weight healthy males who received capsules of caffeine mixed with tea catechins (600 mg), failed to show any effects on their appetite sensations (Gregersen et al., 2009). In obese subjects, the intake of a green tea extract that is rich in catechins (689.9 mg/day) for 12 weeks, without any modifications in lifestyle, induced a reduction in body weight without affecting the food intake (Nagao et al., 2007). In the same sense, in overweight Asian populations, a daily consumption of 500-900 mg of green tea catechins (with low-moderate amounts of caffeine) for 90 days exerted positive effects on the body composition and abdominal fat mass, but a reduction in the body weight was not accompanied by changes in the reported energy intake (Wang et al., 2010). Combinations of tea catechins and other substances have also shown a lack of effect on hunger, e.g., in overweight humans, EGCG together

with NOPE enhanced compliance to a low calorie diet for 4 weeks but did not have any significant effects on weight loss or feelings of hunger (Mangine et al., 2012).

Importantly, some studies have also found increased energy intake due to tea catechin consumption. A population of sedentary, middle-aged, overweight or obese men was given 530 mg decaffeinated green tea extract (DGT) twice daily for 6 weeks during 2 intervention periods. During the first intervention period, the body weight of the placebo group increased while that of the DGT group decreased, without any differences in food intake. During the second treatment period, the DGT showed increased energy intake compared with the placebo group, while the body weight was reduced in both groups, without significant differences due to the DGT treatment (Brown et al., 2011). Increased hunger and prospective food consumption was also observed after green tea treatment in overweight females who were eating a low-energy diet (weight loss intervention). The dose that was administered was 1125 mg tea catechins + 225 mg caffeine/day for 83 days, and no effect of the treatment on the body weight was observed (Diepvens et al., 2005). Increased hunger and lower satiety has been described in response to green tea-caffeine mixtures in overweight and moderately obese subjects who were first subjected to weight loss followed by a weight maintenance period. The dose that was administered, which also promoted weight maintenance, was 270 mg EGCG + 150 mg caffeine/day) (Hursel & Westerterp-Plantenga, 2009). These studies suggest that the effects of tea catechins promote weight maintenance but not weight loss and could lead to increased energy intake.

All of the previous data show that although there are several studies that report the effects of tea catechins on food intake, and some of them support a positive effect, the exact effects and mechanisms remain unresolved. In general, these studies suggest that a role of increasing energy expenditure and fat oxidation as well as inhibition of nutrient absorption might be more relevant than limiting the food intake in the reduction of body weight gain by green tea extract, according to the literature (reviewed in (Rains et al., 2011)).

Other flavanols besides tea catechins

Grapes, especially grape seed and skin, and their derivate beverages, such as wine, are another source of flavanols that have well-defined beneficial health effects (Puiggros et al., 2005; Bladé et al., 2010; Pinent et al., 2012; Terra et al., 2011). The type of flavanols in which grapes, and especially grape seeds and skin, are enriched differ from those found in tea. Grapes contain mainly catechin, epicatechin and their polymerized forms, and the content of EGCG in grapes is low (Quiñones et al., 2013).

For grape flavanols, the effects on food intake have not been studied as extensively as for tea catechins (summarized in Table 3). In fact, most of the studies focus on the effects on body weight and not specifically on food intake control. Several studies, which were mostly on animals, also point to a role of grape flavanols at impairing weight gain. In rats that were fed a hypercaloric diet for 8 weeks, moderate wine consumption (voluntary consumption) prevented an increase in body weight. This result was associated with a decreased food intake (Vadillo et al., 2006). Tebib et al. (1996) first described that feeding grape seed tannins at a dietary level of 7.1 mg per kg bw for a 12-wk period in male Sprague Dawley rats resulted in a reduced body weight gain. The authors

hypothesized that this finding might be due to a delayed absorption that is caused by flavanol polymers because a monomer-enriched diet did not induce such an effect. None of the diets modified the food intake (Tebib et al., 1996). It has been suggested that not only the effects on food absorption but also other mechanisms could lead to a reduction in weight gain. A monomer-rich grape seed extract at 0.5 or 1% in an HFD for 12 weeks reduced the increase in body weight in C57BL/6J mice without modifying the food intake, and the authors point to an increase in fatty acid oxidation as being responsible for the reduced weight gain (Ohyama et al., 2011). Grape seed procyanidins that were administered together with a high-fat diet (1 mg PE/g of feed) prevented the body weight gain that was induced by the diet, without modifying the total energy intake, and it prevented low-grade inflammation (Terra et al., 2011). A grape seed extract of 50.1% total flavanols, 49.08% procyanidins, and 1.02% monomeric flavanols was administered together with a high-fructose diet (at 0.5% and 1%) to male Sprague Dawley rats for 8 weeks. Both of the doses led to a reduced body weight without changes in the food intake. Grape seed procyanidins at a dose that is achievable by the human diet (25 mg/kg bw) and for a subchronic period of time (15 days) reduced the body weight gain in hamsters that were fed either a control or a high-fat diet. Such effects were not explained by a food intake reduction because the food intake was not modified. Instead, increased oxidation and the glycerol/fatty acid cycle in adipose tissue might explain the antiobesity effects (Caimari et al., 2013). In humans, compensatory effects of the polyphenols on thermogenesis and substrate oxidation were also suggested for concord grape juice, which is a source of catechin, myricetin, quercetin, anthocyanidins, and proanthocyanidins; when administered to healthy males for 12 weeks (480 ml/day), there were no differences in the food intake (as reported by the participants) or appetite sensations when compared to a polyphenol-free drink or a non-treatment control. The polyphenol-rich juice also did not lead to significant differences in the weight gain compared to the non-treatment controls, while the polyphenol-free juice did increase the weight gain (Hollis et al., 2009). Additionally, other studies report no effects on food intake while they also fail to find a modulation in the body weight. Grape seed extract (50 mg/kg bw) or fractions that were extracted in different solvents (30 mg/kg) were administered to male and female db/db mice (C57BL/KsJ-lepr^{db}/lepr^{db}) for 8 weeks, but this administration did not modify the body weight or food intake of the diabetic mice (Hwang et al., 2009). Additionally, no changes in the body weight were observed when two grape extracts (of the seed and skin) were tested for their potential toxicity. They were supplied together with the diet to outbred albino rats at 0.63, 1.25, and 2.5% (w/w). No effect on the food consumption was observed in the female rats. Instead, at the 2.5% dose of both extracts (mean equivalent to 1780 mg/kg bw day), the male rats showed a small but significant increase in their food intake from day 7 until the end of the study (3 months) compared to controls (Bentivegna & Whitney, 2002). Although it does not appear that inhibition of energy intake is a mechanism that explains the effects of grape flavanols avoiding weight gain, a short-term study on healthy humans taking grape seed procyanidin extract (300 mg containing > 90% procyanidins, in two intervention periods of 3 consecutive days separated by a washout period) showed a reduced 24-hour energy intake in subjects with an energy requirement ≥ the median of 7.5 MJ/day, without affecting satiety (Vogels et al., 2004).

The interest in defining the beneficial effects of flavanols has led to the study of several other sources that are enriched in such bioactive compounds. Because most of the studies that analyze food intake do aim to demonstrate a putative antiobesity effect, they are conducted in animal models of diet-

induced obesity. The polyphenols of a lingonberry extract (5.8% flavanols, 2.9% flavonols, 1.9% phenolic acids, and 1.5% anthocyanins) were analyzed, and the effect on this extract was assayed in rats that were fed a high-cholesterol, high-fat diet. The effects on the energy intake appeared to be dependent on the dose because the lowest dose (8 mg/day) significantly reduced the energy intake, while the highest dose (50.6 mg/day) increased it. A lower intake correlated with a reduced body weight gain in the low dose, but the high dose did not show any significant difference (Mane et al., 2011). Instead, there are more studies that show the effects of reducing the body weight without altering the food intake. Extracts from acacia bark, which is rich in flavan-3-ols such as robinetinidol and fisetinidol, were assessed in KKAy mice that were fed a high-fat diet, to induce severe obesity. Acacia polyphenols (2.5% or 5% (w/w) for 7 weeks) suppressed HF-induced body weight, and no effect on the food intake was found (Ikarashi et al., 2011). Hop (*Humulus lupulus* L.) is an herb that is used in beer production and contains flavonoids such as procyanidins and prenylflavonoids. Purified hop pomace polyphenols (60% procyanidins, 15% other flavonoids, 3% astragalin, 2% isoquercitrin and 20% unknown phenolic compounds) were administered to OLETF rats (a model of obesity and type 2 diabetes) at 1% for 70 days. Polyphenol-treated rats tended to show reduced final body weight and weight of the mesenteric adipose tissue, while the food intake did not change between the groups (Yui et al., 2013). Additionally, different plant extracts that are rich in polyphenols were tested for their putative antiobesity effects in a model of Wistar rats that were fed a high-fat sucrose diet for 56 or 64 days. Several extracts (apple, cinnamon, hamamelis and birch) lowered the body weight gain and improved the HOMA-IR index, and among them, apple and cinnamon were the most promising for being antiobesogenic because they also prevented the increase in total white adipose tissue, and no changes in the food intake were found (Boqué et al., 2013).

Thus, no clear evidence shows a modulation in the food intake by non-tea flavanols, although the number of studies for each extract was too low to establish firm conclusions. On the other hand, some action of the flavanols that are present in natural sources toward reducing body weight gain are described, and the underlying mechanisms could be similar to those described for tea and grape flavanols, such as a reduction in the fat absorption, a reduction in the inflammation (Boqué et al., 2013) and a regulation of fatty acid metabolism (Yui et al., 2013).

General conclusions

After having collected all of the available studies in which food intake is reported, the main conclusion is that there is a lack of reliable data that allows clear effects to be described. Flavanols are a large group of compounds, and the studies on their effects on food intake and the enteroendocrine system are scarce and diversified. From all of these studies, the involvement in the modulation of food intake from the effects of flavanols is not evident. However, the fact that there are some controversial studies that show positive or negative effects on food intake, together with the ability of some of these compounds to modulate some enterohormones, singles them out as possible candidates for acting through mechanisms that exert regulatory effects on food intake, which is a hypothesis that requires further work to be elucidated.

Table 1. Summary of the different subset of enteroendocrine cells, localization and hormone(s) secreted (adapted from Janssen and Depoortere (2012)

Cell Type	Highest density	Peptide released
G cells	Stomach	Gastrin
D cells	Stomach	Somatostatin
P or X/A cells	Stomach	Ghrelin
I cells	Duodenum	Cholecystokinin (CCK)
K cells	Duodenum	GIP
L cells	Duodenum, and colon	GLP-1, PYY
EC cells	Entire GI tract	5-HT (5-hydroxytryptamin)

Table 2a. Effects on food intake of tea catechins

Compound/ extract	Daily dose (mg/Kg bw) (way of administration)	Time of treatment	Diet	Specie	Effect on food intake	Ref.
EGCG	85 (i.p.)	7 days	Standard	Male Sprague Dawley rats	Reduced	(Kao 2000)
Green tea extract	2300 *estimated* (in the pellet)	2 weeks	High fat	Male Sprague – Dawley rats	No effect	(Choo 2003)
EGCG	92 (i.p.)	8 days	Standard	Male Zucker fatty	Reduced	(Kao 2000)
EGCG	425.6 *estimated* (in the pellet)	16 weeks	High fat	Male C57BL/6J mice	No effect	(Bose et al. 2008)
EGCG	425.6 *estimated* (in the pellet)	17 weeks	High fat	Male C57BL/6 mice	No effect	(Chen et al. 2011)
EGCG	592 *estimated*(in the pellet)	11 months	High fat	Male C57Bl/6J mice	Reduced	(Murase et al. 2002)
Green tea extract	2368 *estimated* (in the pellet)	16 weeks	Standard	Female mice	No effect	(Zheng et al. 2004)
TEAVIGO (94% EGCG)	1.3 (in the pellet)	29 days	High fat diet (corrective)	Male obesity prone NZB mice	No effect	(Klaus et al. 2005)

Table 2b. Effects on human food intake of tea catechins

Catechin mixture	8.5 *estimated* (pill)	1 dose	Standard	Normal weight healthy males	No effect	(Gregersen et al. 2009)
Green tea plus capsaicin	26.8 *estimated* (in the food)	1 day	Positive balance intake	Healthy humans	Reduced	(Reinbach et al. 2009)
Oily complex of EGCG	0.55 *estimated* (pills)	2 months	Restricted energy intake	Overweight humans	Promoted diet compliance	(Rondanelli et al. 2009)
Green tea extract + other agents	17,8 *estimaded* (orally)	8 weeks	Standard	Overweight men	Reduced	(Belza, Frandsen, and Kondrup 2007)
Beverage containing fiber ± caffeine and EGCG	2 *estimated* (orally)	Acute load	Standard	Overweight humans	Satiating effect	(Carter and Drewnowski 2012)
Green tea extract + caffeine	3 *estimated (*mixture with diet)	3 months	Maintenance diet ± high protein	Overweight subjects	Increased hunger and lower satiety	(Hursel and Westerterp-Plantenga 2009)
Green tea extract rich in catechins	9.44 (test beverage)	12 weeks	Standard	Obese subjects	No effect	(Nagao, Hase, and Tokimitsu 2007)
Green tea catechins + caffeine	5-12 *estimated* (test beverage)	90 days	Standard	Overweight subjects	No effect	(Wang et al. 2010)
Green tea extract	10.6 (pill)	6 weeks	Standard	Overweight men	No clear effect	(Brown et al. 2011)
Green tea extract + caffeine	16 *estimated* (synthetic formula)	83 days	low energy diet	Overweight females	Increased hunger	(Diepvens et al. 2005)

Table 3 Effects on food intake of catechins and procyanidins

Compound (extract)	Daily dose (mg/Kg bw) (way of administration)	Time of treatment	Diet	Specie	Effect on food intake	Ref.
Red wine consumption	14.2 *estimated* (oral)	8 weeks	Hypercaloric diet	Male Zucker lean	Decreased	(Vadillo et al. 2006)
Grape seed procyanidins	30 (in the pellet)	19 weeks	High fat diet	Females Wistar	No effect	(Terra et al. 2011)
Grape seed tannins	7 (in the pellet)	12 weeks	Standard	Male Sprague-Dawley rats	No effect	(Tebib, Besançon, and Rouanet 1996)
Two grape extracts (of seed and skin)	1780-2150 (in the pellet)	3 months	Standard	Sprague-Dawley	Increase in males. No effect in females	(Bentivegna and Whitney 2002)
Grape seed procyanidins	25 (in the pellet)	15 days	high fat diet	Male Golden Syrian hamsters	No effect	(Caimari et al. 2013)
Grape seed extract	1.2 *estimated* (in the pellet)	12 weeks	HFD	Male C57BL/6J mice	No effect	(Ohyama et al. 2011)
Grape seed extract	50 (in water, with syringe)	8 weeks	Standard	C57BL/KsJ-lepr^{db}/lepr^{db}	No effect	(Hwang et al. 2009)
Acacia bark	2.5 or 5 *estimated* (in the pellet)	7 weeks	High fat diet	Male mice KKAy	No effect	(Ikarashi et al. 2011)
Polyphenols of a Lingonberry extract	23-48 (in the pellet)	6 weeks	High cholesterol high fat diet	Male Wistar rats	Reduced-increased	(Mane et al. 2011)
Hop pomace polyphenols	200 (in the pellet)	10 weeks	Standard	Male OLETF rats	No effect	(Yui et al. 2013)
Concord grape juice	11.67 *estimated* (juice)	12 weeks	Standard	Healthy human males	No effect	(Hollis et al. 2009)
Grape seed procyanidin extract	12 estimated (3 pills)	3 days	Standard	Healthy humans, some overweight	Reduced in high EI group	(Vogels, Nijs, and Westerterp-Plantenga 2004)

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Joan Serrano López

HYPOTHESIS AND OBJECTIVES

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5. HYPOTHESIS AND OBJECTIVES

Phenolic compounds are pervasive in plants and therefore plentiful in ordinary human diets. Among other health-related effects, the consumption of dietary phenolic compounds has been associated with beneficial effects on body weight, and this has led to a great deal of research into their mechanisms of action in an attempt to improve the well-being of our increasingly overweight society. Of all the phenolic compounds, phenolic acids and flavonoids are the groups most commonly found in the human diet and, therefore, the most widely studied.

In the particular field of body weight regulation, the inhibition of nutrient absorption and the increase in energy expenditure are still the main effectors of dietary phenolic compounds such as tea- and grape-derived compounds.

In some studies in humans, the administration of food-derived phenolic compounds has been shown to change the plasma levels of satiety-related enterohormones such as GLP-1 or ghrelin, although there is no proof that they have a general effect on any enterohormone. In particular, cellular studies in rodents strongly suggest that flavonoids influence GLP-1 secretion, which may play a role in vivo as a satiety signal. Moreover, ghrelin secretion has not been thoroughly studied for grape-seed phenolics, even though some studies on soy isoflavones and green tea extracts have found it to be reduced.

On the basis of the recent finding that the administration of a grape-seed proanthocyanidin extract acutely and chronically increases GLP-1 in rats, **we hypothesized that the administration of grape-seed phenolic compounds influences the release of the main satiety-related enterohormones and, therefore, influences food intake and body weight in rats.** Therefore, the main objective of this thesis was to assess whether grape-seed phenolic compounds can reduce food intake and body weight in rats by changing the release of the main satiety-related enterohormones.

To fulfill this main objective, we established a set of **specific objectives:**

1. To determine whether a grape-seed proanthocyanidin extract affects food intake in rats

Since it is known that a single administration of 1 g/kg body weight (BW) of a grape-seed proanthocyanidin extract increases plasma GLP-1 in rats and GLP-1 is a satiety signal, one straightforward objective is to determine whether this administration can also reduce food intake. Hence, we aimed to discover whether 1 g/kg BW and lower doses of grape-seed proanthocyanidins influence food intake in male and female rats under different patterns of administration and diets, while screening for the effects of other sources of phenolic compounds.

2. To study whether the acute effects of a grape-seed proanthocyanidin extract on food intake could be reproduced in subchronic treatments

Since satiety agents are intended to help maintain or reduce body weight, they must be effective over time in the mid- and long-term. Moreover, grape-seed phenolics may not only affect food intake, but also nutrient absorption and energy expenditure, which may also affect body weight. Hence, we also aimed to discover whether acute effective doses of a grape-seed proanthocyanidin extract are effective in short 8-day subchronic treatments and contribute to changes in body weight, and to compare its effects on food intake with its effects on nutrient absorption and energy expenditure.

3. To study the effects of grape-seed phenolics on enterohormone secretion, and especially ghrelin secretion, and the role of ghrelin and GLP-1 in the possible effects of grape-seed phenolics on food intake

While a grape-seed extract may be a satiating agent per se that can be translated to human studies, a greater understanding of the physiological pathways and the compounds involved in the satiety effect may enable treatments to be improved via new formulations. Hence, we aimed to discover whether acute treatment with grape-seed proanthocyanidin extract affects CCK, GLP-1, PYY and ghrelin plasma levels, and whether the acute effects of a grape-seed proanthocyanidin extract and a selected grape-seed phenolic compound on food intake are specifically driven by GLP-1 signaling.

Given the lack of studies on the effects of grape-seed phenolics on ghrelin release and their possible effects on food intake, we also aimed to study the dose-response effects of the main grape-seed phenolic compounds on ghrelin release and the effect of subchronic treatments with a grape-seed proanthocyanidin extract on ghrelin levels in rats and to analyze whether GLP-1 and ghrelin gene expression in rats are related to food intake after a subchronic treatment with a grape-seed proanthocyanidin extract.

RESULTS

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6. ACUTE EFFECTS OF A GRAPE-SEED PROANTHOCYANIDIN EXTRACT ON FOOD INTAKE AND GASTROINTESTINAL HORMONES IN RATS

Given our interest in unveiling a possible satiating effect of grape-seed phenolic compounds, we first screened the possible effects of a grape-seed phenolic extract in rats.

Herein, we present **manuscript 1** followed by a proof of its **related patent**, where we studied the satiating effects of a grape-seed phenolic extract in rats (**objective 1**) and their concomitant effects on the secretion of four gastrointestinal hormones (**objective 3**).

Given that any possible effect on food intake may present an important applicability per se (as seen in the **related patent**), the causal effects of gastrointestinal hormones in food intake (also part of **objective 3**) were not studied herein, but presented in a later chapter.

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6.1 Manuscript 1

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Acutely administered grape-seed proanthocyanidin extract acts as a satiating agent†

Joan Serrano,‡ Àngela Casanova-Martí,‡ Katherine Gil-Cardoso, M. Teresa Blay, Ximena Terra, Montserrat Pinent* and Anna Ardévol

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Grape-seed proanthocyanidins' role as stimulators of active GLP-1 in rats suggests that they could be effective as satiating agents. Wistar rats were used to study the effects of proanthocyanidins on food intake with different doses, administration times and proanthocyanidin extract compositions. A dose of 423 mg of phenolics per kg body weight (BW) of grape-seed proanthocyanidin extract (GSPE) was necessary to decrease the 12-hour cumulative food intake by 18.7 ± 3.4%. Proanthocyanidins were effective when delivered directly into the gastrointestinal tract one hour before, or simultaneously at the start of the feeding period. Proanthocyanidins without galloyl forms, such as those from cocoa extract, were not as effective as grape-seed derived forms. GSPE increased the portal levels of active GLP-1 and total ghrelin and decreased the CCK levels, simultaneously with a decrease in gastric emptying. In conclusion, grape-seed proanthocyanidins could be useful as a satiating agent under the conditions defined in this study.

Introduction

As a result of increased food consumption, decreased physical activity and changes in the nutritional value of foods, overweight and obesity are today major problems in developed societies.[1] The relationship between overweight and various related diseases, such as high blood pressure, coronary artery disease, stroke, type 2 diabetes mellitus, hyperlipidemia, arthritis/degenerative joint disease, obstructive sleep apnea, infertility, gallstones and depression, among others, is well known. Many strategies have been proposed to solve these problems, and one of these is the administration of functional ingredients.[2,3] Functional ingredients could act against excessive body weight gain, by means of several mechanisms, such as modifying the activity of the digestive enzymes,[4] hampering nutrient absorption and/or inducing thermogenic effects.[3,5] Another strategy for functional ingredients is to reduce food intake by several mechanisms, such as reducing the desire for food, decreasing the feeling of hunger, inducing fullness and early meal termination and maintaining the sensation of fullness between meals.[6]

Proanthocyanidins are a group of polyphenols widely distributed in nature (in fruits, vegetables and their beverage products such as red wine and tea). They have been shown to have protective effects against several diseases[7–10] but their effects on food intake have been scarcely studied.[11] Most studies on proanthocyanidins have focused on their effects on body weight, but not specifically on food intake control. Some animal studies mention a role of grape flavanols in impairing weight gain,[12,13–16] although food intake was neither measured nor modified in many of them. A short-term study of healthy humans consuming grape-seed proanthocyanidin extract (GSPE) showed a reduced 24-hour energy intake in subjects with an energy requirement ≥ the median of 7.5 MJ per day.[17] As regards the mechanisms that may be involved in this reduction of food intake, a recent review shows that some polyphenols appear to have the potential to modulate food intake due to their action on the neuropeptides involved in food intake and satiety, such as NPY, AgRP, POMC and CART, although how the polyphenols affect the function of these peptides in the brain is not fully understood.[18] A direct site of action of proanthocyanidins could be the enteroendocrine system, where enteroendocrine cells secrete peptidic hormones that control digestive processes and food intake, such as the orexigenic hormone ghrelin and the anorexigenic hormones cholecystokinin (CCK), glucagon-like-peptide-1 (GLP-1) and peptide YY (PYY)[19] that could, in turn, affect the neuropeptide system. Proanthocyanidins were recently shown to modulate GLP-1 levels through their capacity to inhibit the GLP-1-degrading protease dipeptidyl-peptidase 4 (DPP4).[20] Proanthocyanidins also increase GLP-1 plasma levels in rats after an oral glucose load,[21] although direct GLP-1 stimulatory effects were

Universitat Rovira i Virgili, Biochemistry and Biotechnology Department, c/Marcel lí Domingo no. 1, 43007, Tarragona, Spain. E-mail: montserrat.pinent@urv.cat

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‡These authors contributed equally to this work.

not found in the STC-1 cell line.[22] Finally proanthocyanidins might also act through alteration of the number of entero-endocrine cells in the intestine.[23] Similarly, a single administration of berry purée (800 mg polyphenols including proanthocyanidins and other flavonoids) together with sucrose in healthy humans tended to increase GLP-1,[24] and the proanthocyanidin cinnamtannin A2 increases GLP-1 in mice.[25] Ghrelin, which is produced mainly in the stomach and which has a main role in the regulation of food intake, is modulated by isoflavones, but there is less information regarding other types of flavonoids. There is even less information on the effects of flavonoids on other enterohormones such as PYY or CCK and, indeed, none at all about proanthocyanidins.[11]

The aim of this study is to analyze whether proanthocyanidins modify food intake and the food intake-controlling enterohormones. Two proanthocyanidin extracts with different compositions from cocoa and grape seeds were compared under standard feeding conditions to test their effects on food intake. GSPE was also tested in two different models of disrupted metabolism very common in human societies under overfeeding situations: a hyperphagic model caused by a tasty diet and an impaired glucose tolerant aged rat model.

Results

Grape-seed proanthocyanidins limit food intake

We hypothesized that the interaction of proanthocyanidins with the enteroendocrine system, previously shown to raise GLP-1 levels,[21] could lead to a reduction in food intake. We thus first tested GSPE, at a dose that had previously been shown to be effective at increasing GLP-1 levels, of 846 mg phenolics per kg BW. The GSPE significantly reduced periodic and cumulative food intake 12 hours (h) after feeding initiation in healthy rats (Fig. 1A and B) and in a glucose-resistant model (Fig. 1C and D). A conditional taste aversion (CTA) test and a pica behavior test were performed and no signs of gastrointestinal malaise were found. In the CTA test, the rats developed aversion to a new vanilla flavor when it was paired with a treatment of the nausea-inducing agent LiCl, while the GSPE dose did not produce this effect (Fig. 2A). In the pica test, the rats reduced their daily cumulative intake of chow and increased

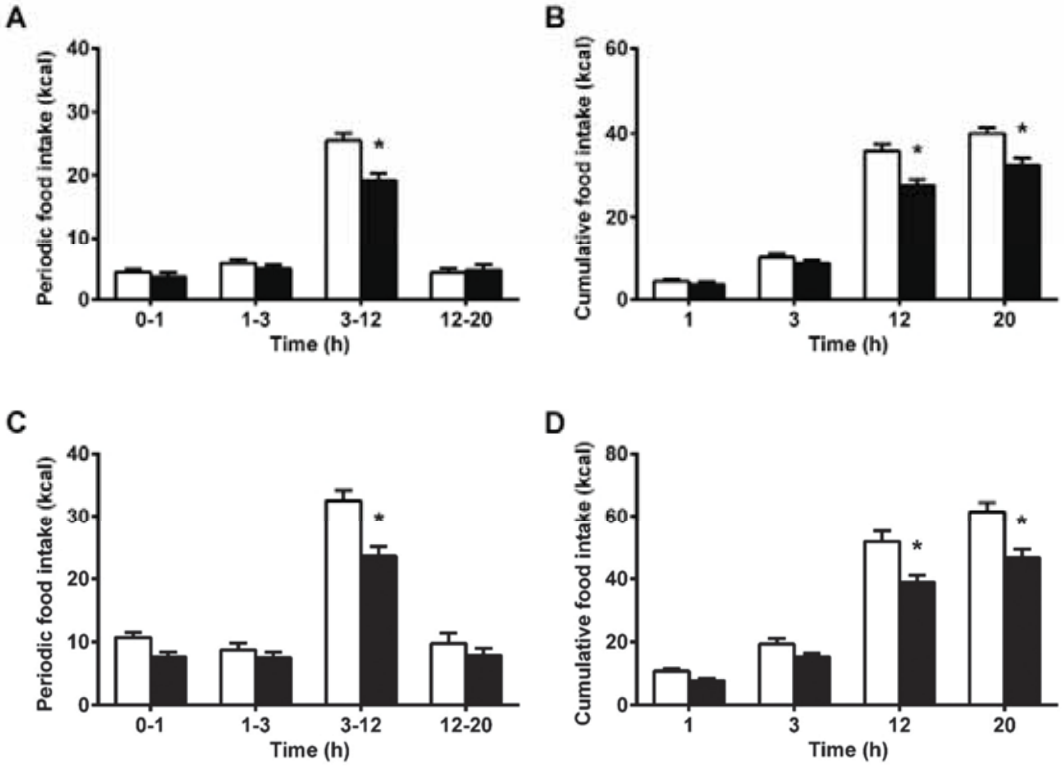

Fig. 1 GSPE effects on food intake in normal and impaired glucose-tolerant rats. Periodic (A) and cumulative (B) food intake in healthy female Wistar rats and in glucose-resistant male rats (C, D). Daily chow intake was measured at each time point after an intragastric dose of GSPE (846 mg per kg BW). Light bars correspond to control (vehicle-administered) animals and dark bars to GSPE-treated animals. A mixed-model two-way ANOVA showed significant time, treatment and interaction terms in both cases ($p < 0.05$). Asterisks represent significant differences in food intake of treated *vs.* control group at each time point, assessed by Bonferroni *post-hoc* tests ($p < 0.0001$).

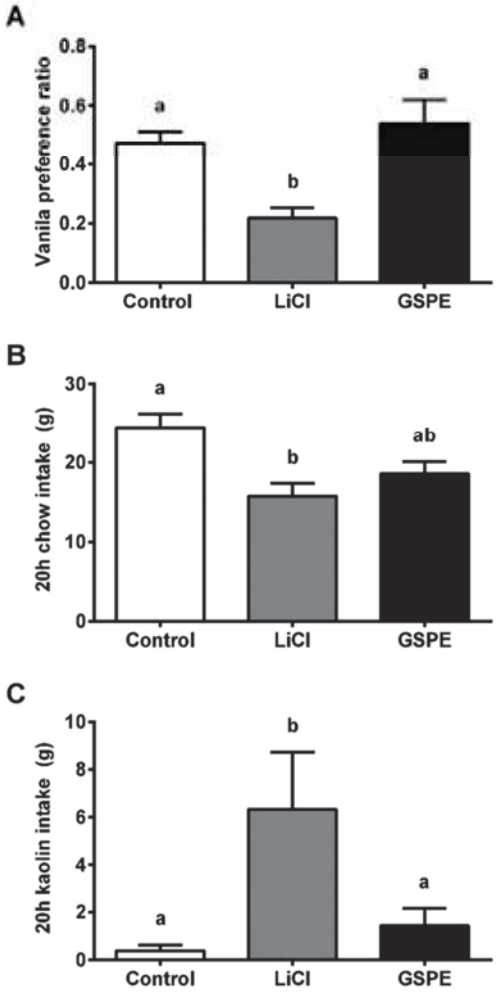

Fig. 2 GSPE effects on the gastrointestinal comfort. Conditional taste aversion test (A) and pica behavior test (B, C) after an intragastric dose of GSPE (846 mg per kg BW). LiCl was used to induce gastrointestinal distress. The different letters represent significant differences between groups assessed by Bonferroni *post-hoc* tests after a significant one-way ANOVA ($p < 0.05$).

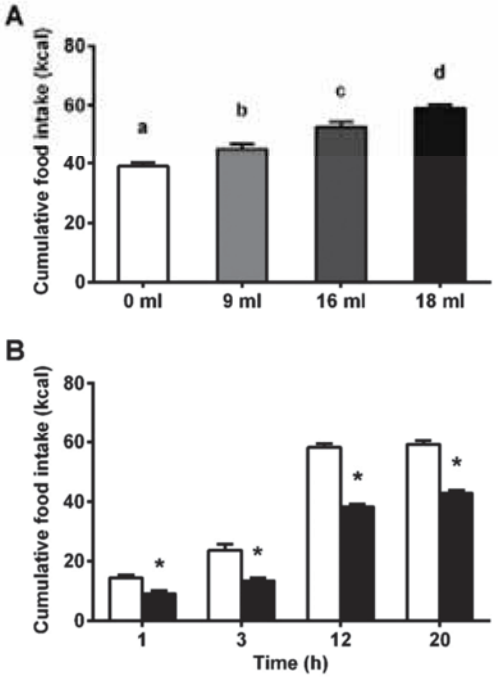

Fig. 3 GSPE effects on food intake under hyperphagic conditions. (A) Effects of the diet supplementation with a palatable hypercaloric solution (HS) on the daily energetic intake. Increased amounts of offered HS proportionally increased the daily energy intake. The different letters represent significant differences between groups assessed by Bonferroni *post-hoc* test after a significant one-way ANOVA ($p < 0.05$). (B) GSPE effects on the cumulative food intake in a hyperphagic paradigm. An intragastric dose of GSPE (846 mg per kg BW) was gavaged and food intake was measured at each time point after a diet supplementation with 16 ml HS. Asterisks represents significant differences in food intake of treated (dark bars) *vs.* control group (light bars) at each time point, assessed by Bonferroni *post-hoc* tests ($p < 0.01$) after significant time, treatment and interaction terms in a two-way ANOVA ($p < 0.05$).

their intake of non-nutritive kaolin pellets when treated with LiCl (Fig. 2B). The GSPE treatment led to a similar reduction in food intake, but it was not accompanied by an increase in kaolin consumption (Fig. 2C).

To disrupt food intake control, we stimulated the animals' energy consumption with a palatable food offer. As shown in Fig. 3A, increasing amounts of a palatable hypercaloric solution proportionally increased energy intake. Fig. 3B shows that under these conditions, food intake was significantly reduced since the beginning of the feeding period. Despite the significant effects on food intake appeared earlier in the hyperphagic model, the % inhibition in food intake in the normophagic model was constant from 1 hour to 20 h. A 2-way ANOVA of experiments with different degrees of hyperphagia discarded any effect of time in the reduction of food intake, but showed an enhancement in the GSPE action proportional to the degree of hyperphagia (Table S1†).

Conditions required for a satiating effect of proanthocyanidins

To better define the satiating effects of the GSPE, we compared different dosages, administration times and animal models. The results are shown in Table 1. In first place, we found that the effects of GSPE on food intake were not affected by gender or age, since a GSPE dose of 846 mg phenolics per kg BW produced similar satiating effects in female and aged male Wistar rats. Afterwards, the importance of the time of administration was studied. We found that the satiating effects of 846 mg phenolics per kg BW were similar when the extract is administered 1 h before food and when the extract is administered at the same time as food. Conversely, there was no effect when the GSPE was administered 1 h after the meal initiation.

Regarding the dosage, we found that 423 mg phenolics per kg BW significantly reduced food intake in a standard feeding

Table 1 Satiating effects of proanthocyanidins under different conditions

Sex	BW (g)	Diet	Time of dose	Extract	Dose	12 h inhibition (%)
Effects of different GSPE moments of administration in both genders						
♀	207	Chow	−1 h	GSPE	846	22.2 ± 3.8**
♂	542	Chow	−1 h	GSPE	846	25.0 ± 4.5**
♀	204	Chow	0 h	GSPE	846	27.7 ± 9.0*
♂	543	Chow	1 h	GSPE	846	16.2 ± 7.1
Effects of lower GSPE doses under standard and hyperphagic conditions						
♀	223	Chow	−1 h	GSPE	423	18.7 ± 3.4***
♀	217	Chow + 11 ml HS	−1 h	GSPE	423	15.9 ± 3.1*
♀	219	Chow + 11 ml HS	−1 h	GSPE	84.6	1.4 ± 5.0
Effects of CCE at doses equivalent to GSPE						
♀	196	Chow	−1 h	CCE	423	−3.86 ± 4.4
♀	196	Chow	−1 h	CCE	846	−3.56 ± 2.0
♂	542	Chow	−1 h	CCE	846	1.8 ± 4.8

Satiating effects of proanthocyanidins observed with different doses, diets, time of administration, animal models, and types of extracts. Time of dose indicates the hour of the extract treatment referred to the food replacement (0 h). Dose is expressed as mg phenolics per kg BW. Food intake data are expressed as 12 h cumulative food intake inhibition (%) of treated *vs.* control group. Asterisks represent significant differences assessed by an unpaired *t*-test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

model and a hyperphagic model. A lower dose of 84.6 mg phenolics per kg BW presented no effect in food intake.

Finally, the importance of the extract composition was studied by treating the rats with a different proanthocyanidin-rich extract, derived from cocoa (CCX), at doses with a total phenolic content and antioxidant capacity equivalent to the assayed GSPE doses (423 or 846 mg phenolics per kg BW). The treatment with CCX did not inhibit food intake.

GSPE causes changes in portal enterohormones and delays gastric emptying

Our next goal was to analyze the hormonal changes induced by 846 mg phenolics per kg BW of GSPE. Twenty minutes (min) after GSPE gavaging, there were no changes in active GLP-1 (3.22 ± 0.37 *vs.* 4.01 ± 0.36 pmol L^{-1}) or in insulin (2.68 ± 0.53 *vs.* 2.57 ± 0.3 μg L^{-1}). Table 2 shows the lack of changes in insulin, CCK or total ghrelin levels sixty min after the GSPE administration. At this point (t = 60 min), we mimicked food intake by an intragastric meal. Twenty min after the intragastric meal (t = 80 min), the GSPE group showed increased active GLP-1 levels (3.84 ± 0.35 *vs.* 8.64 ± 1.00 pmol L^{-1}, $p < 0.05$), which led to an increase in insulinemia observed at the same time point (1.76 ± 0.31 *vs.* 3.29 ± 0.72 μg L^{-1}, $p < 0.05$). Sixty min after the intragastric meal (t = 120 min), the treatment also increased total ghrelin (4.78 ± 0.46 *vs.* 6.63 ± 0.77 μg L^{-1}, $p < 0.05$) and prevented the postprandial rise in CCK observed in the control group (2.00 ± 0.45 *vs.* 0.76 ± 0.05 μg L^{-1}, $p < 0.05$). All these changes were not due to the modulation of mRNA expression (Table 3).

Concomitantly, we found that 1 h after the intragastric meal, the GSPE group retained a larger amount of food in the stomach than the control group (Fig. 4).

Table 2 Portal hormone levels 60 min after the GSPE gavage

Hormone (μg L^{-1})	Control	GSPE
Insulin	0.98 ± 0.33	0.80 ± 0.18
Ghrelin	3.87 ± 0.31	3.45 ± 0.17
CCK	0.67 ± 0.06	0.75 ± 0.06

No significant differences, assessed by *t*-test ($p < 0.05$) were found between control and GSPE.

Table 3 Gene expression of the enterohormones measured in plasma (relative units)

	Control	GSPE
Ghrelin[a]	1.19 ± 0.31	1.35 ± 0.18
CCK[b]	1.00 ± 0.01	1.04 ± 0.15
Proglucagon[c]	1.12 ± 0.22	1.07 ± 0.21

[a] Measured in corpus. [b] Measured in duodenum. [c] Measured in colon. No significant differences, assessed by *t*-test ($p < 0.05$) were found between control and GSPE.

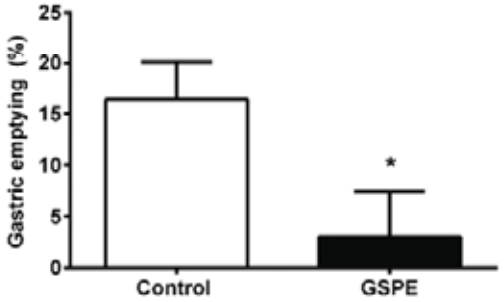

Fig. 4 GSPE effects on gastric emptying. Percentage of food emptied from the stomach 1 h after the gavaging of 1.5 g mashed chow in control rats (light bars) and in rats treated with 846 mg per kg BW GSPE (dark bars). The results are expressed as a % of dry matter relative to a paired oven-dried mashed chow. The asterisk represents significant differences assessed by an unpaired *t*-test ($p < 0.05$).

Discussion

As recently reviewed,[11] few studies have evaluated the effect of flavonoids on food intake, and most of them evaluated it as a secondary issue in studies designed to analyze the effects on body weight and energy balance. In the present study we describe the clear effects of a grape-seed extract on inhibiting food intake, and we also define the effective dose and administration mode.

Our results show that GSPE is effective at reducing food intake when administered under specific conditions that we

will define. We found that the minimum amount of proanthocyanidins to be effective is higher than 86.2 mg phenolics per kg BW. From the doses that we tested, the minimal effective dose was 423 mg phenolics per kg BW. Other studies, focusing mainly on the effects on body weight, report no effects of grape-seed extracts on food intake. This discrepancy might be due to the fact that most of these studies used lower doses.[26,27] Another reason could be the different methods of administration (proanthocyanidins alone or with other food). In most of the studies where no effect on food intake was observed, the extract had been administered with different vehicles, many of which included other nutrients such as standard chow,[16,28] high-fat chow and condensed milk.[15] Our data demonstrate that the satiating effect of proanthocyanidin extract was found when it was administered one h before or just before the beginning of the feeding period, while there was no effect on food intake if the extract was administered 1 h after food initiation. This highlights the need for an almost empty gastrointestinal tract when the GSPE is administered for it to be effective. Some authors have proved that the phenolic compounds interact with food components, *i.e.* proteins.[29] Furthermore, some studies showing an inhibitory effect on food intake attribute it to the discomfort caused in the oral cavity because of its tannic effect (astringency).[30] Proanthocyanidins were mixed with drinking water or food in these studies.[27] In this paper, we avoided this problem by administering the extract intragastrically. The proanthocyanidins thereby reach the stomach and duodenal mucosa directly. We also discarded any alteration in both tissues using histological analysis (results not shown), as well as we made sure that the treatment does not cause gastrointestinal malaise by pica and conditional taste aversion tests.

Another important point is related to the quality of food intake measurements. Measures taken using inadequate methodology could produce controversial results, which create doubts regarding the effectiveness of food intake treatments.[31] This could also bias the food intake results obtained in the previous studies focused on the effects of proanthocyanidins on body weight and metabolic parameters. For this reason, in this study animals and people were specifically trained in order to obtain reliable data, as explained in the Experimental section.

The composition of the extract must also be considered. Our results support the theory that the effectiveness of a grape-seed derived extract is higher than a cocoa derived extract. The GSPE extract used here contains monomeric (epi)-catechins, their gallate forms, dimers and trimers of all these monomeric forms[32] and some higher structures.[33] We do not rule out the presence of some prodelphinidins, as defined by Gu *et al.*[34] We have confirmed that some concordant grape-seed proanthocyanidin extracts also produce an inhibition of food intake (results not shown). Conversely, cocoa extract, which contains proanthocyanidins without galloyl forms,[35] did not cause a clear satiating effect. This suggests that the bioactivity of GSPE in inhibiting food intake might be attributed to the galloyl forms of proanthocyanidins. In this regard, different flavonol structures have different abilities to stimulate the taste receptors located at the enteroendocrine cells, with their galloyl forms being most effective at binding to these taste receptors.[36] The precise link between the differential stimulation of taste receptors by different flavonoid structures and the enteroendocrine signals generated from the gastrointestinal tract in response has not yet been determined. We have previously proved that GLP-1 levels increase after the administration of GSPE at the same doses as those used in this study.[21] Moreover, a revision of this topic[11] shows that flavonoids modulate GLP-1 levels and ghrelin, but there is very little information on their effects on other enterohormones. Importantly, whether such modulation involves effects on food intake was not assessed in any of these studies.

A common mechanism of the anorexigenic action of GLP-1 and CCK consists of the reduction of the gastrointestinal transit, contributing to both meal termination and feeling of fullness.[37] Our results show that GSPE leads to a reduction in gastric emptying in unconscious rats, concomitantly to an increase in active GLP-1. This inhibition of gastric emptying could help explain the inhibition of food intake. A different effect is found for CCK, which only increased after the meal in the control group. Although we are unable to formally rule out an inhibition of CCK release, baseline CCK levels are maintained in the GSPE group, suggesting that CCK levels may not rise in the treated group due to the slower gastric emptying to the duodenum.

Meanwhile, ghrelin, in its acylated form, is the only enterohormone with orexigenic properties.[38] Unexpectedly, we found an increase in total ghrelin after the GSPE treatment. Despite this increase, the food intake data clearly show that the reduction in food intake is sustained over time after the treatment. A possible explanation could be related to the results of Toshinai *et al.* which show that the orexigenic action of ghrelin is abolished with a prior rise of GLP-1 acting on vagal afferents.[39] Our data suggest that this ghrelin desensitization could also occur in the group treated with GSPE, since the stimulation of the GLP-1 release occurred prior to the increase in total ghrelin.

Both effects on portal enterohormone levels occur after the intragastric meal. Although our study was not designed to evaluate the effects of nutrients on the GSPE action, this result suggests that other molecules must be present in the gastrointestinal tract, after GSPE administration, to produce changes in the release of enterohormones. We have confirmed earlier findings that GSPE influences the secretion of active GLP-1 after a nutrient load[21] and extended them to changes in the secretion of CCK and ghrelin.

Finally, we have proved the effectiveness of GSPE administration in healthy animals with an adjusted food intake control, and an even greater effectiveness with a disrupted food intake control, as occurs with a highly palatable diet.[40] This greater effectiveness with the high-sucrose palatable diet could point to a role of GLP-1 through an increased glucose-stimulated GLP-1 release. We have also proved the extract's bioactivity in a model of impaired glucose tolerance (aged

rats), highlighting glucose-intolerant subjects as a potential target. Both results point to the utility of this treatment in individuals with difficulties controlling food intake who usually have concomitant defective glucose tolerance.

Experimental

Proanthocyanidin extracts

The grape-seed extract enriched in proanthocyanidins (GSPE) was obtained from Les Dérivés Résiniques et Terpéniques (Dax, France). According to the manufacturer, the extract contains monomeric (21.3%), dimeric (17.4%), trimeric (16.3%), tetrameric (13.3%) and oligomeric (5–13 units; 31.7%) proanthocyanidins. The small molecules (up to trimers) present in GSPE were characterized in more detail by reverse-phase chromatographic analyses by our research group.[32] Cocoa derived extract (CCX) was kindly provided by Dr Julian Castillo of Nutrafur, S.A. (Murcia, Spain). According to the manufacturers, the extract contains epicatechin (6.84%), catechin (0.86%), B2 (4.15%) and B1 (0.73%). It was possible to obtain a complementary quantification from Quinones *et al.*[35] The phenolic content of both extracts was quantified with the Folin method[41] as 845.5 ± 10.5 mg g^{-1} GSPE and 452 ± 9.94 mg g^{-1} CCE.

Animals

Two sets of female Wistar rats (Harlan, Barcelona, Spain), weighing 180–200 g upon arrival, were used for the conditional taste aversion test (n = 21) and for the food intake studies and blood sampling (n = 21). Two sets of adult male Wistar rats (Harlan, Barcelona), weighing 450–500 g, were used for the pica test (n = 18) and the food intake studies (n = 15), as a model for glucose-impaired tolerance.[42] The studies in male groups took place at the facilities of the Technological Center of Nutrition and Health (http://www.ctns.cat). All of the procedures were approved by the Experimental Animal Ethics Committee of the Universitat Rovira i Virgili.

On arrival, the subjects were single housed at 22 °C under a 12 h light/dark cycle (lights on at 8 am) with access to standard chow pellets (Teklad Global Diets #2014, Harlan, Barcelona) and tap water *ad libitum* during a 1 week adaptation period. Following this adaptation period, the animal sets for the food intake studies were introduced to a daily 4 h food deprivation before the light offset (from 16:00 h until 20:00 h), and blank chow intake measurements were taken on 4 days prior to each experiment, to habituate the subjects to the experimental schedule. One experiment per week was performed in a cross-over design for all the food intake studies. The animal sets for the gastrointestinal malaise tests underwent special adaptation conditions, as described below.

Food intake experiments

Standard diet. To assess the effects of an acute dose of proanthocyanidins on food intake, trained animals were treated with GSPE or CCX doses from 423 to 846 mg phenolics per kg of BW intragastrically (i.g.) 1 h before the dark onset (unless otherwise stated), using tap water as a vehicle, and the chow intake was measured 1, 3, 12 and 20 h after the chow replacement with an accuracy of 0.01 g. Parallel controls were performed by administering the vehicle intragastrically.

Palatable hypercaloric meal. The effect of proanthocyanidins under a hypercaloric meal was tested only in female rats, due to their sensitivity to diet challenges[43] using a palatable high-sucrose emulsion (HS) presented in an independent bottle, containing (by weight) 10% powdered skimmed milk, 40% sucrose, 4% lard and 0.35% xanthan gum as a stabilizer (Sigma-Aldrich, St Louis, MO), with a caloric density of 2.10 kcal g^{-1}.[44] A GSPE dose of 846 mg phenolics per kg of BW (unless otherwise stated) or the vehicle was administered i.g. 1 h before the dark onset, and chow and HS intake were measured 1, 3, 12 and 20 h after the meal presentation. The effect on different hypercaloric situations was assayed by administering different HS volumes (0, 9, 16, 18 ml) in separate experiments.

Gastrointestinal malaise tests

Conditional taste aversion (CTA). Seven days prior to the conditioning day, animals were introduced to a water-restriction schedule, so they were accustomed to drinking the whole daily fluid intake in single 30 min sessions (starting at 12:00).[45] In order to habituate rats to a 2-bottle choice, water was presented in two sipper bottles simultaneously. On the conditioning day, the rats were given access to two bottles of 0.5% vanilla flavored solution (Dr Oetker, Barcelona) and after the 30 min drinking period, the bottles were removed and the subjects were administered i.g. water, i.g. GSPE (846 mg phenolics per kg of BW) or intraperitoneally (i.p.) with the gastrointestinal distress-inducing agent LiCl (0.15 M, 2% body weight). On the first day after conditioning, the rats had 30 min access to water, and on the subsequent day, a two bottle preference test between water and 0.5% vanilla was conducted. The position of the two bottles was counter-balanced between the subjects and fluid consumption was measured with an accuracy of 0.01 g after the 30 min session. The results were expressed as a preference ratio of the drug-paired flavor by dividing the intake of flavored solution to total fluid intake.

Pica behavior test. Human grade kaolin powder (A. Vogel, Barcelona) was mixed with 1% arabic gum (Sigma-Aldrich, St Louis) and tap water, squeezed through a pastry decorator and allowed to dry at 35 °C.[46] The resulting kaolin pellets, similar in shape and size to chow pellets, were introduced to subjects seven days prior to the pica test, placed with chow in the food hoppers. On the test day, the animals were administered i.g. water, i.g. GSPE (846 mg phenolics per kg of BW) or i.p. the gastrointestinal distress-inducing agent LiCl (0.15 M, 2% body weight) just before the dark onset, and the chow and kaolin intake were measured 20 h later.

Portal vein catheterization and intragastric feeding

The female rats from the food intake set were randomly divided into two groups: a control group (n = 10) treated with

tap water, and a proanthocyanidin treated group (n = 11) gavaged with GSPE at 846 mg phenolics per kg BW. The rats were fasted from 15.00 h until 18.00 h before the treatment, and were anesthetized 5 min later with 70 mg per kg BW i.p. of sodium pentobarbital. The abdominal cavity was incised through the midline, and the portal vein was catheterized with a PE tube (I.D. 0.58 mm, O.D. 0.965 mm; Becton Dickinson, Sparks, MD, USA) using a standard procedure. The catheter was fixed with methacrylate and the abdominal cavity was closed with surgical clamps. The body temperature was monitored by an abdominal probe and kept constant at 37.5 ± 0.5 °C by a heated surgical table and overhead lamps. At 60 min after the treatment, 5 ml of mash, containing 1.5 g of standard chow and 25 mg of xanthan gum as a stabilizer, was punctured in the forestomach with an Abbocath-T 18G catheter (Hospira Inc., IL, USA) at a constant rate of 1 ml min^{-1}.

Tissue sampling

Portal blood samples (400 µl) were taken 20, 40, 60, 80 and 120 min after the treatment, and the catheter was refilled with heparinized 0.9% NaCl (600 IU ml^{-1}). The blood was transferred to heparinized tubes and a 1 : 100 volume of a 1 : 1 mix of protease inhibitors was spiked immediately (Pefabloc, Sigma-Aldrich, MO; DPP-IV inhibitor, Millipore, MI). Plasma was collected by centrifugation at 1500g over 15 min at 4 °C and frozen immediately at −80 °C. After the 120 min procedure, the rats were sacrificed by exsanguination of the aortal vein; tissue samples were immediately frozen in liquid nitrogen, and then stored at −80 °C. The stomachs were excised and the stomach content of each individual was collected and dried overnight at 50 °C.

Plasma and tissue quantification

The enterohormones were assayed in duplicate using commercial ELISA kits for insulin (Mercodia, Uppsala, Sweden), GLP-1 3-37 amide (Millipore, Billerica, MA, USA), total CCK (Peninsula Laboratories, San Carlos, CA, USA) and total ghrelin (Phoenix Pharmaceuticals, Burlingame, CA, USA).

Total RNA was extracted using Trizol (Ambion, USA) and trichloromethane–ethanol (Panreac, Barcelona, Spain), and purified using a RNA extraction kit (Qiagen, Hilden, Germany). Complementary DNA was obtained using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Madrid, Spain) and the quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) amplification was performed using TaqMan Universal PCR Master Mix and the respective specific TaqMan probes (Applied Biosystems, Madrid, Spain). The relative expression of each mRNA was calculated against the control group using the $2^{-\Delta\Delta Ct}$ method, with cyclophilin as the reference gene.

Statistical analysis

The data are represented as mean ± SEM. Periodic and cumulative food intake was analyzed using a mixed-model two-way ANOVA, with time as a within factor and treatment as a between factor. The portal hormone concentrations were analyzed using a regular two-way ANOVA. Conditional taste aversion, pica behavior, 12-hour cumulative food intake inhibition, gastric emptying rate and gene expression results were analyzed using a one-way ANOVA or Student's t-test, as appropriate. Bonferroni *post-hoc* tests were performed to assess the differences between groups after significant ANOVA. The statistical analyses were performed with IBM SPSS Statistics 22. P-values < 0.05 were considered significant in all cases.

Conclusions

A grape-seed proanthocyanidin extract reduces food intake in rats when it is intragastrically administered at a dose of 423 mg phenolics per kg BW or higher, preferably before meal initiation. This effect could be partially explained by its interaction with the enteroendocrine system and its effects on reducing gastric emptying. Further work is needed to clearly define the possible role of the enteroendocrine system in this satiating effect. This treatment could be recommended for individuals with difficulties in food intake control, who usually have concomitant defective glucose tolerance.

Conflict of interest

The authors declare that there are no conflicts of interest.

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6.2 Related patent

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(71) Applicant(s):
UNIVERSITAT ROVIRA I VIRGILI [ES/ES]; C. Escorxador, s/n. E-43003 Tarragona (ES) *(for all designated states)*

(72) Inventor(s):
ARDÉVOL GRAU, Anna; C. Escorxador, s/n. E-43003 Tarragona (ES)
PINENT ARMENGOL, Montserrat; C. Escorxador, s/n. E-43003 Tarragona (ES)
BLAY OLIVÉ, Maria Teresa; C. Escorxador, s/n. E-43003 Tarragona (ES)
AROLA FERRER, Lluís; C. Escorxador, s/n. E-43003 Tarragona (ES)
SERRANO LÓPEZ, Joan; C. Escorxador, s/n. E-43003 Tarragona (ES)
CASANOVA MARTI, Angela; C. Escorxador, s/n. E-43003 Tarragona (ES)

(74) Agent(s):
PONTI SALES, Adelaida; C. Consell de Cent, 322 E-08007 Barcelona (ES)

(54) Title (EN): USE OF PROANTHOCYANIDINS FOR SUPPRESSING APPETITE OR INDUCING SATIATION

(54) Title (FR): UTILISATION DE PROANTHOCYANIDINES POUR LA SUPPRESSION DE L'APPÉTIT OU L'INDUCTION DE LA SATIÉTÉ

(54) Title (ES): UTILIZACIÓN DE PROANTOCIANIDINAS PARA LA SUPRESIÓN DEL APETITO O INDUCCIÓN DE LA SACIEDAD

(57) Abstract:

(EN): The invention relates to the use of an extract comprising proanthocyanidins, in the gallated or non-gallated form, together with gallated procyanidins, for producing a solution and/or other forms of administration suitable for suppressing appetite or inducing satiation, wherein said proanthocyanidins do not include proanthocyanidins formed solely by gallated (epi)catechin units.

(FR): La présente invention concerne l'utilisation d'un extrait qui comprend des proanthocyanidines, de forme gallate ou non, en combinaison avec des procyanidines-gallates, pour la préparation d'une solution et/ou d'autres formes d'administration adéquates, destinées à la suppression de l'appétit ou à l'induction de la satiété, lesdites proanthocyanidines n'incluant pas les procyanthocyanidines formées uniquement par des unités d'(épi)catéchine de forme gallate.

(ES): La presente invención se refiere a la utilización de un extracto que comprende proantocianidinas, en forma galada o no galada, junto con procianidinas galadas, para la preparación de una solución y/u otras formas de administración adecuadas para la supresión del apetito o la inducción a la saciedad, en la que dichas proantocianidinas no incluyen las proantocianidinas formadas únicamente por unidades de (epi) catequinano galadas.

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UNIVERSITAT ROVIRA I VIRGILI
SATIATING PROPERTIES OF A GRAPE SEED PROANTHPCYANIDIN EXTRACT
Joan Serrano López

7. SUCHRONIC EFFECTS OF A GRAPE-SEED PROANTHOCYANIDIN EXTRACT ON FOOD INTAKE, NUTRIENT ABSORPTION AND ENERGY EXPENDITURE

Given our interest in finding a proper satiety agent, after the preliminary screening on the acute effects of a grape-seed phenolic extract on food intake we aimed to study whether this extract could be effective in a subchronic paradigm in rats. Herein, we present **manuscript 2**, which fits with the purposes presented in **objective 2**. Hence, in the next study we do not only compare the effects of two grape-seed phenolic extract doses in food intake and body weight in rats, but also compared its effects on food intake with its effects on nutrient absorption and energy expenditure to highlight until which extent the effects on food intake are important in this subchronic paradigm.

UNIVERSITAT ROVIRA I VIRGILI
SATIATING PROPERTIES OF A GRAPE SEED PROANTHPCYANIDIN EXTRACT
Joan Serrano López

7.1 Manuscript 2

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ORIGINAL CONTRIBUTION

A specific dose of grape seed-derived proanthocyanidins to inhibit body weight gain limits food intake and increases energy expenditure in rats

Joan Serrano[1] · Àngela Casanova-Martí[1] · Andreu Gual[1] · Anna Maria Pérez-Vendrell[2] · M. Teresa Blay[1] · Ximena Terra[1] · Anna Ardévol[1] · Montserrat Pinent[1]

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Abstract
Purpose Several studies have suggested that flavanols may have antiobesity effects; however, those effects clearly depend on the experimental conditions. In a previous study, we found that a single acute dose of grape seed proanthocyanidin extract (GSPE) has satiating effects. We therefore hypothesise that satiating doses of GSPE could be used to reduce body weight gain, and our present objective was to define the most effective dose.
Methods We assayed two GSPE doses in aged male Wistar rats. First we performed a subchronic (8-day) treatment by intragastric administration, which was repeated after a washout period. We measured body weight, energy intake and faeces composition; we performed indirect calorimetry; and we analysed the mRNA expression of genes involved in lipid metabolism to determine the target tissue for the GSPE.
Results We observed that 0.5 g GSPE/kg BW significantly reduced food intake and thus the amount of energy absorbed. This dosage also increased lipid oxidation in subcutaneous adipose tissue, thus causing a higher total energy expenditure. These combined effects caused a decrease in body weight. Conversely, 1 g GSPE/kg BW, which also reduced energy absorption after the first treatment, had a rebound effect on body weight gain which resulted in a lower response to the proanthocyanidin extract. That is, after the second treatment, the GSPE did not reduce the energy absorbed or modify energy expenditure and body weight.
Conclusion GSPE at a dose of 0.5 g/kg can reduce body weight by limiting food intake and activating energy expenditure in subcutaneous adipose tissue.

Keywords Proanthocyanidin · Obesity · Food intake · Energy expenditure · Subcutaneous adipose tissue

Introduction

Obesity and overweight, which are increasing dramatically throughout the world [1], are multifactorial problems that require increased knowledge to identify the origin of the problem and define the optimal solution. Currently, reducing energy intake and increasing exercise are pillars of the treatment. However, complementary dietary strategies such as bioactive compounds with antiobesity effects could be useful. In this area, identification of new compounds and their targets is of great interest. From a functional food perspective, the most appropriate manner in which an antiobesity compound is effective is to prevent and/or treat the problem at the beginning. Interesting candidates are grape seed-derived proanthocyanidins (PACs), which have been shown to possess many healthy properties that render them candidates to be components of functional food ingredients [2]. PACs' effects on body weight have recently been revised [3]. There are some discrepancies in

Joan Serrano and Àngela Casanova-Martí have contributed equally to this work.

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✉ Anna Ardévol
anna.ardevol@urv.cat

[1] Departament de Bioquímica i Biotecnologia, Universitat Rovira i Virgili, c/Marcel·lí Domingo, No. 1, 43007 Tarragona, Spain

[2] Monogastric Nutrition, Centre Mas de Bover, IRTA, Ctra. Reus-El Morell Km 3.8, 43120 Constantí, Spain

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the literature; whereas in several experiments, proanthocyanidins decreased body weight gain, in others experiments, no effects were observed. This dichotomy emphasises the fact that a proper optimisation of the dose and treatment conditions is required before claiming an antiobesity effect. Concerning the mechanisms of action, in general, the antiobesity effects of PACs appear to be highly attributable to increased energy expenditure. PACs cause the upregulation of energy expenditure-related genes in skeletal muscle and the liver whilst decreasing fatty acid synthesis and fat uptake in the liver [4]. However, PACs also act to inhibit digestive enzymes such as lipase and amylase, repressing fat and glucose absorption from the gut [5, 6]. At the intestinal level, PACs modulate enteroendocrine secretions [7, 8] that could affect satiety. In fact, our research group has quite recently identified a dosage of grape seed proanthocyanidin extract that acutely limits food intake through its modulation of the enteroendocrine system [9].

In this study, we evaluate whether the acute satiating effects of the above-mentioned extract can also be reproduced in short-term treatment. We also analyse the effects of different doses of the extract on energy metabolism and compare their effectiveness at reducing energy intake, energy expenditure and body weight gain.

Materials and methods

Proanthocyanidin extract

The grape seed extract enriched in proanthocyanidins (GSPE) was obtained from *Les Dérivés Résiniques et Terpéniques* (Dax, France). According to the manufacturer, the extract contains monomeric (21.3 %), dimeric (17.4 %), trimeric (16.3 %), tetrameric (13.3 %) and oligomeric (5–13 U; 31.7 %) proanthocyanidins. The small molecules (up to trimers) present in GSPE were characterised in more detail by reverse-phase chromatographic analyses by our research group [10].

Animals

A group of adult aged (40 weeks) male Wistar rats (Harlan, Barcelona), each weighing 450–500 g, were used for the food intake studies ($n = 15$). The studies in male groups occurred at the facilities of the Technological Center of Nutrition and Health (www.ctns.cat).

Upon arrival, the animals were single-housed at 22 °C in a 12-h light/dark cycle (lights on at 8 am) with access to standard chow (Teklad Global Diets #2014, Harlan, Barcelona; 2.9 kcal/g) and tap water ad libitum during a 1-week adaptation period prior to the experiments. All procedures were approved by the ethical committee.

Food intake experiments

The animal sets for the food intake studies were introduced to a daily 4-h food deprivation before the light offset (from 1600 to 2000 hours), and blank chow intake measurements were taken daily to habituate the animals to the experimental schedule.

First-period treatment

To assess the effects of a short-term chronic dose of proanthocyanidins on food intake, trained animals were divided into three groups: a control group treated with vehicle (tap water) and two GSPE groups (0.5 g GSPE/kg BW and 1 g GSPE/kg BW) treated with 423 and 846 mg phenolics/kg of BW, respectively. The treatments were intragastrically (i.g.) administered 1 h before the dark onset, in 2 ml of tap water, and chow intake was measured 1, 3, 12 and 20 h after the chow replacement with an accuracy of 0.01 g. Treatment was repeated for a total of 8 days. During this period, body weight was measured every other day. On the eighth day, faeces were collected for further analysis. Animals were then left to resume their standard growth pattern for 30 days, during which we continued to measure food intake and body weight.

Second-period treatment

After 30 days of recovery, the identical treatment was repeated. On the seventh day of this second treatment, measurement of respiratory metabolism was taken in a ventilated hood system (Panlab Harvard Apparatus, Barcelona, Spain) from 0800 to 1500 hours. The respiratory quotient (RQ) and energy expenditure were calculated with metabolism 2.1.04 (Panlab Harvard Apparatus, Barcelona, Spain). On the ninth day, after fasting from 1500 to 1800 hours, the animals were anaesthetised with 70 mg/kg BW i.p. of sodium pentobarbital and were killed by exsanguination of the aortal artery. Plasma and tissue samples (liver, subcutaneous and epididymal WAT, muscle, BAT and hypothalamus) were immediately frozen in liquid nitrogen and then stored at −80 °C.

Plasma and tissue mRNA quantification

Plasma β-hydroxybutyrate was analysed by colorimetry (BEN, Mainz, Alemania). Total RNA was extracted using Trizol (Ambion, USA) and trichloromethane–ethanol (Panreac, Barcelona, Spain) and purified using an RNA extraction kit (Qiagen, Hilden, Germany). Complementary DNA was obtained using the high-capacity cDNA reverse transcription kit (Applied Biosystems, Madrid, Spain), and the quantitative reverse transcriptase-polymerase chain

reaction (qRT-PCR) amplification was performed using TaqMan Universal PCR Master Mix and the respective specific TaqMan probes (Applied Biosystems, Madrid, Spain). The relative expression of each mRNA was calculated against the control group using the $2^{-\Delta\Delta Ct}$ method, with cyclophilin used as the reference.

Faeces analysis

Samples were analysed using standard official methods (AOAC, 2000). Crude protein was determined utilising the Dumas procedure based on the complete combustion of the sample and using a Nitrogen Analyzer Leco FP-528 (AOAC method no 968.06). The determination of crude fat content was based on the continuous extraction Soxhlet procedure using an automated Buchi Extraction System B-811 (AOAC method no 920.39). An enzymatic method (AOAC no 996.11) was used to determine the total starch content. The crude fibre content was measured on an Ankom Fibre Analyzer, based on filter bags technology (AOCS method no Ba 6a-05).

Statistical analysis

The data are represented as the mean ± SEM. Statistical comparisons between groups were assessed by ANOVA followed by Bonferroni post hoc tests. Analyses were performed with IBM SPSS Statistics 22. p values <0.05 were considered significant in all cases. In the text, p values <0.05 are indicated as different letters (a, b, c).

Results

A dose of 0.5 g GSPE/kg BW inhibits energy intake, activates energy expenditure and reduces body weight gain

We assayed two GSPE doses with acute satiating effects [9] (corresponding to 423 and 846 mg phenolics/kg of BW) during a short chronic treatment to evaluate their effect on body energetics (first-period treatment). We observed that both doses significantly reduced food intake on each day of the treatment period (data not shown) and resulted in a reduced cumulative food intake throughout the treatment period [control: 100 ± 3 % (a); 0.5 dose: 73 ± 4 % (b); 1 dose: 70 ± 6 % (b)]. Concomitantly, a similar reduction in body weight gain was observed for both doses (Fig. 1). Both doses also diminished feeding efficiency (mg body weight gain per kcal consumed; control: 10.29, 0.5 dose: −17.18, 1 dose: −37.20), which indicated that the reduced food intake was not the only cause of the reduction in body weight.

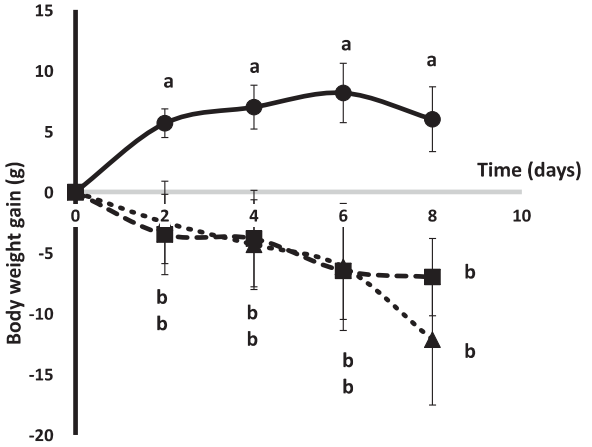

Fig. 1 Body weight gain during first-period treatment. Body weight in aged male Wistar rats was measured every 2 days. *Circles* indicate control (vehicle-administered) animals, *squares* indicate 0.5 g GSPE/kg BW dose, and *triangles* indicate 1 g GSPE/kg BW dose. *Different superscripts* indicate significant differences between groups at each day measurement at $p < 0.05$

Table 1 Energy entrance after first-period treatment

	Control	0.5 g/kg BW	1 g/kg BW
Ingested energy (kcal)	69.01 ± 3.99^a	56.18 ± 1.92^b	54.31 ± 3.43^b
Absorbed energy (kcal)	63.82 ± 3.93^a 44.72 ± 2.81^a	50.49 ± 1.89^b 35.04 ± 1.35^b	49.02 ± 3.37^b 33.89 ± 2.41^b
Absorbed versus control (%)	100 ± 6^b	78 ± 3^b	76 ± 5^b
Absorbed nutrients (%kcal)			
CH	70.62 ± 0.18^a	71.50 ± 0.16^b	71.47 ± 0.29^b
Lipid	11.32 ± 0.07^a	11.43 ± 0.04^a	11.80 ± 0.06^b
Protein	18.06 ± 0.11^a	17.07 ± 0.11^b	16.73 ± 0.23^b
Absorbed nutrients (kcal)			
CH	45.63 ± 2.64^a	37.09 ± 1.27^b	35.84 ± 2.27^b
Lipid	7.33 ± 0.50^a	5.93 ± 0.24^b	5.93 ± 0.43^b
Protein	11.70 ± 0.79^a	8.87 ± 0.38^b	8.44 ± 0.68^b

Different superscripts indicate $p < 0.05$ compared with control group in each treatment. For treatment 1, $n = 6$ for all groups. For treatment 2, $n = 6$ for control group, $n = 5$ for GSPE groups

Ingested energy (kcal) = food ingested (g) × caloric content of food (kcal/g); absorbed energy = ingested energy − faeces energy content

To calculate the amount of energy absorbed, we measured food intake and analysed the composition of faeces during 20 h on the 7th day of the treatment. Both GSPE doses maintained a 20 % decrease in the amount of absorbed energy (Table 1). Thus, the reduction in energy absorption clearly parallels the reduction in food intake although the ratio of absorbed energy compared with

ingested energy shows a slight statistically significant reduction in the GSPE-treated rats, suggesting that GSPE has a slight effect on the digestion process [% absorbed/ingested energy; control: 0.92 ± 0.003 (a); 0.5 dose: 0.90 ± 0.003 (b); 1 dose: 0.90 ± 0.005 (b)].

Analysis of faeces composition indicated that GSPE modified the absorption of the main nutrients (Table 1). Both doses of GSPE had a similar diminishing effect on the digestion of protein (% absorbed/ingested protein for each dose: 0.5: 10.42; 1: 11.67) and of carbohydrates (respectively, 0.25–0.32 %). The 0.5 dose also diminished the percentage of absorbed versus ingested lipids (2.77 %). This higher impairment of protein digestion led to different proportions of macronutrient absorption. GSPE-treated rats absorbed a lower percentage of protein and a higher proportion of carbohydrates. Regarding the lipids, both GSPE doses led to the absorption of the same amount of energy (5.93–5.93 kcal). Regarding absorbed energy, the 0.5 GSPE dose rats ingested a lower percentage of energy in the form of lipids than did the controls and the higher GSPE dose rats.

After the first-period treatment, we continued to regularly measure food intake and body weight. Body weight changes remained for a longer period after both treatments although there were differences between each treatment (Figure S1). A rebound effect on body weight was observed in the 1 g/kg-treated animals, which presented a significantly higher growth rate than the controls and the 0.5 g/kg-treated animals. A month after treatment the animals resumed a normal growth pattern. At that point, we carried out an identical second-period treatment to analyse the effectiveness of repeated treatments.

We observed greater differences between the two GSPE doses during this second-period treatment because the cumulative ingested energy was only reduced by the 0.5 g/kg BW dose [control: 100 ± 4 % (a); 0.5 dose: 86 ± 3 % (b); 1 dose: 89 ± 3 % (a, b)].

During this second-period treatment, we also measured oxygen consumption and CO_2 production. Figure 2a shows that the 0.5 g/kg BW dose caused an increase (approximately 12 %) in the energy consumption of these rats, whereas the 1 g/kg BW dose tended to decrease energy consumption. Food intake and energy expenditure during the second treatment led to clearly observed changes in body weight gain [g; control: −2.67 ± 2.7 (a); 0.5 dose: −12.6 ± 3.3 (b): 1 dose: −7.40 ± 4.7 (a)]. The body weight of the 0.5-treated group decreased 2–4 times as much as the other two groups (control and 1 g/kg BW; detailed body weights in Table 2). Consequently, after the two treatment periods, the 0.5 g/kg BW group had a body weight gain of 40 % compared with 100 % in the control group and the 1 g/kg BW dose group (Fig. 2b).

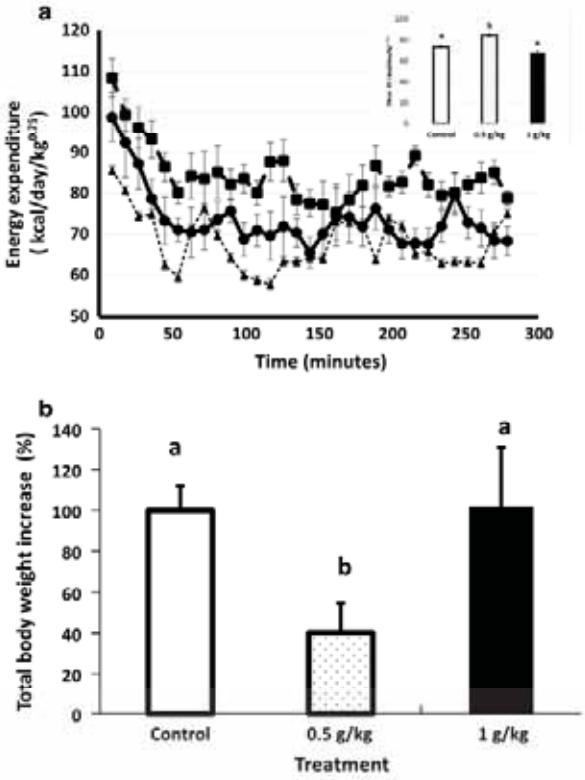

Fig. 2 Energetics in second-period treatment. **a** Energy expenditure. On day 7 of treatment, at 09:00 rats enter indirect calorimeter for respiratory measurements and remain until 15:00. *Circles* indicate control (vehicle-administered) animals, *squares* indicate 0.5 g GSPE/kg BW dose, and *triangles* indicate 1 g GSPE/kg BW dose. Summary of statistics is included in the inserted graph. *Different superscripts* indicate significant differences between groups at $p < 0.05$. **b** Total change in body weight after both periods of treatment. Body weight change was measured from the beginning of treatment period 2 (final BW−initial BW). Body weight change in control group was used as a reference to calculate the effects of the other two treatments. *Different superscripts* indicate significant differences between groups at each day measurement at $p < 0.05$

Subcutaneous adipose tissue is the main target of the 0.5 g GSPE/kg BW

The respirometer data showed that both GSPE doses caused a decreased RQ, i.e. higher lipid oxidation [control: 0.9 ± 0.004 (a); 0.5 dose: 0.81 ± 0.003 (b); 1 dose: 0.83 ± 0.004 (a, b)]. To identify where this higher energy expenditure occurred, we measured β-oxidation at gene expression level in the main candidate tissues. Amongst the main tissues involved in lipid oxidation, including different adipose pads, the lower GSPE dose only increased CPT-1 (carnitine palmitoyltransferase I) expression in subcutaneous WAT (Fig. 3a), which was concomitant with an increased expression of

Table 2 Body weight (g) of animals a the key points of each treatment

	Control	0.5 GSPE	1 GSPE
Start of study	526.83 ± 23.76	482.83 ± 13.18	478.00 ± 12.68
After first treatment	552.17 ± 24.23	496.40 ± 15.04	504.17 ± 16.15
End second treatment	549.50 ± 25.68	483.80 ± 15.83	494.20 ± 18.42

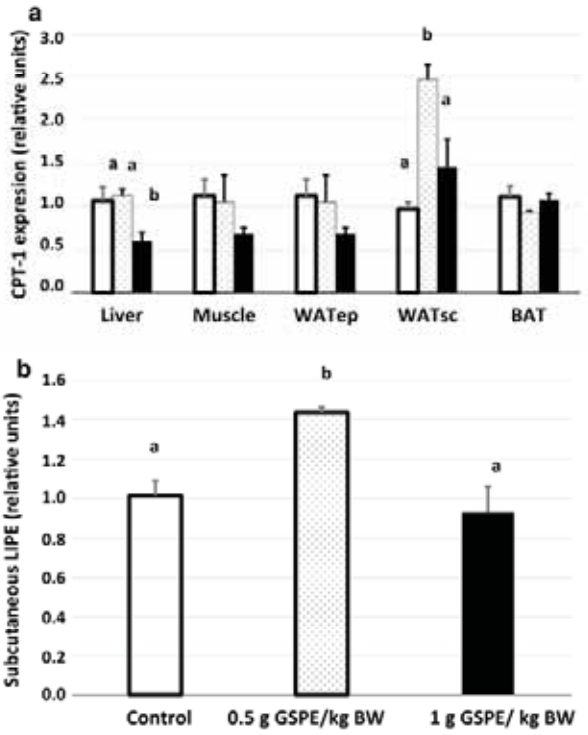

Fig. 3 GSPE treatment effects on mRNA expression. The rats were treated with the vehicle (*white bars*), the 0.5 g GSPE/kg BW (*dotted bar*) or 1 g GSPE/kg BW dose (*dark bars*) during treatment 2. **a** CPT-1 expression in different tissues of GSPE-treated rats. CPT-1 relative mRNA expression levels were calculated using the DDCt method for each tissue. The data are the mean ± SEM ($n = 6$). *Different superscripts* indicate $p < 0.05$; GSPE compared with control in each tissue; calculated using Student's *t* test. WAT (white adipose tissue); WATep (epididymal); WATsc (subcutaneous); BAT (brown adipose tissue). **b** Subcutaneous WAT LIPE expression in different GSPE treatments. LIPE relative mRNA expression levels were calculated using the DDCt method for each tissue. The data are the mean ± SEM ($n = 6$). *Different superscripts* indicate $p < 0.05$ calculated using Student's *t* test

LIPE (hormone-sensitive lipase; Fig. 3b). There were no changes in the lipogenic genes in either the subcutaneous WAT depot or the liver, and UCP2 (uncoupling protein 2) was also unaffected (Table 3). There were no changes in plasma free fatty acids or glycerol between treatments (results not shown).

By contrast, in the 1 g GSPE/kg BW dose, we identified less β-hydroxybutyrate in plasma [mM; control: 0.30 ± 0.03 (a); 0.5 dose: 0.23 ± 0.06 (a); 1 dose: 0.20 ± 0.01 (b)]. Table 3 shows that there were no changes in the use of ketone bodies (3-oxoacid CoA-transferase 1: OXCT1) in either muscle or hypothalamus. There was no change in mRNA responsible of the hepatic ketone bodies production (3-hydroxy-3-methylglutaryl-CoA synthase 2: HMGCS2); however, we did observe decreased mRNA expressions in the liver CPT-1a (Fig. 3a).

Discussion

Proanthocyanidins have previously been linked to body weight reduction, primarily related to their ability to inhibit food intake because of their astringency [11] and to inhibit gastrointestinal enzymes, thereby limiting macronutrient digestion, because of their lipolytic activity [12, 13]. We have recently identified that these compounds of grape seed origin may also limit food intake independent of their astringency [9]. In fact, we posited that the effect on food intake is strongly related to the mode of administration and dependant on the dose, either 0.5 or 1 g GSPE/kg BW effective in an acute single treatment. The present study supports only the 0.5 g GSPE/kg BW dose administered chronically in the short term in repetitive treatments as effective in inhibiting energy intake and activating energy expenditure, which ultimately leads to reduced body weight increase.

We observed that the satiating effect that we had previously observed in an acute treatment [9] could be maintained during a short (8-day) chronic treatment. The assayed doses acted to limit food intake in a similar manner. We discarded the effects of taste on oral perception because we forced GSPE doses intragastrically (further discussed in Serrano et al. [9]). Concerning the possible effects at the digestion level, we showed that GSPE-treated groups absorbed a considerably lower protein content, a slightly lower carbohydrate content and a lower lipid amount in the case of the 0.5 g/kg dose, most likely because of the previously described inhibition of intestinal enzymes (lipase [14], amylase [15], glucosidase [15], protease [16], etc.). The high inhibition of the protein availability resulted in higher proportions of absorbed carbohydrates and lipids. In 1994, Tebib et al. [17] showed a strong inhibition in intestinal enzymes; however, those authors showed that in vivo, at least at the duodenal stage, this effect disappears because of the alkalinity and detergency of the pancreatic biliary secretion, which neutralised the ability of the tannins to inactivate brush-border hydrolase

Table 3 mRNA expression

	mRNA ScWAT		Liver mRNA			Ketone bodies metabolism mRNA		
	FASn	DGAT2	UCP2	FASn	DGAT2	Muscle OXCT1	Hypothalamus OXCT1	Liver HMGCS2
Control	1.04 ± 0.14	1.02 ± 0.10	1.03 ± 0.11	1.15 ± 0.25	1.01 ± 0.05	1.04 ± 0.12	1.00 ± 0.03	1.02 ± 0.02
0.5 g/kg	1.17 ± 0.04	1.24 ± 0.01	1.20 ± 0.02	0.65 ± 0.14	0.99 ± 0.03	0.99 ± 0.15	1.34 ± 0.25	0.90 ± 0.05
1 g/kg	0.94 ± 0.26	1.04 ± 0.14	1.39 ± 0.35	0.92 ± 0.30	1.26 ± 0.16	0.77 ± 0.04	1.03 ± 0.15	0.97 ± 0.01

activities. Our results also support Tebib's evidence that in vivo, the inhibition of intestinal enzymes is nearly negligible because the ratio of energy absorbed compared to energy ingested is quite similar to the controls, supporting the idea that the reduction in energy absorption is because of the reduced food intake.

During the first period of treatment, both GSPE doses acted similarly; immediately afterwards, the differences between them appeared. The 1 g/kg-treated group quickly equalled the control body weight. The repetition of the treatment clearly highlighted the interest of the 0.5 g GSPE/kg BW dose above the 1 g/kg because only this dose continued to inhibit food intake at identical levels, activate energy expenditure and lead to a final net reduction in body weight. Concerning food intake, there are few previous studies showing the effects of this type of proanthocyanidins on food intake. The majority of the studies assaying these doses analysed effects on body weight but not on food intake [18, 19]. Only Bao et al. [20] used the same dose administered intragastrically in quite a different model, a type 2 diabetes mellitus rat model that showed an enhanced food intake. These authors also observed a reduction in food intake of approximately 20 % after 16 weeks of treatment.

Furthermore, we observed strong differences between the treatments in indirect calorimetry measurements. We measured calorimetry in the quietest period of the animal to avoid exercise- and food-linked thermogenesis [21] during a time period that represents approximately 35 % of daily energy expenditure (results are not shown). We observed that the 0.5 dose increased energy expenditure, as previously shown for different flavonoid structures [22, 23–30]. However, this is the first time that this thermogenic effect has been shown for grape seed proanthocyanidins at the assayed doses. A recent revision of Salvadó et al. [12] on proanthocyanidins' effects on body weight control did not show effects on entire energy expenditure although the summarised studies use a maximum dose of 250 mg GSPE/kg BW. In fact, studies in our laboratory with different animal models using the identical extract at lower doses (25–50 mg GSPE/kg BW) did not show increased energy expenditure [31, 32]. Therefore, it appears that grape seed proanthocyanidins' ability to increase energy expenditure is only observed in a narrow range of dosage/treatment.

Conversely, both doses show decreased RQ. This effect has been extensively repeated in the majority of assayed doses in the previously cited works [31, 32], suggesting that proanthocyanidins and several flavonoids increase lipid oxidation compared with carbohydrate oxidation. Previous works have identified this increase in lipid oxidation primarily at retroperitoneal WAT [33] (cafeteria-induced obesity in hamsters, GSPE treatment 25 mg/kg BW). Casanova et al. [31] also observed changes at the muscular level in cafeteria animals using a similar treatment by activating AMPK phosphorylation. A similar dose administered to pregnant rats caused a reduced RQ in the offspring; the authors also showed activation of the oxidation capabilities of lipidic metabolism in muscle [32]. Notably, although the global effect on RQ is identical in both assayed doses, we observed significant differences concerning the target tissues. Margalef et al. [34] showed that after different GSPE doses, the primary flavonoid components that reach liver and adipose tissue are quite different. At lower doses, the liver has a 53 % level of gallic acid, a 1 % level of free flavanols and a 31 % level of methyl-glucuronidation as the primary metabolite. At a 375 mg/kg dose, the closest to our 500 mg kg/BW assayed dose, the liver has 53 % of methyl-glucuronidated forms, 8 % of free flavanols and only 10 % gallic acid. Quite a different situation was observed at the 1000 mg/kg dose when in the liver there were scarcely any gallic acid, 28 % free flavanols and 31 % of methyl-glucuronidated forms. These differences may explain the differences in the metabolic effects on this tissue caused by the different doses. We observed that the lower dose (0.5 g/kg BW) did not modify liver gene expression related to lipid oxidation (CPT-1) and only showed a tendency to decrease the fatty acid synthesis, consistent with the previously described antilipogenic effects of GSPE on this tissue [35]. Instead, at a dose 1 g/kg, we observed an inhibition of CPT-1 without changes in FASn or DGAT2 mRNA levels, suggesting less ability to oxidise fatty acids in the liver. This decrease in fatty acid liver oxidation could explain the lower levels of plasma ketone bodies observed in this dose. At gene expression level, we have not observed signs of muscle β-oxidation, ketone bodies oxidation or signs of brain ketone bodies use. Further research would be necessary to define where this lipid oxidation occurs in the higher dose; however, because these effects are not linked

to increased energy expenditure or reduced body weight gain, we are currently focusing on the effective dose.

At 0.5 mg/kg, we observed increased CPT-1 and LIPE expression in subcutaneous adipose tissue but not in other fat deposits, suggesting that this is the target tissue in current experimental conditions. That subcutaneous adipose tissue composes approximately 80 % of all body fat [36] clearly justifies the higher energy expenditure of lipid origin in these animals. In addition, the characteristics of this tissue, which acts as a metabolic sink in which excess free fatty acids (FFAs) and glycerol are stored as triglycerides (TGs) in adipocytes [37], help explain the higher fatty oxidation observed immediately after the feeding period, when we run indirect calorimetry.

Consistent with the current results in our aged animals, which are glucose intolerant (results not shown), we observed that subcutaneous adipose tissue was the target tissue for GSPE in the glucose-intolerant Zucker obese rat [38]. The same study showed that identical doses target mesenteric adipose tissue in Wistar lean, healthy rats, which also showed different flavanol metabolites in the different adipose tissues. In fact, Margalef et al. [34] showed different presences of flavonoid structures in white adipose tissue, depending on the dose. This could explain the different effects observed depending on the administered dose.

Conclusion

We currently define a 0.5 g GSPE/kg BW dose as effective in short-term, chronic and repeated treatment because the dose causes a reduction in food intake that leads to reduced absorbed energy. In addition, the 0.5 g dose increases lipid oxidation in subcutaneous adipose tissue, producing greater total energy expenditure. Together, these effects cause a decrease in body weight.

By contrast, 1 g GSPE/kg BW, which also produced a lower absorption of energy after one treatment, produces a rebooting effect in body weight. This adverse effect could explain why, after a second treatment, the amount of energy absorbed did not decrease and why whole body organism energy expenditure, corroborated by the lack of b-oxidation in the assayed tissues, did not increase.

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Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest.

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8. EFFECTS OF GRAPE-SEED PHENOLICS ON GASTROINTESTINAL SATIETY SIGNALING

In this last chapter of results, we present three different sections in which we specifically aimed to study whether grape-seed phenolic compounds affect the secretion of ghrelin and the roles of ghrelin and GLP-1 in the observed effects in food intake. Hence, the following results mostly correspond to **objective 3**, but not exclusively.

In **manuscript 3**, we present additional dose-response data of the effects of a grape-seed phenolic extract on food intake, plus emphasized in some confounding factors in food intake experimentation, which corresponds to **objective 1**. Thereafter, we compare the effects of a grape-seed phenolic extract and gallic acid in food intake and the role of GLP-1 in these effects, plus present results on neuropeptide expression and its relationship on food intake, corresponding to **objective 3**.

In **manuscript 4** we studied the effects of grape-seed phenolics on ghrelin release in ghrelin-producing cells, tissue segments and rats, which corresponds solely to **objective 3**.

To finally accomplish **objective 3**, where we aimed to study until which extent GLP-1 and ghrelin are involved in the subchronic effects of a grape-seed phenolic extract in rats, we realized a post-hoc multilinear regression analysis which is presented and discussed in the **statistical addendum**. After fulfilling all our purposed objectives, the results section will end after this numeric analysis.

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SATIATING PROPERTIES OF A GRAPE SEED PROANTHPCYANIDIN EXTRACT
Joan Serrano López

8.1 Manuscript 3

nutrients

MDPI

Article

Defining Conditions for Optimal Inhibition of Food Intake in Rats by a Grape-Seed Derived Proanthocyanidin Extract

Joan Serrano †, Àngela Casanova-Martí †, Mayte Blay, Ximena Terra, Anna Ardévol * and Montserrat Pinent

MoBioFood Research Group, Departament de Bioquímica i Biotecnologia, Universitat Rovira i Virgili, 43007 Tarragona, Spain; joanet70@gmail.com (J.S.); angela.casanova@urv.cat (À.C.-M.); mteresa.blay@urv.cat (M.B.); ximena.terra@urv.cat (X.T.); montserrat.pinent@urv.cat (M.P.)
* Correspondence: anna.ardevol@urv.cat; Tel.: +34-977-55-9566
† These authors contributed equally to this work.

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Abstract: Food intake depends on homeostatic and non-homeostatic factors. In order to use grape seed proanthocyanidins (GSPE) as food intake limiting agents, it is important to define the key characteristics of their bioactivity within this complex function. We treated rats with acute and chronic treatments of GSPE at different doses to identify the importance of eating patterns and GSPE dose and the mechanistic aspects of GSPE. GSPE-induced food intake inhibition must be reproduced under non-stressful conditions and with a stable and synchronized feeding pattern. A minimum dose of around 350 mg GSPE/kg body weight (BW) is needed. GSPE components act by activating the Glucagon-like peptide-1 (GLP-1) receptor because their effect is blocked by Exendin 9-39. GSPE in turn acts on the hypothalamic center of food intake control probably because of increased GLP-1 production in the intestine. To conclude, GSPE inhibits food intake through GLP-1 signaling, but it needs to be dosed under optimal conditions to exert this effect.

Keywords: proanthocyanidins; food intake; dose; GLP-1; hypothalamus; synchronicity

1. Introduction

Food intake plays a key role in body energetics and is one of the most commonly modulated parameters used to counteract obesity related problems, despite being a complex system [1,2]. In humans, the homoeostatic control of appetite is often conceptualized through a series of physiological processes that initiate and terminate feeding (i.e., satiation), and those which suppress inter-meal hunger (i.e., satiety). Collectively, these processes have been termed the Satiety Cascade and they involve psychological and behavioral patterns, peripheral physiological and metabolic events and neural and metabolic interactions in the brain [3–6]. This complex interplay of integrative neural processes within the brain, which are sensitive to a plethora of signals that originate in the periphery, underpin the behavioral expression and conscious experience of appetite and represent the ultimate factors in the control of energy intake [2].

The macronutrient composition of food play a role in the expression of the satiety cascade and appetite related processes [1]. When expressed relative to energy content rather than weight of food, protein exerts the strongest effect on satiety [7], whereas fat exerts the weakest effect intake [8], although they are able to induce ileal brake [9]. There is some controversy regarding fiber effectiveness [10,11].

Polyphenols, a large group of molecules found in a myriad of plant sources, have also been postulated as possible modulators of food intake. Panickar summarized that some polyphenols (resveratrol, apigenin) could act on neuropeptides involved in food intake control,

such as Neuropeptidy Y (NPY), Agouti-related protein (AgRP), Pro-opiomelanocortin (POMC) and Cocaine- and amphetamine-regulated transcript (CART) [12]. However, information about the effect of polyphenols on peripheral tissues is scarce, and there is no clear consensus regarding the effects of the most studied compounds, isoflavones [13]. We have previously reported that flavanols, a type of polyphenol found in abundance in grape seed proanthocyanidin extract (GSPE), have a modulatory effect on food intake. We described a dose that inhibited food intake and stimulated energy expenditure; this dose inhibited body weight gain by 60% after two eight-day periods of treatment [14]. Previously, we have shown that GSPE produces a lipolytic effect, which contributed to the effects on body weight in this study [12,15,16]. We ruled out the possibility that proanthocyanidins were playing an important role in inhibiting the gastrointestinal digestion process [14]. Remarkably, the animals ingested less food, which could be due to the changes induced by the extract on enteroendocrine signals (increased active GLP-1 [17] and decreased acylated ghrelin [18]) and/or by other signals that limit their need to feeding [17]. Bao et al, working with GSPE in diabetic rats and at doses similar to ours, also found an inhibition in food intake after chronic administration [19]. However, the limited human studies conducted to date have yielded controversial results. Some studies reported no effect by flavanols on food intake [20,21]. Törrönen et al. found increases in Glucagon-like peptide-1 (GLP-1) after the administration of a berry purée containing proanthocyanidins, although they reported no effects on food intake [22]. For an improved understanding of the effects of GSPE, it is therefore important to describe more extensively its mechanisms of action and the factors that influence it.

In the present study, we fine-tune the mechanisms of the effects of GSPE on food intake in rats. We describe how reproducibility of this effect is limited by stress, synchronicity and dose. We also prove the importance of GLP-1 in the GSPE effects on food intake and its main target tissues.

2. Materials and Methods

2.1. Materials

The proanthocyanidin-enriched GSPE was obtained from *Les Dérivés Résiniques et Terpéniques* (Dax, France; Batch number: 124029). According to the manufacturer, the extract contains monomeric (21.3%), dimeric (17.4%), trimeric (16.3%), tetrameric (13.3%) and oligomeric (5–13 units; 31.7%) proanthocyanidins. GSPE were characterized in more detail by reverse-phase chromatographic analyses by our research group [23]. Briefly, the main compounds (up to tetramers) found in the GSPE lot used in this study were gallic acid (31 mg/g), (epi)catechin (214 mg/g), epicatechin gallate (21 mg/g), procyanidin dimers (168 mg/g), procyanidin dimer gallate (9 mg/g) and trimers (5 mg/g). The phenolic content of the extracts was quantified using the Folin method [24] as 845.5 $\pm$ 10.5 mg/g GSPE. Gallic acid, exendin 9-39 (Ex9) and 70 kDa fluorescein isothiocyanate (FITC)-dextran were obtained from Sigma (St. Louis, MO, USA). GLP-1 7-36 amide was obtained from PolyPeptide (Limhamn, Sweden).

2.2. Animals

Female Wistar rats (Harlan, Barcelona, Spain), weighing 180–200 g upon arrival, were used for the food intake studies. A group of adult male Wistar rats (Harlan, Barcelona, Spain), weighing 450–500 g, were used for food intake studies, as a model for glucose-impaired tolerance [25]. The male groups were studied at the facilities of the Technological Center of Nutrition and Health (www.ctns.cat). All the procedures were approved by the Experimental Animal Ethics Committee of the Universitat Rovira I Virgili (code: 0152S/4655/2015).

On arrival, the animals were single housed at 22 °C under a 12 h light/dark cycle (lights on at 8 a.m.) with access to standard chow pellets (Teklad Global Diets #2014, Harlan, Barcelona, Spain) and tap water ad libitum for a one-week adaptation period. Following this adaptation period, the animal sets for the food intake studies were deprived of food every day for 4 h before between 16:00 and 20:00, and chow intake measurements were taken on the 4 days prior to each experiment to habituate the

subjects to the experimental schedule. One cross-over experiment per week was performed for all of the food intake studies.

2.3. Food Intake Experiments

Acute treatments. To assess the effects of different acute doses of proanthoacyanidins on food intake, trained animals were treated with intragastric GSPE doses (i.g.) 1 h before the dark onset, using tap water as a vehicle, and food intake was measured 12 h after the initiation of the feeding period. Parallel controls were performed by intragastrically administering the vehicle. This experiment was conducted on animals on a standard chow diet and on animals receiving hypercaloric meals as detailed in [17].

Chronic treatments. For the chronic treatment assays, animals received two periods of treatment as detailed previously [14]. Briefly, in the first-period treatment, trained animals were divided into 3 groups: a control group treated with a vehicle (tap water) and two GSPE groups (0.5 g GSPE/kg body weight (BW) and 1 g GSPE/kg BW) treated with 423 and 846 mg phenolics/kg of BW, respectively. The treatments were intragastrically (i.g.) administered 1 h before the dark onset for 8 consecutive days, and the chow intake was measured. The animals were then left to resume their standard growth pattern for 30 days before an identical treatment period was repeated. On the ninth day, after fasting from 15:00 to 18:00, the animals were anaesthetised with 70 mg/kg BW i.p. of sodium pentobarbital and were sacrificed by exsanguination of the aortal vein. Tissue samples were immediately frozen in liquid nitrogen and then stored at −80 °C.

Gastrointestinal motility. To assess the effects of GSPE on gastrointestinal motility, rats were treated 1 h before light offset with tap water or 0.5 g GSPE/Kg BW using 2.5 mg of 70 kDa FITC-dextran as a vehicle. Chow was returned to the cages at light offset and the rats were sacrificed 20 min after by aortal exsanguination under sodium pentobarbital anesthesia. After the excision of the stomach and caecum, the colon was divided into 2 equal parts, and the small intestine was divided into 10 equal parts. The contents of the stomach, caecum and each intestinal segment were washed in 5 mL PBS and the FITC-dextran of each segment were quantified in clean supernatants after a 5 min centrifugation at 12,000 rpm. To determine the motility of FITC-dextran, the gastrointestinal segments were indexed from 1 (stomach) to 14 (intestine, caecum and colon) and the FITC-dextran content was multiplied by its corresponding index to obtain a mean geometric center of motility [26–28].

Antagonism study. To prevent any stress from interfering with food intake, prior to the experiments with the GLP-1 receptor antagonist Ex9, the rats were specially trained by being handled daily for 10 s at 30 and 15 min before light offset for 2 weeks. A blank food intake measure was taken 3 days prior to an experiment. For the 2 days prior to any experiment, the rats were given a sham dose at 30 and 15 min before light offset, and blank measurements of chow intake were taken. Experiments were performed only if normal food intake was detected after the sham dosage. A crossover experiment was performed once a week. Rats were treated i.p. with saline or Ex9 30 min before light offset and thereafter with intragastric water, gallic acid or GSPE 15 min before light offset. Chow intake was subsequently measured after it was reintroduced [29–31]. The Ex9 dose was studied previously to rule out any effect on food intake and to confirm its antagonistic effect on i.p. injected GLP-1.

2.4. Plasma and Tissue Quantification

Plasma leptin was assayed in duplicate using a commercial ELISA kit (Millipore, St. Charles, MO, USA). Total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA, USA) and trichloromethane-ethanol (Panreac, Barcelona, Spain), and purified using an RNA extraction kit (Qiagen, Hilden, Germany). Complementary DNA was obtained using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Madrid, Spain), and the quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) amplification was performed using TaqMan Universal PCR Master Mix and the respective specific TaqMan probes (Applied Biosystems, Madrid, Spain). The relative expression of each mRNA was calculated against the control group using the $2^{-\Delta\Delta Ct}$ method, with actin and cyclophilin as a reference.

2.5. Statistical Analysis

Single cosinor analysis and Rayleigh *z*-tests were used to assess the synchronicity of food intake as described previously [32–34]. The effects of GSPE on gastrointestinal motility, gene expression, gallic acid effects on food intake and the antagonism study were assessed by Student's *t*-test. The dose-response effects of GSPE on food intake and the stressing effects of Oral Glucose Tolerance Test (OGTT) on food intake were assessed by one-way ANOVA and appropriate post hoc tests. The role of the hypothalamic gene expression of neuropeptides in food intake, glucagon and GLP-1 receptor gene expression in the hypothalamus after the chronic treatment was assessed by multivariate linear regression analysis for each treatment group. The statistical analyses were performed with IBM SPSS Statistics 22 (IBM Corp., Armonk, NY, USA). p-values < 0.05 were considered significant in all cases. The data are represented as mean ± SEM.

3. Results

3.1. The Importance of Stable Eating Patterns for Determining the Effects of Procyanthocyanidins on Food Intake

We first observed that stress removes the inhibitory effect of GSPE on food intake. Figure 1 shows that, in aged males, GSPE had a clear inhibitory effect on food intake that could be observed daily during the first eight days of treatment, but that this effect was lost on the ninth day when animals were subjected to eight hours of overnight fasting and followed by an OGTT, a procedure that involves several stressing situations as overnight fast, forced oral glucose administration and blood sampling from tail tips.

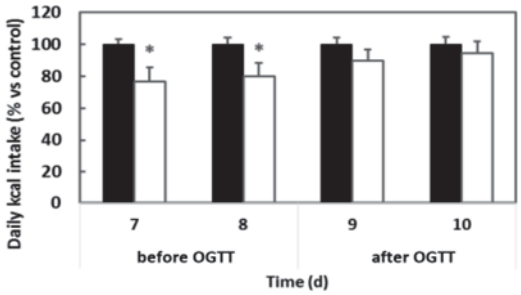


Figure 1. Effect of stressing treatment on food intake measurement. Wistar rats received grape seed proanthocyanidins (GSPE) treatment (0.5 g/kg body weight (BW)) (**white** columns) on the days shown (*x*-axis). Control animals are indicated as **black** columns. Food intake was measured 20 h after the start of the dark-phase start. On day eight after GSPE treatment initiation, rats were overnight fasted and exposed to an Oragl Glucse Tolerance Test (OGTT) the next morning. The results are shown as % vs. control group. $n = 6$ per group; * $p < 0.05$ vs. control group by Bonferroni test after ANOVA.

Another key point in the feeding physiology is the existence of feeding patterns. Food intake has been reported as having a periodicity of close to 3.5 days for male Wistar rats [33]. In the case of female rats, food intake shows a periodicity consistent with the four-day estrous cycle [35]. Single cosinor analysis showed that the food intake of both male and female rats follows a four-day cycle, with a greater minimum to maximum amplitude among females, which could reflect the influence of the estrous cycle (males: 12% ± 1%, females: 25% ± 4%; relative to the mean food intake in the period). We performed Rayleigh a test to check the effects of GSPE on the synchronicity of the food intake patterns [34]. Table 1 shows that the control animals that lived together for more than two weeks are perfectly synchronized in the first treatment study (F1) (critical r for $p < 0.05 = 0.69$). The GSPE-treated rats did not adjust to the same extent as the control animals, suggesting a change in the feeding pattern

of these animals. This effect was even stronger for the highest dose (1000 mg GSPE/kg BW). We found a similar change in the synchronization when we reproduced food intake measurements in the same control group (indicated as C (F2) in Table 1), but this time starting treatment by pairs on sequential days without enough time for synchronization between them.

Table 1. Rayleigh z-test to measure effects on feeding pattern synchronicity.

Treatment	*n*	Vector Size (*r*)
C (F1)	6	0.72 *
500 mg GSPE/kg BW	6	0.56
1000 mg GSPE/kg BW	6	0.34
C (F2)	6	0.68

F1, first treatment study; F2, second treatment study; GSPE, grape seed proanthocyanidin extract; BW, body weight; * $p < 0.05$ vs. control (C) group.

3.2. *The Minimal Dose of GSPE Required to Limit Food Intake*

To identify the minimal dose needed to obtain the inhibitory effect of GSPE on food intake, we assayed several GSPE doses (100, 500 and 1000 mg GSPE/kg BW) using synchronized females on a highly palatable diet. Figure 2a shows that a minimal dose of 500 mg GSPE/kg BW is needed to produce a statistically significant inhibition of food intake in animals fed a highly palatable diet. Since a highly palatable diet broke the normal food pattern of rats fed on standard chow, we also assayed different doses in rats fed on standard chow. Figure 2b shows a statistically significant inhibition of food intake in rats fed on standard chow after the minimum dose of 350 mg GSPE/kg BW.

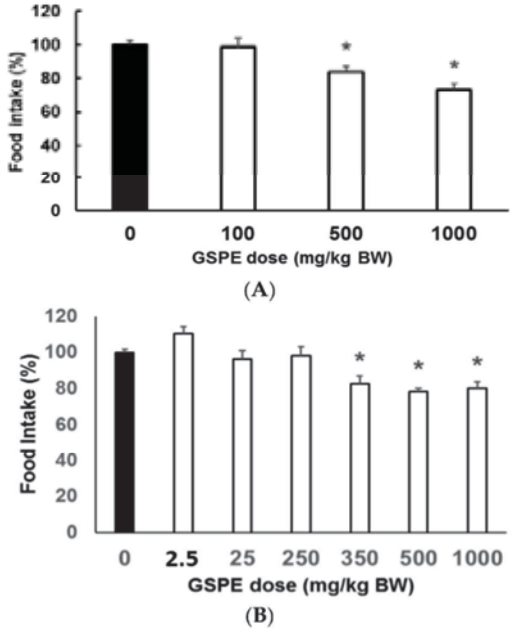
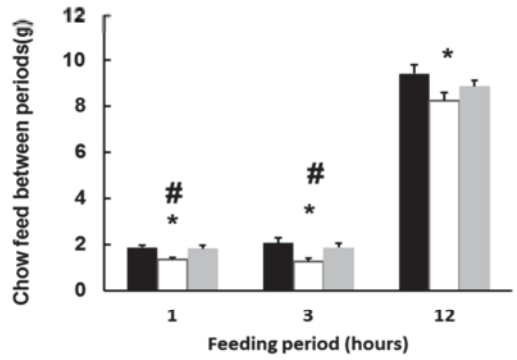


Figure 2. Range of effective doses at which grape seed proanthocyanidin extract (GSPE) inhibits food intake. Wistar rats received one of the various GSPE doses (**white** columns). Control animals are indicated as **black** columns. Food intake was measured twelve hours after the initiation of food intake period. (**A**) rats on a highly palatable diet; and (**B**) rats fed with standard chow. Results are shown as % vs. control group. $n = 6$ per group; * $p < 0.05$ vs. control group by Dunett's test after ANOVA.

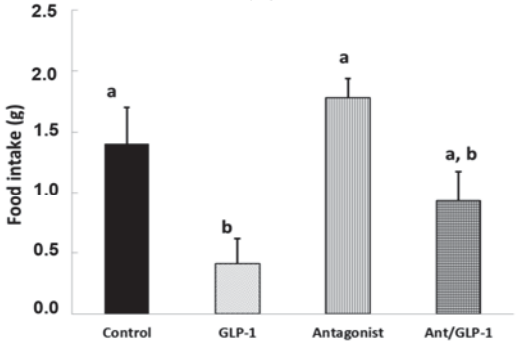
Importantly, the upper effective dose assayed had collateral effect of increasing the caecum size (g of tissue: 1.91 ± 0.12 GSPE group vs. 1.47 ± 0.1 control group; g of content: 8.22 ± 0.39 GSPE group vs. 4.3 ± 0.3 control group).

3.3. GLP-1 Plays a Major Role in Mediating GSPE Inhibitory Effects on Food Intake

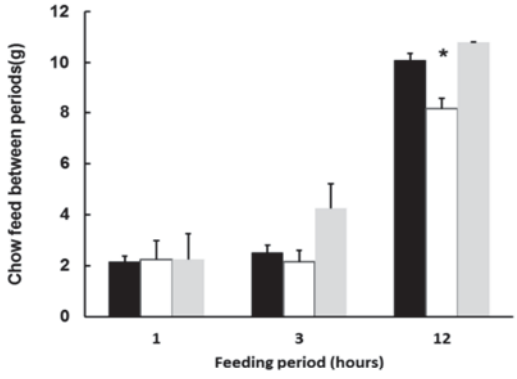
Given that GSPE was effective in increasing active GLP-1 levels [36] at doses inhibiting food intake [17], we checked the role of GLP-1 in this effect on food intake. Figure 3a shows that the short-term effects of GSPE on food intake were antagonized by the GLP-1 receptor (GLP-1-R) antagonist Ex9 [29], as was the case with i.p. GLP-1 treated rats (Figure 3b).



(A)

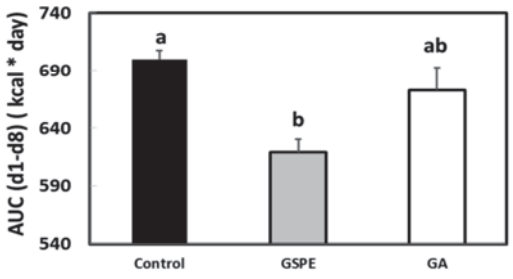


(B)



(C)

Figure 3. *Cont.*



(**D**)

Figure 3. Antagonism of Exendin (9-39) on the effects of grape seed proanthocyanidin extract (GSPE) and gallic acid on food intake. (**A**,**B**) Wistar rats received one acute oral GSPE or i.p. Glucagon-like peptide-1 (GLP-1) treatment, with or without Exendin (9-39) prior to the start of the food intake period. Food intake was measured at the time intervals indicated, counted from the initiation of the dark phase. The **black** column refers to the control group: (**A**) the **white** column refers to GSPE treatment of 0.5 g/kg body weight (BW); the **grey** column indicates those animals that previously received treatment with Exendin (9-39); (**B**) food intake measurements were at 3 h after the food initiation period. $n = 14$ per group. # $p < 0.05$ vs. antagonist. * $p < 0.05$ vs. control group. Different superscripts indicate statistical differences between the treatments by *t*-test; (**C**,**D**) relate to the effects of gallic acid on food intake: (**C**) animals received one dose of treatment. The **white** column refers to gallic acid treatment; the **grey** column indicated those animals that previously received treatment with Exendin (9-39); (**D**) refers to animals treated for eight days with the indicated treatments. $n = 9$ per group * $p < 0.05$ vs. control group by *t*-test. Different superscripts (lowercase in panels B and D) indicate statistical differences between the treatments.

Since we were working with an extract, we were interested in identifying the main ingredient responsible for this effect on food intake. We checked whether gallic acid (for which a food intake inhibitory effect has also been defined [37]), could play a role in the effects of GSPE on food intake. Figure 3c shows that gallic acid administered in the equivalent dose as in the GSPE treatment had an effect on food intake 12 h after the initiation of the feeding period.

Furthermore, the effects of equivalent doses of gallic acid and GSPE on food intake inhibition were different after a chronic treatment. We found that gallic acid exhibited lower effects than the GSPE extract, as shown in Figure 3d.

3.4. Key Organs for the Inhibitory Effect of GSPE on Food Intake

In order to identify where GSPE acts to modulate food intake, we assayed the changes in the main organs involved in controlling food intake. To do this, we analyzed gastrointestinal derived signaling, peripheral signals and the central nervous system.

Figure 4a shows a higher FITC content in the upper gastrointestinal tract, indicating lower gastrointestinal motility as a result of GSPE treatment. To analyze peripheral signaling, we assayed changes to leptinaemia after eight days of GSPE treatment. There were no differences in leptin levels between the groups (0.5 dose: 13.2 ± 5.04; control: 17.78 ± 1.82 (ng/mL)).

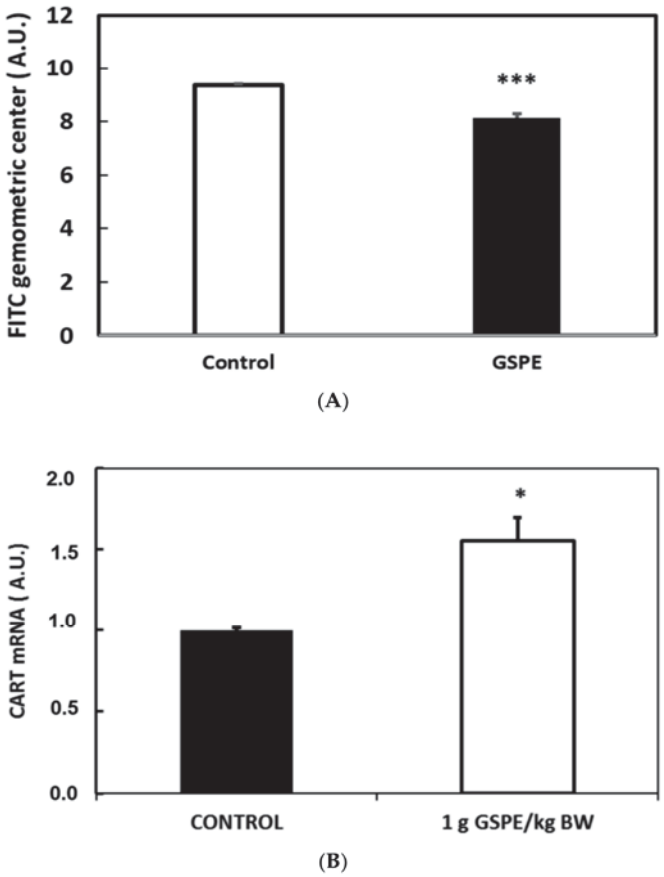


Figure 4. Grape seed proanthocyanidin extract (GSPE) effect on physiological parameters. Wistar rats that received GSPE treatments are indicated as **white** columns. Control animals are indicated as **black** columns. (**A**) rats received a chronic treatment with 0.5 g GSPE /kg body weight (BW). Fluorescein isothiocyanate (FITC) was orally forced and FITC in different gastrointestinal localizations was measured after death; (**B**) rats received an acute treatment of 1 g GSPE/kg BW. After death, hypothalamic CART expression was measured. $n = 6$ per group; * $p < 0.05$; *** $p < 0.001$ vs. control group by Student's *t*-test.

To evaluate effects on the central nervous system, we analyzed changes in the expression levels of the main hypothalamic neuropeptides. We found that in rats who fasted, after an acute dose of 1 g GSPE/kg BW, which increased GLP-1 plasma levels, CART mRNA expression, increased slightly, with no changes in the other assayed neuropeptides (Figure 4b). After chronic treatments, with doses limiting food intake, we found no clear effects on the hypothalamic mRNA levels of GLP-1, GLP-1-R, POMC/CART and NPY/AgRP, but there was a very strong statistical correlation between them. In order to evaluate the effect of GSPE on hypothalamic gene expression, we analyzed the multivariate linear regression between mRNA levels and food intake in these animals. Table 2a shows that 79% of food intake in the control animals was explained statistically by hypothalamic POMC expression (first row). GSPE treatments changed this relation, suggesting an effect on the hypothalamic control of food intake. This effect was more evident in the dose of 1 g GSPE/kg BW, where a statistically significant 79% of food intake could be explained by the hypothalamic GLP-1 expression.

Sisley et al. argued that neuronal GLP-1Rs mediate the anorectic effects of liraglutide, a long-lasting GLP-1 agonist [38]. As shown in Table 2b, we checked if GSPE modulated the relations between the expressions of this receptor and the food intake control neuropeptides (AgRP/POMC/NPY/CART). mRNA expression of GLP-1 receptor fits perfectly (100%) with the expression of CART and NPY in control rats. GSPE treatments also abolished the relationship between NPY and GLP-1-R. Likewise, in the control group the correlation between AgRP and POMC accounts for 94% of the changes in the GLP-1 receptor ($p = 0.059$), in a relationship that is also modified by GSPE. Despite the possibility that 86% of the changes in the GLP-1 receptor after the 0.5 GSPE dose were due to POMC mRNA (Table S1 included as supplementary material), there was a lack of correlation between GLP-1 mRNA levels and the mRNA of the other neuropeptides.

Table 2. Multivariate linear regression after chronic grape seed proanthocyanidin extract (GSPE) treatment.

(**a**)							
Treatment	**a**	**X_1**	**b**	**X_2**	**c**	***p***	**R^2**
Control	55.54	POMC			343.6	0.044 *	0.79
0.5 GSPE	9.08	POMC			373.9	0.426	0.33
1 GSPE	2.67	POMC	2.24	CART	435.5	0.835	0.17
Control	29.85	GLP-1h			444.0	0.345	0.22
0.5 GSPE	−10.22	GLP-1h			423.6	0.899	0.01
1 GSPE	21.18	GLP-1h			425.7	0.045 *	0.79
(**b**)							
Treatment	**a**	**X_1**	**b**	**X_2**	**d**	***p***	**R^2**
Control	0.37	CART	−0.25	NPY	0.37	0.000 *	0.99
0.5 GSPE	2.94	CART	1.06	NPY	−10.55	0.772	0.41
1 GSPE	0.30	CART	0.56	NPY	−0.46	0.389	0.85
Control	0.70	POMC	−0.87	AgRP	0.49	0.059	0.94
0.5 GSPE	0.36	POMC	-	-	0.40	0.022 *	0.86
1 GSPE	−0.01	POMC	1.63	AgRP	−0.36	0.525	0.72

Wistar rats treated for eight days with 0.5 g GSPE/kg body weight (BW) or 1 g/kg were killed under fasting conditions. mRNA levels of POMC, NPY, AgRP, CART, GLP-1 and GLP-1-R were evaluated in the hypothalamus to determine their fit with the multivariate linear regression of $Y = aX_1 + bX_2 + c$. (**a**) Y: food intake from these animals; (**b**) Y: GLP-1 Receptor mRNA in hypothalamus p-value < 0.05 indicates a statistically significant regression. R^2 identifies the degree of fit between related parameters.

4. Discussion

We initially defined the inhibitory effect of GSPE on food intake in female, male, young, aged, normofagic and hyperfagic animals after an acute treatment [17]. Afterwards, we showed that a specific dose is needed to ensure a significant chronic effect on body weight control [14]. We now describe in deeper detail several aspects that should be taken into account if a GSPE treatment is to be effective.

According to our previous results, to obtain an inhibition on food intake, GSPE needs to be administered prior to or simultaneously with food intake [14]. Here, we first showed that to obtain an inhibition on food intake, animals have to be maintained as far as possible in calm, stress-free conditions. Abbott et al. lost the anorectic effect of Peptide YY (PYY) and GLP-1 administration simply by moving the rats from their original cage to a new cage [39]. In our study, we lost the effectiveness of our treatment through overnight fasting, the forced administration of glucose and the tail blood sampling required to carry out for an OGTT.

Regarding the optimal dose, we initially proved that the inhibition on food intake could be obtained by the GSPE dose, which caused an increase in GLP-1 levels, 1 g GSPE/kg BW; we then proved that GSPE at a dose of 500 mg of GSPE extract/kg of body weight is the optimal dose [17]. However, since several studies have failed to find any effect on food intake at lower doses, we

considered it necessary to determine the range of doses that could inhibit food intake. To address this point, it should be recalled that the rats' feeding behavior was perfectly adjusted under a normal food pattern with standard chow, but that this was disrupted under a highly palatable diet [40]; consequently, it was essential to evaluate the effectiveness under both situations. Working with healthy female rats after fifteen days of adaptation to synchronize their estrus cycles before studying food intake [41], we again found that 500 mg/kg BW dose is the minimal amount of GSPE required under a stimulated food intake situation. However, we also found that this dose could be reduced to 350 mg/kg BW for the animals fed standard chow. Bao and et al. [19], working with Diabetes Mellitus animals fed with standard chow, also showed a similar effect working with a similar extract. They also showed the need for a minimal dose, since, in their study, a dose of 125 mg GSPE/kg BW did not reduce food intake, while a dose of 250 produced a 15% food intake inhibition, which increased to 25% with a dose of 500 mg/kg BW. Furthermore, we identified certain undesirable effects when 1000 mg GSPE/kg BW was administered chronically, which leads us to the conclusion that an upper effective limit also needs to be established. This dose did not maintain food intake inhibition after a second treatment period, and it did not increase the animals' energy expenditure [14], probably as a result of the exaggerated effects during the first treatment period. We have showed some differences in gastrointestinal physiology and in the mechanism of action between the assayed doses that could explain the different levels of effectiveness in terms of metabolic parameters. This dose or higher ones have been shown to be nontoxic [42,43]; however, studies on toxicity did not measure food intake after chronic treatment. Consequently, our results lead us to the conclusion that GSPE treatment requires a range of optimal doses to be effective. This could also explain why so few studies have demonstrated a lack of the effects of flavanols on human food intake, since most of studies have used doses lower than suggested for humans in the present study (100 mg GSPE/ Kg BW according to Body Surface Area methodology [44]) [20–22].

Given that food intake control involves several key organs in the body, we tried to identify the main target tissue for GSPE in terms of its effect on food intake. At the gastrointestinal level, we have shown that GSPE modifies several enteroendocrine secretions and delays gastric emptying [17]. We also have shown a delay in intestinal motility after the final dose of an eight-day treatment with 0.5 g/kg BW in animals that were awake, thus reproducing the previously shown effect in anesthetized animals after a single 1 g/kg BW dose with GSPE [17]. This delaying effect is a key mechanism in the short-term limitation of food intake caused by GLP-1 [45,46]. We previously proved that GSPE increased active GLP-1 [36]. In the present study, we have shown that antagonism of the GLP-1-Receptor removes the GSPE effect on food intake, thus suggesting that the increase in GLP-1 is a key mediator regarding the effect of GSPE on food intake. Similar results were obtained by Sisley et al. working with liraglutide (a long-lasting GLP-1 agonist) [38]. From our data, we also identified for the first time that GSPE affects the standard pattern of the feeding profile in male rats. Ribas-Latre et al. showed that acute similar doses of GSPE could have chronobiological properties, depending on the time of administration [47]. In our studies, the time of administration is a constant parameter between the groups. Since GLP-1 levels have also been shown to be sensitive to circadian rhythms [48], it could be possible that the effects of GSPE on feeding patterns are related to the effects of GSPE on GLP-1.

If GLP-1 produced at gastrointestinal level is one of the signaling molecules that mediates the effect of GSPE on food intake, hypothalamic neuronal centers need to be sensitive to this treatment. GLP-1 directly activates POMC/CART neurons and, via GABAergic transmission, indirectly inhibits the neuropeptide Y/agouti-related peptide (NPY/AgRP) neurons, which collectively results in signals that reduce food intake [49]. A previous review showed that there is a lack of information regarding the effects of flavanols on food intake and the neuropeptides controlling food intake [12]. We showed that chronic GSPE treatment for 12 weeks, at a dose without an effect on food intake, concomitant with a cafeteria diet, increased the expression of hypothalamic GLP-1 and decreased GLP-1-receptors [50]. In the present study, we have shown that there is a different pattern between the relations defined for the control animals regarding food intake and the hypothalamic peptides resulting from GSPE

treatment. This effect differs depending on the dose. By comparing the relation between hypothalamic GLP-1-Receptor expression and hypothalamic peptides, we also found that the control group had a different pattern from the GSPE treated animals, all of which suggests that GSPE affects the hypothalamic centers involved in controlling the food intake.

To determine whether peripheral signals that indicate the status of the body's energy storage have a role in food intake control, we analyzed leptinemia and found no changes due to GSPE treatments. Chronic long-term treatment with doses without properties on food intake have proven effective in limiting the increase in leptinemia related to a high-fat diet in hamsters [51,52], but corrective GSPE doses for shorter periods (30 days) did not revert the higher leptinemia [51]. Thus, the different studies, working with different doses, show inconsistent results that prevent clear conclusions from being drawn regarding the effect of GSPE on leptin production.

Another aspect that needs to be addressed is identifying the compound/s in the extract that exert the inhibitory effect on food intake. Through a comparative study between GSPE and Cocoa extract (Cocoanox), we had previously ruled out epicatechin, catechin and their oligomeric and polymeric forms as the main candidates because Cocoa extract, which is rich in these forms, was less effective at inhibiting food intake than GSPE [17]. In contrast to Cocoanox, GSPE contains gallic acid and gallated flavanols. Our results showed that gallic acid, for which effects on food intake have previously been defined [37], could therefore also be involved in the effect of GSPE limiting food intake. In fact, the GLP-1 antagonist Ex9 also inhibits the effects of gallic acid limiting food intake, suggesting that gallic acid needs the participation of GLP-1. In support of this, we have also proved that gallic acid inhibits intestinal Dipeptidyl peptidase-4 (DPP4) activity, thus favoring an increase in GLP-1 intestinal production [36]. However, gallic acid only showed clear effects after several hours of treatment, an aspect that contrasts with the high bioavailability of gallic acid, which has been identified at around 60–90 min after ingestion in rats and humans [53,54]. Therefore, gallated flavanols might be responsible for the short-term effects of GSPE at inhibiting food intake. To support this hypothesis, it should be recalled that bitter compounds directly interact with gastrointestinal smooth muscles to inhibit gastrointestinal motility, thus producing a feeling of fullness and diminishing food intake [55–57]. Since the bitter-sensing proprieties of flavanols increase in the presence of a galloyl moiety in the 3rd position of the C-ring [58], the gallated flavanols in GSPE could play a main role in this short-term inhibitory effects. Proving this hypothesis is severely limited by the difficulties faced in obtaining pure gallated forms for assays in animals.

5. Conclusions

In conclusion, GSPE must be used at an optimal dose under non-stressful conditions with an appropriately defined pattern of administration to act as limiting agent on food intake. The bioactive components of the extract act by modifying the GLP-1 signaling, which acts on the central areas that control food intake. Some more work is needed to adjust their use on humans.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6643/8/10/652/s1, Table S1: Y: Multivariate linear regression. Y: GLP-1 mRNA in hypothalamus.

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Author Contributions: A.A., M.P., A.C.-M. and J.S. conceived and designed the experiments; A.C.-M. and J.S. performed the experiments; A.C.-M., J.S. and X.T. analyzed the data; A.A., M.P., X.T. and M.B. contributed reagents/materials/analysis tools; and A.A., M.B. and J.S. wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest.

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RESEARCH ARTICLE

Subchronic treatment with grape-seed phenolics inhibits ghrelin production despite a short-term stimulation of ghrelin secretion produced by bitter-sensing flavanols

Joan Serrano[1], Àngela Casanova-Martí[1], Inge Depoortere[2], Maria Teresa Blay[1], Ximena Terra[1], Montserrat Pinent[1] and Anna Ardévol[1]

[1] MoBioFood Research Group, Departament de Bioquímica i Biotecnologia, Universitat Rovira i Virgili, Tarragona, Spain
[2] Gut Peptide Research Lab, Translational Research Center for Gastrointestinal Disorders, KU Leuven, Leuven, Belgium

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Scope: Grape-seed phenolic compounds have recently been described as satiating agents in rats when administered as a whole phenolic extract (GSPE). This satiating effect may involve the release of satiating gut hormones such as GLP-1, although a short-term increase in the orexigenic hormone ghrelin was also reported. In this study, we investigated the short- and long-term effects of GSPE in rats, focusing on the role of the main grape-seed phenolics in ghrelin secretion.

Methods and results: GSPE produced a short-term increase in plasma ghrelin in rats after an acute treatment. A mouse ghrelinoma cell line was used to test the effects of the main pure grape-seed phenolic compounds on ghrelin release. Monomeric flavanols stimulated ghrelin secretion by activating bitter taste receptors. In contrast, gallic acid (GA) and oligomeric flavanols inhibited ghrelin release. The ghrelin-inhibiting effects of GA were confirmed in rats and in rat duodenal segments. One day after the last dose of a subchronic treatment, GSPE decreased plasma ghrelin in rats, ghrelin secretion in intestinal segments, and ghrelin mRNA expression in stomach.

Conclusion: The sustained satiating effects of GSPE are related to a long-term decrease in ghrelin expression. GA and oligomeric flavanols play a ghrelin-inhibiting role in this process.

Keywords:
Bitter agonists / Flavanols / Food intake / Gallic acid / Ghrelin

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1 Introduction

Ghrelin is a peptide produced in the X/A-like cells of the gastrointestinal tract [1]. Although ghrelin was first discovered as a growth hormone secretagogue, it soon became clear that ghrelin is a pleiotropic hormone with functions mainly related to the regulation of energy and glucose homeostasis. Ghrelin stimulates gastrointestinal motility, reduces fat utilization and glucose-stimulated insulin release, increases body weight and, notably, increases appetite through the stimulation of NPY/AgRP orexigenic neurons in the hypothalamus [2–6]. In keeping with this role as "hunger hormone", ghrelin blood levels fluctuate with meals [7]. In humans, ghrelin levels rise before every meal through β1 adrenergic neurotransmission and decline thereafter in a magnitude that depends on the macronutrient and caloric composition of the meal [8–10].

To be functional, ghrelin is posttranscriptionally modified with an octanoyl group by the enzyme ghrelin-*O*-acyltransferase (GOAT) [11]. The X/A-like ghrelin-producing cells are mainly found in the gastric mucosa of the stomach as closed cells, but also in a lower proportion as lumen-projected

Correspondence: Dr. Montserrat Pinent
E-mail: montserrat.pinent@urv.cat

Abbreviations: **B2**, procyanidin dimer B2; **B2g**, procyanidin dimer B2 gallate; **CA**, (+)-catechin; **EC**, (−)-epicatechin; **ECg**, (−)-epicatechin gallate; **GA**, gallic acid; **GOAT**, ghrelin-*O*-acyltransferase; **GSPE**, grape-seed phenolic extract; **h**, hours; **min**, minutes; **4F6MF**, 4′-fluoro-6-methoxyflavanone

open cells in the intestine [12]. Several G-protein coupled receptors are expressed in ghrelin-producing cells to stimulate or inhibit ghrelin secretion through neuronal, endocrine, or metabolic signals, including taste receptors [13–15].

We have reported that a grape-seed phenolic extract (GSPE) reduces food intake in rats [16]. This phenolic extract is rich in gallic acid (GA), oligomeric, and polymeric flavanols [17]. The satiating effect was sustained over an 8-day daily treatment which, together with an increased lipid metabolism, resulted in a reduced body weight gain [18]. To explain how GSPE acts as a satiating agent, we postulate that the compounds in GSPE could affect the secretion of (an)orexigenic hormones from enteroendocrine cells. In this regard, we have observed that a GSPE treatment in rats increases the circulating levels of GLP-1, a hormone with known satiating effects that is produced in the L-cells of the lower gut [16].

There is little information regarding the effects of the GSPE phenolic compounds on ghrelin secretion. Gruendel et al. found decreased postprandial ghrelin in humans after the administration of a standardized meal enriched with a carob extract, and increased plasma levels 24 hour (h) later [19,20]. This carob extract was rich in insoluble fiber, but also contained 2.8% phenolic compounds, the majority of them being GA, gallotannins, and flavonol glycosides. In contrast, a diet supplemented with a tea extract rich in monomeric flavanols did not affect fasting ghrelin in different human studies [21–23], although Hsu et al. also reported that after 16 weeks of diet supplementation fasting ghrelin decreased only in the placebo group [24]. Finally, we recently showed that the plasma levels of total ghrelin increased in rats after an acute GSPE gavage [16].

With this information, we hypothesize that the phenolic compounds of GSPE affect ghrelin release. In this study, we describe the short-term and long-term effects of GSPE on ghrelin secretion in vivo in rats and ex vivo in rat intestinal segments. A murine ghrelinoma cell line was used to study the effects of GSPE and the main pure phenolic compounds of GSPE on ghrelin release. Pure phenolic compounds were eventually studied in rats and rat intestinal segments to confirm the effects. Since monomeric flavanols are agonists of the bitter receptors hTAS2R14 and hTAS2R39 [25–28], we used ghrelinoma cells to test the hypothesis that bitter taste signaling is involved in the flavanol-mediated effects on ghrelin release.

2 Materials and methods

2.1 Materials

GSPE was obtained from Les Dérivés Résiniques et Terpéniques (Dax, France). The same lot (#124029) was used in all the studies. It contained 85% penolics [16] and was previously characterized by LC–MS/MS [29]. Its detailed phenolic composition is included in Table 1. (+)-Catechin (CA), (−)-epicatechin (EC), GA, U-73122, and probenecid were obtained from Sigma (St. Louis, USA). (−)-Epicatechin gallate (ECg) and procyanidin dimer B2 (B2) were obtained from Extrasynthese (Genay, France). The procyanidin dimer B2-gallate (B2g) was obtained from TransMTT (Gießen, Germany). Trimer C1 (C1) was obtained from Phytolab (Vestenbergsgreuth, Germany). 4'-fluoro-6-methoxyflavanone (4F6MF) was obtained from VitasM (Narva, Estonia). For all the studies, stocks were prepared in DMSO and further diluted in HEPES buffer. All the compounds were from high purity (>90%) and were tested for cytotoxicity using a LDH assay (Promega, Madison, USA); only nontoxic doses were assayed.

Table 1. Main phenolic compounds of the GSPE used in this study, adapted from Ref. [17]

Compound	Concentration (μmol/g)
GA	182.64 ± 0.47
Protocatechuic acid	8.69 ± 0.13
Vanillic acid	4.58 ± 0.24
CA	417.96 ± 11.75
EC	321.91 ± 14.71
ECg	47.57 ± 2.44
Epigallocatechin	0.88 ± 0.10
Epigallocatechin gallate	0.07 ± 0.00
Procyanidin dimer B1	153.50 ± 5.98
Procyanidin dimer B2	57.46 ± 2.40
Procyanidin dimer B3	209.71 ± 5.89
Gallated dimers	12.13 ± 0.19
Trimers	6.65 ± 0.54
Tetramers	0.04 ± 0.01

2.2 Animals

Adult (30 weeks) male and female Wistar rats were obtained from Harlan (Barcelona, Spain). Animals were single housed at 22°C under a 12 h (h) light/dark cycle (lights on at 8 am) with access to standard chow (Teklad Global Diets #2014, Harlan) and tap water ad libitum during a 1 week adaptation period. According to the manufacturer, this chow contains a small amount of phenolic compounds, allowing us to test the effects of a phenolic overdietary supplementation on a standard diet. All the experimental procedures were carried out in accordance with the ethical guidelines of the ethical committee for animal experiments of Universitat Rovira i Virgili.

2.3 Experimental design

2.3.1 Acute study

To assess the acute effects of GSPE in fasting conditions, female rats ($n = 5$) were overnight fasted (12 h) and gavaged with 1 g/kg GSPE at the end of the dark period and portal

blood was obtained 60 minutes (min) after under sodium pentobarbital anesthesia prior to sacrifice.

2.3.2 Subchronic studies

For the subchronic treatments, rats were introduced (1 week) to a daily 4 h fasting and chow was replaced in the dark onset. After the adaptation, rats were daily treated during 8 days with GSPE or GA 1 h prior to chow replacement by gavage, using tap water as vehicle. Male rats ($n = 6$) were sacrificed 24 h after the last dose. After checking the gender-independent effects of GSPE on food intake, female rats ($n = 9$) were used to study the long-term effects of GSPE on ex vivo ghrelin secretion and to study the short-term effects of GSPE or GA after a subchronic treatment. Female rats were further sacrificed 80 min after the last dose. Animals were sacrificed by aortal exsanguination and the heparinized and acidified plasma (0.1 M HCl) was stored at −80°C.

2.4 Ghrelin release from ghrelinoma cells

MGN3-1 cells (gift from Professor Hiroshi Iwakura, Kyoto University Hospital, Japan), derived from a mice gastric ghrelinoma were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C in 5% CO_2 at the Targid Lab in Leuven [30]. Cells from 25 to 35 passages were plated in 12-well plates (6×10^5 cells/well) 24 h before the experiments. For the dose–response studies, cells were incubated for 3 h with individual phenolic compounds or GSPE using HEPES buffer 0.1% DMSO as vehicle. Bitter agonism was studied with the ubiquitous bitter blocker probenecid (1 mM) [31], the specific hTAS2R39 blocker 4F6MF (200 μM) [26] and the PLC inhibitor U-73122 (10 μM) [32, 33]. For these studies, cells were preincubated for 1 h with the respective antagonist, following 3 h incubation with a mixture of antagonist and ECg or GSPE, using HEPES buffer 0.1% DMSO as vehicle. After the incubation the supernatant was collected, acidified (0.1 M HCl), and stored at −80°C.

2.5 Ghrelin release from intestinal segments

Female rats from subchronic study were sacrificed and segments (0.5 × 1.5 cm) of proximal duodenum and ascending colon were dissected in ice-cold HBSS. After a 10 min washing period in HBSS, segments were incubated during 2 h at 37°C in Krebs buffer 0.1% DMSO containing the compounds to be tested. Duodenal segments were treated with GSPE or GA and colonic segments were treated with the phenolic cecal content of GSPE-treated rats 80 min after the GSPE gavage, using the phenolic cecal content of naïve rats as control. The cecal mass (1 g) was dissolved in 10 mL/g PBS (pH 2), extracted twice with 10 mL/g ethyl acetate and the organic fraction was nitrogen dried overnight and reconstituted in 3 mL Krebs buffer 0.1% DMSO for the treatments [34, 35]. At this time point, most of the nonabsorbed catechins (34%) were found in the cecum, as determined by spectrophotometry at $\lambda = 280$ and subtracting the background found in naïve rats [36].

2.6 Ghrelin quantification

Total ghrelin from plasma samples have been analyzed with an extraction-free total ghrelin enzyme immunoassay (Phoenix Pharmaceuticals, Burlingame, USA) [16]. Active ghrelin from plasma samples and intestinal segments were analyzed with a specific octanoyl ghrelin enzyme immunoassay (Millipore, Billerica, USA). Total and octanoyl ghrelin from subchronically treated male rats and MGN3-1 cells were analyzed by radioimmunoassay after a C18 extraction, as previously described [37], in the Targid Lab in Leuven.

2.7 Gene expression

RNA was isolated with Qiagen RNeasy kit (Qiagen, Hilden, Germany). Complementary DNA was obtained using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA, USA). For ghrelin and GOAT gene expression, the quantitative RT-PCR was performed using specific TaqMan probes (Applied Biosystems) and the relative expression of each gene was calculated against the control group using the $2^{-\Delta\Delta Ct}$ method, with cyclophilin A (PPIA) as reference. To confirm the expression of bitter taste receptors in MGN3-1 cells, the RT-PCR was performed with EvaGreen Sybr chemistry (Bio-Rad, Hercules, CA, USA) using PPIA as a positive control of the reaction, using previously validated primers as follows: PPIA forward GCATACAGGTCCTGGCATCT, reverse TTACAGGACATTGCGAGCAG; mTAS2R140 forward TGCCAAAGACTCCAGAGACC, reverse GCATGACAAGTGCCAGAAAG; mTAS2R139 forward TGAATTAATGCCCTGGCTTC, reverse AGCTGGGTAGACAGGCAGAA.

2.8 Statistical analysis

Results are presented as mean ± SEM. Data were analyzed with SPSS (IBM, Chicago, IL, USA). Data from intestinal segments and plasma ghrelin with a single experimental case were analyzed by Student *t*-tests. Dose–response studies were analyzed by one-way ANOVA followed by Dunnet's test against control, with additional Student's *t*-tests when stated. The effects of GSPE and ECg in the presence of antagonists were analyzed by two-way ANOVA followed by Bonferroni tests. Significance was accepted over 5%.

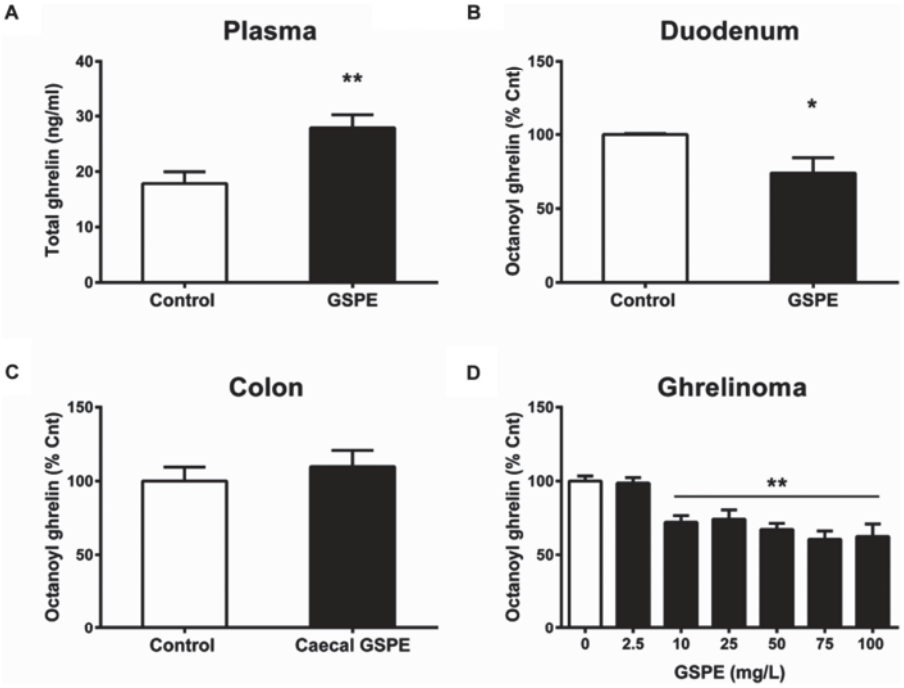

Figure 1. Acute effects of GSPE on ghrelin secretion. (A) Plasma levels of total ghrelin 80 min after the last intragastric gavage of 0.5 g/kg GSPE in 8-day treated Wistar rats, using vehicle (tap water) as control ($n = 7$). (B) Octanoyl-ghrelin secretion in tissue segments of rat duodenum after a 2-h treatment with 200 mg/L GSPE, using vehicle (Krebbs buffer 0.1% DMSO) as control ($n = 10$). (C) Octanoyl-ghrelin secretion of tissue segments of rat colon after a 2-h treatment with the nonabsorbed cecal phenolic compounds of GSPE 80 min after the GSPE gavage, using cecal phenolic extract of naïve rats as control (control: 95.15 ± 8.84 mg phenolics/L; GSPE: 188.44 ± 20.64 mg phenolics/L) ($n = 10$). (D) Octanoyl-ghrelin secretion in MGN3-1 cells after a 3-h treatment with GSPE, using vehicle (HEPES 0.1% DMSO) as control ($n = 3$ independent experiments performed in triplicate). *$p < 0.05$ versus control; **$p < 0.01$ versus control. Cnt, control group.

3 Results

3.1 Acute doses of GSPE increase ghrelin secretion in vivo, but not ex vivo or in vitro

The intragastric administration of GSPE (1 g/kg) to overnight-fasted rats tended ($p = 0.061$) to increase total ghrelin levels 60 min after the gavage (control: 3.9 ± 0.5 versus GSPE 5.5 ± 0.5; $n = 4$-5). The acute effect (80 min after the last dose) observed on the 8th day of a subchronic treatment with a lower dose (0.5 g/kg) increased total ghrelin (Fig. 1A) and produced a similar but nonsignificant increase in octanoyl ghrelin (control: 326 ± 29 pg/mL versus GSPE: 428 ± 79 pg/mL; $p = 0.219$).

Conversely, the ex vivo duodenal octanoyl ghrelin secretion of rats was reduced after an acute GSPE treatment (Fig. 1B), meanwhile a GSPE-digested cecal phenolic extract produced no effect on the colonic octanoyl ghrelin secretion (Fig. 1C). Basal release was similar between duodenum (303 ± 42 pg/g of tissue) and colon (259 ± 61 pg/g of tissue). An inhibitory effect on octanoyl ghrelin secretion was also observed in vitro when MGN3-1 mouse ghrelinoma cells were treated with GSPE (10–100 mg/L) (Fig. 1D and Supporting Information Fig. 1A).

3.2 Monomeric flavanols stimulate in vitro ghrelin secretion via activation of bitter taste receptors while oligomeric flavanols inhibit in vitro ghrelin secretion

The effects of the main available phenolic compounds in GSPE were tested in vitro on MGN3-1 cells using high purity compounds to ensure confident results. CA and EC increased in vitro octanoyl-ghrelin secretion by 10 and 30%, respectively, at 500 μM, but only EC treatment presented a significant ANOVA (Fig. 2A). ECg presented a stronger effect, increasing the secretion of octanoyl ghrelin by 70–100% from 100 to 500 μM (Fig. 2B). ECg presented a dual effect, tending to reduce octanoyl ghrelin secretion at 10 μM. The secretion of octanoyl ghrelin was more sensitive than the secretion of total ghrelin for all these compounds (Supporting Information Fig. 1B), but the ratio between both forms of ghrelin was not significantly changed by CA or EC (data not

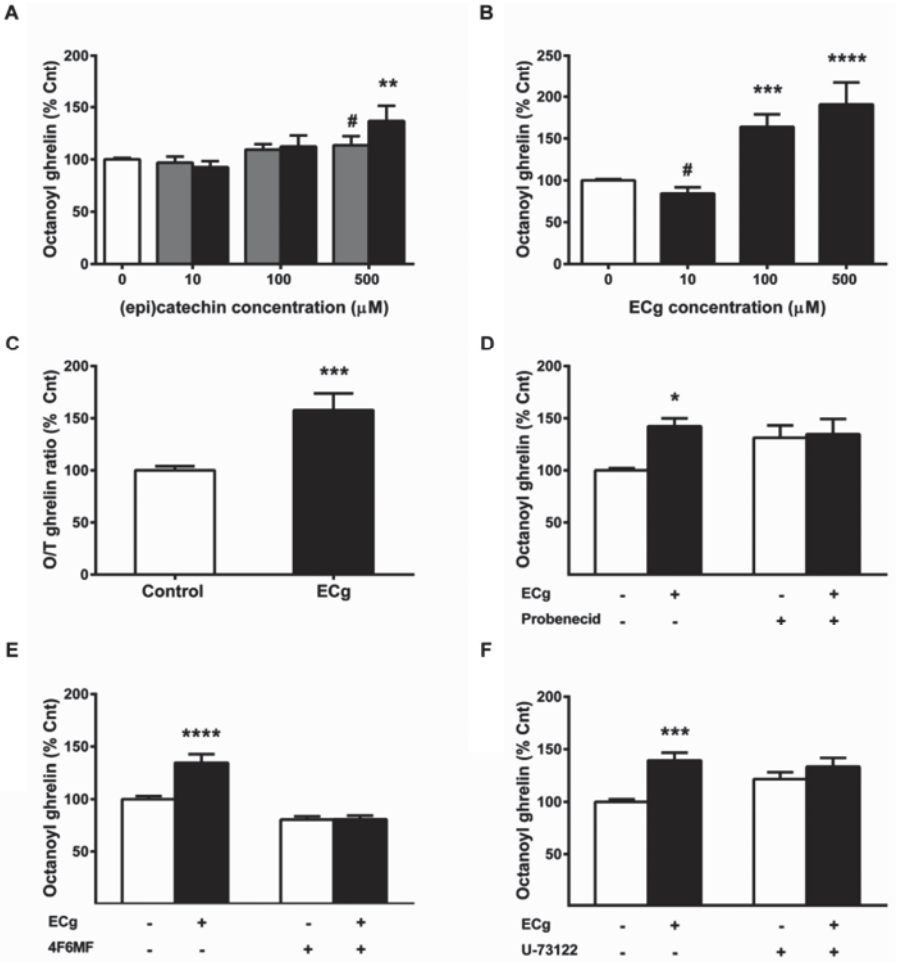

Figure 2. Stimulation of ghrelin release by monomeric flavanols in MGN3-1 cells. (A) Concentration-dependent effect of CA (gray bars) and EC (black bars) on octanoyl ghrelin release after a 3-h stimulation of MGN3-1 cells, using HEPES buffer 0.1% DMSO as vehicle (n = 5 independent experiments performed in triplicate). (B) Concentration-dependent effect of ECg on octanoyl ghrelin release after a 3-h stimulation of MGN3-1 cells, using HEPES buffer 0.1% DMSO as vehicle (n = 5 independent experiments performed in triplicate). (C) Proportion of octanoyl to total ghrelin secreted after incubation of MGN3-1 cells with 100 μM ECg, using HEPES buffer 0.1% DMSO as vehicle (n = 2 independent experiments performed in triplicate). (D–F) Secretion of octanoyl ghrelin stimulated by 100 μM ECg (black bars) after preincubation of MGN3-1 cells with 1 mM probenecid (D), 200 μM 4F6MF (E), and 10 μM U-73122 (F), using HEPES buffer 0.1% DMSO as vehicle (n = 4 independent experiments performed in triplicate). Mean octanoyl ghrelin ranged from 21 to 30 pg/10^5 cells. *p < 0.05 versus control; **p < 0.01 versus control; ***p < 0.001 versus control; ****p < 0.0001 versus control; $^{\#}p$ < 0.05 versus control by unpaired t-test. Two-way ANOVA revealed a significant treatment (ECg 100 μM) factor in the experiments with probenecid and a significant interaction (ECg 100 μM × blocker) factor in the experiments with 4F6MF and U-73122 (p < 0.05). Cnt control group.

shown). Only ECg significantly increased the proportion of octanoyl ghrelin secreted (Fig. 2C).

To check the specific interaction between flavanols and bitter taste receptors, we first confirmed the expression of the mouse orthologs of human bitter flavanol receptors, mTAS2R140 and mTAS2R139, in MGN3-1 cells by RT-PCR (threshold cycles: TAS2R140 = 38 ± 0; mTAS2R139 = 32 ± 0). Both the ambiguous bitter receptor blocker probenecid and the specific hTAS2R39 antagonist 4F6MF completely blocked the stimulatory effect of ECg on MGN3-1 cells (Fig 2D and E). A similar effect was obtained with the PLC inhibitor U-73122 (Fig. 2F) confirming the canonical intracellular bitter signaling through PLCβ2 [38].

The effects of flavanols on ghrelin secretion shifted as their degree of polymerization increased. Dimers B2 and B2g did not significantly affect ghrelin secretion, when analyzed by ANOVA (Fig. 3A), while B2g inhibited the secretion of total ghrelin (Fig. 3B) and the trimer C1 reduced the secretion of both forms of ghrelin by 40% at 500 μM (Fig. 3C and D).

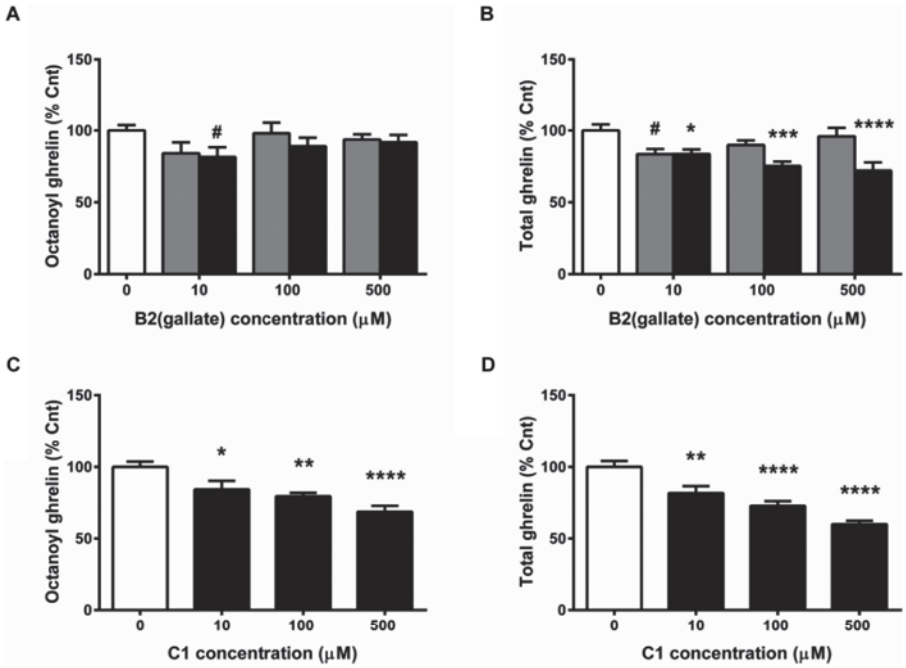

Figure 3. Inhibition of ghrelin release by oligomeric flavanols in MGN3-1 cells. (A and B) Effects of dimer B2 (gray bars) and dimer B2 gallate (black bars) on the octanoyl ghrelin (A) and total ghrelin (B) secreted by MGN3-1 cells after 3 h incubation, using HEPES buffer 0.1% DMSO as vehicle ($n = 4$ independent experiments performed in triplicate). (C and D) Dose–response inhibition of octanoyl ghrelin (C) and total ghrelin (D) by trimer C1, using HEPES buffer 0.1% DMSO as vehicle ($n = 4$ independent experiments performed in triplicate). Basal octanoyl ghrelin secretion ranged from 26 to 34 pg/10^5 cells, basal total ghrelin secretion ranged from 390 to 440 pg/10^5 cells. *$p < 0.05$ versus control; **$p < 0.01$ versus control; ***$p < 0.001$ versus control; ****$p < 0.0001$ versus control; $^{\#}p < 0.05$ versus control by unpaired *t*-test. C1, procyanidin trimer C1; Cnt, control group.

3.3 GA reduces ghrelin secretion in vitro, ex vivo and in vivo

When pure phenolic compounds were screened for their effects on MGN3-1 cells, it was found that GA diminished the secretion of octanoyl ghrelin at 10 μM (Fig. 4A) and decreased the proportion of octanoyl ghrelin released (Fig. 4B). This reduction in octanoyl ghrelin secretion was studied further ex vivo and in vivo. Octanoyl ghrelin secretion in rat duodenal tissue segments was reduced after an acute GA treatment (Fig. 4C). To analyze GA effects in vivo we subchronically treated rats for 8 days with a dose of GA equivalent to the amount found in a 0.5 g/kg GSPE treatment. These animals showed reduced octanoyl ghrelin levels 80 min after the last dose of GA (Fig. 4D).

3.4 A subchronic treatment with GSPE reduces octanoyl ghrelin in rats and inhibits its production

We have previously shown that an 8-day GSPE treatment produced a sustained reduction in food intake [18]. Twenty four hours after the last dose with 0.5 or 1 g/kg GSPE we found lower plasma levels of octanoyl ghrelin (Fig. 5A). Likewise, the basal ex vivo ghrelin secretion was notably reduced in tissue segments of duodenum and colon of rats treated for 8 days with 0.5 g/kg GSPE (Fig. 5B). In contrast, the plasma levels of total ghrelin were not significantly changed, but tended to decrease in a dose-dependent manner (control: 77 ± 11 pg/mL; 0.5 g/kg: 64 ± 11 pg/mL; 1 g/kg: 59 ± 15 pg/mL). The resulting percentage of octanoyl ghrelin over total ghrelin was significantly reduced in the 0.5 g/kg group. At the gene expression level there was a clear reduced expression of ghrelin gene expression in the gastric body for both doses of GSPE (Fig. 5D). The changes in GOAT expression were only found in colon in the 1 g/kg dose (Fig 5C). The gene expression levels of GOAT and ghrelin in the gastric body inversely correlated with food intake during the treatment (Supporting Information Table 1), with *p*-values of 0.014 and 0.016, respectively.

Since these animals showed a limited orexigenic signal before meal initiation, we measured food intake 24 h after the last treatment with GSPE. In line with the expected lower ghrelin gene expression and plasma levels, these animals showed reduced food intake during the first 3 h after meal initiation. This reduction at the beginning of the feeding period was sustained for the 5 days following the last dose of

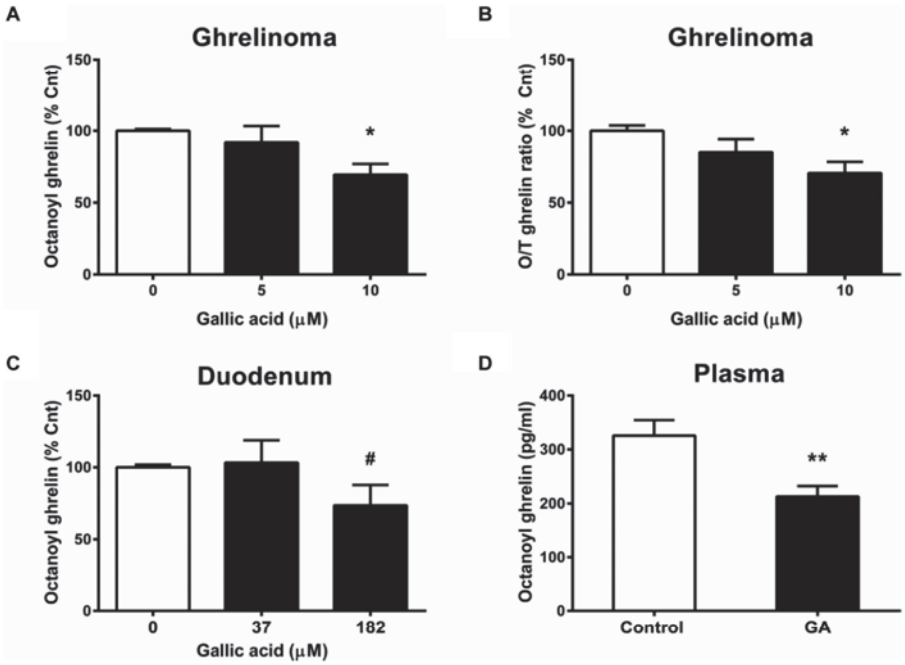

Figure 4. Effects of GA on ghrelin release. (A and B) Inhibitory effects of GA on the octanoyl ghrelin (A) and the octanoyl to total ghrelin ratio (B) secreted by MGN3-1 cells after 3-h incubation, using HEPES 0.1% DMSO as vehicle ($n = 3$ independent experiments performed in triplicate). Mean basal secretion of octanoyl and total ghrelin was 23 and 435 pg/10^5 cells, respectively. (C) Octanoyl-ghrelin secretion in tissue segments of rat duodenum after a 2-h treatment with GA, using Krebs buffer 0.1% DMSO as vehicle ($n = 6$–9). (D) Rat plasma levels of octanoyl ghrelin 80 min after the intragastric gavage of 15.5 mg/kg GA, using rats gavaged with vehicle (tap water) as control ($n = 8$). $^*p < 0.05$ versus control; $^{**}p < 0.01$ versus control; $^{\#}p < 0.05$ versus control by unpaired *t*-test. Cnt, control group.

GSPE (Fig. 5E), although the total daily food intake recovered immediately (Fig. 5F).

4 Discussion

There is little information available regarding the effects of phenolic compounds on ghrelin secretion [39,40]. Our in vivo studies show that GSPE causes an acute stimulating effect on plasma ghrelin levels. This moderate short-term effect of GSPE after a single acute dose in vivo remained similar after the last dose of a subchronic treatment. Conversely, GSPE decreased long-term ghrelin synthesis and plasma levels 24 h after the last dose of a subchronic treatment in vivo. In contrast to this, we found a marked decrease in ghrelin secretion in vitro and ex vivo after acute treatments with GSPE. Given that the ghrelin-producing cells in the intestine, but not those in the stomach, directly sense the gastrointestinal content [12], and that GSPE is composed of several different molecules with different bioavailability [41], we initially hypothesized that converse effects observed in vivo over time may be due to the different molecules in GSPE having opposite effects on ghrelin secretion.

The in vitro studies with the main available molecules in GSPE confirmed various disparate effects, with monomeric flavanols stimulating ghrelin secretion and oligomeric flavanols and GA inhibiting ghrelin secretion. In keeping with this, the gavage of GA reduced the plasma levels of octanoyl ghrelin and a cocoa extract rich in monomeric flavanols and without GA tended to increase food intake in rats [16]. These results also coincide with studies in humans that report a decrease in ghrelin after the administration of a carob extract containing GA and a prevention in ghrelin decrease after the administration of tea extracts containing monomeric flavanols [19, 24].

The fact that GSPE produced a dual effect in vivo could reflect differences between monomeric flavanols and the ghrelin-inhibiting compounds in terms of digestion and bioavailability. Given that Rios et al. found similar gastric emptying of monomeric and oligomeric flavanols [42], we can rule out the possibility that the converse effects observed over time are caused by the monomeric flavanols acting on the open-type cells directly after the GSPE gavage. In fact, the stomach is the main producing site for circulating ghrelin, so changes in plasma ghrelin would likely reflect changes in ghrelin release from the stomach [43]. Therefore, differences in the bioavailability of these molecules could play a main role in vivo. GA, monomeric flavanols and the lower flavanol oligomers are all absorbed in the upper gastrointestinal tract and reach their maximum plasma levels 1 h after ingestion;

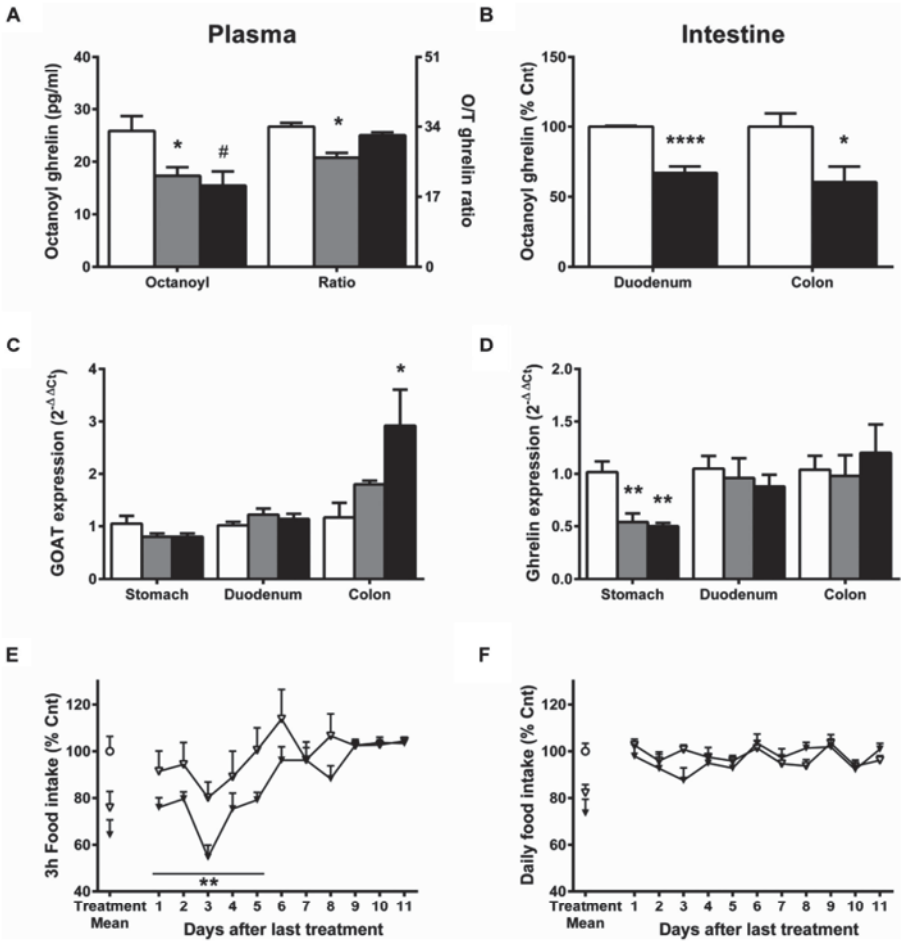

Figure 5. Effects of 8-day GSPE treatments in rats. (A) Plasma octanoyl ghrelin and octanoyl to total ghrelin ratio 24 h after the last dose of an 8-day treatment with 0.5 g/kg GSPE (gray bars) or 1 g/kg GSPE (black bars) ($n = 5$–6). (B) Basal octanoyl ghrelin secretion in tissue explants of duodenum and colon after an 8-day treatment with 0.5 g/kg GSPE ($n = 7$–10). (C) GOAT and (D) ghrelin gene expression in different segments of the gastrointestinal tract after an 8-day treatment with 0.5 g/kg GSPE (gray bars) or 1 g/kg GSPE (black bars) in rats ($n = 5$–6). Food intake during the first 3-h period (E) or the whole daily period (F) as a cumulative mean during the treatment (stated as treatment mean) and in each of the subsequent days after the treatment with 0.5 g/kg GSPE (empty triangles) or 1 g/kg GSPE (black triangles) ($n = 6$). The control group (circles) is omitted in the representation after the treatment. Vehicle (tap water) treated rats were used as a control in all the cases. Doses are stated as kilogram of body weight. $^{\#}p = 0.053$ versus control; $^{*}p < 0.05$ versus control; $^{**}p < 0.01$ versus control; $^{****}p < 0.0001$ versus control. Cnt, control group.

however, the low absorption rate of oligomeric flavanols results in plasma concentrations two orders of magnitude below those of monomeric flavanols [41, 44]. This impaired absorption means that the proportion of monomeric flavanols in the plasma is much higher and could explain the acute stimulatory effect of GSPE observed in vivo.

Importantly, we have also shown that the release-promoting effect of monomeric flavanols is caused by bitter-sensing mechanisms. It was previously shown that the bitter properties of monomeric flavanols are related to a direct agonism with the human bitter receptors hTAS2R14 and hTAS2R39 in which the activity thresholds range from 32 to 500 μM, depending on the compound, the receptor and the methodology used, ECg being the strongest agonist [25–28]. Our studies with monomeric flavanols in MGN3-1 cells showed a similar activity range. The fact that the ECg effects on these cells are blocked by the specific hTAS2R39 antagonist 4F6MF or the downstream PLCβ2 signaling indicates that the ghrelin-releasing effect of monomeric flavanols is produced by bitter-sensing mechanisms [26, 38]. The biological role of an increase in postpandrial ghrelin has been reported in several published studies but it is generally not discussed [45]. Regarding bitter tastants, it was previously shown in mice that the gavage of different bitter agonists

increased the circulating levels of ghrelin through bitter taste signaling, thus increasing food intake in the first moments of the meal [37]. This increase was followed by a notable decrease in food intake and gastric emptying, an effect also observed in humans for the bitter agonist denatonium benzoate [46] and in rats after a GSPE load [16]. Although it was argued that the ghrelin increase could promote taste aversion to these bitter, potentially toxic substances, we observed no significant gastrointestinal discomfort or conditioned taste aversion to a paired new flavor caused by GSPE [16].

Furthermore, this plasma ghrelin increase could be complementarily explained by parallel mechanisms that stimulate ghrelin secretion such as β-adrenergic signaling [8]. Monomeric flavanols are substrates of the epinephrine-degrading enzyme COMT, raising the levels of norepinephrine in vivo [47, 48]. Since norepinephrine is a potent stimulator of ghrelin release, the increased β-adrenergic tonus could also play a role in the effects of GSPE after gavage.

Despite the short-term increase in ghrelin, our results show that GSPE produces a sustained reduction in food intake, which correlates with plasma ghrelin levels and ghrelin production in the stomach. After 8 days of treatment, GSPE reduced ghrelin circulating levels and ex vivo ghrelin secretion in duodenum and colon. The in vitro studies suggest that this decrease in ghrelin release could be attributed to oligomeric flavanols and GA. It should be recalled that GA is also released from the unabsorbed flavanol polymers due to the action of microbiota, together with other phenolic and benzoic molecules such as vanillic, hypuric, or 3-hydroxybenzoic acids that appear in plasma up to 24 h after the dose [41]. In this regard, it is noteworthy that the unabsorbed GSPE compounds extracted from cecum 80 min after the dose did not affect colonic ghrelin secretion, thus suggesting that the microbial products of the unabsorbed GSPE remnant are the main ghrelin inhibitors in the colon.

The lower plasma levels of octanoyl ghrelin may also be also explained by inhibited ghrelin expression given that both doses of GSPE reduced ghrelin gene expression in the stomach, where ghrelin is mostly synthetized [12]. Since ghrelin's active form depends on its acylation process, a lower GOAT expression or activity in stomach could also play a role reducing the octanoyl ghrelin found in plasma. The long-term effects of GSPE on ghrelin and GOAT mRNA in stomach may explain the decrease on plasma acylated ghrelin. We can not discard an inhibitory effect on GOAT activity as suggested by MGN3-1 experiments, where GA reduced the proportion of octanoyl/total ghrelin secreted, so that the absorbed GA may have a role in in GOAT activity and/or expression. The low amount of ghrelin-producing cells in the colon [12] makes difficult to evaluate the overall effect of the higher GOAT gene expression found in the colon of rats treated with the higher GSPE dose. Our results do not give a clear explanation for this colon-specific enhancement of GOAT gene expression. A possible explanation could be a counter regulatory mechanism against potentially microbial-released GOAT inhibitors such as GA [49]. This highest dose also increased the cecum size, where there is a high percentage of open-type ghrelin-producing cells [12, 18].

Finally, a complementary explanation might be that GLP-1 and insulin have an indirect action on ghrelin, given that they are known to play a role in reducing ghrelin secretion [50]. We discarded the possibility that the effects of GSPE in MGN3-1 cells are caused by a direct activation of the insulin receptor, as it has previously been described in 3T3-L1 cells [51], by working with the insulin receptor inhibitor HNMPA-$(AM)_3$ (results not shown). Furthermore, it has been recently reported that the ghrelin peaks before meals prime the L-cells for a nutrient-induced GLP-1 release, in order to prepare the body for the incoming meal [52]. Therefore, the GSPE acute increase in ghrelin could enhance the effects of GSPE on GLP-1, which are only observed after a meal or glucose load [53]. This would help to explain why GSPE was more effective as a satiating agent when it was delivered before meal initiation [16, 53]. By the same token, the higher GLP-1 levels and insulinemia could contribute to the observed reduction in ghrelin 24 h after the GSPE gavage.

To conclude, GSPE stimulates ghrelin release due to the interaction between monomeric flavanols and bitter receptors. Conversely, subchronic GSPE treatment reduces ghrelin production by acting on its secretion and/or synthesis. GA and oligomeric flavanols inhibit ghrelin secretion and could play a main role in the in vivo long-term inhibition observed for GSPE. These effects on the ghrelin system help to explain the satiating effects of GSPE. The GSPE ingredients may be used as a dietary supplement to control food intake. More studies are needed to understand the molecular mechanisms behind ghrelin inhibition.

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A.A., A.C., M.P., M.T.B., J.S., and X.T. designed the animal studies; A.C. and J.S. performed the in vivo and ex vivo studies; A.A., I.D., and J.S. designed the in vitro studies; JS performed the in vitro studies; A.A., I.D., J.S., and M.P. drafted or critically revised this document.

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The authors have declared no conflict of interest.

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8.3 Statistical addendum

Modeling of relationships between enterohormones, neuropeptides, lipid metabolism and food intake in male rats after a subchronic treatment with GSPE

Aims of the chapter

The evidences presented in the previous sections of this thesis point that the satiating effect of GSPE in rats is concomitant to its effects on the enteroendocrine system. **Manuscript 2** highlights the importance of the GSPE dose in its satiating effects and its concomitancy with lipolytic activity. In **manuscript 3** we support that the acute satiating effects of GSPE and gallic acid are mediated by GLP-1 signaling, while exploring the subchronic satiating effects of GSPE using regression models. These regression models, which focused in hypothalamic gene expressions, showed that GSPE treatments affect how food intake and GLP-1 receptor gene expression in hypothalamus are related to neuropeptide gene expressions. In addition, in **manuscript 4** we describe a down-regulation of ghrelin expression in the subchronic treatments with GSPE. This section was conceived as a statistical addendum to core manuscripts presented in this thesis. It is intended to explore the possible relationships between GLP-1 and ghrelin with other gene expressions and cumulative food intake after a subchronic treatment with GSPE. With this addendum, we aim to enlighten until which extent the long-term satiating effects of GSPE are driven by GLP-1 and/or ghrelin signaling.

Statistical design

The data modelled herein was obtained from the experiments described in **manuscripts 2, 3 and 4**, corresponding to an 8-day subchronic treatment with 0.5 and 1g/kg GSPE in male Wistar rats fed on standard diet, using IBM SPSS Statistics 22. The analysis were performed with all the data available, including the most scattered, to preserve the original correlation of variables within each subject. The relationships between variables were firstly inspected using Pearsons' correlations in each treatment group. Multiple linear regression models were constructed with up to 3 predictors and followed a stepwise backwards elimination of the non-significant variables ($p>0.05$). The most significant model is presented for each dependent variable, regardless of its R^2 value. For the sake of a straightforward interpretation of the models presented, the sign of each coefficient was checked to coincide with the sign of the Pearson's coefficient of each pair of variables.

Note on food intake modelling interpretation: Cumulative food intake during the 8 days of treatment was used as a dependent variable to explore a main effect of (along) the treatment. Needless to say, it is not possible to measure food intake in a subject after gene expression measurement, neither is possible to measure cumulative gene expression along the treatment. Since the prediction of a past event may be a linguistic paradox, we suggest readers may substitute the common statistical term "predictor" for the term "descriptor" if they wish.

Abbreviations used in the tables and figures

Tissues: d duodenum **c** colon, **h** hypothalamus, **s** stomach; **Genes: PPG** preproglucagon, **GHS** ghrelin, **HSL** hormone-sensitive lipase, **NPY** neuropeptide Y, **AgRP** agouti-related peptide, **POMC** pro-opiomelanocortin, **CART** cocaine and amphetamine-regulated transcript, **GLP1R** GLP-1 receptor; **Others: FI** food intake

Results and discussion

Food intake as explained by preproglucagon-related gene expressions

We have shown that an acute 1g/kg GSPE load increases CART expression 1h after its single administration and that CART do not explain *per se* the variability in food intake after a subchronic treatment with 0.5 or 1g/kg GSPE (**Manuscript 3**). By its way, hypothalamic preproglucagon expression was only partially explaining food intake variability in the 1g/kg group (r^2=0.79), while the expression of GLP-1 receptor was unrelated. Even so, the visual inspection of Pearson's correlations showed high relationships between those variables in the 0.5g/kg group. Hence, we first modelled food intake using these GLP-1-related variables.

The selection of hypothalamic preproglucagon, CART and GLP-1 receptor fails to explain the variability in food intake in the control group (p>0.05; **table m1**), where only POMC gene expression explains food intake (**Manuscript 3**). In the 0.5g/kg treated group CART, hypothalamic preproglucagon and GLP-1 receptor explain all (r^2=1) the variability in food intake, where it must be stressed out the negative sign in both preproglucagon and CART B coefficients (**table m1**). In contrast, hypothalamic preproglucagon expression is positively explaining food intake in the 1g/kg group, with a strong tendency to statistical significance (p=0.051; **table m1**).

Table m1: FI predicted by hypothalamic GLP-1-related expressions

	B coefficients					
Group	**Constant**	**hPPG**	**CART**	**hGLP1R**	**R^2**	**p-value**
Control	428.056		16.639		0.542	0.095
0.5g/kg	763.532	-28.453	-127.627	76.885	1.000	0.022
1g/kg	360.651	68.4			0.769	0.051

Thereafter, we modelled CART and the GLP-1 receptor with its possible effectors, hypothalamic preproglucagon and gastrointestinal enterohormones. We found out that the variability in the expression of both variables is negatively explained by ghrelin and hypothalamic preproglucagon in the 0.5g/kg group (**tables m2 and m3**). In the 1g/kg group, ghrelin expression is not significativelly related to the expression of the GLP-1 receptor (**table m2**) but in contrast, CART is explained by both enterohormones and hypothalamic preproglucagon in this group (**table m3**).

Table m2: hGLP1R predicted by enterohormones and hPPG

	B coefficients					
Group	**Constant**	**cPPG**	**sGHS**	**hPPG**	**R^2**	**p-value**
Control	-0.561		1.785		0.318	0.244
0.5g/kg	3.835		-3.013	-0.202	0.965	0.035
1g/kg	4.871		-7.847		0.66	0.095

Table m3: CART predicted by enterohormones and hPPG

	B coefficients					
Group	**Constant**	**cPPG**	**sGHS**	**hPPG**	**R^2**	**p-value**
Control	-2.367		5.33		0.324	0.238
0.5g/kg	5.533		-2.803	-0.322	0.998	0.002
1g/kg	19.242	-3.005	-29.505	0.35	0.999	0.031

The negative correlations between ghrelin expression and the GLP-1 receptor and CART could be expected from the known GABA-mediated desensitization produced by ghrelin on the POMC/CART neurons [1], but the negative signs of preproglucagon predictors may be unexpected and point to a counter-regulatory mechanism. In this sense, it is woth to mention that the larger effect of GSPE in the neuropeptide expression of this subchronic treatment was found in the 1g/kg group for the GLP-1 receptor and CART, which were 20% diminished from the control group (**data not shown**). It is also noteworthy that previous experiments with GSPE leaded to a significant diminished expression of the hypothalamic GLP-1 receptor [2].

It can also be inferred from **tables m2 and m3** that the relevance of colonic preproglucagon expression is relatively low at explaining the variability in CART and the GLP-1 receptor expressions in hypothalamus. Since intestinal GLP-1 is more likely signaled to hypothalamus via vagus nerve and NTS projections, we modelled the hypothalamic preproglucagon directly with colonic preproglucagon expression (**table m4**). Despite hypothalamic preproglucagon is highly explained (93%) by its colonic gene expression in the 0.5g/kg group, this is unrelated to the hypothalamic expressions in the control and the 1g/kg group.

Table m4: hPPG predicted by colonic GLP-1

	B coefficients			
Group	**Constant**	**cPPG**	**R^2**	**p-value**
Control	1.030	0.121	0.010	0.849
0.5g/kg	-7.612	10.254	0.931	0.008
1g/kg	1.800	-0.083	0.000	0.978

We excluded ghrelin as an independent variable in the models of **table m4** due to the observed high correlations between ghrelin expressions and colonic preproglucagon. As shown in **table m5**, ghrelin is highly correlated with GLP-1 in all the groups (r2=0.7-0.8), despite the relationship between duodenal ghrelin expression and colonic preproglucagon expression in the 1g/kg group was the only significant model.

Table m5: Colonic GLP-1 predicted by GHS expressions

	B coefficients				
Group	**Constant**	**sGHS**	**dGHS**	**R^2**	**p-value**
Control	5.001	-2.384	-1.382	0.840	0.064
0.5g/kg	2.424	-2.396		0.680	0.086
1g/kg	1.986		-1.289	0.820	0.034

Food intake as explained by enterohormone expressions

The results in the previous section point to GLP-1 and ghrelin as mediators of the GSPE effects in the 0.5g/kg group, via hypothalamic-related gene expressions. A direct modelling of food intake by enterohormone expressions is not significant in this group, despite directly described a 70% of the variation in food intake (**table m6**). In contrast, ghrelin expression is directly related to food intake in the 1g/kg group, where it also stands out the counterintuitive sign in its B coefficient (**table m6**). It is hence an outstanding contrast that in the group with the lower ghrelin levels, rats with the lower ghrelin expression are those eating the most, where we must sum that ghrelin expression within this paradox explain all (100%) the variability in food intake in this group. Paradoxal or not, this result highlight the direct relationship of ghrelin to food intake in this group.

Table m6: FI predicted by enterohormones

	B coefficients					
Group	**Constant**	**cPPG**	**sGHS**	**dGHS**	**R^2**	**p-value**
Control	450.451	27.423			0.031	0.738
0.5g/kg	483.047	-65.062			0.691	0.081
1g/kg	777.101		-761.037	33.879	1.000	0.000

It should be mentioned out that plasmatic active ghrelin is negatively correlated to ghrelin gene expression in the stomach of 1g/kg treated rats, but without statistical significance. Hence, it cannot be confirmed or denied whether ghrelin gene expression in this group reflects a per se down regulation after reaching its transcription peak. In addition, plasma active ghrelin was not a significant predictor when introduced to the model of **table m6**.

Relationships of enterohormones and neuropeptides on metabolic gene expressions

Both ghrelin and GLP-1 influence energy metabolism, which may had play a role in the subchronic experiments presented in manuscript 2. CPT1 expression from brown adipose tissue and from subcutaneous white adipose tissue was not correlated with any neuropeptide gene expression combination (**data not shown**).

When HSL was modeled with the enterohormones and the hypothalamic preproglucagon gene expression it was found no relation of hypothalamic preproglucagon in any group, but a relation of ghrelin gene expression and HSL gene expression in the control and 1g/kg groups (**table m7**). Hence, rats with lower ghrelin levels express lower HSL, but treatment with 0.5g/kg GSPE increases HSL expression despite the reduction in ghrelin. In contrast, the normal relationship between ghrelin and HSL is reestablished in the 1g/kg group, in consistency with the lower HSL expression levels in this group. Similar effects were found when substituting hypothalamic preproglucagon by CART or POMC gene expression (**data not shown**).

Table m7: HSL predicted by enterohormones and hPPG

	B coefficients					
Group	**Constant**	**cPPG**	**sGHS**	**hPPG**	**R^2**	**p-value**
Control	-0.115		1.12		0.886	0.005
0.5g/kg	1.352			0.008	0.260	0.380
1g/kg	-1.814		5.771		0.799	0.041

With these results, we hypothesized whether HSL expression could be under the influence of top-down sympathetic tone via GLP-1 signaling in the 0.5g/kg GSPE group. When we modeled HSL with the sympathetic activator CART [3], the sympathetic inhibitor NPY [4], and the GLP-1 receptor as a parameter of POMC/CART neurons sensitivity, we found out that this model explains all the variability in HSL expression in the 0.5g/kg group (**table m8**), being CART the predictor with the greatest standardized coefficient (**data not shown**). It is interesting to mention that the expressions of hypothalamic GLP-1, GLP1 receptor, NPY and CART are increased in the 0.5g/kg group from 6 to 20%, despite non-significantly different from control (**data not shown**).

Table m8: HSL predicted by sympathetic modulators and GLP1-R

	B coefficients					
Group	**Constant**	**GLP1R**	**CART**	**NPY**	**R^2**	**p-value**
Control	0.546			0.458	0.320	0.242
0.5g/kg	0.989	-0.341	0.250	0.090	0.999	0.042
1g/kg	1.467	-0.412			0.380	0.268

Hence, the variability in HSL is related to gene expressions of the POMC/CART neurons in the 0.5g/kg group and to ghrelin gene expression in the 1g/kg group. Since hypothalamic gene expressions are highly counter-regulated, we speculated whether its integration to HSL gene expression could per se be used as a predictor of food intake.

Modelled alone, HSL did not explain per se the variability of food intake in any group (**data not shown**). In the case of the 0.5g/kg group, the addition of HSL as a predictor allows the use of ghrelin as a predictor when modeled with colonic preproglucagon, so food intake in this group can also be explained by ghrelin and HSL (**table m9**). When modelled with GLP-1 related neuropeptides (**table m10**) HSL (r=1) do not explain more variability than ghrelin gene expression (r=1, **data not shown**). These results are in line with our proposed role of HSL as an integrative variable of the hypothalamic gene expressions. In the case of the 1g/kg group, HSL is also a significant predictor of food intake, likely reflecting redundant data with its highly-correlated ghrelin expression (**tables m9 and m10**).

Table m9: FI predicted by enterohormones and HSL

	B coefficients					
Group	**Constant**	**cPPG**	**sGHS**	**HSL**	**R^2**	**p-value**
Control	496.96			-17.71	0.010	0.851
0.5g/kg	702.35		169.725	-278.7	0.996	0.004
1g/kg	776.105	-38.118	-552.327	-38.118	1.000	0.009

Table m10: FI predicted by neuropeptides and HSL

	B coefficients					
Group	**Constant**	**hPPG**	**CART**	**HSL**	**R^2**	**p-value**
Control	428.056		16.639		0.542	0.095
0.5g/kg	991.943	-19.733	-61.363	-243.507	1.000	0.012
1g/kg	453.481		17.541	-66.093	0.993	0.007

Highlights

The results in this addendum points to bottom-up GLP-1 signaling as the main satiety pathway produced by GSPE after a subchronic treatment in standard-fed rats, which is accentuated by the concomitant inhibition of ghrelin signalling.

Based in the physiological pathways involving the variables studied, several causal mechanisms could be hypothesized, as follows: Treatment with 0.5g/kg GSPE reduces ghrelin synthesis in stomach, which is likely to produce a lower ghrelin signaling in the vagus nerve and the AgRP/NPY neurons or GABA interneurons of the hypothalamus. This lower ghrelin signaling may have hampered the normal ghrelin-mediated inactivation of anorexigenic signals at a vagal or hypothalamic level, hence increasing the sensitivity to satiety signals such as GLP-1. In turn, the increased GLP-1 signaling in the hypothalamus appears to be a key mechanism to reduce food intake together with increased CART expression. Furthermore, the higher sympathetic tone produced by CART likely stimulates HSL expression to increase lipolysis and energy expenditure (**figure m1**).

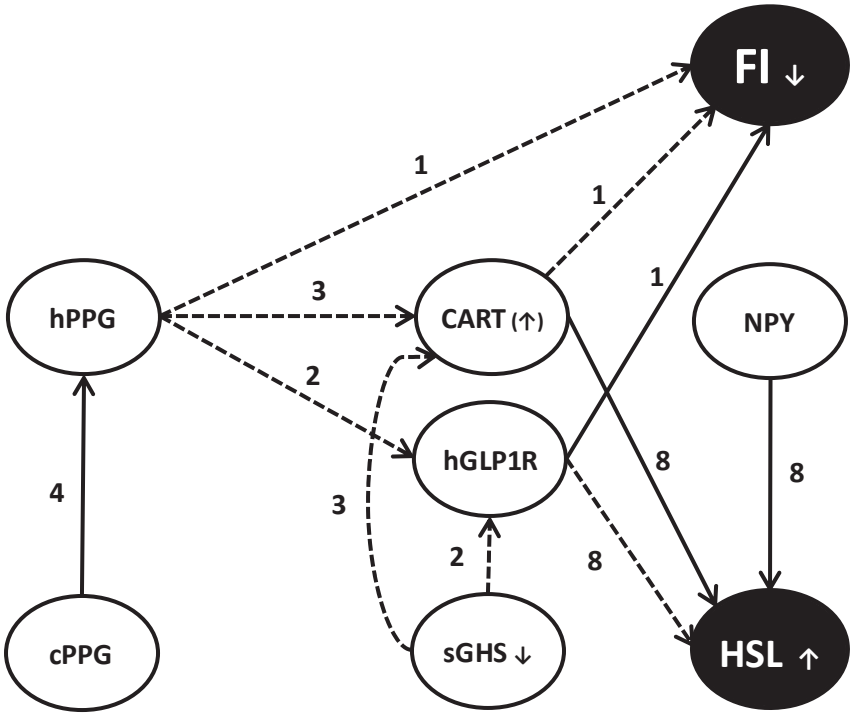

Figure m1: Diagram of modeled relationships between enterohormones, neuropeptides, lipid metabolism and food intake in male rats after a subchronic treatment with 0.5g/kg GSPE (from models in **tables m1-m8**). Solid lines represent a positive contribution to the dependent variable, while dashed lines represent a negative contribution. Numbers refer to the statistical model used in each case (number 1 for model 1, collected in **table m1**, and so on), so the contributions with the same number should be interpreted altogether. Arrows inside a variable bubble represent a significant increase or decrease in that variable, as found in **manuscripts 2, 3 and 4**, while the arrow within brackets represent a non-significant increase in CART gene expression.

The hypothalamic GLP-1 signaling also seems to produce a counter-regulatory effect in the POMC/CART neurons (**figures m1 and m2**). A total desensitization to GLP-1 likely occurred in the 1g/kg group due to its very low ghrelin synthesis, which may have increased the hypothalamic GLP-1 signaling until reducing GLP-1R and CART expressions. We propose that in this group the subjects more sensitive to the GSPE treatment present the lower ghrelin levels but also the lower CART levels and, thereafter, the higher food intake. On the top of everything, the down-regulation of POMC/CART neurons seems the key physiological mechanism beyond drug resistance to both, the satiating and lipolytic effects of GSPE in this group of rats (**figure m2**).

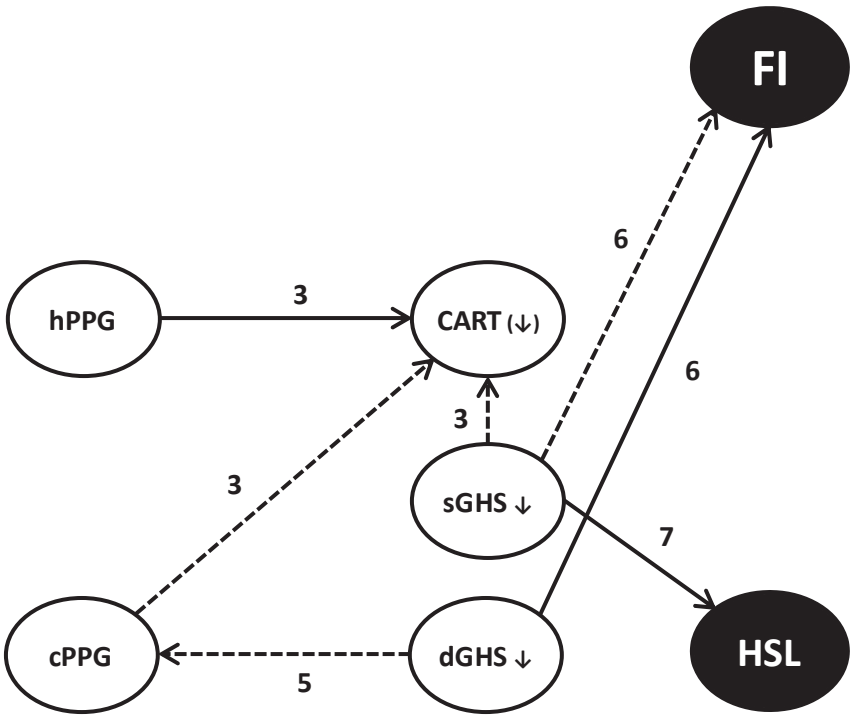

Figure m2: Diagram of modeled relationships between enterohormones, neuropeptides, lipid metabolism and food intake in male rats after a subchronic treatment with 1g/kg GSPE (from models in **tables m1-m8**). Solid lines represent a positive contribution to the dependent variable, while dashed lines represent a negative contribution. Numbers refer to the statistical model used in each case (number 1 for model 1, collected in **table m1**, and so on), so the contributions with the same number should be interpreted altogether. Arrows inside a variable bubble represent a significant decrease in that variable, as found in **manuscript 4**, while the arrow within brackets represent a non-significant decrease in CART gene expression of roughly 20%.

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DISCUSSION

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9. THESIS SUMMARY AND GENERAL DISCUSSION

The evidence provided by this thesis supports the hypothesis that GSPE is a food intake inhibitor in rats, which is related to changes in GLP-1 and ghrelin. In acute treatments, these effects can be assessed from a 350 mg/kg BW dose and extended to the larger 1 g/kg BW dose when administered intragastrically once a day, before meal initiation. These effects were largely confounded by stress, between-subject variability and rhythmical within-subject variability, which may vary food intake by as much as 25%. Acute effects are similar whether rats are on a normal or a hypercaloric diet; in this thesis, we have focused on the subchronic effects of GSPE on a normal diet.

The considerable variation in food intake due to rhythmicity and stress interaction may have veiled a modest effect size in this and other studies which focused on lower doses of GSPE. In unpublished studies by our group, female rats orally treated with a dietary 25 mg/kg BW GSPE dose daily before meal initiation tended to reduce food intake with a new cafeteria diet, although not significantly. Iin a similar preventive study carried out in male rats, GSPE between 25 and 200 mg/kg BW did not affect food intake with a new cafeteria diet which was available ad libitum before the dosage [1]. On the other hand, the same group recently reported a significant reduction in food intake after a 25 mg/kg BW dose of GSPE when it was given correctively once rats had adapted to a cafeteria diet [2]. Also in a recent paper, 250 and 500 mg/kg BW GSPE, but not 125 mg/kg BW GSPE, reduced food intake over a 16-week period when administered intragastrically once a day in streptozotocin-induced male diabetic rats [3]. Although the clear metabolic differences between the subjects of these studies may influence the sensitivity to GSPE, our data also suggest that GSPE doses may need to be high (> 200 mg/kg BW) to overcome the stress and measurement confounders of food intake and confidently reduce food intake across different experimental designs. We also suggest that after thorough consideration of any stressing factor, the rhythmic patterns in food intake could be used to perform before-after within-subject food intake experiments, which may reveal a lower effective dose of GSPE or any other satiating agent, especially in acute and subchronic designs.

As we have hypothesized, the acute increase in GLP-1 after treatment with GSPE is an effective satiety signal in rats. First, as in previous experiments, 1 g/kg BW GSPE acutely increased GLP-1 levels, and we observed a concomitant reduction in food intake and gastric emptying. Further studies with a dose of 0.5 g/kg BW also showed a reduction in intestinal motility, but more importantly a complete annulment of the satiating GSPE action after the blockade of the GLP-1 receptor with exendin. These results support the idea that an enhancement in GLP-1 signaling is responsible for the acute satiating effects of GSPE.

After GSPE had been observed to have acute satiating effects, we had to respond to two important questions before we considered to study any mechanistic issue. First, could this satiating effect be a byproduct of some kind of discomfort? And second, could this satiating effect be sustained on a daily basis in a chronic or subchronic treatment? Plasma GLP-1 and CART gene expressions increase after our treatments and are also known to be mediators of conditioned taste aversion. Indeed, the effects of the gold-standard malaise-inducing agent LiCl seem to be mediated by the GLP-1 receptor itself [4,5]. Although in this sense malaise may be an extreme feature of GLP-1 related satiety, the fact that the higher 1 g/kg BW GSPE dose does not have this side effect suggested

that GSPE can be used on a daily basis. On the other hand, we observed that both 1 g/kg BW and 0.5 g/kg BW doses had very similar effects on food intake and body weight gain throughout the initial 8-day treatment, which calls into question the need for a 1 g/kg BW dose. Indeed, the 1 g/kg BW dose was not only not necessary but counterproductive, because it had a rebound effect on body weight after the treatment, non-significant satiating effects during a second 8-day treatment, and a final body weight equivalent to that of the control group.

In this subchronic paradigm, it was important to discern to what extent the novel satiating effect of GSPE plays a role in body weight reduction versus the effects that phenolic compounds have on digestive impairment and increased energy expenditure. Fecal analysis showed that GSPE significantly impaired digestion, but accounted only for 2% of the ingested energy, which will not solely account for the overall decrease in body weight. On the other hand, it may be hypothesized that the fact that more nutrients reach the lower gut may enhance the secretion of GLP-1 and therefore play a role in the general effects of GSPE. This hypothesis seems to be reinforced by the fact that GSPE is more effective when administered before meal initiation, when it can impair the digestion process from the beginning, but this issue requires further study. We should also point out that the energy expenditure for the 0.5 g/kg BW dose increased in the second subchronic treatment, and that this increase may have played an important role in body weight reduction, since rats spent 12% more energy than the control group (in the light phase time-bin) even when they ate 20% less.

Focusing on the effects of GSPE on enterohormone production, both an acute dose of 1 g/kg BW and a subchronic dose of 0.5 g/kg BW of GSPE also produced a short-term increase in plasma ghrelin, but this increase did not seem to affect food intake. An increase in ghrelin is expected to desensitize vagal afferents to GLP-1 and downregulate the activity of POMC/CART neurons [6,7], but the successful blocking of the effects of GSPE by exendin indicates that the short-term increase in ghrelin may not effectively produce such a desensitization. The fact that GLP-1 rose 40 min before ghrelin in these acute situations may have prevented ghrelin from having its orexigenic effects. One of the main outcomes of the subchronic treatments with GSPE is the considerable reduction in ghrelin expression and its circulating levels 24h after the dosage. This may help to explain the long-term satiating effects of GSPE. According to other experiments, long-term GSPE treatments may also induce GLP-1 synthesis [8], even though its colonic gene expression presented large between-subject variation in our subchronic experiments. All in all, it was interesting to analyze the extent to which both hormones are related to the satiating effects of GSPE.

As a preliminary exploratory analysis, multiple linear regressions were performed in the subchronic studies, under the assumption that small changes in an outcome may be differently related to other variables in each treatment group. Although this analysis does not show a cause and effect relation per se, we were able to hypothesize about the pathways involved in the observed effects of GSPE. Especially in the 0.5 g/kg BW group, in which the treatment effectively reduced food intake, it can be postulated that the decrease in ghrelin sensitizes the POMC/CART neurons to GLP-1 stimuli, with hypothalamic preproglucagon and CART mediating the changes in gastrointestinal GLP-1 and ghrelin to reduce food intake. It is worth mentioning that the nature of preproglucagon-expressing neurons in the hypothalamus has yet to be described, so our data do not reveal whether this hypothalamic preproglucagon gene expression corresponds to intermediate hypothalamic neurons receiving information from the brainstem or if the hypothalamic preproglucagon gene expression

directly corresponds to an axonal translation of preproglucagon mRNA of brainstem neurons, a rare process but one that has been described for other mRNAs [9]. Either way, it should be pointed out that hypothalamic preproglucagon is inversely related to GLP-1 receptor, which may point to the POMC/CART neurons being desensitized to the higher GLP-1 stimuli via GLP-1 receptor expression.

Many of the relationships explored for the 1 g/kg BW group are similar to those of the control group, and confirm the paradox that, at the moment of sacrifice, the rats with lower ghrelin and higher CART and hypothalamic preproglucagon expressions are also the ones with the higher food intake. This suggests that POMC/CART neurons in the more sensitive rats are desensitized earlier to GLP-1 stimuli, which explains the late resistance of these rats to GSPE effects after a second subchronic treatment.

Serendipitously, these preliminary statistical analyses also suggest that the same gut-brain axis pathway is responsible for the subcutaneous hormone-sensitive lipase activity via CART-driven sympathetic response. On the basis of in vitro reports of catechol-O-methyltransferase (COMT) inhibition by flavonoids and increased norepinephrine-mediated BAT respiration rate by tea catechins [10,11], increased adrenergic signaling is often claimed to be responsible for the well-known lipolytic effect of flavanols but the in vivo extent of these mechanisms is not known. In this regard, our statistical results provide an alternative preliminary explanation for the in vivo lipolytic effects of polyphenols, and point to the gut-brain axis as being responsible for the effect. All in all, this hypothetical signaling pathway should be studied further in the future to improve the formulation and therapeutic window of phenolic compounds as satiating and thermogenic agents.

The central role of GLP-1 and ghrelin in the observed effects on food intake supports the idea of the enteroendocrine cells being one of GSPE's main targets. Our studies of these interactions focus on ghrelin-producing cells, in which mainly epicatechin-gallate stimulated ghrelin release and larger proanthocyanidins and gallic acid reduced ghrelin release. Although we have stated that the increased ghrelin produced by epicatechin-gallate is mediated by bitter-sensing mechanisms, we have been unable to describe a mechanistic pathway for the GSPE-driven inhibition, which stresses the need for further mechanistic studies in the future. Interestingly, the G-protein coupled receptors FFA2, FFA4 and CaSR inhibit ghrelin release and stimulate GLP-1 release when activated [12], so they may be interesting candidates to explore in regard to the mechanistic effects of GSPE on enteroendocrine cells.

A parallel step to understanding the mechanistic effects of GSPE is to unravel its bioactive compounds. The lack of effects of a cocoa-derived extract on rats plus the very potent ghrelin-inhibitory effects of gallic acid in the ghrelinoma cell line, gastrointestinal segments and in the short term in rats after a subchronic treatment indicated that gallic acid was an important bioactive GSPE compound. Indeed, gallic acid inhibited food intake in a similar way to an equivalent GSPE dose in an acute administration. Nevertheless, GSPE still has an effect if it is administered subchronically whereas gallic acid alone failed to sustain a reduction in food intake throughout an 8-day subchronic treatment. In this sense, it should be noted that all the gallated proanthocyanidins release gallic acid during their microbial processing [13], and this may should be taken into account when adjusting the dose and establishing the dosage administration pattern so that it is really

equivalent to GSPE. In addition, we have observed a reduction in ghrelin release in a postprandial state right after gallic acid administration, but lack information on the long-term (24h) effects of gallic acid in ghrelin preprandial levels, which may be related to its subchronic effects on food intake. On the other hand, other compounds in GSPE may play a role in maintaining the satiating effects of the gallic acid contained in GSPE once administered on a daily basis. We speculatively propose that flavanols of GSPE may acts as prebiotics, as previously described in the literature [14,15], with increased SCFA as a possible candidate to sustain the satiating effects of gallic acid over time.

Setting aside any mechanistic considerations, it can be inferred from our bibliographic research that effective dietary interventions using food ingredients are mostly associated with decreases in preprandial ghrelin, and increases in satiating enterohormones are reported in some cases. In this regard, GSPE seems to be unique because of the combination of a postprandial increase in GLP-1 and a decrease in preprandial ghrelin, although our studies were performed solely on rats.

In our studies, rats are given one dose of GSPE a day and the dosage extrapolation to humans is not straightforward, since we are not able to tell a priori if this dose will be equally effective in different intakes. The equivalent human dose of a 0.5 g/kg BW dose in rats is 80 mg/kg -BW, according to the Food and Drug Administration (FDA) guidance, which is 5.6 g of GSPE/day for a 70-kg person [16]. Since GSPE should be administered before meals to be effective, studies would have to be made to determine whether it should be administered once a day or thrice a day, in 1.9 g shots or greater. Studies in humans suggest doses of green tea extract and GSPE between 800 and 1500 mg/day of extract, which are far lower than our extrapolation. For green tea extract, only the higher doses reduced ghrelin secretion, but food intake was not assessed [17]. For GSPE, food intake reduction was only observed in subjects with high energy requirements [18]. Altogether, our results indicate that this dose needs to be increased in humans if there is to be any clear effect on food intake. Future work on the bioactive compounds in GSPE may help to improve our extrapolated dosage to humans.

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CONCLUSIONS

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10. SUMMING UP: MAIN CONCLUSIONS

The acute administration of a grape-seed proanthocyanidin extract reduces food intake in rats

- when intragastrically given before/on starting food intake;
- in male and female subjects of different ages;
- with a standard or a tasty hypercaloric diet;
- from a minimum dose of 0.35 g/kg BW in a standard diet; and
- without producing gastrointestinal malaise.

The subchronic administration of a grape-seed proanthocyanidin extract reduces food intake and body weight gain in rats, although different doses can have different effects.

- In the initial 8-day subchronic treatment, doses of 0.5 and 1 g/kg BW reduce food intake and nutrient absorption by a similar amount (-30% and -2%, respectively).
- However, in the second 8-day subchronic treatment, doses of 0.5 g/kg BW reduce food intake and body weight gain by -14% and -60%, respectively, and increase energy expenditure by +12%, but doses of 1 g/kg BW have no effect. This shows that **doses need to be properly adjusted to prevent drug resistance.**

Grape-seed phenolic compounds affect ghrelin and GLP-1 secretion, which are related to the changes observed in food intake in rats.

- GLP-1 signaling is the mediator of the acute satiating effects of a grape-seed proanthocyanidin extract and gallic acid.
- Catechins, and especially (-)-epicatechin-gallate stimulate ghrelin release in ghrelin-producing cells via a bitter-sensing pathway, which also increases in plasma after acute treatments with a grape-seed phenolic extract in rats.
- Proanthocyanidins, gallic acid and a grape-seed phenolic extract are inhibitors of ghrelin release in ghrelin-producing cells, which also decreases in plasma 80 min after the administration of gallic acid and 24 h after the administration of a grape-seed phenolic extract in rats.
- GLP-1 and ghrelin signaling are related to food intake after a subchronic treatment with a grape-seed proanthocyanidin extract in rats via hypothalamic CART signaling.

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