Gut induced Biomarkers of Appetite and Satiety

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1 Summary

Obesity has reached pandemic proportions; worldwide, since 1980, it has more than doubled. The current therapy options are limited: Lifestyle modification results in only modest weight loss; few pharmacological treatments are available, but they are also far from effective. The only adequate management for obesity is currently bariatric surgery; however, the perioperative risks, the limited availability of surgical expertise and the financial cost restrict access to a wide population. Thus, there is need for safe and more effective, non-surgical treatment options.

During the last decades, the gastrointestinal tract received growing attention as a control parameter for the regulation of appetite and food intake. Obesity is basically caused by an imbalance between food intake and energy expenditure. It is well documented that the gastrointestinal tract plays a key role in the control of food intake, but the regulatory circuits and their interactions are complex. The understanding of each gastrointestinal mechanism that might be involved in the process of eating provides a basis for the assessment of the potential of the gastrointestinal tract in the fight against obesity.

The basic understanding on the role of the gut starts with notion “we are what we eat”. Food enters the gastrointestinal tract, which then trigger specific mechanisms or a sensing machinery that respond to specific components of food. The anatomical bases for the sensing machinery are enteroendocrine cells in the small intestine, which act as neural triggers or as intestinal satiation peptide secreting cells. These cells express chemosensory receptors that respond to luminal stimuli (nutrients as well as non-nutrients). This thesis addresses specific mechanisms regarding enteroendocrine cells and how nutrient components interact with this machinery to stimulate and regulate the secretion of gut peptides, which play a key role in the regulation of food intake and a wide range of metabolic functions.

In a first set of experiments, we investigated the involvement of two potential targets of peptide release, such as glucagon-like peptide 1 (GLP-1), peptide tyrosine tyrosine (PYY) and cholecystokinin (CCK): i) bile acids (BAs) as possible TGR5 agonists and ii) glucose stimulating the sweet receptor T1R2/T1R3.

To investigate the physiological role of BAs, subjects received intraduodenal infusions of different loads of chenodeoxycholic acid (CDCA, a primary BA in humans) in comparison to sodium-oleate (a potent secretagogue for the peptides mentioned above) or vehicle as a

control. Administration of CDCA resulted in a significant increase of both plasma GLP-1 and CCK levels; however, the stimulatory potency was small, if we compare the magnitude of the GLP-1 and CCK responses to other well-known secretagogues such as glucose or fatty acids.

To investigate the physiological role of T1R2/T1R3 in the secretion of intestinal satiation peptides we used lactisole, a T1R2/T1R3 receptor antagonist. Subjects received i) intragastric and intraduodenal infusions of glucose and ii) intragastric and intraduodenal infusions of a liquid mixed meal, both with and without lactisole. Lactisole induced a significant reduction of plasma GLP-1 levels in both, the intragastric and intraduodenal glucose-stimulated parts. However, we observed no effect of lactisole on gastrointestinal peptide secretion in the mixed liquid meal-stimulated parts. The liquid meal consisted beside glucose also of proteins, fats and other complex carbohydrates. The lack of effect of lactisole suggests that these nutrients induced the release of gastrointestinal peptides probably via other receptor mechanisms and thus outweighed the effect of T1R2/T1R3 blockade. These findings indicate that the receptor is not alone responsible for peptide secretion; it is rather a complex interaction between the multiplicities of different receptor mechanisms. In addition, we found that the inhibitory effect of lactisole on the secretion of GLP-1 was greater in response to intragastric glucose administration compared to the intraduodenal infusion. These results let assume interaction mechanisms between gastric signals and signals from the small intestine and indicate a relevant contribution of the stomach in the regulation of gastrointestinal peptide secretion.

Indeed, several studies in animals and humans suggest that gastric and intestinal signals interact to elicit optimal satiation and adequate control of eating. In humans, little information is available on the underlying mechanisms of this interaction. In addition, uncertainties exist about the role of both gastric and intestinal parameters, as well as their interaction in the control of satiation in relation to body mass. In a second set of experience, we investigated the reciprocal control between gastric functions and intestinal parameters in the control of appetite in lean as well as in obese persons.

To investigate this potential interaction, lean subjects received either a rapid intragastric load or a continuous intraduodenal infusion of glucose or a mixed liquid meal. We found that infusions of glucose directly into the small intestine elicit only weak effects on appetite and the secretion of GLP-1 and PYY. In contrast, identical amounts of glucose delivered into the stomach markedly suppressed appetite paralleled by significantly greater plasma levels of GLP-1 and PYY. Administration of the mixed liquid meal showed a similar outcome. It seems that an initial more rapid rate of duodenal delivery after intragastric infusions account for the accelerated

secretion of GLP-1 and PYY. These findings suggest again a role of the stomach in the control of appetite and indicate interaction mechanisms between gastric emptying rates and the release of intestinal satiation peptides.

In a last series of experiments, we compared gastric emptying, intestinal peptide release and satiation parameters in response to nutrients between normal weight and obese healthy subjects. We found that gastric emptying rates were delayed in obese subjects, possible due to impaired gastric sensory functions. In addition, the increase in post-prandial plasma GLP-1 and PYY levels was reduced and the caloric intake was higher in obese compared to lean subjects. These results document once more the importance of gastric signals in the control of appetite.

Together, chemosensing receptors like T1R2/T1R3 are involved in the secretion of gastrointestinal peptides, however each receptor by itself is probably not alone responsible for peptide release – it is rather a complex interaction between different receptor mechanisms. In addition, complex interactions between different gastrointestinal signals are responsible for the control of eating. The understanding of each of these signals and interaction mechanisms is essential and could constitute a promising therapeutic approach for the treatment of obesity.

2 General Introduction

There is no doubt that the obesity epidemic continues to increase in industrialized nations as well as in developing countries, such as India, South Africa or Argentina. The world health organization (WHO) estimates the world prevalence of overweight (BMI > 25) adults at more than 1.6 billion, with more than 400 million considered as obese (BMI > 30). For 2015, the WHO predicts that more than 2.3 billion adults will be overweight, with more than 700 million obese individuals worldwide. [1, 2] The list of obesity-associated complications is tremendous and projections cite direct, obesity-related health care costs will more than double every decade [3, 4].

The current treatment options are limited. Lifestyle modifications, such as food intake restriction, exercise and behavior modification, result in only modest weight loss; poor adherence and recidivism are significant problems [5]. Pharmacological treatments are recommended for obese patients who are not responsive to lifestyle interventions alone. However, only one drug (Orlistat) has been approved for the long-term treatment by the US Food and Drug Administration (FDA); other pharmacotherapies, including rimonabant and sibutramine, have been withdrawn from the market due to safety concerns. To date, the most effective treatments for obesity are bariatric surgical therapies [6, 7]; however, the perioperative risks, limited availability of surgical expertise and financial cost allow accessibility to only a limited number of patients. [8]

Taken together, these facts demonstrate the need for effective, safer and palatable treatment options and they have encouraged research to investigate the role of gastrointestinal (GI) peptides in the control of eating and body weight. The eating-inhibitory effect of GI peptides is of great clinical interest, as their action is not only seen in healthy lean subjects, but also in obese subjects and type II diabetes patients.

But, not only GI peptides are of interest. The understanding of the integral control process of eating is important to have a rational basis for the fight against obesity: the eating process is starting with the thought about food (cephalic phase) and the first bites that evolve smell and taste (oral phase); this first phase is followed by the passage of food into the stomach with consecutive gastric distention, as well as the gastric emptying of ingested food into the small intestine (gastric and intestinal phases); finally, all these phases interact with each other, but

moreover, there is an important communication of these phases with the central nervous system (CNS).

This thesis primarily deals with the gastric and intestinal phases of eating control. Different mechanisms of GI peptide secretion were examined and possible interactions between gastric and intestinal phase signals in the control of eating were evaluated.

The introduction provides a detailed overview about the current state of knowledge concerning the process of eating control (chapter 2.1) and introduces into the different mechanisms of GI peptide secretion (chapter 2.2).

2.1 Process of eating control

Food intake provides energy (in form of calories) as well as all macronutrients (proteins, fats and carbohydrates), vitamins and minerals, which are necessary for living. Energy intake and energy expenditure (exercise) are precisely coupled over long intervals, which results in stable body weight. The processes that regulate these behaviors are collectively called energy homeostasis [9]. The two main processes are the short-term and the long-term regulation of energy homeostasis: i) “short-term, situational and meal-related signals” originate from various internal sources (e.g. GI peptide secretions), from the environment (e.g. food-related cues) and from “higher centers” (e.g. emotional and cognitive factors) and are responsible for maintaining meal size such that the daily regulation of energy intake is coordinated with energy expenditure [10, 11]; ii) “long-term, adiposity-related signals” (e.g. insulin and leptin), whose concentrations are proportional to body fat mass, couple energy intake to energy homeostasis by modulating the sensitivity of the brain to afferent inputs generated in response to the short-term factors. [10, 11] The focus of this thesis work is mainly on short-term regulation of energy homeostasis.

The initiation of a meal is influenced by non-homeostatic signals, such as food availability, social conventions and learned associations, as well as by homeostatic signals, such as the orexigenic peptide ghrelin [12]. Together these signals induce hunger and subsequently trigger food intake. Traditionally, the response of the GI tract to the entry of nutrients is separated into cephalic, gastric and intestinal phases [13]. The cephalic phase includes mainly organoleptic properties, such as sight, smell, taste and texture of the food; the gastric phase includes gastric distension and therefore the volume of a meal; the intestinal phase includes neural and hormonal signals, mainly satiation peptides, which are released in response to the specific macronutrients of a food. Whereas cephalic phase signals, especially at the beginning, contribute rather to meal ingestion, gastric and intestinal phase signals are responsible for the termination of a meal. This process of terminating food consumption is termed satiation.

Satiation (intra-meal satiety) develops during a meal and tends to bring the period of eating to an end, thus limiting meal size [14]. In contrast, satiety (inter-meal satiety) develops after food has been ingested and delays the onset of the next meal, thus regulating meal frequency [15].

Throughout the GI tract exists chemo- and mechanoreceptors that transmit the information about the nutrient content, mainly via peripheral nerves, but also via the bloodstream, to the CNS. To place the role of cephalic, gastric and intestinal phase signals on energy homeostasis into the overall context it is appropriate to briefly describe the CNS and its role in the control of eating.

2.1.1 CNS circuits of eating control

The brain receives and integrates a variety of homeostatic signals regarding energy status with non-homeostatic signals to produce an overall response of hunger or satiation [16, 17]. The main areas that are involved include the corticolimbic system, the hypothalamus and the caudal brainstem. Figure 1 provides a schematic overview about the three brain areas, as well as the homeostatic and non-homeostatic inputs.

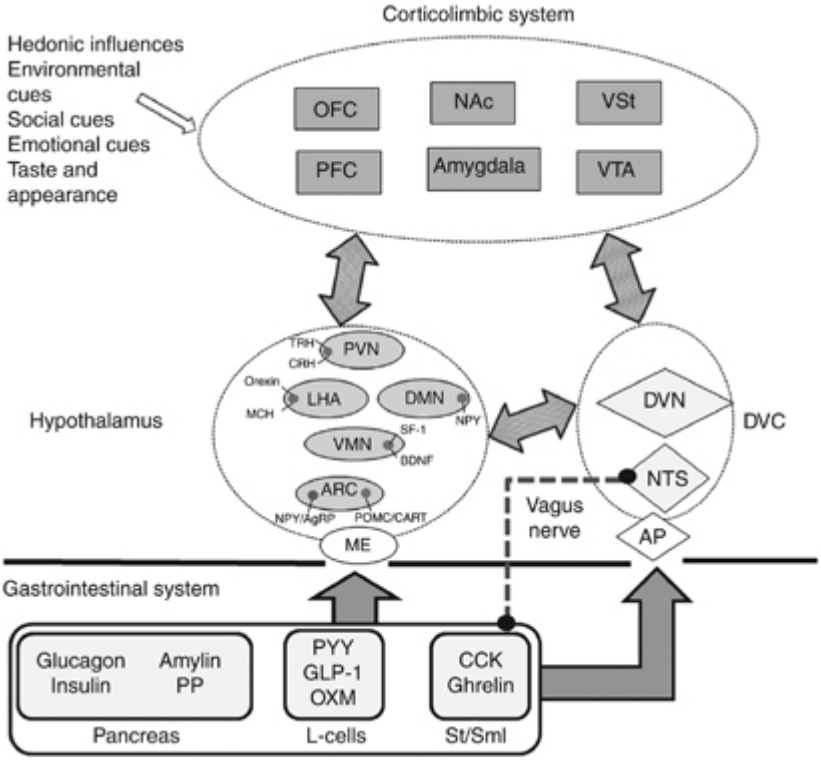

Figure 1 Neuronal network between the hypothalamus, the brainstem and the corticolimbic system, as well as homeostatic and non-homeostatic inputs [18].

Corticolimbic system

The corticolimbic system includes structures such as the prefrontal cortex (PFC), the orbitofrontal cortex (OFC), the nucleus accumbens (NAc), the ventral striatum (VSt) and the amygdala. Its main function is to process non-homeostatic inputs, including environmental cues, social and emotional cues, as well as gustatory, olfactory and somatosensory inputs [18]. Important connections between non-homeostatic and homeostatic signals were found, which indicates that the homeostatic eating control systems in the hypothalamus and caudal brainstem are modulated by non-homeostatic signals from the corticolimbic system [17].

Hypothalamus

The hypothalamus plays a prominent role in coordinating the central control of eating. It is subdivided into different nuclei, including the paraventricular nucleus (PVN), the lateral hypothalamic area (LHA), the perifornical area (PFA), the dorsomedial nucleus (DMN), the ventromedial nucleus (VMN) and the arcuate nucleus (ARC), which is one of the main nuclei in the control of eating [19]. The ARC is anatomically related to the median eminence (ME), a region that lacks a complete blood-brain barrier, which allows peripheral signals, such as hormones or nutrients, to gain access to the CNS. Within the ARC two neurons are prominently implicated in the control of feeding and are considered as first-order neurons in the hypothalamic response to peripheral signals (Figure 2A). One neuron coexpresses the anorexigenic (eating-inhibitory) peptides cocaine- and amphetamine-related transcript (CART) and pro-opiomelanocortin (POMC). The other neuron coexpresses the orexigenic (eating-stimulatory) peptides neuropeptide Y (NPY) and agouti-related protein (AgRP). Both populations communicate with i) other hypothalamic nuclei, including PVN, LHA and PFA, which contain second-order neurons (Figure 2B) and ii) other brain areas, including the nucleus of the tractus solitaries (NTS) in the caudal brainstem. The second-order neurons in the PVN are activated by POMC and α-melanocyte-stimulating hormone (α-MSH, a POMC product) and express the anorexigenic peptides thyrotropin-releasing hormone (TRH), corticotropin-releasing hormone (CRH) and oxytocin (OXY). In contrast, NPY/AgRP suppresses the second-order neurons in the PVN by decreasing POMC expression and α-MSH interaction with the MCR3/MCR4 receptor. Second-order neurons in the PFA and LHA are activated by NPY/AgRP and express the orexigenic peptides orexin and melanin-concentrating hormone (MCH). In contrast POMC and CART suppress the second-order neurons in the PFA and LHA. [16, 18, 20]

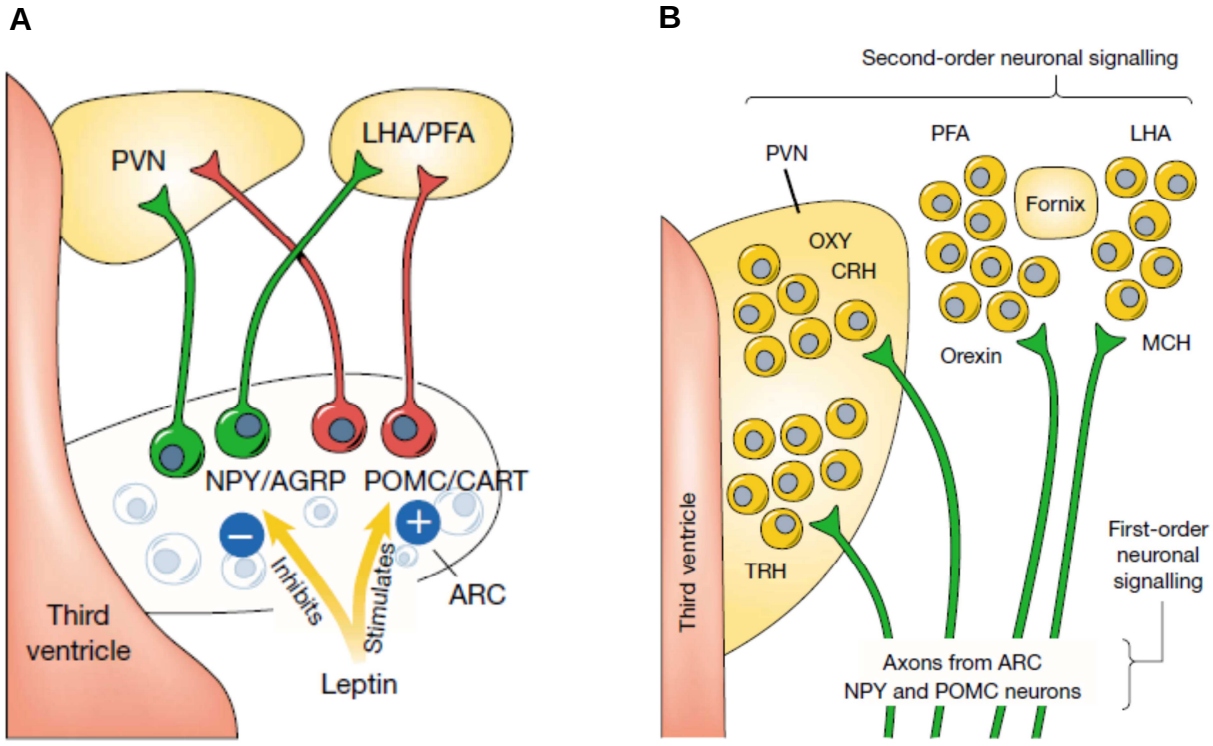

Figure 2 First-order neurons in the ARC response to peripheral signals and project to the PVN, LHA and PFA. **B** Interaction between first- and second-order neurons in the PVN, LHA and PFA. Adapted from Schwartz *et al.* [16].

A role for these hypothalamus areas in the control of eating was shown in stimulation and lesioning studies. A synthetic agonist of the MCR3/MCR4 receptor suppressed food intake, whereas a synthetic antagonist induced the opposite effect [21]. AgRP, identified as antagonist of the MCR3/MCR4 receptor [22], caused hyperphagia when administered intracerebrocentricularly [23] and furthermore, mice lacking the MC4 receptor were obese [24]. Injections of NPY into the hypothalamus of rats stimulated food intake, and repeated central administration led to obesity [25, 26]. Furthermore, PVN lesions caused hyperphagia, whereas LHA lesions caused anorexia and weight loss [3, 27, 28].

The ARC receives the input for eating control predominantly from the adiposity related signals leptin and insulin. Injections of leptin into the ARC triggered an anorexigenic response [29] and leptin was unable to reduce food intake after the ARC was destroyed [30, 31]. Receptor expression for leptin and insulin was found in both NPY/AgRP and POMC/CART neurons [32-34]. NPY/AgRP neurons were inhibited by leptin and were activated when leptin levels were low [16]. Similar effects were seen with insulin: a deficiency seems to activate these neurons, which resulted in an orexigenic response [35]. In contrast, POMC and CART expression was inhibited by reduced leptin or insulin levels, whereas increased levels of these hormones activated POMC/CART neurons [36-38]. In addition to leptin and insulin receptors, receptors for many GI

peptides (described in 2.1.2.3), including glucagon-like peptide-1 (GLP-1), peptide tyrosine tyrosine (PYY), cholecystokinin (CCK) and ghrelin, were found in the ARC; however, because these peptides may also be expressed within the brain, the role of peripheral peptides in the activation of these ARC located receptors is not completely understood to date [39].

Caudal Brainstem

Whereas the hypothalamus plays a major role in mediating the response to adiposity signals, the caudal brainstem is mainly the site that processes GI signals. In the caudal brainstem, the dorsal vagal complex (DVC), consisting of the NTS, the area postrema (AP) and the dorsovagal neurons (DVN), is responsible for the communication with the periphery in the control of eating (Figure 3) [18, 40].

The NTS receives neural and humoral signals from the GI tract. The major neural link between the GI tract and the NTS is the vagus nerve that transmits the mechanical and chemical information of ingested food [20]. Humoral signals (GI satiation peptides) can act directly on the AP (the area lacks a complete blood-brain barrier) and the signals are further transmitted to the NTS [20].

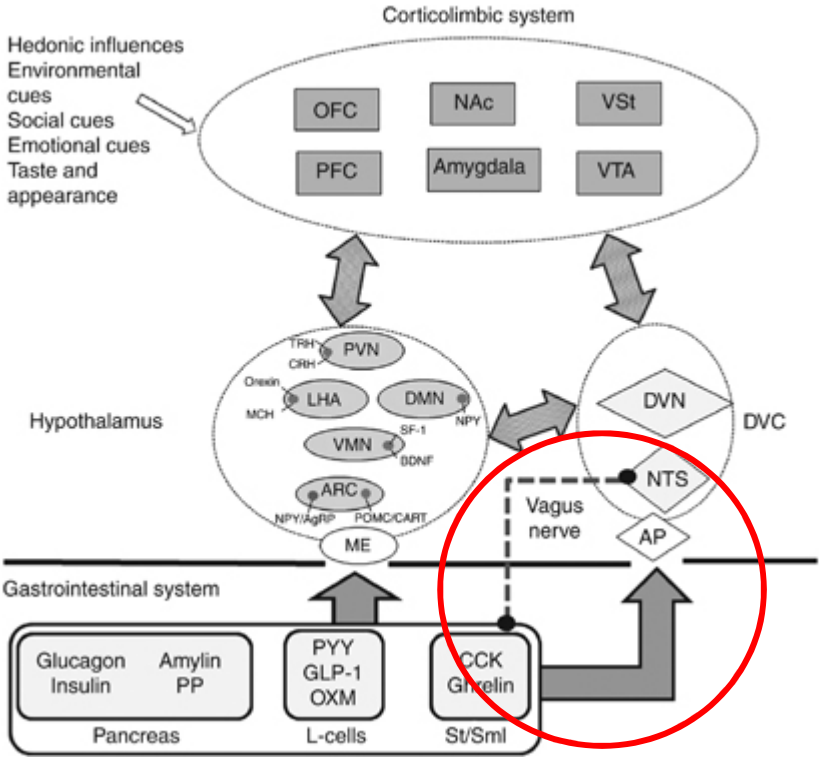

Figure 3 Simplified overview of the GI tract-brainstem axis (red circle). Adapted from Hussein *et al.* [18].

Beside signals from the GI tract, the NTS receives gustatory inputs (also via the vagus nerve), including taste and texture information from ingested food [40]. Furthermore, the NTS contains elements of leptinergic and melanocortinergic signaling systems: leptin, POMC and MC4 receptors were found in neurons of the NTS [41].

In addition to the communication with the GI tract, the caudal brainstem is reciprocally connected with the hypothalamus and the limbic system. Together they form a complex neuronal network that allows an interchange between signals from the corticolimbic system, adiposity signals and GI satiation signals [18]. However, beside these complex interactions between the different brain areas there is evidence from experiments with decerebrated rats that an isolated brainstem can terminate a meal independently from the other brain areas [42]. This indicates that a GI-brainstem communication is sufficient for the control of single meal intake.

2.1.2 Gastrointestinal phase of eating control

The GI tract is key for the control of eating: Beside its action in digestion and absorption of nutrients, the GI tract is the origin for a wide range of signals that control food intake and satiation. Traditionally, the response of the GI tract to the entry of nutrients is separated into cephalic, gastric and intestinal phase. Each of these phases is a source of satiation signals and there is ample experimental evidence that these signals interact in order to elicit an optimal satiation process and adequate control of food intake.

2.1.2.1 Cephalic phase of eating control

The term “cephalic” refers to the period of time that precedes the intake and absorption of ingested foods. The cephalic phase is separated into two different phases, which both include the stimulation of cephalic signals: The initial cephalic phase starts before ingestion with the thought about food and the anticipation of food ingestion and food reward; this phase ends with swallowing the first bites or sips. The second phase occurs after meal initiation and includes continued cephalic stimulation by the smell, taste and touch of ingested food. [43]

The gustatory and trigeminal sensors in the mouth are regarded as “gate keepers” at the entrance to the alimentary canal [44]. The gustatory sensory system includes specific receptor and transduction mechanisms for the four classical taste modalities: sweet, bitter, sour and umami. The trigeminal sensory system includes mechano- and temperature-sensors, which recognize additional attributes of ingested foods, such as creaminess and crunchiness.

Together these taste and texture informations are projected by the vagus nerve to the NTS. [40, 45, 46]

The responses to these sensory cues are collectively called cephalic phase responses (CPRs). CPRs function to prepare the GI tract for optimal digestion and absorption of nutrients [43] and include the release of saliva, gastric acid, pancreatic exocrine enzymes, as well as hormones (such as insulin or ghrelin).

2.1.2.2 Gastric phase of eating control

The second part of the cephalic phase overlaps with the start of the gastric phase. The fundus (proximal part of the stomach) accommodates the ingested food by reduction of its tone, which results in an increase in proximal and distal (antrum) stomach volume – mixing and digestion are initiated. Gastric emptying starts with the liquid phase of ingested meal; solids empty selectively when the particle size is sufficiently reduced to about 1-2 mm [47]. In general, the magnitude and timing of each gastric step are dependent on meal composition parameters such as volume, consistency, energy value and macronutrient content [13].

A role for gastric parameters such as gastric distension, gastric hormones and gastric emptying in the control of food intake has been acknowledged many times and will be discussed below. Other gastric parameters, such as pH and pyloric function are also possible gastric phase targets [48, 49]; however, these have rarely been studied in relation to food intake. [13] Evidence for a contribution of the stomach in feeding control derives from experiments involving cuffs that can reversibly close the pylorus and thus prevent the delivery of ingested food into the small intestine. Studies with this method indicated that mainly gastric distension and thus the volume of the food contributes to the termination of food ingestion [50, 51]. Phillips and Powely suggested that satiation produced by food in the stomach depends on mechanical stimulation of ingested food rather than chemical stimulation by food constituents [52]. In contrast, there is evidence that the pylorus detects the energy content of food: McHugh *et al.* showed that a fixed number of calories are delivered into the duodenum per unit of time, regardless of the composition of the food. In support, Degen *et al.* suggested that the stomach could sense the nutrient composition of an oral preload: preloads with different contents had variable effects on satiation and food intake [53]. Thus, to date, there is an open question concerning whether the stomach can sense only food volume or energy and nutrient composition as well.

The volume of ingested food is detected by vagal afferents in the external muscle layers that are sensitive to stretch and tension [54]. The following different vagal afferent endings exist: i)

intraganglionic laminar vagal afferent endings (IGLEs) found in large numbers throughout the esophagus and GI tract [55, 56]; they respond to muscle tension and generate vagal afferent tone for balanced awareness and emotional well-being (thus, they especially play a role in meal frequency) [57]; ii) intramuscular arrays (IMAs) are mostly located in gastric longitudinal and circular muscle layers [55, 58]; they respond to muscle stretch and are the prime candidates for generating the negative feedback of a full stomach [40]; iii) vagal afferent fibers that innervate the gastric mucosa and probably detect paracrine and endocrine substances such as gastrin releasing peptide (GRP), ghrelin or leptin [12, 59]. The output of these vagal sensory nerves is relayed to the CNS [50, 60], using neurotransmitters and neuromodulators such as glutamate, acetylcholine, nitric oxide, calcitonin-gene-related peptide, substance P, galanin, and cocaine-and-amphetamine-related transcripts [50, 51].

Gastric distension

The most potent gastric signals that control food intake are probably those reflecting gastric distension. Animal studies showed that gastric distension decreases the vagal firing rate for food intake and there is evidence that vagotomy blocks this satiating effect of gastric distension [61]. In humans, an early work from Geliebter *et al.* indicated that meal intake was lower and hunger ratings decreased when a gastric balloon with a volume of 400 mL or more was inflated [62]; this was confirmed in several other balloon studies [63-65] or in studies using ultrasound or magnet resonance imaging (MRI) [66-68]. Shide *et al.* reported that slow intragastric infusion of nutrients over 3.5 hours did not suppress food intake, while rapid, 15 min intragastric infusion of nutrients reduced food intake [69]. The latter induced a clear gastric distension whereas the slow infusion over several hours did not. Whether gastric distension induces its satiation effects only direct via vagal afferent fibers or also indirect via the stimulation of satiation peptide release is not known to date. Pure “mechanical” gastric distension using a gastric balloon during intestinal nutrient infusions caused not a further rise in plasma peptide levels (PYY or CCK) [70, 71]. However, to the best of our knowledge, no studies have examined the isolated effects of “nutrient” gastric distention with concomitant intestinal nutrient infusions.

Gastric hormones

The stomach produces a variety of signaling molecules that might participate in the satiation process. GRP and somatostatin are released from the antrum of the stomach and both peptides have been shown to have satiating effects in animals and humans [72, 73].

Leptin was found in rodent and human gastric mucosa; the peptide was rapidly mobilized by feeding [59]. It activated vagal afferents [74]; in addition, small doses of leptin into the celiac

artery decreased food intake; this effect was not observed in rats with subdiaphragmatic vagotomy [75].

Ghrelin is mainly secreted by X- or G-cells in the fundus of the stomach, and also by neurons in the CNS [76, 77]. It is the first and only peripherally secreted hormone with a stimulatory effect on eating. Plasma ghrelin levels rise prior to a meal when the stomach is empty and rapidly decreases upon the ingestion of food [78], thus ghrelin plays a role in meal initiation. In humans, intravenous infusion of ghrelin significantly increased hunger ratings and meal size [79]. There is evidence that ghrelin stimulates eating by the inhibition of the activity of vagal afferents; the eating-stimulating effect was abolished in rats with subdiaphragmatic vagotomy [80] and in patients with surgical procedures involving vagotomy [81]. However, in a more recent study in rats it was demonstrated that vagal afferents are not necessary for the eating-stimulatory effect, which suggests a direct action of circulating ghrelin on the brain [82]. Indeed, ghrelin receptors were found in the hypothalamus [83] and ghrelin transport across the blood brain barrier has been demonstrated [84]. The controls of ghrelin secretion are not completely understood: ingested food in the stomach was not a sufficient stimulus for decreasing the secretion of ghrelin [85]. Furthermore, small intestinal nutrient infusions were equally effective in reducing ghrelin secretion, which indicates an indirect control that may be neurally or hormonally mediated [86]. A role for CCK in suppressing ghrelin secretion was suggested: administration of a CCK antagonist blocked the ability of intraduodenal lipids to inhibit ghrelin levels [87]. Whether other intestinal peptides contribute to meal-induced decrease of plasma ghrelin levels has not been adequately investigated [88]. Ghrelin levels seem to be affected by body weight: they rise with weight loss and tend to be lower in obese compared to lean individuals [78]. This indicates a role for ghrelin in both the short-term as well as the long-term control of food intake.

Gastric emptying

Gastric emptying is influenced by several characteristics of ingested food, including nutritive, chemical and physical properties, which result in a selective emptying of the different meal components [89]. Inert liquids (water or isotonic saline) follow a single exponential curve, which means that the volume of a liquid load that is emptied in a given time period is a constant fraction [90]. Glucose or other nutrient-containing liquids show an initial more rapid phase of emptying (gastric emptying during gastric filling) [91, 92]. A subsequent linear rate of nutrient delivery has been reported by Brener *et al.* [93] and others [91, 94]; in contrast, Schirra *et al.* [95] found that gastric emptying of glucose is not constant but declines exponentially over time. Liquids with a high caloric density empty more slowly than those having fewer calories per unit volume [96]. Gastric emptying of solids exhibit an initial lag phase (mixing and retropulsion of

the food), during which only small amounts of ingested food leave the stomach, followed by a linear emptying phase [97]. In humans, solid food is delivered to the duodenum not before the particles are reduced to a size of about 1-2 mm [47].

The role of gastric emptying in the control of eating is most likely due to its counter regulatory properties. On the one hand, gastric emptying affects the magnitude and duration of gastric distension and thus the rate of nutrient delivery into the small intestine as well as the secretion of intestinal satiation peptides [13, 98-101]. On the other hand, gastric emptying is affected by the volume of a meal [102] and probably most of all by the intestinal feedback in response to specific nutrients in the small intestine [13]. Indeed, intestinal satiation peptides such as CCK, GLP-1 and PYY that are released in response to ingested nutrients are well known to delay or inhibit gastric emptying [103-105]. Therefore, gastric emptying should not be considered as solely a gastric phenomenon but more as an intestine mediated response.

2.1.2.3 Intestinal phase of eating control

The small intestine, like the stomach, contains both IGLEs and IMAs, which respond to the local volume or stretch of ingested food [50]. In addition, Berthoud *et al.* [106, 107] demonstrated in different studies that vagal afferent fibers are present in the lamina propria of duodenal and jejunal villi and crypts. These vagal afferents, however, do not extend from the lamina propria into the basal membrane to innervate the epithelial layer and therefore, they are not in a position to sense luminal nutrient content directly. These findings suggest that intestinal nutrients may activate vagal afferents by triggering the secretion of neuroactive substances from the intestinal epithelium [50]. The ability of the gut to sense changes in the luminal content and respond to it by releasing chemical signals was already described early in the twentieth century. In 1902, Bayliss and Starling [108] observed that increasing the acidity in the enervated proximal small intestine elicited pancreatic fluid secretion. They suggested that this could not be mediated by the nervous system but by a substance secreted by epithelial cells, which stimulated the pancreas via the bloodstream. They called this secreted compound ‘secretin’ – the birth of the first GI peptide. To date, more than 20 gut derived hormones (Figure 4 shows a selection) are described that are released and act either in a paracrine fashion (act locally and activate afferent nerves) or in a endocrine mode, that means, they enter the blood circulation to act directly on other organ systems including the brain. Together with gastric distension, nutrient stimulated peptide release from the intestine is considered to play a major role in the control of eating.

It is not easy to document that a gut peptide is a physiological regulator of a digestive function including food intake. Several criteria must be fulfilled before such a peptide can be accepted as a physiologically relevant satiation signal [79, 109]: i) endogenous secretion of the signal should be induced by food intake and the pharmacological profile should be related to the ingestion of the meal; ii) receptors for the signal should be expressed at its site of action; iii) exogenously administration of the signal (in amounts that reproduce endogenous secretion) should reproduce the physiological patterns of the endogenous signal, e.g. induce reduction on meal size; iv) it should evoke the opposite effect when the respective signal or receptor is removed (*knockout*) and exogenous replacement of the signal (at physiological doses) should normalize the effect; v) exogenous administration of a selective and potent antagonist of the signal should prevent the effect of the endogenous or exogenous given signal and evoke the opposite effect; vi) the inhibitory eating effect of the signal should not be the consequence of illness or malaise.

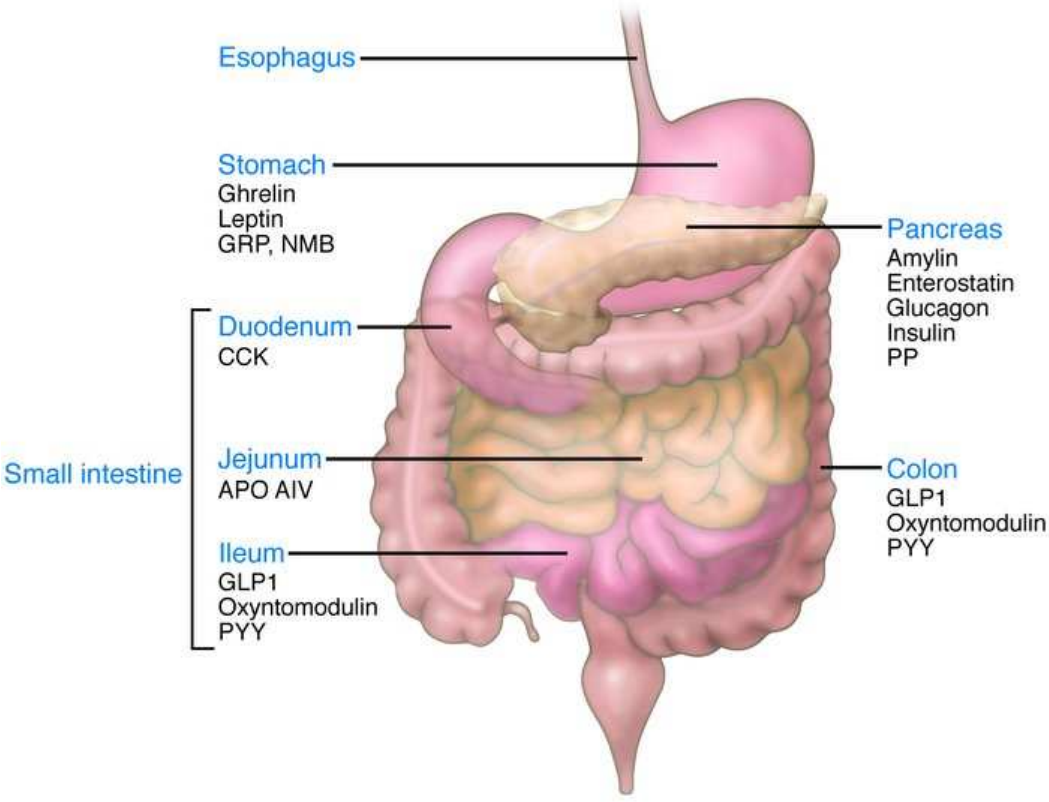

Figure 4 The main secretion sites for each intestinal peptide implicated in the satiation process. Most of these hormones are also detectable in smaller amounts at other sites of the intestine [51].

This section introduces the relevant intestinal satiation peptides for the present thesis: CCK, GLP-1 and PYY.

Cholecystokinin (CCK)

CCK derives from a 115 amino acid precursor and was first identified by Mutt and Jorpes as a peptide of 33 amino acids [110]. To date it is known that CCK exists in several bioactive forms, including CCK-8, CCK-22, CCK-33 and CCK-58, and has a number of regulatory functions, both centrally and peripherally. In the brain, CCK (mainly CCK-8) was found to act as neurotransmitter [111]. In the periphery, CCK is widely distributed throughout the GI tract, but is mainly synthesized by I-cells of the duodenum and the jejunum [112, 113] and by enteric nerves [114]. It is secreted within 15 minutes after meal initiation (especially in response to fats and proteins) [115, 116] and exerts local regulatory effects including the stimulation of gallbladder contraction [115], pancreatic enzyme secretion [115] and the inhibition of gastric emptying [103]. CCK interacts with two receptors expressed in the brain and gut: CCK-1 receptors (formerly termed CCK-A receptor for 'alimentary') are predominantly expressed in the GI tract and CCK-2 receptors (formerly termed CCK-B receptor for 'brain') occur predominantly in the brain [117, 118]. *In vitro* studies showed that the affinity of the different CCK isoforms to CCK receptors is similar [116].

CCK was the first intestinal peptide for which effects on satiation were documented. Gibbs *et al.* reported that intraperitoneal injections of CCK into rats reduced meal size in a dose-dependent manner [119]. This effect appears to be mediated by the CCK-1 receptor: administration of a selective CCK-1 receptor antagonist to rats reversed the inhibitory effect of intraperitoneal administered CCK on food intake [120] and rats with a CCK-1 receptor null mutation were hyperphagic and obese [121]. CCK is thought to act on vagal afferents near the site of CCK secretion. Indeed, CCK-1 receptor expression was found on vagal afferents and peripheral CCK administration increased vagal afferents firing in the hindbrain [122]. Abdominal vagotomy attenuated the anorectic effect of peripherally administered CCK [123]. These findings suggest a vagal pathway for CCK induced satiation.

In humans, many studies have shown that intravenous infusion of CCK-8 suppressed food intake [124, 125]. Both, Ballinger *et al.* [126] and Lieverse *et al.* [127] reported that CCK infusions at physiological doses were sufficient to receive a decrease in food intake of about 20%. Further, blocking the CCK-1 receptors with MK-329 or loxiglumide diminished the satiation effect of CCK [127-130]. These findings clearly demonstrate a role for CCK in the control of eating.

Despite this effect of CCK in the short-term control of eating, its importance in long-term body-weight regulation and its therapeutic potential for the use in the treatment of obesity are

questionable. Repeated, long-term chronic [131] or intermittent [132] administration of CCK to rats had no effect on weight loss. When CCK reduced the size of a meal, animals compensated by increasing meal frequency [132, 133]. Furthermore, the anorectic effects of continuously infused CCK were lost after 24 h [131]. The half-life of CCK is only 1-2 minutes and if administered more than 15 minutes before a meal it failed to reduce meal size; these characteristics are barriers to the therapeutic utility of CCK [134]. In contrast, CCK appears to interact with long-term signals such as leptin and insulin: the anorectic effects of CCK can be augmented by coadministration of leptin or insulin [10]. Furthermore, molecular forms of CCK larger than CCK-8, such as CCK-33 and CCK-58, decreased meal size and delayed the frequency to the next meal [135]. These findings may revive interest in the therapeutic potential of CCK for the treatment of obesity.

Glucagon-like peptide-1 (GLP-1)

The 160 amino acid prohormone proglucagon undergoes differential, tissue-specific cleavage: in the pancreatic α-cells the major cleavage product is glucagon, in the intestine and CNS, the products are oxyntomodulin, GLP-1 and GLP-2 [136, 137]. Although all of these peptides are implicated in satiation, the available evidence is strongest for GLP-1. In the intestine, GLP-1 is produced by enteroendocrine L-cells, primarily in the ileum and colon [138]. It is secreted as GLP-1(7-37) and GLP-1(7-36) [139], two equipotent bioactive forms, which both are rapidly degraded by dipeptidyl peptidase-IV (DPP-IV) to the inactive analogs GLP-1(9-37) or GLP-1(9-36-NH2) [140, 141]. GLP-1 is secreted in response to ingested nutrients, whereas carbohydrates or lipids are the most potent stimulants [51]. The secretion occurs as a biphasic pattern: an early peak within 5-15 min after meal initiation and a prolonged second phase from 60 min up to 2 or 3 hours [13, 142]. It was often discussed that indirect mechanisms for the release of GLP-1 are required to explain this rapid onset of the meal response, because nutrients do not reach the distal intestine (where the majority of L-cells are present) within 30 min after ingestion. One proposed mechanism was a neurohumoral ‘proximal to distal loop’ [95, 143], another suggestion was that CCK and GIP secreted from the proximal small intestine mediate the stimulation of distal L-cells [143, 144]. However, Holst [138] recently mentioned that although the density of L-cells is higher in the ileum and colon, there are numerous L-cells in the proximal jejunum that very well may be responsible for the early response. Indeed, recent studies have suggested that the limited number of L-cells present in the proximal part are sufficient for the early phase secretion, which suggests that nutrients may direct stimulate the secretion of GLP-1 [145, 146]. The second peak of GLP-1 release is probably triggered by a direct effect of nutrients on the L cell in the distal intestinal lumen.

One of the major actions of GLP-1 is its role as incretin hormone, which means, that GLP-1 stimulates glucose dependent insulin secretion and decreases glucagon release [141, 147-150]. Further biological effects include the inhibition of GI motility, gastric acid secretion and the attenuation of gastric emptying, indicating that GLP-1 is a major component of the “ileal brake”. The ileal brake is an inhibitory feedback mechanism, which can be defined as a distal to proximal feedback mechanism to control transit of a meal through the gastrointestinal tract in order to optimize nutrient digestion, absorption and food intake. The ileal brake is activated by undigested food reaching the distal small intestine; it generates intestinal signals that inhibit proximal GI motility, GI secretion and gastric emptying, which in turn leads to a behavioral brake on eating and thus limits the rate of nutrients entering the bloodstream [10, 104, 151].

A role for GLP-1 in the control of eating was first described by Bloom and co-workers: GLP-1 injected directly into the brain was shown to inhibit feeding in rats [152]. Later studies confirmed these initial findings in numerous studies in rodents: systemically [153, 154] or intracerebroventricularily [155] injected GLP-1 or exendin-4 (a DDP-IV resistant GLP-1 agonist) powerfully reduced food intake. Moreover, repeated administration of GLP-1 induced a reduction in body weight [155], whereas administration of exendin 9-39 (antagonist of GLP-1 receptor) increased feeding and body weight [152, 155]. In humans, different groups proposed a role for GLP-1 in the control of food intake [156-158]. Studies in healthy humans, obese persons or type II diabetic subjects showed that peripherally administration of GLP-1 suppressed hunger and energy intake [157-159]. Moreover, oral administration of GLP-1 showed marked effects on appetite with enhanced fullness at meal onset and reduced energy intake in healthy humans [160].

The mechanism by which GLP-1 mediates its anorectic effects is not completely understood but involves vagal and possibly direct central pathways. GLP-1 receptors (GLP-1Rs) have been found in various tissues, including the pancreas, the stomach, the gut and the brain [138, 161]. Further, GLP-1Rs are expressed in vagal afferents neurons, and total subdiaphragmatic or specific afferent vagotomy abolished the anorectic effects of GLP-1 [143, 153]. This indicates that peripherally administered GLP-1 acts on vagal afferents in close to the enteroendocrine L-cells. GLP-1 can cross the blood-brain barrier; however, whether peripheral GLP-1 also function through central receptors is questionable. The short half-life period (1-2 min) through rapid peripheral degradation by DPP-IV makes it difficult that physiologically relevant quantities of peripheral GLP-1 can reach the brain [51, 138].

Not all data support a role for GLP-1 in the regulation of food intake. Studies in *GLP-1R*$^{-/-}$ mice

have documented increased levels of blood glucose following oral glucose challenge in association with diminished levels of circulating insulin. Intracerebroventricular administration of GLP-1 inhibited feeding in wild-type mice but not in *GLP-1R*$^{-/-}$ mice; more important, no evidence for abnormal body weight or feeding behavior was observed in *GLP-1R*$^{-/-}$ mice. These observations demonstrate a regulatory function for GLP-1 in the regulation of glycemia; however, disruption of GLP-1/GLP-1R signaling in the CNS does not seem to be associated with perturbation of feeding behavior or obesity in vivo [162]. Studies in humans addressing this question have not been performed yet. At this stage it is unclear whether the effect of exogenous GLP-1 or its analogues represent a physiological or a pharmacological effect.

GLP-1 levels are lower in obese than in lean persons [163, 164] and because GLP-1 both stimulates insulin secretion and reduces food intake, it is a promising candidate as therapeutic agent in the treatment of type II diabetes and obesity. Indeed, exendin-4 (Exenatide), the long-acting GLP-1R agonist, is already in use for the treatment of type II diabetes and showed improved glycemic control and weight loss in type II diabetes patients [165]. Furthermore, longer acting DPP-IV resistant GLP-1 analogues are currently under investigation for the treatment of obesity: Liraglutide, a once-daily GLP-1 mimetic demonstrated sustained weight loss without any major safety concerns [166].

Peptide tyrosine tyrosine (PYY)

PYY is a 36 amino acid peptide and belongs to the pancreatic polypeptide family that also includes pancreatic polypeptide (PP) and NPY. Like GLP-1, PYY is synthesized by L-cells in the distal ileum and colon [167]. PYY is secreted as PYY1-36 and is rapidly degraded by DPP-IV to the most circulating form PYY3-36 [168, 169]. The postprandial secretion of PYY is influenced by caloric load, macronutrient composition and food consistency [167, 170, 171]. The secretion follows a biphasic pattern with an increase of PYY levels within the first 15 min after meal initiation and a peak at approximately 60 to 90 min that remains elevated for up to 6 hours [167]. As with GLP-1, it is proposed that the secretion of PYY could be stimulated by indirect effects, like the ‘proximal to distal loop’, as well as by direct contact of ingested foods with L-cells of the distal small intestine [51, 142]. Further, CCK has been demonstrated to play a role in the secretion of PYY: exogenous CCK increased plasma PYY levels [172] whereas an administration of a CCK antagonist blocked the ability of duodenal lipids to stimulate the secretion of PYY [87].

PYY delays gastric emptying and thus has (together with GLP-1) been implicated as a major component of the ileal brake [105]. A role for PYY as satiation peptide was first mentioned by

Batterham and co-workers [173]. Peripheral and central administration of PYY3-36 reduced food intake and body weight in rodents [153, 173, 174]. In humans, intravenous infusion of PYY3-36 at physiological doses reduced hunger feelings and decreased food intake by 36% [173]. Subsequent studies confirmed an anorectic action of PYY3-36 in healthy humans and obese persons [175-177].

All of the pancreatic polypeptide proteins exert their effects through the NPY receptor family: Y1, Y2, Y4, Y5 and Y6. Whereas PYY1-36 activates mainly three different receptors (Y1, Y2 and Y5) and increases food intake through the activation of the orexigenic Y1 and Y5 receptors [178], PYY3-36 induces its anorectic effect through the selective activation of Y2 receptors [179]. Expression of Y2 receptors was found in NPY neurons of the ARC. Injections of PYY3-36 into this brain region decreased food intake [180]. The inhibitory effect of PYY3-36 was attenuated in rats treated with Y2 receptor antagonist [173, 181] and abolished in Y2 receptor *knockout* mice [173]. Y2 receptors were also found on vagal afferents, which led to the assumption that PYY3-36 may influence appetite via vagal afferent fibers projecting to the NTS in the brain [182, 183]. Indeed, vagotomy as well as transectioning of the brainstem-hypothalamic pathway abolished the anorectic effects of peripheral PYY3-36 [20, 153, 182].

PYY3-36 levels are lower in obese than in lean persons and in both, the anorectic effect of peripherally administered PYY3-36 was observed [176, 184]. In contrast to the anorectic adiposity hormones leptin and insulin, it seems that there is no obesity-related resistance to PYY3-36 and indicates a therapeutic potential of PYY in the treatment of obesity.

2.1.2.4 Interaction of GI satiation signals

The sections above demonstrated that satiation and meal termination mainly occur in response to nutrients-induced gastric and intestinal signals. It was shown that the second cephalic phase is simultaneously the start of the gastric phase and during this process of meal ingestion, gastric emptying starts with the delivery of nutrients into the small intestine. Therefore, strong evidence exists that pregastric, gastric and intestinal satiation signals interact in order to increase satiation and limit meal size.

In vivo animal studies, in which feeding experiments were combined with the subsequent withdrawal of food from the stomach showed that gastric distension in the presence of intestinal infusion of nutrients is necessary for the control of eating [185, 186].

In humans, Cecil and colleagues [187] investigated the combined effects of signals arising from different regions of the GI tract and found that oral ingestion of a soup induced a greater

reduction in hunger feelings than when the soup was infused directly into the stomach. Moreover, both of these induced a stronger satiation effect than the perfusion directly into the small intestine (given at a rate proportional to the oral rate of gastric emptying) [187]. Indeed, a number of studies by Cecil *et al.* showed that infusions directly into the small intestine induced no suppression of hunger feelings or increase in fullness [187-189]. However, when a test meal was offered at the end of the infusion, food intake was reduced by the nutrient infusion [188, 189]. Furthermore, Feinle *et al.* demonstrated that sensory responses to gastric distension using an inflatable bag were altered by duodenal infusions: whereas bag inflation in combination with duodenal infusions of saline induced epigastric pressure or bloating, the combination of gastric distension with duodenal infusions of nutrients was associated with more 'meal like' sensations of fullness [190]. Several studies using traditional preload paradigms showed an increase in the satiation effect when intestinal stimulation and gastric distension were combined [129, 188, 191]. In addition, a combination of oral preloads with exogenous administration or endogenous stimulation of intestinal satiation peptides, like CCK or GLP-1, synergistically increased satiation and reduced food intake in humans [53, 129, 192]. Together these findings indicate the importance of the stomach in the short-term control of satiation and suggest that intestinal signals act to potentiate signals arising higher in the GI tract [193]. This interaction between gastric and intestinal signals seems to be necessary to elicit optimal satiation and adequate control of eating.

To date, the exact mechanism of this interaction is only poorly understood in healthy humans. In addition, uncertainties exist about the role of both gastric and intestinal parameters, as well as their interaction in the control of satiation in relation to body mass. The projects 3 (see 5.3) and 4 (see 5.4) in the present thesis concern these themes.

2.2 Mechanisms for intestinal peptide secretion

During the last few decades the understanding of the mechanisms by which the gut sense luminal nutrients and induces peptide secretion has greatly improved. Specific receptor- and transporter- systems, which are located in the membrane of specialized chemosensing cells sense ingested food in the intestinal lumen. This sensing triggers the release of intestinal peptides, like GLP-1, PYY and CCK.

This chapter describes the full machinery of intestinal chemosensing, including cells, receptors and ligands for the receptors.

2.2.1 Intestinal chemosensing cells

As mentioned above, vagal afferent fibers in the lamina propria do not extend into the basal membrane and therefore, are not in a position to sense luminal nutrient content directly. The common assumption is that intestinal nutrients activate vagal afferents by triggering the secretion of peptides from the intestinal epithelium. Indeed, specialized epithelial cells, such as tuft cells or enteroendocrine cells, are able to sense nutrients in the gut lumen and in response to that secrete neuroactive peptides into the lamina propria.

2.2.1.1 Tuft cells (brush or caveolated cells)

Tuft cells in the GI tract were first described in 1956 by Jarvi *et al.* [194]. The cells contain caveolae in the apical cytoplasm and a tuft of microvilli extending towards the lumen [194, 195]. They differ morphologically from other secretory cells in the gut as they lack electron-dense secretory granules [196]. Tuft cells have recently been demonstrated to form a distinct lineage of secretory cells in the GI tract [197]. Furthermore, it was shown that they express components of the taste signaling system, including α-gustducin and TRPM5, which indicates a role as chemosensory cells in the gut [198]. The direct sensing of macronutrients by tuft cells and their influence on other chemosensory cells in the gut remains to be proven.

2.2.1.2 Enteroendocrine cells (EECs)

EECs are dispersed among mucosal cells of the GI tract and although they represent less than 1% of the gut epithelial population they constitute the largest endocrine organ of the human body [199]. They produce and secrete more than twenty different peptides or signaling molecules [200]. The secretion products are located in secretory granules and after stimulation they are secreted by exocytosis at the basolateral membrane into the lamina propria where they either act locally on vagal afferents (paracrine manner) or enter directly into the bloodstream (endocrine manner) [201]. The stimulation of the exocytosis is mediated through the activation of G protein-coupled receptors (located at the apical membrane of EECs) by ingested foods [199]. There are at least ten discrete cell types (Table 1 shows a selection) that make up the enteroendocrine family – each classically described by a distinct localization along the gut and a different hormonal profile [202]. Many EECs include microvilli extending to the lumen, leading to their description as ‘open cells’, which enable to directly sense luminal factors. In contrast, ‘closed cells’ (like enterochromaffin-like cells) do not reach the lumen and are believed to respond to luminal content indirectly through mechanical stimuli and neuronal or humoral factors. Together, EECs can be regarded as primary chemoreceptors, capable of responding to luminal contents by releasing secretory peptides, which act locally, centrally and in the periphery. [203, 204]

Table 1 Selected enteroendocrine cells (EECs), including location sites and secretory products.

Cell type	Primary location site	Released peptide
G-cells	Stomach	Gastrin
X-cells	Stomach	Ghrelin
Unnamed-cells	Stomach and duodenum	Gastrin releasing peptide (GRP)
I-cells	Duodenum and jejunum	Cholecystokinin (CCK)
K-cells	Duodenum and jejunum	Glucose-dependent insulinotropic polypeptide (GIP)
L-cells	Ileum and colon	Glucagon-like peptide-1 (GLP-1) Peptide tyrosine tyrosine (PYY)
Unnamed-cells	Colon	Pancreatic polypeptide (PP)

Adapted from Steinert *et al.* [142].

The scarcity of EECs in the GI-tract and the difficulty in identifying them accurately limit detailed molecular studies about the chemosensory pathways of these cells. Mostly, knowledge has come from work using cell lines such as GLUTag [205] and NCI-H716 [206] (murine and human models for GLP-1 secretion, respectively), STC-1 (secrete a variety of small intestinal hormones, including CCK and GLP1) [207], and BON (model for enterochromaffin cells) [208]. However, recently, the generation of transgenic mice expressing fluorescent proteins under the control of hormonal promoters (proglucagon, GIP, CCK and ghrelin), which results in cell-specific labeling of L-, K-, I-, and X/A- cells, respectively, provides new opportunities for the understanding of the chemosensory pathways of EECs [209-211].

2.2.2 Intestinal chemosensing receptor- and transporter- systems

Different receptor- or transporter systems that are located in the membrane of chemosensing cells are “gate keepers” for luminal nutrients and their breakdown products, including fatty acids, glucose, amino acids and proteolytic products. The activation of these receptors and transporters causes the release of intestinal peptides. To date nutrient sensory systems that are mostly involved in this secretion of peptides include G protein-coupled receptors and solute transporters.

2.2.2.1 G protein-coupled receptors

In recent years, G protein-coupled receptors (GPCR) have been identified as obvious nutrient sensors in the intestine [212]. GPCRs, also known as seven-transmembrane receptors, are subdivided into six families based on sequence homology and functional similarity: family A

(rhodopsin-like receptors), family B (secretin receptors), family C (metabotropic glutamate receptors), family D (fungal mating pheromone receptors), family E (cyclic AMP receptors) and family F (frizzled/smoothed receptors) [213-215]. A role in nutrient sensing was shown for family A and C receptors [216, 217]. Family A receptors include the GPR93 (responsive to protein degradation products) and the free fatty acid receptors (FFAR1-3) [204]. Family C receptors are characterized by a large extracellular Venus flytrap ligand-binding domain and include metabotropic glutamate (mGlut) receptors, calcium-sensing receptors (CaR), family 1 taste receptors (T1Rs) and G protein-coupled receptors family C, group 6, subtype A (GPRC6A) [204].

Extracellular binding of nutrients or their breakdown products to GPCRs induces conformational changes of the receptor that results in a separation of the membrane-bound heterotrimeric guanine nucleotide-binding G-protein subunits (α, β and γ). In the following, these subunits stimulate their respective effector pathways (Figure 5): i) stimulation of phospholipase C (PLC), leading to the generation of inositol triphosphate (IP3) and diacylglycerol (DAG), which activate protein kinase (PK) C; ii) stimulation of adenylate cylase (AC), which results in an elevation of cyclic adenosine monophosphate (cAMP) concentrations; iii) stimulation of phospholipase C β2 (PLC β2). Each pathway results in an increase of cytoplasmatic Ca^{2+} that activates transient receptor potential channel type 5 (Trpm5) and with this the entry of cations. The resulting depolarization triggers the generation of action potentials and in turn the release of gut peptides. [204, 218]

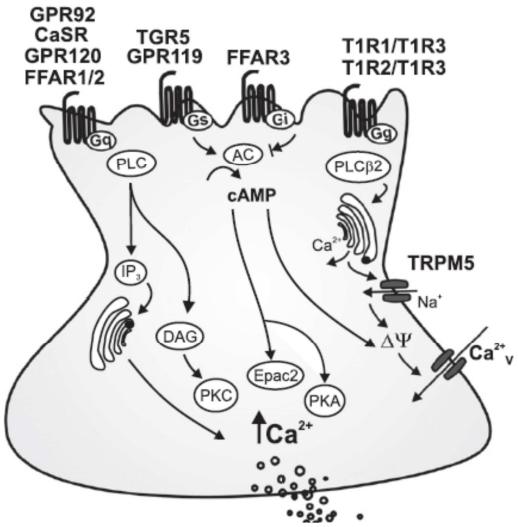

Figure 5 G protein-coupled receptor-mediated nutrient sensing in EECs [204].

2.2.2.2 Solute carrier (SLC) transporters

The intestinal brush border membrane is rich in SLC transporters, which control the uptake and efflux of nutrients (fatty acids, glucose, amino acids), ions and drugs. The SLC family includes passive transporters, ion coupled transporters and exchangers; in humans these transporters are subdivided into 47 families and include 248 transporter genes [219]. Passive transporters allow passage of substances across membranes down their electrochemical gradients. Ion coupled transporters and exchangers transport the substances against their concentration gradient, utilizing the energy gradient of the coupled ion or solute.

A role in the release of satiation peptides was found by different transporter subtypes. The sodium-coupled glucose transporter (SGLT1) generates pathways capable of triggering action potentials and Ca^{2+} entry [220]. Amino acids have been shown to depolarize EECs as direct consequence of their electrogenic uptake via sodium-coupled transporters, resulting in Ca^{2+} entry and Ca^{2+}-dependent stimulation of secretion [221, 222]. Furthermore, di- and tripeptides were found to stimulate SCT-1 cells transfected with the peptide transporter PEPT1 [223]. Together these findings suggest that transporters have additional, receptor-like activity and trigger intracellular signaling pathways, potentially coupled to a change in membrane potential, which results in peptide secretion.

2.2.3 Ligands for intestinal chemosensing receptors

As mentioned above, the main ligands for the receptor- and transporter- systems in the gut are the three macronutrients (proteins, fats and carbohydrates) of ingested foods. Beside these nutrient stimuli, there is evidence that non-nutrient stimuli, such as bile acids, also activate receptors in the gut and induce peptide secretion.

2.2.3.1 Proteins

Proteins in the GI lumen are broken down by digestive enzymes, resulting in amino acids, di/tripeptides and larger oligopeptides. Both, GPCR and SLC transporter pathways have been proposed for the detection of these molecules with downstream signaling that induces the secretion of GI peptides from EECs.

GPCRs

Among the GPCRs, the CaR is currently the strongest candidate for amino acid sensing. This receptor detects mainly aromatic L-amino acids, such as L-phenylalanine and L-tryptophan, followed by aliphatic and polar amino acids [224]. Expression of the CaR was found along the small and large intestine [225-227], particularly in CCK secreting I-cells [211, 228]. Recently it

was shown that CCK secretion induced by various protein hydrolysates was reduced by CaR antagonists in STC-1 cells [229]. In addition, in the small intestine of rats was demonstrated that the secretion of GLP-1 and PYY was modulated by the CaR: Mace *et al.* were able to inhibit L-amino acid stimulated release of GLP-1 and PYY with inhibitors of the CaR [230]. These results indicate a significant role of this receptor as a sensor for dietary peptides to stimulate GI peptide secretion from EECs. GPR93 was described as receptor responsive to peptones and has been proposed as the missing link between peptone stimulation and increased CCK expression and release from I-cells [231]. Activation of GPR93-overexpressed STC-1 cells led to G protein-signaling cascades that promoted *CCK* mRNA expression and CCK secretion [232]. Although both, the T1R1/T1R3 taste receptor [233, 234] and also the GPRC6A [235, 236] were reported to be expressed throughout the gut and respond to amino acids, to date no reports exist regarding their role in peptide secretion.

Transporter systems

Beside the GPCRs, peptide transporters, such as PEPT1 and PEPT2 (SLC15A1 and SLC15A2), and sodium-coupled amino acid transporters are potentially able to secrete GI peptides from EECs. Studies by Matsumura *et al.* showed that transfection of PEPT1 in STC-1 cells evoked di-peptide-stimulated hormone secretion in a pH-dependent manner [223]. Tolhurst *et al.* proposed that glutamine stimulated GLP-1 release from GLUTag cells occurred in part via the sodium-dependent amino acid transporters SLC38A2 and SLC6A19 [222, 237].

2.2.3.2 Carbohydrates

Carbohydrates found commonly in food include the complex carbohydrates, such as starch, cellulose (both from plant products) and glycogen (from animal products), disaccharides, such as sucrose and lactose, and monosaccharides, such as glucose, galactose and fructose. Complex carbohydrates and disaccharides are broken down to monosaccharides by specific digestive enzymes (such as α-amylase and β-glucosidase). Glucose in the intestinal lumen induces the release of a number of GI peptides.

Closure of K_{ATP} channels

The closure of K_{ATP} channels in the cell membrane could be one possible mechanism by which glucose is sensed in EECs. Metabolic regulation of K_{ATP} channel activity has been described in glucose-sensitive tissues, such as pancreatic β-cells and the brain. In pancreatic β-cells metabolism of glucose generates ATP, which results in the closure of K_{ATP} channels. The reduction in K^+ triggers membrane depolarization, action potential generation, voltage-gated Ca^{2+} entry and subsequently the release of insulin [238]. This pathway was also described in

GLUTag and NCLH716 cells, both of which secrete GLP-1 [220, 239]. Studies with native EECs isolated from mouse intestine confirmed a role for K_{ATP} channels in the release of GLP-1 and GIP [209, 210]. Moreover, the expression of the K_{ATP} channel subunit Kir6.2 was detected by immunostaining in L- and K-cells of the human intestine [145, 240]. However, despite the clear presence of K_{ATP} channels in human K- and L-cells, there is little evidence that K_{ATP} channel closure causes the secretion of GLP-1 and GIP: sulphonylureas, which stimulate insulin release through closing of K_{ATP} channels in type II diabetes patients failed to trigger the release of GLP-1 or GIP [203, 241, 242].

GPCRs

Another glucose-sensing mechanism identified in the intestine involves G-protein coupled taste receptors. It has proposed that populations of intestinal cells can “taste” sugars or sweet compounds by sensory pathways similar to those described in lingual taste cells (Figure 6). In the gustatory buds of the tongue, the sensation of sweet taste is mediated by the GPCR heterodimer T1R2/T1R3 (sweet taste receptor). Glucose and artificial sweeteners bind to T1R2/T1R3 that is coupled to the heterotrimeric G-protein (α-gustducin, Gβ3 and Gγ13) [243, 244]. Through activation, the G-protein subunits separate to α-gustducin and βγ-unit (Gβ3-Gγ13); the latter activates PLCβ2 to generate IP3 and DAG [245]. IP3 activates type III IP3 receptors, which results in the release of Ca^{2+} from internal stores [246] and in turn in the activation of TRPM5 [247]. The influx of cations induces taste cell depolarization and subsequent signaling to other taste cells and gustatory afferent nerves.

The expression of signaling components of the sweet taste receptor in extra-gustatory regions was first described by Höfer *et al*. [248]. They found that α-gustducin is expressed in the stomach, duodenum and pancreatic duct of rats. In mice it was shown, that the α-gustducin-containing cells are scattered throughout the epithelium of the gut and show structural features similar to the cells of the tongue [249]. In 2005, the expressions of T1R2, T1R3 and α-gustducin in mouse duodenum and small intestine at mRNA and protein levels were noted [250]. In humans, Rozengurt *et al.* showed that α-gustducin is localized to enteroendocrine L-cells in the colonic mucosa and found expression of T1R3 and α-gustducin in the human colon [251]. Furthermore, most α-gustducin positive cells contain chromogranin A, a marker of EECs, which indicates that α-gustducin is expressed in EECs; the observed colocalization of α-gustducin with PYY and GLP-1 in human colonic cells confirms these findings [251]. In human duodenal biopsy sections, α-gustducin expression was shown by indirect immunofluorescence in GLP-1 expressing L-cells, GIP-expressing K-cells and GIP and GLP-1 coexpressing K/L-cells [146]. Confirmation of the expression of α-gustducin in human enteroendocrine K- and L-cells came

from laser capture followed by reverse transcriptase-polymerase chain reaction [146]. In addition, T1R2, T1R3, Gβ3, Gγ13, PLCβ2 and TRPM5 were found to be coexpressed with α-gustducin and GLP-1 in human duodenal L-cells [146]. The expression pattern of the taste-signaling elements in the human gut was shown by several groups [233, 252, 253]: Whereas the expression of T1R3 is nearly equally throughout the gut, α-gustducin predominantly exists in the proximal part of the GI-tract and expression for T1R2 is only low; no sex specific differences were found [253]. The sweet receptor subunit T1R3 has recently been shown to be present in the human stomach [233, 252].

To date a number of findings indicate that sweet taste receptors may underlie glucose sensing in gut EECs, particularly those releasing GLP-1 and PYY. Activation of GLUTag and NCI-H716 with glucose, sucrose or the artificial sweetener sucralose resulted in the secretion of GLP-1; this release was blocked by the sweet taste receptor antagonists gurmarin and lactisole [146, 254]. *In vivo*, Jang *et al.* showed that plasma GLP-1 levels following glucose gavage were reduced in α-gustducin (*α-gust*$^{-/-}$) or T1R3 (*T1R3*$^{-/-}$) *knockout* mice compared to wild-type mice. In addition, an impaired insulin release and elevated blood glucose levels were found. [146] Recently, these results were confirmed for *T1R3*$^{-/-}$ *knockout* mice, but interestingly *T1R2*$^{-/-}$ mice showed normal glycemic control and partial glucose stimulated GLP-1 secretion [255]. This let assume that T1R3 can mediate GI peptide secretion without T1R2.

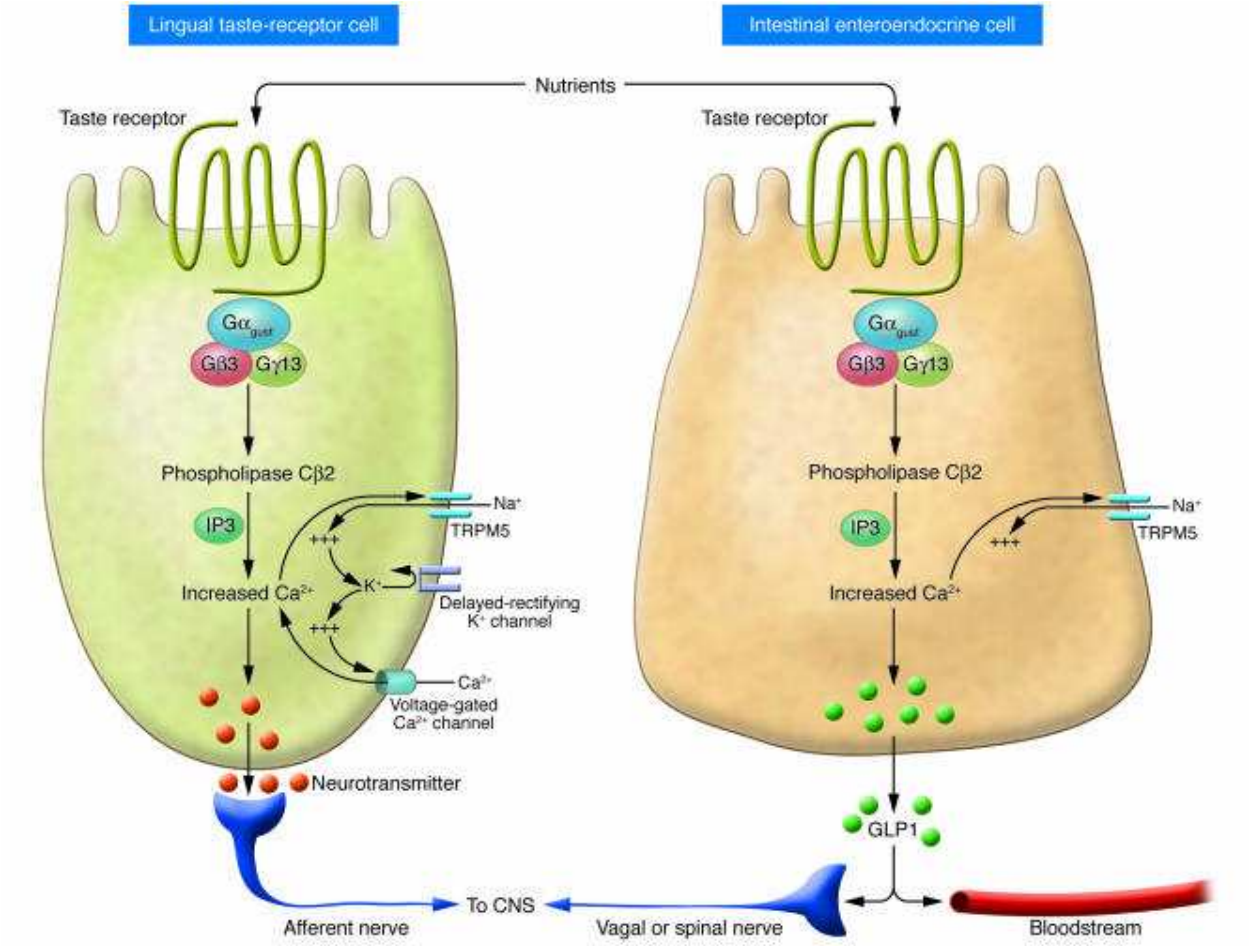

Figure 6 Similarities in nutrient-sensing mechanisms used by taste receptor cells of the tongue and EECs of the intestine [51].

Although these findings indicate that EECs sense sugars and artificial sweeteners via the α-gustducin coupled sweet taste receptor, which in turn leads to the secretion of satiation peptides, artificial sweeteners recently failed to stimulate the secretion of GLP-1 from murine primary intestinal cultures [210]. Moreover, no enrichment of *T1R2, T1R3 or α-gustducin* mRNA was found in purified murine K- and L-cells [209, 210]. In addition, *in vivo* studies could not detect significant increases in plasma incretin levels in response to artificial sweeteners, neither in rodents [256, 257] nor humans [258]. These findings were supported by Steinert *et al.*: neither sucralose nor aspartame and acesulfam K led to a stimulation of GLP-1 secretion in humans [259]. Thus, further work is needed to clarify the role of sweet taste receptors in nutrient induced secretion of GI peptides from EECs. Project 2 (see 5.2) of the present thesis concerns this theme.

Transporter systems

A number of monosaccharide transporters, both sodium-coupled (such as SGLT1, SGLT3) and facilitative (such as GLUT2) mechanisms were suggested as glucose sensors. The role of

SGLT1 in glucose sensing by EECs was demonstrated in GLP-1 secreting GLUTag cells: Gribble *et al.* showed that these cells expressed mRNA for SGLT1 and that glucose triggered the secretion of GLP-1; the release of GLP-1 was blocked by phloridzin, the specific antagonist of SGLT1 [220]. The same was shown *in vivo* in mice, co-administration of phloridzin with glucose in the upper intestine blocked glucose absorption and glucose-induced secretion of GLP-1 and GIP [257]. Together these findings provide evidence for a role SGLT1 in glucose sensing and subsequent GI peptide secretion. The role of SGLT3 in intestinal glucose sensing is less clear. Its expression has been shown in GLUTag cells and in the intestinal epithelium of rodents [220, 260]. Diez-Sampedro *et al.* [261] reported that human SGLT3 lacks glucose transport capacity and rather acts as glucose sensor. In fact, when human SGLT3 was transfected into oocytes, glucose was not transported but depolarized the membrane – this depolarization was blocked by phloridzin. A possible involvement of GLUT2 in glucose induced secretion of GI peptides was described by Cani *et al.* – *GLUT2*$^{-/-}$ *knockout* mice showed impaired GLP-1 secretion in response to oral glucose [262]. Furthermore, glucose stimulated release of GLP-1 and PYY in the small intestine of rats was reduced with inhibitors of GLUT2 [230].

2.2.3.3 Lipids

Fat digestion by pancreatic lipases in the lumen is a strong stimulus for the secretion of GI peptides. Long-chain fatty acids (LCFA, chain length > C 12) trigger the release of CCK, GLP-1 and GIP [115, 263], but also short-chain fatty acids (SCFA, chain length < C 6), which are produced in the distal gut by fermentation of dietary fibers, have been shown to stimulate the secretion of GLP-1 and PYY [264].

GPCRs

The underling mechanisms for these peptide secretions are mainly based on four GPCRs, which respond to free fatty acids (FFA): FFAR1 (GPR40), GPR120, FFAR2 (GPR43) and FFAR3 (GPR41). FFAR1 and GPR120 are responsive to medium-chain and LCFA, whereas FFAR2 and FFAR3 are stimulated by SCFA. [265, 266] *FFAR1* and *GPR120* mRNAs are enriched in cells expressing GLP-1, GIP and CCK [209, 210, 267] and FFAR1 proteins were identified in EECs colocalized with GLP-1 and GIP [268]. In addition, FFAR1 *knockout* mice showed a reduced GLP-1 and GIP response to a high-fat diet [268] and impaired linoleic-acid stimulated CCK secretion *in vitro* [267]. GPR120 stimulation promoted secretion of GLP-1 and CCK in STC-1 cells, in consistent, *knockout* experiments of these cells showed reduced FFA-triggered secretion of GLP-1 and CCK [266, 269]. Together these findings indicate a role for these receptors in FFA stimulated peptide secretion. FFAR2 and FFAR3 were found to be

expressed in colonic L-cells [270], which is consistent with the findings that SCFA enhanced GLP-1 secretion [264]. SCFAs induced activation of FFAR2 enhanced intracellular Ca^{2+} concentrations in L-cells, and *knockout* of FFAR2 reduced SCFA-triggered GLP-1 secretion *in vitro* and *in vivo* [271]. The exact physiological role of FFAR3 in EEC fatty acid sensing remains to be established.

Transporter systems

Whether the two FFAs transporter systems, CD36 (FAT/CD36) and fatty acid transport protein 4 (FATP), play a role in EEC fatty acid sensing is not clear to date. Both are membrane transporters for LCFA [272]. An involvement for CD36 in oral LCFA detection was shown by Laugerette *et al.* [273]; a possible link to fat detection in the small intestine was described by Schwartz *et al.* [274].

Besides FFAs, compounds that are elevated after a lipid rich meal are also able to stimulate intestinal receptor systems: oleoylethanolamide (OEA, fatty acid derivative [274]) or bile acids activate GRP119 and TGR5, respectively [275-277]. GRP119 has been identified in intestinal L- and K- cells [209, 210], which is consistent with the findings that GRP119 agonists, such as OEA and a synthetic GPR119 ligand, stimulated GLP-1 and GIP secretion *in vitro* and *in vivo* [278, 279]. In addition, GPR119 *knockout* mice exhibited an impairment of nutrient-stimulated GLP-1 release [280].

2.2.3.4 Bile acids (BAs)

In addition to their role in dietary lipid absorption and cholesterol homeostasis, BAs have been shown to act as signaling molecules informing the peripheral tissue that meal has been ingested and that energy will be available [281]. The most abundant BAs in humans include the primary BAs, cholic acid (CA) and chenodeoxycholic acid (CDCA), and their respective secondary bile acids, deoxycholic acid (DCA) and lithocholic acid (LCA). That BAs could play a role as signaling molecules was already described in 1995: it was shown that they promote GLP-1 release in the isolated vasculary perfused rat colon [282]. Furthermore, PYY release in the isolated vasculary perfused rabbit colon was also stimulated by BAs [283, 284]. Recently it was shown that enteral BA treatment increased GLP-1 levels in neonatal pigs [285]. In humans, infusions of DCA into the colon increased the secretion of PYY and enteroglucagon [286]. In addition, intrarectal infusion of BAs has been shown to increase plasma GLP-1 and PYY levels in obese, type II diabetic subjects [44]. In the early nineties, Miyasaka *et al.* [287] reported

effects of some BAs on the release of CCK in rats. In humans, Koop and co-workers [288] observed a small increase in CCK levels after CDCA administration.

GPCRs

TGR5, a member of the rhodopsin-like subfamily of GPCRs, is expressed in the gall bladder, in brown adipose tissue, in liver and areas of the CNS. In 2002, the expression of TGR5 was also found in EEC lines of mouse (STC-1 and GLUTag) and human (NCI-H716) origin [276]. Originally considered an orphan GPCR, TGR5 was recently classified as cell surface receptor responsive to BAs [276, 277]. In 2005, Katsuma *et al.* first described that LCA and DCA promote GLP-1 secretion through TGR5 in enteroendocrine STC-1 cells [289]. These findings were confirmed by Parker *et al.*: BAs triggered GLP-1 secretion from GLUTag cells was TGR5 dependent [290]. Activation of TGR5 (Figure 7) leads to the separation of the α-subunit from the G-protein and subsequently to the activation of AC, which results in increased intracellular concentration of cAMP. It is proposed that this increase in cAMP induces the secretion of GLP-1 in enterendocrine L-cells [291]. Maruyama *et al.* examined the effect of endogenous BAs in the activation of TGR5 and showed that LCA is the most potent one followed by DCA, CDCA and CA, respectively [276].

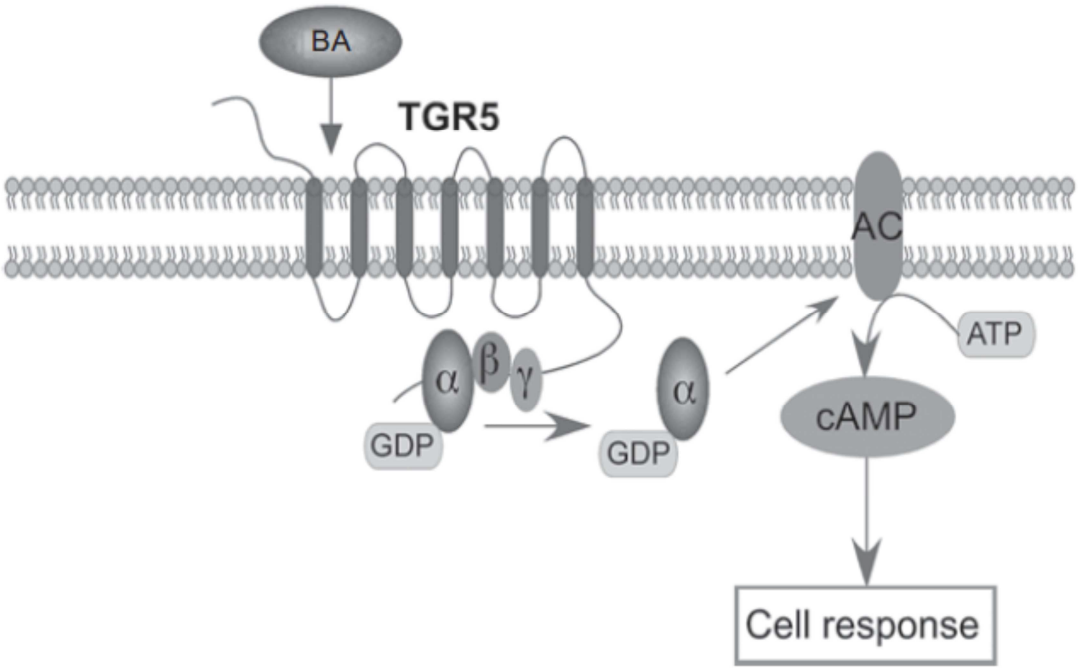

Figure 7 Simplified overview of the TGR5 signaling pathway. Adapted from Pols *et al.* [292].

Together, activation of TGR5 by BAs represents a key pathway for the regulation of intestinal peptide release and indicates that TGR5 activation could play a role in glucose homeostasis. This assumption is in line with findings by Thomas *et al.* [291]: using obese and insulin resistant mouse models, they showed that mice with a gain-of-function of TGR5 were more glucose-tolerant, whereas *TGR5*$^{-/-}$ mice had an impaired glucose clearance. These observation may be related to GLP-1 secretion, which was reduced in *TGR5*$^{-/-}$ and increased in TGR5-transgenic mice.

To date, the most described properties of TGR5 were observed in cell models and animal studies, which indicate that TGR5 could be linked to the beneficial properties of BAs in humans. In healthy subjects it was shown that postprandial GLP-1 and PYY responses were positively correlated with serum BAs [293]. However, our recent findings in humans who underwent bariatric surgery do not support these results – no correlation was found between postprandial plasma BA and the secretion of intestinal peptides (Steinert *et al.*, unpublished data). Moreover, treatment studies with BA sequestrants – thought to improve glucose control through increased secretion of GLP-1 [294-298] – exhibited no correlation between markers of insulin resistance/glucose metabolism and BA metabolism in type II diabetes patients [299, 300]. Thus, further studies with humans are necessary to clarify the role of BAs in GI peptide secretion. Project 1 (see 5.1) of the present thesis concerns this theme.

Transporter systems

The only evidence for a role of transporter systems in BA induced secretion of GI peptides was recently mentioned by Mace *et al.* [230]. They suggested that BA could trigger peptide release via the facilitative glucose transporter GLUT2: using an inhibitor of GLUT2, Mace and co-workers were able to supress a BA induced secretion of GLP-1 and PYY in animals.

3 Aims

The need for effective, safer, and palatable treatment options for obesity has encouraged research to investigate the role of gastrointestinal (GI) peptides in the control of eating and body weight. The eating-inhibitory effect of these peptides is of great clinical interest and direct stimulation could constitute a promising therapeutic approach for the treatment of obesity. During the last decades, enteroendocrine cells (EECs) in the small intestine were identified as intestinal peptide secreting cells and a number of chemosensory receptors that respond to ingested nutrients were found to be expressed in these cells. The first aim of the present thesis was to investigate the involvement of two potential targets of peptide release, such as glucagon-like peptide 1 (GLP-1), peptide tyrosine tyrosine (PYY) and cholecystokinin (CCK): i) bile acids (BAs) as possible TGR5 agonists and ii) glucose stimulating the sweet receptor T1R2/T1R3.

In project 1 (see 5.1) we investigated the physiological role of BAs in the secretion of intestinal peptides by using a paradigm in that subjects received intraduodenal (ID) infusions of different loads of chenodeoxycholic acid (CDCA, a primary BA in humans) in comparison to sodium-oleate or vehicle as a control.

In project 2 (see 5.2) we used the T1R2/T1R3 antagonist lactisole as probe to investigate the physiological role of T1R2/T1R3 in the secretion of intestinal peptides. Two different approaches were applied in that subjects received i) intragastric (IG) and ID infusions of glucose with and without lactisole and ii) IG and ID infusions of a mixed liquid meal with and without lactisole.

Beside the intestinal phase of peptide secretion, the process of eating is controlled by a number of other mechanisms, including gastric phase signals (such as gastric distension or gastric emptying). Several studies in animals and humans suggest that gastric and intestinal signals interact to elicit optimal satiation and adequate control of eating. In humans, little information is available on the underlying mechanisms of this interaction. The second aim of the present thesis was to evaluate the reciprocal control between gastric functions and intestinal parameters in the control of appetite.

In project 3 (see 5.3) we used a paradigm in that lean subjects received either an IG load or an ID infusion of nutrients. Interaction mechanisms between gastric and intestinal phase signals in the control of appetite and the secretion of satiation peptides were examined.

In project 4 (see 5.4) we investigated the interaction between gastric and intestinal parameters in the control of eating in obese subjects, as we compared gastric emptying, intestinal peptide release and satiation parameters in response to nutrients between normal and obese healthy volunteers.

4 General Methods

4.1 Performing clinical studies

Although there are many definitions for clinical trials, they are generally considered to be biomedical or health-related research studies in humans. Clinical studies are based on a protocol that is carefully designed to safeguard the health of the subjects as well as answer specific research questions. [301]

All our study protocols were submitted to and approved by the State Ethical Committee of Basel (EKBB). The purpose of the EKBB is to evaluate the conformity of the proposed research protocols with the ethical and scientific standards as they are established in the Declaration of Helsinki, the International Ethical Guidelines for Biomedical Research Involving Human Subjects and the ICH (International Conference on Harmonisation) Guidelines for Good Clinical Practice (GCP). [302]

The Declaration of Helsinki was developed in 1964 by the World Medical Association (WMA) as a statement of ethical principles for medical research involving human subjects [303]. These principles include informed consent, minimizing risk and adherence to an approved research protocol [304].

The Council for International Organizations of Medical Sciences (CIOMS) is an international, non-governmental, non-profit organization established in 1949 by the WHO and the UNESCO (United Nations Educational, Scientific and Cultural Organization). The contribution of CIOMS to medical research was the issuance of international guidelines for the application of ethical principles, such as the International Ethical Guidelines for Biomedical Research Involving Human Subjects. [305]

GCP is an international ethical and scientific quality standard for designing, conducting, recording and reporting trials that involve the participation of human subjects. The guideline for GCPs was provided by the ICH with the object for a unified standard for the European Union (EU), Japan and the United States. [306]

4.2 Experimental procedure

In our experiments, subjects received (except of Project 4 (see 5.4)) IG or ID infusions of nutrients. The infusion directly into the GI tract bypasses cognitive cues and therefore enables to examine the direct effect of food on GI peptide secretions and appetite. [193]

The experimental procedure is described in detail within the respective project.

4.3 Blood sample collection and Laboratory analysis

In the present thesis, blood samples were collected into tubes and kept on ice. Tubes contained EDTA (6 µmol/L), aprotinin (500 kIU/L) and a DPP-IV inhibitor (for the measurement of the active forms of GLP-1 and PYY). After centrifugation at 4 ℃ (10 min, 3000 rpm; Labofuge 400R, Heraeus, Osterode Germany) plasma samples were separated into different aliquots. For analysis of CCK in Project 1 (see 5.1), blood was 1:10 diluted in an ice-cold buffer (pH 3.6) containing 0.1 M ammonium acetate, 0.5 M sodiumchloride and a protease inhibitor cocktail (Complete®, Roche Diagnostics GmbH, Mannheim, Germany). Diluted blood was centrifuged at 4℃ (10 min, 3000 rpm) and the supernatant collected. All aliquots were stored at -70 ℃ until analysis.

The amount of GI peptides in biological fluids can be measured by several biochemical analytical methods including chromatographic techniques, such as high-performance liquid chromatography (HPLC), or techniques based on the immunoreactivity of the peptide [13]. The most common immunoassay methods are radioimmunoassays (RIA), enzyme linked immunosorbent assays (ELISA), enzyme immunoassays (EIA) and fluorescence immunoassays (FIA). In the present thesis analysis were performed using ELISA kits as well as RIA kits.

Active GLP-1 was measured using a specific ELISA kit. Prior to measurements, GLP-1 was extracted from 1 mL plasma using reversed solid phase extraction (C18-silica cartridges). The extraction method is based on the separation of GLP-1 due to its polarity. GLP-1 was subsequently eluted with acetonitrile and the eluent dried under nitrogen atmosphere. The concentrated samples were resolved in 250 µL ELISA buffer for analysis (concentration factor of 4). The basic steps of the ELISA kit include: i) capture of active GLP-1 from sample by a monoclonal antibody, immobilized in the wells of a microwell plate that binds specifically to the N-terminal region of active GLP-1 molecule, ii) washing to remove unbound materials, iii) binding of an anti GLP-1-alkaline phosphatase detection conjugate to the immobilized GLP-1, iv) washing off unbound conjugate and v) quantification of bound detection conjugate by adding methyl umbelliferyl phosphate, which in the presence of alkaline phosphatase forms the fluorescent product umbelliferone. Since the amount of fluorescence generated is directly

proportional to the concentration of active GLP-1 in the unknown sample, the latter can be derived by interpolation from a standard calibration curve generated in the same assay with reference standards of known concentrations of active GLP-1.

Plasma concentrations of *ghrelin*, *CCK*, *total* and *active PYY* and *insulin* were measured using specific RIA kits. The principle of this assay is based on the competition between a fixed amount of radioactive antigen (labeled antigen, tracer) and a non-radioactive antigen (unlabeled antigen, standards or samples) for the limited and constant number of antigen binding sites on the antibody bound on the solid phase of coated tubes. Separation of the antibody-antigen complexes from unbound tracer is achieved by a washing step or by precipitation of the antibody-antigen complexes with a secondary antibody against the species specific immunoglobulins of the primary antibody. After centrifugation, the unbound tracer is in the supernatant and can be removed. The amount of antibody-tracer complexes is counted by a radioactivity counter; it decreases as the concentration of unlabeled antigen increases. The amount of unlabeled antigen in samples can be derived by interpolation from a standard calibration curve generated in the same assay with reference standards of known concentrations of the antigen. Further details to the specific RIA kits are described in the respective projects.
Prior to CCK measurements in Project 1 (see 5.1) the peptide was extracted from the above described supernatant by using a reversed solid phase extraction (C18-silica cartridges). The extraction method is based on the separation of CCK due to its polarity. CCK was subsequently eluted with acetonitrile and the eluent dried under nitrogen atmosphere. The concentrated samples were resolved in RIA buffer for analysis (volume corresponding to original plasma volume).

Plasma glucose was measured by a commercially available glucoseoxidase method. This method is highly specific for glucose in serum or plasma and is based on an enzymatic reaction [307].

Plasma BAs were analyzed in collaboration with the Department of Nutritional Physiology, Institute of Nutrition, Friedrich Schiller University, Jena in Germany. For further details see Project 1 (5.1).

4.4 Assessment of appetite

In research, appetite is divided into two components: hunger and satiety. 'Hunger' describes the sensation that signifies food deprivation and promotes food consumption, whereas 'satiety', as described above, is separated into two functionally different terms: 'satiation' (intra-meal satiety)

and satiety ('between-meal satiety') [304]. Numerous different measurements are established for the assessment of appetite and are described in detail by Blundell and colleagues [308, 309].

An advantage of human studies, compared to animal models, in the measurement of appetite is that human subjects can be asked structured questions about their motivation to eat or not to eat, which type of food and what amount they would like to eat [310]. A number of different self-report scales, such as uni- and bipolar structured and unstructured lines, verbal categories and numerical scorings, were used to ask subjects the specific questions relating to aspects of their motivation to eat [310-312].

The most common and productive method is the use of visual analogue scales (VAS). VAS consist of a horizontal, unstructured, 10 cm line with words anchored at each end, expressing the most positive and most negative rating [313]. The recommended primary scales for self-reported appetite in healthy adults are the following [308]:

Hunger scale
Question: *How hungry are you?*
Answers: *a) 'Not at all' or b) 'Extremely'/'As hungry as I have ever felt')*
Fullness scale
Question: *How full are you?*
Answers: *a) 'Not at all' or b) 'Extremely'/'As full as I have ever felt'*
Satiety scale
Question: *How satiated are you?*
Answers: *a) 'Not all' or b) 'Extremely'*
Desire scale
Question: *How strong is your desire to eat?*
Answers: *a) 'Very weak' or b) 'Very strong'*
Prospective food consumption scale (quantity)
Question: *How much do you think you could eat?*
Answers: *a) 'Nothing at all' or b) 'A very large amount'*

Subjects are asked to assign a vertical mark across the line to index the magnitude of their subjective sensation at the present time point. Quantification of the measurement is made by measuring the distance from the left end of the line to the mark. [313]

Qualities of these scales were recently summarized by Blundell and colleagues [308]:

i) Easily applied and unambiguously interpreted by investigators as well as by subjects
ii) Demonstrated repeat-reliability
iii) Convergent validity with other, similar scales
iv) Known sensitivity to relevant manipulations
v) Suitability for relevant mathematical and statistical handling

Beside these advantages it should be noted that the results obtained from VAS are neither objective nor strictly quantitative. The subjectively rated motivation to eat is not an inevitable outcome of underlying physiological processes, it is rather the subject's own interpretation of own sensations and motivations, which are influenced by a number of physiological processes [310, 314]. Therefore, to enable reliable and valid results, it is recommendable that subjects take enough time and rate their appetite sensation as precisely as possible.

4.5 Assessment of gastric emptying

The 'gold standard' for the measurement of gastric emptying is scintigraphy; however, this technique requires radionuclides and a gamma camera, and is to date only used by some specific research groups. The currently most widely used methods for gastric emptying are ultrasonography, paracetamol absorption test, magnetic resonance imaging (MRI) and stable isotope breath test (^{13}C-breath test). All these techniques have their advantages and limitations; the chosen approach therefore depends on the study population (type of subjects, number of subjects), the type of meal (liquid or solid), the meal constituents and the availability and feasibility of the methodology [13]. We used in our projects ^{13}C-breath tests, which are very easy tests to perform and well validated with excellent correlations with gastric emptying parameters of the scintigraphic technique [315, 316]. The test is reproducible [317, 318] and can be applied for measuring gastric empting of liquids (using ^{13}C-acetate or ^{13}C-octanoate depending on the nutrient of interest) and solids (using ^{13}C-octanoate). Whereas the ^{13}C-acetate is more water-soluble and mostly represents the water-soluble compounds of the meal, the ^{13}C-octanoate is less water-soluble and mostly represents the fat-soluble compounds of the meal [13]. The exact procedure of the assessment of the gastric emptying is described in the respective projects.

4.6 Statistical analysis

All data analyses were performed using the statistical software package SPSS for Windows V. 14.0 and V. 19.0 (SPSS Inc., Chicago, Ill, USA). All values are reported as means ± SEM. Differences are considered statistically significant with $P < 0.05$. For detailed description see respective projects.

5 Projects

5.1 Effect of bile acids on the secretion of GLP-1 and CCK in healthy humans

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5.1.1 Abstract

Background/Objective: Bile acids (BAs) have been appreciated for their signaling properties. In vitro cell studies indicated that BAs induce the release of GLP-1. A stimulatory effect of different BAs on the secretion of gut peptides was also demonstrated in animal and human studies. We hypothesized that intraduodenal (ID) infusions of chenodeoxycholic acid (CDCA), the major primary BA in the human bile, stimulate the secretion of intestinal peptides, including, GLP-1, PYY and CCK, and induces beneficial effects on glucose homeostasis in humans.

Methods: Twelve healthy male volunteers received ID perfusions (2.0 mL/min for 180 min) of different CDCA concentrations (5 and 15 mmol/L), a fatty acid or placebo. After 60 min subjects performed an oral glucose tolerance test (oGTT).

Results: 1) CDCA induced a significant increase in GLP-1 and CCK secretion within the first 60 minutes ($P = 0.016$ and $P = 0.011$). 2) Plasma insulin and glucose were not affected within the first 60 minutes; an attenuated insulin release was observed after the oGTT ($P = 0.011$). 3) Total plasma BAs were significantly increased after administration of CDCA. 4) CDCA induced a significant rise in the plasma levels of the fibroblast growth factor 19 (FGF19).

Conclusions: CDCA can modulate the secretion of GLP-1 and CCK. The effect does, however, not influence blood glucose level. The marked increase in plasma BAs and the attenuated insulin release after the oGTT indicate a role of BAs in the control of glucose homeostasis independently of the incretin axis. Key words: GI-physiology, CDCA, bile acids, satiation peptides

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Conflict of interest: All authors disclose that they do not have any financial and personal relationships with other people, or organizations, that could inappropriately influence (bias) their work.

5.1.2 Introduction

Apart from their role in cholesterol homeostasis and dietary lipid absorption, bile acids (BAs) have been appreciated for their signaling properties. Secretion studies with enteroendocrine STC-1 and GLUTag cells indicated that BAs induce the release of GLP-1 [289-291]. A stimulatory effect of BAs on intestinal secretion of GLP-1 and PYY has also been demonstrated in animal models [282-285] and in humans – early studies performed by Adrian and co-workers demonstrated that infusions of deoxycholic acid into the colon increase the secretion of PYY and enteroglucagon [286]. In addition, intrarectal infusions of taurocholic acid have been shown to increase plasma GLP-1 and PYY levels in obese, type 2 diabetic subjects [44]. In the early nineties, Miyasaka *et al.* also reported effects of some BA on CCK release in rats [287]. In humans, Koop and co-workers [288] observed a small increase in CCK levels after CDCA administration, however, these data were not supported by Ooteghem *et al.* [319] and Portincasa *et al.* [320].

GLP-1, PYY and CCK are released from enteroendocrine cells of the small intestine in response to food intake. GLP-1 effects include increase of glucose-stimulated insulin release, inhibition of glucagon secretion and thus control of glucose homeostasis in humans; PYY and CCK, apart from digestive functions, both reduce food intake and appetite feelings [51, 124, 138, 177, 321]. Based on these pharmacological features, direct stimulation of these peptides could constitute a promising therapeutic approach for the treatment of metabolic diseases.

We therefore sought to investigate whether ID infusions of CDCA, the major primary BA in the human bile, 1) stimulate the secretion of intestinal peptides including, GLP-1, PYY and CCK, and 2) induces beneficial effects on glucose homeostasis in healthy humans.

5.1.3 Methods

Overall study design

The study was conducted as a randomized, double-blind, placebo-controlled, crossover trial. The protocol was submitted and approved by the State Ethical Committee of Basel and the study was carried out in accordance with the principles of the Declaration of Helsinki. Each subject gave written informed consent for the study. Exclusion included smoking, substance abuse, regular intake of medications, medical or psychiatric illness and any abnormalities detected on physical examination or laboratory screening. None of the subjects had a history of gastrointestinal disorders, food allergies or dietary restrictions. Anthropometric measurements including weight, height, BMI, as well as heart rate and blood pressure were recorded for all participants. Subjects were instructed to abstain from alcohol, caffeine, black- and green tee, coke, chocolate and strenuous exercise for 24 hours before each treatment and furthermore to abstain from sprouts, broccoli and grapefruit for the whole study participation. The day before each study day, subjects consumed a restricted simple-carbohydrate standard dinner before 8 p.m. and fasted from 12 PM. On each study day, subjects were admitted to the Phase 1 Research Unit of the University Hospital of Basel at ~ 7 AM. Subjects swallowed a radiopaque polyvinyl feeding tube (external diameter 8 french). The tube was inserted into the duodenum using a guide wire. The intraduodenal position was verified by fluoroscopy. The feeding tubes were firmly attached behind the ear to prevent further progression during the experiments. An antecubital vein catheter was inserted into a forearm vein for blood collection. The test trials were identical in design except for the ID perfusions. The perfusions were different concentrations of a principal primary BA in humans (sodium- chenodeoxycholic acid (CDCA)), a fatty acid (sodium-oleate, positive control) or placebo (saline, negative control). Subjects participated at five occasions with at least three days apart.

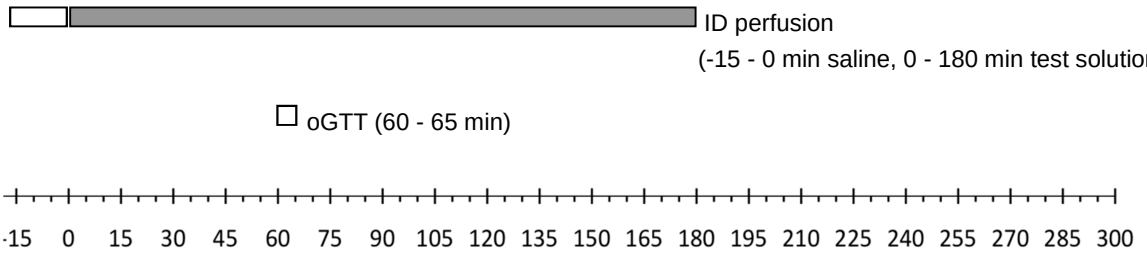
Subjects

Twelve male volunteers (mean age: 22.6 ± 0.5 years, range 19-25 years) participated in the study. All subjects were healthy, with weight within the normal range in relation to age, sex and height (mean BMI: 22.7 ± 0.3 kg/m^2, range 20.5 – 24.1 kg/m^2).

Experimental Procedure

The experimental procedure and the different test solutions are described in Table 2. The different test solutions were perfused intraduodenally in random order for 180 min (t = 0 – 180 min) following a 15 min saline prephase. The perfusion rate was 2.0 mL/min. After 60 min subjects performed an oral glucose tolerance test (oGTT, 75 g glucose in 300 mL tab water) within 5 min or less (t = 60 – 65 min).

Table 2 Experimental procedure and different test solutions used during all study sessions.



Blood samples: -15, -1, 15, 30, 45, 60, 75, 90, 105, 120, 150, 180 min
Visual analogue scales: -15, -1, 15, 30, 45, 60, 75, 90, 105, 120, 150, 180 min

Visit A: ID saline + lecithin	**Visit D:** ID saline + lecithin + sodium-oleate 20 mmol/L (7.2mmol)
Visit B: ID saline + lecithin + CDCA 5 mmol/L (1.8 mmol)	**Visit E:** ID saline + lecithin + sodium-oleate 20 mmol/L (7.2 mmol) + CDCA 5 mmol/L (1.8 mmol)
Visit C: ID saline + lecithin + CDCA 15 mmol/L (5.4 mmol)	

CDCA, chenodeoxycholic acid - sodium salt (>95% pure). Concentrations of the test solutions are expressed in mmol/L, with total amounts in parentheses. Concentration of lecithin was 2.5 mmol/L (0.9 mmol) for all study sessions. Subject received the different test solutions in random order. n = 12.

BAs and sodium-oleate were administered in physiological amounts according to previous experiments [322, 323]. Based on the study by Penagini and co-workers lecithin was added to the test solution to dissolve CDCA and to mimic the endogenous input of BAs which occurs in the physicochemical state of mixed micelles [322]. All test solutions were adjusted to pH 7 and at 37℃ when administered.

At regular time intervals blood samples were collected on ice into tubes containing EDTA (6 µmol/L), aprotinin (500 kIU/mL) and a DPP-IV inhibitor. Tubes were centrifuged at 4℃ at 3 000 rpm for 10 min and plasma samples were processed into different aliquots. For analysis of CCK, blood was 1:10 diluted in an ice-cold buffer (pH 3.6) containing 0.1 mol/L ammonium acetate (Sigma-Aldrich, Steinheim, Germany), 0.5 mol/L sodiumchloride (Sigma-Aldrich, Steinheim, Germany) and a protease inhibitor cocktail (Complete, Roche, Mannheim, Germany). Diluted blood was centrifuged (4℃ at 3 000 rpm for 10 min) and the supernatant collected. The method

has been described recently in more detail by Stengel *et al.* [324]. All samples were stored at -70 ℃ until analysis of plasma GLP-1, PYY, insulin, glucose, BAs, fibroblast growth factor 19 (FGF19) and CCK.

Immediately after each blood collection, appetite perceptions, such as feelings of hunger, prospective food consumption, fullness and satiety were recorded using visual analogue scales (VAS). The scales and scores have previously been described in detail [313, 325].

Vital signs (blood pressure, heart rate) were measured before and after each study day.

Materials

Chenodeoxycholic acid - sodium salt (>95 % pure) was purchased from Calbiochem (affiliate of Merck KGaA), Darmstadt, Germany, and lecithin (L-alpha-phosphatidylcholin) from Sigma-Aldrich, Schnelldorf, Germany. Sodium-oleate in the form of sodium salt was purchased from Chemie Brunschwig AG, Basel, Switzerland and glucose from Hänseler AG, Herisau, Switzerland.

Laboratory analysis

Active GLP-1 was measured with a commercially available ELISA kit (Millipore Corporation, Billerica, Massachusetts, USA). The intra- and inter-assay coefficient of variation for this assay is below 9.0% and 13.0%, respectively.

Total PYY (tPYY) and CCK were measured with commercially available RIA kits (Millipore Corporation, Billerica, Massachusetts, USA; Euro-Diagnostica AB, Malmö, Sweden). The intra- and inter-assay coefficients of variation for these assays are below 9.4% and 8.5% (tPYY) and below 5.5% and 13.7% (CCK), respectively. Prior to CCK measurements, CCK aliquots were i) extracted from supernatant by using a reversed solid phase extraction (C18-silica cartridges, Waters AG, Baden-Dättwil, Switzerland), ii) dried under nitrogen atmosphere and iii) dissolved in a diluent (volume corresponding to original plasma volume).

Insulin was measured with a commercially available RIA kit (CIS bio international, Bagnols, France). The intra- and inter-assay coefficient of variation for this assay is below 12.2% and 9.0%, respectively.

Plasma glucose concentration was measured by a commercially available glucoseoxidase-method (Bayer Consumer Care AG, Basel, Switzerland). The lowest level of glucose that can be detected by this assay is 0.6 mmol/L.

All methods have been described recently in more detail [326, 327].

Plasma bile acids were measured according to an established method using gas chromatography-mass spectrometry. The method was modified for application to human plasma; prior to measurements, BAs were extracted from plasma and after derivatization into trimethyl silyl ether methyl ester they were analysed on the base of a BA-specific ion in the multi ion current mode. [328]

FGF19 was measured with a commercially available ELISA kit (BioVendor, Brno, Czech Republic). The intra- and inter-assay coefficient of variation for this assay is below 7.0% and 8.5%, respectively.

Statistical analysis

Descriptive statistics were used for demographic variables such as age, weight, height, and BMI.

Hormone, glucose, plasma bile acid and FGF19 profiles were analyzed by calculating area under the concentration-time curve (AUC) from baseline values and maximal plasma concentrations (C_{max}). The parameters were tested for normality by the Shapiro-Wilk test method. The General Linear Model procedure of repeated measures ANOVA, using simple contrast, was used to test for significant differences between placebo and the single treatment groups.

VAS ratings were statistically analyzed by calculating AUC from baseline values. These data were compared between placebo and the single treatment groups by the General Linear Model procedure of repeated measures ANOVA, using simple contrast.

All statistical analysis was done using the statistical software package SPSS for Windows Version 19.0 (SPSS Inc., Chicago, USA). Values were reported as mean ± SEM. Differences were considered to be significant $p \leq 0.05$.

5.1.4 Results

Intestinal peptides:

ID perfusion of CDCA induced a dose-dependent increase in the secretion of GLP-1 and CCK in comparison to placebo (Figure 8A and B). AUCs of plasma GLP-1 and CCK were significantly increased with ID perfusion of the high concentration of CDCA (15 mmol/L) in the first 60 min (AUC 0-60 min: $P = 0.016$ and $P = 0.011$, respectively). In contrast, no significant increase in CCK and GLP-1 was observed with ID perfusion of the lower concentration of CDCA (5 mmol/L). The secretion of PYY was also not significantly affected by CDCA (5 mmol/L as well as 15 mmol/L) within the first 60 min (Figure 8C).

ID perfusion of sodium-oleate (positive control) induced a significant increase in the secretion of plasma GLP-1, CCK and PYY compared to placebo within the first 60 min (AUC 0-60 min: $P = 0.007$, $P < 0.001$ and $P < 0.001$, respectively; Figure 8A, B and C). Coadministration of sodium-oleate plus CDCA (5 mmol/L) had no additional effect on the secretion of GLP-1, PYY and CCK compared to sodium-oleate alone (data not shown).

As expected, oral glucose administration (at $t = 60$ min) in form of the oGTT caused a marked and rapid increase in GLP-1, PYY and CCK secretions in all study arms, with no significant differences between ID perfusions of CDCA, sodium-oleate and placebo (Figure 8).

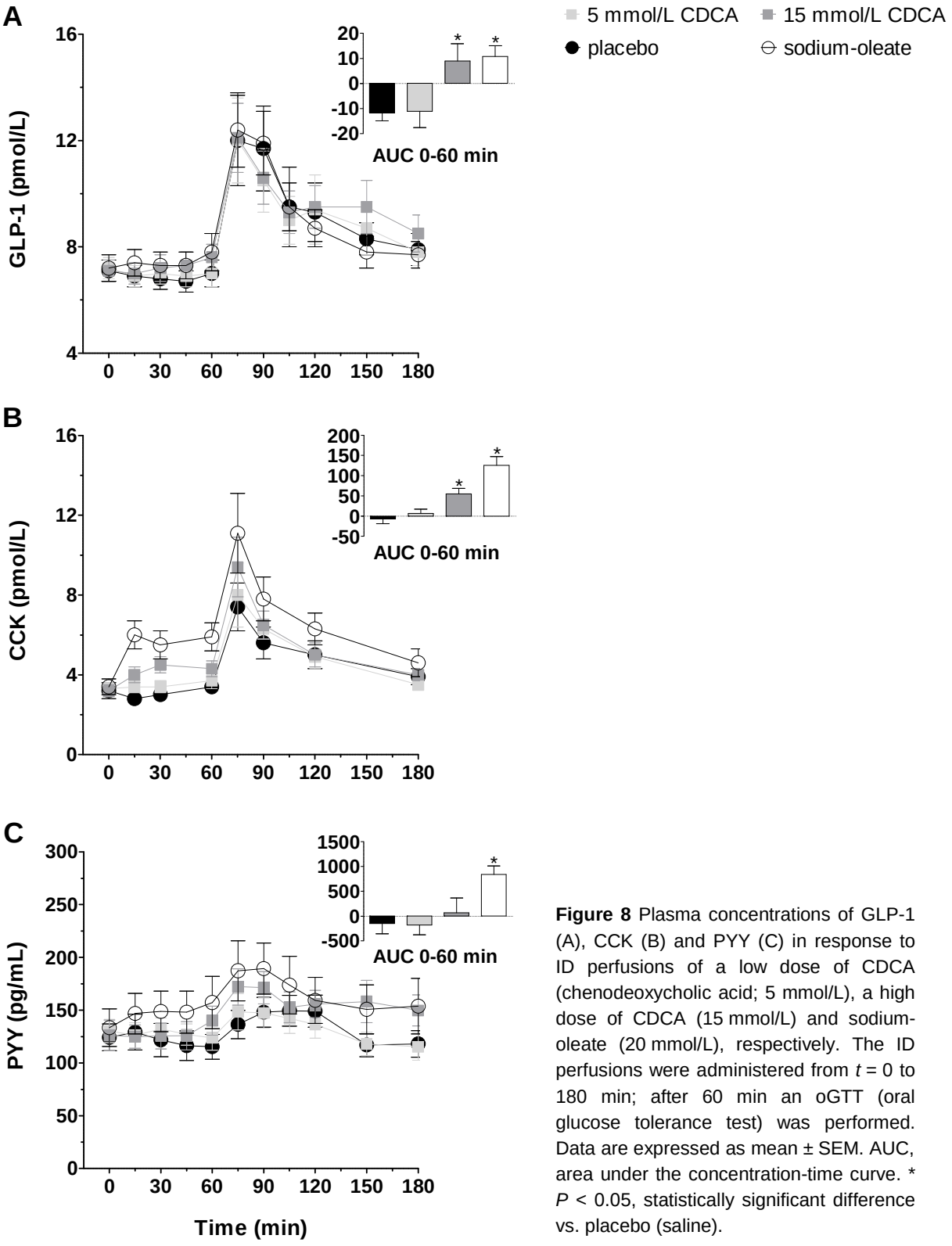


Figure 8 Plasma concentrations of GLP-1 (A), CCK (B) and PYY (C) in response to ID perfusions of a low dose of CDCA (chenodeoxycholic acid; 5 mmol/L), a high dose of CDCA (15 mmol/L) and sodium-oleate (20 mmol/L), respectively. The ID perfusions were administered from $t = 0$ to 180 min; after 60 min an oGTT (oral glucose tolerance test) was performed. Data are expressed as mean ± SEM. AUC, area under the concentration-time curve. * $P < 0.05$, statistically significant difference vs. placebo (saline).

Insulin and glucose:

Plasma insulin (Figure 9) and glucose (Figure 10) were not affected by CDCA perfusions (5 mmol/L as well as 15 mmol/L) within the first 60 min. After the oGTT, a significantly attenuated insulin release compared to placebo was observed with the high concentration of CDCA (*P* = 0.011; Figure 9).

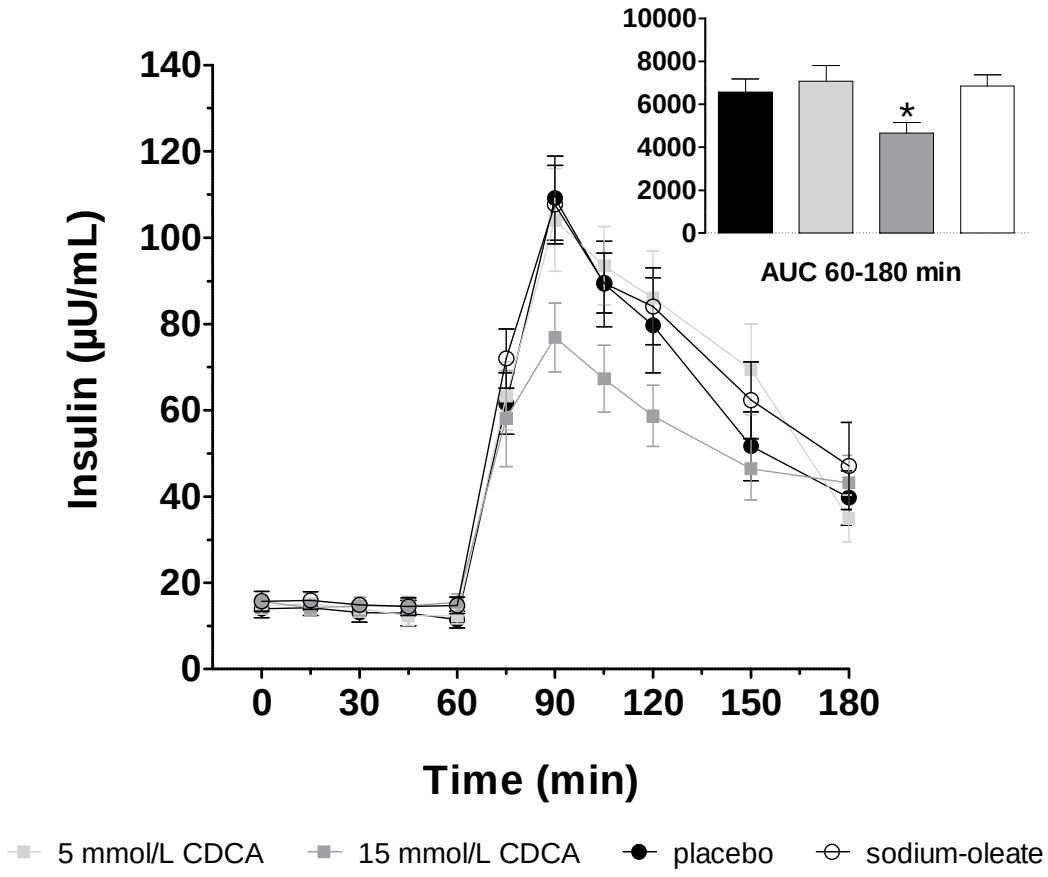


Figure 9 Plasma concentration of insulin in response to ID perfusions of a low dose of CDCA (chenodeoxycholic aicd; 5 mmol/L), a high dose of CDCA (15 mmol/L) and sodium-oleate (20 mmol/L), respectively. The ID perfusions were administered from *t* = 0 to 180 min; after 60 min an oGTT (oral glucose tolerance test) was performed. Data are expressed as mean ± SEM. AUC, area under the concentration-time curve. * *P* < 0.05, statistically significant difference vs. placebo (saline).

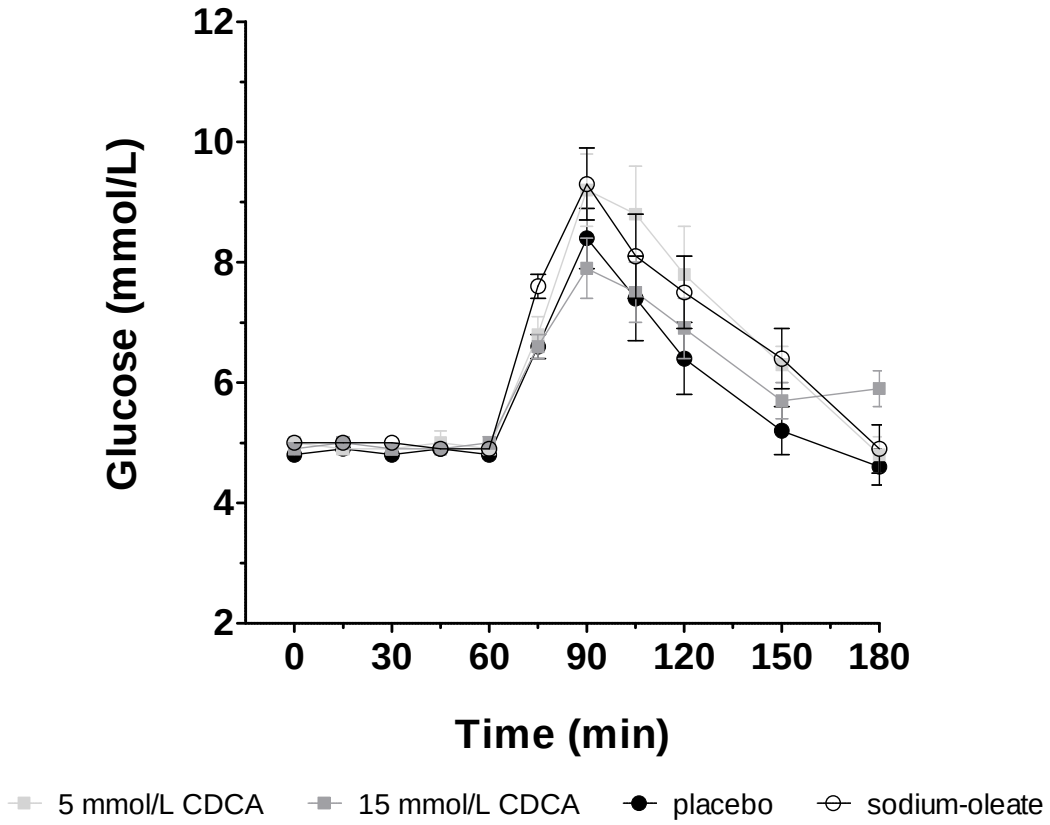


Figure 10 Plasma concentration of glucose in response to ID perfusions of a low dose of CDCA (chenodeoxycholic aicd; 5 mmol/L), a high dose of CDCA (15 mmol/L) and sodium-oleate (20 mmol/L), respectively. The ID perfusions were administered from *t* = 0 to 180 min; after 60 min an oGTT (oral glucose tolerance test) was performed. Data are expressed as mean ± SEM.

Plasma BAs:

CDCA induced a dose-dependent rise in plasma BAs (Figure 11). Compared to placebo. AUC of plasma BAs were significantly increased after the ID perfusion of CDCA, both with 5 mmol/L and 15 mmol/L CDCA (*P* = 0.001, respectively). Coadministration of sodium-oleate plus CDCA (5 mmol/L) induced significantly increased plasma BAs levels compared to placebo (AUC 0-180 min: *P* = 0.003; Figure 11); an additive effect of the combination was observed compared to CDCA (5 mmol/L) alone and sodium-oleate alone.

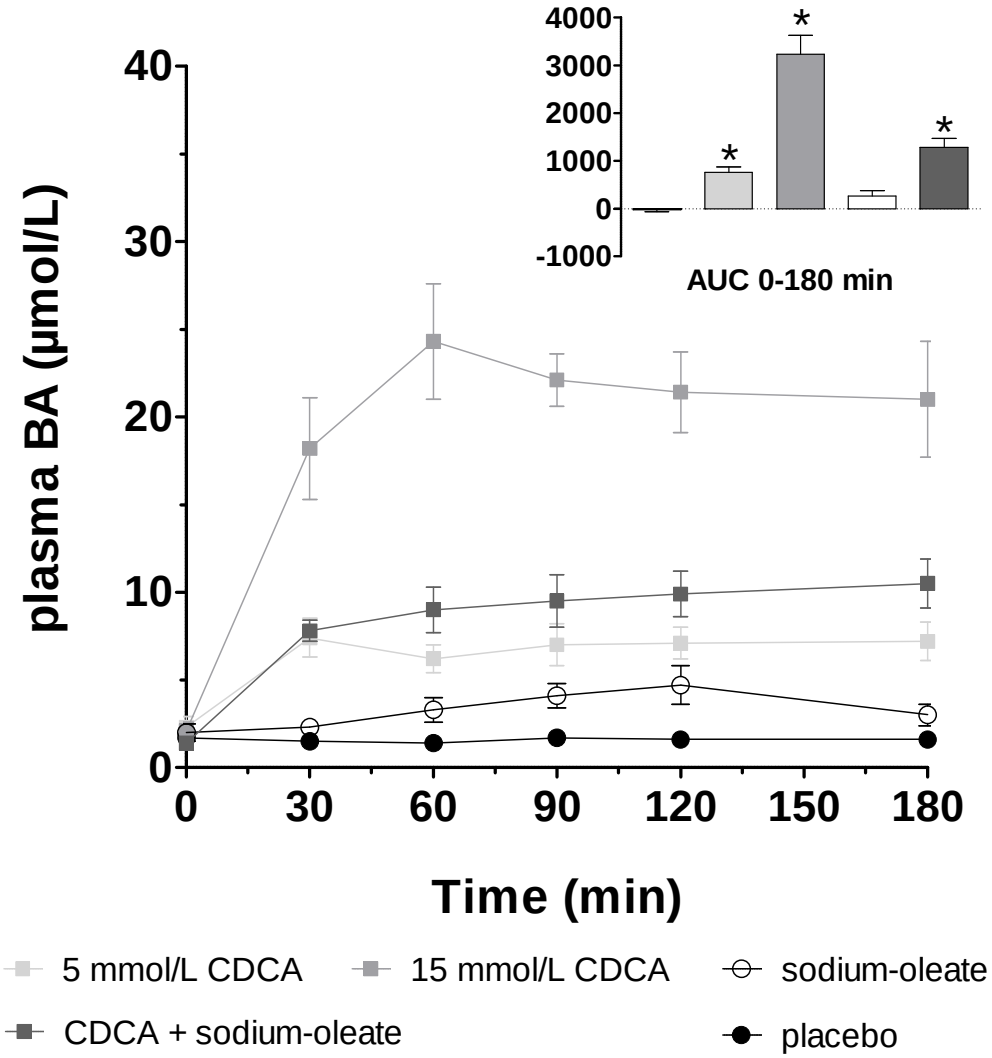


Figure 11 Plasma BA concentrations in response to ID perfusions of a low dose of CDCA (chenodeoxycholic acid; 5 mmol/L), a high dose of CDCA (15 mmol/L), sodium-oleate (20 mmol/L) and a combination (sodium-oleate plus CDCA (5 mmol/L)). The ID perfusions were administered from *t* = 0 to 180 min; after 60 min an oGTT (oral glucose tolerance test) was performed. Data are expressed as mean ± SEM. AUC, area under the concentration-time curve. * *P* < 0.05, statistically significant difference vs. placebo (saline).

Plasma FGF19

CDCA induced a dose-dependent rise in plasma FGF19 after 60 min of administration (Figure 12). Compared to placebo, AUC of plasma FGF19 was significantly increased after the ID perfusion of the high concentration of CDCA (15 mmol/L; AUC 0-180 min: $P < 0.001$). Co-administration of sodium-oleate plus CDCA (5 mmol/L) induced significantly increased FGF19 levels compared to placebo (AUC 0-180 min: $P = 0.01$; Figure 12); an additive effect of the combination was observed compared to CDCA (5 mmol/L) alone and sodium-oleate alone.

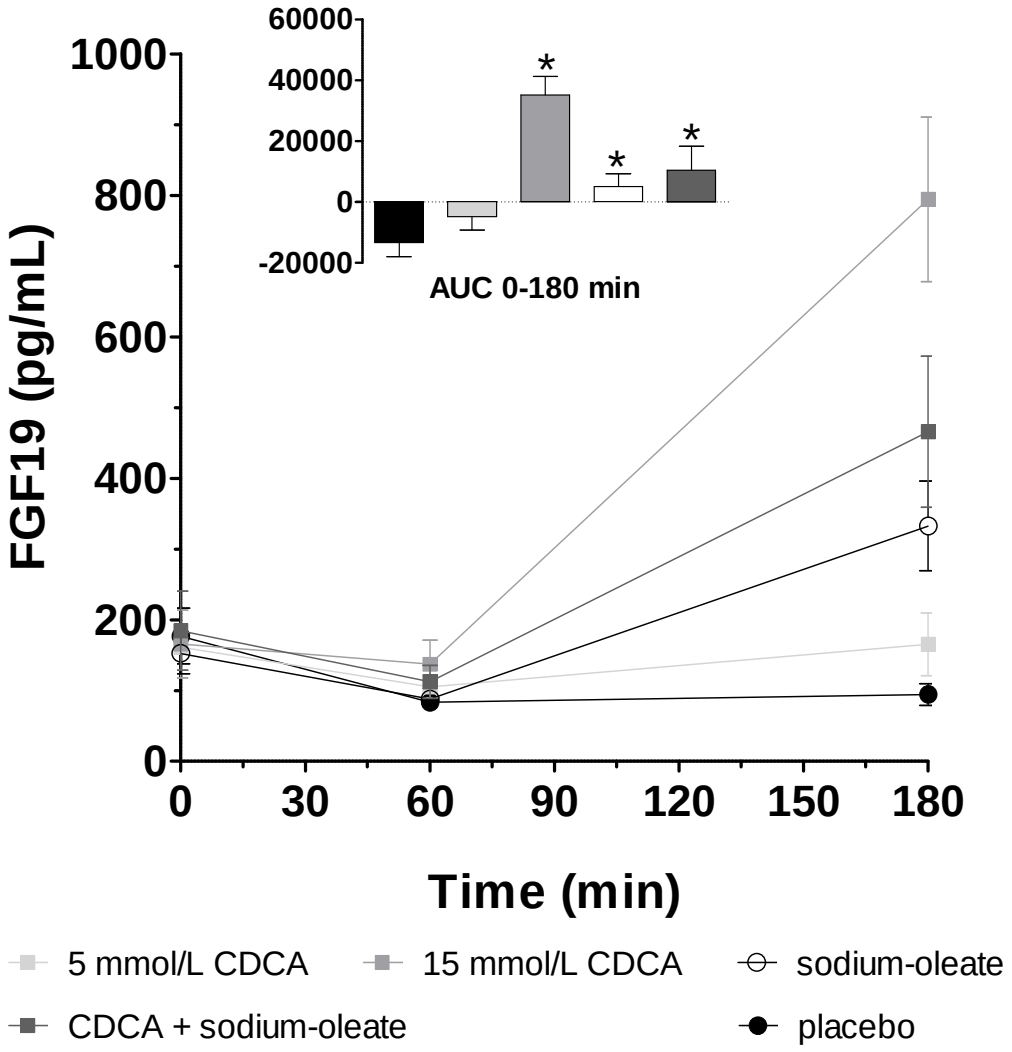


Figure 12 FGF19 concentrations in response to ID perfusions of a low dose of CDCA (chenodeoxycholic acid; 5 mmol/L), a high dose of CDCA (15 mmol/L), sodium-oleate (20 mmol/L) and a combination (sodium-oleate plus CDCA (5 mmol/L)). The ID perfusions were administered from $t = 0$ to 180 min; after 60 min an oGTT (oral glucose tolerance test) was performed. Data are expressed as mean ± SEM. AUC, area under the concentration-time curve. * $P < 0.05$, statistically significant difference vs. placebo (saline).

Appetite perceptions:

ID perfusion of CDCA (5mmol/L and 15 mmol/L) did not affect appetite perceptions, neither within the first 60 min nor after the oGTT (data not shown).

5.1.5 Discussion

Eating behavior is a complex process, which is not just defined by metabolic requirements. One important element in this complex regulatory process is the brain-gut communication through gut peptide hormones. Three major gut satiety peptides include CCK, GLP-1 and PYY. All three peptides are stimulated by nutrients, but recent evidence, largely from animal studies, suggests that other factors such as BAs participate in their release [290]. We therefore hypothesized that ID perfusions of CDCA stimulate the secretion of CCK, GLP-1 and PYY in humans with consequences for glucose metabolism.

The main findings can be summarized in brief: 1) Intestinal infusion of CDCA mimicking physiological concentrations resulted in significant increases of both GLP-1 and CCK secretion (P = 0.016 and 0.011, respectively). 2) Plasma insulin and glucose were, however, not affected by CDCA in the first 60 minutes of CDCA administration; an attenuated insulin release was observed after the oral glucose load (P = 0.011). 3) Total plasma BAs were dose-dependently increased after administration of CDCA (5 mmol/L and 15 mmol/L). 4) CDCA (15 mmol/L) induced a marked rise in the FGF19 levels.

Already in 1995, Plaisancié and co-workers had shown that BAs promote GLP-1 release in isolated vasculary perfused rat colon [282]; in support, infusions of taurochenodeoxycholic acid and deoxycholic acid were shown to increase secretion of PYY in rabbits [283, 284]. Also in humans, it has been demonstrated that infusion of deoxycholic acid into the colon results in increased secretion of enteroglucagon and PYY in healthy subjects [286], furthermore, intrarectal infusion of taurocholic acid was shown to increase plasma GLP-1 and PYY levels in obese, type 2 diabetic subjects [44]. Finally, Roberts and co-workers reported that postprandial GLP-1 and PYY responses were positively correlated with serum BAs in healthy subjects [293].

Apart from the stimulatory effects of BAs on enteroendocrine L-cells secreting GLP-1 and/or PYY, an early study by Miyasaka *et al.* has demonstrated that some conjugated BAs stimulate CCK release in rats [287].

Our findings confirm in healthy human males the stimulatory effects of BAs on the secretion of these peptides. The stimulatory potency of CDCA on CCK and GLP-1 was, however, small, if we compare the magnitude of the GLP-1 and CCK responses to other well-known secretagogues such as glucose or long-chain fatty acids. Indeed, ID CDCA perfusions induced a GLP-1 response, which amounted to roughly 25% of what we generally have observed after a standard glucose stimulation. As a consequence, the small effect of CDCA on GLP-1 was

without effect on plasma glucose and insulin release within the first 60 min. In concordance, CDCA had no influence on appetite perceptions.

Whether perfusions of other BAs, conjugated BAs, or other concentrations of BAs into the duodenum would induce different effects in humans is unknown. CDCA is, however, one of the major primary BAs; the plasma BA levels obtained in our experiment are at the upper limit of the physiological range. We infer from these observations that a physiological load of CDCA is a rather weak stimulus for GLP-1 and CCK secretion with no insulinotropic GLP-1 dependent effects on glucose homeostasis and appetite perceptions. Our results are in agreement with our observations in patients after bariatric surgery: no correlation was found between postprandial plasma BAs and the secretion of satiation peptides (unpublished data). Moreover, treatment studies with BA sequestrants – thought to improve glucose control through increased secretion of GLP-1 [297, 298] – exhibited no correlation between markers of glucose metabolism and BA metabolism in type II diabetes patients [300].

Interestingly, we observed a significantly attenuated insulin response after the oGTT with 15 mmol/L CDCA. This observation is in line with recent findings by Shaham and co-workers who showed a correlation between BA levels and insulin sensitivity in humans [329]. The results indicate a role for BAs in glycemic control and suggest an improvement in insulin resistance independent of the incretin axis. The mechanism might involve BA induced activation of farnesoid X receptor (FXR) signalling pathways. FXR was identified as nuclear receptor of BAs [330] and strong experimental evidence supports a role of FXR receptors in glycemic control and glucose homeostasis [281, 331]. As mentioned above, we observed that the perfusion of physiological loads of CDCA resulted in an impressive dose-dependent increase in plasma BAs. These plasma BA levels might have activated a FXR dependent cascade. Indeed, we observed a dose-dependent increase in plasma levels of FGF19 after ID administration of CDCA. FGF19 is a postprandial hormone released from the small intestine. The expression of FGF19 occurs primarily in the ileum and is directly induced through the activation of FXR [319, 332, 333]. FGF19 exhibit insulin-like effects, which are mediated by signaling pathways distinct from those employed by insulin [319]. To what extend the CDCA induced increase in FGF19 could play a role in the observed attenuated insulin release needs further investigation.

Finally we found that CDCA induces the secretion of CCK. Although the negative feedback control of postprandial CCK release by ID BAs is well established [288, 334-336], the relationship between BAs and CCK in the fasting state is less clear. Whereas Ooteghem *et al.* [319] and Portincasa *et al.* [320] reported that ID bile salts exert negative feedback control on

gall bladder volume in the fasting state by a CCK independent mechanism (as they observed no changes in CCK release), Koop and co-workers [288] observed a small increase in CCK levels after CDCA administration. The latter is in line with our observations. A possible explanation for BAs stimulating CCK secretion could be a direct effect on enteroendocrine I-cells via a TGR5 (membrane receptor of BAs [276, 277]) dependent mechanism; however, the expression of TGR5 on I-cells has not been reported yet and further research is required to evaluate the role of BA in CCK secretion.

In conclusion, we have observed that CDCA mimicking physiological doses of BAs can modulate the secretion of GLP-1 and CCK in humans. Under our experimental conditions, the effect is, however, small and, therefore, does not influence blood glucose level. The marked dose-dependent increase in plasma BAs after ID CDCA and the attenuated insulin release after the oGTT may indicate a role of BAs in the control of glucose homeostasis independently of the incretin axis. To date, the peptide secreting properties of BAs are mainly observed in cell models and animal studies; therefore further studies with more potent BAs or pharmacological doses of BAs could clarify to what extent the described relationship between BAs and gut peptide secretion in cells and animals can be translated into the human situation.

5.2 The role of the gut sweet taste receptor in regulating GLP-1, PYY and CCK release in humans

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5.2.1 Abstract

Background and aims: The recent identification of sweet taste receptors in the gastrointestinal (GI) tract has important implications in the control of food intake and glucose homeostasis. Lactisole can inhibit the sweet taste receptor T1R2/T1R3. The objective was to use lactisole as probe to investigate the physiological role of T1R2/T1R3 by assessing the effect of T1R2/T1R3 blockade on GLP-1, PYY and CCK release in response to: i) intragastric (IG) administration of nutrients, or II) intraduodenal (ID) perfusion of nutrients.

Methods: The study was performed as randomized, double-blind, placebo-controlled, crossover study, including 35 healthy subjects. In part I, subjects received IG 75 g glucose in 300 mL water or 500 mL of a mixed liquid meal with or without lactisole. In part II, subjects received an ID perfusion of glucose (29.3 g glucose per 100 mL; rate: 2.5 mL/min for 180 min) or a mixed liquid meal (same rate) with or without lactisole.

Results: 1) Lactisole induced a significant reduction in GLP-1 and PYY, but not CCK secretion in both, the IG and the ID glucose-stimulated parts ($P \leq 0.05$). 2) Comparison of the inhibitory effect of lactisole showed a significantly greater suppression of the hormone response in the IG part ($P = 0.023$). 3) Lactisole had no effect on liquid meal-stimulated parameters.

Conclusions: We conclude that T1R2/T1R3 is involved in glucose-dependent secretion of satiation peptides. However, the results of the liquid meal-stimulated parts show that the receptor is not alone responsible for peptide secretion.

Key words: T1R2/T1R3, gastric emptying, gut, lactisole, nutrient sensing

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Conflict of interest: All authors disclose that they do not have any financial and personal relationships with other people, or organizations, that could inappropriately influence (bias) their work.

5.2.2 Introduction

Enteroendocrine cells (EECs) in the human gut sense chemical components of ingested food and secrete a number of GI satiation peptides, including GLP-1, PYY or CCK [199]. All of them have demonstrated to influence gastric emptying, increase satiety and reduce food intake [103, 105, 124, 158, 177, 337]. Recently it has been shown that G-protein-coupled receptors that sense chemical components of food on the tongue – including T1R2/T1R3 as well as key elements, like α-gustducin, phospholipase Cβ2 (PLC β2) and transient receptor potential channel type 5 (Trmp5) – also exist in EECs of the gut [146, 233, 250, 251].

It is known that glucose is an activator for the sweet taste receptor on the tongue; in addition, glucose is a strong stimulus of the secretion of GI peptides. In case that the sweet taste receptor system in the gut is involved in the secretion of GI peptides, lactisole – the sodium salt of 2-(4-methoxyphenoxy)-propionic acid – should attenuate the glucose-stimulated peptide secretion. *In vivo* lactisole suppresses the sweet taste perception on the tongue; the inhibitory effect is specific to humans and other primates. Furthermore, *in vitro*, lactisole antagonizes the effects of sucralose-stimulated GLP-1 release from human EECs (NCI-H716). [146, 338, 339]

In a preliminary study, we were able to show, that in healthy humans the secretion of GLP-1 and PYY in response to glucose was reduced by lactisole [253]. These data suggest that lactisole could be an excellent tool to investigate the regulatory role of the sweet receptor's dependent physiological functions in humans.

The objectives were to use lactisole as a probe to investigate the physiological role of the sweet taste receptor by assessing the effect of the sweet receptor blockade on GLP-1, PYY and CCK release in response to i) IG administration of nutrients, or ii) in response to ID perfusion of nutrients. Furthermore we were interested to determine the consequences on glucose-homeostasis and the effect of sweet taste receptor blockade on appetite perceptions and on gastric emptying rates.

5.2.3 Methods

Subjects

Thirty-five volunteers (20 male and 15 female; mean age: 24 ± 0.4 years, range 19-30 years) participated in the study. All subjects were healthy, with weight within the normal range in relation to age, sex and height (mean BMI: 22.3 ± 0.3 kg/m^2, range 19.0-25.9 kg/m^2).

The protocol was submitted and approved by the State Ethical Committee of Basel and the study was carried out in accordance with the principles of the Declaration of Helsinki. Each subject gave written informed consent for the study. Before acceptance, each participant was required to complete a screening and medical interview, received a full physical examination and participated in an initial laboratory screening. The criteria for exclusion were smoking, substance abuse, regular intake of medications (except for oral contraceptives), medical or psychiatric illness and any abnormalities detected on physical examination or laboratory screening. None of the subjects had a history of GI disorders, food allergies or dietary restrictions. Subjects were instructed to abstain from alcohol, caffeine and strenuous exercise for 24 hours before each treatment.

Experimental Procedure

The study consisted of two experimental parts. Both parts were performed as a randomized, double-blind, placebo-controlled, crossover study, with each subject studied on two (part I) or five (part II) occasions at least three days apart. Subjects food intake on the preceding day of each study day was standardized: they consumed a restricted simple-carbohydrate standard dinner before 8 PM and fast from 10 PM. On each study day, subjects were admitted to the Phase 1 Research Unit of the University Hospital of Basel between 7:00 AM and 8:30 AM.

Part I: Effect of sweet taste receptor blockade in response to IG administered nutrients.

At each occasion, a radiopaque polyvinyl feeding tube (external diameter 8 french) was inserted into the stomach through an anesthetized nostril. The IG position of the tube was confirmed by rapid injection of 10 mL air and auscultation of the upper abdomen. An antecubital vein catheter was inserted into a forearm vein for blood collection. The treatments were identical in design except for the IG infusions.

In a first series, 26 subjects (13 male and 13 female) received an IG infusion of 75 g glucose alone (300 kcal) or glucose together with 450 ppm lactisole (45 mg/100 mL) dissolved in 300 mL tap water within two minutes (t = 0-2 min). In addition, the test solution of 10 subjects was labeled with 50 mg ^{13}C-sodium acetate for determination of gastric emptying rate.

In a second series, 16 subjects (8 male and 8 female) received an IG infusion of a complex liquid meal (500 mL Ensure Plus®, 17% protein, 30% fat and 53% carbohydrate, caloric load: 600 kcal) with 450 ppm (45 mg/100 mL) lactisole dissolved. All test solutions were labeled with 50 mg ^{13}C-sodium acetate for determination of gastric emptying. The placebo treatment was the test solution without lactisole.

The chosen doses of lactisole derive from previous experiments [253, 340]. The IG infusions were freshly prepared each morning of the study and were at room temperature when administered. The feeding tube was removed immediately after the infusion was completed.

At regular time intervals, 10 ml blood samples were collected on ice into tubes containing EDTA (6 µmol/l), aprotinin (500 kIU/ml) and a DPP-IV inhibitor. After centrifugation (3000 rpm, 10 min at 4℃), plasma samples were processed into differe nt aliquots and kept frozen at -70 ℃ until analysis. Immediately after each blood collection, appetite perceptions, such as feelings of hunger, prospective food consumption, fullness and satiety were recorded.

For determination of the gastric emptying rates, end-expiratory breath samples were taken at fixed time intervals after instillation of the test solution.

Vital signs (blood pressure, heart rate) were measured before and after each study day.

Part II: Effect of sweet taste receptor blockade in response to ID perfused nutrients.

Ten male volunteers were included. Similar to part I, each subject swallowed a radiopaque polyvinyl feeding tube (external diameter 8 french). The tube was transported to the second part of the duodenum within 30 min; the correct position was verified by fluoroscopy. The feeding tube was firmly attached to the skin behind the ear to prevent further progression of the tube during the experiment. An antecubital vein catheter was inserted into a forearm vein for blood collection. The treatments were identical in design except for the ID infusions.

At each occasion, subjects first received a forerun (30 min at 2.5 mL/min), consisting of a saline solution (0.9%) with or without 300 ppm (30 mg/100 mL) lactisole. Subsequently, the different test solutions were perfused for 180 min at 2.5 mL/min. The glucose solution contained 29.3 g glucose per 100 mL tap water, which delivers a caloric load of 3.0 kcal/min into the duodenum (total glucose load per 180 min: 131.7 g). The complex liquid meal (Ensure Plus®) contained 17% protein, 30% fat and 53% carbohydrate, which also delivers a caloric load of 3.0 kcal/min into the duodenum (total caloric load per 180 min: 540 kcal). Both treatments (glucose and

liquid meal) were administered either with or without 450 ppm lactisole. Finally, saline (0.9%) together with 450 ppm lactisole was perfused as control solution.

The chosen doses of lactisole derive from previous experiments [253, 340]. The test solutions were freshly prepared each morning of the study and were at room temperature when administered.

Similar to part I, 10 ml blood samples were collected, centrifuged, processed and frozen at regular time intervals. Immediately after each blood collection, appetite perceptions, such as hunger, prospective food consumption, fullness and satiety were recorded.

Vital signs (blood pressure, heart rate) were measured before and after each study day.

Materials

Lactisole was a friendly gift of Domino Sugar Corporation, New York, USA. The complex liquid meal (Ensure Plus®) and glucose were purchased at Abbott AG, Baar, Switzerland and Hänseler AG, Herisau, Switzerland, respectively. ^{13}C-sodium acetate was purchased at Wagner Analysen Technik GmbH, Bremen, Germany and ReseaChem GmbH, Burgdorf, Switzerland.

Assessment of appetite perceptions

VAS were used to rate the subjective sensations of hunger, prospective food consumption, fullness and satiety. The scales and scores have previously been designed and described [308, 313]. In brief, VAS consist of a horizontal, unstructured, 10 cm line with words anchored at each end, expressing the most positive and most negative rating. Subjects assign a vertical mark across the line to index the magnitude of their subjective sensation at the present time point. To ensure reliable and valid results, subjects took enough time and rated their appetite sensation as precisely as possible. In addition, they could not refer to their previous ratings when marking the VAS. Subjects were allowed to talk, relax and read with the exception that they could not discuss or compare their ratings. Quantification of the measurement was made by measuring the distance from the left end of the line to the mark.

Assessment of gastric emptying

Gastric emptying rate was determined using a ^{13}C-sodium acetate breath test. This test is an accurate, non-invasive, simple method, without radiation exposure, and represents a reliable alternative to scintigraphy, the gold standard for measuring gastric emptying [341, 342]. Test solutions were labeled with 50 mg ^{13}C-sodium acetate, which is rapidly absorbed in the duodenum, transported to the liver and metabolized to $^{13}CO_2$ [343]. Subjects were asked to

exhale through a mouth-piece to collect an end-expiratory breath sample into a 100 mL foil bag. The $^{13}CO_2$ breath content was then determined by non-dispersive infrared spectroscopy using an isotope ratio mass spectrophotometer (IRIS; Wagner Analysen Technik, Bremen, Germany). The $^{13}CO_2/^{12}CO_2$ ratio in progress of time was used as a parameter for gastric emptying. Mathematical analysis were performed according to the methods described by Sanaka et al. [341].

Hormones

GLP-1 was measured with a commercially available ELISA kit (Millipore Corporation, Billerica, Massachusetts, USA). This kit is for non-radioactive quantification of GLP-1 (7-36) in serum and EDTA-plasma samples; it is high specific and does not detect other forms of GLP-1. The lowest level of GLP-1 that can be detected by this assay is 0.5 pmol/L when using a 100 µl plasma sample. Prior to measurements, GLP-1 was extracted from 1 mL plasma by using a reversed solid phase extraction (C18-silica cartridges, Waters AG, Baden-Dättwil, Switzerland).

PYY was measured with a commercially available RIA kit (LINCO Research, St. Charles, Missouri, USA). The anti-PYY-antibody used in this kit is raised in guinea pigs and displays 100% cross-reactivity with human PYY1-36 and human PYY3-36, but no cross-reactivity with human pancreatic polypeptide, NPY and unrelated peptides such as leptin and ghrelin. The intra- and inter-assay coefficient of variation for this assay is below 9.4% and 8.5%, respectively. The lowest level of PYY that can be detected by this assay is 10 pg/mL when using a 100 µL plasma sample.

CCK concentrations were measured by a commercially available RIA kit (Euro-Diagnostica AB, Malmö, Sweden). This kit is for assay of CCK in plasma by using an antiserum raised against sulfated CCK-8 N-terminally conjugated to bovine albumin. The antiserum displays 100% cross-reactivity with CCK-8 sulphate, but no relevant cross-reactivity with sulfated gastrin. The intra- and inter-assay coefficient of variation for this assay is below 5.5% and 13.7%, respectively. The lowest detectable concentration is 0.3 pmol/L when using a 200 µL plasma sample. Prior to measurements, CCK was extracted from 1 mL plasma by using an ethanol extraction method.

Insulin was measured with a commercially available RIA (CIS bio international, Bagnols, France). This kit is for quantitative determination of insulin in human serum and plasma (EDTA). It is highly specific for insulin and shows no cross-reactivity with other peptides, e.g. c-peptide or glucagon. The intra- and inter-assay coefficient of variation for this assay is below 12.2% and 9.0%, respectively. The lowest level of insulin that can be detected by this assay is 4.6 µU/mL.

Plasma glucose concentration was measured by a commercially available glucoseoxidase-method (Bayer Consumer Care AG, Basel, Switzerland). This method is highly specific for measurement of glucose in serum or plasma. The lowest level of glucose that can be detected by this assay is 0.6 mmol/L.

Statistical analysis

Descriptive statistics were used for demographic variables such as age, weight, height, and BMI.

Hormone and glucose profiles were analyzed by calculating pharmacodynamic parameters (area under the concentration-time curve (AUC), delta AUC, maximal plasma concentration (C_{max}) and time to maximal plasma concentration (T_{max})). The parameters were tested for normality by the Shapiro-Wilk test. To test for significant differences between the treatment groups within part I and part II, AUC, C_{max} and T_{max} were compared using student's paired t-test. To test for significant differences between part I (IG infusions) and part II (ID perfusions), delta AUC were assessed using the student's unpaired t-test.

VAS ratings were statistically analyzed by calculating AUC (0-120 min) from baseline. These data were compared between the treatments using the non-parametric Wilcoxon's signed-rank test due to high variability and non-normal distribution (Shapiro-Wilk test).

All statistical analysis was done using the statistical software package SPSS for Windows Version 14.0 (SPSS Inc., Chicago, USA). Values were reported as mean ± SEM. All test were two-tailed with $P \leq 0.05$ considered statistically significant.

5.2.4 Results

Effect of sweet taste receptor blockade in response to IG nutrients.

GLP-1, PYY and CCK:

Lactisole induced a reduction in IG glucose-stimulated secretion of GLP-1 and PYY (24.3 ± 7.7 % and 15.5 ± 6.1 %, respectively) (Figure 13A and B). AUCs were significantly decreased by lactisole in comparison to the administration of glucose alone (P = 0.007 and 0.012; Table 3). In contrast, lactisole showed no significant effect on IG administered mixed liquid meal-stimulated secretions of GLP-1 and PYY (Figure 13D and E). Plasma CCK levels were not affected by lactisole, neither after IG glucose nor after mixed liquid meal administration (Figure 13C and F).

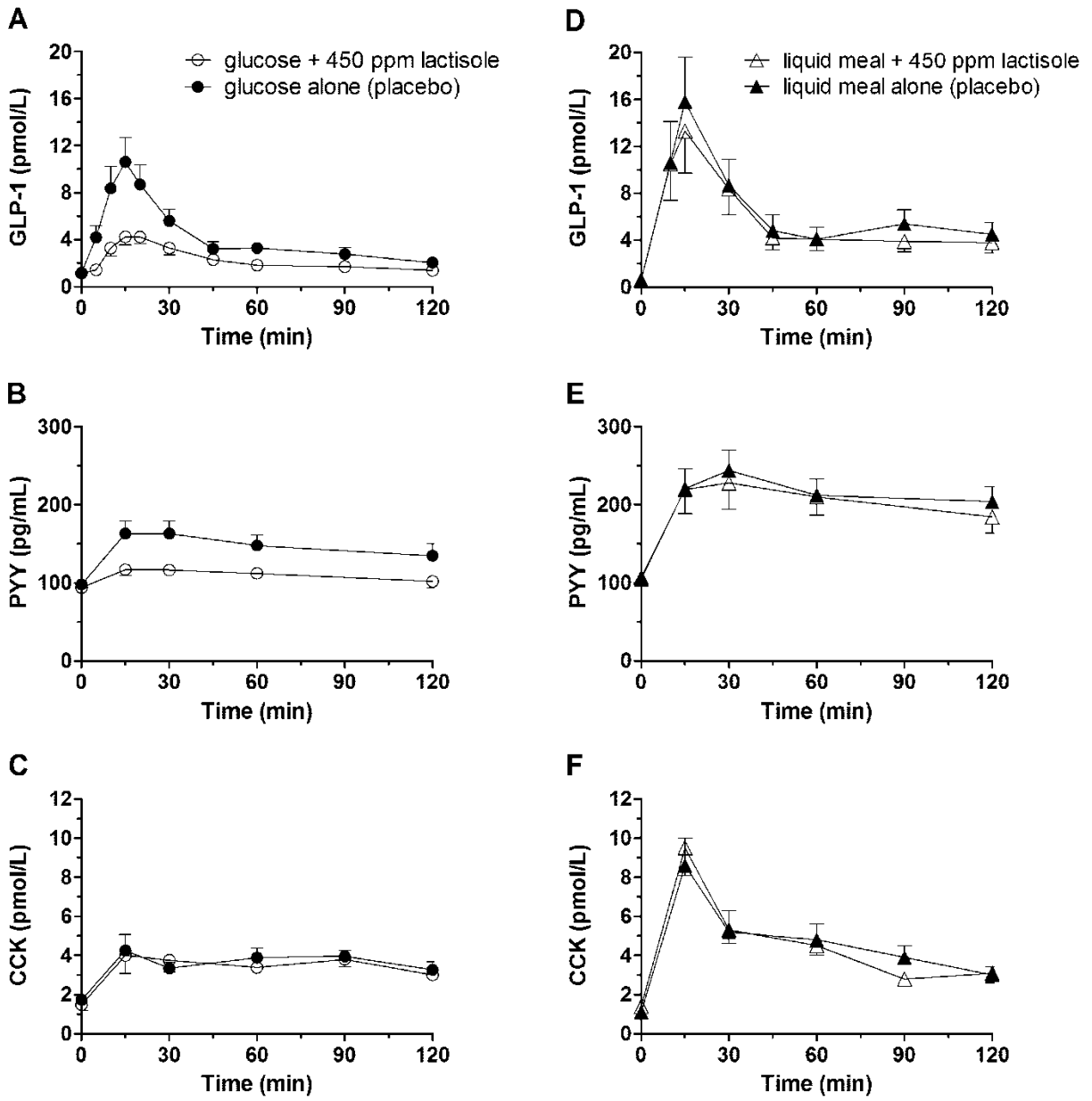

Figure 13 Plasma concentrations of GLP-1, PYY and CCK after an IG load of 75 g glucose (A, B and C) or an IG infusion of a mixed liquid meal (500 mL) (D, E and F), both with or without 450 ppm lactisole. The IG infusions were administered at t = 0 min. Data are expressed as mean ± SEM.

Table 3 Effect of lactisole (450 ppm) on IG glucose-stimulated secretion of GLP-1, PYY, insulin and glucose in healthy subjects.

	glucose alone (placebo)	**glucose + 450 ppm lactisole**
GLP-1		
AUC (0-120 min) (pmol x min/L)	489.2 ± 76.3	267.3 ± 29.0 *(P = 0.007)*
C_{max} (pmol/L)	12.2 ± 2.3	5.3 ± 0.8 *(P = 0.007)*
PYY		
AUC (0-120 min) (pg x min/mL)	17,488.4 ± 1637.8	13,170.5 ± 787.7 *(P = 0.0012)*
C_{max} (pg/mL)	173.4 ± 17.2	128.2 ± 7.8 *(P = 0.012)*
Insulin		
C_{max} (0-30 min) (µU/mL)	118.0 ± 8.0	100.4 ± 7.0 *(P = 0.048)*
Glucose		
AUC (0-120 min) (mmol x min/L)	759.9 ± 24.4	830.6 ± 34.3 *(P = 0.029)*
C_{max} (mmol/L)	9.1 ± 0.4	9.5 ± 0.4 *(NS)*

AUC, area under the concentration-time curve; C_{max}, maximum plasma concentration. Data are expressed as mean ± SEM. n = 26. *P* values are given in parentheses and represent comparisons vs. glucose alone (placebo): $P \leq 0.05$, statistically significant difference vs. glucose alone; NS, not statistically significant vs. glucose alone.

Glucose and Insulin:

The AUCs for plasma glucose were significantly increased by lactisole when glucose was IG infused (P = 0.029; Figure 14A; Table 3). Plasma insulin was significantly reduced by lactisole in the first 30 min (C_{max}; $P \leq 0.05$), although no significant differences were seen in the later time course of insulin compared to administration of glucose alone (Figure 14B; Table 3). In contrast, neither plasma glucose levels nor insulin release were affected by IG liquid meal administration (Figure 14C and D).

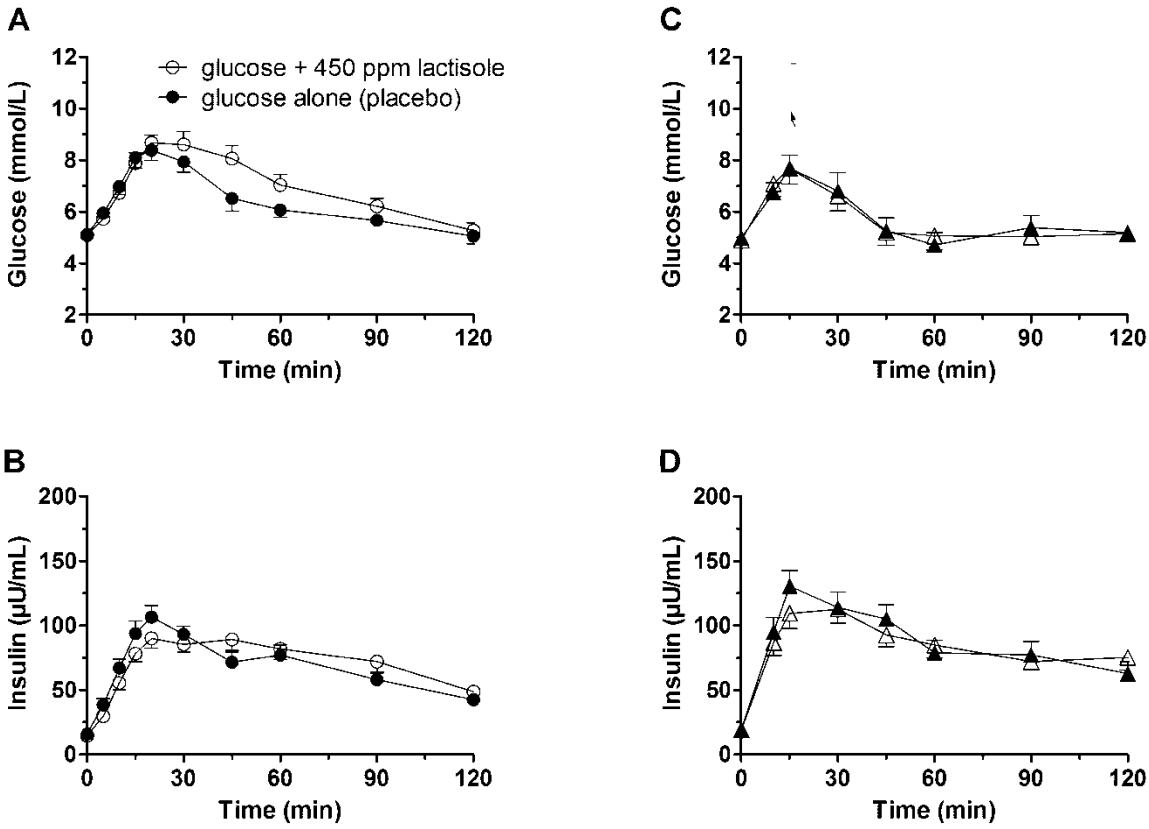

Figure 14 Plasma concentrations of glucose and insulin after an IG load of 75 g glucose (A and B) or an IG infusion of a mixed liquid meal (500 mL) (C and D), both with or without 450 ppm lactisole. The IG infusions were administered at t = 0 min. Data are expressed as mean ± SEM.

Appetite perceptions:

We observed a reduction in fullness by lactisole when glucose was IG infused, however, the effects did not reach the level of statistical significance (Figure 15A). In contrast, no effect was observed after liquid meal administration (Figure 15B).

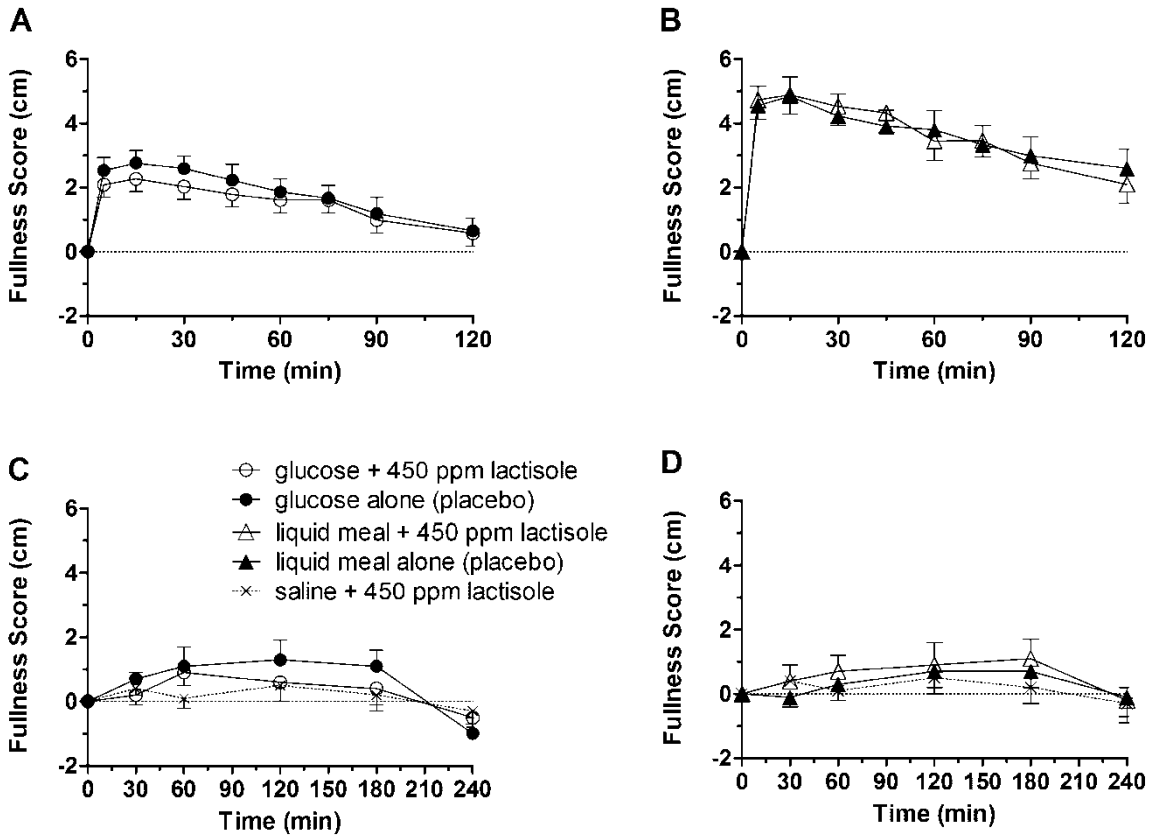

Figure 15 Subjective ratings of fullness after administration of an IG load of 75 g glucose (A), an IG load of 500 mL mixed liquid meal (B), an ID perfusion of glucose (3 kcal/min) (C), and an ID perfusion of mixed liquid meal (3 kcal/min) (D) with or without 450 ppm lactisole. The IG load was administered at t = 0 min, respectively. The ID perfusion was administered at t = 0-180 min, respectively. Data are expressed as mean ± SEM.

Gastric emptying:

The rate of gastric emptying was not affected by lactisole, neither after glucose nor after mixed liquid meal administration (Figure 16).

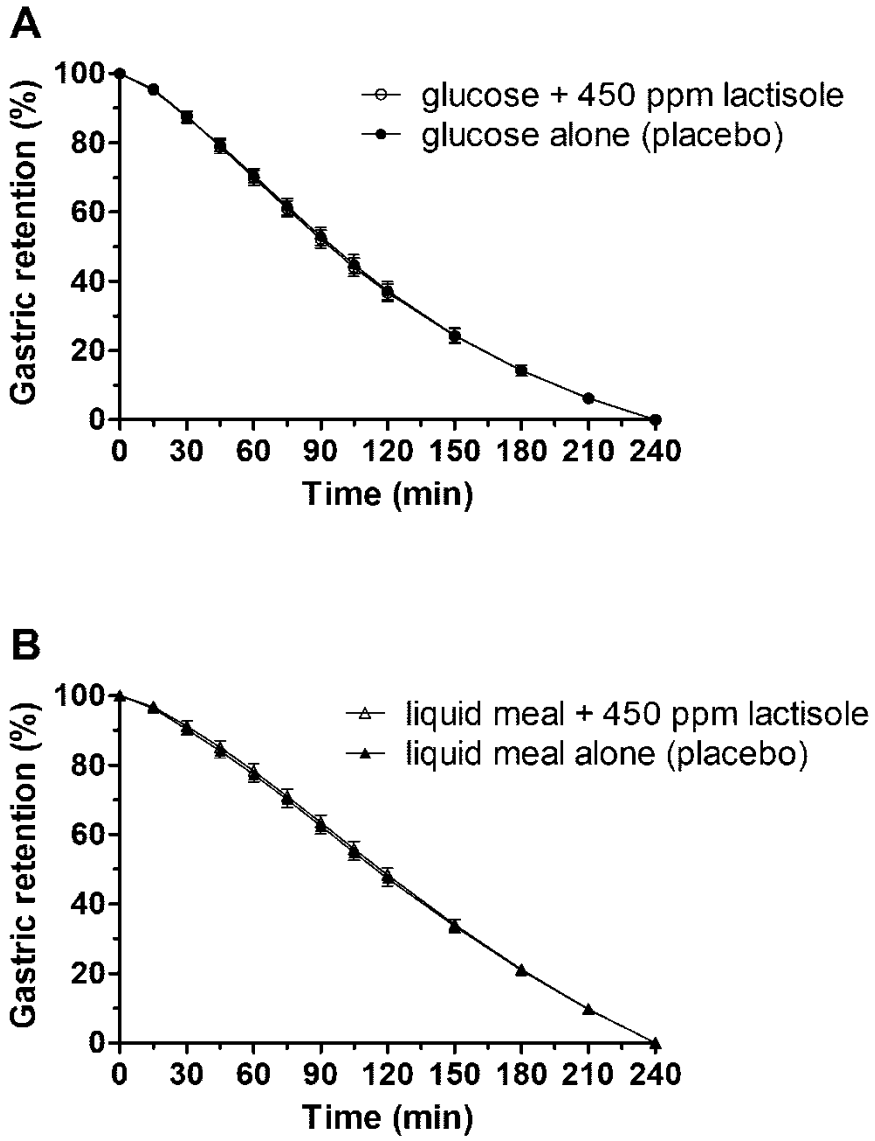

Figure 16 Gastric emptying in response to glucose-stimulation (75 g) (A) and mixed liquid meal-stimulation (500 mL) (B), both with and without 450 ppm lactisole. The IG load was administered at t = 0 min. Data are expressed as mean ± SEM.

Effect of sweet taste receptor blockade in response to ID nutrients.

GLP-1, PYY and CCK:

During ID infusions of glucose, lactisole induced a reduction (11.9 ± 4.2 %) in GLP-1 secretion: AUC (0-120 min) for GLP-1 was significantly decreased by lactisole in comparison to the administration of glucose alone (P = 0.031; Figure 17A). In contrast, PYY and CCK secretions were not significantly reduced (Figure 17B; for CCK: data not shown). In parallel to the IG part, lactisole showed no significant effect on ID liquid meal-stimulated secretions of GLP-1 and PYY (Figure 17C and D).

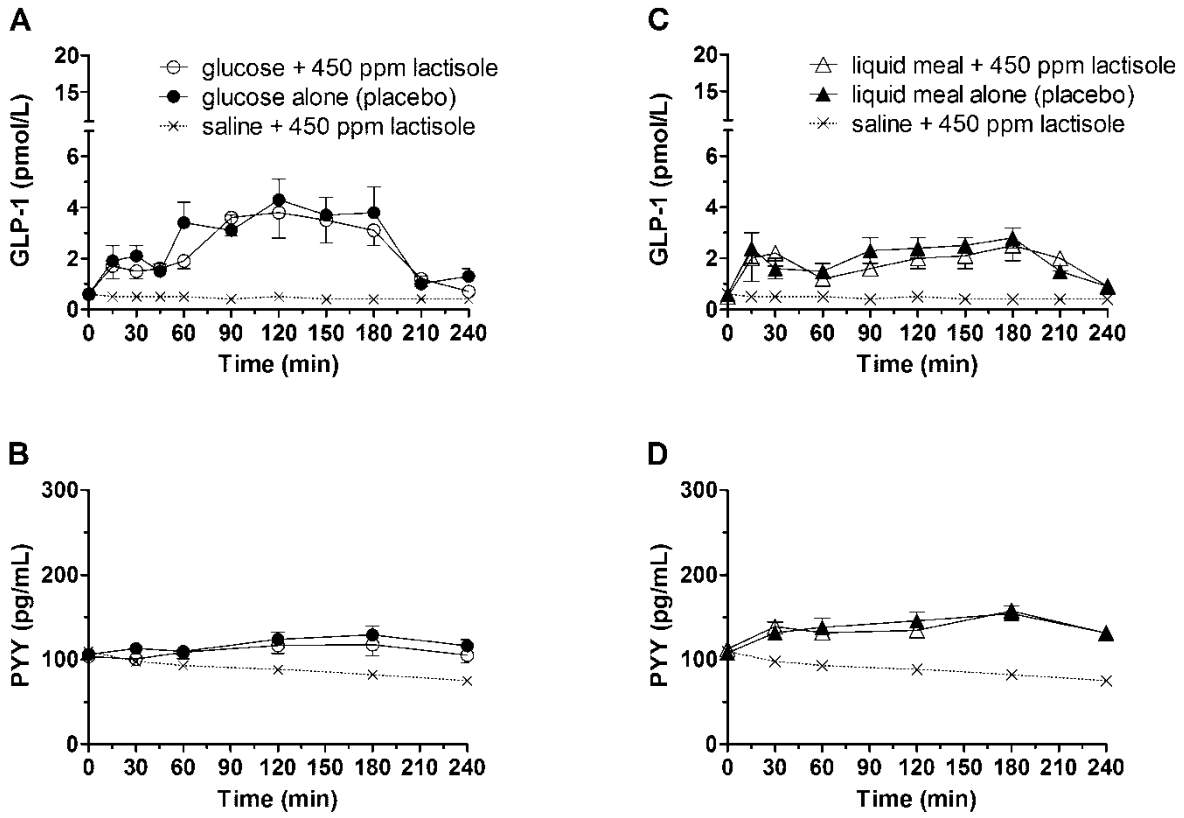

Figure 17 Plasma concentrations of GLP-1 and PYY after an ID perfusion of glucose (3 kcal/min) (A and B) or an ID perfusion of a mixed liquid meal (3kcal/min) (C and D), both with or without 450 ppm lactisole. The ID perfusion was administered from t = 0 to 180 min. Data are expressed as mean ± SEM

Glucose and Insulin:

Plasma glucose and insulin concentrations were not affected by lactisole when glucose or a liquid meal were ID infused (Figure 18). However, glucose and insulin levels in the glucose-stimulated part trended towards a glucose/insulin profile, which was already observed after administration of glucose (Figure 14A and B).

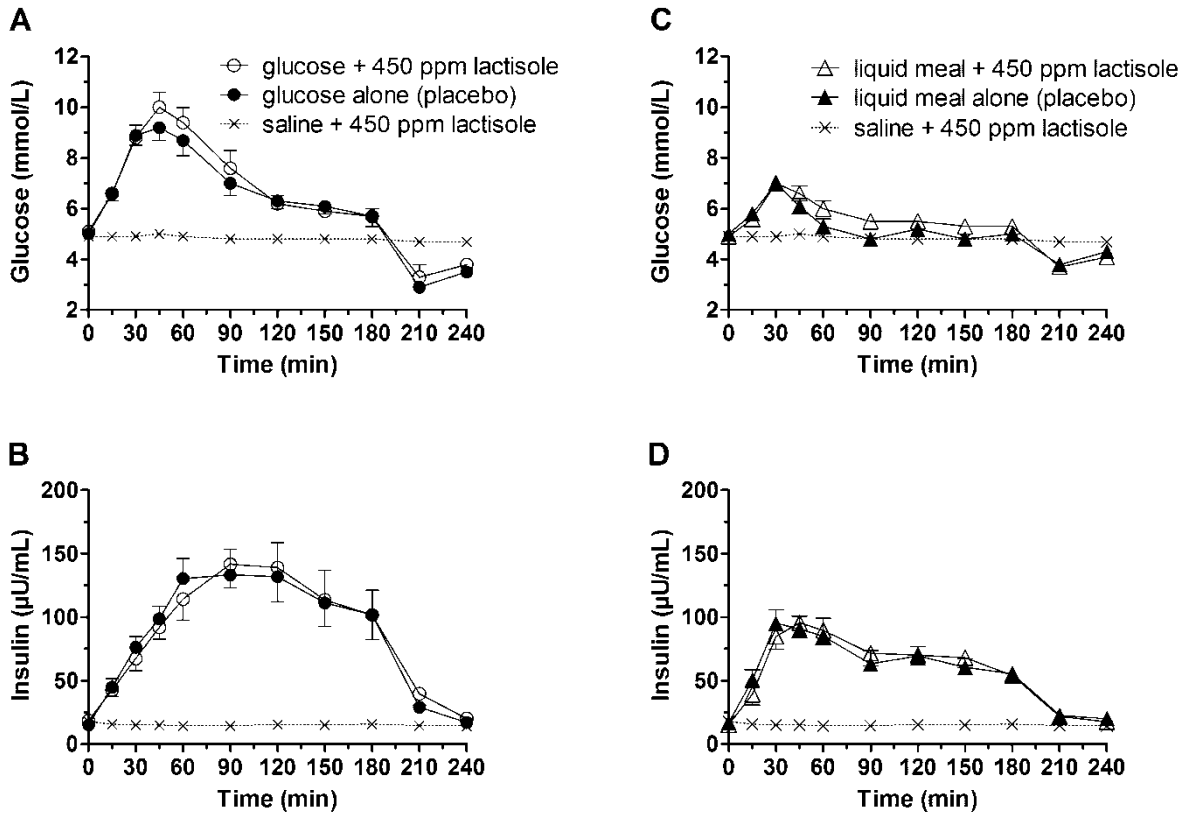

Figure 18 Plasma concentrations of glucose and insulin after an ID perfusion of glucose (3 kcal/min) (A and B) or an ID perfusion of a mixed liquid meal (3kcal/min) (C and D), both with or without 450 ppm lactisole. The ID perfusion was administered from t = 0 to 180 min. Data are expressed as mean ± SEM

Appetite perceptions:

A slight reduction in feelings of fullness was observed when glucose was ID infused; however, these effects did not reach the level of statistical significance (Figure 15C). In parallel to the IG part, no effect in fullness was seen during the liquid meal infusion (Figure 15D).

Comparison of the IG and ID inhibitory effect of lactisole on peptide secretion.

The inhibitory effect of lactisole on GLP-1 secretion was significantly greater after IG administration of glucose than after ID perfusion of glucose (*P* = 0.023) (Figure 19).

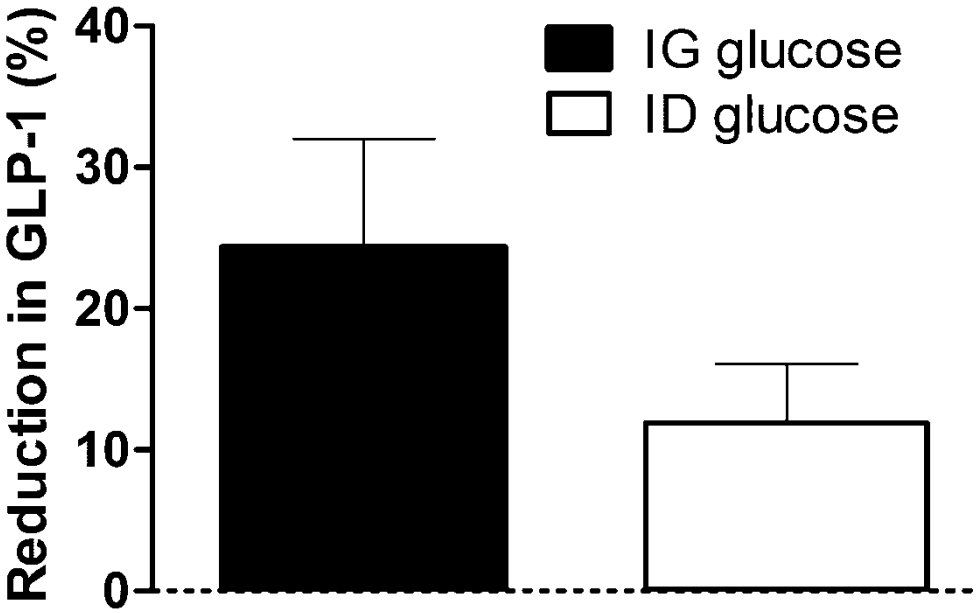

Figure 19 Comparison of the inhibitory effect of lactisole on the secretion of GLP-1 in response to IG vs. ID administration of glucose. Data are expressed as mean ± SEM.

No differences in the inhibitory effect of lactisole were seen between the IG versus ID administration of the mixed liquid meal.

5.2.5 Discussion

The recent identification of the sweet taste receptor T1R2/T1R3 and the detection of α-gustducin, PLC β2 and TRPM5 in EECs of the human gut let one anticipate a taste system in the GI tract comparable to that in the mouth [233, 250, 251]. The discovery of such a “new” receptor system raises the question whether and how this system contributes to glucose sensing and metabolism. By defining activators and inhibitors of this system, we sought to define the importance of sweet taste receptors in the secretion of satiation peptides and appetite control.

In previous studies in humans it was shown that glucose act as an activator of this sweet taste receptor system with regard to peptide secretion, whereas artificial sweeteners showed no such effect.[146, 258, 259] Lactisole, the sodium salt of 2-(4-methoxyphenoxy)-propionic acid, is a potential inhibitor of this receptor system. *In vitro*, lactisole has been shown to inhibit the effects of sucralose-stimulated GLP-1 release from human EECs (NCI-H16) [146]. *In vivo*, it suppresses the sweet taste on the tongue; the inhibition is only observed when sweeteners and lactisole are mixed prior to tasting, and appears to be a competitive inhibition [340]. Lactisole inhibits the T1R2/T1R3 human sweet taste receptor by binding to the transmembrane domain of T1R3 [338]. In a pilot study, we were able to show that the secretion of GLP-1 and PYY in response to glucose was reduced by lactisole in healthy human subject. These data suggest that lactisole could be an excellent tool to investigate the regulatory role of the gut-expressed sweet receptor’s dependent physiological functions in humans.

Two different approaches were used to establish the role of sweet taste receptor blockade on the secretion of GI satiation peptides, including GLP-1, PYY and CCK, and the influence on glucose-homeostasis. First, an IG administration of two liquid meals (glucose and a mixed meal) with and without lactisole and second, ID perfusion of the same meals, in which for both, the effect of lactisole on glucose- and liquid meal-stimulated secretion of satiation peptides was examined.

The results can be summarized as follows: 1) Lactisole induced a significant reduction in GLP-1 and PYY, but not CCK secretion in the IG glucose-stimulated part ($P = 0.007$ and 0.012); as consequence plasma insulin levels were significantly decreased in the early postprandial state (C_{max}; $P \leq 0.05$), and subsequently glucose levels significantly increased ($P = 0.029$). Appetite perceptions showed a trend for reduced feelings of fullness. 2) Lactisole induced a smaller, albeit still significant ($P = 0.031$) reduction in GLP-1 secretion in the ID glucose-stimulated part; PYY and CCK secretions were, however, not significantly reduced. 3) Comparison of the

inhibitory effect of lactisole on the secretion of GLP-1 in response to IG versus ID administration of glucose showed a significantly greater suppression of the hormone response in the IG part (P = 0.023). 4) Lactisole had no effect on mixed liquid meal stimulated parameters, neither in the IG nor in the ID part. 5) The gastric emptying rates were not affected by lactisole, neither with glucose nor with mixed liquid meal stimulation.

The results are in line with previous *in vitro* and *in vivo* animal studies [146, 338] showing that lactisole is an effective antagonist of the sweet taste receptor system to glucose stimulation. In a previous study we have established an inhibition of lactisole on glucose-stimulated GLP-1 release in healthy male subjects [253]. The current results confirm these results and show again, that blockade of the sweet taste receptor in the human gut not only significantly reduces the secretion of GLP-1, but also reduces PYY release. Previous *in vitro* or animal studies have not suggested such an effect for PYY. Therefore, in addition to GLP-1, PYY secretion must be taken into account whenever the sweet taste receptor system in the human gut is evaluated. Our results confirm the findings that the release of CCK is, beside fats and proteins, also stimulated by the presence of glucose in the proximal small intestine [115, 344]. The lack of effect of lactisole on CCK release let us assume that glucose-induced CCK secretion is not mediated by the sweet taste receptor T1R2/T1R3 and that other glucose-sensing receptors must be involved.

The current results were to a certain extent surprising, as we did not anticipate a smaller effect of sweet taste receptor blockade in the ID perfusion studies. Interaction of nutrients with the small intestine plays an important role in the regulation of glucose homeostasis. Furthermore, the presence of glucose in the small intestine is a well-established stimulus for GLP-1 secretion, leading (together with GIP) to glucose-dependent insulin secretion from the β-cells and a feedback that regulates gastric emptying [95]. Direct exposure of carbohydrate to the mucosa of the small intestine appears to be an essential requirement for GLP-1 secretion [345], and the magnitude is dependent on the rate of duodenal glucose entry [99, 101]. Although the sweet receptor subunit T1R3 has been shown to be present in both the human stomach and the small intestine, it is predominantly expressed in duodenum and jejunum, and further, the expression of the sweet receptor subunit T1R2 was not found in the stomach [233, 252, 346]. Based on these findings, we would infer a greater effect of sweet receptor blockade after duodenal meal perfusion. As this was not the case, an interaction of the stomach with signals from the small intestine is a more likely interpretation.

Furthermore, the observed smaller effect of lactisole on the release of satiation peptides in the ID part suggests a relevant contribution of the stomach in the regulation of these hormones. One assumption is that the sweet receptor subunit T1R3 in the stomach plays a role in detection of nutrients, which in turn initiates a hormonal or neural cascade pathway crucial in the secretion of satiation peptides [346, 347]. Another potential mechanism could be the influence of gastric distension. In the IG study, glucose was given as bolus within two minutes, whereas in the ID study, glucose was continuously perfused over three hours. Therefore, in the IG study, gastric distension was increased, which may have an influence on secretion of satiation peptides and subsequently on the inhibitory effect of lactisole. However, previous studies showed that satiation peptides, like CCK and PYY, were not altered by mechanical gastric distension [71]. A further explanation could be that the gastric emptying rates had an influence on the effectiveness of lactisole. Although several investigators have suggested that a key mechanism of GLP-1 with respect to glucose control is based on its effect on gastric emptying [348], this hypothesis is based on the results of studies using exogenous administration of GLP - 1. In the present study, we document an identical pattern of postprandial gastric emptying with and without lactisole suggesting that endogenous GLP-1 has no detectable effect on the rate of passage of glucose from the stomach to the duodenum. The lack of effect of lactisole to alter $^{13}CO_2$ appearance in the breath is similar to what has been reported previously in studies with GLP-1 receptor blockade in healthy human subjects, in patients with type II diabetes mellitus, and in nonhuman primates given liquid glucose solutions [349-351]. In the present study, we show that blocking the sweet receptor, which induced an attenuated GLP-1 response, has no effect on gastric emptying rates; our data stand against an important physiologic role for GLP - 1 in the regulation of prandial gastric emptying in humans. Not all data are consistent with this conclusion: Deane *et al.* have recently published evidence that endogenous GLP-1 delays gastric emptying in healthy subjects after a solid carbohydrate meal [352]. These findings could, however, not be confirmed by Nicolaus *et al.*, who reported that blockade of the GLP-1 receptor by the specific receptor antagonist exendin 9-39 had no effect on gastric emptying of a mixed semisolid oral meal [353]. Both investigators used scintigraphy, the gold standard for measuring gastric emptying. The reasons for these discrepancies remain unclear, but the available evidence suggests that changes of gastric emptying mediated by GLP-1 probably do not play a major role in the regulation of glucose homeostasis in humans.

In contrast to our expectations, lactisole had no effect on parameters stimulated by a mixed liquid meal, neither in the IG nor in the ID part. The liquid meal consists beside glucose also of proteins, fats and other complex carbohydrates. The lack of effect of lactisole suggests that these nutrients induce the release of satiation peptides via other mechanisms; more important

these mechanisms seem to outweigh the effect of sweet receptor blockade. This raises the question on the physiological importance of the sweet taste receptor system in regulating GLP-1 release and associated functions. Long-chain fatty acids, apart from glucose, are also potent luminal secretagogues for GLP-1 release [144]. Thus, the fast response to glucose is based on tasting and activation of the sweet receptor system, whereas the effects of a mixed meal are also mediated by lipids and, perhaps proteins. A potential mechanism by which lipids stimulate the secretion of GI satiation peptides is through activation of GPR120, a specific receptor for medium and long chain free fatty acids [266, 269]. In addition, several receptors exist, which are possible to act as amino acid sensors, like T1R1/T1R3, the extracellular Ca^{2+}-sensing receptor (CaR) or the Na^{+}-coupled neutral amino acid transporter 2 (SNAT2) [234, 354-356]. Finally, additional sensing mechanisms for carbohydrates have been proposed, which are different from the sweet taste receptor T1R2/T1R3 [357]. The multiplicity of different receptors therefore suggests that the sweet taste receptor T1R2/T1R3 is not alone responsible in the regulation of GLP-1 release and associated functions.

We have not measured whether the presence of lactisole in the duodenum affects the expression of T1R3, T1R2 or α-gustducin. Young *et al.* previously showed that the expression of T1R2 was decreased by jejunal glucose perfusion in mice [252]. These results suggest the existence of a mechanism, which can down regulate the sweet taste receptor in the presence of agonists. Whether an antagonist, like lactisole, has comparable effects on the regulation of receptor expression is not known. If so, the preloading of lactisole in our second study part could have resulted in a reduced availability of the receptor to ID stimuli, which would have affected the secretion of GLP-1 and PYY.

In conclusion, the present results show that the sweet taste receptor system is involved in the secretion of GI satiation peptides with potential effects on glucose homeostasis. The study raises questions with respect to the functional involvement of sweet taste receptors expressed in the stomach and the role of the stomach in the release of satiation peptides. The physiological conclusions of these findings seem to indicate that the sweet taste receptor in the gut is of limited importance and not alone responsible for peptide release; it is rather a complex interaction between different receptor mechanisms.

5.3 The role of the stomach in the control of appetite and the secretion of satiation peptides

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5.3.1 Abstract

It is widely accepted that gastric parameters such as gastric distention provide a direct negative feedback signal to inhibit eating; moreover, gastric and intestinal signals have been reported to synergize to promote satiation. There are, however, only few human data exploring the potential interaction effects of gastric and intestinal signals in the short-term control of appetite and the secretion of satiation peptides. We performed experiments in healthy subjects receiving either a rapid intragastric (IG) load or a continuous intraduodenal (ID) infusion of glucose or a mixed liquid meal. ID infusions (3 kcal/min) were at rates comparable to the duodenal delivery of these nutrients under physiological conditions. ID infusions of glucose elicited only weak effects on appetite and the secretion of GLP-1 and PYY. In contrast, identical amounts of glucose delivered IG markedly suppressed appetite ($P < 0.05$) paralleled by greatly increased plasma levels of GLP-1 and PYY (up to 3-fold, $P < 0.05$). Administration of the mixed liquid meal showed a comparable phenomenon. In contrast to GLP-1 and PYY, plasma ghrelin was suppressed to a similar degree both with IG and ID nutrients. Our data confirm that the stomach is an important element in the short-term control of appetite and suggest that gastric and intestinal signals interact to mediate early fullness and satiation, potentially by increased GLP-1 and PYY secretions.

Key words: satiation, stomach, small intestine, humans, satiation peptides

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Conflict of interest: All authors disclose that they do not have any financial and personal relationships with other people, or organizations, that could inappropriately influence (bias) their work.

5.3.2 Introduction

Eating is organized into discrete meals and determined by meal size and meal frequency. Multiple regulatory pathways seem to promote or inhibit eating and thus regulate energy balance. Gastrointestinal (GI) satiation signals such as CCK, GLP-1 or PYY as examples are secreted from the small and large intestine in response to food ingestion and signal to the brain via neural or endocrine pathways to inhibit eating [358]. Their plasma levels are low in the fasting state and rise during a meal, which is consistent with the satiating effect observed when peripherally infused in rats and humans [359-361]. In contrast, the peptide ghrelin is secreted from the stomach during fasting and plasma level fall shortly after meals consistent with a hunger-inducing action observed in rats and humans [362, 363].

Gastric parameters such as stomach distension are another important source of negative feedback [364]. Feelings of hunger and satiety have long been associated with gastric motor and sensory functions, and more importantly, gastric and intestinal signals have been reported to interact [67, 365, 366]. We and other groups could show that in humans oral preloads combined with (exogenously administered or endogenously stimulated) CCK or GLP-1 synergistically increase satiation and reduce food intake [53, 367, 368]. Furthermore, Feinle *et al.* [190] demonstrated that sensory responses to gastric distension are altered by duodenal infusions of nutrients, with more ‘meal like’ sensations of fullness after gastric distension plus ID nutrients. Animal studies support these concepts [185, 186, 369, 370] and suggest that a combination of gastric signals and intestinal nutrient stimulation is necessary to elicit optimal satiation and adequate control of eating. In humans, however, little information is available on the mechanisms of interaction between gastric and intestinal signals to modulate appetite and the secretion of satiation peptides. We therefore sought to investigate potential interaction effects by using a paradigm in which subjects received either an IG load or an ID infusion of glucose or a mixed liquid meal. Direct nutrient infusions into different areas of the GI tract bypasses cognitive cues, and thus provide information on the isolated properties of nutrients and the relative role of a specific GI segment in the secretion of satiation peptides and the short-term control of appetite.

5.3.3 Methods

Overall study design

The study was conducted as a randomized, double-blind, placebo-controlled, parallel-designed trial. The protocol was submitted and approved by the State Ethical Committee of Basel and the study was carried out in accordance with the principles of the Declaration of Helsinki. Each subject gave written informed consent for the study. The criteria for exclusion were smoking, substance abuse, regular intake of medications (except for oral contraceptives), medical or psychiatric illness and any abnormalities detected on physical examination or laboratory screening. None of the subjects had a history of GI disorders, food allergies or dietary restrictions. Subjects were instructed to abstain from alcohol, caffeine and strenuous exercise for 24 hours before each treatment. The day before each study day, subjects consumed a restricted simple-carbohydrate standard dinner before 8 PM and fasted from 10 PM. On each study day, subjects were admitted to the Phase 1 Research Unit of the University Hospital of Basel at ~ 7:00 AM. Subjects swallowed a radiopaque polyvinyl feeding tube (external diameter 8 french), which was either positioned in the stomach or duodenum. The IG position was confirmed by rapid injection of 10 mL air and auscultation of the upper abdomen. The ID position was verified by fluoroscopy. Due to radiation exposure during fluoroscopy, female subjects were excluded from ID experiments. The feeding tubes were firmly attached behind the ear to prevent further progression during the experiments. An antecubital vein catheter was inserted into a forearm vein for blood collection.

Experimental procedure

Part A: IG vs. ID administration of glucose.

Part A included 34 healthy, normal weight, male and female volunteers (mean age: 23.4 ± 0.4 years, range 19-30 years; mean BMI: 22.3 ± 0.3 kg/m^2, range 19.0-25.0 kg/m^2). Twenty-four subjects (13 female, 11 male) received an IG load of 75 g glucose (Hänseler AG, Herisau, Switzerland) dissolved in 300 mL tap water (total caloric load 300 kcal) within two minutes (t = 0-2 min). Ten male subjects received an ID infusion of glucose at a rate of 3.0 kcal/min and 2.5 mL/min for 120 min. With this infusion rate the total caloric load at 100 min was equal to the IG glucose load (300 kcal). The test solutions were freshly prepared each morning of the study and were at room temperature when administered.

Part B: IG vs. ID administration of a mixed liquid meal.

Part B included 26 healthy, normal weight, male and female volunteers (mean age: 24.0 ± 0.4 years, range 20-28 years; mean BMI: 22.0 ± 0.3 kg/m^2, range 19.1-24.9 kg/m^2). Sixteen subjects (8 female, 8 male) received an IG load of 500 mL of a mixed liquid meal (Ensure

Plus®, 17% protein, 30% fat and 53% carbohydrates, total caloric load 600 kcal, Abbott AG, Baar, Switzerland) and 10 male subjects an ID infusion of the mixed liquid meal at a rate of 3.0 kcal/min and 2.5 mL/min for 120 min. With this infusion rate the total caloric load at 100 min was the half of the IG load (300 kcal vs. 600 kcal). The test solutions were freshly prepared each morning of the study and were at room temperature when administered.

Blood sample collection

At regular time intervals blood samples were collected on ice into tubes containing EDTA (6 µmol/L), aprotinin (500 kIU/mL) and a DPP-IV inhibitor (50 µmol/L). The tubes were centrifuged at 4℃ at 3000 rpm for 10 min. After ce ntrifugation, the plasma samples were processed into different aliquots and stored at -70 ℃ until analysis.

Appetite measurements

Appetite perceptions (feelings of hunger, fullness and satiety) were assessed immediately after each blood collection using VAS. VAS consisted of a horizontal, unstructured, 100 mm line with words anchored at each end describing the extremes of a unipolar question (most positive and most negative rating). Subjects assign a vertical mark across the line to index the magnitude of their subjective sensation. To ensure reliable and valid results, subjects could not refer to their previous ratings. In addition, they were instructed to take enough time to rate their appetite sensation as precisely as possible. Quantifications of the measurements were made by measuring the distance from the left end of the line to the mark. The scales and scores have previously been designed and described in more detail [308].

Laboratory analyses

Active GLP-1 was measured with a commercially available ELISA kit (Millipore Corporation, Billerica, MA, USA). The intra- and interassay coefficients of variation were <9.0% and <13.0%, respectively. *Active PYY, total ghrelin* and *insulin* were measured with commercially available RIA kits (Millipore Corporation, Billerica, MA, USA; LINCO Research, St. Charles, MO, USA; Cisbio International, Bagnols, France). The intraassay coefficients of variation were <15% for active PYY, <10.0% for total ghrelin and <12.2% for insulin. The interassay coefficients of variation were <11% for active PYY, <14.7% for total ghrelin and <9.0% for insulin. The methods have been described recently in more detail [371-373]. Plasma glucose concentration was measured using the glucose oxidase method (Bayer Consumer Care AG, Basel, Switzerland).

Assessment of gastric emptying

In a subset of 16 subjects (part A: 4 female/4 male, mean BMI: 21.7 ± 0.6 kg/m^2; mean age: 22.5 ± 0.7 years; part B: 5 female/3 male, mean BMI: 21.7 ± 0.4 kg/m^2, mean age: 24.3 ± 0.8

years) gastric emptying rates were assessed using the ^{13}C-sodium acetate breath test. This test is an accurate, non-invasive, simple method without radiation exposure, and represents a reliable alternative to scintigraphy, the gold standard for measuring gastric emptying [342, 374]. The test solutions were labeled with 50 mg ^{13}C-sodium acetate; the substrate is rapidly absorbed in the proximal small intestine, metabolized in the liver with the production of $^{13}CO_2$, which is exhaled rapidly, thus, reflecting gastric emptying of nutrients [342, 374]. Subjects were asked to exhale through a mouth-piece to collect an end-expiratory breath sample into a 100 mL foil bag at certain time intervals. The $^{13}CO_2$ breath content was then determined by non-dispersive infrared spectroscopy using an isotope ratio mass spectrophotometer (IRIS; Wagner Analysen Technik, Bremen, Germany). ^{13}C-abundance in breath was expressed as relative difference (δ ‰) from the universal reference standard (carbon from Pee Dee Belemnite limestone). ^{13}C-enrichment was defined as the difference between preprandial ^{13}C-abundance in breath and ^{13}C-abundance at the defined time points postprandially and was given in δ over basal (DOB, ‰). Based on these values, time to reach maximal emptying speed and AUC of the responses were calculated.

Statistical analysis

Descriptive statistics were used for demographic variables such as age, weight, height, and BMI. Hormone and glucose profiles were analyzed by calculating areas under the curve (AUC). To test for significant differences between the treatments, AUCs were compared using student's unpaired t-test. VAS ratings were analyzed by calculating AUCs from baseline. Student's unpaired t-tests were used to test for differences between treatments. All parameters were tested for normality using the Shapiro-Wilk and Kolmogorov-Smirnov test methods. All statistical analysis was done using the statistical software package SPSS for Windows Version 14.0 (SPSS Inc., Chicago, USA). Values were reported as mean ± SEM. Differences were considered statistically significant with $P < 0.05$.

5.3.4 Results

Part A: IG vs. ID administration of glucose

Plasma glucose and insulin:

Blood glucose level increased more rapidly in response to IG administered glucose in the early postprandial phase. In the later postprandial phase, blood glucose levels were clearly higher with ID perfused glucose ($P < 0.05$, Figure 20A), which also resulted in significantly greater insulin secretions. ($P < 0.05$, Figure 20B). Total blood glucose and insulin excursion over 100 min were significantly higher with ID perfused glucose ($P < 0.05$, Figure 20A and B).

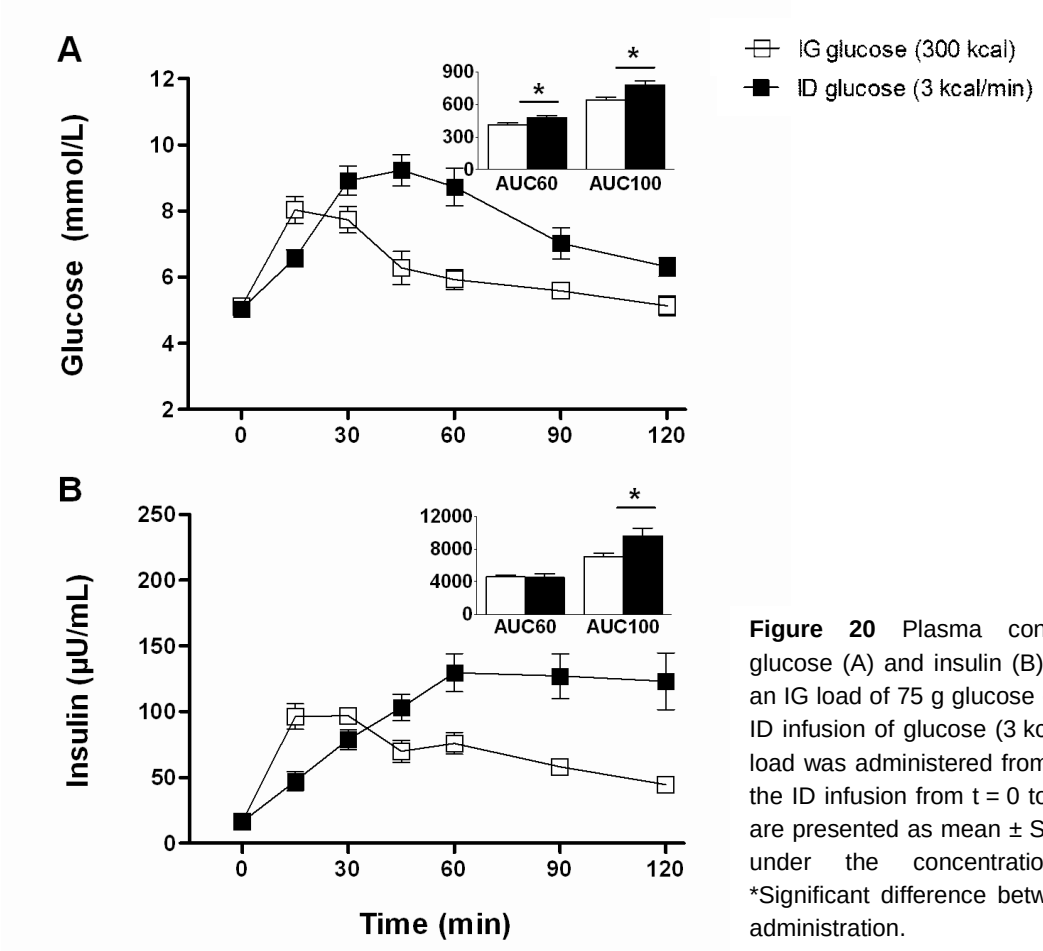

Figure 20 Plasma concentrations of glucose (A) and insulin (B) in response to an IG load of 75 g glucose (300 kcal) or an ID infusion of glucose (3 kcal/min). The IG load was administered from t = 0 to 2 min; the ID infusion from t = 0 to 120 min. Data are presented as mean ± SEM. AUC, area under the concentration-time curve. *Significant difference between IG and ID administration.

Plasma GLP-1, PYY and ghrelin:

IG administration of glucose resulted in markedly higher active GLP-1 and active PYY releases than equicaloric ID glucose infusions. AUCs were significantly greater in particular in the early postprandial phase (0 to 60 min) for both hormones after IG administered glucose ($P < 0.05$, Figure 21A and B). In contrast, both suppressed comparable amounts of plasma ghrelin, IG and ID administered glucose (Figure 21C).

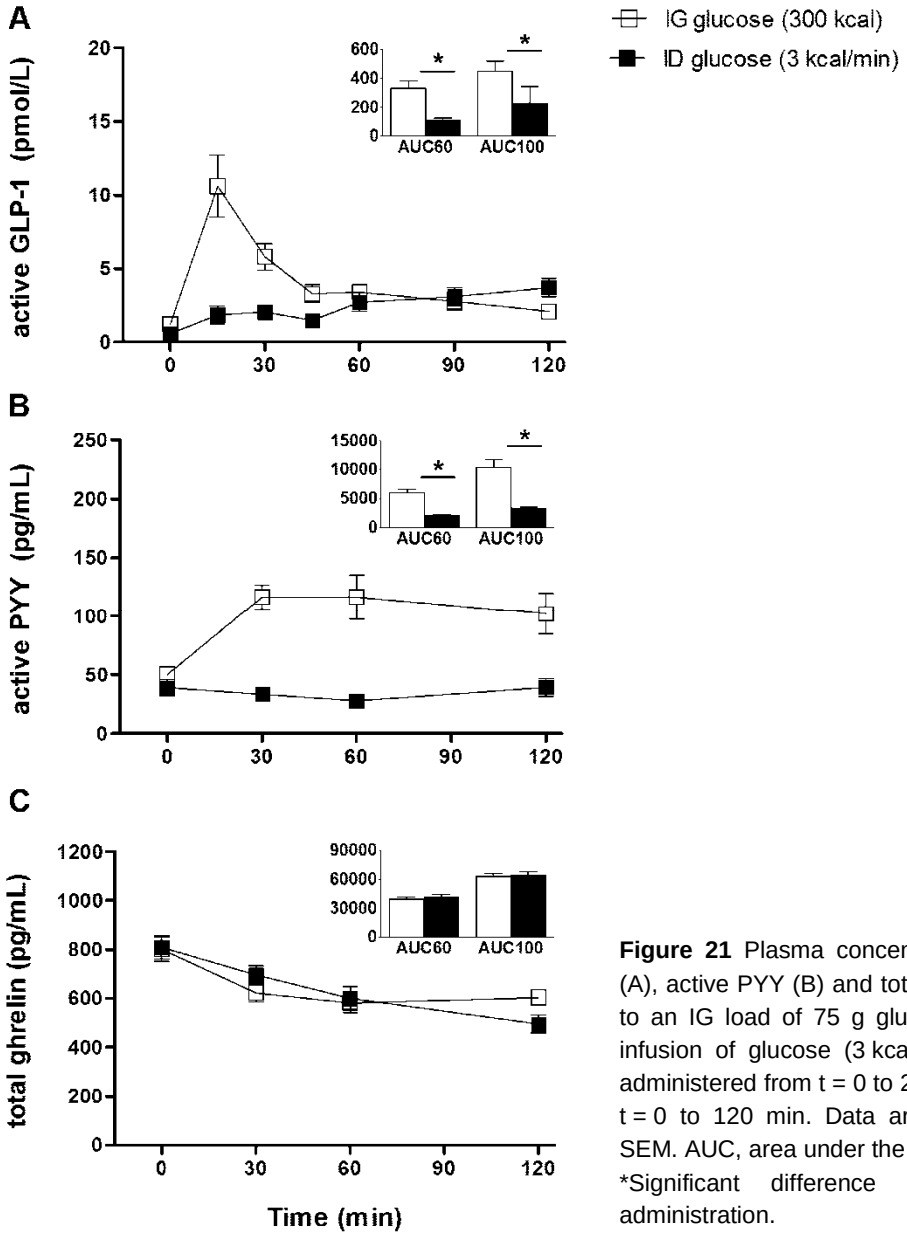

Figure 21 Plasma concentrations of active GLP-1 (A), active PYY (B) and total ghrelin (C) in response to an IG load of 75 g glucose (300 kcal) or an ID infusion of glucose (3 kcal/min). The IG load was administered from t = 0 to 2 min; the ID infusion from t = 0 to 120 min. Data are presented as mean ± SEM. AUC, area under the concentration-time curve. *Significant difference between IG and ID administration.

Appetite measurements:

IG administration of glucose clearly increased fullness and satiety feelings and reduced hunger; in contrast, equicaloric ID glucose infusions over 100 min resulted in only small effects on appetite. AUCs were significantly higher particular in the early postprandial phase (0 to 60 min) for fullness and satiety and significantly lower for hunger after IG administered glucose ($P < 0.05$, Figure 22A, B and C).

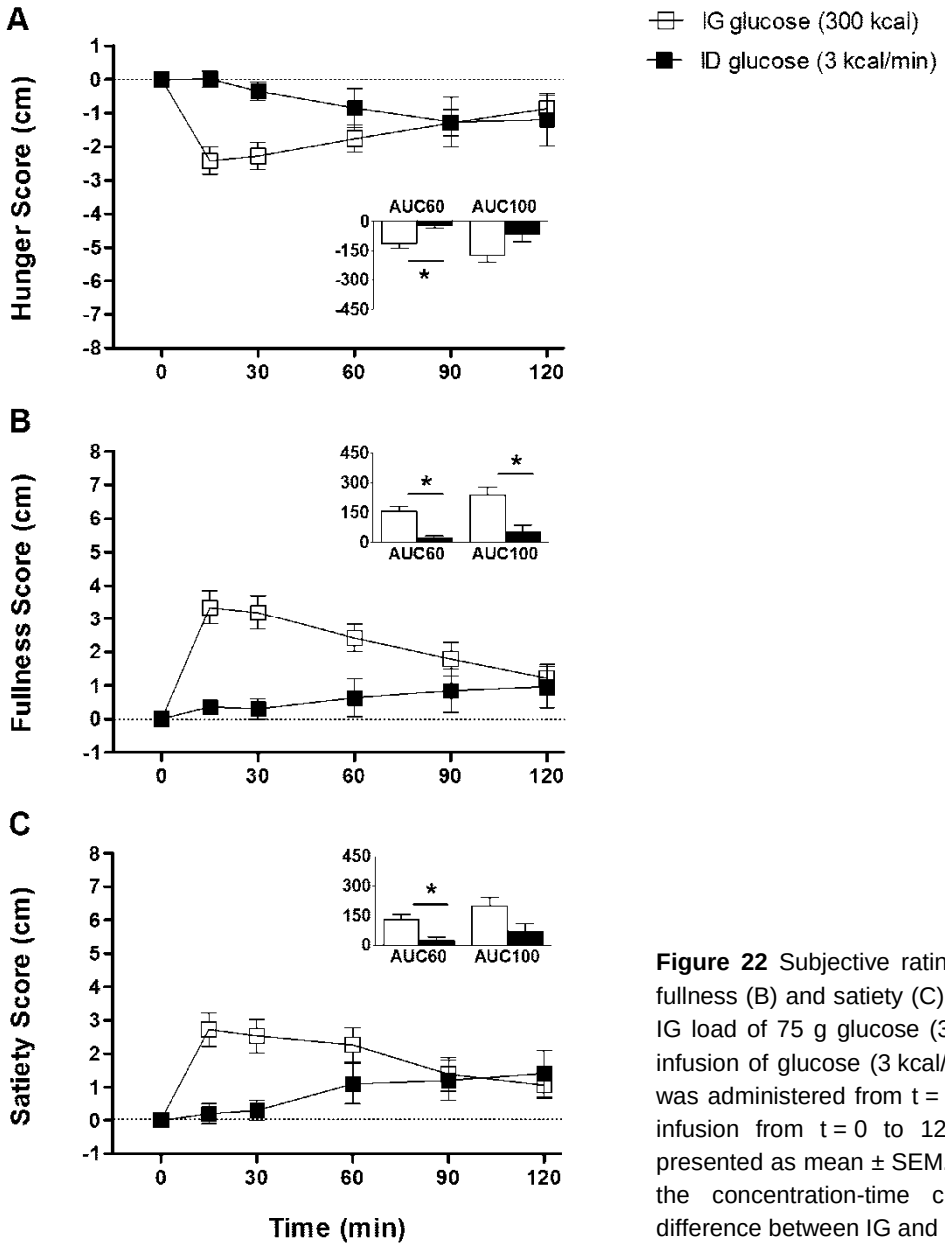

Figure 22 Subjective ratings of hunger (A), fullness (B) and satiety (C) in response to an IG load of 75 g glucose (300 kcal) or an ID infusion of glucose (3 kcal/min). The IG load was administered from t = 0 to 2 min; the ID infusion from t = 0 to 120 min. Data are presented as mean ± SEM. AUC, area under the concentration-time curve. *Significant difference between IG and ID administration.

No sex differences were observed in the IG part for all measurements.

Part B: IG vs. ID administration of the mixed liquid meal

Plasma glucose and insulin:

In analogy to part A, blood glucose levels increased more rapidly in response to the IG administered mixed liquid meal in the early postprandial phase, which also resulted in significantly greater insulin levels ($P < 0.05$, Figure 23A and B). In the later postprandial phase, blood glucose and insulin levels were not significantly different from the ID perfused mixed liquid meal (Figure 23A and B). Total blood glucose and insulin excursion over 100 min were not significantly different with the IG and ID administered mixed liquid meal (Figure 23A and B).

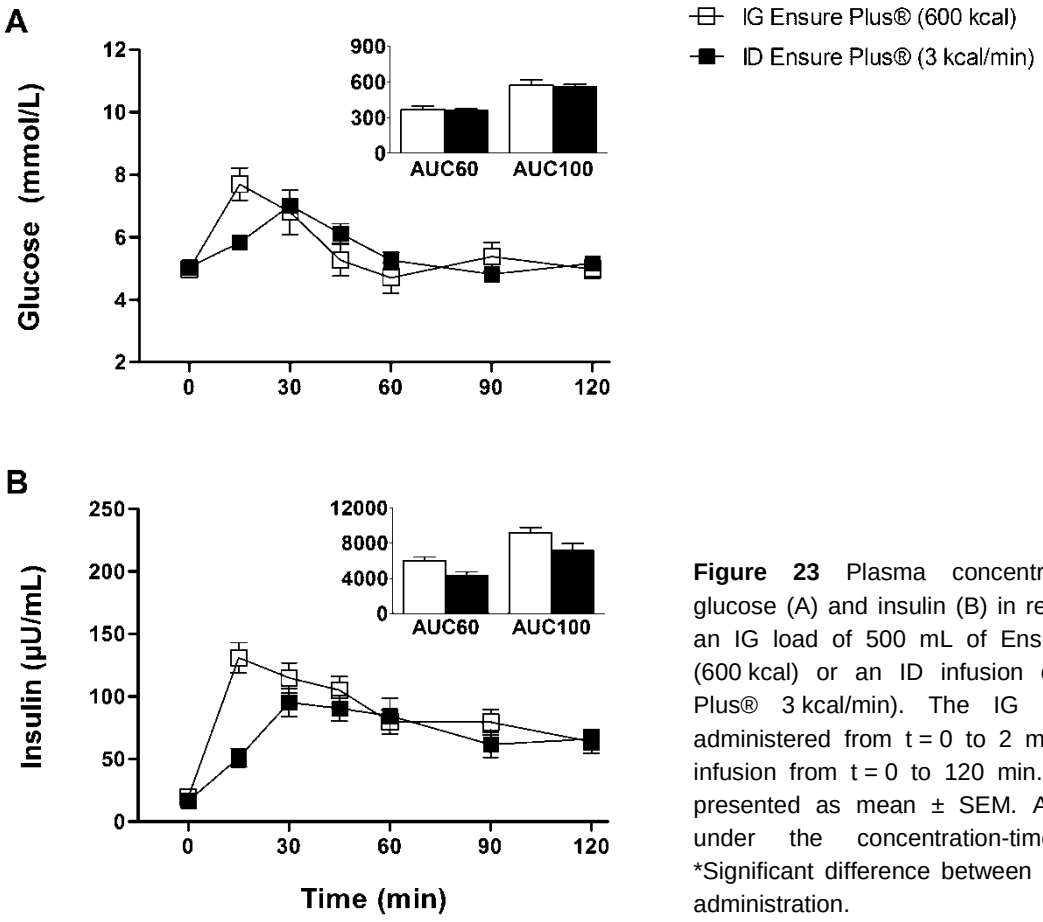

Figure 23 Plasma concentrations of glucose (A) and insulin (B) in response to an IG load of 500 mL of Ensure Plus® (600 kcal) or an ID infusion of Ensure Plus® 3 kcal/min). The IG load was administered from t = 0 to 2 min; the ID infusion from t = 0 to 120 min. Data are presented as mean ± SEM. AUC, area under the concentration-time curve. *Significant difference between IG and ID administration.

Plasma GLP-1, PYY and ghrelin:

IG administration of the mixed liquid meal resulted in a substantial increase in active GLP-1 and active PYY compared to only small responses after the ID perfused mixed liquid meal. AUCs after the IG administered mixed liquid meal were significantly higher for both GLP-1 and PYY ($P < 0.05$, Figure 24A and B). In contrast, comparable amounts of plasma ghrelin were suppressed by both routes of administration (Figure 24C).

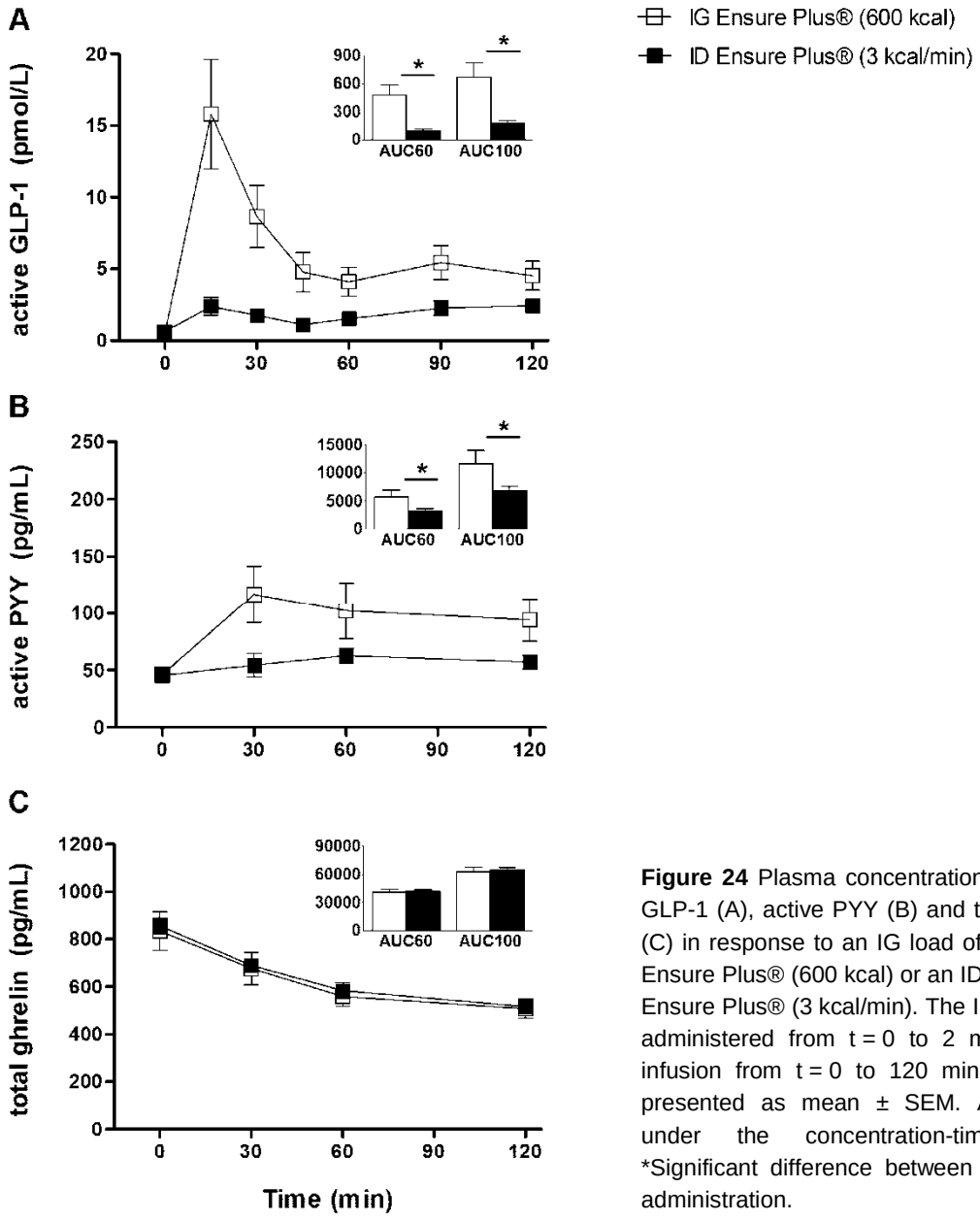

Figure 24 Plasma concentrations of active GLP-1 (A), active PYY (B) and total ghrelin (C) in response to an IG load of 500 mL of Ensure Plus® (600 kcal) or an ID infusion of Ensure Plus® (3 kcal/min). The IG load was administered from t = 0 to 2 min; the ID infusion from t = 0 to 120 min. Data are presented as mean ± SEM. AUC, area under the concentration-time curve. *Significant difference between IG and ID administration.

Appetite measurements:

IG administration of the mixed liquid meal largely increased fullness and satiety and reduced hunger, in contrast to ID infusions of the mixed liquid meal over 100 min, which resulted in only small effects on appetite. AUC were significantly greater for fullness and satiety and significantly lower for hunger after IG administered glucose ($P < 0.05$, Figure 25A, B and C).

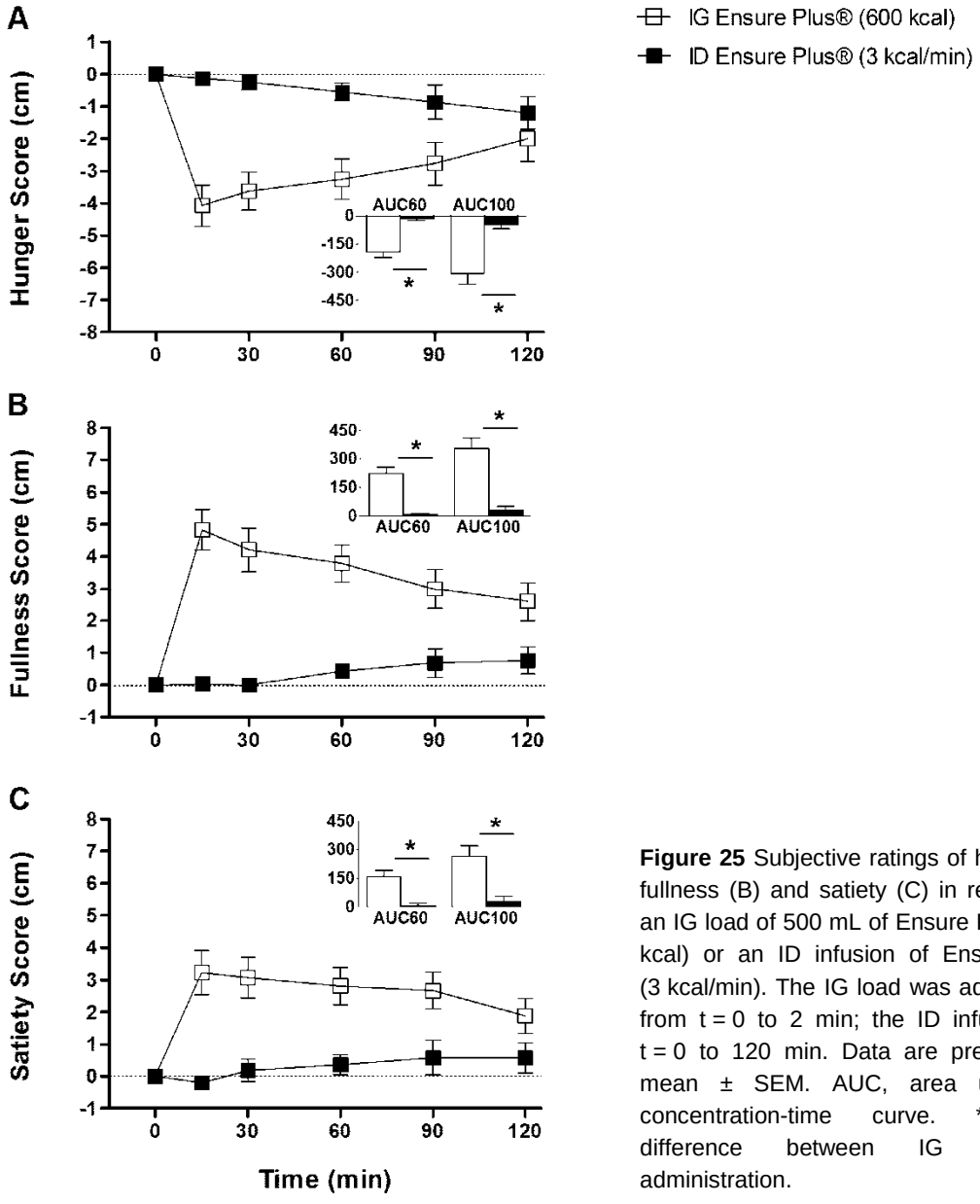

Figure 25 Subjective ratings of hunger (A), fullness (B) and satiety (C) in response to an IG load of 500 mL of Ensure Plus® (600 kcal) or an ID infusion of Ensure Plus® (3 kcal/min). The IG load was administered from t = 0 to 2 min; the ID infusion from t = 0 to 120 min. Data are presented as mean ± SEM. AUC, area under the concentration-time curve. *Significant difference between IG and ID administration.

No sex differences were observed in the IG part for all measurements.

Gastric emptying of glucose and the mixed liquid meal

The mixed liquid meal emptied more slowly than glucose. Time to maximum emptying speed was significantly delayed (glucose 63.8 ± 3.8; mixed liquid meal 116.3 ± 11.9; $P < 0.05$) and the AUC of the 120 min responses significantly reduced compared to the glucose meal ($P < 0.05$, Figure 26A and B).

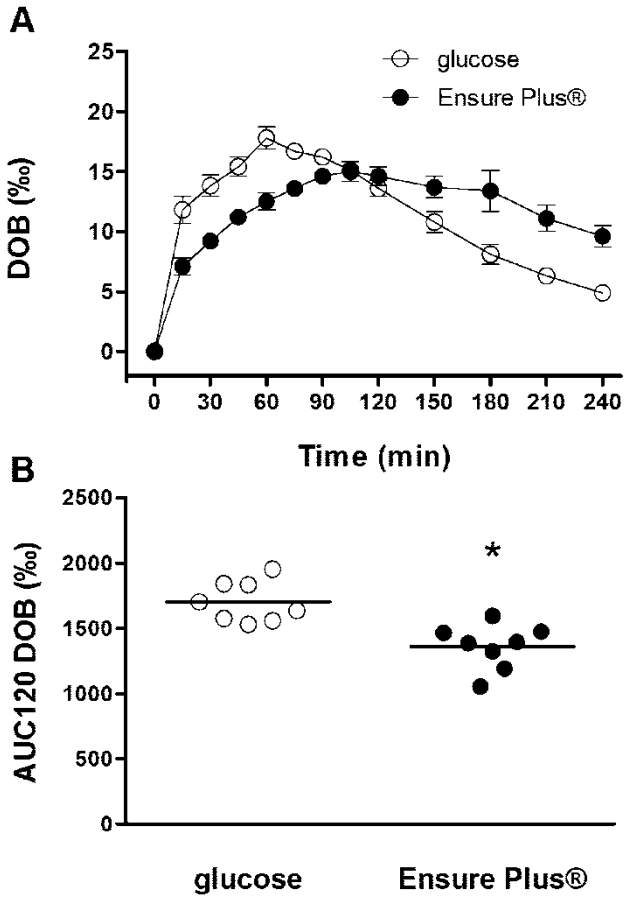

Figure 26 Gastric emptying of glucose or Ensure Plus®. (A) This part depicts the mean ± SEM δ over basal (DOB) responses for each test meal; (B) shows the AUC of the 120 min DOB responses for each individual with the horizontal lines representing the mean DOB response. AUC, area under the concentration-time curve. *Significant difference between IG glucose and IG ensure.

5.3.5 Discussion

The GI tract plays a major role in the control of appetite and food intake and it is accepted that the stomach participates in this process by conveying satiation signals to the brain. There are, however, only few human data exploring the potential interactions between the stomach and the small intestine in appetite control and the secretion of satiation peptides. We performed a randomized, double-blind, parallel-designed study in healthy subjects receiving either a rapid IG load or a continuous ID infusion of glucose or a mixed liquid meal. ID infusions (3 kcal/min) were at rates comparable to the mean duodenal delivery of these nutrients under physiological conditions [93, 94, 375], and in the glucose experiments, the total caloric load at 100 min was equal after both ID and IG administration (300 kcal). In order to examine the interaction effects between the stomach and the small intestine, we compared early postprandial appetite and hormone responses of subjects having received the rapid IG loads (stomach distension + gastric and intestinal nutrient stimulation) *versus* subjects having received the direct ID infusions (intestinal nutrient stimulation only). We found that infusions of glucose directly into the small intestine elicit only weak effects on appetite and the secretion of GLP-1 and PYY. In contrast, identical amounts of glucose delivered into the stomach markedly suppressed appetite paralleled by significantly greater plasma levels of GLP-1 and PYY. Administration of the mixed liquid meal (IG *versus* ID) showed a comparable phenomenon with even augmented effects on appetite and hormone secretion after IG administration. However, interpretation of these data is limited given the different caloric loads (600 vs. 300 kcal). Finally, and in contrast to GLP-1 and PYY secretions, we found that plasma ghrelin levels were suppressed to a similar degree both with IG and ID nutrient administration. There are several explanations for these observations and we will consider them in the following in the context of the available literature.

1) IG nutrient administration is more potent in suppressing appetite than ID nutrient infusions. Studies in animals and humans have documented that gastric distension makes an important contribution to early satiation. These observations are based on experimental procedures such as balloon distension, pyloric occlusion studies or gastric fistulae experiments [51, 62, 91, 96]. Gastric satiation has been found to be volumetric with the stomach monitoring meal volumes based on mechanoreceptors innervated by multiple vagal branches [365, 376, 377]. In addition, in the 1970's and 80's, Smith & Gibbs [378, 379] and Welch *et al.* [380, 381] coined the term "intestinal satiety", which was based on their observation that satiation could be elicited by infusion of food to the duodenum. In contrast to gastric satiation, intestinal satiation is largely nutrient-dependent and mediated by neural and endocrine signals. The gut hormones

CCK, GLP-1 or PYY3-36 were found to be associated with the nutrient stimulated inhibition of food intake [359, 360, 365, 367].

Interactions between gastric and intestinal signals in the control of short-term satiation have been a focus of work in many laboratories. In studies performed in rhesus monkeys and rats, Wirth & Mc Hugh [186] and Kaplan *et al.* [185] combined feeding experiments with the subsequent withdrawal of food from the stomach. The experiments document the importance of gastric distension for short-term satiation in the presence of post-pyloric nutrient administration. In humans, most of the studies have used traditional preload paradigms to demonstrate that the potency of the signal to stop eating is increased when intestinal stimulation is combined with gastric distention [188, 191, 367]. Interaction effects between gastric and intestinal signals have also been demonstrated for CCK and GLP-1 when exogenously administered in combination with a preload in both animals and humans [53, 368-370, 382].

Our data are in line with the body of the literature and support that gastric distention makes a potent contribution to short-term satiation. Moreover, our data are complementary to earlier reports by Cecil and colleagues [187, 193] who found that direct infusion of a soup into the small intestine had no significant effect on hunger, desire to eat or fullness, but the same soup fed orally or IG significantly suppressed appetite over time. We extend these findings by showing that IG nutrient administration (compared to ID administered nutrients) exerts significantly greater effects on appetite, which is paralleled by a significantly higher secretion of satiation peptides.

2. IG nutrient administration is more potent in stimulating the secretion of GLP-1 and PYY but not ghrelin than ID nutrient infusions. The augmented hormone responses after IG administration compared to ID infusions may suggest the existence of interaction effects between gastric and intestinal signals in stimulating the secretion of GLP-1 and PYY. Neural links between the stomach and the small intestine could potentiate peptide responses and thereby influence appetite. Such a notion is also supported by data from Schirra *et al.* [375]. They also found that oral administration of glucose results in markedly higher GLP-1, but not GIP release compared to identical duodenal glucose infusions.

However, we and others have shown that pure mechanical gastric distension by itself or during ID nutrient infusions does not cause a further rise in plasma PYY or CCK [70, 383]; thus at present, no data are available supporting the existence of a “mechanistic” gastric phase of gut peptide secretion. To the best of our knowledge, no studies have, however, examined the isolated effects of “nutrient” gastric distention with concomitant ID nutrient infusions. Early

studies by Deutsch and co-workers [384] have proposed that gastric satiation is at least in part nutritive but such a hypothesis still awaits further proof. The results of these authors were criticized by many, and the large body of experimental evidence points to gastric satiation being triggered by mechanical distension rather than nutrient or chemical stimulation (for review see Powley and Phillips [365]).

An alternative explanation for the different effects of IG and ID nutrients on the secretion of GLP-1 and PYY could be a different initial rate of duodenal delivery of nutrients. It has been well documented that the secretion of gut peptides such as GLP-1 or PYY critically depends on nutrient entry into the small intestine [98-101].

Under physiological conditions, gastric emptying of glucose and other nutrient liquids is closely regulated [92, 96, 385]; in humans emptying approximates an overall rate of 1-3 kcal/min [93, 94, 375]. The early phase (gastric emptying during gastric fill) is usually more rapid, presumably, due to the time for nutrients to initiate the intestinal inhibitory feedback [92, 386, 387]. A subsequent linear rate of nutrient delivery has been reported by Brener *et al.* [93] and others [94, 386]. In contrast, Schirra and colleagues [375] found that gastric emptying of glucose is not constant but declines exponentially over time.

Here we selected a duodenal infusion rate, which most likely mirrored the reported physiological ranges. Moreover, in order to compare the rate of ID infusions (3 kcal/min) with gastric emptying under physiological conditions (IG infusions), we measured gastric emptying using the ^{13}C-acetate breath technique. Consistent with the literature, the data show that the mixed liquid meal empties more slowly than glucose, presumably relating to the different material properties, macronutrient composition and subsequent gut receptor responses. We could, however, not directly compare the speed of gastric emptying (following IG infusions) with the ID infusion rates as the ^{13}C-sodium acetate method is only an indirect measurement and not applicable for such calculations. The fact that we found blood glucose levels increased more rapidly in response to IG glucose, however, suggests that gastric emptying slightly exceeded duodenal glucose infusions in the early postprandial phase (**Fehler! Verweisquelle konnte nicht gefunden werden.**A). The initial more rapid rate of duodenal delivery after IG infusions may, thus, account for the accelerated secretion of GLP-1 and PYY. This would be in line with data from Feinle-Bisset, Horowitz and others [98-101, 388] who report the impact of changes in the rate of glucose entry into the small intestine on the secretion of incretins, insulin and blood glucose. Horowitz *et al.* found that in normal subjects gastric emptying accounts for about 34% of the variance in peak plasma glucose after a 75 g oral glucose load [98]. In addition, they performed

elegant experiments, in which subjects received an ID glucose infusion for 120 min: on one day the infusion rate was variable with a rapid initial rate (between 3 and 6 kcal/min) for ~15 min and a slower rate of 1 kcal/min subsequently. On the other day, the infusion rate was constant at 1 kcal/min [99-101]. They found that an initial more rapid small intestinal glucose delivery increases glycemic, insulinemic and incretin responses supporting the notion that the rate of duodenal delivery of nutrients may be critical for peptide secretion.

Finally, we also found that IG and ID nutrients suppressed comparable amounts of plasma ghrelin, which is in line with earlier studies [85, 389]. We infer from these observations that, in contrast, to GLP-1 and PYY, only intestinal stimulation is responsible for ghrelin suppression and that this effect is independent of the rate of duodenal delivery. We did, however, not measure active ghrelin and, thus, may have missed significant differences in the active form.

3. Conclusion: Our experiments confirm that the stomach is essential in the short-term control of appetite and suggest that gastric and intestinal signals interact to mediate early fullness and satiation, potentially by increased GLP-1 and PYY secretions. Further research is, however, required to determine whether gastric and intestinal signals truly interact to modulate the secretion of satiation peptides. When extrapolating our findings to normal physiological eating conditions, one has to consider that also cognitive and sensory factors may influence the outcome.

5.4 Gastric and intestinal satiation in obese and normal weight healthy people

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5.4.1 Abstract

The gastrointestinal (GI) tract plays a key role in feelings of satiety. It is known that there is a reciprocal interaction between the stomach and intestine, but it is not known which factors are of gastric origin and which are intestinal. This three-step study therefore sought to provide illumination on satiety parameters with respect to body mass, and included a total of 36 normal weight and 29 obese healthy volunteers. In the first phase, the time needed to reach maximal satiation and total caloric intake was calculated after subjects imbibed a standardized nutrient drink. The second phase evaluated the gastric emptying of solids using the ^{13}C-octanoic acid breath test. And in the third, fasting and postprandial plasma GLP-1, PYY and ghrelin levels were measured after a standardized nutrient drink. Our results show that, when compared to those of normal weight, obese subjects reached maximal satiation sooner (P = 0.006), their total intake of calories was higher (P = 0.013), and their gastric emptying rates were delayed (P < 0.001). Furthermore, their postprandial increase in plasma GLP-1 and PYY was reduced, (P < 0.001 for both), as was their ghrelin suppression (P = 0.001). We conclude that, in the severely overweight, the delay in gastric emptying leads to an impaired interaction of nutrients with the intestine which, in turn, results in decreased GLP-1 and PYY secretion. As a consequence, obese subjects require more calories before their maximal satiation is reached and they stop eating.

Key words: glucagon-like peptide-1, peptide tyrosine tyrosine, ghrelin, gastric emptying, stomach

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Conflict of interest: All authors disclose that they do not have any financial and personal relationships with other people, or organizations, that could inappropriately influence (bias) their work.

5.4.2 Introduction

Obesity has reached pandemic proportions; worldwide, since 1980, it has more than doubled. Obesity-associated complications are extensive and expensive; projections cite direct, obesity-related health care costs will more than double every decade.

Current therapy options are limited. Lifestyle modification results in only modest weight loss; poor adherence and recidivism are significant problems [390]. Few pharmacological treatments are available, though they are also far from effective (< 5 kg at one year) [391]. Currently, the only adequate management for obesity is bariatric surgery (mean excess weight loss of 60-75%) [7], however, the perioperative risks, the limited availability of surgical expertise and the financial cost restrict access to a wide population [8]. The need for alternative effective and safer treatment options underscores the importance of an improved understanding of the pathogenesis of obesity.

The combination of genetic, environmental, and behavioral factors seems to influence the balance between food intake and energy expenditure. With respect to food intake, the GI tract plays a key role in the control of hunger and satiation – where gastric and intestinal satiation parameters are especially crucial. It seems that, in response to food consumed, gastric and intestinal signals interact in order to increase satiation and to limit meal size; gastric parameters in particular are decisive in the short-term control of appetite. We recently showed that infusions of glucose directly into the small intestine elicit only weak effects on appetite and the secretion of GLP-1 and PYY. In contrast, identical amounts of glucose delivered intragastrically (IG) markedly suppressed appetite [325].

To date, uncertainties exist as to the role of both gastric (e.g., distention and emptying) and intestinal (e.g., satiation peptides) parameters in the control of satiation in relation to body mass. Gastric emptying has been evaluated in normal weight and obese subjects, but contradictory results showing accelerated [392-394], normal [382, 395] or even delayed [396] gastric emptying rates in the obese. For GLP-1, most studies [382, 397] report no difference in fasting concentrations between obese and normal weight individuals, though some do stress a postprandial attenuated GLP-1 response in the obese [163, 164]. Likewise ambiguous is PYY: Most studies show a negative correlation between fasting PYY levels and adiposity markers (such as BMI), and that the postprandial PYY response is attenuated in the obese [176, 398, 399]. Again here, there are contradictory results: for example, Vazquez Roque and co-workers found no significant differences in fasting and postprandial PYY levels between obese and normal weight subjects [382]. To improve the understanding of the reciprocal control between

gastric functions and intestinal parameters in the development of satiation in obese subjects, we compared satiation parameters, gastric emptying and plasma GLP-1, PYY and ghrelin levels between normal and obese healthy volunteers.

5.4.3 Methods

Subjects

A total of 36 normal weight (mean BMI: 22.0 ± 0.3 kg/m^2, range 18.3 – 25.0 kg/m^2) volunteers (16 males and 20 females; mean age: 29.6 ± 1.4 years, range 20-48 years) and 29 obese (mean BMI: 38.7 ± 0.9 kg/m^2, range 30.6 – 55.9 kg/m^2) subjects (10 males and 19 females; mean age: 37.4 ± 1.7 years, range 22-62 years) took part in the study. All participants were healthy. The study consisted of three experimental parts. From among the above-mentioned volunteers, 20 normal weight (mean age: 33.7 ± 1.9 years; mean BMI: 22.2 ± 0.4 kg/m^2) and 20 obese subjects (mean age: 36.4 ± 2.1 years; mean BMI: 39.6 ± 0.7 kg/m^2) participated in parts I and III of the study; the remaining 16 normal weight (mean age: 24.5 ± 1.0 years; mean BMI: 21.8 ± 0.5 kg/m^2) and 9 obese (mean age: 39.6 ± 3.2 years; mean BMI: 36.6 ± 2.6 kg/m^2) volunteers participated in part II of the study.

The protocol was submitted and approved by the State Ethical Committee of Basel and the study was carried out in accordance with the principles of the Declaration of Helsinki. Each subject provided written informed consent for the study. Before acceptance, each participant was required to complete a screening and medical interview, received a full physical examination and underwent an initial laboratory screening. The criteria for exclusion were smoking, substance abuse, regular intake of medications (except for oral contraceptives), medical or psychiatric illness and any abnormalities detected during physical examination or laboratory screening. None of the subjects had a history of GI disorders, food allergies or dietary restrictions. Subjects were instructed to abstain from alcohol, caffeine and strenuous exercise for 24 hours before each treatment.

Experimental procedure

On each study day, subjects were admitted to the Phase 1 Research Unit of the University Hospital of Basel in the morning after a 10 h overnight fast. Vital signs (blood pressure, heart rate) were measured before and after study-related procedures.

Part I: Satiation from a standardized nutrient drink

A standardized nutrient drink (Ensure Plus®; 17% protein, 29% fat and 54% carbohydrate; 1.5 kcal/mL; Abbott AG, Baar, Switzerland) test was used to measure satiation in 20 normal weight and 20 obese subjects. While drinking, the subjective sensation of satiation was measured every three minutes using a VAS. The scales and scores have previously been described in detail [313]. In brief, the VAS consists of a horizontal, unstructured, 10 cm line with words anchored at each end, describing the extremes ('not at all' or 'extremely') of the unipolar

question, 'How satiated are you right now?' To ensure reliable and valid results, subjects rated their feeling of satiation as precisely as possible, and they could not refer to their previous ratings when marking the VAS. Nutrient intake was stopped at t_{max}, the time needed to reach a maximal level of satiation (maximum satiation). The volume ingested was then recorded and caloric intake calculated.

Part II: Gastric emptying

Gastric emptying of solids was measured using the ^{13}C-octanoic acid breath test, an accurate, non-invasive method, without radiation exposure, and a reliable alternative to scintigraphy, the “gold standard” for measuring gastric emptying [374]. Sixteen normal weight and nine obese subjects received a standardized meal, consisting of two scrambled eggs (cooked with 10 g butter), placed on two slices of whole wheat bread and 200 mL of milk (total: 468 kcal). The test meal was labeled with 100 mg ^{13}C-octanoic acid (Wagner Analysen Technik GmbH, Bremen, Germany) for determination of gastric emptying. Subjects were asked to eat the meal within 5-10 minutes. ^{13}C-octanoic acid is rapidly absorbed in the proximal small intestine, transported to the liver and metabolized to $^{13}CO_2$, which is then exhaled rapidly [374]. At fixed time intervals, end-expiratory breath samples were taken into a 100 mL foil bag. The ^{13}C-exhalation was then determined by non-dispersive infrared spectroscopy using an isotope ratio mass spectrophotometer (IRIS; Wagner Analysen Technik, Bremen, Germany), and expressed as the relative difference (δ ‰) from the universal reference standard (carbon from Pee Dee Belemnite limestone). ^{13}C-enrichment was defined as the difference between pre-prandial ^{13}C-exhalation and postprandial ^{13}C-exhalation at defined time points, δ over basal (DOB, ‰); DOB indirectly reflects gastric emptying of nutrients.

Part III: Hormone profiles after a standardized nutrient drink

Within 5 minutes, 20 normal weight and 20 obese subjects ingested 500 mL of a complex nutrient drink (Ensure Plus®, specified above). At regular time intervals, fasting and postprandial blood samples were collected on ice into tubes containing EDTA (6 µmol/L), aprotinin (500 kIU/mL) and a DPP-IV inhibitor. The tubes were centrifuged at 4℃ at 3000 rpm for 10 min. After centrifugation, the plasma samples were processed into different aliquots and stored at -70 ℃ until analysis of plasma GLP-1, PY Y and ghrelin.

Laboratory analysis

Active GLP-1 was measured with a commercially-available ELISA kit (Millipore Corporation, Billerica, Massachusetts, USA). The intra- and inter-assay coefficient of variation for this assay is below 9.0% and 13.0%, respectively. *Total PYY* and *total ghrelin* were measured with

commercially-available RIA kits (LINCO Research, St. Charles, Missouri, USA). The intra- and inter-assay coefficients of variation for these assays are below 9.4% and 8.5% (PYY), and below 10.0% and 17.8% (ghrelin). The methods have been described recently in more detail [327].

Statistical analysis

Descriptive statistics were used for demographic variables such as age, weight, height, and BMI.

The significant differences between healthy normal weight and obese subjects in time needed to reach the maximal level of satiation (t_{max}), total caloric intake and calories per minute (kcals/min) were analyzed using Student's unpaired t-test.

Gastric emptying rates and hormone profiles were calculated using pharmacodynamic parameters (area under the concentration-time curve (AUC) and maximum plasma concentration (C_{max})). To test for significant differences between the two treatment groups, the AUC of gastric emptying was compared using a one-way analysis of covariance adjusted for age and gender. AUC from baseline and C_{max} of the hormone profiles were compared using Student's unpaired t-test.

Linear regression analysis was used to determine a possible correlation between the BMI (independent variable) and t_{max}, total caloric intake and hormone profiles (dependent variables).

All parameters were tested for normality by the Shapiro-Wilk test. All statistical analysis was done using the statistical software package SPSS for Windows Version 14.0 (SPSS Inc., Chicago, USA). Values were reported as mean ± SEM. All tests were two-tailed with $P \leq 0.05$ considered statistically significant.

5.4.4 Results

All subjects tolerated the study procedures well. No volunteer experienced any side effects, such as nausea, abdominal discomfort or vomiting. Four subjects were excluded from the analysis due to incomplete datasets, either in the assessment of satiation parameters, of gastric emptying rate (insufficient end-expiratory breath) or of hormone profiles (missing blood samples due to poor blood flow). No differences between male and female subjects were found.

Part I: Satiation from the nutrient drink

Satiation in the fasting state before ingestion of the nutrient drink did not differ significantly between normal weight and obese subjects. After the nutrient drink, obese subjects reached maximal satiation sooner (t_{max}: 10.3 ± 0.9 min vs. 13.8 ± 0.7 min; *P* = 0.006; Figure 27A), the total intake of calories was higher (786.8 ± 43.2 kcal vs. 647.2 ± 31.1 kcal; *P* = 0.013; Figure 27B) and they consumed significantly more calories per minute than normal weight subjects (86.2 ± 9.1 kcal/min vs. 50.3 ± 4.0 kcal/min; *P* = 0.001; Figure 27C).

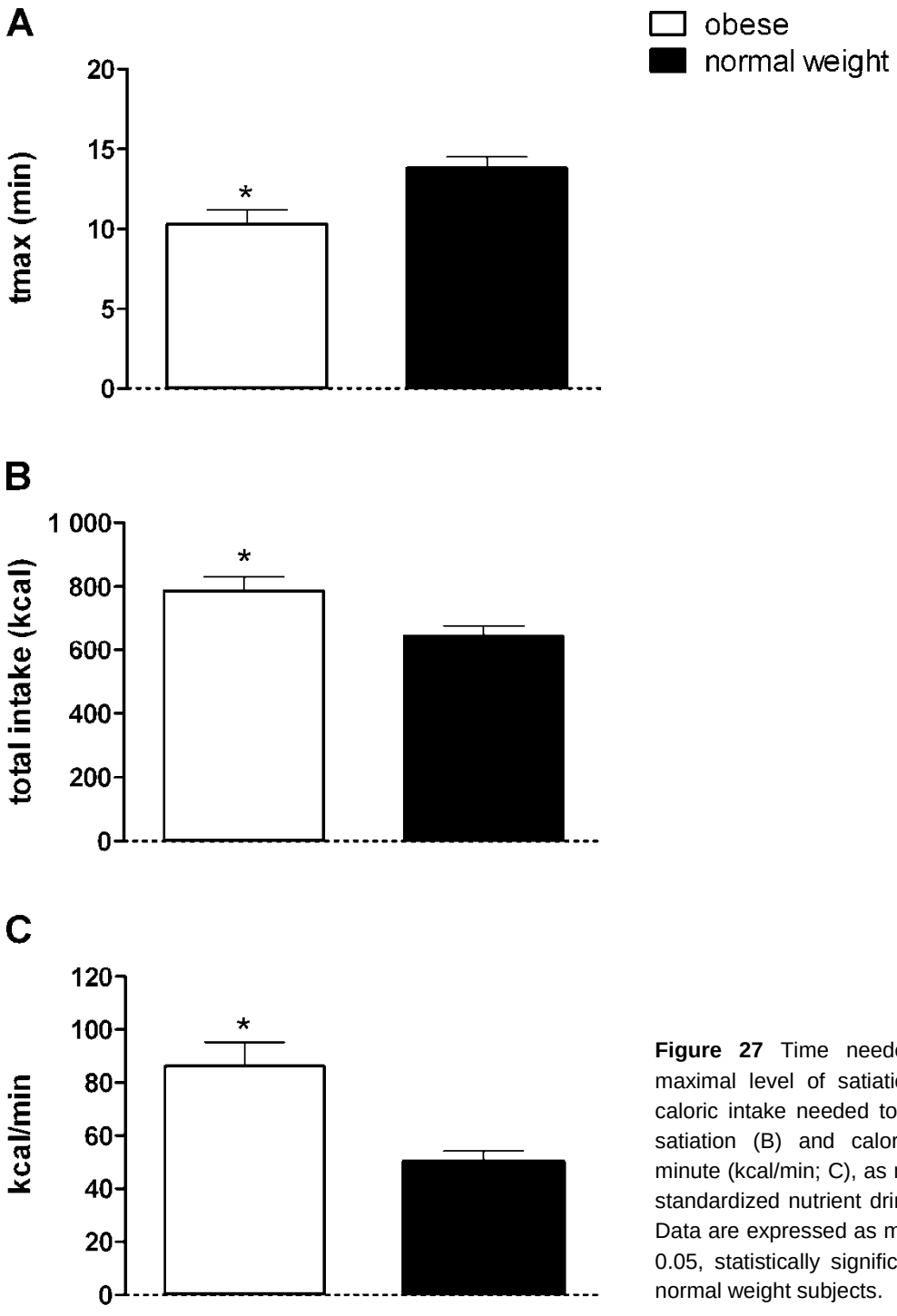

Figure 27 Time needed to reach the maximal level of satiation (t_{max}; A), total caloric intake needed to achieve maximal satiation (B) and calories ingested per minute (kcal/min; C), as measured with the standardized nutrient drink, Ensure Plus®. Data are expressed as mean ± SEM. * $P < 0.05$, statistically significant difference vs. normal weight subjects.

These results confirm the positive correlation between BMI and total caloric intake ($P = 0.010$; Figure 28).

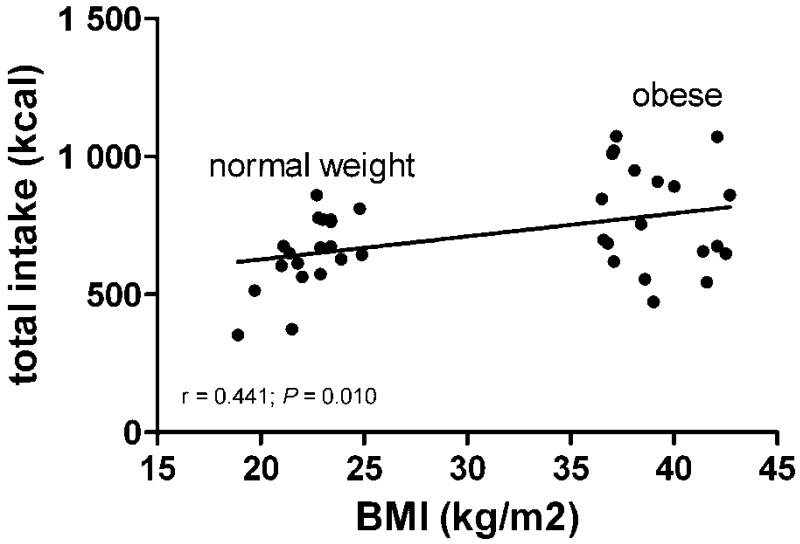

Figure 28 Correlation between BMI and total caloric intake in obese and normal weight subjects. The standardized nutrient drink was Ensure Plus®. The line represents a linear regression between the two variables.

Part II: Gastric functions

Gastric emptying rates of a solid meal were clearly delayed in obese subjects ($P < 0.001$). Although the time to reach maximal emptying speed was comparable between the groups (obese: 200 ± 8.7 min and normal weight: 184 ± 10.5 min), the AUC of ^{13}C-exhalation was significantly reduced in the obese group (AUC 0-240 min: 1,443 ± 41 vs. 2,325 ± 118 in normal weight; $P < 0.001$; Figure 29.

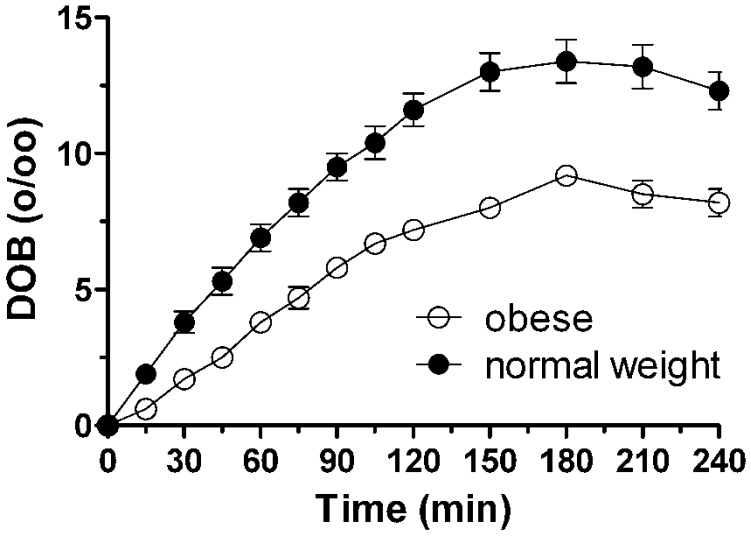

Figure 29 ^{13}C-exhalation – expressed as delta over basal (DOB) – indirectly reflects gastric emptying of nutrients. Data are expressed as mean ± SEM.

Part III: Fasting and postprandial hormonal responses

In response to the nutrient drink given at time 0, the increase in plasma GLP-1 and PYY levels was markedly reduced in obese subjects (Figure 30A and B). Incremental AUCs were significantly lower in obese subjects than in the normal weight (for both: $P < 0.001$; Table 4). Of note, in obese subjects PYY release was virtually absent. These results concur with a negative correlation between BMI and plasma GLP-1 and PYY levels ($P < 0.001$; Figure 31A and B).

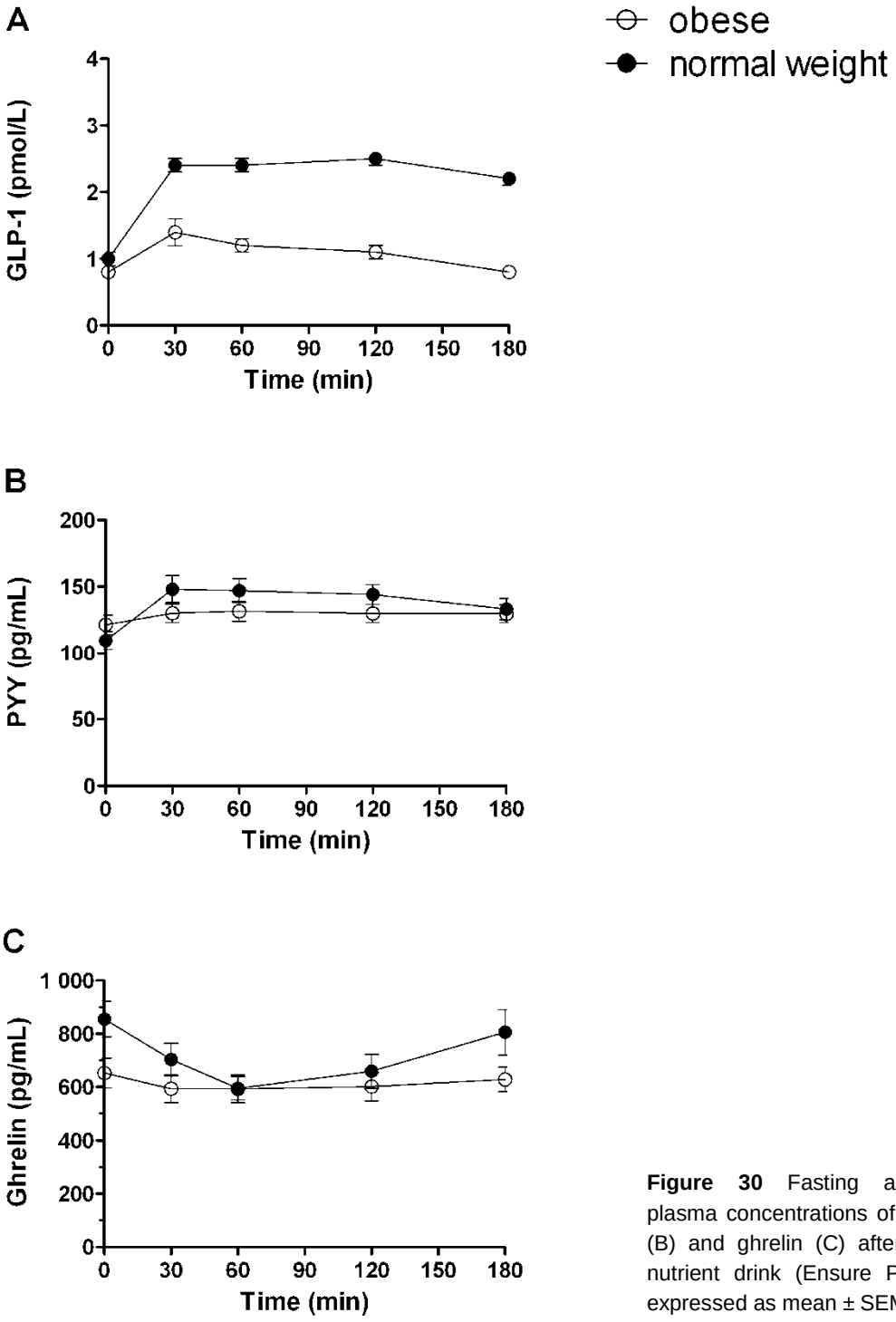

Figure 30 Fasting and postprandial plasma concentrations of GLP-1 (A), PYY (B) and ghrelin (C) after a standardized nutrient drink (Ensure Plus®). Data are expressed as mean ± SEM.

Table 4 Hormone profiles in normal weight and obese subjects in response to a standardized nutrient drink (Ensure Plus®).

	normal weight	**obese**
GLP-1		
AUC (0-180 min) (pmol x min/L)	221.4 ± 17.7	57.8 ± 17.0 *(P < 0.001)*
C_{max} (pmol/L)	2.7 ± 0.1	1.6 ± 0.2 *(P <0.001)*
PYY		
AUC (0-180 min) (pg x min/mL)	5,620 ± 691	1,480 ± 590 *(P < 0.001)*
C_{max} (pg/mL)	171.8 ± 7.6	143.8 ± 6.7 *(P = 0.009)*
ghrelin		
AUC (0-180 min) (pg x min/mL)	-29,585 ± 5,248	-8,299 ± 2,382 *(P = 0.001)*
C_{max} (pg/mL)	930.5 ± 81.8	675.1 ± 53.3 *(P = 0.014)*

AUC, area under the concentration-time curve, was calculated from baseline; C_{max}, maximum plasma concentration. Data are expressed as mean ± SEM. n = 20 obese subjects and 20 normal weight subjects. *P* values are given in parentheses and represent comparisons vs. normal weight subjects: $P \leq 0.05$, statistically significant difference vs. normal weight subjects.

Obese subjects had lower fasting ghrelin levels than their normal-weight counterparts (*P* = 0.027). The suppression of plasma ghrelin after the nutrient drink was significantly less in the obese group (AUC: *P* = 0.001; Figure 30C and Table 4), which is in line with the positive correlation between BMI and plasma ghrelin (*P* = 0.001; Figure 31C).

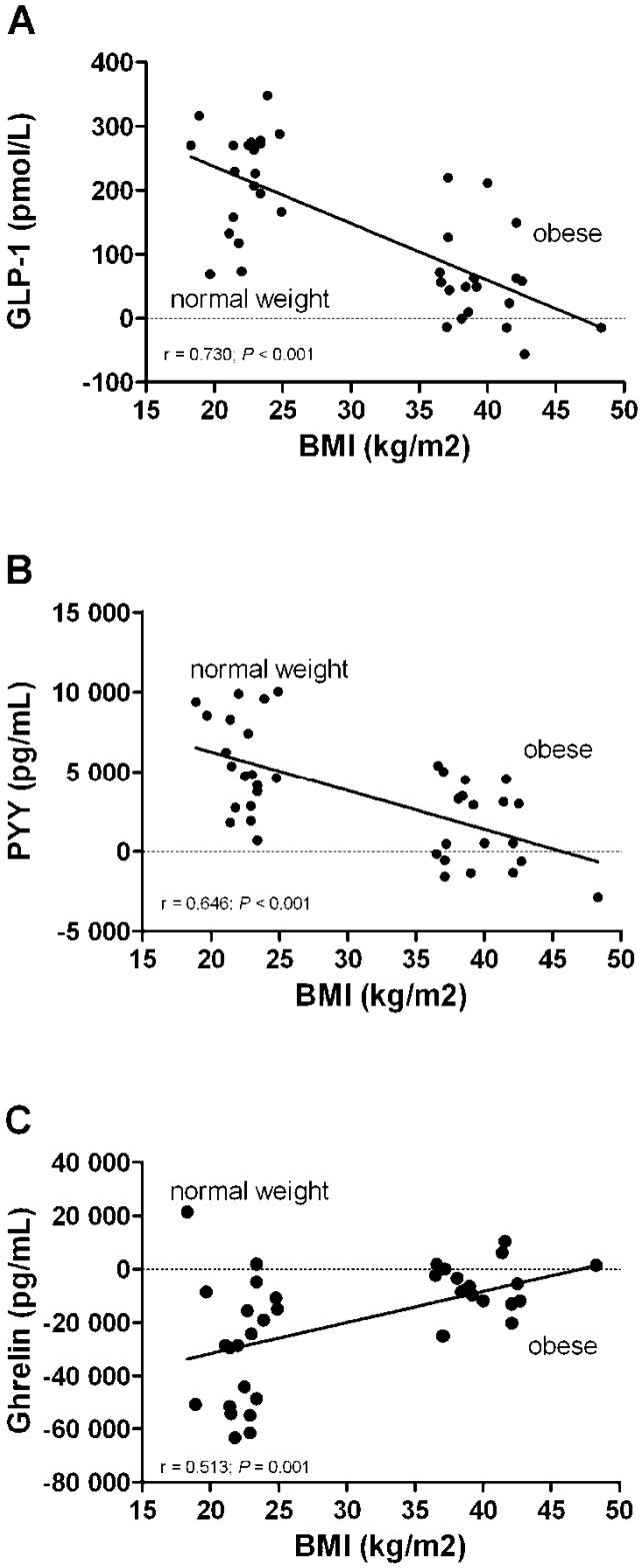

Figure 31 Correlations of BMI and plasma GLP-1 (A), PYY (B) and ghrelin (C) levels in obese and normal weight subjects after a standardized nutrient drink (Ensure Plus®). The lines represent linear regression between the two variables.

5.4.5 Discussion

The increasing prevalence, progressive natural history, and complications of obesity, with pandemic proportions, is associated with exploding health care costs. Current treatment options are, however, limited in scope and effectiveness. Bariatric surgery is currently the most effective treatment option, but it is an invasive therapy and associated with complications and costs. Safe and successful alternative therapies are needed that will target different aspects of the multifactorial nature of obesity. To achieve this goal an improved understanding of the pathogenesis of obesity is key.

Human obesity basically results from an imbalance between energy intake and expenditure; energy intake largely depends on food intake, the latter being strongly influenced by the GI tract. Gastric parameters (gastric distention and gastric emptying) and intestinal signals (satiation peptides, such as GLP-1 and PYY) play key roles in the control of hunger and satiation and they have been studied in relation to body mass, both in normal weight and in obese subjects [163, 176, 394, 400].

To better understand the reciprocal control between gastric functions and the release of satiation hormones in the process of satiation as a pathophysiological mechanism in obesity, we compared satiation parameters, gastric emptying rates and hormone release (plasma GLP-1, PYY and ghrelin levels) in normal and obese healthy volunteers.

Our results show that obese subjects reached maximal satiation sooner ($P = 0.006$) and their total caloric intake per minute was higher ($P = 0.013$), while their gastric emptying rates were delayed ($P < 0.001$). The increase in postprandial plasma GLP-1 and PYY levels was reduced in obese subjects ($P < 0.001$, *for both*) while suppression of physiological ghrelin levels was significantly smaller compared to normal weight subjects ($P = 0.001$). We infer from these observations that obese persons have a disturbed balance in regulatory factors associated with digestive functions and appetite control.

The observed higher total caloric intake in obese subjects coincides with a previous study [401]. Delgado-Aros *et al*. showed that the obese require approximately 225 kcal more than normal weight people to reach maximal satiation. In our experimental set-up, obese subjects consumed approximately 140 kcal more calories than the controls, a factor which may contribute for their being overweight: 100 kcal more than the daily requirement is sufficient to cause weight gain [402]. Similarly, in line with a previous study [403], we observed that obese subjects consumed more calories per minute than did normal weight subjects.

Gastric and intestinal signals seem to interact to mediate satiety in healthy and normal-weight subjects. We have recently reported that a direct infusion of glucose into the small intestine elicited only weak effects on appetite, whereas identical amounts of glucose delivered IG suppressed appetite [325]. These results support the concept of an important interaction between gastric signals and small intestinal nutrient stimulation in the short-term control of food intake [188, 404]; furthermore, they underscore that the stomach is an essential component in this control circuit. Along this line, Cecil *et al*. showed that IG infusion of tomato soup suppressed appetite, whereas ID infusions did not [405]. In support, Feinle and co-workers documented that “meal-like” feelings of satiation were only induced when intra-intestinal infusions were combined with stomach distension [190]. Several studies have shown that gastric satiation is elicited by increased gastric volume mediated by mechanoreceptors in the stomach, rather than by increased nutrient intake [406-408].

In obesity, a continued increased gastric distention may result in poor tone of the gastric fundus, with an ensuing reduced sensitivity of mechanoreceptors [409]. This, in turn, may reduce feelings of satiety and lead to increased food intake. As a consequence of larger gastric volumes, gastric emptying is delayed [102]. These assumptions are in line with our observations documenting delayed gastric emptying rates in obese subjects. Accordingly, Jackson *et al*. also showed a prolonged lag phase and delayed gastric emptying in obese subjects using the same methodology (^{13}C-octanoic acid breath test) [400]. Whether gastric capacity is increased in obese individuals and contributes to delayed satiation is not yet clear. Two studies that used intragastric latex balloons showed larger gastric capacities in the obese [62, 410]: They reached maximal satiation sooner and had a 21.6% (139.6 kcal) higher mean caloric intake, though this may just be a reflection of their greater gastric capacity. Furthermore, Delgado-Aros and co-workers found larger fasting gastric volumes and a delayed satiation in response to a constant rate of a liquid meal [401]. However, this and other studies using barostat or SPECT techniques did not detect differences in the maximal gastric capacity between obese and lean subjects [401, 411].

In addition to assessing satiation parameters and gastric emptying rates, we also characterized the postprandial responses of ghrelin and the satiation peptides, GLP-1 and PYY, discriminated by body mass. The reduced physiological suppression of postprandial ghrelin in the obese has been shown previously and suggests an adaptation to a long-term positive energy balance [412, 413]. Ghrelin is the only known circulating orexigen: Its lower degree of suppression in obese subjects may contribute to reduced feelings of satiation and increased food intake. The attenuated postprandial increase in both GLP-1 and PYY in the obese was anticipated from

previous reports [163, 164, 176, 398], though its cause is still unknown. The delay in gastric emptying, probably from a reduced gastric mechanoreceptor sensitivity, may lead to impaired and delayed interaction of nutrients with the intestine, eventually resulting in decreased secretion of GLP-1 and PYY. Even relatively minor changes in the rate of food delivery into the small intestine exert major effects on the secretion of satiation peptides [101, 325]. We thus see that delayed gastric emptying may result in decreased postprandial secretion of satiation peptides from the small intestine, and hence reduced satiation; it may therefore take conspicuously more food to induce the same degree of satiation and to suppress food intake.

In conclusion, this study shows that gastric emptying is delayed in obese subjects, probably due to altered gastric sensory functions. We suggest that the delay in gastric emptying leads to impaired and delayed interactions of ingested nutrients with the small and large intestine, thereby resulting in decreased secretion of the satiation peptides, GLP-1 and PYY. As a consequence, obese subjects require more calories to reach a maximal satiation and to stop nutrient intake. These results document once again the importance of the stomach in the control of appetite and satiation. Altered gastric sensory and motor functions can be considered as potential contributing factors in the development of obesity. Recently, Torra *et al.* showed that meal size can be decreased in obese subjects by pharmacologically accelerating gastric emptying [414]. Further research is thus needed to evaluate whether changes in gastric parameters may present useful targets in preventing and treating obesity.

6 General Discussion and Conclusion

The present thesis investigates aspects of the regulation of food intake focusing on the gastric and intestinal phases of eating control: different gastrointestinal (GI) peptide secretion mechanisms were examined and possible interactions between gastric and intestinal phase signals in the control of appetite were evaluated.

Enteroendocrine cells (EECs) in the small intestine have been identified and characterized as intestinal peptide secreting cells and a number of chemosensory receptors that respond to luminal stimuli (nutrients as well as non-nutrients) were found to be expressed on these cells. The first aim of the present thesis was to investigate the involvement of two potential targets of peptide release: i) bile acids (BAs) as possible TGR5 agonists and ii) glucose stimulating the sweet receptor T1R2/T1R3.

Project 1 (see 5.1): Secretion studies with enteroendocrine STC-1 and GLUTag cells indicated that BAs induce the release of GLP-1 [289-291]. A stimulatory effect of BAs on intestinal peptide secretion has also been demonstrated in animal models [282-285, 287]. In humans, infusions of BAs into the colon or rectum induced secretion of GLP-1 and PYY [44, 286]. In addition, Koop and co-workers [288] observed a small increase in CCK levels after CDCA administration. We investigated the physiological role of BAs in stimulating the secretion of intestinal peptides by using a paradigm in that subjects received intraduodenal (ID) infusions of different loads of chenodeoxycholic acid (CDCA, a primary BA in humans) in comparison to sodium-oleate or vehicle as a control. Infusion of CDCA (15 mmol/L) mimicking physiological concentrations resulted in a significant increase of plasma GLP-1 and CCK levels. The stimulatory potency was, however, small, if we compare the magnitude of the GLP-1 and CCK responses to other well-known secretagogues such as glucose or fatty acids. Furthermore, there was no effect of CDCA on the secretion of PYY, and the small effect of CDCA on GLP-1 induced no effect on plasma glucose levels and insulin release. A physiological load of CDCA is, therefore, a rather weak stimulus for GLP-1 and CCK secretion with no insulinotropic GLP-1 dependent effects on glucose homeostasis in humans.

Project 2 (see 5.2): The sweet taste receptor T1R2/T1R3 is expressed on human enteroendocrine L-cells [250, 251]; there is strong experimental evidence documenting that T1R2/T1R3 senses glucose in the gut lumen [146, 254]. In addition, ingestion of glucose by

T1R3 *knockout* mice induced a reduction in plasma GLP-1 levels with consequent deficiencies in the regulation of plasma insulin and glucose [146]. These findings indicate a role for the sweet taste receptor in GI peptide secretion. To further characterize the role of the sweet taste receptor in this regulatory process we used lactisole, a T1R2/T1R3 antagonist [146, 338]. Two different approaches were applied to healthy subjects: they received i) intragastric (IG) and ID infusions of glucose and ii) IG and ID infusions of a liquid mixed meal, both with and without lactisole. In the IG glucose-stimulated part, lactisole induced a dose-dependent and significant reduction of GLP-1 and PYY levels. As a consequence, plasma insulin levels were decreased and glucose levels increased. In the ID glucose-stimulated part, lactisole induced also a reduction in GLP-1 secretion, but had no effect on the release of PYY. Moreover, the comparison of the inhibitory effect of lactisole on the secretion of GLP-1 in response to IG versus ID administration of glucose showed a significantly greater suppression of the hormone response in the IG part. These findings suggest interaction mechanisms between gastric signals and signals from the small intestine; they also indicate a relevant contribution of the stomach in the regulation of GI peptide secretion. One possible mechanism could be that the sweet receptor subunit T1R3 in the stomach plays a role in the detection of nutrients, which in turn initiates either hormonal or neural cascades which are crucial for the secretion of intestinal peptides [255, 346, 347]. In contrast to the glucose-stimulated meal, lactisole had no effects on GI peptide secretion in both, the IG and ID mixed liquid meal stimulation. Of note the liquid meal consisted beside glucose also of proteins, fats and other complex carbohydrates. The lack of lactisole to reduce peptide secretion in response to a mixed meal suggests that apart from glucose other nutrients induced the release of GI peptides via different receptor mechanisms, which outweighed the effect of sweet receptor blockade. As already mentioned above, long-chain fatty acids (LCFAs) are also potent luminal secretagogues for GLP-1 release [144]. Thus, the fast response to glucose is based on the activation of the sweet receptor system, whereas the effects of a mixed meal are mediated by lipids, proteins and probably BAs.

In summary, the role of BAs in intestinal peptide secretion is probably of minor importance; further studies with other BAs or pharmacological manipulation of BAs could clarify to what extent the described relationship between BAs and gut peptide secretion in cells and animals can be translated into the human situation.

The sweet taste receptor T1R2/T1R3 seems to be involved in the secretion of GI peptides; however, the results of the mixed liquid meal indicate that 1) the effect is restricted to glucose stimulation and 2) the receptor is not alone responsible for peptide secretion. It is rather a complex interaction between the multiplicities of different receptor mechanisms. Moreover, the smaller effect of lactisole on the suppression of peptide secretion in the ID glucose-stimulated

part compared to IG stimulation let assume a relevant contribution of the stomach (with other, probably neural mechanisms involved) and further suggests interaction mechanisms between gastric and intestinal signals.

Indeed, several studies in animals [185, 186] and humans [187-189] have documented that gastric and intestinal signals interact to elicit optimal satiation and adequate control of eating processes. In humans, little information is available on the underlying mechanisms of this interaction. In addition, uncertainties exist about the role of both gastric and intestinal parameters, as well as their interaction in the control of satiation in relation to body mass. The second aim of the present thesis was therefore to evaluate the reciprocal control between gastric functions and intestinal parameters in the control of appetite in lean as well as in obese persons.

Project 3 (see 5.3): In this part of the study we investigated potential interactions of regulatory circuits of appetite control by comparing different models in normal weight persons. We applied a paradigm in that lean subjects received either a rapid IG load (gastric distension + gastric and intestinal nutrient stimulation) or a continuous ID infusion (intestinal nutrient stimulation only) of glucose or a mixed liquid meal. We found that infusions of glucose directly into the small intestine elicit only weak effects on appetite and the secretion of GLP-1 and PYY. In contrast, identical amounts of glucose delivered into the stomach markedly suppressed appetite paralleled by significantly greater plasma levels of GLP-1 and PYY. Administration of the mixed liquid meal (IG *vs.* ID) showed a similar outcome. These results indicated that: i) IG nutrient administration is more potent in suppressing appetite than ID nutrient infusion; these data are in line with previous work by Cecil *et al.*, who found that direct infusion of a soup into the small intestine had no effect on appetite, but the same soup as IG load suppressed appetite [187-189]; ii) IG nutrient administration is more potent in stimulating the secretion of GLP-1 and PYY than ID nutrient infusion. These findings could probably be related to the gastric emptying of the nutrients. Gastric emptying of liquid nutrients is closely regulated with an initial more rapid rate of duodenal delivery [92, 96, 385]. In our study design, we selected a duodenal infusion rate, which most likely mirrored the reported physiological range (1-3 kcal/min) [93, 94, 375]. However, we found a more rapid increase in blood glucose levels in response to the IG glucose load, which let assume that gastric emptying presumably slightly exceeded ID glucose infusion in the early postprandial phase. This initial more rapid rate of duodenal delivery after IG infusions might account for the accelerated secretion of GLP-1 and PYY. Indeed, it is well documented that the secretion of intestinal peptides critically depends on nutrient entry into the small intestine [98-101].

Project 4 (see 5.4): In project 4, we compared regulatory circuits in normal weight and obese healthy subjects. We found that gastric emptying rates were delayed in obese subjects and the increase in postprandial plasma GLP-1 and PYY levels was reduced. It is well documented that an increase in gastric volume is mediated by mechanoreceptors in the stomach [54]. In obesity, a continued increased gastric distention, due to the amount of food consumed at any time, might result in reduced sensitivity of mechanoreceptors [409]. This, in turn, might reduce feelings of satiety and lead to increased food intake; as a consequence of larger gastric volumes, gastric emptying could be delayed [102]. This delay in gastric emptying could lead to impaired and delayed interaction of nutrients with the small intestine, which in turn would result in decreased secretion of GLP-1 and PYY. As mentioned above, the secretion of intestinal peptides critically depends on nutrient entry into the small intestine and relatively minor changes in the gastric emptying rates of food could exert major effects on the secretion of intestinal peptides [98-101]. Decreased postprandial secretion of intestinal satiation peptides would result in reduced satiation; it may therefore take more food to induce the same degree of satiation in order to suppress food intake. This assumption is in line with our results: the total caloric intake was higher in obese persons compared to lean subjects.

In summary, gastric and intestinal signals interact to mediate early satiation probably, at least in part, by increased GLP-1 and PYY secretions. In addition, project 2 as well as project 3 indicate a relevant contribution of the stomach in the regulation of GI peptide secretion and confirm the essential role of the stomach in the short-term control of appetite. In project 4 we observed delayed gastric emptying in obese subjects that caused a decrease in peptide secretion and an increase in calorie intake; these findings support the importance of gastric signals in the control of appetite.

To conclude, chemosensing receptors, such as T1R2/T1R3 to give an example, induce GI peptide secretion in response to nutrients. A single receptor is probably not alone responsible for peptide release – it is rather a complex interaction between different receptor mechanisms. In addition, appetite control is a complex interaction between a plenty of different GI signals, neural and hormonal, with a relevant contribution of the stomach. It is, therefore, not possible to restrain the complexity of eating control to the assessment of only one or two mechanisms. Nonetheless, the understanding of each individual mechanism by its own is essential to understand the complexity and the integral process of eating control. Perhaps in the very next future, this understanding could enable to exploit the full potential of the GI tract in the treatment and fight of obesity.

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Abbreviations

AC	adenylate cylase
AgRP	agouti-related protein
AM	ante meridiem
ANOVA	Analysis of Variance between groups
AP	area postrema
APO A-IV	apolipoprotein A-IV
ARC	arcuate nucleus
ATP	adenosine triphosphate
AUC	area under the curve
BA	bile acid
BMI	body mass index
CA	cholic acid
Ca^{2+}	calcium
cAMP	cyclic adenosine monophosphate
CaR	calcium-sensing receptor
CART	cocaine- and amphetamine-related transcript
CCK	cholecystokinin
CDCA	chenodeoxycholic acid
CD36	free fatty acid transporter system
CIOMS	Council for International Organizations of Medical Science
C_{max}	maximal plasma concentration
CNS	central nervous system
CPRs	cephalic phase responses
CRH	corticotropin-releasing hormone

DAG	diacylglycerol
dAUC	delta area under the curve
DCA	deoxycholic acid
DMN	dorsomedial nucleus
DPP-IV	dipeptidyl peptidase-IV
DVC	dorsal vagal complex
DVN	dorsovagal neurons
EEC	enteroendocrine cells
EDTA	ethylenediaminetetraacetic acid
e.g.	exempli gratia
EIA	enzyme immunoassay
EKBB	The State Ethical Committee of Basel
ELISA	enzyme linked immunosorbent assay
EU	European Union
FATP4	fatty acid transport protein 4
FDA	American Food and Drug Administration
FFAR1-3	free fatty acid receptor 1-3
FIA	fluorescence immunoassay
FXR	farnesoid X receptor
GCP	Good Clinical Practice
GDP	guanosine diphosphate
GI	gastrointestinal
GIP	glucose-dependent insulinotropic peptide
GLP-1	glucagon-like peptide-1
GLP-2	glucagon-like peptide-2
GLUT2	glucose transporter 2
GPCR	G protein-coupled receptor

GPR40	G protein-coupled receptor 40
GPR41	G protein-coupled receptor 41
GPR43	G protein-coupled receptor 43
GPR93	G protein-coupled receptor 93
GPR120	G protein-coupled receptor 120
GPRC6A	G protein-coupled receptors family C, group 6, subtype A
GRP	gastrin-releasing peptide
h	hour
ICH	International Conference on Harmonisation
ID	intraduodenal
IG	intragastric
IGLEs	intraganglionic laminar vagal afferent endings
IMAs	intramuscular arrays
IP3	inositol triphosphate
K^+	potassium
kcal	kilocalorie
Kir6.2	K_{ATP} channel subunit
kIU	kilo international units
LCA	lithocholic acid
LCFA	long-chain fatty acid
LHA	lateral hypothalamic area
MCH	melanin-concentrating hormone
ME	median eminence
min	minute
mM	millimolar (millimole per liter)
mmol	millimole
MRI	magnetic resonance imaging

mRNA	messenger ribonucleic acid
NAc	nucleus accumbens
Na^+	sodium
NMB	neuromedin B
NPY	neuropeptide Y
NS	not significant
NTS	nucleus of the tractus solitaries
OA	oleanolic acid
OEA	oleoylethanolamide
OFC	orbifrontal cortex
OXM	oxyntomodulin
OXY	oxytocin
P	probability
PEPT1	peptide transporter 1
PEPT2	peptide transporter 2
PFA	perifornical area
PFC	prefrontal cortex
pg	picogram
pH	pondus hydrogenii
PK	protein kinase
PLC	phospholipase C (PLC),
PLC β2	phospholipase C β2
PM	post meridiem
pmol	picomol
POMC	pro-opiomelanocortin
ppm	parts per million
PVN	paraventricular nucleus

PYY	peptide tyrosine tyrosine
RIA	radioimmunoassay
rpm	revolutions per minute
SCFA	short-chain fatty acid
SCL	solute carrier transporter
SEM	Standard Error of the Mean
SGLT1	soldium-coupled glucose transporter 1
SGLT3	soldium-coupled glucose transporter 3
SLC6A19	sodium-dependent amino acid transporter
SLC38A2	sodium-dependent amino acid transporter
SPSS	Statistical Package for the Social Sciences
St/SmI	stomach and small intestine
t	time
tmax	time needed to reach a maximal level of satiation
Tmax	time to maximal plasma concentration
tPYY	total peptide tyrosine tyrosine
TRH	thyrotropin-releasing hormone
Trpm5	transient receptor potential channel type 5
T1Rs	family 1 taste receptors
T1R2/T1R3	sweet taste receptor
UNESCO	United Nations Educational, Scientific and Cultural Organization
US	United States
VAS	visual analogue scale
VMN	ventromedial nucleus
vs.	versus
VSt	ventral striatum
VTA	ventral tegmental area

WHO	World Health Organization
WMA	World Medical Association
α-MSH	α-melanocyte-stimulating hormone
µL	microliter
µmol	micromol
µU	microunits
^{13}C	stable isotope of carbon
℃	degree celsius
%	percentage

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