Small intestinal targets involved in food intake regulation

'From nutrient to satiety signal'

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Abstract

Background and aim: The worldwide increasing prevalence of overweight and obesity raises concerns for health. There is a clear need for preventive strategies, because current preventative interventions have proven to be unsuccessful in the long term. New strategies may be developed based on targets in the small intestine by activating satiety signals. The thesis aimed to investigate small intestinal targets contributing to food intake regulation. These targets included serotonin, the vagal nerve and the intestinal brake mechanism.

Methods: The effects of ileal stimulation with safflower oil (lipid mixture), casein (protein), sucrose (carbohydrate) and rebaudioside A (non-caloric sweetener) on GLP-1 and PYY release were investigated by applying an porcine *ex vivo* intestinal segment model. The same model was also used to investigate if serotonin is involved in (non-) nutritional-induced GLP-1 and PYY release.

The contribution to satiation of GLP-1 and CCK receptors at the vagal nerve, was studied by investigating the effects of GLP-1 and CCK receptor antagonists on *ad libitum* food intake in a pig model of subdiaphragmatic vagotomy.

Two placebo controlled randomized crossover studies were performed in healthy volunteers to investigate the effects of small intestinal macronutrient delivery on *ad libitum* food intake and satiety signals. The first study compared the effects of duodenal, jejunal and ileal casein delivery on *ad libitum* food intake and satiety signals. The second study investigated if ileal delivery of all three macronutrients results in activation of satiety signals and reduction in *ad libitum* food intake. In addition, it was investigated if ileal delivery of native casein is efficiently digested and absorbed and does not result in adverse effects. In both studies the nutrients were delivered to the small intestine by inserting a nasointestinal feeding tube in healthy volunteers.

Results: All macronutrients and rebaudioside A stimulated GLP-1 and PYY release from ileal tissue segments. Protein and fat stimulated serotonin release. Inhibiting the reuptake of serotonin resulted in enhanced nutrient induced GLP-1, PYY and CCK release. Serotonin stimulated GLP-1 release from enteroendocrine cells via a serotonin receptor mediated process.

Results of the *in vivo* pig study showed that antagonism of the CCK receptor increased food intake in both vagotomized and sham operated pigs. Blocking the GLP-1 receptor did not affect food intake in both groups.

The human studies showed that ileal protein delivery inhibited food intake and activated satiety signals as compared to duodenal or jejunal protein delivery. Also, ileal delivery of small quantities (51.7 kcal) of each macronutrient decreased food intake and activated satiety signals. In addition, it was shown that ileal delivery of native casein resulted in a time and concentration depended increase in plasma concentrations of amino acids and did not result in activation of immune responses nor in gastrointestinal complaints.

Conclusions: The data presented in this thesis show that ileal delivery of all macronutrients results in activation of satiety signals and reduction of food intake. Stimulation of the ileum resulted in the strongest activation of satiety signals and inhibition of food intake compared to duodenal and jejunal stimulation. Besides direct nutrient-receptor interaction, the ileum senses (non-)nutritional stimuli via serotonin mediated processes resulting in GLP-1 release. In conclusion, these results demonstrate that targeting the ileum with small amounts of macronutrients is safe and has potential as a weight management strategy.

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Chapter 1

General introduction

Worldwide, the prevalence of obesity has more than doubled since 1980. In 2014, more than 1.9 billion adults were overweight of which 600 million were obese (1). In the Netherlands the number of adults being overweight and obese has increased as well. Between 1985 and 2014 the number of overweight people increased from 33.1% to 43.8%, of which 12.2% were obese. By comparison, in 1985 the number of people that were obese in the Netherlands was only 5.1% (2).

The excessive accumulation of body fat is associated with various health risks such type II diabetes, cardiovascular diseases and cancer (3). These health risks are associated with increased all-cause mortality (4). Because of the established health risks and substantial increases in prevalence, obesity has become a major global health challenge (5).

What is obesity?

According to the world health organization (WHO) overweight and obesity are defined as 'abnormal or excessive fat accumulation that may impair health.

A body mass index (**BMI**), which is the weight in kilograms divided by the square of the height in meters (kg/m^2), of equal to or more than **25** is defined as **overweight**, and a BMI equal to or more than **30** is defined as **obese** (1)

Not surprisingly there is a clear need for preventive strategies, as well as the need for therapies and non-surgical strategies that lead to a reduction in energy intake, weight loss and remission of diseases such as type II diabetes. To find such strategies and therapies it is crucial to understand how body weight is regulated and food intake is controlled. It is well established that when average caloric intake exceeds total energy expenditure, this result in a positive energy balance and finally results in excessive accumulation of body fat (6). Despite the fact that the rude causes of weight gain are relatively well understood, it has proven difficult to find strategies for weight management.

Energy balance and appetite regulation

Energy balance is the balance between energy intake and total energy expenditure. When energy intake repeatedly exceeds energy expenditure, this results in weight gain (7). To find targets that help people to reduce their energy intake it is important to understand how the process of food intake and meal termination is regulated by the body. The drive for energy intake depends on both external and internal factors. External factors include conditioned and environmental factors of which many are highly variable throughout the day the day (8). Humans do not only eat to satisfy their energy demand and appetite, but also for many other reasons such as sensory hedonics, sensory stimulation, tension reduction, social pressure and boredom. Internal factors for food intake include sensory, cognitive, postingestive and postabsoptive signals (7).

The first event in food intake regulation, which is initiated before actual food ingestion starts is the so-called cephalic phase response. During this phase, physiological signals are generated by the sight, smell, and oromucosal sensation of food. Cephalic phase responses are occurring in many parts of the gastrointestinal tract (GI) and their presumed functions are to anticipate to the ingestion of food. During food ingestion itself, two processes are activated that bring eating to a halt and suppress hunger. The first is satiation, which is the process that leads to meal termination and therefore controls meal size. The second is satiety. Satiety can be seen as a behavioral state including the post- and preprandial phase. Satiety coordinates our eating behavior and controls the frequency of eating episodes (7, 9). Satiety and satiation are activated by different kinds of satiety signals triggered by the ingestion of food. In figure 1.1 the satiety cascade as originally proposed by Blundell in 1994 is shown, illustrating how satiation and satiety coordinate our eating behavior (7, 10).

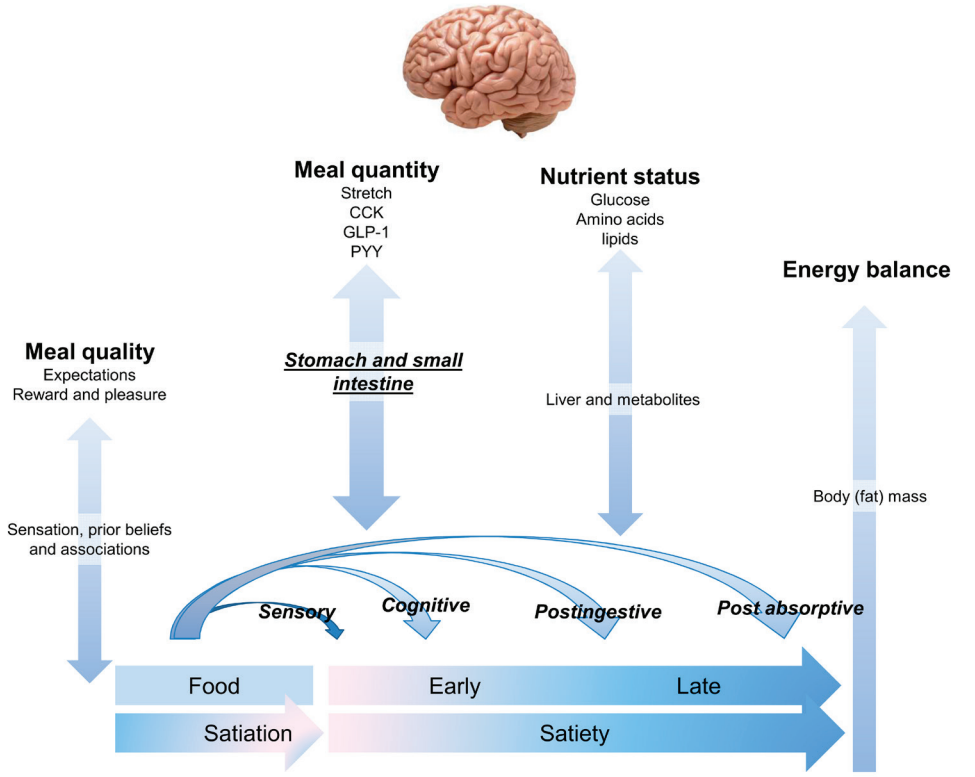

Figure 1.1 The satiety cascade, showing the expression of appetite by the relation between physiological and behavioral events and physiological and metabolic events (*Figure adapted from Blundell et al. 1994*).

Various organs (e.g. the brain, adipose tissue, liver, muscles, pancreas and central nervous system) contribute to the signals generated during this satiety cascade. The GI tract is

one of the main organ systems involved in the generation of physiological signals that promote satiation and satiety. One of the main functions of the GI tract is to digest ingested food and to absorb nutrients, vitamins and other important building blocks for the body. Simultaneously, the GI tract senses the ingestion of food and generates signals to activate satiation and thereafter satiety. Various chemosensory mechanisms along the entire GI tract are continuously monitoring the concentration of nutrients, digestion products and microbial metabolites. Together with their effect on GI hormone secretion, these chemosensory processes are often referred to as 'nutrient sensing'. Nutrient sensing involves the action of different receptors and transporters located on membranes or within the cytoplasm of enterocytes, brush cells and enteroendocrine cells (EEC) (11). The primary sensor of ingested food is the enteroendocrine system. This system is responsible for secreting an array of gut hormones, which act in concert to modulate multiple physiological responses such as gastrointestinal motility and secretion, glucose homeostasis, satiation and satiety (12). The GI tract represents the largest endocrine organ in the human body. Enteroendocrine cells are located throughout the GI tract. These cells constitute <1% of the cell population in the intestinal epithelium, but play a critical physiological role and represent an important component of the gut-brain axis (13). At least 15 types of EECs have been described, capable of releasing gut hormones that influence processes such as gut motility, gastric acid secretion and energy intake (13, 14).

Nutrient sensing by G-protein coupled receptors

The enteroendocrine system interacts with and reacts to individual dietary components and nutrients intra- and extracellular (15). Several nutrient transporters and G-protein coupled receptors (GPCRs) that are expressed on enteroendocrine cells sense macronutrients such as lipids, carbohydrates and proteins. Activation of these transporters and GPCRs results in downstream signaling and several studies showed that activation of these receptors results in the release of gut hormones such as glucagon-like peptide 1 (GLP-1), cholecystokinin (CCK) and peptide YY (PYY) (12).

Lipids activate several GPCRs expressed on EECs which result in the release of GLP-1 (16, 17). EECs express GPCRs such as GPR120 (or free fatty acid receptor 4 FFAR4) and GRP40 (FFAR1) both activated by long chain fatty acids (18), whereas short chain fatty acids activate FFAR2 and FFAR3 (19, 20). Another GPCR involved in lipid mediated GLP-1 release is GPR119, which is known to be activated by oleoylethanolamine (OEA) (21).

Carbohydrates are predominantly sensed in the form of glucose. The proposed receptor involved in glucose uptake is the Na^+ dependent sodium/glucose cotransporter member 1 (SGLT-1) (12). There is also evidence for a so-called sweet taste receptor, a G-protein coupled heterodimer (T1R2/T1R3). It has been proposed that activation of this receptor by glucose and non-caloric sweeteners induces the release of satiety hormones

(22). However, if activation of this sweet taste receptor results in GLP-1 release is also subject to debate. Although glucose ingestion increases GLP-1 concentrations in rats (23) and humans (24), the ingestion of non-caloric sweeteners did not stimulate GLP-1 release *in vivo (23, 25)*.

Proteins are digested to- and mainly sensed as amino acids or di-and tripeptides. Amino acid-induced gut hormone release is associated with activation of the G-coupled calcium sensing receptor (CaSR) (26-28) and the umami taste receptor dimer T1R1/T1R3 (29) as well as GPRC6A (30). Larger protein fragments such as di-and tripeptides have been linked to activation of the peptide transporter 1 (PEPT1) (31-33).

Satiety signals released by the enteroendocrine system

Satiation depends on short term satiety signals such as stomach distension as well as on the release of gut hormones such as GLP-1, CCK and PYY (8). These hormones have in common that they are released from enteroendocrine cells in the small intestine. They are referred to as satiating peptides since exogenous administration of physiological concentrations of these hormones dose dependently results in decreased food intake without affecting illness (34).

GLP-1 derives from the proglucagon gene which is highly conserved among mammals. After transcription, GLP-1_{1-36} is n-terminally truncated to the active forms GLP-1_{7-36} and GLP-1_{7-37} by posttranslational processing in enteroendocrine L-cells(35). At physiological concentrations the active forms of GLP-1 stimulate insulin secretion (36, 37) , insulin gene transcription (38) and improve pancreatic β-cell glucose responsiveness (39). Once released, the bioactive forms GLP-1_{7-36} and GLP-1_{7-37} are very rapidly cleaved by dipeptidyl peptidase IV (DPPIV) into the inactive form GLP-1_{9-36} (40). Besides its incretin effects, GLP-1 contributes to satiety and satiation. Intravenous infusion of GLP-1_{7-36} decreases gastric emptying and reduces of food intake, suppresses hungerand increases the period of postprandial satiety (41-45). GLP-1 is found in enteroendocrine L-cells in the jejunum, ileum and colon (46). Other hormones which contribute to satiation and satiety are PYY and CCK. PYY is predominantly located in the distal ileum and colon, whereas CCK is found in the duodenum and jejunum (14, 47).

CCK is found in EECs present in the duodenal and jejunal mucosa. Intestinal CCK is secreted in response to luminal nutrients such as proteins and lipids. The CCK prepropeptide is processed by endoproteolytic cleavage into at least six peptides, ranging from 8 to 83 amino acids in length. The multiple bioactive forms share a common carboxy-terminal octapeptide with an O-sulfated tyrosine. The major circulating forms of CCK are CCK_8, CCK_{22}, CCK_{44} and CCK_{58} (34). CCK has a large variety of physiological functions in the human body, such as stimulation of gall bladder contraction, pancreatic enzyme secretion, intestinal motor activity and inhibition of gastric emptying. When CCK is secreted, the gallbladder and pancreas are stimulated to excrete their juices containing digestion enzymes and bile (34, 48). Besides its function

to stimulate pancreas juices, CCK contributes to satiation and satiety. Intravenous infusion of CCK suppresses food intake (49-51).
Similar to GLP-1, PYY is produced by enteroendocrine L-cells, most of which co-express GLP-1. PYY is secreted postprandially in proportion to caloric load. Once released from L-cells PYY_{1-36} is rapidly proteolyzed by DPPIV into $PYY_{3-36,}$ and unlike GLP-1, the cleaved product PYY_{3-36} is bioactive. Similar to CCK and GLP-1, intravenous infusion of PYY_{3-36} results in decreased hunger and ad libitum food intake, without causing nausea, affecting food palatability or altering fluid intake (52).
Receptors of GLP-1, PYY and CCK are expressed in various organs including the pancreas, intestine and the brain (47, 53). Once released, GLP-1, CCK and PYY may act paracrinely, neurocrinely or endocrinely. Secreted GLP-1, CCK and PYY diffuse into the lamina propria and enter the systemic circulation via the hepatic portal vein. Systemic circulating concentrations rise within 15 minutes of food intake. Once released from EECs these hormones may act neurocrine as well by interacting with receptors expressed at the nodose ganglion on afferent sensory vagal nerve fibers (13, 54, 55). Vagal afferent fibers transmit information such as gastric distension, ingested dietary composition and water content to the nucleus of the solitary tract located in the brainstem. This feedback mechanism by vagal afferent fibers is part of the so-called gut-brain axis and contributes to satiation as well.

Potential targets and strategies for weigh management

The increase in knowledge about physiological mechanisms that control eating and body weight is in contrast to the lack of available nutritional and pharmacological therapies leading to safe efficient and long lasting body weight reduction (56). The most effective treatment for severe obesity is bariatric surgery. Bariatric surgery is normally indicated for people who have a BMI of $\geq$40 kg/m^2 or a BMI of $\geq$35 kg/m^2 plus comorbid conditions that will be improved by weight loss (57). Bariatric surgery is effective both in terms of achieved and maintained weight loss and with regard to improvement of obesity related comorbidities (58, 59). One of the most frequently performed bariatric operation worldwide is Roux-en-Y gastric bypass. This surgery results in body weight reduction of about 20% - 30% and this weight loss is maintained for several years after surgery (58-60). By applying this technique, nutrients bypass the duodenum and proximal jejunum and as a consequence nutrients are directly delivered into the distal small intestine (57). In addition to weight loss, a considerable advantage of RYGB is the immediate improvement in insulin sensitivity, which acts in concert with increased release of hormones such as GLP-1 and PYY. This immediate effect on gut hormone release and remission of type II diabetes is typical for RYGB, since other surgeries such as gastric banding typically show these effects only after a longer period of weight loss. The mechanism by which RYGB surgery decreases body weight and improves metabolic health is not completely understood yet. Although restrictive or

malabsorption has been often proposed to be involved in weight loss after RYGB, there is more evidence available showing that the underlying mechanisms are metabolic (57). One proposed mechanism by which RYGB may have its effect is enhanced nutrient stimulation of EECs in the distal small intestine, resulting in the release of gut hormones such as GLP-1 and PYY (61). Although RYGB is very effective in causing weight loss and immediately improves metabolic health of obese people, there is a need for nonsurgical strategies that help people limiting their daily energy intake.
Similar to RYGB, a non-surgical intervention by which intact nutrients are shunted into the distal ileum would be by activation of the so called ileal brake. Activation of this feedback loop slows or 'brakes' gastric emptying and duodenal-jejunal motility. The ileal brake is activated when energy containing nutrients, mainly lipids, are delivered beyond the duodenum and jejunum into the ileum, as is the case after RYGB. Infusion of lipids into the ileum results in enhanced satiety signals, satiation and a reduction in of energy intake (62-64).

Aims and outlines this thesis

Previous research shows that satiation is regulated by satiety signals released from the enteroendocrine system after the ingestion of food. This system in the small intestine has proven to be a potential target for surgical weight management strategies. However, to prevent normal weight and overweight people to become obese, other nonsurgical weight management strategies are needed. Before such strategies can be invented several knowledge gaps remain to be filled. By addressing how potential targets may activate satiety signals, more effective nonsurgical or nutritional strategies may be invented to help people limiting their daily energy intake.
The aim of this thesis is therefore: to investigate potential small intestinal targets that may contribute to satiation. How the chapters contribute to this aim is shown in figure 1.2

Small intestinal hormone release

There are studies investigating the effects of macronutrients such as glucose on the release GLP-1, PYY and CCK. We were interested if other dietary components also affect the release of GLP-1 and PYY. In **chapter 2** it has been investigated if the *Stevia rebaudiana* derived non-caloric sweetener rebaudioside A also stimulates the release of GLP-1 and PYY. For this study a previously described *ex vivo* porcine model was used (65).
Sensing nutrients via GPCRs on EECs results in the release of satiety hormones. It has been proposed by others that EECs express GPCRs which are responsible for nutrient-induced GLP-1, PYY and CCK release. Undigested macronutrients, and other dietary compounds such as rebaudioside A also stimulate GLP-1 release. The exact mechanism

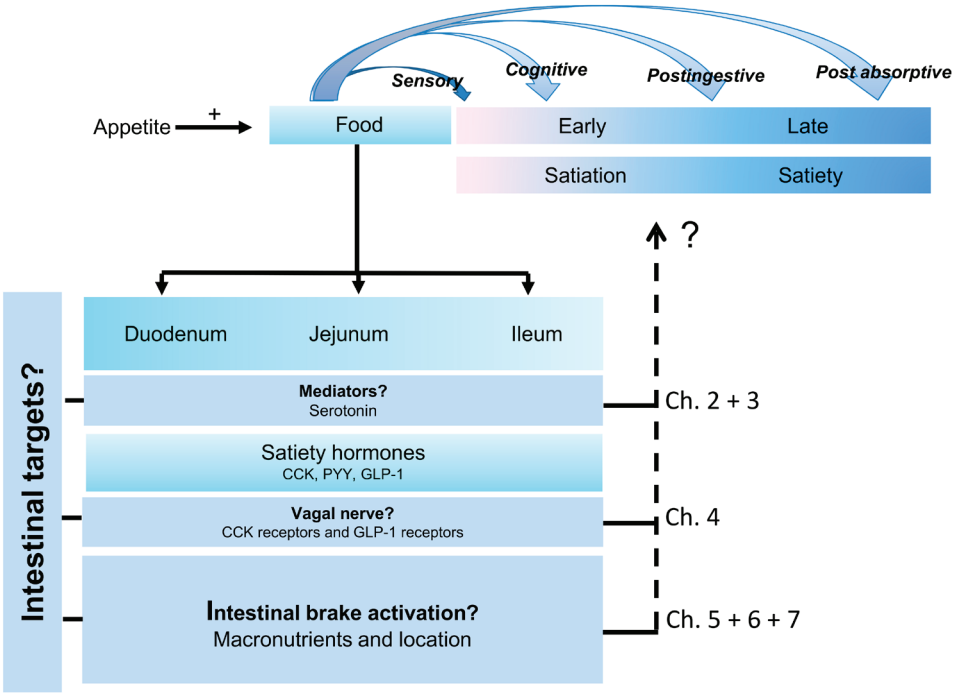

Figure 1.2. Schematic overview of the aims of this thesis and the contribution of the chapters to these aims.

behind undigested macronutrient or rebaudioside A-induced GLP-1 release, is still unknown. A potential small intestinal molecular target involved in food intake regulation is serotonin. Approximately 90% of serotonin is stored in enterochromaffin cells in the small intestine, and modulation of the serotonergic system does affect food intake. In **chapter 3** we investigated if serotonin is involved in nutrient and rebaudioside A induced GLP-1 release. In addition, we investigated the effects of macronutrient and rebaudioside A on the release of serotonin. This was studied in the same *ex vivo* porcine intestinal segment model as which was used in chapter 2.

Neural contribution to satiation by receptors located at the vagal nerve

When GLP-1 and CCK are released from EECs they can act either endocrinely via the blood to activate receptors, paracrine to act on neighboring cells or neurocrine via receptors located at the vagal nerve endings. In **chapter 4** we investigated the contribution of neurocrine CCK and GLP-1 signaling to satiation. This was studied by investigating the effects of CCK and GLP-1 receptor antagonist on ad libitum food intake in a pig model of subdiaphragmatic vagotomy. In this study we also explored the effects of subdiaphragmatic vagotomy on postprandial release of GLP-1 and CCK.

Targeting the intestinal brake as a tool for weight management

Previous research has shown that ileal brake activation by intraileal lipid infusion results in increased satiation and decreased energy intake. This mechanism may therefore be considered as a potential target for weight management. Whether other macronutrients such as protein and carbohydrate also activate the ileal brake, and whether the ileal brake is more effective in reducing food intake as compared to duodenal or jejunal brake remains to be investigated. In **chapter 5** we compared the effects of intraduodenal, intrajejunal and intraileal protein infusion on satiation. In this chapter the effect of intraduodenal, intrajejunal and intraileal protein infusion on GLP-1, PYY and CCK are evaluated as well. In **chapter 6** we investigated whether different macronutrients infused into the ileum increase satiation and decrease energy intake. In this chapter the effects of intra-ileal infusion on gut hormone release and appetite ratings have been studied as well. In **chapter 7** we investigated if native protein infused into the ileum does not result in immediate reverse reactions. In this chapter it was also investigated if amino acids present in this native protein infused do recover in plasma during ileal infusion in healthy human subjects.

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Chapter 2

Steviol glycoside rebaudioside A induces glucagon-like peptide-1 and peptide YY release in a porcine ex vivo intestinal model

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Abstract

Glucagon-like peptide-1 (GLP-1) and peptide YY (PYY) are hormones important for satiation and are involved in the process called "ileal brake". The aim of this study was to investigate the GLP-1- and PYY-stimulating efficacy of rebaudioside A, casein, and sucrose. This was studied using tissue segments collected from various regions of the pig small intestine. GLP-1 release was strongest from the distal ileum. There, control release was 0.06 ± 0.01 (GLP-1) and 0.07 ± 0.01 (PYY) pmol/cm^2 of tissue. Rebaudioside A (2.5, 12.5, and 25 mM) stimulated GLP-1 release (0.14 ± 0.02, 0.16 ± 0.02, and 0.13 ± 0.02 pmol/cm^2 of tissue, $p<0.001$) and PYY release (0.19 ± 0.02, 0.42 ± 0.06, and 0.27 ± 0.03 pmol/cm^2 of tissue, $p<0.001$). Sucrose stimulated GLP-1 release (0.08 ± 0.01 pmol/cm^2 of tissue, $p<0.05$) only at 10 mM. Casein (0.5%, 1%, and 2.5%, w/v) stimulated GLP-1 release (0.15 ± 0.03, 0.13 ± 0.02, and 0.14 ± 0.01 pmol/cm^2 of tissue, $p<0.001$) and PYY release (0.13 ± 0.02, 0.20 ± 0.03, and 0.27 ± 0.03 pmol/cm^2 of tissue, $p<0.01$). These findings may help in developing dietary approaches for weight management.

Introduction

The presence of nutrients and other molecules in the small intestine stimulates the release of satiety hormones, such as Cholecystokinin (CCK), Glucagon-Like Peptide-1 (GLP-1), and Peptide YY (PYY) (1, 2). The release of these hormones results in decreased gastric emptying and activation of the so-called intestinal brake. This poorly explored mechanism refers to a physiological feedback mechanism that is triggered by nutrients at a specific location in the intestine resulting in reduced appetite and food intake (3, 4). Lipids have been widely researched and are known to activate this ileal brake mechanism resulting in the release of satiety hormones and reduced food intake (5-8). However, whether other macronutrients such as carbohydrates and proteins could also activate the ileal brake mechanism is still unknown (4). Knowledge of the satiety-hormone-inducing properties of macronutrients is relevant to better understanding the intestinal response to ingested food and may contribute to the development of products with satiating properties.

The efficacies of non-caloric sweeteners to induce the release of satiety hormones are also partly unknown. However, a study by Anton et al. (2010) showed that preloads containing the sweetener stevia reduced daily caloric intake by 301 kcal and reduced postprandial glucose and insulin levels compared to preloads containing sucrose (9). Such a reduced caloric intake as well as glucose and insulin responses may be caused by reduced caloric content of the preload but may also be caused by other mechanisms such as GLP-1 release. The glycoside rebaudioside A is the one of the most abundant sweet compound extracted from the leaves of *Stevia rebaudiana bertoni*. Rebaudioside A is reported to be the most potent sweetener among the steviol glycosides (10) with a relative sweetness of 200 – 400 times greater than that of sucrose (11). Rebaudioside A is increasingly used in food products as a natural noncaloric sweetener. It has been approved in the United States since 2008 (12), and in the European Union since 2010 (13).

It is unknown if ileal delivery of macronutrients other than lipids and sweeteners could also stimulate the release of satiety hormones and consequently activate the ileal brake. Stimulation of GLP-1 and PYY release from isolated ileal tissue has been demonstrated for lipids including unsaturated fatty acids. This was shown using a recently described ex vivo porcine intestinal model (14). For other macronutrients such as sucrose and casein such studies have not been performed yet, although their efficacy to induce gut hormone release has been shown in cell cultures (15-17). The pig was chosen as a model because the gastrointestinal tract of the pig shows a high degree of macroscopic and microscopic resemblance with that of humans (18-20). The present study primarily focused on the GLP-1 and PYY inducing efficacy of casein, sucrose and the non-caloric sweetener rebaudioside A at various locations of the small intestine. We hypothesize that rebaudioside A, the protein casein, and the carbohydrate sucrose are effective

stimulators of GLP-1 and PYY release from various locations of the small intestine, and thus may be effective activators of the ileal brake *in vivo*.

Materials and methods

Chemicals

Casein protein (food grade, sodium caseinate purity >82% batch number 158156) was obtained from Dutch Protein Services BV, Tiel, The Netherlands. Rebaudioside A (**Figure 2.1**, food grade, purity >97% batch number 20110301, ChemSpider ID: 5294031) was obtained from SteviJa Natuurlijk BV, Drachten, The Netherlands. Sucrose (food grade, purity >99.9%) was supplied by Suiker Unie, Oud Gastel, The Netherlands. Safflower oil (food grade, composed of 3-8% C16:0, 2-5% C18:0, 9-15% C18:1 and 75-85% of C18:2) was provided by De Wit Specialty oils, De Waal, The Netherlands. Xantham Gum and Guar gum were supplied by GF Supplies, Amsterdam, The Netherlands. Chemicals to make the Krebs Ringer bicarbonate (KRB) buffer (D-glucose 1.8 gram/L, Magnesium Chloride 0.0468 gram/L, Potassium chloride 0.34 gram/L, Sodium Chloride 7.0 gram/L, Sodium Phosphate Dibasic 0.1 gram/L, Sodium Phosphate Monobasic 0.18 gram/L, HEPES 5,579 gram/L) were obtained from Sigma, Zwijndrecht, The Netherlands. The pH of the KRB buffer was adjusted to pH 7.4.

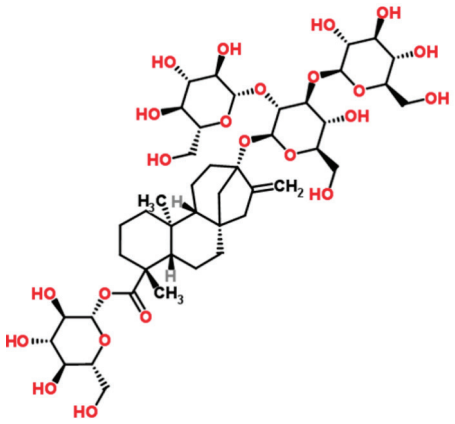

Figure 2.1 Chemical structure of Rebaudioside A **(***Adapted from ChemSpider ID: 5294031)*

Collection of porcine intestinal tissues

Intestinal tissues were obtained from ten healthy male pigs (*Sus scrofa domesticus*). The pigs were considered healthy after evaluation by a veterinarian. The protocol for this study was approved by the Animal Ethics Committee Utrecht (Ethics Committee permit number 2012.I.09.096, Utrecht, The Netherlands). Pigs had a mean (± SEM) body weight of 141 ± 2 kg, and mean (± SEM) age of 6 ± 0.1 months. They were housed in

groups and were fed *ad libitum* with standard pig diet (vitaal P 003103, de Heus, Ede, The Netherlands) and free access to water. Within 10 minutes after the pigs were sacrificed, intestines were excised and segments of different anatomical regions were stored in ice-cold oxygenated (95% O_2, 5% CO_2) KRB buffer. Jejunum (4.5 meter proximal to the ileocecal junction) and proximal, mid and distal ileum (0.5 meter of intestine sampled from 1.5, 1 and 0.5 meter proximal to the ileocecal junction, respectively) were collected for the experiments. Tissues were transported to the laboratory and immediately used for *ex vivo* experiments. Time between excision and start of the experiments was approximately 30 minutes.

Nutrient exposure of intestinal tissue and viability tests

Incubation with casein (0.5, 1 and 2.5% w/v), rebaudioside A (2.5, 12.5 and 25 mM), sucrose (2.5, 5 and 10 mM), and safflower oil (3.35% w/v) were performed with porcine intestinal tissue as described previously (14) with some minor adaptations. In brief, upon arrival at the laboratory, the intestine was rinsed with KRB buffer and cut open in a longitudinal direction. The outer muscle layers were carefully stripped off with the basolateral side upwards. The mucosal tissue was placed on a gauze (pores = 250 μm, Sefar Nitex 03-250/50, Sefar Heiden Switzerland) with the apical side upward, and circles of tissue with a diameter of 11.8 mm (about 1.0 cm^2) were punched out using a biopsy punch. The intestinal segments were randomized, per region, in a beaker glass and transferred to a 24-wells plate, filled with 500 μl ice cold KRB buffer/well, and kept on ice until start of the experiment. Thereafter, tissues were brought to room temperature within 30 min, followed by an pre-incubation step at 37°C for 1 hour in a humidified incubator (5% v/v CO_2). To study the release of GLP-1 and PYY, incubations were initiated by replacing the KRB buffer solution with 500 μl pre-warmed (37°C) KRB without D-glucose containing the test compounds. KRB buffer without D-glucose was used as a control since D-glucose might induce gastrointestinal hormone release. Then the tissue was put in a humidified incubator for 1 hour at 37°C at 5% v/v CO_2. After incubation, solutions were collected, mixed with 100 μM phenylmethanesulfonyl floride (PMSF) (Sigma Aldrich, Zwijndrecht, The Netherlands) to inactivate dipeptidyl peptidase IV, aliquoted and stored at 4°C for further analysis of lactate dehydrogenase (LDH), or stored at -80°C for further analysis of GLP-1 and PYY.

Tissue viability was checked by measuring leakage of intracellular LDH in supernatant. LDH, a stable cytosolic enzyme, was analyzed using a LDH kit (Sigma Aldrich, Zwijndrecht, The Netherlands). LDH activity in supernatant was expressed as a percentage of the total LDH activity present in control tissue collected before incubation. Total tissue levels of LDH were determined per region in 1.0 cm^2 tissue samples, which were homogenized in ice-cold KRB buffer with a Potter-Elvehjem-type Teflon pestle tissue grinder (Braun, Melsungen, Germany) for 5 minutes at 200 rounds per minute. Samples were only included for further analysis when LDH leakage did not

exceed 10% of the total LDH tissue content (14, 21). No samples had to be excluded applying this criterion.

Analysis of satiety hormones GLP-1 and PYY

GLP-1 active levels were analyzed using commercially available ELISA assay kits obtained from Millipore (Billerica, MA, USA) and used according to the manufacturer's instructions using a BioTek Synergy HT fluorescence microplate reader (BioTek Instruments Inc., Winooski Vermont, USA). Since the GLP-1 hormone gene sequence is highly preserved, this kit could be applied for analyzing porcine GLP-1. The range of this assay is approximately 2 – 100 pM. The inter-assay coefficient of variation is 11%, and the intra-assay variation is 6%. When necessary, samples were diluted in assay buffer from the kit to be in this range.

PYY release was measured from distal ileum only, since PYY is known to be co-localized with GLP-1 (22). PYY concentrations were measured using a PYY ELISA kit for total PYY (Bachem, Penisula Laboratories, San Carlos, CA, USA) according to the manufacturer's instructions using a BioTek Synergy HT microplate reader (BioTek Instruments Inc., Winooski Vermont, USA). This kit measures porcine PYY, which is identical to human PYY[23]. The range of this assay is approximately 0.32 – 5 ng/ml (4.6 – 1150 pM). The inter assay coefficient of variation for this assay is 6% and the intra assay coefficient of variation is 3%. The lower detection limit of this kit was 20 pg/ml (4.6 pM) It was not necessary to dilute samples for this assay.

Statistical analysis

Statistical analysis was performed using the SAS statistical software package (SAS version 9; SAS institute, Cary, NC, USA). Both variables GLP-1 and PYY were compared with a mixed analysis of variance model (ANOVA). GLP-1 and PYY were both log transformed to meet the requirements for ANOVA. The ANOVA model included the fixed factors dose solution (control, sucrose 2.5, 5 and 10 mM, casein 0.5, 1 and 2.5% w/v, rebaudioside A 2.5, 12 and 25 mM and safflower oil 3.35% (w/v), intestinal location (jejunum, and proximal mid and distal ileum) and the number of incubations per condition (incubations were performed in triplicate). The factor pig, which specifies the individual pig, was added to the model as random factor. The Tukey post hoc test was used to correct for multiple comparisons. Data are presented as the mean ± SEM and considered significant at $p<.05$.

Results

Effects of rebaudioside A and other compounds / mixtures on GLP-1 release

Table 2.1 shows the GLP-1 concentrations in the medium following incubation with the test compounds or mixtures using tissues taken from different locations. In all parts of the small intestine investigated, rebaudioside A caused a significant release of GLP-1 already at the lowest concentration tested (2.5 mM) ($p<0.01$), except for the jejunum where the release was significant at a concentration of 12.5 mM ($p<0.05$). The highest relative increase of GLP-1 after incubation with rebaudioside A was seen in the distal ileum ($p<0.001$). The release at 25 mM was not higher than at 12.5 mM. As also shown in **Table 2.1**, safflower oil (3.35% w/v) significantly increased GLP-1 release from the proximal ileum ($p<0.05$) and even more from the distal ileum ($p<0.001$). Safflower oil did not increase GLP-1 release from the mid-ileum and only marginally from the jejunum. At the concentrations tested sucrose did not stimulate GLP-1 release from jejunal and mid ileal tissue. The only significant stimulation was seen in tissues from the distal ileum at a sucrose concentration of 10 mM ($p<0.05$).
Casein, at 1 and 2.5 % (w/v) increased GLP-1 release ($p<0.001$) from tissues collected from the jejunum, whereas 0.5% (w/v) casein did not. Both 0.5 and 2.5% (w/v) casein

Table 2.1 GLP-1 concentrations released from jejunal, proximal, mid and distal ileum intestinal tissue segments after exposure to various compounds.

		GLP-1 release (pmol/cm² tissue)			
Solution	Concentration	Jejunum	Proximal ileum	Mid ileum	Distal ileum
Control		0.04± 0.01	0.07 ± 0.02	0.07 ± 0.01	0.06 ± 0.01
Safflower oil (%)	3.35%	0.07± 0.02	0.11±0.03 [a]	0.09±0.02	0.13 ±0.02 [c]
Sucrose (mM)	2.5	0.05±0.01	0.10±0.02	0.07±0.01	0.06±0.01
	5	0.05±0.01	0.17±0.05	0.08±0.01	0.07±0.01
	10	0.06±0.01	0.07±0.01	0.08±0.01	0.08±0.01 [a]
Casein (%)	0.5	0.06±0.01	0.12±0.02 [c]	0.09±0.01	0.15±0.03 [c]
	1	0.09±0.01 [c]	0.09±0.02 [b]	0.08±0.01	0.13±0.02 [c]
	2.5	0.08±0.02 [c]	0.13±0.03 [c]	0.08±0.01	0.14±0.01 [c]
Rebaudioside A (mM)	2.5	0.07±0.01	0.14±0.02 [c]	0.13±0.02 [b]	0.14±0.02 [c]
	12.5	0.07±0.01 [b]	0.11±0.02 [c]	0.18±0.04 [c]	0.16±0.02 [c]
	25	0.07±0.01 [b]	0.14±0.02 [c]	0.13±0.02 [b]	0.13±0.02 [c]

[a] $p<0.05$, [b] $p<0.01$, [c] $p<0.001$; p-values are based on comparison to control (ANOVA + Tukey correction). The incubations were performed in triplicate (n=6 for jejunum, n=10 for proximal, mid and distal ileum

stimulated GLP-1 release from the proximal ileum ($p<0.001$). A concentration of 1% (w/v) casein stimulated GLP-1 release from proximal ileum ($p<0.010$), but exposure to 0.5% and 2.5% (w/v) casein caused a higher GLP-1 release. None of the casein concentrations, 0.5, 1 and 2.5% (w/v), stimulated GLP-1 release from the mid ileum. All three casein concentrations stimulated GLP-1 release from the distal ileum ($p<0.001$). Similar to rebaudioside A, the most pronounced stimulation of GLP-1 by casein was observed in the distal ileum.

Regional differences in GLP-1 release

Exposing intestinal tissue to the control did not result in regional differences in control GLP-1 release. However, after exposure to the nutrients, regional differences were observed. For GLP-1 both an overall incubation and location effect ($p<0.001$), and an interaction effect between incubation and location was found ($p<0.05$). Overall GLP-1 release from all ileal locations (distal, mid and proximal ileum), was higher than GLP-1 release from the jejunal locations ($p<0.001$). Furthermore, GLP-1 release from the distal ileum was higher than GLP-1 release from the mid ileum ($p<0.001$). Neither hormone release from mid and proximal ileum differed, nor did release from proximal and distal ileum ($p<0.08$).

Table 2.2 PYY concentrations released distal ileum intestinal tissue segments after exposure to various compounds

		PYY (pmol/cm² tissue)
Solution	Concentration	Distal ileum
Control		0.07±0.01
Safflower oil (%)	3.35	0.17±0.01 [b]
Sucrose (mM)	2.5	0.14±0.03
	5	0.12±0.02
	10	0.08±0.01
Casein (%)	0.5	0.13±0.02 [a]
	1	0.20±0.03 [b]
	2.5	0.27±0.03 [b]
Rebaudioside A (mM)	2.5	0.19±0.02 [b]
	12.5	0.42±0.06 [b]
	25	0.27±0.03 [b]

[a] $p<0.01$, [b] $p<0.001$; p-values are based on comparison to control (ANOVA + Tukey correction).The incubations were performed in triplicate (n=10).

Effects of compounds and mixtures on PYY release

The compounds and mixtures stimulating GLP-1 release were also tested for their PYY releasing capacity from the distal ileum (**Table 2.2**). Safflower oil was found to stimulate PYY release, whereas sucrose at the concentrations tested did not. Casein stimulated PYY release from the distal ileum at all three concentrations tested. Release after exposure to a concentration of 1% (w/v) was higher than with 0.5% (w/v) casein ($p<0.05$). Rebaudioside A stimulated PYY release from distal ileum ($p<0.001$). The effect at 12.5 mM rebaudioside A was higher than that at 2.5 mM ($p<0.05$).

Discussion

Our study confirmed our hypothesis that rebaudioside A and casein induce GLP-1 and PYY release from porcine intestinal tissue segments. Remarkably, rebaudioside A was found to stimulate GLP-1 release from all ileal and jejunal regions investigated, whereas sucrose (10 mM) only caused GLP-1 release from the distal ileum. Rebaudioside was effective in stimulating hormone release from more locations of the small intestine than casein. Therefore, taking into account that the model and the tested concentrations make a direct comparison difficult, our results suggest that rebaudioside A and the protein casein are candidate substances that may activate the ileal brake *in vivo* via the release of GLP-1 and PYY.

Some sweeteners including sucralose were found not to affect satiety hormone release *in vivo (*24, 25). For *Stevia* Anton *et al.* (2010) demonstrated satiating inducing properties which may be due to increased satiety hormone release. However, since satiety hormones were not measured in that study, this association cannot be made from these findings. To our knowledge no reports have shown the satiety hormone-inducing capacity of the non-caloric sweetener rebaudioside A as was done in our study.

Nutrients, such as casein and sucrose, could stimulate satiety hormone release following interaction with G-protein coupled receptors and some solute carrier transporters located on enteroendocrine cells in the intestinal epithelium (26). A potential mechanism by which rebaudioside A could stimulate GLP-1 and PYY release is via the sweet taste receptor. Recently, it was found that rebaudioside A has affinity for the sweet taste receptor TAS1R2/TASR3 (10). This receptor is involved in glucose dependent release of GLP-1 and PYY *in vivo* (27). Furthermore, TAS1R2/TASR3 is present on brush cells and enteroendocrine cells (28). However, other mechanisms than via the taste receptors by which rebaudioside A and casein could induce satiety hormone release cannot be excluded and should be further studied.

Already more than 100 compounds have been identified in *Stevia rebaudiana.* However, the steviol glycosides stevioside and rebaudioside A have been found to be the most abundant. Steviol glycosides are metabolized into steviol upon their arrival in the colon and are absorbed thereafter (29). Incubation of the murine enteroendocrine STC-1

cells with *Stevia* preparations resulted in higher GLP-1 and CCK release compared to incubation with sucrose (17), which is in line with our findings. The same study also demonstrated GLP-1 and CCK release after incubation with sucralose. However, only relatively high concentrations of 150 and 300 mM sucralose and of 600 mM sucrose were able to stimulate GLP-1 and CCK release from STC-1 cells. In our study 2.5 mM rebaudioside A resulted in higher GLP-1 release compared to 2.5 mM sucrose (0.14 ± 0.02 *vs.* 0.06 ± 0.01 $p<0.001$, distal ileum). Concentrations of sucrose and rebaudioside A in this study were in the same range (0-25 mM). Further *in vitro* studies should explore dose-effect relationships of these compounds.

Safflower oil was used as a positive control in our study. Safflower oil (3.35%) was found to activate the ileal brake *in vivo* and to increase satiety and gastrointestinal hormone concentrations (5, 7). As expected safflower oil effectively stimulated GLP-1 release from the ileum mainly from the distal part whereas no such stimulation occurred from the mid ileum. This difference may relate to the enteroendocrine cell distribution. Enteroendocrine L-cells are most abundant in the distal part of the small intestine (30) corresponding to the intestinal tissue content of GLP-1 (14). The protein casein increases satiety *in vivo* (31) and *in vitro* it stimulates GLP-1 release from enteroendocrine murine STC-1 and human NCI-H716 cells (15, 16, 32). In our study casein induced GLP-1 and PYY release which confirms these previous findings and suggests that casein is a candidate to induce the ileal brake *in vivo*.

Apart from the differences in efficacy of various nutrients to stimulate GLP-1 and PYY some other questions remain. An important question is to what extent the effects on satiety hormone release found in our model can be reproduced in *in vivo* models and specifically in humans. Another relevant question is whether the capacity to induce satiety hormone release will lead into satiation *in vivo*. In one study of Veldhorst *et al.*(2009) it was shown that breakfast with higher casein content (25% *vs.* 10%) increased VAS scores in fullness and satiety whereas subsequent energy intake was not reduced and GLP-1 plasma concentrations did not differ per condition (33). Food intake is regulated by a whole cascade of responses which have to be taken into account if studying satiation *in vivo*. Therefore, knowledge of the satiety hormone releasing capacity of macronutrients and non-caloric sweeteners could be useful to understand this so called 'satiety cascade' (34).

The current *in vivo* data are based on lipid infusions mainly. Those data could be extended into infusion studies using rebaudioside A and casein since the results from this study suggest that the satiety hormone releasing effect of sweeteners and proteins might be at least as effective as fat. These human interventions will then further substantiate the relevance of this *ex vivo* intestinal model.

One could question whether the concentrations of the selected compounds are relevant in view of those reached in the gut after dietary intake. Nutrient concentrations in the small intestine depend on the rate of gastric emptying of a meal, the rate of absorption,

and (or) digestion of the nutrients the meal contained (35-37). Data on actual concentrations of the selected products are very scarce. There is only one animal study showing that luminal glucose concentrations after a meal are varying between 0.2 and 48 mM depending on postprandial time and the small intestinal region (38). Although the data are limited one could estimate intestinal concentrations on the following assumptions; 1 cup of coffee or tea (200 ml) with 0.3 gram of rebaudioside A (based on ADI of 0.4 mg/kg BW/day), or 1 cube of sugar (1 gram), in a total volume of 300 ml (100 ml gastric juice is added in the stomach) (17). These assumptions would yield an estimate of approximate concentrations of 10 mM sucrose and 1 mM rebaudioside A for the small intestine. The estimate for casein is based on the assumption that 1 glass of milk is consumed (total volume including gastric juice 300 ml) with a casein concentration of 80% of total milk protein content which is approximately 35 g/L. This means that a concentration of approximately 3% may initially be reached in the small intestine. Although these values are estimates only they suggest that the concentrations used in this study may be relevant from a dietary perspective.

The hormone secreting capacity of nutrients and other compounds is often studied *in vitro* using enteroendocrine cell lines including murine STC-1 (15, 39-41), GLUTag cells (42), and the human NCI-H716 cell line (43). The advantage of the current *ex vivo* model compared to cell lines is that this model consist of all different cell types present in the gut. Another model to study the GLP-1 and PYY inducing capacity of nutrients is the Ussing chamber technology using animal or human biopsies (44). It is however, challenging to obtain human biopsies especially from the ileum which makes the *ex vivo* intestinal porcine segment system a valuable alternative. However, there are still some technical limitations such as tissue viability and intra-subject variation. In this study tissue viability was evaluated by LDH leakage as before (14) and LDH leakage did not exceed 10%. Also, hormone release in the control condition was low. Both conditions indicate a good tissue viability. However, we cannot exclude that hormone release may be due to aspecific processes like cell lysis. Also, a high number of replicates was needed because of a high intra-subject variation. This variation may be caused by the relatively low number of enteroendocrine cells (reported to account for only 1% of the epithelial cell population in the small intestine) and these cells may be unevenly distributed (2, 28).

In conclusion, the present study showed that rebaudioside A and casein, but not sucrose, induced satiety hormone release at various locations of the small intestine using an *ex vivo* model. These findings may contribute to the development of dietary approaches improving weight management and glucose control. However, *in vivo* studies are necessary to confirm the potential satiety inducing properties of rebaudioside A and casein *in vivo*.

Competing Interest

The authors have declared that no competing interests exist.

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Chapter 3

Nutrient-induced glucagon like peptide-1 release is modulated by serotonin

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Abstract

Glucagon like peptide-1 (GLP-1) and serotonin are both involved in food intake regulation. GLP-1 release is stimulated upon nutrient interaction with G-protein coupled receptors by enteroendocrine cells (EEC), whereas serotonin is released from enterochromaffin cells (ECC). The central hypothesis for the current study was that nutrient-induced GLP-1 release from EECs is modulated by serotonin through a process involving serotonin receptor interaction.

This was studied by assessing the effects of serotonin reuptake inhibition by fluoxetine on nutrient-induced GLP-1, PYY and CCK release from isolated pig intestinal segments. Next, serotonin-induced GLP-1 release was studied in enteroendocrine STC-1 cells, where effects of serotonin receptor inhibition were studied using specific and non-specific antagonists.

Casein (1% w/v), safflower oil (3.35% w/v), sucrose (50 mM) and rebaudioside A (12.5 mM) stimulated GLP-1 release from intestinal segments, whereas casein only stimulated PYY and CCK release. Combining nutrients with fluoxetine further increased nutrient-induced GLP-1, PYY and CCK release.

Serotonin release from intestinal tissue segments was stimulated by casein and safflower oil while sucrose and rebaudioside A had no effect. The combination with fluoxetine (0.155 μM) further enhanced casein and safflower oil induced-serotonin release.

Exposure of ileal tissue segments to serotonin (30 μM) stimulated GLP-1 release whereas it did not induce PYY and CCK release. Serotonin (30 and 100 μM) also stimulated GLP-1 release from STC-1 cells, which was inhibited by the non-specific 5HT receptor antagonist asenapine (1 and 10 μM).

These data suggest that nutrient-induced GLP-1 release is modulated by serotonin through a receptor mediated process.

Introduction

Gastro-intestinal (GI) hormones play major roles in the regulation of food intake, gastric emptying and GI motility. In the small intestine hormones are secreted by enteroendocrine cells (EEC) (1), and are involved in modulating feelings of satiety and satiation using feed-back mechanisms to reduce food intake (1, 2). Well-known satiety inducing hormones are cholecystokinin (CCK), peptide YY (PYY) and glucagon like peptide-1 (GLP-1). The release of these hormones involves interaction between nutrients and G-protein coupled receptors (GPCR) or ion-dependent transporters that are expressed on EECs (1).

Recently it was found by our group that nutritional and non-nutritional stimuli such as a lipid mixture (safflower oil), carbohydrate (sucrose), protein (casein) and a non-caloric sweetener (rebaudioside A) induce GLP-1 and PYY release from ileal tissue segments (3). The mechanism of how these different nutrients cause satiety hormone release remains to be investigated.

It is known that lipids are sensed in the form of long and short chain fatty acids by free fatty acid receptors. Carbohydrates are predominantly sensed as glucose, possibly via the Na^+ dependent sodium/glucose cotransporter member 1 (SGLT-1) or by the so-called sweet taste receptor, a G-protein coupled heterodimer (T1R2/T1R3). Proteins are mainly detected after being digested into amino acids, di- and tripeptides by the G-coupled calcium sensing receptor (CaSR), umami taste receptor dimer T1R1/T1R3, GPCR6A, and peptide transporter 1 (PEPT1) (1, 4). However, not for all nutrients stimulating GLP-1, PYY or CCK release, a receptor is known. There may be additional mechanisms allowing to respond to fluctuations in intestinal nutrient levels (5). One such mechanism may involve paracrine cell-cell interaction via serotonin release from enterochromaffin cells (ECC).

Although it is known that serotonin is involved in food intake regulation (6), it is still unknown how serotonin stored in the small intestine contributes to food intake regulation. Serotonin is present throughout the gut in the duodenum, jejunum and ileum and stored in dense core granules located in the basal and apical part of ECCs. These cells are responsible for the production and storage of approximately 90% of the total pool of serotonin in the body (3, 12, 44). The release of serotonin from ECCs is stimulated by some dietary compounds *in vitro* (7) and several serotonin receptor subtypes are expressed in the small intestine. Enteric neurons and enterocytes express 5-HT_1, 5-HT_2, 5-HT_3, 5-HT_4 and 5-HT_7 receptors. Activation of these receptors has various effects such as neurotransmitter release, controlling motility, intestinal secretion and contraction or relaxation (8).

The present study aimed to investigate to what extent serotonin is involved in nutrient-stimulated GLP-1 release. It was hypothesized that nutrients stimulate serotonin release from ECCs, which consequently stimulates GLP-1 secretion via a serotonin receptor

at EECs. This was studied by analyzing serotonin, GLP-1, PYY and CCK release from intestinal tissue segments after exposure to a lipid mixture (safflower oil), protein (casein), carbohydrate (sucrose) and a non-caloric sweetener (rebaudioside A).
Next, the role of serotonin on GLP-1 release from intestinal segments was studied using the selective serotonin re-uptake inhibitor (SSRI) fluoxetine. Fluoxetine inhibits serotonin re-uptake that is mediated by the membrane embedded serotonin reuptake transporter (9). Two different models were used; an *ex vivo* and *in vitro* model. First, porcine intestinal segments were applied because these conglomerates of multiple cell types such as EECs, ECCs, and absorptive enterocytes offer a suitable system to study cellular interactions involved in serotonin and GLP-1 release. Second, serotonin-stimulated GLP-1 release was studied using the murine enteroendocrine STC-1 cell line (10). STC-1 cells were exposed to serotonin and the non-specific receptor antagonist asenapine or the specific 5-HT_3 receptor antagonist ondansetron.

Material and Methods

Chemicals

Casein (food grade containing sodium, purity 88%, batch number 15156) was obtained from Dutch Protein Services BV (Tiel, The Netherlands). Rebaudioside A (food grade, purity >97%, batch number 20110301, Chemspider ID 5294031) was obtained from SteviJa Natuurlijk BV (Drachten, The Netherlands). Sucrose (food grade, purity >99.9%) was supplied by Suiker Unie (Oud Gastel, The Netherlands). Safflower oil (food grade, composed of 3% C16:0, 2-5% C18:0, 9-15% C18:1 and 75-85% C18:2) was provided by De Wit Specialty Oils (De Waal, The Netherlands). Safflower oil was emulsified as described previously (3, 11) using potassium caseinate (2.5% w/v), xanthan gum and guar gum (both 0.1% w/v, GF supplies, Amsterdam, The Netherlands). Asenapine maleate (purity >98%), 5-hydroxytryptamine hydrochloride (serotonin, purity >98%), fluoxetine hydrochloride, L-ascorbic acid (purity >99%), pargyline hydrochloride and phenylmethanesulfonyl fluoride (PMSF) were obtained from Sigma Aldrich (Zwijndrecht, The Netherlands). Chemicals to prepare the Krebs-Ringer bicarbonate (KRB) buffer were: D-glucose (1.8 g/L), magnesium chloride (0.0468 g/L), potassium chloride (0.34 g/L) sodium chloride (7.0 g/L), sodium phosphate dibasic (0.1 g/L) sodium phosphate monobasic (0.18 g/L), and HEPES (5.95 g/L) were all obtained from Sigma Aldrich (Zwijndrecht, The Netherlands). Hanks' Balanced Salt Solution (HBSS) was obtained from Life technologies Europe BV (Bleiswijk, The Netherlands) and was supplemented with 10mM HEPES (Merck Millipore, Darmstadt, Germany). Lonza (Verviers SPRL, Belgium) provided Dulbecco's Modified Eagle Medium (DMEM), fetal calf serum (FCS). The pH of both the buffers was adjusted to pH 7.4 and both buffers contained 1 mM L-ascorbic acid and 100 μM pargyline to prevent oxidation and metabolism of serotonin by monoamine oxidase as described previously (12).

Collection of porcine intestinal tissues

Intestinal tissues were obtained from 10 male pigs (*Sus scrofa domesticus*), considered healthy after evaluation by a veterinarian. The protocol for this study was approved by the Animal Ethics Committee Utrecht (Ethics Committee Permit Numbers 2014.I.01.001 and 2014.I.06.037, Utrecht, The Netherlands). The pigs had a mean (± SEM) body mass of 88 ± 4 kg and a mean age of 6 months. Pigs were housed in groups, fed *ad libitum* with a standard pig diet (Vitaal P 003103, de Heus, Ede The Netherlands) with free access to water. The pigs were killed by a veterinarian through an intracardial injection of Euthanimal (pentobarbital 40%, 30 ml per pig) (Alfasan, Woerden, NL). Within approximately 5 minutes after the pigs were killed, the intestines were excised, and intestinal tissue of about 1 meter length of different anatomical regions were collected and stored in ice-cold oxygenated (95% O_2, 5% CO_2) KRB buffer. Duodenal tissue was collected at 10 cm below the pylorus, jejunal tissue was collected 4.5 meters proximal from the ileocaecal junction and ileal tissue was collected at 1 meter proximal from the ileocaecal junction. Tissues were transported to the laboratory and immediately used for *ex vivo* experiments.

The time between excision and start of the experiments did not exceed 30 minutes. To determine the intracellular concentrations of serotonin, CCK, GLP-1 and PYY whole tissue segments (0.5 cm^2) from duodenal, jejunal and ileal tissue were immediately snap frozen and stored below -70°C.

Stimulation of intestinal segments with casein, safflower oil, sucrose and rebaudioside A

Concentrations of the nutrient stimuli chosen are within the physiological range and these concentrations previously showed to stimulate GLP-1 release in the same porcine intestinal segment model (3). Incubations of porcine intestinal tissue segments with casein (1% w/v), rebaudioside A (12.5 mM), sucrose (50 mM), safflower oil (3.35% w/v), serotonin (30 μM) and fluoxetine (0.155 μM) alone, or in the presence of fluoxetine (0.155 μM) were performed as described previously (3). In brief, upon arrival at the laboratory the intestine was rinsed with ice-cold oxygenated KRB buffer and cut open in a longitudinal direction. The outer muscle layers were carefully stripped off with the basolateral side upward. The mucosal tissue was placed with the apical side upward, and circles of tissue with a diameter of 8 mm (about 0.5 cm^2) were punched out using a disposable biopsy punch (Medline Industries, Inc. Mundelein, Illinois, USA). Intestinal segments of duodenal and ileal tissue were used for the incubation experiments. The intestinal segments were transferred to a 48-wells plate which was filled with 500 μL ice-cold KRB buffer per well, and kept on ice until the start of the experiment. Thereafter, the tissues were brought to room temperature within 30 minutes, followed by a preheating step at 37°C for 1 hour in a humidified incubator (95% O_2, 5% v/v CO_2). Within the time of this preheating step all tissue segments exposed to the SSRI fluoxetine were pre-incubated with fluoxetine, 30 minutes before the actual incubation

started, by replacing the KRB buffer with KRB buffer in which fluoxetine had been dissolved. The concentrations fluoxetine (0.155 μM) and serotonin (30 μM) used were selected based on pilot experiments analyzing tissue uptake of radiolabeled serotonin after exposure to various concentrations of fluoxetine and serotonin, and on a previous study showing an IC_{50} for fluoxetine of 0.0155 μM in Caco-2 cells (12).
To study the release of serotonin, CCK, PYY and GLP-1, incubations were initiated by replacing the KRB buffer solution with preheated (37°C) isosmotic KRB buffer without D-glucose containing the test compounds. KRB buffer without D-glucose was used as a control since D-glucose might induce gastrointestinal hormone release. Next, the tissue was put in a humidified incubator for either 5, 10, 20 or 60 minutes at 37°C (5% (v/v) CO_2). After incubation (5, 10, 20 and 60 minutes) samples of incubation media were collected, mixed with 100 μM PMSF to inactivate dipeptidyl peptidase IV, aliquoted and either stored at 4°C for further analysis of lactate dehydrogenase (LDH) or stored below -70°C for further analysis of serotonin, CCK, GLP-1 and PYY.
Tissue viability was checked by measuring the leakage of intracellular LDH as described before (3, 13) using an LDH kit (Sigma Aldrich, Zwijndrecht, the Netherlands). LDH activity in the supernatant was expressed as a percentage of the total LDH activity present in control tissue collected before incubation. Total tissue levels of LDH and intracellular serotonin, CCK, GLP-1 and PYY were determined in 0.5 cm^2 tissue segments of duodenum, jejunum, and ileum, which were homogenized in ice-cold KRB buffer containing 100 μmol/L PMSF with a Potter Elvehjem type Teflon pestle tissue grinder (Braun, Melsungen, Germany) for 5 minutes at 200 rounds per minute. Samples were only included for further analysis when LDH leakage did not exceed 10% of the total LDH tissue content. No samples had to be excluded on the basis of this criterion.

Incubation of STC-1 cells with serotonin

STC-1 cells, a murine pluripotent enteroendocrine cell-line, were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf's serum (Life Technologies Europe BV., Bleiswijk, NL) and 1% Penicillin-Streptomycin (Lonza, Verviers, BE). For the GLP-1 secretion experiments the cells were grown in a 24-wells plate. Upon reaching 80% confluence, medium was washed and replaced by Hank's balanced salt solution (HBSS) (Life Technologies Europe BV., Bleiswijk, NL) with serotonin (0, 10, 30 and 100 μM). STC-1 cells were incubated with serotonin (0, 30 μM) in the presence and absence of asenapine (0, 1 and 10 μM) a non-specific serotonin receptor antagonist (14) and in the presence and absence of ondansetron (0, 1 and 10 μM) a specific 5-HT_3 receptor antagonist (15, 16) to investigate whether serotonin-stimulated GLP-1 release is mediated through a receptor-mediated process. The concentrations asenapine and ondansetron were chosen based on literature data (14-16). Cells were pre-incubated with either asenapine or ondansetron for 30 minutes in a humidified incubator (37°C; 95% O_2, 5% v/v CO_2). After pre-incubation, medium was replaced by HBSS containing

test compounds and incubated for 2 hours. Media samples were collected, PMSF (100 μM) was added and samples were stored at -20°C for GLP-1 analysis. Cell viability was analyzed by measuring leakage of intracellular lactate dehydrogenase (LDH) into the media, and expressed as a percentage of total leakage induced by 1% Triton-X100 incubation. LDH leakage did not exceed 10%. Furthermore, cell viability was checked by neutral red assay (17). None of the test conditions affected cell viability.

Serotonin, CCK, GLP-1 and PYY analysis

Serotonin was analyzed according to instructions of the manufacturer by a commercially available enzyme-linked immunosorbent assay (ELISA) (BA E-5900; Labor Diagnostika Nord GmbH & Co. Germany), using a BioTek Synergy HT absorbance microplate reader (BioTek Instruments, Inc. Winooski, VT, USA). The detection range of this assay is 0.015 – 2.5 ng/mL. The inter-assay coefficient of variation (CV) of this kit is 18% and the intra-assay variation is 14%. If necessary, samples were diluted with assay buffer to obtain concentrations within the detection range of this kit.
Concentrations of CCK-8 (CCK 26-33) were analyzed with an optimized and validated commercial human RIA kit (EURIA-CCK, RB302, Euro-diagnostica, Malmö, Sweden). An identical sequence of CCK-8 has been found for most mammals, among them pig and man. This improved assay system has been optimized to reach a high sensitivity of 0.05 pM and does not have cross-reactivity with gastrin-17 or sulfated gastrin. The intra-assay CV was 8.9% at a concentration of 0.84 pM and 4.9% at a concentration of 1.98 pM. Radioactivity of [^{125}I]-CCK-8 was measured by liquid scintillation counting using a gamma counter (EC&G, Breda, The Netherlands).
Active GLP-1 (7-36) was determined using a Glucagon Like Pepide-1 (Active) ELISA kit (EGLP-35K, Millipore, Linco Research, Billerica, MA, USA) according to the manufacturer's instructions. Since the GLP-1 hormone gene sequence is highly preserved, this kit can be applied for analyzing porcine GLP-1. The range of this assay is 2-100 pM. The inter-assay CV is 11% and the intra-assay CV is 6%. When necessary, samples were diluted in assay buffer from the kit to be within the detection range of this assay.
PYY release was analyzed by a commercially available ELISA kit for total porcine PYY (Bachem, Penisula Laboratories, San Carlos, CA, USA) according to the manufacturer's instructions. This kit measures porcine PYY, and the range of this assay is approximately 4.6 - 150 pM. The inter-assay CV is 6% and the intra-assay CV is 3%. It was not necessary to dilute samples for this assay.

Statistical analysis

Statistical analysis was performed using the SAS statistical software package (SAS version 9; SAS Institute, Cary, NC, USA). All variables, serotonin, CCK, GLP-1 and PYY were compared with a mixed analysis of variance (ANOVA) model. Data

were visually checked on normality and on constant variance of residuals by plots of residuals vs. corresponding predicted values. If data was not normally distributed, log transformation was applied for further analysis of the data as was the case for CCK, GLP-1 and serotonin. For the intestinal segment experiments the statistical model included the fixed factors time (5, 10, 20 and 60 minutes), location (duodenum and ileum) and treatment (control, casein, safflower oil, sucrose, rebaudioside A, serotonin, fluoxetine, casein and fluoxetine, safflower oil and fluoxetine, sucrose and fluoxetine, rebaudioside A and fluoxetine, serotonin and fluoxetine). Because all incubations were performed using intestinal tissue obtained from the same pig, intervention effects within the intestine of one pig were compared by including the random factor pig, which specifies the individual pig. For the STC-1 cell experiments the statistical model included fixed factors serotonin (0 and 30 μM) and asenapine (0, 1 and 10 μM). A post hoc test with Tukey-Kramer adjustment was used if an significant effect occurred. Data are presented as the mean ± SEM and differences are considered signifcant at $p<0.05$.

Results

Intestinal hormone concentrations in tissue segments and effects of nutrients and rebaudioside A on GLP-1, PYY and CCK release

GLP-1 tissue concentrations were highest in ileum followed by jejunum and duodenum (0.3±0.1, 6.7±0.7, 10.5±1.2 pmol/cm^2 tissue in duodenum, jejunum, and ileum, respectively). PYY concentrations in tissue were highest in jejunum followed by ileum and duodenum (1.1±0.4, 7.7±1.0, 4.3±0.8 pmol/cm^2 tissue, for duodenum, jejunum and ileum, respectively) and CCK intestinal tissue concentrations were highest in duodenum followed by jejunum and ileum (12.8±1.6, 1.5±0.3, and 0.27±0.1 pmol/cm^2 tissue, for duodenum, jejunum and ileum, respectively).

The effects of casein, sucrose, safflower oil and rebaudioside A on GLP-1, PYY and CCK release from ileal and duodenal segments are presented in **Figure 3.1**. The effects on GLP-1 release from ileal segments were the most pronounced **(Figure 3.1A**). Casein, sucrose and rebaudioside A all induced a marked GLP-1 increase ($p<0.001$). The effect of safflower oil was less pronounced ($p<0.05$). Casein and rebaudioside A also increased ileal GLP-1 release as compared to safflower oil ($p<0.05$). Nutrients and rebaudioside A did not stimulate GLP-1 release from duodenal segments (data not shown).

None of the nutrients stimulated PYY release from ileal segments as compared to control. Casein increased ileal PYY release as compared to sucrose and safflower oil ($p<0.05$; (**Figure 3.1B**)). Nutrients and rebaudioside A did not stimulate PYY release from duodenal segments (data not shown).

None of the nutrients stimulated CCK release from duodenal segments as compared to control. Casein and rebaudioside A increased CCK release (**Figure 3.1C**) as compared to

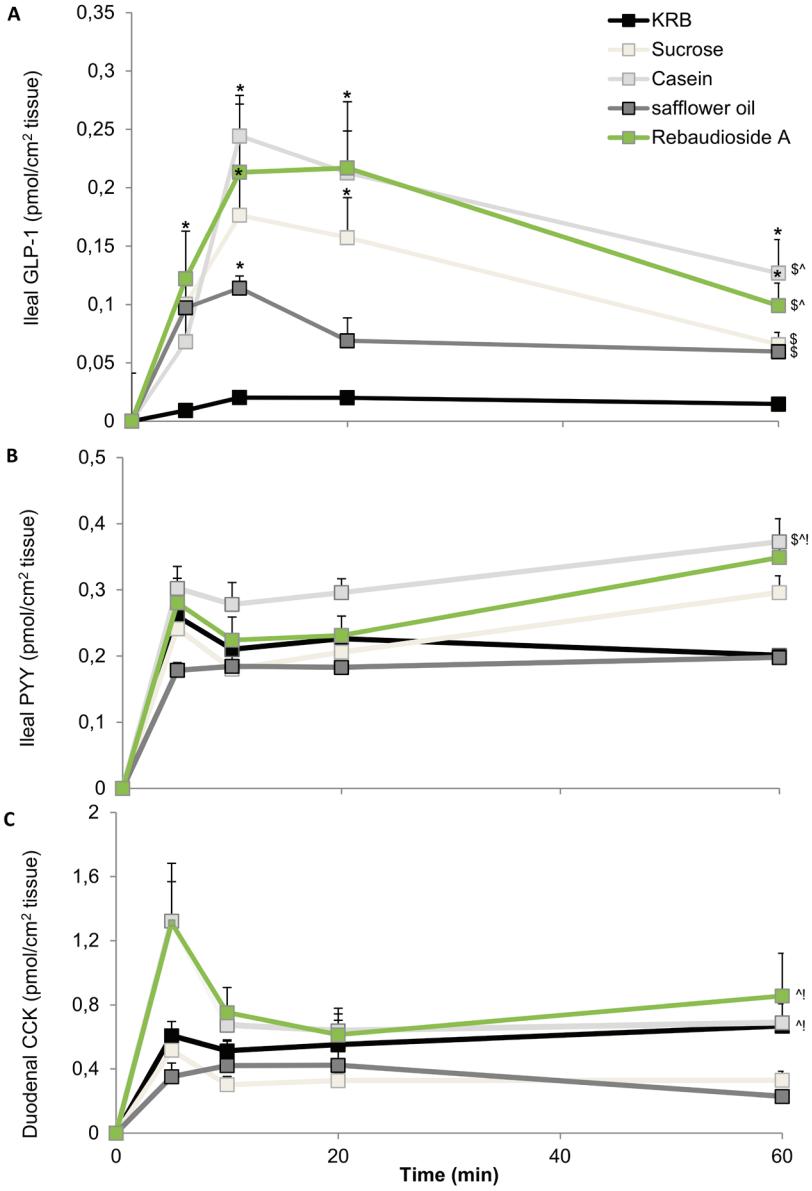

Figure 3.1 GLP-1, PYY and CCK released from intestinal tissue segments after exposure to various nutrients and rebaudioside A**.** Ileal GLP-1 (A), ileal PYY (B) and duodenal CCK (C) released from intestinal segments after exposure to KRB buffer, sucrose, casein, safflower oil or rebaudioside A. $^{\$}p<0.05$ release is increased over time as compared to KRB buffer, ^p<0.05 release is increased over time as compared to safflower oil, $^{!}p<0.05$ release is increased over time as compared to sucrose, *p<0.001 release is increased as compared to KRB buffer at the same time point, #p<0.05 release is increased as compared to safflower oil at the same time point.

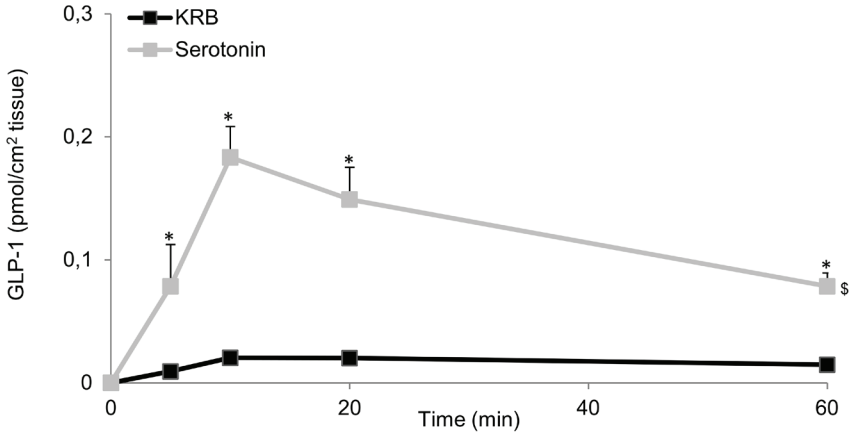

Figure 3.2 GLP-1 released from ileal tissue segments after exposure to serotonin. $^{\$}p<0.05$ GLP-1 release is increased over time as compared to KRB buffer. *$p<0.001$ significant increased GLP-1 release as compared to KRB buffer at the same time point.

exposure to sucrose and safflower oil ($p<0.05$). Nutrients and rebaudioside A exposure did not stimulate ileal CCK release (data not shown).

Serotonin stimulates GLP-1 release from ileal tissue segments and enteroendocrine cells

Serotonin increased GLP-1 release from ileal segments several fold over time ($p<0.001$) (**Figure 3.2A**). Incubation with serotonin did not change PYY release nor CCK released from both ileal and duodenal tissue segments (data not shown).
The role of serotonin on GLP-1 release was further explored in STC-1 cells experiments. Serotonin (30 and 100 μM) stimulated GLP-1 release from STC-1 cells as compared to control and to 10 μM serotonin ($p<0.001$) (**Figure 3.3A**). Serotonin-stimulated GLP-1 release was inhibited by the non-specific serotonin receptor antagonist asenapine (**Figure 3.3B**). Effects were significant at 10 μM asenapine ($p<0.05$) but not at 1 μM asenapine ($p=0.07$). Exposure of STC-1 cells to asenapine alone did not affect GLP-1 release. Ondansetron (1 and 10 μM) a specific 5-HT_3 receptor antagonist had no effect on serotonin-stimulated GLP-1 release (data not shown).

Serotonin intestinal tissue concentrations and induction of it's release following exposure to casein and safflower oil

Tissue concentrations of serotonin showed a different distribution pattern over the small intestine compared to that of GLP-1, PYY and CCK. Tissue concentrations of serotonin were highest in duodenum, followed by ileum and jejunum (2463±363, 884±202, and 1371±154 pmol/cm^2 tissue for duodenum, jejunum and ileum, respectively).
To study the effect of (non-)nutritional stimuli on serotonin release from intestinal epithelium, concentrations of serotonin secreted in the medium were analyzed after

exposure of the tissues to casein, safflower oil, sucrose and rebaudioside A. Casein and safflower oil caused increased serotonin concentrations in media over time ($p<0.001$) (**Figure 3.4**), with media concentrations from duodenum approximately 10 times higher as compared to media concentrations from ileum ($p<0.001$). Duodenal and ileal serotonin concentrations in medium did not change by exposure to sucrose and rebaudioside A (**Figure 3.4**).

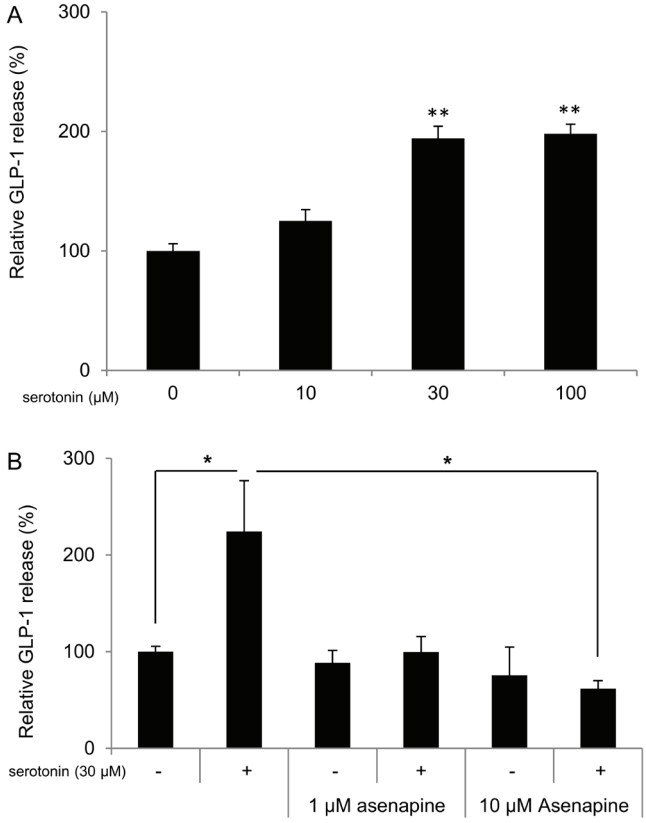

Figure 3.3 GLP-1 released from STC-1 cells after exposure to serotonin (A), GLP-1 released in the absence and presence of the non-specific serotonin receptor antagonist asenapine (B) (n=3).

**$p<0.001$ increased GLP-1 release as compared to 0 and 10 μM serotonin. *$p<0.01$ GLP-1 release is decreased as compared to 30 μM serotonin. #$p=0.07$ 1 μM asenapine tended to inhibit serotonin-stimulated GLP-1 release.

SERT inhibition augments casein- and safflower-stimulated serotonin release

Blocking the cellular reuptake of serotonin with fluoxetine resulted in increased serotonin concentrations in the medium from duodenal segments after 60 minutes ($p<0.001$) (**Figure 3.5A**). Fluoxetine induced a smaller effect on serotonin concentrations in medium from ileal tissue segments, these concentrations being increased after 20 minutes only ($p<0.05$) (**Figure 3.5B**).

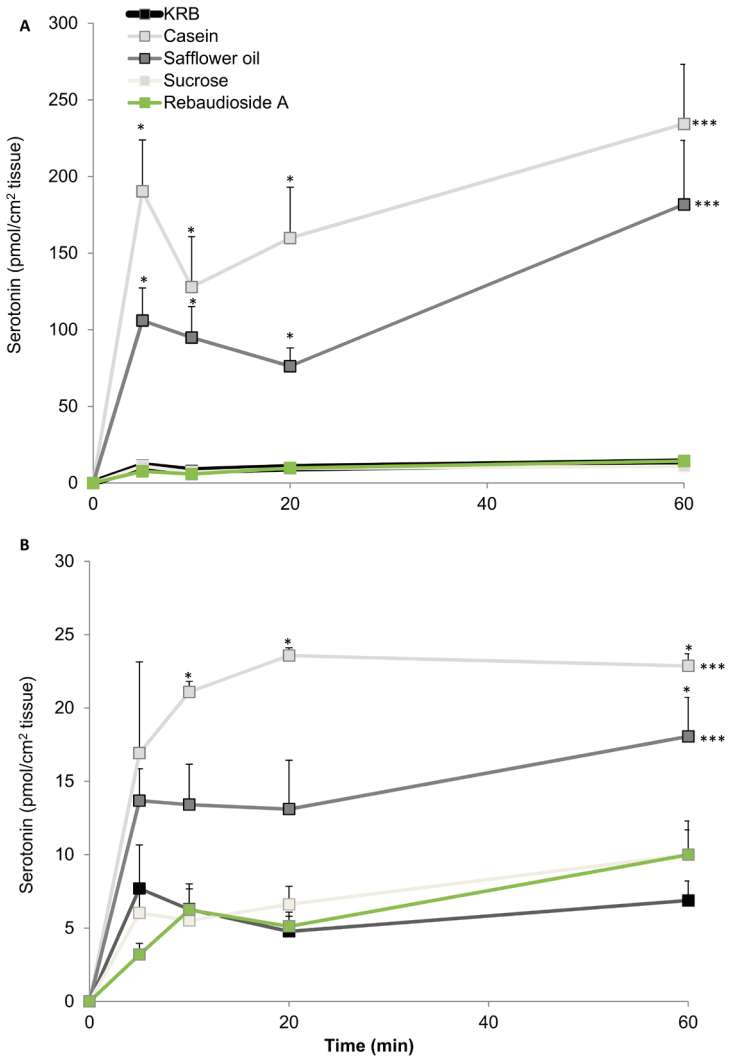

Figure 3.4 Serotonin released from duodenal (A) and ileal (B) tissue segments after exposure to various nutrients and rebaudioside A. Duodenal (A) and ileal (B) serotonin released from intestinal segments after exposure to KRB buffer, sucrose, casein, safflower oil or rebaudioside A. ***$p<0.001$ release is increased over time as compared to KRB buffer, *$p<0.001$ release is increased as compared to KRB buffer at the same time point.

The combination of fluoxetine and casein increased serotonin medium concentrations from duodenal and ileal tissue incubations as compared to fluoxetine alone ($p<0.001$) and casein alone ($p<0.05$ at 10 minutes) (Figure 5). Combining fluoxetine with safflower oil increased serotonin medium concentrations from duodenum and ileum as compared to fluoxetine alone ($p<0.001$) (**Figure 3.5**).

The combination of rebaudioside A and fluoxetine increased duodenal serotonin concentrations in medium only after 10 minutes as compared to rebaudioside A ($p<0.001$). However, serotonin concentrations in medium of ileum did not change after exposure to the combination of rebaudioside A and fluoxetine (data not shown). The combination of sucrose and fluoxetine did not change serotonin concentrations in the media of both duodenal and ileal segments (data not shown).

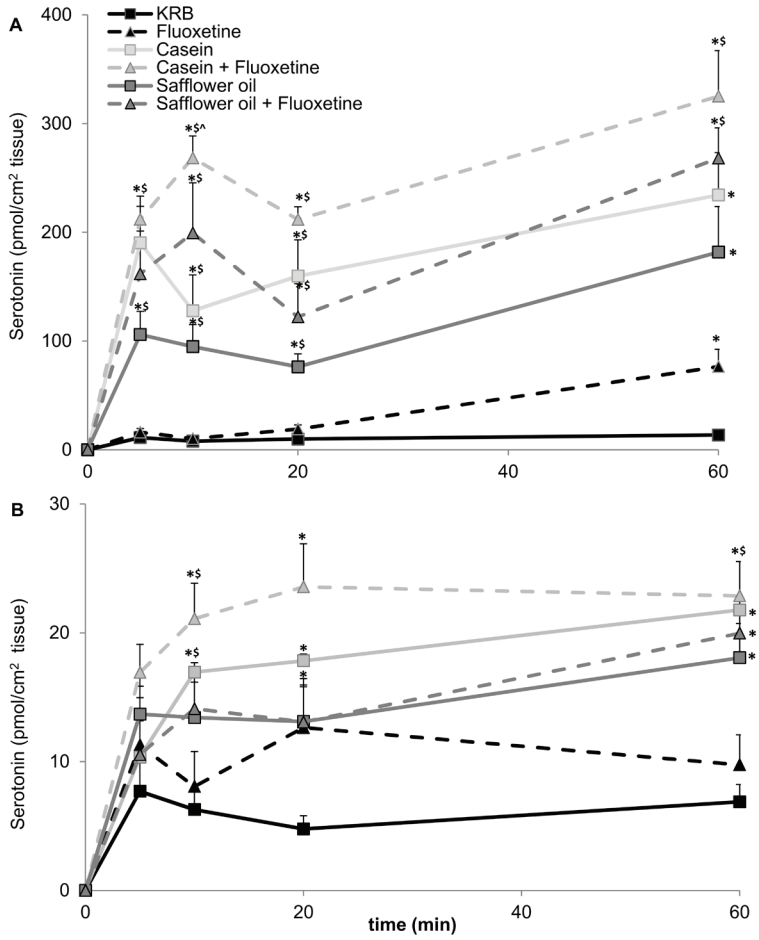

Figure 3.5 Serotonin released from duodenal (A) and ileal (B) tissue segments after exposure to casein and safflower oil in the presence and absence of fluoxetine. Duodenal (A) and Ileal (B) serotonin concentrations in incubation media after exposure to KRB, fluoxetine, casein, casein + fluoxetine, safflower oil and safflower oil + fluoxetine. *$p<0.001$ increased as compared to KRB buffer at the same time point, $^{\$}p<0.001$ increased as compared to fluoxetine at the same time point, ^$p<0.001$ increased as compared to casein at the same time point.

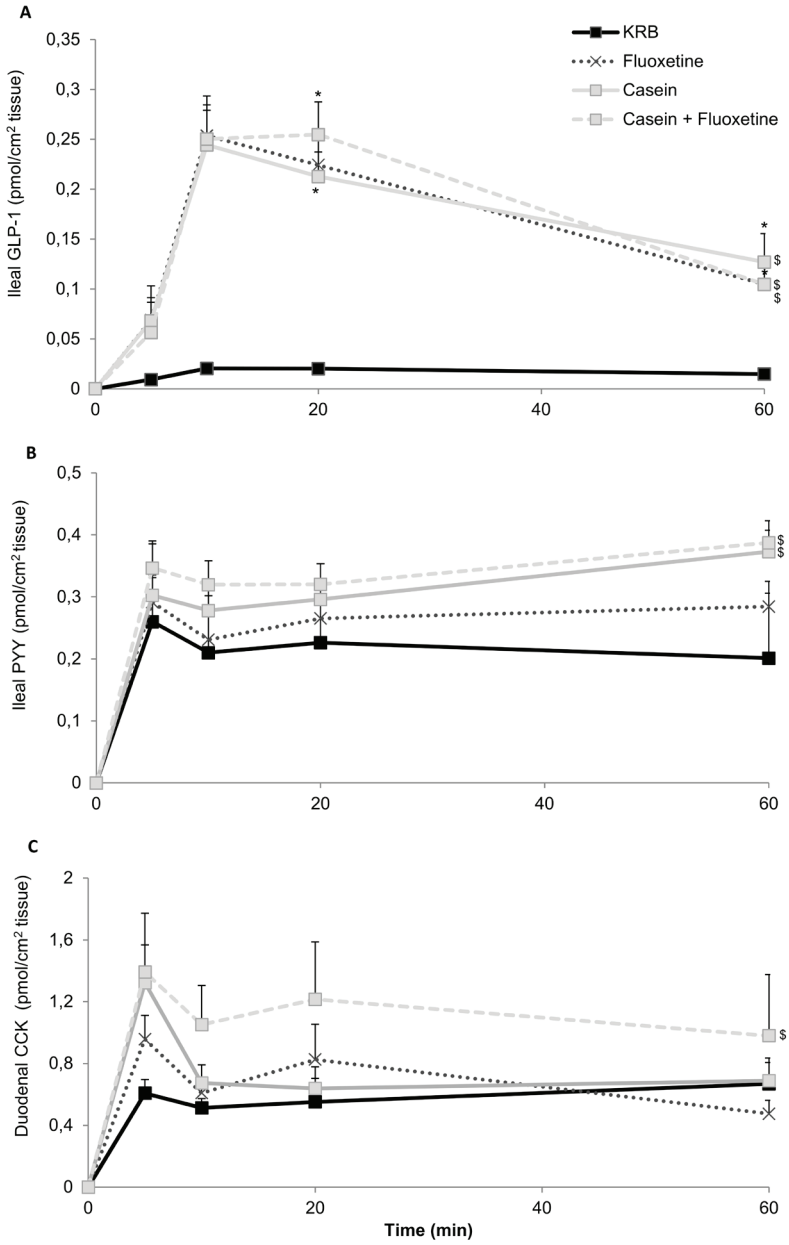

Figure 3.6 GLP-1, PYY and CCK released from intestinal tissue segments after exposure to casein in the presence and absence of fluoxetine. Ileal GLP-1 (A), ileal PYY (B) and duodenal CCK (C) released after exposure to KRB buffer, fluoxetine, casein or the combination of casein and fluoxetine. $^{\$}p<0.05$ release is increased over time as compared to KRB buffer. *$p<0.001$ increased release as compared to KRB buffer at the same time point.

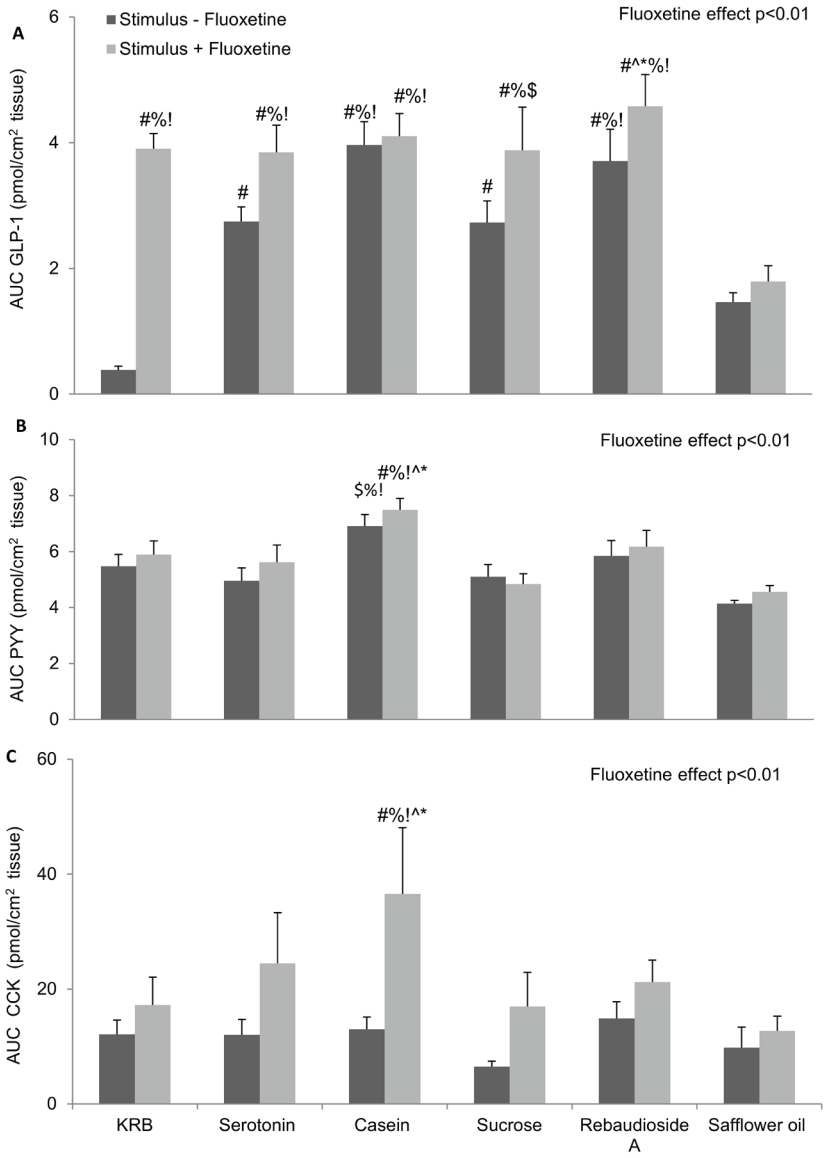

Figure 3.7 GLP-1, PYY and CCK released from intestinal segments after exposure to various nutrients and rebaudioside A in the presence and absence of fluoxetine. Area under the curves (AUCs) of ileal GLP-1 (A), ileal PYY (B) and duodenal CCK (C) released after exposure to various stimuli with and without fluoxetine. The AUCs of CCK, PYY and GLP-1 are increased by SERT inhibition as compared to stimuli exposure alone (fluoxetine effect $p<0.01$). $^{\#}p<0.05$ increased compared to KRB buffer, $^{\%}p<0.05$ increased compared to safflower oil, $^{!}p<0.05$ increased compared to safflower oil + fluoxetine, $^{\wedge}p<0.05$ increased compared to serotonin, $^{*}p<0.05$ increased compared to sucrose, $^{\$}p<0.05$ increased compared to sucrose + fluoxetine.

SERT inhibition increases nutrient- and rebaudioside A-stimulated GLP-1, PYY and CCK release

Fluoxetine alone increased GLP-1 release ($p<0.001$), whereas ileal PYY and duodenal CCK release were not changed. The combination of casein and fluoxetine increased GLP-1 ($p<0.01$), PYY and CCK release over time ($p<0.001$) (**Figure 3.6**). For the other combinations of nutrients and sweetener with fluoxetine no time and treatment interactions were found. However, since the release of GLP-1, PYY and CCK tended to be increased by the addition of fluoxetine as compared to nutrient exposure alone, total area under the curves (AUC) were calculated. Overall, the combination of fluoxetine with the nutrients tested and with rebaudioside A resulted in increased AUCs of GLP-1 (**Figure 3.7**), PYY and CCK release vs nutrients alone ($p<0.01$).

Discussion

In our study we showed that GLP-1 release from intestinal tissue following stimulation by different (non-)nutritional stimuli could be further augmented by adding the serotonin reuptake inhibitor fluoxetine. Next to this, serotonin itself stimulated GLP-1 release from ileal tissue segments and from STC-1 cells. The latter was inhibited by the non-specific serotonin receptor antagonist asenapine suggesting a receptor-mediated process.

All nutrients and mixtures tested as well as the non-caloric sweetener rebaudioside A stimulated GLP-1 release from intestinal tissues, which confirmed our previous findings using the same intestinal segment model (3). These data correspond to findings *in vivo* as well, since ileal stimulation with the same macronutrients resulted in increased satiation and satiety hormone release (11). For rebaudioside A, however, it is not known if this stimulates GLP-1 release *in vivo* as well and hence induces satiation. Another study demonstrated satiating properties of stevia *in vivo* (18) whereas other sweeteners including sucralose did not affect satiety hormone release *in vivo* (19, 20).

In the experiments described here, time-dependency of satiety hormone release was studied. Compared to our previous study we found a slightly higher PYY release from ileal segments after exposure to buffer only. It is unlikely that this effect is due to tissue damage, since LDH leakage was below 10%. CCK release from duodenal segments was analyzed for the first time using this *ex vivo* model. Surprisingly, rebaudioside A and casein stimulated CCK release only when compared to sucrose and safflower oil, but not when compared to control. Safflower oil was expected to increase CCK, as it was shown before that intraduodenal lipid infusions resulted in increased CCK levels *in vivo* (21). In the latter study, lipid infusions of mixtures of linoleic, oleic and palmitic acid were used.

Overall, the results of the present study illustrate that the *ex vivo* model being a conglomerate of multiple cell types such as EECs, ECCs and absorptive enterocytes,

offers a suitable system to study the cellular interactions involved in serotonin and GLP-1 release.

It is known that nutrient sensing GPCRs and ion-dependent nutrient transporters at EECs play a major role in nutrient-stimulated GLP-1, PYY and CCK release (15, 21). However, not for all nutrients stimulating GLP-1, PYY or CCK release, a receptor is known. We hypothesized that nutrient-receptor interaction results in serotonin release, and that this released serotonin enhances nutrient-stimulated GLP-1 release. A hypothetic model for this effect is depicted in **Figure 3.8**.

The proposed mechanism by which serotonin may contribute to receptor-mediated GLP-1 release is; 1) nutrients stimulate serotonin release from ECCs via interaction with GPCR receptors on ECCs (7), subsequently 2) this released serotonin stimulates the release of GLP-1 from EECs via a serotonin receptor-mediated process. This hypothesis is supported by the data found in the present study. We showed that nutrients stimulated serotonin release from ileal tissue segments and that serotonin stimulated GLP-1 release from ileal tissue segments. Also in the enteroendocrine STC-1cell model, serotonin stimulated GLP-1 release which could be inhibited by a non-specific serotonin receptor agonist asenapine.

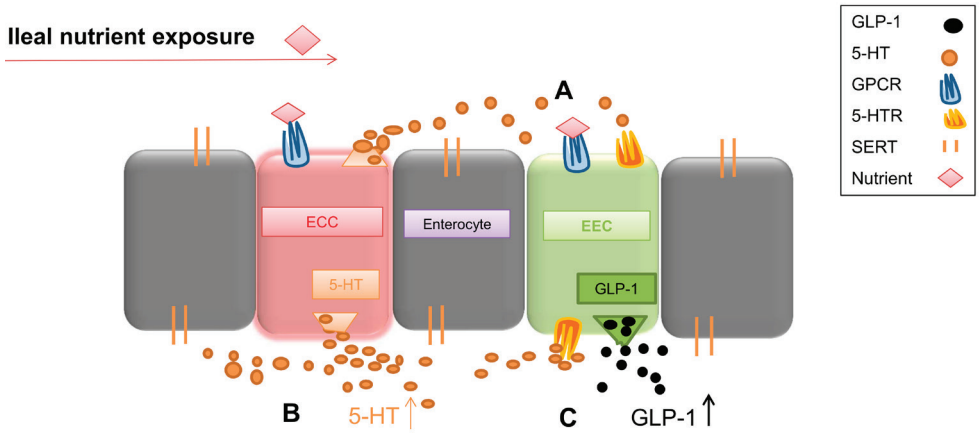

Figure 3.8 Proposed mechanism of serotonin modulating GLP-1 release from the ileum

This cartoon illustrates the following hypothesis; dietary compounds such as casein, safflower oil, sucrose or rebaudioside A may stimulate GLP-1 release from an EEC via interaction with a GPCR (A), simultaneously casein may stimulate extra-intestinal serotonin concentrations from ECC via interaction with a GPCR (B). Consequently this induced serotonin release may stimulate (additional) GLP-1 release via interaction with an 5-HT receptor on EEC (C).

Asenapine has binding affinities for various serotonin receptors including the $5HT_{1a}$, $5\text{-}HT_{1b}$, $5\text{-}HT_{2a}$, $5\text{-}HT_{2b}$, $5\text{-}HT_{2c}$, 5-HT5, $5\text{-}HT_{6}$ and $5\text{-}HT_{7}$ receptor (14). It should be noted that this antagonist has binding affinities for other receptors such as adrenoreceptors and dopamine receptors as well (14). However, because asenapine inhibited GLP-1 release after stimulation with serotonin, we believe this effect to be

mediated via serotonin receptors. In contrast to asenapine, ondansetron, a specific 5-HT_3 receptor antagonist did not inhibit serotonin-stimulated GLP-1 release from STC-1 cells. This suggests that receptors other than the 5-HT_3 receptor mediate serotonin-stimulated GLP-1 release. A potential candidate may be the 5-HT_{1b} receptor. Asenapine has affinity for this 5-HT_{1b} receptor. A recent report also showed that stimulation of 5-HT_{1b} receptors with specific 5-HT_{1b} agonists enhanced GLP-1 plasma concentrations in mice (22). Further studies are needed to identify the specific serotonin receptors involved in serotonin-stimulated GLP-1 release.

This study has also limitations that warrant discussion. First, the data presented support the idea that serotonin contributes to nutrient-stimulated GLP-1 release, whereas the effects on CCK and PYY were less convincing. Such an effect may relate to serotonin receptor distribution in the small intestine. Possibly, serotonin receptors on GLP-1 containing L-cells in the ileum are more abundant than those on EECs containing PYY, or CCK in the duodenum. Remarkable though was that serotonin release and tissue concentrations were highest in duodenum. Therefore, more studies are needed to investigate serotonin receptor expression on EECs and to study the relation between activation of specific serotonin receptors and GLP-1, PYY and CCK release.

Second, sucrose and rebaudioside A both stimulated GLP-1 release and this GLP-1 release was further stimulated after inhibition of serotonin reuptake. However, serotonin release was not stimulated by these compounds. It was expected that sucrose and rebaudioside A would result in serotonin release since other studies showed that odorants and tastants other than rebaudioside A stimulated serotonin release from enterochromaffin cells (7, 23). Possibly, the turn-over of serotonin is so high that quantities of serotonin accumulated in the incubation media were too low to be detected. It would be interesting to use alternative methods to provide more insight in the timing and sequence of the release of serotonin and satiety hormones. One interesting method is amperometrical recording (24), which has been applied to measure serotonin release from rabbit ileal mucosa and from ECCs (23, 25).

Third, the combination of nutrients and fluoxetine further enhanced nutrient-stimulated GLP-1, PYY and CCK release from intestinal segments. However, the additional effect of fluoxetine was only small in comparison to hormone release induced by the nutrients alone, possibly due to the fact that there was already a copious release of serotonin in the epithelial space. Further studies are needed to investigate the magnitude of serotonin's contribution to nutrient-stimulated GLP-1 release.

Collectively, our data suggest an additional mechanism for nutrient-stimulated GLP-1 release via the release of serotonin by ECCs. Serotonin present in the small intestine contributes to the enhancement of GLP-1 release by responding to macronutrients, possibly via the activation of serotonin receptors on EECs. This additional mechanism contributes to the idea that nutrient sensing in the small intestine is regulated by

interaction of multiple cells that respond to nutrient fluctuations in order to optimize food digestion and regulation of food intake.

Competing Interest

The authors have declared that no competing interests exist.

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Chapter 4

Cholecystokinin regulates satiation independently of the abdominal vagal nerve in a pig model of total subdiaphragmatic vagotomy

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Abstract

The vagal nerve and gut hormones CCK and GLP-1 play important roles in the control of food intake. However, it is not clear to what extent CCK and GLP-1 increase satiation by stimulating receptors located on abdominal vagal nerve endings or via receptors located elsewhere. This study aimed to further explore the relative contribution of the abdominal vagal nerve in mediating the satiating effects of endogenous CCK and GLP-1. Total subdiaphragmatic vagotomy or sham operation was combined with administration of CCK_1 and GLP-1 receptor antagonists devazepide and exendin (9-39) in 12 pigs, applying an unbalanced Latin Square within-subject design. Furthermore, effects of vagotomy on preprandial and postprandial acetaminophen absorption, glucose, insulin, GLP-1 and CCK plasma concentrations were investigated.

Ad libitum liquid meal intake (mean ± SEM) was similar in sham and vagotomized pigs (4180 ± 435 and 3760 ± 810 gram/meal). Intake increased by about 20 % after blockade of CCK_1 receptors, independently of the abdominal vagal nerve. Food intake did not increase after blockade of GLP-1 receptors. Blockade of CCK_1 and GLP-1 receptors increased circulating CCK and GLP-1 concentrations in sham pigs only, suggesting the existence of a vagal reflex mechanism in the regulation of plasma CCK_1 and GLP-1 concentrations. Vagotomy decreased acetaminophen absorption and changed glucose, insulin, CCK and GLP-1 concentrations indicating a delay in gastric emptying. Our data show that at liquid feeding, satiation is decreased effectively by pharmacological blockade of CCK_1 receptors. We conclude that regulation of liquid meal intake appears to be primarily regulated by CCK_1 receptors not located on abdominal vagal nerve endings.

Introduction

The regulation of appetite and food intake is complex and consists of several processes, each involving combinations of different and interacting regulatory mechanisms (1). A better understanding of these processes is not only relevant to prevent overweight, but also relevant in relation to unintentional weight loss as for example in cachexia or sarcopenia. Two key processes involved in limiting food intake are satiety and satiation. Satiety refers to the feeling of fullness after a meal, which decreases in time and ultimately leads to initiation of a new meal (2, 3). Satiation refers to processes that promote meal termination by which meal size is limited (4). Nutrient sensing in the gastrointestinal tract is one of the processes involved in generating satiation responses. This process may be defined as the activation of neural and hormonal signals in response to nutrients passing the gastrointestinal tract.

The vagal nerve is involved in the neural regulation of satiation (5). The vagal nerve by its efferent fibers controls intestinal motility and as a consequence the intestinal transit time of nutrients. As such the vagal nerve contributes to the exposure time of the nutrients to the enteroendocrine cells located in the small intestine (6).

Gastrointestinal hormones such as cholecystokinin (CCK) and glucagon-like peptide 1 (GLP-1) are playing important roles in generating satiation. These hormones are released when nutrients are sensed by receptors located on enteroendocrine cells which are distributed over the small intestine. CCK is mainly released by enteroendocrine I-cells which are relatively abundant in the duodenum, whereas GLP-1 is released by enteroendocrine L-cells mainly distributed over the distal jejunum, ileum and colon (7, 8). CCK is mainly secreted in response to dietary fat and protein (8). The largest bioactive form is CCK-83, which corresponds to the amidated sequence 1-83 of proCCK. This sequence is cleaved variably at four monobasic sites to release CCK-58, CCK-33, CCK-22 and CCK-8, all of which have the same C-terminal heptapeptide amide sequence which is necessary for receptor binding (9-11). There are two types of CCK receptors, CCK_1 receptors and CCK_2 receptors. The vagal nerve expresses CCK_1 receptors. Studies where this receptor is blocked using specific CCK_1 antagonists, such as devazepide, resulted in increased food intake (12). These results show that this CCK_1 receptor is important for the regulation of food intake.

The GLP-1 receptor is expressed on enteric nerves, vagal nerve endings, and in the pancreas, stomach, small and large intestine, adipose tissue and brain (7, 13). Similar to CCK, GLP-1 levels in plasma increase after a meal (14). GLP-1 exists in multiple forms, the majority (at least 80%) of circulating biologically active GLP-1 in humans is the COOH terminally amidated form, GLP-1 (7–36) amide, with lesser amounts of the minor glycine extended form, GLP-1 (7–37). Other forms of GLP-1 are 9-36 and 9-37 which arise after the action of the serine protease dipeptidyl peptidase IV (DPP-IV) (7, 15).

It has been shown that peripheral administration of exogenous GLP-1 and CCK reduces food intake in humans and experimental animals (16-20). Studies, mostly in rodents, suggest that this effect is mediated via vagal afferent nerves (12, 21-24). However there is also evidence from studies using pharmacological receptor blockade that endogenous CCK stimulates satiety not only via vagal afferent nerves but also via CCK1 receptors beyond the blood brain barrier independently of the vagal nerve (25, 26). For this reason in the present study it was investigated whether CCK and GLP-1 affect food intake via their receptors located at the abdominal vagal nerve, or by receptors elsewhere. In this study we have used the pig as experimental model for food intake regulation. Pigs resemble humans in many ways with respect to the omnivorous character of food intake, digestion, endocrine regulation of metabolism and neurophysiologic modulation of food intake (27, 28).
To determine whether the effects on food intake of endogenous CCK and GLP-1 are mediated via receptors located at the abdominal vagal nerves, or receptors located elsewhere, ad libitum liquid food intake was studied after blocking the CCK_1 receptors, GLP-1 receptors or both, in a group of sham operated and a group of total subdiaphragmatic vagotomized pigs. These effects were further studied by evaluating pre- and postprandial hormone responses and portal-peripheral hormone gradients.

Material and methods

Ethical approval

All procedures involving animal handling and testing were reviewed and approved by the ASG-Lelystad Animal Care and Ethics Committee (Ethics Committee permit number 2011143 Lelystad, The Netherlands).

Design

Twelve domestic 4 months old, crossbred (Yorkshire x Landrace) male pigs, weighing 49 ± 10.2 kg (Wageningen UR-Swine Innovation Centre, Sterksel, The Netherlands) were used in the study. Two weeks before surgery the pigs were kept in specially designed metabolic pens (1.15 x 1.35 m) and adapted to the light/dark cycle (lights on at 05:00 h and off at 19:00 h) and the feeding schedule. During the whole study period pigs were fed twice a day at 08:00 AM and 04:00 PM at a restricted feeding level of 2.8 times maintenance requirements (MR) for metabolisable energy (MR=293 kJ/kg $BW^{0.75}$). For a pig of 60 kg this amount is approximately 16 MJ gross energy per meal which is practically close to ad libitum access to food (29). The diet (Milkiwean®, Trouw Nutrition, Putten, The Netherlands) was based on a standard pig nutrition, containing all required macronutrients and vitamins (**Table 4.1**), but fed in a liquid form (thin slurry 1:2 (w:v) ratio). During the whole study period water was available overnight from 05:00 PM to 07:00 AM.

Table 4.1 Chemical composition of Milkiwean® diet

Name	Unit	Amount/kg
Dry matter	(g)	920
Moist	(g)	80
Protein	(g)	200
Fat	(g)	100
Starch	(g)	250
Sugars	(g)	80
Lactose	(g)	150
Ash	(g)	50
Non-starch polysaccharides	(g)	20
Linoleic acid C18:2	(g)	40
Digestible phosphorus	(g)	4.6
Ileal digestible lysine	(g)	14
Ileal digestible methionine	(g)	4.6
Ileal digestible methionine + cysteine	(g)	7.3
Ileal digestible threonine	(g)	8.5
Ileal digestible tryptophan	(g)	2.5
Metabolizable energy	MJ	15.8

Pigs underwent either sham surgery (further indicated as sham pigs) (n=6) or total abdominal subdiaphragmatic vagotomy (further indicated as vagotomized pigs) (n=6) under general sevoflurane anaesthesia. Using laparoscopy, in the latter both afferent and efferent subdiaphragmatic vagal nerves were interrupted by removal of approximately 3 cm length of both vagal nerves along the esophagus in between the diaphragm and the stomach, as close as possible to the diaphragm in order to bypass the interconnections between the dorsal and ventral trunks posterior to the heart. Vagal nerve interruption was confirmed at necropsy; all nerve-like tissue at the level of the esophagus and diaphragm was interrupted only in the vagotomized group (**Figure 4.7**). In the sham group exactly the same procedure was done without vagal nerve interruption. During and three days after surgery pigs were administered flunixin twice daily (2.2 mg/kg; Finadyne 50 mg/ml, Schering-Plough Animal Health, Brussels, Belgium) to ease postsurgical pain. The pigs also received an antibiotic combination (once a day 50 µl/kg pig; 200.000 IU/ml procaïne penicillin G and neomycin 100mg/ml, Neopen, Intervet, Boxmeer, The Netherlands) to prevent bacterial infections. After a 7 day postsurgical recovery period, catheterization surgery was performed. Under general sevoflurane anaesthesia, Tygon catheters (i.d. 1.27 mm, o.d. 2.29 mm, length 1.25 m; Access Technologies, Skokie, Illinois, USA) were placed into the right carotid artery, the right external jugular vein and the portal vein according to a procedure described previously(1, 2) Seven days after catheterization surgery the first study day started.

Neural and hormonal mechanisms of satiation and satiety were investigated in a within-subject design. Pigs were unequally divided in six groups with different treatment orders according to the unbalanced Latin Square design. Allocation per treatment order was

randomized according to weight of the pigs. Each treatment was studied on 4 separate study days with 2 day wash out periods. Total study duration period was six weeks.

Experimental design of a study day

At 8 AM (t = 0) a standardized liquid morning meal (1.4 times maintenance requirements for energy, based on metabolic weight of the pigs) was presented to the pigs. This meal was mixed with acetaminophen (0.02 g/kg pig) (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) to monitor gastric emptying(3-5). At t = 120 min, an intravenous infusion via the jugular vein catheter was started at a rate of 20 ml/h and continued for 2 hours, with either placebo (saline), exendin (9-39) a selective GLP-1 receptor antagonist (American Peptide Company, Sunnyval, CA, USA) at 0.5 nmol/kg.min (dose based on human studies(6-8)), devazepide a selective CCK_1 receptor antagonist (Tocris Bioscience, R&D Systems, Abindon, Oxon UK) at 2 nmol/kg.min (dose based on pig and rat studies(9-11)), or a combination of both exendin (9-39) and devazepide. Sixty minutes after initiation of the infusion (t=180 min) an *ad libitum* meal was presented to the pigs. Food intake was measured and eating behavior and time of meal termination (ingestion time) were observed. At 240 min after the standardized liquid morning meal the study day ended. Portal vein and peripheral blood samples were taken at t = -45, -15, 30, 60, 90, 120, 150 and 180 min.

Necropsy

Four days after the last study day, pigs were sacrificed exactly 180 minutes after they received a standardized liquid morning meal (1.4 times maintenance requirements for energy, based on metabolic weight of the pigs) after overnight fasting. The stomach and intestine were excised and total length and food content of the small intestine, which was divided into three parts, were measured. Bladder content and liver weight were also measured.

Plasma analysis

Portal and peripheral blood samples were collected in cooled EDTA vacutainers^R containing dipeptidyl peptidase IV inhibitor (DPPIV) (Millipore, Billerica, MA, USA) with EDTA free protease inhibitor tablets (Roche Applied Sciences, Penzberg, Germany) to inhibit enzymatic degradation of the bioactive hormones. Glucose levels were measured instantaneously using portable glucose analyzers (Medisense Precision Xceed, Abbott Diabetes Care Ltd. Witney, Oxon, UK) and glucose strips (Freestyle Precision, Abbott Diabetes Care Ltd. Witney, Oxon, UK). Blood samples were gently shaken and kept on ice and centrifuged at 4°C for 10 min at 2000 g within 10 minutes. Plasma samples were aliquoted and kept at -80°C until further analysis. Commercially available ELISA assay kits for active GLP-1 were obtained from Millipore (Billerica, MA, USA) and utilized

according to the manufacturer's instructions using a BioTek Synergy HT fluorescence microplate reader (BioTek Instruments Inc., Winooski Vermont, USA). ELISA assay kits to analyze porcine insulin levels were obtained from Alpco Diagnostics (Salem, NH, USA) and were used according to the manufacturer's instructions. Peripheral CCK levels were analyzed using an validated commercial available RIA kit for human plasma (Euro Diagnostica Malmö, Sweden) optimized for pig plasma. This kit uses rabbit antiserum raised against CCK 26-33 sulphate and is found to cross react with CCK 26-33 sulphate (100%) and CCK-33 sulphate (134%). Peripheral blood samples were analyzed for acetaminophen using a commercially available kit according to the manufacturer's instructions (Immunalysis, Oxfordshire, UK).

The change in plasma concentrations after the morning meal was calculated by subtracting the preprandial concentrations of glucose, insulin, CCK and GLP-1 from each concentration on the various time points until the drug infusion started (t=120). This value was statistically analysed to determine the effect of vagotomy on postprandial glucose, insulin, CCK and GLP-1 responses.

The portal-peripheral gradient was calculated by subtracting the peripheral concentrations of glucose, insulin and GLP-1 for each time point from the portal levels.

The drug responses were analyzed by taking the time interval beginning at the start point of the infusion (t=120) until the ad libitum meal started (t=180). This interval was further used in the statistical analysis to determine the effect of the CCK and GLP-1 receptor blockade on circulating blood concentrations. The changes in plasma concentrations after drug infusion were calculated by subtracting the concentrations of glucose, insulin, GLP-1 and CCK at the start of the drug infusion (t=120) from the time points at the start of the ad libitum meal (t=180).

Data and statistical analysis

Statistical analyses were performed using the SAS statistical software package (SAS version 9; SAS institute, Cary, NC, USA). Proc Gplots were used to test outcome variables for normality of distribution. If data were not normally distributed, log transformation was applied for further analysis of the data as was the case for insulin, GLP-1, CCK and acetaminophen.

A mixed model (ANOVA) with a co-variance structure, was used to analyze the effects of vagotomy and receptor blockade on food intake. Fixed factors in this model are vagotomy (yes/no) , devazepide (yes/no), exendin (9-39) (yes/no), combination (yes/no) and treatment order to correct for possible treatment order effects. No treatment order effects were observed. Time curves of plasma parameters were compared with a mixed analysis of variance model using a co-variance structure, that included the fixed factors treatment (sham vs vagotomy), drug (placebo, exendin 9-39, devazepide or combination) and the interaction of drug treatment and vagotomy. To exclude possible treatment order effects, the treatment order was added to the model. No treatment order

effects were observed. For the plasma parameters, time and the interaction between treatment and time, drug and time, and the interaction time, treatment and drug were added to the model. The factor entry number, which specifies the pig number, was added to the model as a random factor. A post hoc Tukey's test was used to analyze differences between groups. Data is presented as the mean ± SEM and considered significant at $p<0.05$. Necropsy data was analyzed by using the two-tailed independent samples t-test.

Results

All 12 pigs rapidly recovered from surgery and showed normal pig behavior as judged by feed intake, weight gain and the absence of any clinical symptoms. Weight of the pigs at the start of the study period was 49 ± 10.2 kg. Weight gain during the 6-week study was comparable between groups (20 ± 1.7 kg sham vs 19 ± 1.9 kg vagotomy). Data obtained during the last study day of one vagotomized pig was excluded from analysis because the pig did not eat the standardized liquid morning meal.

Effects of vagotomy and CCK, GLP-1 receptor blockade on ad libitum liquid food intake

Vagotomy did not affect food intake (4180 ± 440 and 3760 ± 810 g/meal $p= 0.794$) (**Figure 4.1**). As compared to placebo, blocking CCK receptors using devazepide resulted in a significant increase ($p<0.01$) in food intake in both groups (**Figure 4.1**). Administration of exendin (9-39) to block GLP-1 receptors did not affect food intake in both groups. The combination of a CCK and GLP-1 receptor antagonist resulted in an increased meal size ($p<0.05$). There was no interaction between vagotomy and devazepide, vagotomy and exendin (9-39) or vagotomy and combination, suggesting

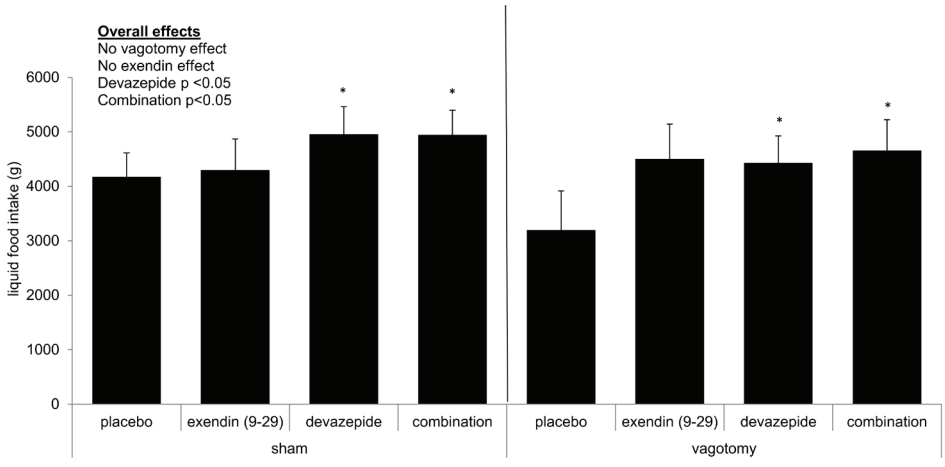

Figure 4.1 *Ad libitum* liquid food intake for both groups after various treatments. The results are shown as the mean ± SEM. *$p<0.05$ food intake increased as compared to placebo.

that the effect of devazepide and combination treatment was independent of the vagotomy. Ingestion time was unaffected by vagotomy (6.9 ± 0.8 min sham vs 6.8 ± 0.9 min vagotomized) and the various drug treatments (7.0 ± 0.7 min exendin (9-39) vs 6.7 ± 0.8 min devazepide vs 7.6 ± 0.4 min combination).

Effects of vagotomy on preprandial plasma glucose, insulin, CCK and GLP-1 concentrations

Plasma concentrations of glucose, insulin, CCK and GLP-1 in sham and vagotomized pigs were analyzed after overnight fasting on four separate study days. Preprandial peripheral and portal plasma concentrations of insulin, glucose and CCK were significantly higher in vagotomized pigs compared to sham pigs (**Table 4.2**), but preprandial peripheral and portal GLP-1 concentrations did not differ between the two groups. The preprandial portal-peripheral glucose gradient was elevated in the vagotomy group (0.01 ± 0.1 mmol/L sham vs 0.56 ± 0.12 vagotomized $p<0.01$). Preprandial portal-peripheral gradients of insulin and GLP-1 were not affected by vagotomy.

Table 4.2. Preprandial plasma concentrations of glucose, insulin, GLP-1 and CCK. The effects on preprandial plasma concentrations are based on 48 observations per group (6 pigs, 4 study days, 2 time points). ND not determined

		Sham	Vagotomy	p
Glucose(mmol/L)	peripheral	5.12 ± 0.06	5.91 ± 0.08	p<0.001
	portal	5.11 ± 0.09	6.37 ± 0.14	p<0.01
Insulin (ng/ml)	peripheral	0.10 ± 0.02	0.17 ± 0.03	p<0.01
	portal	0.14 ± 0.04	0.29 ± 0.08	p<0.05
CCK (pmol/L)	peripheral	0.48 ± 0.13	0.80 ± 0.06	p<0.01
	portal	ND	ND	
GLP-1 (pmol/L)	peripheral	9.98 ± 1.17	14.93 ± 1.74	p<0.42
	portal	14.69 ± 1.00	16.65 ± 1.48	p<0.59

Effects of vagotomy on postprandial glucose, insulin, CCK and GLP-1 responses

Plasma levels of glucose, insulin, CCK and GLP-1 were analyzed to monitor the effects of vagotomy on postprandial responses after a standardized liquid morning meal. The effects of vagotomy on postprandial peripheral glucose and insulin levels are shown in **Figure 4.2A and 4.2C**. An overall time effect ($p<0.01$ peripheral, $p<0.01$ portal) and an interaction between time and vagotomy ($p<0.01$ peripheral, $p<0.01$ portal) was found for postprandial glucose and insulin levels. Peak levels for glucose and insulin were reached 30 minutes after the standardized liquid morning meal. Vagotomy did not affect peak peripheral glucose level (7.39 ± 0.25 mmol/L sham vs 7.22 ± 0.22 mmol/L vagotomy) but portal peak glucose tended to be decreased after vagotomy (10.65 ± 0.49 mmol/L sham vs 8.68 ± 29 mmol/L vagotomy $p<0.08$). Peak peripheral insulin level was decreased after vagotomy (1.39 ± 0.23 ng/ml sham vs 0.53 ± 0.06 ng/ml

vagotomy p<0.05). Portal peak insulin did not differ significantly between the sham and vagotomized pigs (1.09 ± 0.11 ng/ml sham and 0.81 ± 0.12 ng/ml vagotomy). The change in peripheral glucose and insulin after a standardized liquid morning meal was greater in the sham group as compared to the vagotomy group (p<0.007) (**Figure 4.2B, 4.2D**).

The plasma concentrations for peripheral CCK and GLP-1 are shown in **Figure 4.3 and 4.4**. A time effect (p<0.001) and an interaction between time and vagotomy (p<0.001) were found for postprandial peripheral CCK, however individual time points did not differ significantly between sham and vagotomized pigs. For postprandial GLP-1 concentrations a time effect (p<0.001 peripheral and portal) and interaction between time and vagotomy (p<0.05 peripheral p<0.01 portal) were found, however individual time points did not differ significantly between sham and vagotomized pigs.

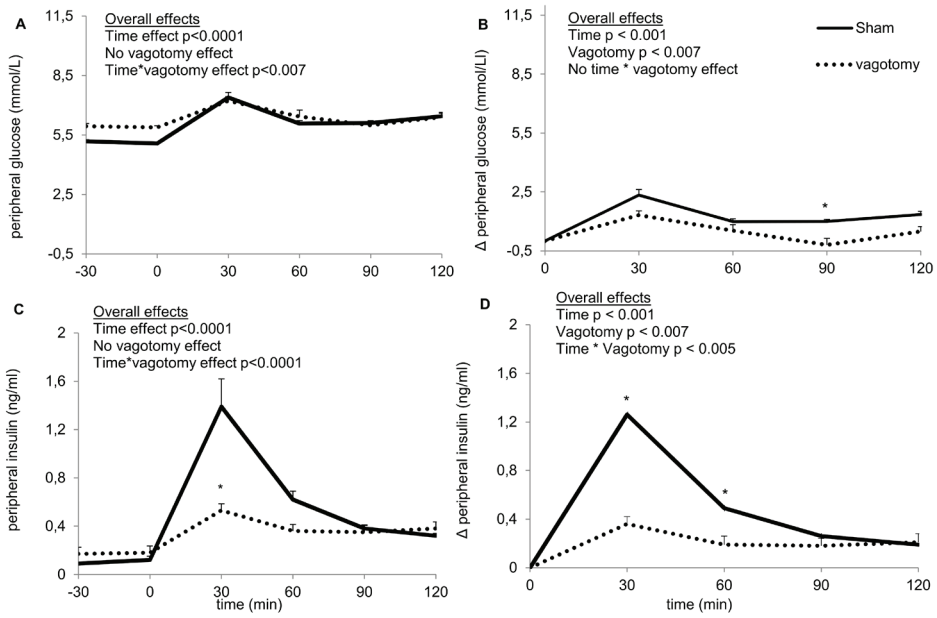

Figure 4.2 Peripheral glucose (A) and insulin (C) concentrations and changes (Δ) in peripheral glucose (B) and insulin (D) concentrations after a standardized morning meal. The results are shown as the mean ± SEM. The solid line represents the sham group (24 observations per time point) the dotted line represents the vagotomy group (24 observations per time point). *p<0.05 specific time point is significant different between the sham and vagotomy group.

Effects of CCK and GLP-1 receptor blockade in combination with vagotomy on CCK and GLP-1 plasma concentrations

Both peripheral and portal insulin and glucose concentrations were not affected by the various drug treatments. An overall drug (devazepide and exendin (9-39)) effect (p<0.001) was found for both CCK (**Figure 4.3**) and GLP-1 concentrations (**Figure**

4.4). Peripheral CCK concentrations were increased after blockade of the CCK receptor by devazepide in the sham group ($p<0.01$) and tended to be increased after combination treatment in the sham group ($p = 0.06$). The effect of devazepide and combination treatment on peripheral CCK concentrations was absent in the vagotomized pigs. The change in peripheral CCK concentrations after the various drug treatments is shown in **Figure 4.3B**. For the change in CCK concentrations an overall vagotomy effect was found ($p<0.05$) as well as an overall drug effect ($p<0.001$). CCK concentrations increased more after devazepide ($p<0.05$) as compared to placebo treatment in the sham operated group.

Peripheral GLP-1 was increased after blocking the GLP-1 receptor using exendin (9-39) in the sham group ($p<0.01$). Vagotomy abolished the effect of exendin (9-39) on peripheral GLP-1 concentrations. Combined drug treatment resulted in increased GLP-1 in the sham group ($p<0.001$) and in the vagotomized group ($p<0.05$). The

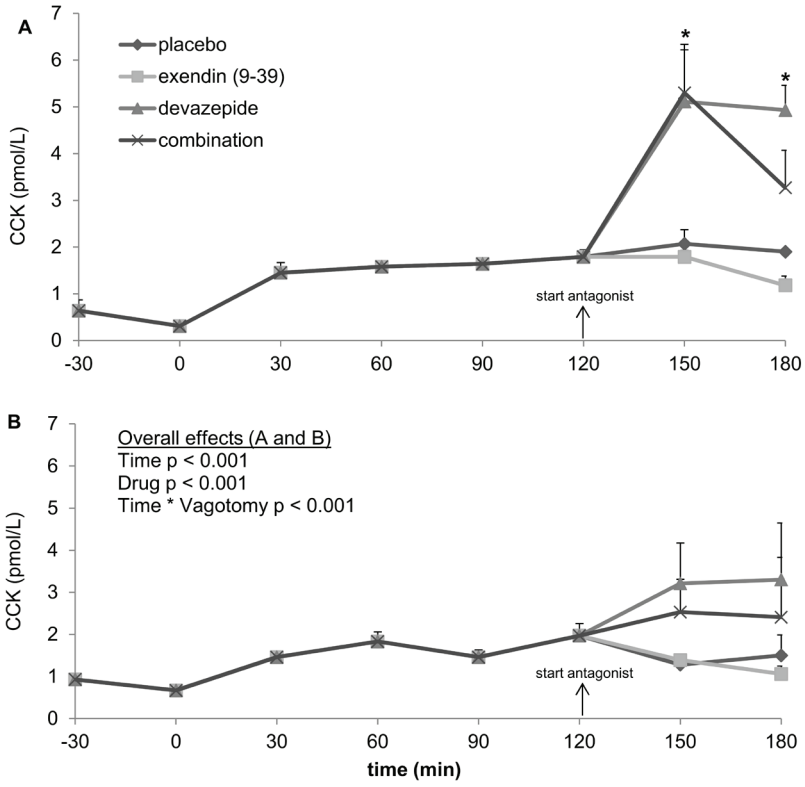

Figure 4.3A Peripheral CCK responses in the sham group (A) and the vagotomized group (B), after a standardized morning (t=0 min) meal and various drug treatments (t=120 min). The CCK concentrations (mean ± SEM) of the sham group (A) and the vagotomy group (B) after a standardized liquid morning meal (0 minutes) and concentrations after the drug infusions started (t=120 minutes). *$p<0.05$ specific time point is significant different as compared to placebo treatment within the group.

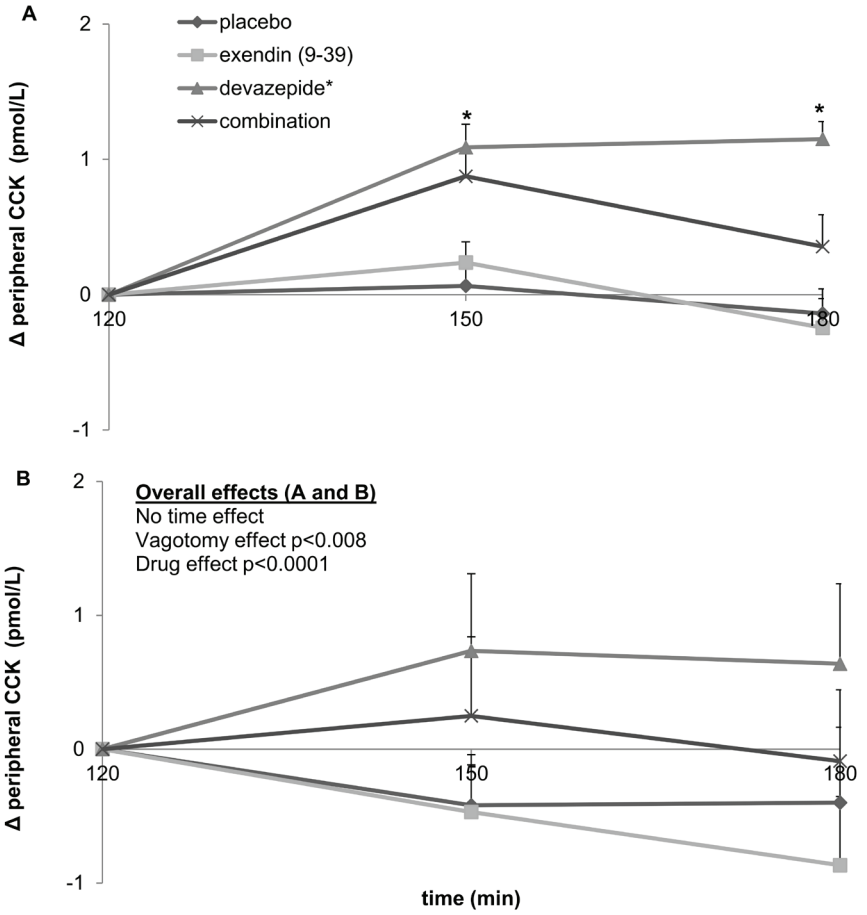

Figure 4.3B Changes (Δ) in peripheral CCK concentrations in the sham group (A) and the vagotomized group (B), after various drug treatments (t=120 min). The changes in CCK concentrations (mean ± SEM) of the sham group (A) and the vagotomy group (B) after the drug infusions started (t=120 minutes). *p<0.05 The change in CCK concentration is significant different compared to placebo treatment within the group.

change in both peripheral and portal GLP-1 concentrations is shown in **Figure 4.4B**. For both peripheral and portal GLP-1 concentrations an overall time effect ($p<0.05$) and an overall drug effect ($p<0.001$) was found. Vagotomy did not affect the changes in peripheral and portal GLP-1 concentrations. The changes in peripheral GLP-1 concentrations were larger after the exendin (9-39) ($p<0.05$) and combination ($p<0.05$) treatment as compared to placebo. The changes in portal GLP-1 concentrations were larger after exendin (9-39) ($p<0.05$) and combination ($p<0.05$) treatment in the sham group only.

Portal-peripheral gradients

The portal – peripheral gradients for glucose and GLP-1 are shown in **Figure 4.5**. For glucose a time effect ($p<0.001$) and interaction between time and vagotomy treatment ($p<0.001$) were found, with a decreased glucose gradient in the vagotomized pigs. Also for GLP-1 a time effect ($p<0.001$) and interaction between time and vagotomy treatment ($p<0.05$) were found, however individual time points did not differ significantly between sham and vagotomized pigs. The GLP-1 gradient was decreased after vagotomy. The portal-peripheral insulin gradient was not affected by vagotomy and portal CCK was not analyzed.

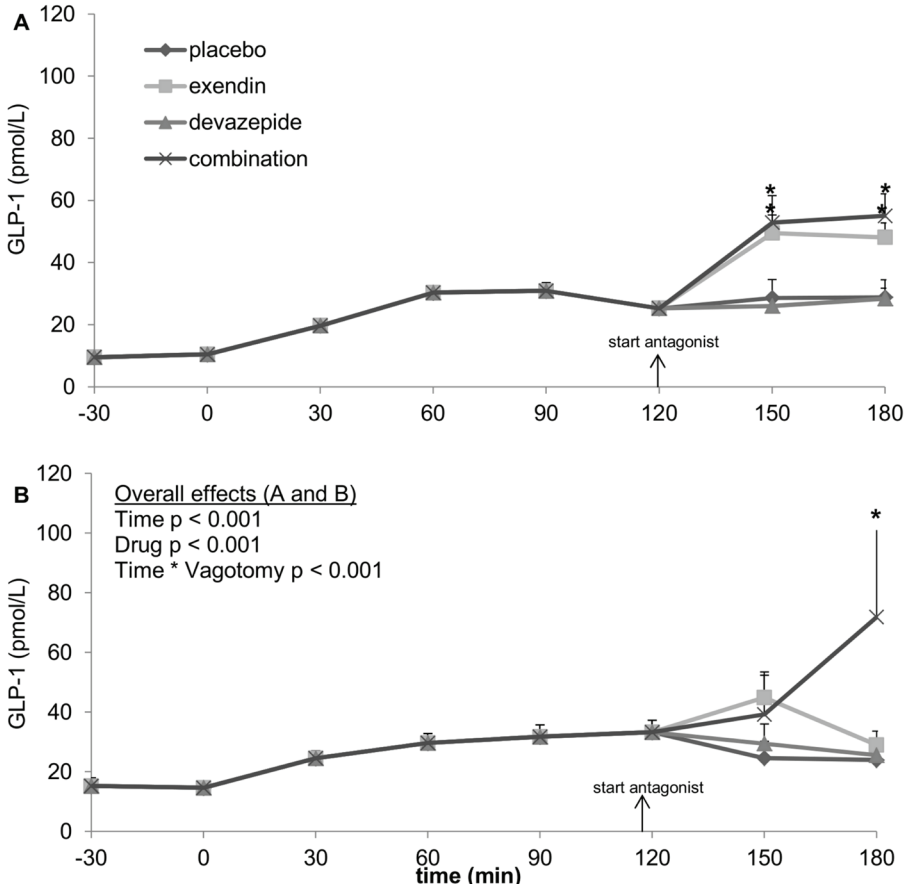

Figure 4.4A Peripheral GLP-1 responses in the sham group (A) and the vagotomized group (B) after a standardized morning (t=0 min) meal and various drug treatments (t=120 min).The GLP-1 plasma concentrations are shown as the mean ± SEM. The GLP-1 concentrations (mean ± SEM) of the sham group (A) and the vagotomy group (B) after a standardized liquid morning meal (0 minutes), and concentrations after the drug infusions started (t=120 minutes). *$p<0.05$ specific time point is significant different as compared to placebo treatment within the group.

The various drug treatments did not affect peripheral and portal glucose and insulin levels in both sham and vagotomized pigs (data not shown). Portal-peripheral GLP-1 gradient was increased after combination treatment in both groups ($p<0.05$), single drug treatments did not affect the portal-peripheral GLP-1 gradient.

Effects of vagotomy on small intestinal motility and gastric emptying

The peripheral plasma acetaminophen concentrations (μg/ml) were measured over time to monitor gastric emptying after the standardized liquid morning meal (**Figure 4.6**). A time effect ($p<0.001$), vagotomy effect ($p<0.001$) and interaction between time and vagotomy ($p<0.001$) were found. The acetaminophen concentration was higher in the sham group 30 minutes after the standardized liquid morning meal (4.1 ± 0.6 μg/ml sham vs 1.7 ± 0.2 μg/ml vagotomy $p<0.01$), and the maximum acetaminophen plasma concentrations (Cmax) was higher in the sham group (11.6 ± 1.0 μg/ml sham vs 4.1 ± 0.5 μg/ml vagotomy $p<0.001$). The time that the maximum acetaminophen concentrations were reached (Tmax) did not differ between groups (154 ± 8 min sham vs 146 ± 9 min vagotomy).

Stomach content 180 minutes after a standardized liquid morning meal (postmortem examination) was larger ($p<0.001$) in vagotomized pigs (5320 ± 430 g) as compared to the sham group (1770 ± 750 g). Content of the first 1/3 part of the small intestine

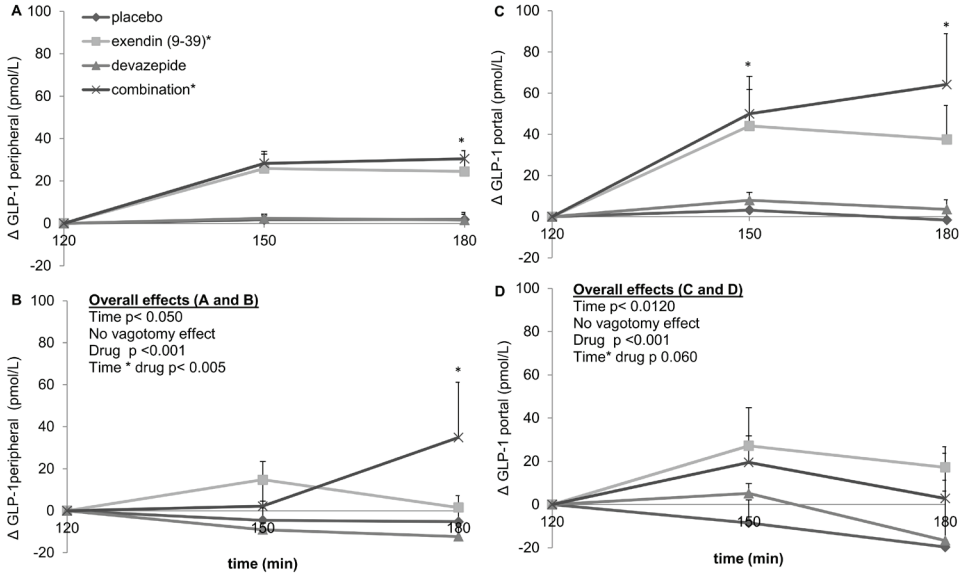

Figure 4.4B Changes (Δ) in peripheral and portal GLP-1 concentrations in the sham group (A and C) and the vagotomized group (B and D), after various drug treatments (t=120 min). Changes in GLP-1 concentrations (mean ± SEM) of the sham group (peripheral A, portal C) and the vagotomy group (peripheral B, portal D) after the drug infusions started (t=120 minutes). *$p<0.05$ The change in GLP-1 concentration is significant different compared to placebo treatment within the group.

was larger in vagotomized pigs as compared to sham pigs (325 ± 65 g sham vs 430 ± 85 g vagotomy, p<0.001). Length of small intestine in vagotomized pigs was greater as compared to small intestinal length in sham pigs (13 ± 0.2 meter sham vs 15 ± 0.5 meter vagotomy, p<0.001). Content of the second and third part of the small intestine did not differ between groups, neither did liver weight nor bladder content.

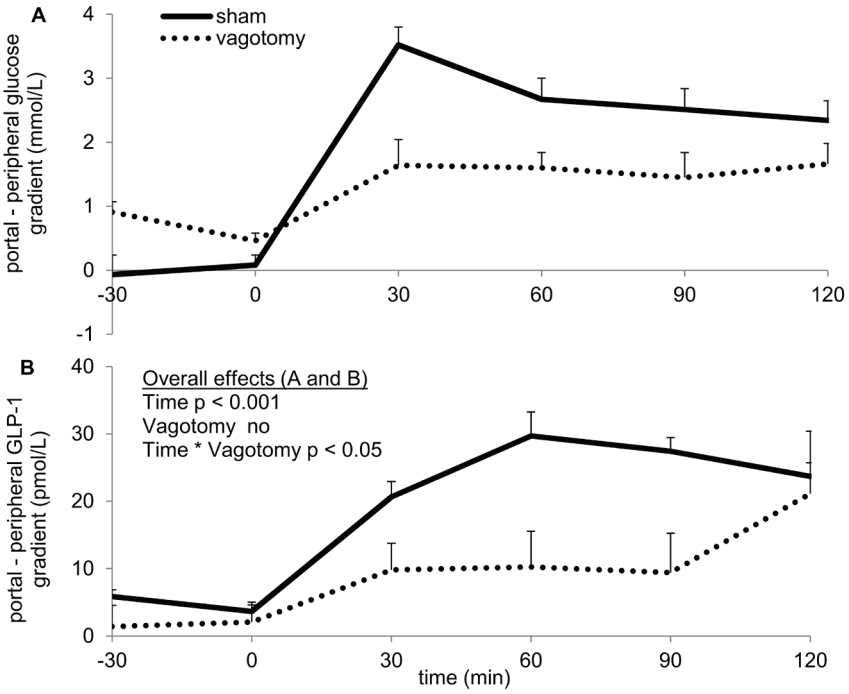

Figure 4.5 Preprandial and postprandial portal-peripheral gradients of glucose (A) and GLP-1 (B). The portal – peripheral gradients are shown for glucose and GLP-1. The sham group is represented by the solid line (24 observations per time point) and the vagotomized group is represented by the dotted line (20 observations per time point).

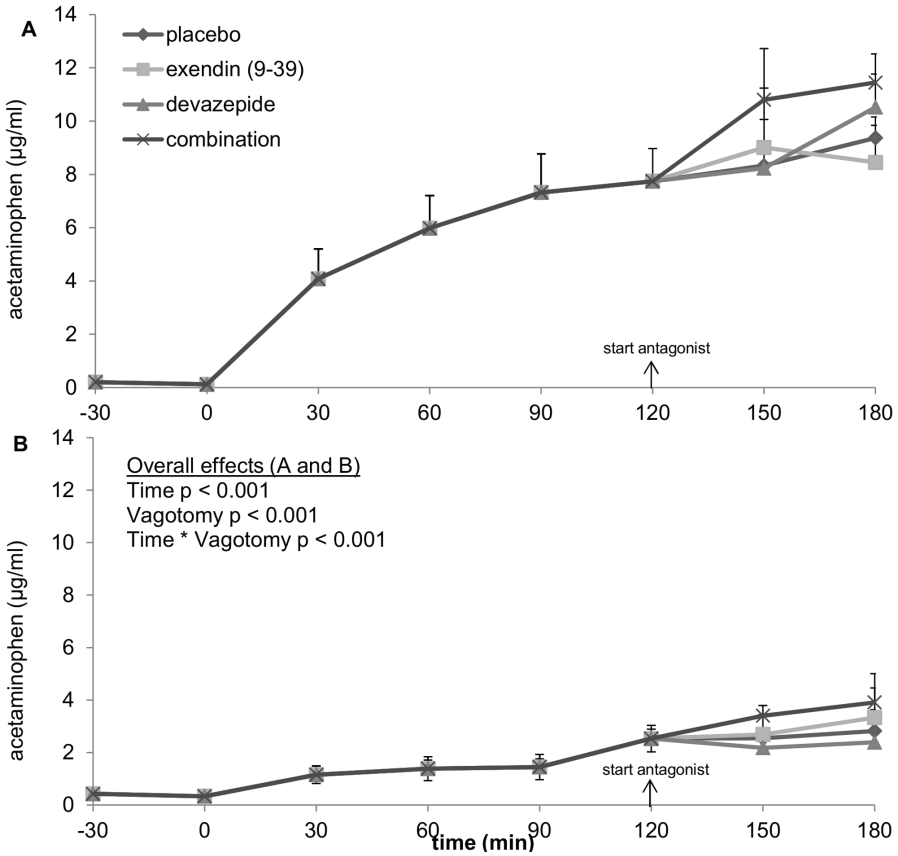

Figure 4.6 Appearance of acetaminophen in plasma after standardized acetaminophen containing liquid morning meal in the sham (A) and vagotomized group (B**)**. Plasma concentrations of acetaminophen (µg/ml) in time in peripheral blood is shown.

Discussion

The present study showed that blockade of CCK_1 receptors using devazepide, and a combination of CCK_1 and GLP-1 receptor blockade resulted in increased liquid food intake. This was observed both in the sham and vagotomized pigs which indicates that this effect was independent of CCK_1 receptors located on the abdominal vagal nerve. These results suggest that under the test conditions CCK mainly induces satiation via central CCK_1 receptors and not via CCK_1 receptors located at the abdominal vagal nerve. Simultaneous blocking of both CCK_1 and GLP-1 receptors did not enhance the effect on food intake compared to CCK_1 receptor blockade alone, suggesting the absence of synergy between the CCK and GLP-1 pathways. Interruption of abdominal vagal signal transduction by subdiaphragmatic vagotomy did not affect ad libitum liquid meal size in pigs.

Our results on CCK are in line with previous studies in which the intravenous administration of devazepide (17.5-140 μg/kg) in pigs resulted in a dose dependent increase in food intake (38), and also confirm rat studies showing that satiety in rats is mediated in part by an endogenous CCK action that is independent of the vagal nerve (25). In a more recent study (26) it was concluded that endogenous CCK acts through CCK_1 receptors beyond the blood brain barrier and through CCK_1 receptor-mediated mechanisms involving abdominal vagal nerves. This was studied in rats by using two antagonists of which one does not cross the blood brain barrier, whereas the other devazepide does. This rat study showed that CCK increases food intake via CCK_1 receptors beyond the blood brain barrier. This finding was confirmed in our pig study showing that devazepide is effective in increasing food intake in vagotomized pigs. Furthermore, Reidelberger et al.(26) found that endogenous CCK can also act on mechanisms involving abdominal vagal nerves to inhibit food intake. The latter mechanism seems to play no major role in the regulation of liquid food intake in pigs because vagotomy did not alter the efficacy of devazepide in our study.

In our study GLP-1 receptor blockade with exendin (9-39) did not affect food liquid food intake. This is in line with another rat study in which the intravenous administration of 10 or 30 nmol/kg body weight exendin (9-39) failed to increase spontaneous meal size (40).

However, the effects of GLP-1 receptor blockade on food intake are inconsistent. In a study of Williams et al.(41) blockade of the GLP-1 receptor by exendin (9-39) in rats increased food intake. This effect was only observed when food intake was normally low. In another rat study food intake decreased after administration of another GLP-1 receptor antagonist (exendin-4[desHis-1, Glu-9]) prior to a premeal peak of GLP-1 (42). In this rat study food intake increased when the antagonist was administered after the GLP-1 premeal peak but prior to food presentation (42). These results suggest that studying the effects of GLP-1 receptor blockade on food intake depend on the timing of the antagonist administration. In our pig study the timing of antagonist administration and ad libitum food intake was optimized to avoid any anticipatory or conditioned release of GLP-1 prior to the ad libitum meal. Pigs were normally fed around 8:00 AM and 4:00 PM whereas during the study days they were fed at 08:00 AM (morning meal) and at 11:00 AM (ad libitum meal). Although the time between the morning meal and the ad libitum meal at the study days was only 3 hours, the drive of the pigs to consume the (second) ad libitum meal might have overruled the effect of the GLP-1 receptor blockade.

Another reason for the lack of effect of exendin (9-39) might be the high ingestion rate of liquid feeding. Liquid feeding does not require chewing and the rate of ingestion is therefore high. Solid food is usually ingested in about 30 minutes, whereas in this study the liquid meals were completed within 6.9 ± 0.6 min. It is known that CCK is produced by enteroendocrine I cells which are most abundant in proximal part of the

small intestine located at duodenal and jejunal mucosa (14) whereas GLP-1 is released from enteroendocrine L-cells which show a maximum density in the ileum for most species (7, 14). It is therefore conceivable that the more pronounced effect of CCK receptor blockade is due to the short period of eating and rapid passage of nutrients into the duodenum. Also, there was a large variability in food intake. Therefore the power of this study might be a limitation.

Total subdiaphragmatic vagotomy was conducted by endoscopic laparoscopy which has been used before (43, 44). By using this technique, pigs recovered rapidly without complications from their surgery. Total subdiaphragmatic vagotomy was achieved by removal of approximately 3 cm length of both vagal nerves and confirmed by inspection at necropsy (**Figure 4.7**).

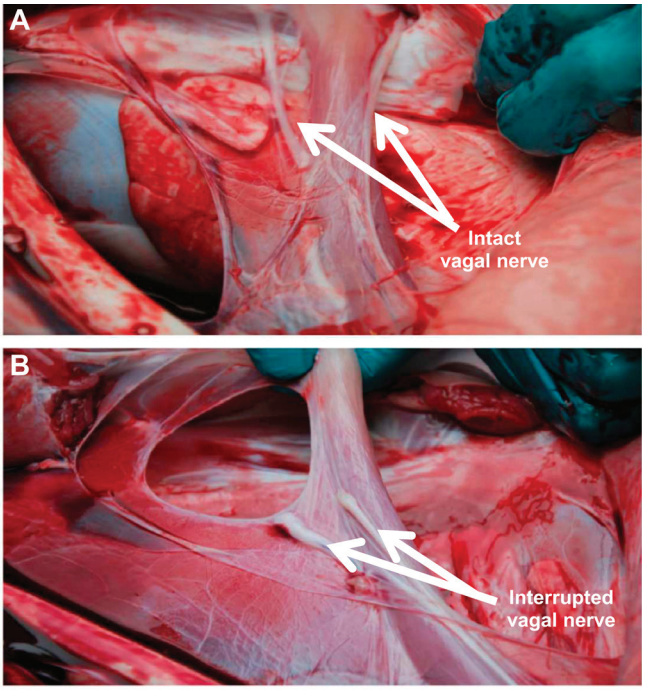

Figure 4.7 Confirmation of subdiaphragmatic abdominal vagotomy at necropsy in the sham showing the intact vagal nerves (A) and vagotomized group showing the interrupted vagal nerves (B).

Although liquid food was chosen to avoid effects on gastric emptying in the vagotomized pigs, an effect on gastric emptying cannot be excluded, since the acetaminophen Cmax was lower in the vagotomy group as compared to the sham group. Plasma acetaminophen concentrations were determined by small intestinal absorption of acetaminophen, which is dependent on gastric emptying rate (32, 33, 45, 46). The decreased plasma acetaminophen concentrations in the vagotomized pigs thus indicate a delay in gastric

emptying. A decreased gastric emptying rate is supported by the observation that animals from the vagotomy group showed a larger stomach content at autopsy.
In our study vagotomy did neither affect food intake, nor body weight. The reports on the effects of vagotomy on food intake in previously conducted studies are contradictory (20, 25, 26, 47). This may be the result of types and characteristics of food and(or) feeding regimens, different vagotomy techniques and species differences. Liquid meals used in other vagotomy studies were often provided as sucrose solutions which lack other essential nutrients and possibly sucrose intake is more related to taste than to dietary satiation. In the present study we have used a complete liquid diet containing all macronutrients required for pig food.
The present study showed the effects of total subdiaphragmatic vagotomy on basal and postprandial responses of glucose, insulin, GLP-1 and CCK. It was found that postprandial responses of glucose, insulin and CCK were decreased after vagotomy, whereas basal levels were increased. A difficulty in the interpretation for these findings is the delay in gastric emptying which might have caused the differences in both the basal and postprandial responses of the measured plasma parameters in the vagotomy group. Despite the effect of gastric emptying, the vagal nerves are involved in detecting the presence and volume of ingested food (48-53). This may also have contributed to the increased basal and decreased postprandial concentrations in the vagotomy group.
In the present study we investigated the role of CCK and GLP-1 by blocking their receptors which is expected to interrupt the action of endogenously released hormones. Studies in which CCK and GLP-1 are exogenously administered lead to concentrations outside the normal physiological range, locations which do not correspond to their endogenous sites of secretion and time profiles which may be out of balance with the fasting – feeding cycles. Blocking the receptors as is done in the present study, allows to study the effects of physiologically released CCK and GLP-1 at their normal sites of action, since both antagonist are able to penetrate the blood brain barrier (26, 54). Some other studies using receptor blockade have been done in rats, but to the best of our knowledge we are the first to report the effects of the abdominal vagal nerve interruption in combination with GLP-1 and CCK receptor blockade on food intake regulation. Also effects of vagotomy on postprandial responses of plasma acetaminophen, glucose, insulin, CCK and GLP-1 have not been reported elsewhere. The novel finding of increased CCK and GLP-1 concentrations after receptor blockade in our study mostly in sham pigs suggests the existence of a feed-back mechanism involving a sensing and/or regulatory role of the vagal nerve in CCK and GLP-1 plasma concentrations. This vagal feed-back mechanism may consist of the vago-vagal reflex, as described previously (55).
In our study CCK_1 receptor blockade resulted in increased food intake. For pharmaceutical applications, this would argue for the further development of CCK_1 receptor agonists or antagonists to either inhibit or stimulate food intake. In practice the exogenous

administration of CCK_1 receptor agonists has been hampered by difficulties in dosing, route of administration and timing with respect to feeding. Indeed, CCK agonists have not delivered any useful weight management drugs so far. While human trials with such compounds indeed often showed lower food-intake during meals, participants tended to compensate with increased intake in between meals (56). On the other hand, pharmaceutical or dietary intervention strategies to increase the endogenous release of CCK may not suffer from the drawbacks of exogenously administered CCK and could therefore still provide interesting approaches for weight reduction. At the same time, pharmaceutical and dietary interventions aiming at a reduction of endogenous CCK secretion or CCK receptor blockade may stimulate (liquid) food intake and thereby offer novel therapeutic possibilities for patients suffering from delayed gastric emptying or disease related malnutrition (57).

In conclusion, our data contribute to a better understanding of the balance between neural and humoral regulation of satiation by CCK and GLP-1. They underline the importance of the vagal nerve in regulating the gastric emptying, and meal transit which are important determinants for blood glucose, insulin, CCK and GLP-1 concentrations. Remarkably, in case of CCK, CCK_1 receptors elsewhere in the body appear to be more important than the CCK_1 receptors located at the abdominal vagal nerve for the regulation of liquid meal size.

Competing Interest

The authors have declared that no competing interests exist.

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Chapter 5

Small intestinal protein infusion: evidence for a location specific gradient in braking efficacy

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Manuscript submitted

Abstract

Background: Protein infusion in the small intestine results in intestinal brake activation: a negative feedback mechanism that is mediated by the release of GI peptides and results in a reduction in food intake. It has been proposed that duodenum, jejunum and ileum may respond differently to proteins.

Objective: To investigate differences in *ad libitum* food intake, feelings of hunger and satiety and the systemic levels of cholecystokinin (CCK), glucagon-like peptide-1 (GLP-1), peptide YY (PYY), glucose and insulin after intraduodenal, intrajejunal and intraileal protein infusion.

Methods: Fourteen subjects (4 male, mean age: 23 ± 2.1 years, mean body mass index: 21,6 ± 1,8 kg/m^2) were intubated with a naso-ileal catheter and participated in this double blind, randomized, placebo-controlled crossover study. Test days started with the ingestion of a standardized breakfast, followed by the infusion of 15 g of protein in either the duodenum, jejunum or ileum over a period of 60 minutes. Food intake was measured by offering an *ad libitum* meal and Visual Analogue Scale (VAS) scores were used to score feelings of hunger and satiety. Blood samples were drawn at regular intervals for CCK, GLP-1, PYY, glucose and insulin analyses.

Results: Intraileal protein infusion resulted in a decrease in *ad libitum* food intake compared with both intraduodenal and placebo infusion (Ileum: 628.5 ± 63 kcal vs. Duodenum: 733.6 ± 50 kcal at $p<0.01$ and Placebo: 712.2 ± 53 kcal at $p<0.05$). GLP-1 concentrations were increased after ileal infusion compared with duodenal, jejunal and placebo infusion, while CCK concentrations were only increased after intraileal protein infusion compared with placebo. None of the treatments affected VAS scores for hunger and satiety nor plasma concentrations of PYY and glucose.

Conclusion: Protein infusion in the ileum results in a more potent increase in the release of GLP-1 and a decrease in food intake compared with infusion into the duodenum or jejunum, or compared with placebo, respectively.

Introduction

Protein is known to be more satiating than an isocaloric amount of either fat or carbohydrate (1, 2). An increase in the amount of dietary protein results in satiating effects with significant long-term weight loss (3). It has been suggested that increasing protein ingestion is an effective weight management tool (2). The mechanisms underlying the satiating effect of protein are multifactorial, but it has been well established that the gastrointestinal (GI) tract plays an important role in generating signals that mediate satiety.

It has been shown previously that intraduodenal infusion of protein results in a more pronounced increase in plasma cholecystokinin (CCK) levels and decrease in food intake compared with oral ingestion of the same amount of protein (4). This difference may be explained by the activation of the so-called duodenal brake. Undigested nutrients in the duodenum activate this negative feedback mechanism that modulates not only proximal gastrointestinal motility, secretion and mucosal peptide release but also feelings of satiety and food intake (5). In recent years, several human studies provided evidence for the inhibitory effects of intraduodenal infusion of protein on satiety and food intake (6-9). However, it has been suggested that infusion of nutrients into the ileum results in an even greater effect on satiety and food intake when compared with infusion into the more proximal parts of the small intestine (10).

Under physiological conditions, only small amounts of undigested nutrients reach the distal small intestine (11, 12). Most evidence for a more pronounced ileal versus duodenal or jejunal brake effect is derived from surgical procedures such as the Roux-en-Y Gastric Bypass (RYGB) and ileal transposition (IT). RYGB is one of the procedures that bypass the proximal intestine, herewith increasing the exposure of the distal small intestine to undigested nutrients (13). Also, intraileal fat infusion applying intestinal feeding catheters results in a significant reduction in appetite, a significant increase in fullness and a more delayed GI motility as compared with intraduodenal fat infusion (14). Human studies comparing the responsiveness of different parts of the small intestine to protein are still lacking. Aim of the present study was therefore to investigate differences in *ad libitum* food intake, feelings of hunger and satiety and the systemic levels of cholecystokinin (CCK), glucagon-like peptide-1 (GLP-1), peptide YY (PYY), glucose and insulin after intraduodenal, intrajejunal and intraileal protein infusion, respectively. We hypothesized that intraileal protein infusion results in a more potent decrease in food intake and a stronger increase in both feelings of satiety and plasma GI peptide levels as compared with intraduodenal and intrajejunal protein infusion.

Materials & Methods

This study was approved by the Medical Ethics Committee of the Maastricht University Medical Center+ (MUMC), Maastricht, the Netherlands, and performed in full

accordance with the Declaration of Helsinki (latest amendment by the World Medical Association in 2013). All participants gave their written informed consent prior to participation. The study has been registered in the US National Library of Medicine (http://www.clinicaltrials.gov, NCT02500069).

Subjects

Healthy, lean volunteers were recruited by local advertisement. Subjects were screened using a standard health questionnaire and a physical examination. Exclusion criteria included: medical or surgical history that could have affected study outcome, medication use (except contraceptives), smoking and excessive alcohol consumption (>2 units per day). All participants were weight stable for at least two months prior to participation, and were not following any energy restriction- or food supplementation diets. Power calculation showed that fourteen study completers were needed, to reach sufficient statistical power.

Study design

In this double-blind randomized placebo-controlled crossover study, subjects were studied on 4 randomly assigned consecutive test days. On these occasions, 15 g of casein was infused using a naso-ileal feeding catheter over a period of 60 minutes into one of the three different locations of the small intestine: 1) the duodenum, 2) the jejunum and 3) the ileum, or no protein was infused (placebo). During each intervention, protein infusion was accompanied by simultaneous infusion of tap water in the other two locations. For example, intraduodenal protein infusion was accompanied by simultaneous tap water infusion in jejunum and ileum, respectively. The placebo treatment consisted of tap water infusion in all three locations.

Naso-ileal catheter

Subjects were intubated with a 290 cm long silicon 9-channel (8-lumen, 1 balloon inflation channel, outer diameter of 3.5 mm) custom-made catheter (Dentsleeve International, Mui Scientific, Missisauga, Canada). The catheter contained three sideholes per channel with 3-cm interspacing between consecutive side holes, and had an inflatable balloon (maximum inflation capacity 10mL) integrated into the distal tip. After local anaesthesia of a nostril (xylocaine 10% spray; AstraZeneca, Zoetermeer, The Netherlands), the catheter was introduced into the stomach. Subsequently, the tip of the catheter was positioned in the duodenum under intermittent fluoroscopic control. Progression of the catheter into the ileum was performed as described previously (15). Correct positioning of the catheter was checked under fluoroscopy before starting each test day. The catheter includes radio-opaque markers at all sideholes, which enabled us to accurately determine the position of the catheter. The radio-opaque markers made it

possible to select the infusion channel from this multi-lumen catheter that was located at the infusion location (duodenum, jejenum or ileum). Infusion into the duodenum took place 5 cm distal to the pylorus, while infusion into the jejunum took place 40-50 cm distal of the pylorus. Intraileal infusion was performed at least 120 cm distal to the pylorus.

Protein and placebo infusions

Casein (energy density: 3.4 kcal/g, Dutch Protein Services, the Netherlands) was used as the protein source in this study. Fifteen gram of casein was dissolved in 180 mL of tap water and infused at a speed of 3 mL/min over a 60 min period. During placebo treatment, tap water was infused simultaneously in all three locations.

Protocol

During four consecutive test days, subjects arrived at our laboratory at 8:00 AM after an overnight fast. After checking correct positioning of the catheter as described above, test days started with the insertion of an intravenous catheter in a forearm vein for collection of blood samples. At 8:30 AM basal blood samples and VAS scores for hunger and satiety were taken. Hereafter, subjects consumed a standard fixed sized liquid breakfast meal (150 kcal, Goedemorgen Drink Yoghurt, Friesland Campina, the Netherlands). One hundred fifty minutes after the ingestion of the breakfast meal (11:00 AM), infusion of protein or placebo into the small intestine was started. Intestinal infusion took place over 60 min, at a rate of 3 mL/min. Thirty minutes after ending the infusion, volunteers received a standard *ad libitum* pasta lunch (Lasagne Bolognese, t=240 min). After ingestion of the meal, the test day was finished (see **Figure 5.1** for design of test day)

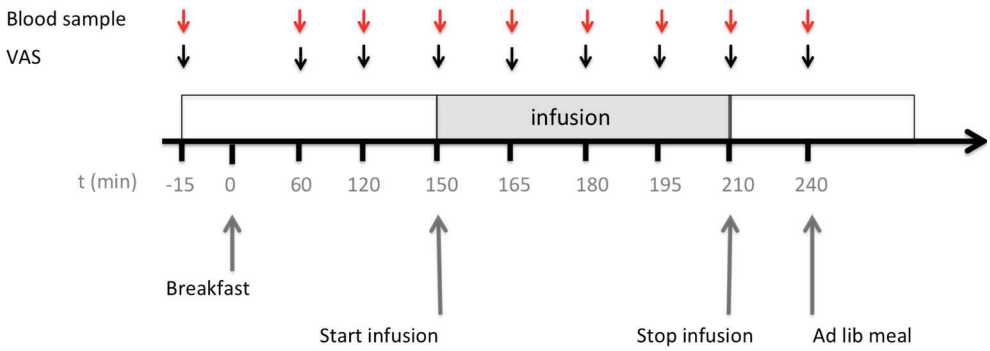

Figure 5.1 Timeline of test day. Intestinal infusion started 150 minutes after ingestion of the breakfast and took sixty minutes (until t=210 min). Blood samples and VAS scores were collected at several time points.

Gastrointestinal peptides

Venous blood samples were drawn at regular intervals (baseline (0), 60, 120, 150, 165, 180, 195, 210, 240 min). For GLP-1 (7-36), PYY and CCK measurements blood was collected in ice chilled EDTA aprotonin coated tubes (Becton & Dickinson, New Jersey, USA) and 10 µL Dipeptidyl peptidase-4 inhibitor (DPP4-010, Merck Millipore, Massachusetts, USA) per 1 mL of whole blood was immediately added after blood collection to prevent proteolytic cleavage. Tubes were centrifuged at a rate of 3000 revolutions per minutes (rpm), 4° C for 15 minutes and plasma was transferred into aliquots and stored at -80° C. Active GLP-1 (7-36) was determined using a Glucagon Like Peptide-1 (Active) ELISA kit with a range of 2-100 pmol/L, an inter-assay coefficient variation (CV) of 11%, and an intra-assay CV of 6% (EGLP- 35K, Merck Millipore, Massachusetts, USA). Total PYY (includes both peptide YY 1-36 and peptide YY 3-36) was measured using a human PYY (Total) ELISA kit with a range of 10-2000 pg/ml, an inter-assay CV of 6% and an intra-assay CV of 3% (EZHPYYT66K, Merck Millipore, Massachusetts, USA). Plasma cholecystokinin-8 (cholecystokinin 26–33) concentrations were measured with an optimized and validated commercial human RIA kit (EURIA CCK, RB302, Euro-Diagnostica, Malmö, Sweden) (14). This improved assay system has been optimized to reach a high sensitivity of 0.05 pmol/L and to have no cross-reactivity to gastrin-17 or sulfated gastrin. The intra-assay CV was 8.9% at a concentration of 0.84 pmol/L and 4.9% at a concentration of 1.98 pmol/L.

Glucose and insulin

Sodium fluoride- and SST II Plus gold tubes (Becton & Dickinson, New Jersey, USA) were used for determination of glucose and insulin, respectively. SST II Plus gold tubes were stored at room temperature for 30 minutes before centrifugation at 3000 rpm, 20° C for 15 minutes. Glucose measurements were performed on a Roche Cobas C701 analyzer (GLUC3, Roche, Mannheim, Germany) with an inter-assay variation of 0.02 mmol/L at glucose concentration 3.27 mmol/l. Serum insulin was measured using the Immulite 1000 Insulin Kit (LKIN5) on the Immulite 1000 (Siemens Medical Solutions Diagnostics, Los Angeles, CA) with an inter-assay variation of 4.09 pmol/L at insulin concentration 51.23 pmol/L.

Visual Analogue Scales (VAS) for hunger and fullness

Feelings of hunger, satiety, fullness and prospective food consumption were measured using Visual Analogue Scales (VAS, 0 to 100 mm) anchored at the low end with the most negative or lowest intensity feelings (e.g., extremely unpleasant, not at all), and with opposing terms at the high end (e.g., extremely pleasant, very high, extreme) (15).

Statistical analyses

Statistical analyses were performed using the SAS statistical software package (SAS version 9; SAS institute, Cary, NC, USA). Data were visually checked for normality and for constant variance of residuals by plots of residuals vs. corresponding predicted values. If data were not normally distributed, log transformation was applied for further analysis of the data, as was the case for CCK, GLP-1 (7-36), and PYY. Regarding food intake, statistical analysis was performed on the amount of food eaten in kcal. VAS scores for hunger and satiety were corrected for the values obtained at the onset of infusion and are displayed from the start of the substrate infusion (t=150 min) until the start of the *ad libitum* meal (t=225 min). CCK, GLP-1 (7-36) and PYY are displayed from the start of the test day (t=-15 min) until the last blood sample collected before the start of the *ad libitum* meal (t=240 min). The effects of each intervention on the levels of GI peptides were determined by analyzing the peptide levels from the start of the test day until ingestion of the *ad libitum* meal.

All variables were compared with a mixed analysis of variance model that included the fixed factors treatment (ileum, jejunum, duodenum and placebo). For the VAS and plasma parameters, time and the interaction between treatment and time were added to the model. Because of the crossover design, intervention effects within subjects were compared by including the random factor subject. If an intervention effect occurred, a post hoc Dunnett test was used to analyze differences in *ad libitum* meal intake. A post hoc Tukey-Kramer test was used to analyze differences in VAS scores and GI peptides (CCK, GLP-1 and PYY). Data are presented as the mean ± SEM (unless specified otherwise) and considered significant at $p<0.05$.

Results

Sixteen healthy volunteers were included. Two volunteers dropped out because of discomfort induced by the feeding catheter; fourteen volunteers (4 male, mean age: 23 ± 2.1 years, mean BMI: 21,6 ± 1,8 kg/m^2) completed the study.

Food intake

Intraileal protein infusion decreased *ad libitum* meal intake compared with placebo infusion and compared with duodenal protein infusion (ileum: 628.5 ± 63 kcal vs. placebo: 712.2 ± 53 kcal; $p<0.05$ and duodenum: 733.6 ± 50 kcal; $p<0.01$) (**Figure 5.2**). Food intake did not differ between jejunal and ileal protein infusion.

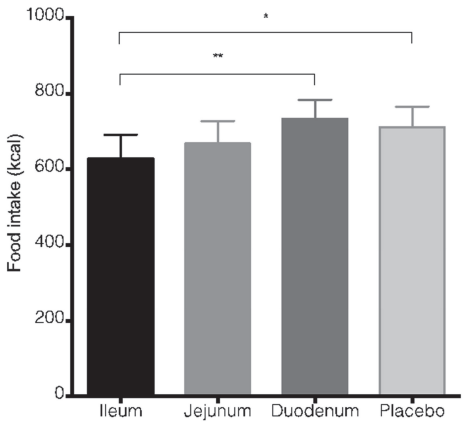

Figure 5.2 Food intake in kcal (mean+SEM) of an *ad libitum* lunch ingested 30 min after ending the protein infusion into the ileum, jejunum, duodenum or placebo, respectively, over a 60 min period (n=15). A significant reduction in food intake was observed after ileal protein infusion compared to duodenal protein infusion ($p<0.01$, **) and placebo infusion ($p<0.05$, *) based on a mixed analysis of variance model with a post-hoc Dunnett correction.

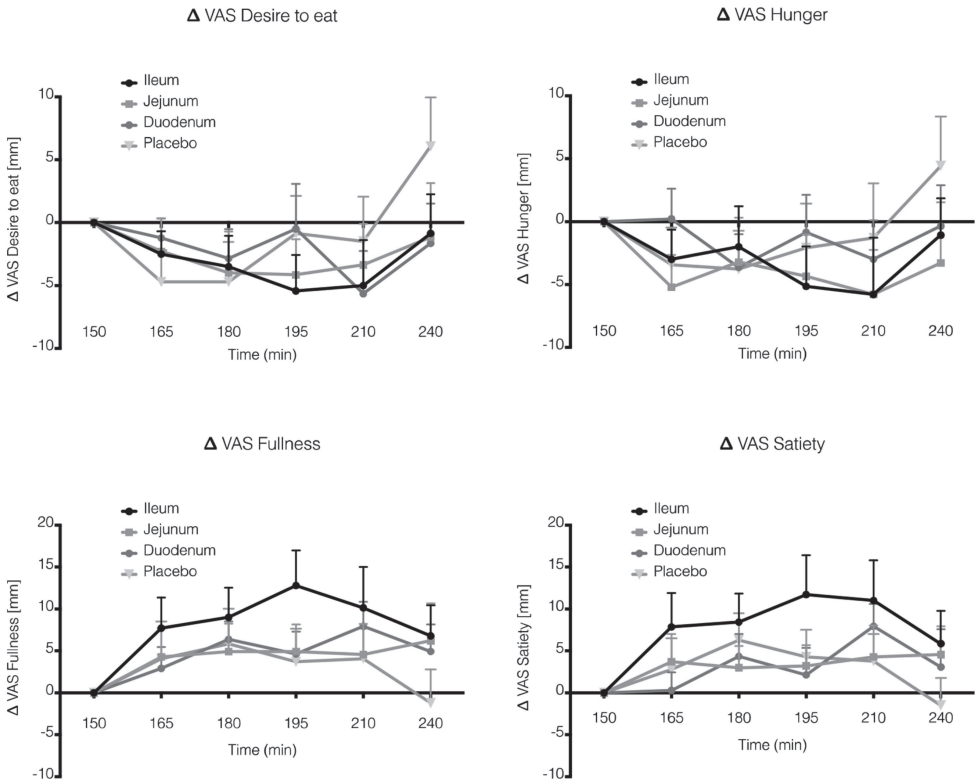

Figure 5.3 VAS Desire to eat, hunger, fullness and satiety (mean+SEM) during and after protein infusion in the ileum, jejunum, duodenum and placebo, respectively. Intestinal infusion started 150 minutes after breakfast ingestion and lasted for 60 minutes. No differences in VAS scores were observed. These results were analyzed with a mixed analysis of variance model with a post-hoc Tukey-Kramer correction.

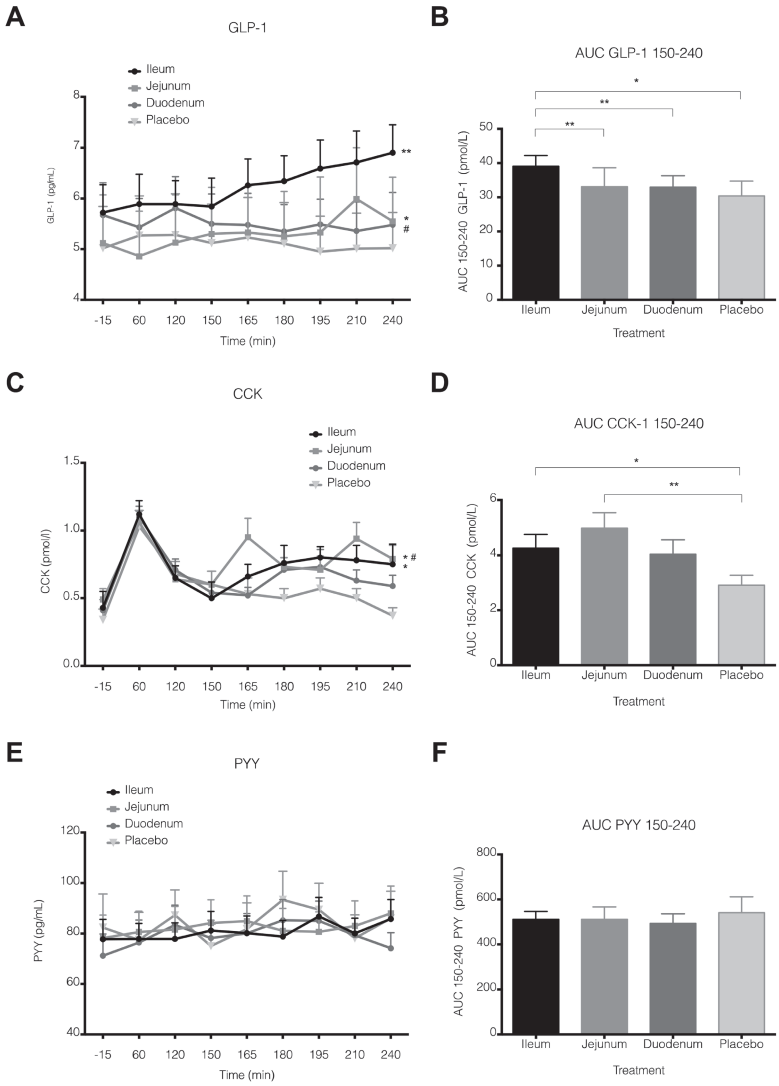

Figure 5.4 Plasma concentrations of GLP-1, CCK and PYY (mean+SEM) and GLP-1, CCK and PYY areas under the curve (AUC 150-240 min) during and after protein infusion in the ileum, jejunum, duodenum and placebo infusion, respectively. Intestinal infusion started 150 minutes after breakfast ingestion and lasted for 60 minutes. A) Significantly higher GLP-1 levels were observed after intraileal infusion of protein versus intrajejunal, intraduodenal and placebo infusion (all $p<0.001$ **). Intrajejunal and intraduodenal protein infusion increased GLP-1 concentrations compared with placebo infusion (both $p<0.02$, *) and # B) AUC (150-240 min) for GLP-1 was significantly increased after ileal protein infusion compared to jejunal ($p<0.05$, **) duodenal ($p<0.05$, **) and placebo infusion ($p<0.01$, *). C) Significantly higher CCK levels were observed after intraileal and intrajejunal infusion of protein versus placebo infusion; ($p<0.001$, *). Intrajejunal infusion resulted in an increase in CCK levels compared with duodenal infusion; ($p<0.05$, #). D) AUC (150-240 min) of CCK during ileal and jejunal infusion was significantly increased compared with placebo infusion ($p<0.05$, * and $P<0.001$, **, respectively). E+F) No differences in the release of PYY were observed. These results were analyzed with a mixed analysis of variance model with a post-hoc Tukey-Kramer correction.

Appetite

Mean VAS scores for desire to eat, hunger, fullness and satiety are presented in **Figure 5.3**. Consumption of the liquid breakfast at time t=0 min resulted in a decrease in desire to eat and hunger and an increase in fullness and satiety in all treatments (data not shown). VAS scores for desire to eat, hunger, fullness and satiety were not different after ileal protein infusion compared with duodenal or jejunal protein infusion or compared with placebo.

GLP-1, CCK and PYY

The effects of ileal, jejunal and duodenal infusion on plasma concentrations of GLP-1, CCK and PYY and AUCs (150-240 min) are presented in **Figure 5.4**. Fasted plasma concentrations of CCK, GLP-1 and PYY did not differ between test days. CCK concentrations increased after ingestion of the liquid breakfast, whereas GLP-1 and PYY concentrations did not change. Ileal protein infusion increased GLP-1 concentrations compared with duodenal, jejunal and placebo infusion, respectively ($p<0.001$, **Figure 5.4A**). Both jejunal and duodenal protein infusion increased GLP-1 concentrations compared with placebo infusion (both $p<0.01$, **Figure 5.4A**). The AUC (150-240 min) for GLP-1 was increased after ileal protein infusion compared with jejunal, duodenal and placebo infusion, respectively (**Figure 5.4B**) CCK concentrations were also increased after intraileal and intrajejunal protein infusion compared with placebo ($p<0.05$). Furthermore, an increase in CCK was observed after intrajejunal infusion compared with duodenal infusion ($p<0.05$, **Figure 5.4C**). Ileal and jejunal protein infusion increased the AUC (150-240 min) for CCK compared with placebo

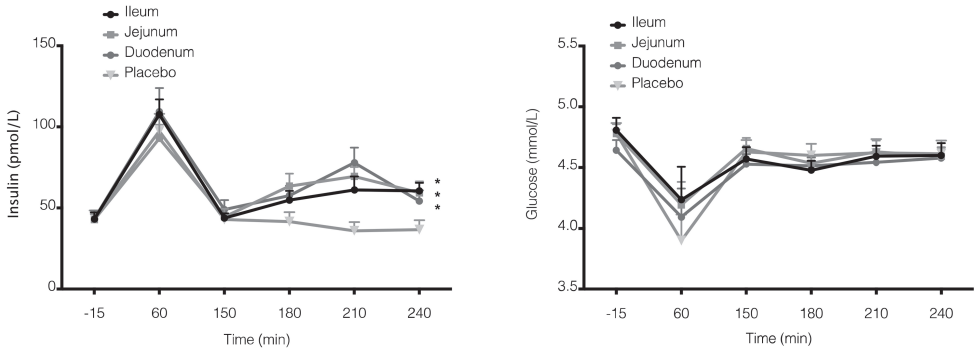

Figure 5.5 Glucose and insulin concentrations *(*mean+SEM) during and after protein infusion in the ileum, jejunum, duodenum and placebo infusion, respectively. Intestinal infusion was started 150 minutes after breakfast ingestion and lasted for 60 minutes. Intraileal, intrajejunal and intraduodenal infusion of protein resulted in an increase in insulin levels compared to placebo infusion ($p<0.0001$*). No differences in glucose concentrations were seen. These results were analyzed with a mixed analysis of variance model with a post-hoc Tukey-kramer correction.

infusion (**Figure 5.4D)** None of the treatments affected plasma concentrations of PYY (**Figure 5.4E+F**).

Glucose and insulin

Ingestion of the breakfast resulted in an increase in plasma insulin concentration, followed by a decrease in plasma glucose in all treatments (**Figure 5.5**). Insulin levels increased after intraileal, intrajejunal and intraduodenal protein infusion compared with placebo infusion ($p<0.0001$). No differences in glucose levels were observed.

Discussion

This is the first study to demonstrate that ileal protein infusion significantly reduces food intake compared with intraduodenal infusion of protein and placebo, while no differences between intraileal and intrajejunal infusion were observed. Herewith we confirm our hypothesis and show differences in responsiveness to undigested protein infusion between the duodenum, jejunum and ileum.

It has been suggested that increasing the exposure of the distal small intestine to nutrients may result in the most potent brake effect. However, the mechanism of action for such an effect is not completely understood. Here we show an increase in the release of the GI peptide GLP-1 after intraileal infusion compared with duodenal and jejunal infusion of protein, respectively. Interestingly, an increase in GLP-1 is also seen after RYGB and IT procedures (16-18). Based on the changes seen after these procedures, it was hypothesized that a higher density of L-cells, found in the mucosa of the distal small intestine could account for such a GLP-1 increase (19). These variations in density and absolute number of L-cells over the small intestine, may also serve as an explanation for the observed differences in food intake and GI peptide release between the duodenum, jejunum and ileum in the current study (10).

In the present study we found a mean decrease in food intake of 90 kcal after intraileal infusion. In another study by our group, we showed that infusion of a similar amount (15 g) of casein into the ileum resulted in a reduction in food intake of 130 kcal (15). This difference may be explained by differences in study design. The set-up of the present study was based on the ingestion of a liquid breakfast in the morning, whereas a solid breakfast was consumed in the previous study. The current design was chosen to ensure that the stomach had emptied any food residues and that the breakfast would have been absorbed at the start of the infusion. As such we excluded intestinal exposure to breakfast-originated nutrients and were able to assess the effects of intestinally infused protein only. Consequently, subjects had an empty and non-distended stomach during the intestinal nutrient infusion in the present study, whereas subjects had a partially filled stomach at the time of intestinal infusion in the previous study. Since it is known that gastric distension plays an important role in the regulation of appetite and food intake,

this may explain the differences in mean reduction of food intake between the two studies (20-22). Furthermore, it has been shown that intestinal infusion of nutrients, with simultaneous distension of the stomach, more potently increases feelings of satiety compared with intestinal infusion alone (22). This may also have contributed to the absence of differences in VAS scores for satiety and fullness in the current study, which were present in the previous study.

The increase in GLP-1 after ileal protein infusion compared with jejunal, duodenal and placebo infusion was unexpected since GLP-1 is primarily stimulated by carbohydrates and fats (23). However, this is not the first study to show the GLP-1 stimulating ability of protein. Ryan et al. showed the load-dependent effect of intraduodenal whey infusion on GLP-1 release and found that a protein load as little as 0.5 kcal/min transiently stimulated the release of GLP-1 (6). In our study, GLP-1 did not increase after intraduodenal or intrajejunal infusion, while protein was infused at a higher rate (0.85 kcal/min). This difference could be explained by the type of protein infused, since Ryan et al used whey protein while we used casein. Casein and whey both contain all essential amino acids but they differ in digestion and absorption (24). Some studies propose that whey is more satiating and results in a greater release of GI peptides than casein (25-28). However, these data are mainly based on studies looking into oral ingestion of casein and whey. We chose casein because we previously established significant effects of intraileal infusion of casein on food intake (15).

GLP-1 is known to increase insulin sensitivity (29). Postprandial GLP-1 levels were previously shown to increase after RYGB and to enhance insulin secretion (30). In the current study, we found an increase in insulin after ileal, jejunal and duodenal protein infusion compared with placebo. However, no differences with regard to insulin secretion between the ileum and other locations were found. These results were not in line with a recent study by Salinari et al. in which bypassing the duodenum and proximal jejunum, hereby infusing a mixed meal (Nutrison, 524 kcal) in the mid jejunum, enhanced insulin sensitivity and decreased insulin levels in glucose-tolerant obese subjects (31). It is not clear why, despite the increase in GLP-1, no differences in insulin levels were seen after intraileal protein infusion compared with the other locations in the current study. Several factors may be involved. First, our study was performed in lean, healthy and young adults, while studies investigating RYGB often include morbidly obese diabetics. The difference in glucose homeostasis between these groups might account for the discrepancy in insulin secretion. Second, the incretin effect on insulin secretion after glucose ingestion was shown to be dose-dependent (32). As we only infused 15 g of protein (50 kcal) in the current study, it is possible that this amount was too small to establish effects on insulin levels.

We found an increase in plasma CCK concentrations over time after intestinal protein infusion compared with placebo, while this increase was shown to be independent of the location of protein infusion (duodenum, jejunum or ileum). CCK is considered to

be a peptide hormone originating from the proximal gut, but CCK-secreting I-cells are also expressed in the distal small intestine (33). Our group previously showed CCK release after intra-ileal fat infusion and the current study adds to this evidence that the same holds true for ileal protein infusion (34).

Distal L-cells secrete PYY in proportion to caloric load, with the lowest macronutrient potency for protein. Ryan et al infused protein in the duodenum and found that a threshold for the infusion rate of protein exists in order to induce PYY release. A load of 0.5 and 1.5 kcal/min did not result in the release of PYY whereas a load of 3 kcal/min did. We infused protein at a rate of 0.85 kcal/min in several parts of the small intestine and found no increase in the release of PYY. It is therefore reasonable to postulate that protein infusion in the distal small intestine may have a higher PYY-inducing threshold compared with that of duodenal infusion.

Our study has some limitations that need to be addressed. First, volunteers were intubated with a naso-ileal catheter for five consecutive days and this could have induced feelings of discomfort, hereby possibly affecting study outcome parameters despite the randomized design of this study. Second, we infused protein directly into the distal small intestine, hereby bypassing regions that are important for protein digestion. It is not clear whether distal protein infusion could lead to protein malabsorption and subsequently to increased colonic protein fermentation. If this would be the case, this could have detrimental effects for the host's physiology and health, possibly affecting outcome parameters in this study (35). Noteworthy, in the current study we did not observe adverse effects in any of the subjects after distal protein infusion. A recent study by Bojsen-Møller et al also showed that protein digestion was not impaired after RYGB (36). This suggests that the distal small intestine is capable of effectively digesting and subsequently absorbing protein and its metabolites.

This study provides evidence for location specific responsiveness in the small intestine to protein infusion with regard to intestinal brake activation. These results have implications for the design of future nutritional and/or surgical strategies for overweight and obesity. These strategies should aim to deliver nutrients to the distal small intestine in order to exert the most potent brake effect.

In conclusion, this study shows the effects of a protein infusion into different locations in the gastrointestinal tract. Ileal protein infusion results in the most pronounced and potent increase in GLP-1 and decrease in food intake.

Competing Interest

The authors have declared that no competing interests exist.

Financial disclosure

The research was funded by TI Food and Nutrition, a public-private partnership on pre-competitive research in food and nutrition. All funders had an input in the study design , whereas study conduct, data collection and analysis, as well as manuscript writing were the sole responsibility of the academic partners.

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Chapter 6

Ileal brake activation: macronutrient specific effects on eating behavior?

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Abstract

Background: Activation of the ileal brake, by infusing lipid directly into the distal part of the small intestine, alters gastrointestinal motility and inhibits food intake. The ileal brake effect on eating behavior of the other macronutrients is currently unknown.
Objective: The objective of this study was to investigate the effects of ileal infusion of sucrose and casein on food intake, release of gastrointestinal peptides, gastric emptying rate and small bowel transit time with safflower oil as positive control.
Design: This randomized, single-blind, crossover study was performed in 13 healthy subjects (6 male; mean age 26.4±2.9 years; mean BMI 22.8±0.4 kg/m2) who were intubated with a naso-ileal catheter. Thirty minutes after the intake of a standardized breakfast participants received an ileal infusion, containing control (saline[C]), safflower oil (51.7 kcal[HL]), low-dose casein (17.2 kcal[LP]) or high-dose casein (51.7 kcal[HP]), low-dose sucrose (17.2 kcal[LC]), high-dose sucrose (51.7 kcal[HC]), over a period of 90 min. Food intake was determined during an ad libitum meal. VAS questionnaires for hunger and satiety and blood samples were collected at regular intervals.
Results: Ileal infusion of lipid, protein and carbohydrate resulted in a significant reduction in food intake compared to control (HL: 464.3±90.7 kcal $p<0.001$, HP: 458.0±78.6 kcal $p<0.005$, HC: 399.0±57.0 kcal $p<0.0001$ vs. control: 586.7±70,2 kcal respectively, $p<0.001$). A reduction in energy intake was still apparent when the caloric amount of infused nutrients was added to the amount eaten during the *ad libitum* meal. Secretion of CCK and PYY but not of GLP-1 (7-36) was increased during ileal perfusion of fat, carbohydrates and protein. During ileal perfusion of all macronutrients a delay in gastric emptying and intestinal transit was observed, but differences were not significant compared to control.
Conclusion: Apart from lipids also sucrose and casein reduce food intake upon ileal infusion, thereby activating the ileal brake. In addition to food intake, also satiety and gastrointestinal peptide secretion were affected.

Introduction

Worldwide the incidence of overweight and obesity is rapidly expanding with tremendous negative impact on health and health care costs (1, 2). Up to now various nutritional and pharmacological strategies for overweight have failed and new treatment modalities in the battle against obesity are urgently needed. An interesting but still poorly explored mechanism is to reduce caloric intake via activation of the so-called intestinal brake, in particular the ileal brake. The brake refers to an intestinal feedback mechanism that is triggered by nutrients at a specific location in the intestine, resulting not only in modulation of gastrointestinal secretions and motility but also of food intake and hunger (3, 4). This concept was first seen in ileal transposition (IT) studies performed in rats. Koopmans and Sclafani were the first to describe this model in 1981 and together with several others it was shown that IT in rats resulted in a reduction in food intake and weight loss on the long term (5, 6).

A few years later, Welch et al demonstrated in humans that ileal infusion of a high amount of lipid delayed gastric emptying, induced satiation and also reduced food intake (7, 8). Recently, several studies have confirmed these findings with much smaller amounts of intact lipids (9-12).

While 'ileal brake' inducing effects on satiety and food intake have been explored in more detail with respect to lipids, little is known about ileal brake induced satiating effects of the other macronutrients, carbohydrates and proteins. Ileal infusion of carbohydrates is known to delay gastric emptying rate, decrease intestinal motility and enhance release of PYY and GLP-1 (13-15), gut peptides associated with induction of satiation and food intake. When administered intraduodenally, potent inhibitory effects of carbohydrates on energy intake have been demonstrated (16-18) but effects of
carbohydrates on food intake and satiety in humans upon intra-ileal infusion have not been assessed.

Dietary proteins are commonly regarded as the most satiating macronutrients (19). However, data on effects of intestinal protein infusion on satiety are scarce. It has been demonstrated that ileal protein infusion resulted in a delay in gastrointestinal motility (20). Intraduodenal administration of pea protein was shown to induce a more pronounced inhibitory effect on food intake compared to oral ingestion of the same amount of pea protein (21).

Up to now, human data on effects of ileal exposure to carbohydrates and proteins on food intake and satiety are lacking. This study was undertaken to investigate effects of ileal infusion of different doses of carbohydrates and proteins on ad libitum food intake in comparison to placebo (control) and to ileal infusion of an equicaloric amount of lipids, as positive control. In addition to food intake also satiety, gastric emptying, small intestinal transit time and gastrointestinal (GI) peptide secretion were measured. Previous studies with duodenal infusion of macronutrients did not show major

differences in satiety and food intake between macronutrients (18, 22). We therefore hypothesized that ileal infusion of carbohydrates and proteins will result in an equal, dose dependent reduction of food intake, and in equally enhanced satiety compared to ileal infusion of equicaloric amounts of lipids.

Methods

Participants

Healthy men and women, aged between 18 and 55 years with a BMI between 18 and 25 kg/m^2 were recruited by local advertisement to participate in this study. Smoking, consumption of >100 g alcohol/wk, medical history, active symptoms, medication use (apart from oral contraceptives) were exclusion criteria. All participants reported to be weight stable for at least two months prior to participation, to be unrestrained eaters (assessed by the Dutch eating behavior questionnaire) and were on a normal caloric diet (23). Written informed consent was obtained from each individual before inclusion in the study. This study was approved by the Medical Ethics Committee of the Maastricht University Medical Center+ (MUMC), Maastricht, the Netherlands, and performed in full accordance with the Declaration of Helsinki. The study has been registered in the US National Library of Medicine (http://www.clinicaltrials.gov, NCT01509469).
According to our power calculation thirteen subjects were needed to complete the study. This number was based on the difference in food intake observed in previous work (7, 10). Fifteen subjects met the inclusion criteria. Due to discomfort induced by the ileal catheter, two subjects did not complete all experiments and dropped out of the study. Thirteen healthy subjects (6 male, age 26.4 ± 2.9 yrs, BMI of 22.8 ± 0.4 kg/m^2) completed the study.

Study outline

This single-blind randomized placebo controlled study compared the effects of six different interventions: 1) saline (control [C]); 2) lipid emulsion (6 g safflower oil [HL], 51.7 kcal); 3) protein low dose (5 g casein [LP], 17.2 kcal); 4) protein high dose (15 g casein [HP], 51.7 kcal); 5) carbohydrates low dose (4.3 g sucrose [LC], 17.2 kcal); 6) carbohydrates high dose (12.9 g sucrose [HC], 51.7 kcal), respectively. Each of the substances was infused directly into the ileum over a 90 min period, on separate test days. Test days were randomly assigned (by using Research randomizer, www.randomizer.org) and subjects were tested in two consecutive weeks, with three test days planned in each week.

Catheter positionning

We used a 270 cm long silicon 9-channel (8-channels, 1 balloon inflation channel, outer diameter 3.5 mm) custom-made catheter (Dentsleeve international, Mui scientific, Mississauga, Canada). The catheter contained three sideholes per channel with 3-cm interspacing between consecutive side holes, and had an inflatable balloon (maximum inflation capacity 10 ml) integrated into the distal tip. Nutrients were directly infused into the ileum.

On the day of catheter introduction (Monday), subjects were allowed to consume a light breakfast in the morning (ingested before 08.00 AM). After local anaesthesia of the nasal mucosa (xylocaine 10% spray; AstraZeneca, Zoetermeer, The Netherlands), the catheter was introduced transnasally into the stomach. Under intermittent fluoroscopic control, the catheter was positioned with the tube tip located in the proximal small intestine. Further progression of the catheter into the ileum was attained as described previously (11). Participants returned to the department at 8:00 AM the next three days for test days 1, 2 and 3 (Tuesday, Wednesday and Thursday) and a week later for test days 4, 5 and 6, respectively. Before the start of each test day the position of the catheter was checked by fluoroscopy. In all subjects the tip of the catheter was placed at least 120cm distal to the pylorus (24).

Nutrient infusions

In this study we infused protein, carbohydrate and lipid directly into the ileum. Casein (energy density: 3.45 kcal/g, Dutch Protein Services, the Netherlands) was used as protein source. Sucrose (energy density: 4 kcal/g, van Gilse Automatensuiker, the Netherlands) was used as carbohydrate source. Safflower oil (6 g; energy density: 8.6 kcal/g, de Wit Specialty Oils, the Netherlands) was used as *positive* control, as it was shown repeatedly that safflower oil appears to be the most potent lipid in ileal brake activation (9). All nutrients were dissolved in a total volume of 180 ml water and administered at a rate of 2 ml/min (total infusion time 90 min).

Experimental design

Each subject participated in six test days. On all test days an intravenous catheter was placed in a forearm vein for collection of blood samples. At 8.30 AM a basal blood sample, Visual Analogue Scores (VAS) for hunger and satiety and breath samples were obtained. Subsequently a standardized breakfast meal, consisting of a sandwich and an egg (sunny side up, 210 kcal), was consumed. Since intraileal infusion of nutrients is known to delay gastric emptying and intestinal transit, gastric emptying rate of the breakfast meal, determined by using the ^{13}C stable isotope breath test (25), and duodenocecal transit time, measured by hydrogen breath testing were included as GI transport parameters (26). At the start of the ileal infusion (at t=30), 6 g of lactulose was administered

directly into the duodenum to enable measurement of the small bowel transit time. Ileal substrate infusion was scheduled from t=30-120 min after breakfast ingestion. One hour after ending the infusion, the volunteer received a standardized *ad libitum* lunch meal (sandwiches with egg salad (energy density: 2.2 kcal/g), t=180). Sandwiches were randomly cut in different sized pieces, to mask the number of sandwiches eaten. After ingestion of the lunch meal, the test day was finished and subjects could return home.

Gastrointestinal peptides

Venous blood samples were drawn at regular intervals. For Glucagon-like Peptide-1 (GLP-1 (7-36)), Peptide YY (PYY) and Cholecystokinin (CCK) measurements blood was collected in ice chilled aprotinin-coated tubes (Becton & Dickinson, New Jersey, USA). Immediately after blood collection, 10 µl of DPP-IV inhibitor (Millipore, Massachusetts, USA) per 1 ml of whole blood was added to the tubes to prevent proteolytic cleavage. Tubes were immediately centrifuged at a rate of 3000 rpm and 4° C for 15 min. Plasma was transferred into aliquots and stored on dry ice for the rest of the test day. At the end of the test day samples were stored at -80° C.
Active GLP-1 (7-36) was determined using a Glucagon Like Peptide-1 (Active) ELISA kit with a range of 2-100 pM, an inter-assay coefficient variation (CV) of 11%, and an intra-assay CV of 6% (EGLP- 35K, Millipore, Linco Research). Total PYY (includes both peptide YY1-36 and peptide YY3-36) was measured using a Human PYY (Total) ELISA kit with an inter-assay CV of 6% and an intra-assay CV of 3% (EZHPYYT66K, Millipore, Linco Research). Plasma cholecystokinin-8 (cholecystokinin 26–33) concentrations were measured with an optimized and validated commercial human RIA kit (EURIA CCK, RB302, Euro-Diagnostica, Malmö, Sweden). This improved assay system has been optimized to reach a high sensitivity of 0.05 pmol/L and to have no cross-reactivity to gastrin-17 or sulfated gastrin. The intra-assay CV was 8.9% at a concentration of 0.84 pmol/L and 4.9% at a concentration of 1.98 pmol/L.
The effects of each intervention on peptide secretion were determined by analyzing the peptide levels at the onset of ileal infusion until ingestion of the *ad libitum* meal. All data were corrected for the values obtained at the onset of infusion.

Satiety and hunger scores

Scores for hunger and satiety feelings (e.g., satiety, fullness, hunger, desire to eat, desire to snack) were measured using Visual Analogue Scales (VAS, 0 to 100 mm) anchored at the low end with the most negative or lowest intensity feelings (e.g., extremely unpleasant, not at all), and with opposing terms at the high end (e.g., extremely pleasant, very high, extreme) (27).

Gastric emptying

^{13}C-octanoic acid (100 mg, Campro Scientific bv, Veenendaal, the Netherlands) was mixed into the standardized breakfast meal ingested at t=0. Breath samples of $^{13}CO_2$ were obtained as described previously (12). Samples were analyzed by using Isotope Ratio Mass Spectometry (IRIS, Wagner, Bremen, Germany).

Small bowel transit time

Duodenocecal transit time was determined by the lactulose hydrogen breath test, as described by Ledeboer et al (26). Via an opening of the catheter located in the duodenum, 6 g of lactulose (Legendal, Inpharzam, Amersfoort) was administered at the start of ileal infusion of the substrates. Breath samples were taken at 15 min intervals and analyzed using a handheld hydrogen breath test unit (Gastyrolyzer, Bedfont Scientific, Kent, United Kingdom). Small bowel transit time was defined as the time between lactulose administration and the onset of a sustained rise in breath hydrogen concentration of at least 10 parts per million (ppm) above basal level.

Statistical analyses

Statistical analyses were performed using the SAS statistical software package (SAS version 9; SAS institute, Cary, NC, USA). Proc Gplots were used to test outcome variables for normality of distribution. If data were not normally distributed, log transformation was applied for further analysis of the data, as was the case for CCK, GLP-1 (7-36), and PYY.

Regarding food intake, statistical analysis was performed on the amount of food eaten in kcal. CCK, GLP-1 (7-36) and PYY are displayed from the start of the infusion (t=30 min) until the last blood sample collected before the start of the *ad libitum* meal (t=180 min). All variables were compared with a mixed analysis of variance model that included the fixed factors treatment (C, HL, LP, HP, LC and HC). For the plasma parameters, time and the interaction between treatment and time were added to the model. The factor subject was added to the model as random factor. A post hoc Tukey test was used to analyze differences between treatments. Data are presented as the mean ± SEM (unless specified otherwise) and considered significant at $p<0.05$.

Results

Food intake

Ileal infusion of lipid, high-dose protein and high-dose carbohydrates resulted in a significantly lower energy intake during the *ad libitum* meal compared to control (HL: 464.3 ± 90.7 kcal $p<0.001$, HP: 458.0 ± 78.6 kcal $p<0.005$, HC: 399.0 ± 57.0 kcal $p<0.0001$ vs. control: 586.7 ± 70,2 kcal respectively). (**Figure 6.1**) There were no statistically significant differences in food intake between the different nutrient infusions HL, HP and HC. No effect of LP and LC over control on food intake was observed (LP: 528.4 ± 86.1 and LC: 491.4 ± 77.5 kcal). Even after adding the caloric amount of infused nutrients to the amount eaten during the *ad libitum* meal (in kcal), the reduction in energy intake was significant (HL and HC vs. C, $p<0.05$ and $p<0.005$ respectively).

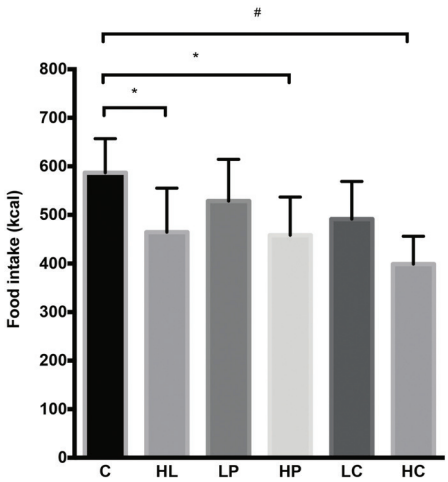

Figure 6.1 Food intake in kcal (mean+SEM) of the *ad libitum* lunch ingested 60 min after ending the intraileal infusion of control (C), safflower oil (HL), low-dose casein (LP), high-dose casein (HP), low-dose sucrose (LC) and high-dose sucrose (HC), respectively. *$p<0.005$ and #$p<0.0001$.

Satiety and hunger scores

Fasting scores for hunger and fullness at the start of the experiments did not differ among the six interventions. Ingestion of the breakfast meal resulted in a significant decrease in hunger and an increase in fullness scores in all six treatments (data not shown). Significant differences in hunger scores were observed only after start of intra-ileal infusion of high-dose protein (from 30-180 min) ($p<0.0001$), but not of the other interventions compared to control (**Figure 6.2A+B**). After ingestion of the breakfast, fullness scores increased in all experiments. **Figure 6.2C+D** shows the integrated fullness scores from the start of ileal infusion up to the intake of the ad libitum meal. No

significant differences were observed in fullness scores between the various treatments and control (**Figure 6.2C+D**).

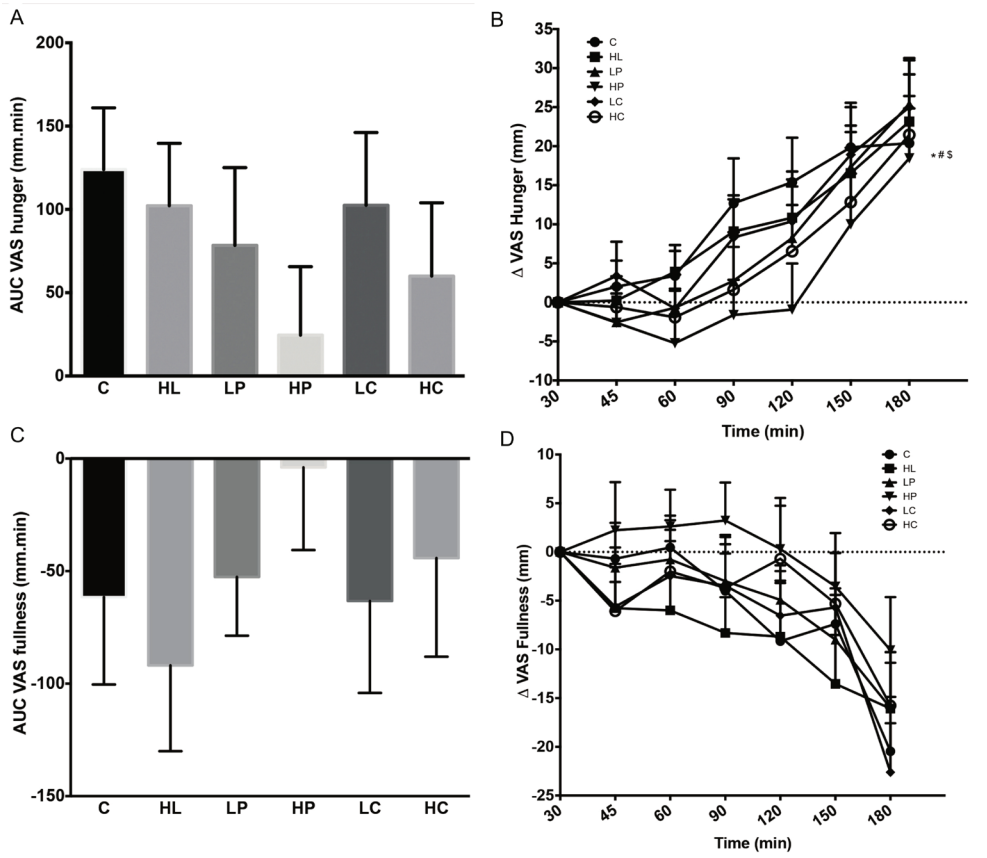

Figure 6.2 Hunger **(**mean+SEM) (**A+C)** and Fullness (mean+SEM) (**B+D)**. VAS Hunger concentrations (**B)** and areas under the curve (AUC 30-180 min **A)** and VAS Fullness concentrations (**D)** and areas under the curve (AUC 30-180 min **C**) during intra-ileal infusion of control (C), safflower oil (HL), low-dose casein (LP), high-dose casein (HP), low-dose sucrose (LC) or high-dose sucrose (HC). Ileal infusion was started at t=30 (30 min after breakfast consumption) and continued for 90 minutes. An *ad libitum* lunch was offered at t=180 min. AUCs were calculated by using the trapezoid rule. *Significantly different from C ($p<0.005$), #Significantly different from HL ($p<0.05$), $Significantly different from LC ($p<0.05$).

Gastrointestinal peptides

CCK

Plasma CCK levels were measured for the C, HL, HP and HC interventions but not for LP and LC. Baseline plasma CCK concentrations did not differ between interventions. The breakfast meal, ingested 30 min prior to starting the ileal infusions, induced an

increase in CCK concentration in all four measured treatments from 0.30 ± 0.06 pmol/L at baseline to 0.74 ± 0.05 pmol/L at 30 min after breakfast intake ($p<0.0001$) (**Supplementary table 1)**. Data are corrected for the CCK levels at t=30 min, when ileal infusions started. **Figure 3A** shows that, after an initial postprandial increase, the plasma CCK levels decline from 45 or 60 minutes after breakfast intake onwards. Ileal infusion of lipid, high-dose protein and high-dose carbohydrates all resulted in higher CCK levels following ileal infusions compared to control. Consequently, the negative AUC of CCK concentrations over time, corrected for the CCK levels at the start of ileal infusions, was smaller after lipid and high-dose protein intervention ($p<0.05$ and $p<0.005$, respectively) **(Figure 3B),** while this did not reach statistical significance for the high-dose carbohydrate treatment.

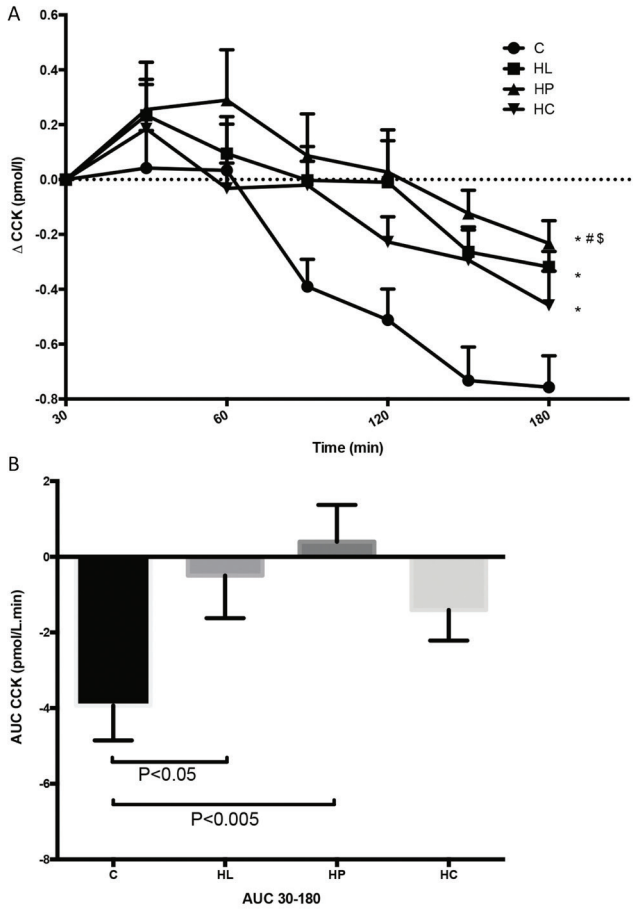

Figure 6.3 *CCK* (mean+SEM). Δ CCK concentrations (**A**) and areas under the curve (AUCs) (**B**). Intraileal infusion of control (C), safflower oil (HL), high-dose casein (HP), or high-dose sucrose (HC) was scheduled from 30-120 min. AUCs were calculated by using the trapezoid rule. *Significantly different from C ($p<0.0005$), #Significantly different from HL ($p<0.05$), $Significantly different from HC ($p<0.0005$).

GLP-1 (7-36)

Baseline plasma GLP-1 (7-36) concentrations did not differ between study days. The breakfast meal induced an increase in GLP-1 (7-36) concentration in all six treatments from 2.63 ± 0.26 pmol/L at baseline to 3.84 ± 0.25 pmol/L at 30 min after breakfast intake ($p<0.005$) (**Supplementary table 1)**

The AUC GLP-1 (7-36) (30–180 min) from start of ileal infusion to onset of meal intake did not significantly differ between any of the treatments (**Figure 6.4B)**. Ileal infusion of high-dose protein resulted in a larger increase in plasma GLP-1 (7-36) when compared to low-dose carbohydrates **(Figure 6.4A).**

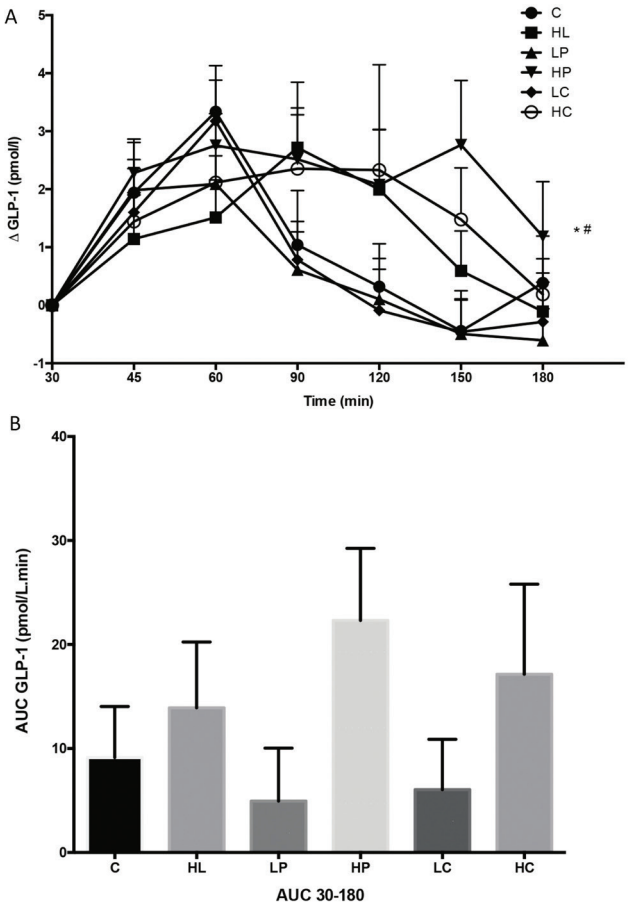

Figure 6.4 *GLP-1 (7-36)* (mean+SEM) Δ GLP-1 (7-36) concentrations (**A**) and areas under the curve (AUCs) (**B**). Intraileal infusion of control (C), safflower oil (HL), low-dose casein (LP), high-dose casein (HP), low-dose sucrose (LC) or high-dose sucrose (HC) was scheduled from 30-120 min. AUCs were calculated by using the trapezoid rule. *Significantly different from LP ($p<0.005$), #Significantly different from LC ($p<0.005$).

PYY

Baseline plasma PYY concentrations did not differ between study days. The breakfast meal induced an increase in PYY concentration in all six treatments from 51.36 ± 2.98 pmol/L at baseline to 60.66 ± 2.98 pmol/L at 30 min after breakfast intake ($p<0.05$) (**Supplementary table 6.1)**The 30-180 min AUCs from the start of ileal infusion to onset of meal intake did not significantly differ between any of the treatments (**Figure 6.5B)**. Infusing high-dose carbohydrates and lipid into the ileum resulted in a significantly larger increase in plasma PYY when compared to control, and of high dose carbohydrates versus low-dose carbohydrates, respectively **(Figure 6.5A)**. The same was true for high-dose protein vs low dose of protein but not vs control.

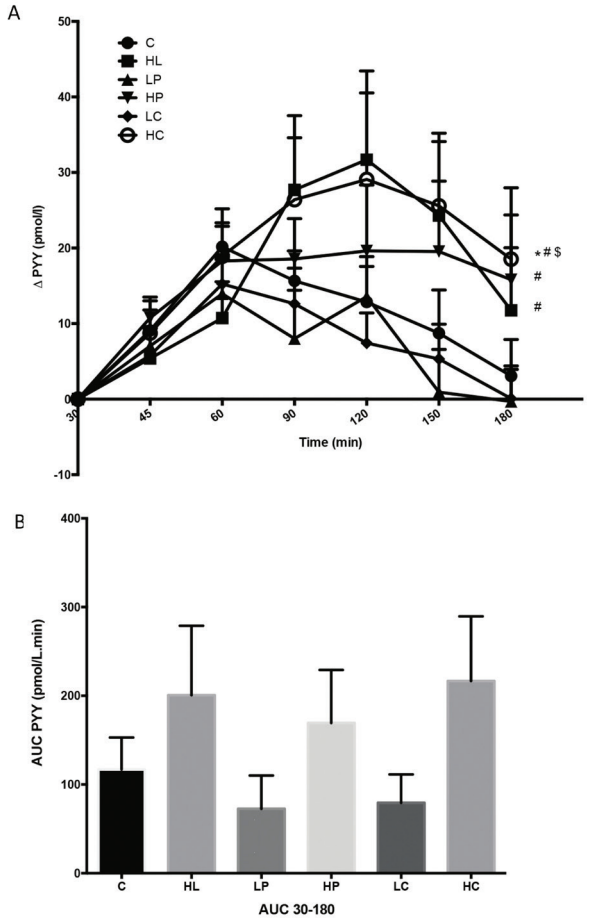

Figure 6.5 PYY **(**mean+SEM). Δ PYY concentrations (**A**) and areas under the curve (AUCs) (**B**). Intraileal infusion of control (C), safflower oil (HL), low-dose casein (LP), high-dose casein (HP), low-dose sucrose (LC) and high-dose sucrose (HC) was scheduled from 30-120 min. AUCs were calculated by using the trapezoid rule. * Significantly different from C ($p<0.005$), #Significantly different from LP ($p<0.005$), $ Significantly different from LC ($p<0.005$).

Gut peptides and food intake

Energy intake during the *ad libitum* meal intake (in addition to the amount of kcal infused) was inversely related to plasma GLP-1 (7-36) AUC ($r=-0.4$, $p<0.0005$) and CCK AUC ($r=-0.4$, $p<0.005$), respectively. No significant correlation between food intake and PYY was found.

GI transport

Ileal nutrient infusion resulted in a tendency to a slower gastric emptying half time ($t^1/_2$) however differences were not statistically significant vs control. This was also true for small bowel transit time (SBTT) compared to control (saline infusion); non-significant differences (**Table 6.1**).

Table 6.1 GI transport. All values are means ± SEM. Gastric emptying half time (GE ½) of the breakfast meal ingested 30 min prior to, and small intestinal transit time during intraileal infusion of control (C), safflower oil (HL), low-dose casein (LP), high-dose casein (HP), low-dose sucrose (LC) and high-dose sucrose (HC), respectively.

	C	L	LP	HP	LC	HC	p
GE 1/2 (min)	155.5 ± 13.8	179.4 ± 20.0	139.9 ± 8.3	166.1 ± 9.7	137.3 ± 9.9	156.2 ± 7.9	ns
SBTT (min)	154.6 ± 13.5	173.1 ± 11.2	180.0 ± 12.4	186.9 ± 12.8	184.6 ± 12.2	186.9 ± 13.8	ns

Discussion

We have shown that ileal infusion of proteins and carbohydrates in healthy volunteers suppresses food intake to the same extent as an equicaloric amount of lipids. Lower doses of proteins and carbohydrates did not affect food intake or satiety/satiation. Thus, the three macronutrients all affected the ileal brake and associated eating behavior to the same extent, while low concentrations may not reach the sensing threshold to induce such effects.

Macronutrients and its metabolites are sensed by various receptors in the gastrointestinal tract. Each macronutrient activates different receptors, mainly present on I- and L cells. Lipids trigger the release of CCK, GLP-1 and PYY primarily via activation of several fatty acid receptors (FFARs, GPR120) while recent evidence suggests that carbohydrates exert their effects on GI peptide release via a possible interaction between the sweet taste receptor (T1R2-T1R3) and the sodium-glucose cotransporter 1 (SGLT1) (28, 29). Proteins and their metabolites are able to trigger the peptone (LPAR5) or the umami receptor (T1R1-T1R3). Activation of these receptors by macronutrients results in the release of a variety of GI peptides, which exert their actions through endocrine, paracrine and neurocrine pathways (30).

Welch et al were the first to demonstrate that infusion of lipid in the form of corn oil in the ileum caused a significant reduction in food intake in healthy volunteers (7). We infused a much smaller amount of lipid but still observed a significant decrease in food intake, confirming results of previous studies from Welch et al and others (7, 10). However, in other studies applying ileal intubations no effects of safflower oil on food intake could be demonstrated (9, 11, 12). This lack of effect may have been caused by differences in study design, leading to a longer time interval between the end of the infusion and start of the *ad libitum* test meal (31).

Effects of intra-ileal infusion of carbohydrates or proteins on food intake and appetite have not been studied previously. Intraduodenal glucose infusion was shown to induce a reduction in food intake (16-18, 32). The caloric content of infused carbohydrates varied from 180-480 kcal in these studies and was not added to the total energy intake during the *ad libitum* meal. None of these studies showed a significant reduction in energy intake when intraduodenal infused calories were added to the ingested calories during the *ad libitum* meal. With regard to intraduodenal protein infusion, comparable amounts of calories, 180-210 kcal, were used. It was shown that activation of the *duodenal* brake by pea or whey protein infusion resulted in a significant decrease in food intake (21, 22, 33). Compared to these previously mentioned studies we have infused far less calories (52 vs. 180-210 kcal) but still found a significant decrease in food intake. Furthermore, we showed that adding the amount of ileal delivered calories to the calories ingested during the *ad libitum* meal after ileal brake induction still resulted in a significant decrease in overall energy intake. This decrease in overall net energy intake may seem rather small (HL: 70 kcal, HC: 135 kcal). However, it should be noted that this acute effect was achieved with a single infusion. Furthermore, it was shown that a positive energy balance may become negative already with very small daily reductions in energy intake of approximately 100 kcal/day (34).

With respect to satiety feelings, only infusion of high-dose protein resulted in a significant decrease in hunger. Infusions of lipids or high dose carbohydrates did not significantly affect feelings of hunger and satiety. The absence of such effects may have been caused by certain aspects in our study design, such as feeding status prior to intestinal brake induction and timing of substrate infusion. However, a significant effect was found in the high-dose protein treatment. This was not unexpected since it is well established that protein is more satiating than carbohydrates or lipid (35). Furthermore it is possible that certain amino acids contribute to the perception of satiety (36).

We observed a significant increase in the release of gastrointestinal peptides to the systemic circulation after ileal infusion with safflower oil, casein and sucrose. While release of CCK after intraduodenal protein, carbohydrates and lipid infusion has been well documented (18, 22, 33), less is known on CCK release in response to *ileal* infusion of macronutrients. CCK was regarded as proximal GI peptide (37).We and others have shown that CCK is also released upon distal, *ileal* nutrient infusion. We cannot

differentiate between a direct effect of ileal nutrient on I cells or an indirect effect via paracrine or neurocrine mechanisms, by feed back signaling to the more proximal parts of the small intestine (38).

GLP-1 release by distal L-cells after ileal infusion of triolein, sodium oleate, starch and maltose, but not after peptone infusion was previously reported (14). Here, we demonstrate a clear increase in GLP-1 (7-36) release after infusion of casein. In fact we confirm previous data, as it has been reported that intestinal exposure to intact proteins induces a stronger effect on GLP-1 release compared to protein hydrolysates (39). In our study food intake was inversely correlated to both CCK and GLP-1 (7-36) plasma levels, confirming the hypothesis that indeed these GI peptides are involved in the regulation of food intake (22).

We also observed an increase in PYY secretion following lipid- and carbohydrates infusion, which is in line with previous observations on ileal exposure to lipid or rice starch with glucose infusion (40, 41). Infusion of the low dosages of casein and sucrose did not result in enhanced GLP-1 (7-36) or PYY release.

Ileal infusion of lipids and carbohydrates is known to delay gastric emptying and small bowel transit time (8, 11, 15, 20, 24). The nutrient dependent delays in GI transit data found in this study was of the same magnitude as found in other studies but was not statistically significant, due to study design with activation of ileal brake 30 min after onset of gastric emptying and transit time measurement (42). Some limitations of our study should be acknowledged. First, effects of macronutrients were studied applying naso-intestinal intubations in healthy individuals. The intubation with a naso-ileal catheter for several consecutive days may have induced discomfort and changes in total wellbeing, thus affecting study outcome parameters. However, sequence of test days did not influence food intake during the *ad libitum* meal. Second, the various nutrients were infused during three consecutive days, resulting in a possible carry-over effect between infusions, although the randomized control design prevented that this effect did influence study outcome. Third, only healthy lean men and women were included in this study. Therefore, these results cannot directly be applied to overweight or obese individuals, since some studies showed a less pronounced suppression of food intake after intraduodenal lipid infusion in obese subjects compared to lean individuals (43, 44). However it is not clear whether this reduced gastrointestinal sensitivity also applies for different infusion locations and more importantly other macronutrients.

We are the first to demonstrate that ileal infusion of all three macronutrients induces a decrease in food intake. Furthermore, we showed that this effect was dose-dependent. The reduction in food intake confirms the findings in IT in rats and shows the potential of the ileal brake as a target for food-based strategies in the prevention or treatment of overweight and obesity (3, 45). Conducting a proof of principle study in overweight/obese individuals would contribute to a better understanding of the effect of ileal brake activation on food intake in obese subjects. Therefore, reliable dietary encapsulation- or

slow release strategies are needed to investigate the application of ileal brake activation in weight management strategies.

In conclusion, we have shown that an ileal brake satiating effect leading to a decrease in food intake is obtained with small amounts of lipid, protein and carbohydrates. Ileal infusion of equicaloric amounts of these macronutrients modulates food intake, gastrointestinal peptide release (CCK, GLP-1 (7-36) or PYY), and feelings of hunger.

Supplementary table 6.1 GI peptides before and after breakfast. All values are means ± SEM. CCK (pmol/L), GLP-1 (pmol/L) and PYY (pmol/L) levels, baseline (before) and 30 minutes after the ingestion of the breakfast meal (after) .

	Before	After	p
CCK (pmol/L)	0.30 ± 0.06	0.74 ± 0.05	p<0.0001
GLP-1 (pmol/L)	2.63 ± 0.26	3.84 ± 0.25	p<0.005
PYY (pmol/L)	51.36 ± 2.98	60.66 ± 2.98	p<0.05

Competing Interest

The authors have declared that no competing interests exist.

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Chapter 7

Intraileal casein infusion increases plasma concentrations of amino acids in humans: a randomized crossover trial

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Abstract

Background: Activation of the ileal brake by casein induces satiety signals and reduces energy intake. However, adverse effects of intraileal casein administration have not been studied before. These adverse effects may include impaired amino acid digestion, absorption and immune activation.

Objective: To investigate the effects of intraileal infusion of native casein on plasma amino acid appearance, immune activation and gastrointestinal (GI) symptoms.

Design: A randomized single-blind cross over study was performed in 13 healthy subjects (6 male; mean age 26 ± 2.9 years; mean body mass index 22.8 ± 0.4 kg m^{-2}), who were intubated with a naso-ileal feeding catheter. Thirty minutes after intake of a standardized breakfast, participants received an ileal infusion, containing either control (C) consisting of saline, a low-dose (17.2 kcal) casein (LP) or a high-dose (51.7 kcal) of casein (HP) over a period of 90 min. Blood samples were collected for analysis of amino acids (AAs), C-reactive protein (CRP), pro-inflammatory cytokines and oxylipins at regular intervals. Furthermore, GI symptom questionnaires were collected before, during and after ileal infusion.

Results: None of the subjects reported any GI symptoms before, during or after ileal infusion of C, LP and HP. Plasma concentrations of all AAs analyzed were significantly increased after infusion of HP as compared to C ($p<0.001$), and most AAs were increased after infusion of LP ($p<0.001$). In total, 12.49 ± 1.73 and 3.18 ± 0.87 g AAs were found in plasma after intraileal infusion of HP and LP, corresponding to 93 ± 13% (HP) and 72 ± 20% (LP) of AAs infused as casein, respectively. Ileal casein infusion did not affect plasma concentrations of CRP, IL-6, IL-8, IL-1β and TNF-α. Infusion of HP resulted in a decreased concentration of 11,12-dihydroxyeicosatrienoic acid whereas none of the other oxylipins analyzed were affected.

Conclusions: A single intraileal infusion of native casein results in a concentration and time dependent increase of AAs in plasma, suggesting an effective digestion and absorption of AAs present in casein. Also, ileal infusion did not result in immune activation nor in GI symptoms.

Introduction

The increasing prevalence of overweight causes increased concerns for health and health care costs worldwide. There is a clear need for therapeutic and preventive strategies, as current strategies has not proven to be successful on the long term.

One potential strategy to reduce energy intake is activation of the so-called ileal brake. The ileal brake is a feedback mechanism that slows or 'brakes' the process of proximal gastrointestinal digestion and absorption, and food intake. The ileal brake is activated when energy-containing macronutrients reach the ileum. Ileal brake activation induces enhanced satiety signals, satiation and reduction of energy intake (1, 2). Recently, we have shown that not only ileal appearance of lipid but also of the other macronutrients, carbohydrate (sucrose) and protein (casein), results in increased satiation and a reduction of food intake (3). This reduction in food intake underlines the relevance of the ileal brake as potential food based strategy in the prevention or treatment of overweight and obesity. Despite its potential, it should be investigated whether undigested native protein infused into the ileum does not result in adverse effects such as protein malabsorption or immune activation.

Intraileal infusion bypasses the proximal parts of the gastro-intestinal (GI) tract as is also achieved by bariatric surgery. One of the most frequently applied bariatric procedures worldwide is the Roux-en-Y gastric bypass (RYGB). RYGB effectively results in weight loss and improvement of type II diabetes (4). Although the mechanism is not completely understood yet, the proposed mechanism by which RYGB may exert its beneficial effects is enhanced stimulation of enteroendocrine cells in the distal small intestine by undigested nutrients, resulting in the release of the L-cell products glucagon-like peptide 1 (GLP-1) and peptide YY (PYY) (5). It has been suggested that weight loss after RYGB may be attributable to limited food intake which can be partly explained by gastric restriction and malabsorption. However, evidence has increased that the positive health effects induced by RYGB may be due to altered GI physiology (4) and not due to gastric restriction and malabsorption. RYGB results in rapid emptying of gastric content with prolonged small intestinal transit times (6) and only a small proportion of the reduction in energy intake after RYGB is due to malabsorption (7). Malabsorption may result in an increased risk of developing nutritional deficiencies, such as protein deficiency (8). Under physiological conditions, gastric acid activates the conversion of pepsinogen into the proteolytic enzyme pepsin which denatures orally ingested protein. Protein is further digested by trypsin in the small intestine. In response to orally ingested protein, amino acids in the form of peptides or free amino acids are found in the intraluminal content of the jejunum and ileum (9).

Recently it was found by Bojsen-Møller et al. (2015) that RYGB accelerates caseinate digestion and amino acid absorption, and it was suggested that protein digestion is not impaired after RYGB (10). However, it remains to be investigated whether native

casein, directly infused into the ileum and thus bypassing the proximal parts of the small intestine results in malabsorption. If this is the case, protein may be available for colonic fermentation resulting in potential harmful effects for the host's health (11). Such effect could manifest itself by immune activation (12). This might be shown by the production and release of pro-inflammatory cytokines such as interleukin-1β (IL-1β), IL-6, IL-8 and tumor necrosis factor-α (TNFα) (12), or other pro-inflammatory mediators such as c-reactive protein (CRP) and eicosanoids, a subset of oxylipins. The release of these lipid mediators can be triggered by a variety of stimuli. They have been associated to be involved in pain and fever and are known to be potent regulators of the host immune response (13, 14).

The aim of this study was to investigate the effects of intraileal native protein infusion on digestion, AA absorption and immune activation. This was done in a study evaluating the effects of intraileal infusion of native protein on food intake previously performed by our group (3). It was hypothesized that ileal infusion of native casein results in plasma appearance of AAs in casein and does not result in acute adverse effects such as incomplete digestion of casein protein or inflammation. This was investigated by analyzing plasma AAs at several time points after intraileal infusion of both a low-dose and high-dose of casein in a randomized single-blind cross over study as reported by Avesaat et al. (2014) (3). Inflammatory mediators such as pro-inflammatory cytokines and oxylipins were analyzed to investigate acute adverse inflammatory effects.

Material and methods

Study design

In the present study plasma samples from a previously published single blind randomized placebo controlled cross over study were analyzed to investigate the effects of intraileal protein infusion on plasma profiles of AAs and inflammation markers (3). Thirteen subjects (6 male, 7 female, age 26 ± 2.9 years, BMI of 22.8 ± 0.4 kg m^{-2}) completed the study.

The effects on AAs and inflammation markers of the following interventions were investigated 1) saline infusion (control (C)), 2) low dose protein infusion (5 g casein (LP), 17.2 kcal) and 3) high dose protein infusion (15 g casein (HP), 51.7 kcal). Each treatment was infused into the ileum over a 90-min period, on separate test days. Order of interventions were randomly assigned to each participant and based on a randomization protocol (via randomizer.org) defined prior to the start of the study.

Casein (energy density: 3.45 kcal g^{-1}, Dutch Protein & Services, Tiel, The Netherlands) was used as protein source. The AA composition of casein is shown in **Table 1** (Eurofins food testing Netherlands B.V., Heerenveen, The Netherlands). Casein was dissolved in a total volume of 180 ml tap water and infused into the ileum a rate of 2 ml min^{-1} (0.6 kcal/min; total infusion time 90 min).

Experimental design

Each subject participated in all three test days. On all test days an intravenous catheter was placed in a forearm vein for blood collection. At 8.30 AM, a basal blood sample was obtained. Subsequently, a standardized breakfast meal consisting of a sandwich and an egg (sunny side up, 210 kcal), was consumed. Ileal substrate infusion was performed from t=30 to t=120 minutes after breakfast ingestion. Participants were blinded to the infusion treatment. One hour after ending the infusion (t=180 minutes), the volunteer received a standardized *ad libitum* lunch meal (sandwiches with egg salad (energy density: 2.2 kcal g^{-1}). At t=240 the last blood sample was taken and the test day was finished.

Table 7.1 Amino acid composition of native casein expressed as % (w/w)

Amino Acid	**%** (w/w)
Glutamic acid*	18.60
Proline	9.21
Leucine	8.09
Lysine	6.95
Aspartic acid*	6.11
Valine	5.55
Serine	4.89
Tyrosine	4.57
Phenylalanine	4.35
Isoleucine	4.33
Threonine	3.68
Arginine	3.08
Alanine	2.62
Histidine	2.40
Methionine	2.22
Glycine	1.56
Tryptophan	1.15
Cysteine	0.39
Total	89.75

*The amino acid composition of casein was analyzed by acid protein hydrolysis followed by amino acid analysis using ninhydrin according to ISO13903:2005, This analytical method is unable to separate aspartic acid from asparagine and glutamic acid from glutamine. Tryptophan and cysteine were analyzed according the EU152/2008 (F) and ISO 13903:2005 protocol.

Questionnaires

GI symptoms were evaluated using a questionnaire addressing complaints such as headache, nausea, stomach-ache, diarrhea, and other symptoms. Symptoms were graded

on a 4-point scale with the grade 0 representing 'not present' to 4 'strongly present'. Subjects were asked to mark how they felt at the moment before (t=15 and t=30), during (t=60 and t=90) and after the infusion stopped (t=120 and t=240).

Sample collection

Venous blood samples were drawn 15 minutes before breakfast (t=-15 min), and at 30, 45, 60, 90, 120, 150, 180, 210 and 240 minutes after breakfast, respectively. Blood was collected in ice-chilled EDTA coated tubes (Becton & Dickinson, Franklin Lakes, NJ, USA). Immediately after blood collection, tubes were centrifuged at a rate of 3000 revolutions per minute at 4°C for 10 minutes. Plasma was transferred into aliquots and stored on dry ice for the rest of the test day. At the end of the test day, samples were stored at -80°C until further analysis of AAs, cytokines and oxylipins.

Plasma amino acid profiles

Plasma AA profiles were analyzed including the AAs present in casein (Table 1, except cysteine), as well as other AAs and amines, i.e. 1-methylhistidine, 3-methylhistidine, asymmetric dimethylarginine, citrulline, DL-3-amino isobutylene, glycyl-glycine, homo-arginine, hydroxyl-lysine, amino adipic acid, α-amino-n-butyric acid, kynurenine, L-4-hydroxy-proline, L-glutamic acid, homo-serine, trimethylamine, phosphor-ethanolamine, putrescine, symmetrical dimethylarginine, sarcosine, serotonin, taurine, gamma-aminobutyrine, gamma-l-glutamyl amine, glycine, ethanol, glutamine, o-acetyl-l-serine. AAs and amines were analyzed using a LC-MS method as described previously (16).

Plasma profiles of pro-inflammatory cytokines and C-reactive protein

Plasma profiles of CRP, TNF-α, IL-1β, IL-6, IL-8 were analyzed using an in-house developed and validated multiplex immunoassay based on Luminex technology (xMAP, Luminex, Austin TX USA). The assay was performed as described previously(17, 18). Aspecific heterophilic immunoglobulins were pre-absorbed from all samples with heteroblock (Omega biologicals Bozeman MT, USA). Acquisition was performed with the Biorad FlexMAP3D (Biorad laboratories, Hercules USA) in combination with xPONENT software version 4.2 (Luminex). Data was analyzed by 5-parametric curve fitting using Bio-Plex Manager software, version 6.1.1 (Biorad).
For TNF-α the range was 1.2-5000 pg/ml and the limit of detection (LOD) for TNF-α was 0.7 pg/ml, with an inter-assay coefficient of variation (CV) of 3.0 ± 1.8% and an intra-assay CV of 7.6 ± 0.6%. For IL-1β the range of the assay was 1.2-5000 pg/ml and the LOD was 0.4 pg/ml with an intra-assay CV of 3.4 ± 2.9 % and an inter-assay of 3.5 ± 2.5%. For IL-6 the range of the assay was 2.4 – 10000 pg/ml and the LOD for IL-6 was 0.9 pg/ml, with an intra-assay CV of 4.4 ± 2.7% and an inter-assay CV of

11.0 ± 4.6%. For IL-8 the range of the assay was 2.4-10000 pg/ml and the LOD was 1.3 pg/ml with an intra-assay CV of 5.8 ± 3.6% and inter-assay CV of 13.7 ± 6.8%. For CRP the range was 12.2 – 50000 pg/ml and the LOD was 11.7 pg/ml whereas the upper limit of detection was 42691.6 pg/ml. The samples were diluted to be within the range of the assay.

Not all cytokine concentrations were above the lower limit of quantification (LLOQ) of the assay. For TNF–α , 63% of the samples was below the LLOQ (1.2 pg/ml). For IL-1β, 25% of the samples was below the LLOQ (2.2 pg/ml). For IL-6, 11% of the samples was below LLOQ (2.8 pg/ml). For IL-8, 46% of the samples was below LLOQ (4.5 pg/ml). Since these samples were below the detection range of the assay, they were excluded from the data analysis.

Plasma profiles of oxylipins

The oxylipin platform (**Table 2**) covers classical and non-classical eicosanoids from different polyunsaturated fatty acids, including ω-6 and ω-3 PUFAs such as linoleic acid, arachidonic acid and dihomo-γ-linoleic acid (all ω-6), eicosapentaenoic acid and docosahexaenoic acid (both ω-3). The samples were analyzed by liquid chromatography tandem mass spectrometry as described previously (19).

Data analysis

Intervention effects on plasma AA concentrations, pro-inflammatory cytokines and oxylipins were analyzed within participants with a mixed model analysis of variance (ANOVA) including the fixed factors treatment (infusion of C, infusion of LP and infusion of HP), time and the interaction between treatment and time. Because of the crossover design, intervention effects within subjects were compared by including the random factor subject.

All statistical analyses were performed using the SAS statistical software package (SAS version 9; SAS institute, Cary, NC, USA). Data were visually checked on normality and on constant variance of residuals by plots of residuals vs. corresponding predicted values. All data met the criteria for ANOVA assumptions. If an intervention effect occurred post hoc comparisons were made using Tukey-Kramer adjustment to correct for multiple testing. Data are presented as the mean ± SEM (unless specified otherwise) and considered significant at $p<0.05$.

Since infused amounts of native casein (5 gram LP vs. 15 gram HP) were known, the amount of AAs infused as casein was calculated based on the AA composition of the native casein infused (Table 1). To calculate the absolute amount of AA in gram, plasma AA concentrations (μmol/L) were corrected for the total blood volume (female 65 ml/kg, male 75 ml/kg (20)). To estimate the increase in AAs due to LP and HP infusion, the total area under the curves (AUCs) during the infusion period (t=30 until t=180) were calculated by applying the trapezoid method. The AUC after C treatment was subtracted from AUCs after LP and HP infusion per individual to correct for control

Table 7.2. Overview of oxylipins analyzed assigned to their precursor fatty acids

	Arachidonic acid	Dihomo-γ-linoleic acid	Docosahexa-enoic acid	Eicosapenta-enoic acid	Linoleic acid
Prostanoids/ Thromboids	TXB2 PGF2α PGE2 13,14-dihydro-PGF2α	PGF1α			
Diols	14,15-DiHETrE 11,12-DiHETrE 8,9-DiHETrE 5,6-DiHETrE		19,20-DiHDPA	17,18-DiHETE 14,15-DiHETE	12,13-DiHOME 9,10-DiHOME
Epoxides					12(13)-EpOME 9,10-EpOME
Alcohols	12-HETE 5-HETE 15-HETE 20-HHETE 11-HETE		17-HDoHE 14-HDoHE 10-HDoHE	12-HEPE 5-HEPE	9-HODE 13-HODE
Ketones					13-KODE 9-KODE
Triols					9,12,13-TriHOME 9,10,13-TriHOME

AA concentrations. In case the AUC was negative after placebo correction, the value was excluded from further analysis (n=3 for LP and n=1 for HP).

Results

Questionnaires

Subjects did not report any feelings of nausea, intestinal cramps, diarrhea, headache, heartburn, belching or other parameters of impaired wellbeing before, during or after infusion of C, LP or HP.

Amino acid plasma concentrations

Baseline AA concentrations were not different between treatments. Overall, a treatment and time interaction effect was found ($p<0.001$); AAs present in native casein increased after LP and HP over time as compared to C ($p<0.001$), whereas amines or metabolites not present in casein such as taurine did not (**Figure 1** and supplemental data Table 1 and 2). Post-hoc analysis showed that both LP and HP resulted in increased plasma concentrations over time as compared to C of 15 out of 19 analyzed AAs in casein, namely alanine, arginine, asparagine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine ($p<0.001$). Aspartic acid was only increased after HP ($p<0.0001$). Concentrations of glutamic acid could not be assessed due to high plasma glutamic acid concentrations. Therefore these results are shown in relative peak areas as compared to an internal standard of glutamic acid concentration (**Figure 1C**). Glutamic acid was increased after HP and LP as compared to C ($p<0.005$). Plasma concentrations of glutamine and glycine did not change after LP and HP infusion.

HP resulted in a further increase of the following AAs as compared to LP; aspartic acid, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, tyrosine, tryptophan and valine ($p<0.001$). After infusion of LP and HP the AAs present in native casein increased when the infusion started ($t=30$), and peak plasma concentrations were reached at the time the infusion stopped ($t=120$) (**Figure 1A, 1B**). All other amines and AAs not present in casein, such as taurine (**Figure 1D**), did not show a change in plasma concentrations after LP, HP or C.

In **Table 3** the mean AUCs (g) are shown per AA for LP and HP. Also the total amount of AAs found in plasma after intraileal infusion of LP and HP was calculated as a percentage of amount of AAs infused as casein (4.44 g for LP and 13.38 g for HP). In total, 3.18 ± 0.87 g and 12.49 ± 1.73 g AAs were found in plasma after intraileal infusion of LP and HP, respectively. These numbers correspond to $72 \pm 20\%$ (LP) and $93 \pm 13\%$ (HP) of the amount of AAs infused as casein.

Cytokines and CRP

Plasma concentrations of IL-6, IL-8, IL-1β, TNF-α and CRP were not affected by either LP, HP or C (**Figure 2**). Plasma concentrations of the cytokines analyzed did not change over time and no interaction effect between time and protein infusion was found.

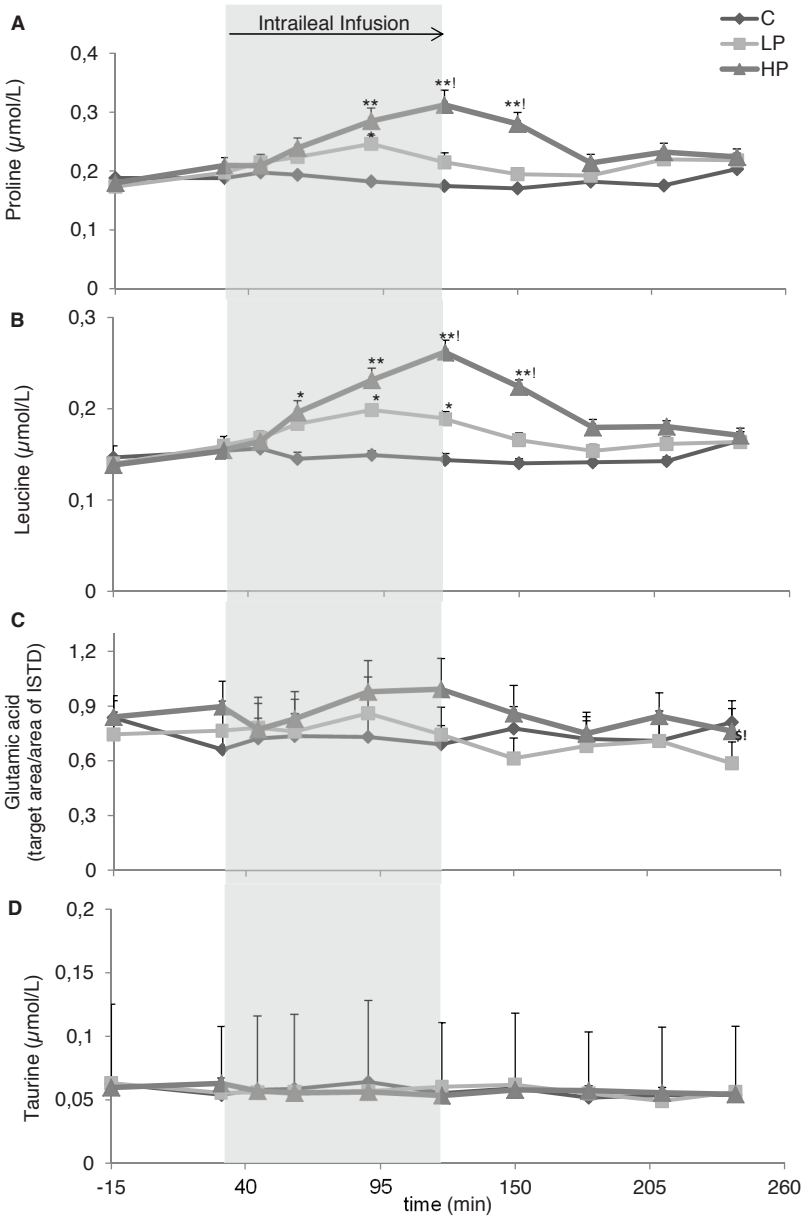

Figure 7.1 Plasma profiles of proline (A), leucine (B), glutamic acid (C) and taurine (D) after intraileal infusion (infusion time t= 30-120 min) of control (C), low-dose casein protein (LP) or high-dose casein (HP) (n=13). An interaction effect (treatment*time) for proline **(A)** and leucine **(B)** was observed. Proline and leucine concentrations increased after HP infusion ($p<0.0001$) and LP infusion ($p<0.0001$). Glutamic acid **(C)** increased after HP infusion ($^{\$!}$ treatment effect $p<0.005$) as compared with both placebo and LP infusion (this panel represents ratios of the peak area's/ internal standard peak areas). Taurine concentrations **(D)** were not affected by HP or LP infusion. *$p<0.05$ compared with placebo at the same time point, **$p<0.0001$ compared with placebo at the same time point, $^{!}p<0.001$ compared with LP at the same time point. Note: in supplemental data Table 1 the results of all amino acids are presented.

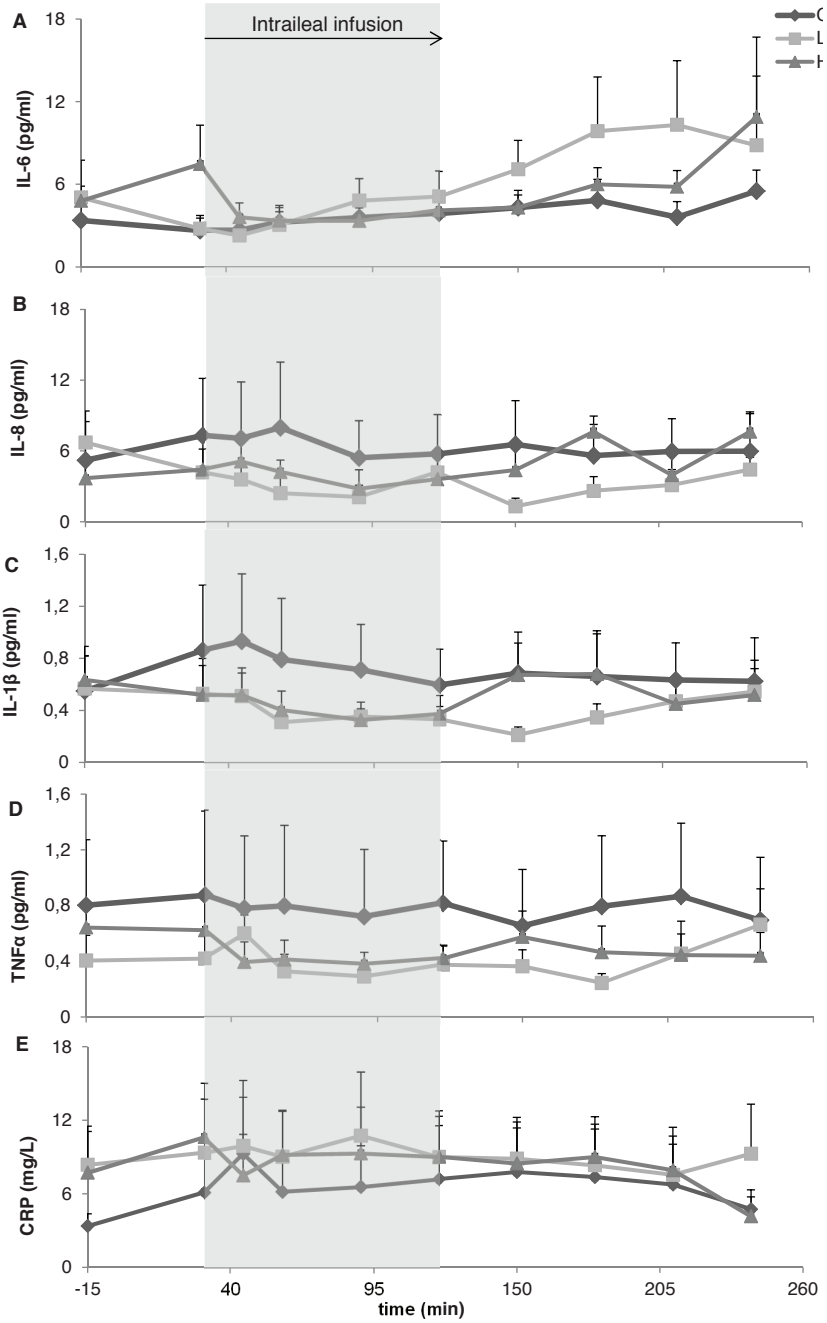

Figure 7.2 Plasma concentrations of IL-6 (A), IL-8 (B), IL-1β (C),TNFα (D) and CRP after intraileal infusion (infusion time t=30-120 min) with control (C), low-dose casein (LP) or high-dose casein (HP)

None of the treatments affected plasma concentrations of the cytokines analyzed. For IL-6, 2 subjects plasma concentrations were below the LLOQ (n=11). For IL-8, 8 subjects plasma concentrations were below the LLOQ (n=5). For IL-1β, 3 subjects had plasma concentrations below the LLOQ (n=10) and for TNFα 8 subjects had plasma concentrations below the LLOQ (n=5).

Table 7.3 AAs in plasma AUCs (30-180 min) after C, LP and HP

	LP (n=10)				HP (n=12)				
	Infused AA (as casein)	AUC plasma AA			Infused AA (as casein)	AUC plasma AA			
Amino Acid	Mean (g)	Mean (g)	SEM	p-value*	Mean (g)	Mean (g)	SEM	p-value*	p-value#
Alanine	0.13	0.44	0.18	ns	0.39	0.88	0.35	<0.05	ns
Arginine	0.15	0.56	0.12	<0.005	0.46	0.72	0.15	<0.0001	ns
Aspartic acid	0.31	0.03	0.01	ns	0.92	0.09	0.04	<0.05	ns
Asparagine		0.06	0.05	ns		0.14	0.05	ns	ns
Glutamic acid	0.90	-0.49	0.90	ns	2.79	0.31	0.89	ns	ns
Glutamine				ns				ns	ns
Glycine	0.08	0.18	0.12	ns	0.23	0.26	0.10	ns	ns
Histidine	0.12	0.31	0.12	<0.05	0.36	0.40	0.10	0.0034	ns
Isoleucine	0.22	0.23	0.08	ns	0.65	0.81	0.12	<0.0001	<0.0001
Leucine	0.40	0.38	0.13	ns	1.21	1.52	0.30	<0.0001	<0.0001
Lysine	0.35	0.47	0.29	ns	1.04	2.35	0.59	0.0003	<0.005
Methionine	0.11	0.04	0.03	ns	0.33	0.28	0.04	<0.0001	<0.0001
Phenylalanine	0.22	0.17	0.07	ns	0.65	0.28	0.06	<0.005	ns
Proline	0.46	0.26	0.18	ns	1.38	0.26	0.18	<0.005	<0.005
Serine	0.24	0.12	0.12	ns	0.73	1.11	0.25	<0.05	ns
Threonine	0.18	0.13	0.09	ns	0.55	0.55	0.14	0.0005	<0.05
Tryptophan	0.06	0.09	0.03	ns	0.17	0.33	0.07	<0.0001	<0.05
Tyrosine	0.23	0.08	0.06	ns	0.69	0.73	0.15	<0.0001	<0.0001
Valine	0.28	0.44	0.23	ns	0.83	1.45	0.26	<0.0001	<0.005
Total	4.44	3.18	0.87	ns	13.38	12.49	1.73	<0.0001	<0.0001

*p-value LP or HP as compared to C, #p-value as compared LP, ns; not significant

Oxylipins

Only an effect of infusion ($p<0.001$), time ($p<0.001$) and interaction between infusion and time was found for 11,12-DiHETrE ($p<0.05$) (**Figure 3**). Concentrations of 11,12-DiHETrE decreased after HP ($p<0.001$) and LP ($p<0.01$) as compared to C. Plasma concentrations of 11,12-DiHETrE did not differ between LP as compared to HP.

An infusion and time effect ($p<0.05$) was found for 5,6-DiHETrE and 19,20-DiHDPA. These oxylipins decreased after HP as compared to C, and LP ($p<0.05$). However, no interaction between time and treatment was found. None of the other oxylipins presented in **Table 2** were affected by ileal infusion of either LP, HP or C.

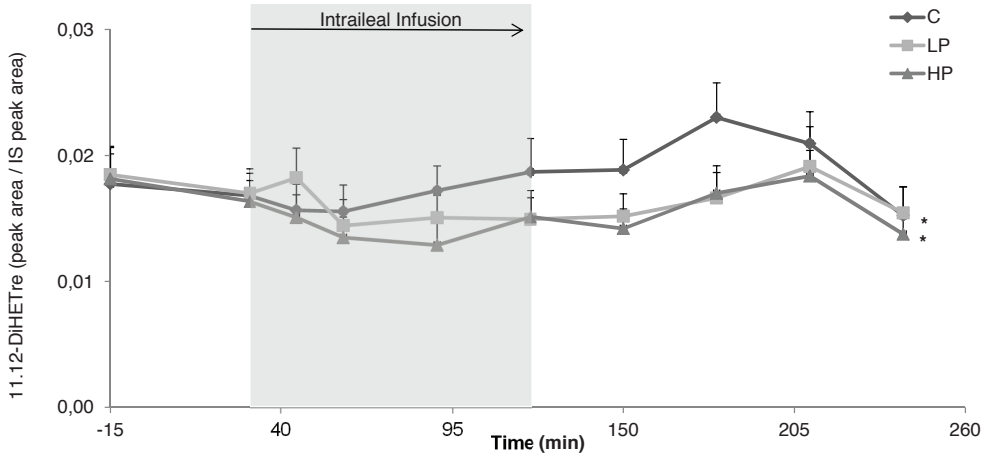

Figure 7.3 Results of 11,12-DiHETre after intraileal infusion (infusion time t=30-120 min) with control (C), low-dose casein (LP) or high-dose casein (HP) (n=13)

An effect for time ($p<0.001$), infusion ($p<0.001$) and interaction between time and infusion ($p<0.001$) was found for 11.12-DiHETre. *$p<0.05$ both LP and HP resulted in decreased 11.12-DiHETre.

Discussion

To our knowledge this is the first study showing that intraileal infusion of native casein results in AA plasma recoveries of approximately 72-93%. These results suggest that ileal infusion of native protein casein at 5 and 15 g may result in near complete digestion and absorption of its AAs. Pro-inflammatory mediators or GI symptoms did not increase in response to intraileal protein infusion, indicating that intraileal casein infusion does not induce adverse effects in healthy subjects.

The present study aimed to investigate adverse effects of intraileal protein infusion. This was done by 1) estimating AA digestion and absorption after LP and HP reducing the possibility of casein malabsorption and 2) evaluating immune activation after ileal protein infusion.

For casein specific AAs, plasma recoveries of 72 ± 20% and 93± 13% were estimated after LP and HP. It cannot be excluded that part of the increased AA concentrations after LP and HP result from endogenous protein breakdown. However the following arguments suggest that the increase in casein specific AAs was due to intraileal casein infusion; 1) data of the present study show reproducible and dose-dependent AA concentration patterns after infusion of both LP and HP for all AAs present in casein. In contrast, plasma concentrations of amines (e.g. taurine) not present in casein did not change after infusion of both LP and HP; 2) Starting ileal protein infusion resulted in an immediate increase in plasma AA concentrations, whereas these concentrations decreased immediately after ending the infusion; 3) Effects on AA plasma concentrations were studied within subjects making direct comparisons between undigested casein and saline possible.

In this study we showed that pro-inflammatory markers were not affected by protein infusion, nor were GI symptoms. Subjects did not report any feelings of nausea, intestinal cramps, diarrhea, headache, heartburn, belching or other overall GI symptoms before, during or after C, LP or HP. The results on subjective feelings of wellbeing during intestinal protein infusion are in line previous studies. In studies of Geraerts (2011) and Ryan et al.(2013) it was shown that intraduodenally infused protein did not result in any GI complaints (21)·(22).

Recently Neurath et al., proposed that factors in the diet such as dietary protein may contribute to an impaired intestinal barrier function, resulting in the release of pro-inflammatory cytokines (12). Another possible trigger for pro-inflammatory responses when protein is not efficiently digested may be protein fermentation in the colon (11). Therefore, it was reasonable to postulate that ileal protein infusion could induce an inflammatory response. However, in our study none of the pro-inflammatory cytokines evaluated were affected by ileal infusion of 5 or 15 g casein. Furthermore, almost the total amount of AAs infused as casein was found in blood plasma suggesting that casein was already digested before entering the colon. Additionally, none of the oxylipins analyzed were induced by protein infusion, except for 11,12-DiHETrE, which plasma concentrations decreased after infusion of HP. Recently, increased plasma concentrations of 11,12-DiHETrE have been found in patients with nonalcoholic steatohepatitis (NASH), making it a candidate biomarker for the non-invasive detection of NASH (23). However, in our study the decrease of 11,12-DiHETrE was rather small and the exact function of this oxylipin is still unknown. Therefore we believe this effect is has little, if any, biological significance.

Several proteolytic enzymes, as well as the expression of peptide- and amino acid transporters (e.g. peptide transporter 1 and B^0AT1) are known to be present in the ileum (24-27). These enzymes and transporters may be involved in the digestion and absorption of casein in the ileum. It was hypothesized that intraileal infusion of native casein results in efficient casein digestion and absorption of casein specific AAs. It was

found that LP and HP resulted in recoveries of 72 ± 20% and 93 ± 13% for casein specific AAs, suggesting that these AAs were indeed efficiently digested and absorbed. To our knowledge, this is the first study investigating the effects of undigested proteins in the human ileum on its digestibility. Previous studies evaluated postprandial kinetics and digestibility of oral protein intake in healthy volunteers (9, 28-30), as well as casein digestibility after RYGB surgery(10). Our estimated AA recoveries after ileal protein infusion are in line with AA recoveries reported by Mahé et al. In that study orally ingested [^{15}N] casein was recovered in the jejunum mainly in the form of peptides and subsequently efficiently absorbed in the upper part of the small intestine (28). Luminal jejunal recovery of orally ingested casein was 82.6 ± 9.5%. In another study by Gaudichon et al. (2002) ingestion of a meal containing milk protein resulted in calculated true digestibility percentages of AAs between 90 and 100% (30). Although the recovery percentages in these studies are in the same range as our findings on AA recoveries, direct comparisons are difficult. Both studies investigated the ingestion of oral protein loads containing more protein as compared to the amounts of protein infused in the present study e.g. 30 g milk protein (30) and 368 mmol casein (28) as compared to 5 or 15 g of casein in the present study. Although the AA recovery of 15 g of ileal casein infusion was approximately 80%, more studies are needed to confirm efficient protein digestion and subsequent AA absorption after intraileal delivery of higher amounts of protein.

This study has limitations that need to be considered. First, the present study design was not optimal to draw conclusions on amino acid digestion and absorption. Since we did not used labeled casein protein we cannot prove that the increased AAs concentrations were due to digestion and absorption of ileal infused casein. Also we cannot exclude a possible interaction effect between breakfast ingestion and casein infusion. Therefore, we can only conclude that it is highly likely that the increased amino acid plasma concentrations originated from casein digestion. Secondly, data of the present study only concern acute responses. The effects of repeatedly targeting the ileum with protein on adverse effects remain to be investigated. However based on available literature of patients undergoing RYGB it seems unlikely that repeatedly targeting the ileum results in adverse effects or protein malabsorption. A recent report showed that ingestion of caseinate by RYGB patients results in efficient and even accelerated protein digestion and absorption as compared to protein ingestion before RYGB (10). Furthermore, it has been proposed that malabsorption contributes only a small proportion to the reduction in net energy absorption which is observed after RYGB (4, 7). These data suggest that it is not very likely that repeatedly targeting the ileum with native casein results in adverse effects in healthy subjects. Thirdly, the present study was performed with healthy subjects with a normal BMI and may not give the same results in other groups, such as subjects with comorbidities or overweight individuals. Fourthly, we

only measured a selection of inflammation parameters and therefore it is possible that effects could have been missed.
Our group previously showed that intra ileal infusion of casein, sucrose and safflower oil results in increased satiation and decreased food intake (3). A reduction in food intake by ileal protein infusion indicates that the ileal brake may be a target in the prevention of overweight and obesity. The data of the present study show that ileal casein infusion results in high plasma recoveries of casein specific AA and does not induce acute adverse effects. These data provide evidence that targeting the ileal brake may not only be a potent but also a safe target for weight management.

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Competing Interest

The authors have declared that no competing interests exist.

Financial disclosure

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Chapter 8

General discussion

The research presented in this thesis adds to our knowledge on the role of the small intestinal tract in the development of satiation. Potential targets that were studied included intestinal brake activation, GLP-1 and CCK signaling via the vagal nerve and the role of mediators such as serotonin that may be involved in nutrient-induced GLP-1 release. An important question addressed was whether stimulation of the distal small intestine with macronutrients other than lipids, such as undigested native protein (casein) and carbohydrate (sucrose) would result in the release of satiety hormones and reduction of food intake. Additionally, the hypothesis of a serotonin mediated mechanism for (non-)nutritional induced GLP-1 release was investigated. This final chapter starts with summarizing the main findings presented in this thesis. Next, these results are discussed in relation to other studies and implications and suggestions for future research are presented.

Main findings

The data presented in this thesis show that of the potential strategies investigated, ileal delivery of (non-)nutritional stimuli is likely to present the best approach for weight management. It was found that ileal stimulation with relatively small amounts of macronutrients increases satiety signaling and reduces energy intake. In addition, the effects of ileal brake activation on satiation were stronger in comparison to activation of the duodenal and jejunal brake **(chapter 5)**. Activation of the ileal brake was found to be independent of the type of macronutrient, since ileal stimulation with all macronutrients tested; lipids (safflower oil), protein (casein) and carbohydrate (sucrose) effectively increased satiety signals and reduced energy intake **(chapter 6)**.

It was found that the same macronutrients, as well as rebaudioside A also stimulated the release of GLP-1 and PYY from ileal tissue segments **(chapter 2**). There are several mechanisms in the ileum which sense (non-)nutritional stimuli, resulting in GLP-1 release. It was found that serotonin

Ileal macronutrient delivery has potential as a weight management strategy because:

The ileum senses all nutrients efficiently; all macronutrients delivered to the ileum stimulate GLP-1 release resulting in satiation via at least two processes 1) nutrient interactions with GPCRs and 2) via serotonin-mediated processes

It does not result in adverse effects

Macronutrients are efficiently digested in-, and absorbed from the ileum

Ileal stimulation more effectively activates satiation in comparison to jejunal or duodenal stimulation

A small amount of macronutrients (51.7 kcal) already results in a significant reduction of energy intake (80-130 kcal)

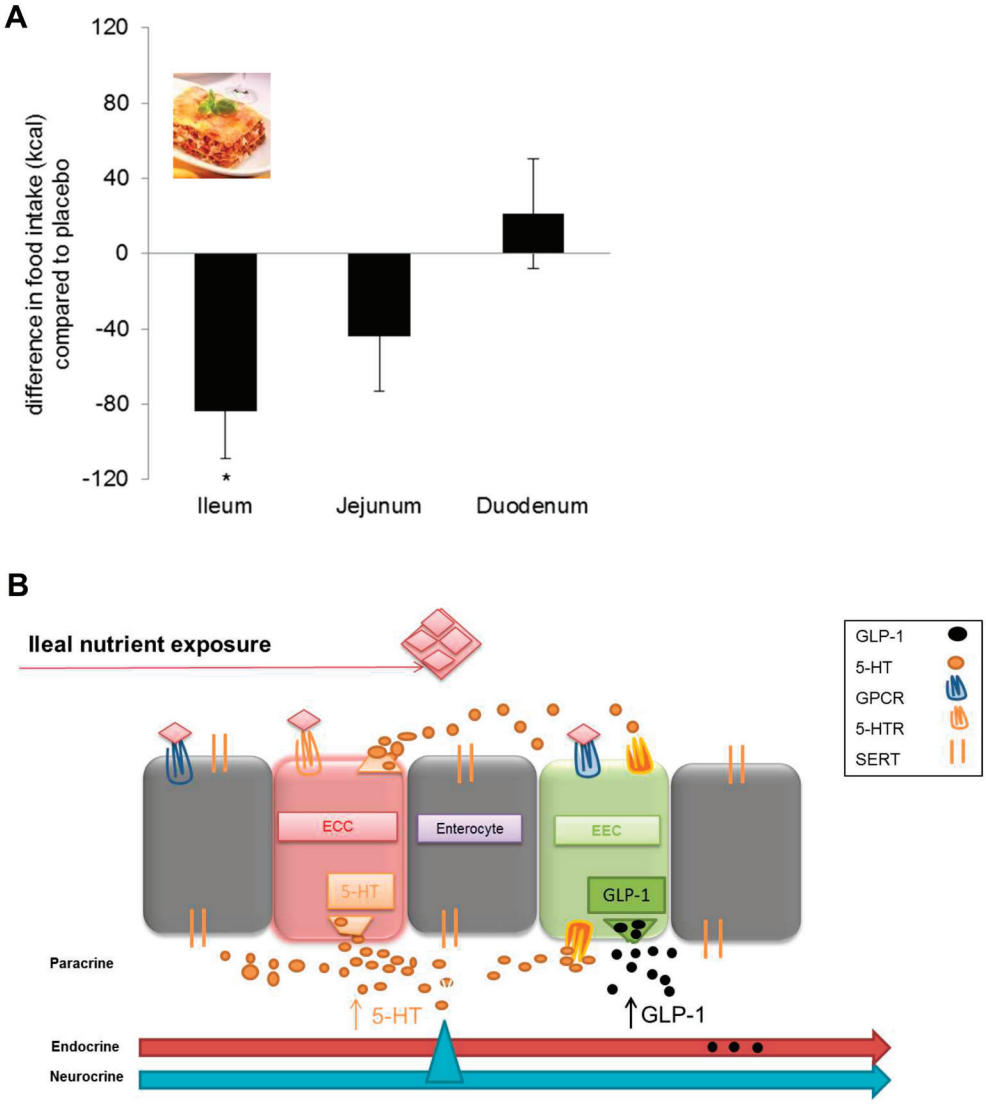

Figure 8.1 Overview main findings this thesis

(A) Ileal exposure results in the most effective reduction in food intake as compared to jejunal or duodenal release. **(B)** Stimulation of ileal tissue segments with casein, sucrose, safflower oil and rebaudioside A results in GLP-1 release. Serotonin contributes to this (non-)nutrient-induced GLP-1 release possibly via serotonin receptors on enteroendocrine cells.

released from enterochromaffin cells (EECs) mediates nutrient-induced GLP-1 release from enteroendocrine cells (EECs) via the activation of serotonin receptors on EECs **(chapter 3)**. Furthermore, the data presented in this thesis show that ileal delivery of native undigested protein does not result in acute adverse effects since ileal delivered

casein is efficiently digested and absorbed, does not activate immune responses, and does not result in gastrointestinal complaints by healthy subjects **(chapter 7)**.
Additionally it was found that the vagal nerve does not contribute to satiation in pigs, and that CCK regulates satiation independently of CCK receptors located on the vagal nerve. Complete interruption of the abdominal vagal nerve in pigs did not affect food intake, and antagonism of the CCK receptor increased food intake in both sham and vagotomized pigs **(chapter 4).** Therefore, the contribution of endocrine and paracrine CCK and GLP-1 signaling to satiation seems to be more important than signaling via CCK and GLP-1 receptors located on the vagal nerve.

Internal validity

For this thesis, a translational approach was used, involving different models and methods. Studies performed in animals or with animal tissues used the pig as model species. Pigs show a high degree of resemblance to human beings in terms of their intestinal anatomy and physiology and the regulation of appetite and energy metabolism. Like humans, pigs are omnivorous animals (1-4). The research strategies and techniques used were based on an *ex vivo* porcine intestinal segment model, an STC-1 cell line, an *in vivo* porcine model of subdiaphragmatic vagotomy, and an *in vivo* intubation technique in healthy volunteers **(figure 8.2)**. In this paragraph the consistency of the data derived from the various methods will be discussed.

In vivo porcine model of subdiaphragmatic vagotomy

To study the role of the abdominal vagal nerve in food intake regulation, total subdiaphragmatic vagotomy was applied by blocking both the efferent and the afferent vagal nerves via endoscopic laparoscopy. Pigs recovered rapidly and without complications from their surgery. The vagal nerve was interrupted by removal of approximately 3 cm length of both afferent and efferent vagal nerves, as was confirmed with inspection at necroscopy. The advantage of total subdiaphragmatic vagotomy is that all vagal nerve signaling under the diaphragm was excluded, making it possible to investigate the contribution of the total abdominal vagal nerve to food intake regulation. By using the pig for this model we were also able to control the feeding regime during the whole study period, thereby reducing variation in food intake between individual animals.
For interpretation of the results of this study, the limitations of total subdiaphragmatic vagotomy should be considered as well. First, care should be taken with the interpretation of the results since this model does not represent healthy physiology. Second, vagotomy decreased the intestinal transit time of food. It resulted in a decreased gastric emptying rate and attenuated postprandial responses of glucose and insulin. At necropsy stomach contents were found to be increased. These results make it difficult to pinpoint whether

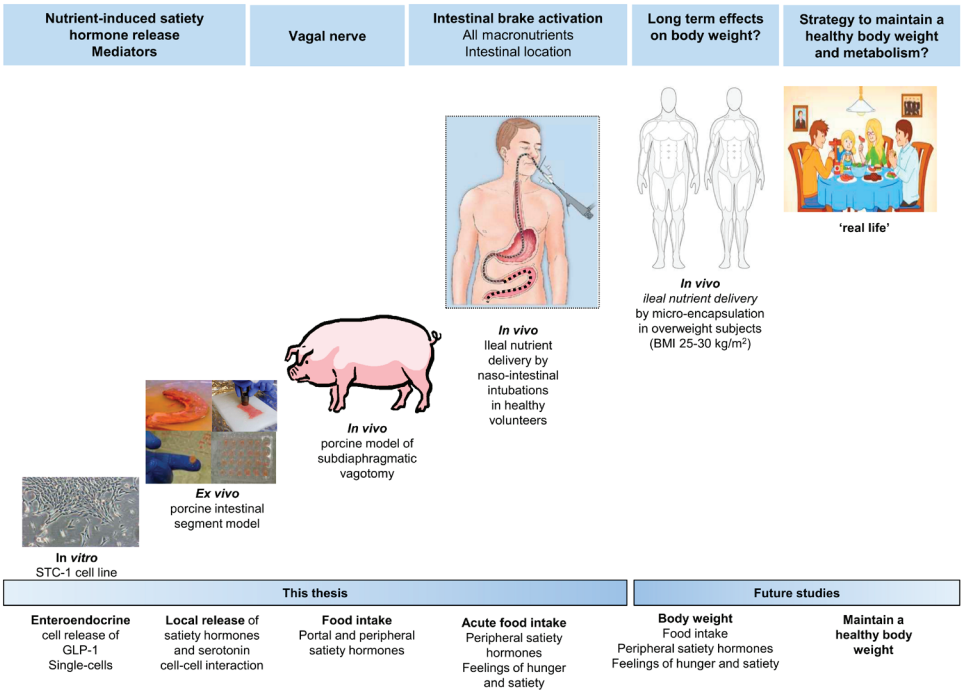

Figure 8.2 Overview of the research and different research models presented in this thesis

The STC-1 cell line was used to study the effects of serotonin induced GLP-1 release from enteroendocrine cells. The *ex vivo* intestinal segment model was applied to study the effects and mechanisms of intestinal nutrient stimulation on the release of satiety hormones. An *in vivo* porcine model of subdiaphragmatic vagotomy was used to study the contribution of the vagal nerve and CCK and GLP-1 receptors located at the vagal nerve to satiation. *In vivo* studies with healthy volunteers were conducted to study the effects of intestinal macronutrient delivery on satiation. **Future studies;** Applications for nutrient delivery, other than intubation, should be developed in order to study the effects of daily ileal nutrient delivery on body weight and satiation. If the latter is successful, it should be investigated if ileal nutrient delivery before a meal helps people to maintain their healthy body weight.

the effects of vagotomy on food intake and postprandial hormone responses are caused by vagal nerve interruption or whether they are a consequence of the decreased gastrointestinal transit time. Despite the differences in gastrointestinal transit time no effects on food intake between vagotomy or sham operated pigs were found, suggesting that in pigs the vagal nerve does not contribute to satiation. To analyze the contribution of neural signaling via the vagal nerve to satiation other, more precise technologies able to measure real time vagal nerve activity should be applied. Others applied vagal electrodes to minipigs and rats to stimulate vagal nerve activity. These authors showed that stimulation of the vagal nerve resulted in decreased weight gain and reduced food intake (5, 6). It would thus be very interesting to develop technologies to analyze real time local vagal nerve activity *in vivo*.

Third, due to different vagotomy techniques, species differences and types and characteristics of food and (or) feeding regimens, it was difficult to compare the results from our study with data from other studies (7-10).
Despite the limitations of this model of total subdiaphragmatic vagotomy, it was shown that interruption of the total vagal nerve in pigs does neither affect food intake nor body weight. Overall, the results of this study show that the vagal nerve is involved in regulation of intestinal transit of food, but does not contribute to the regulation of satiation, whereas other targets such as CCK receptors did show to affect satiation.

Ex vivo and in vitro models to study (non-)nutrient-induced GLP-1, PYY and CCK release

Another model used for the research presented in this thesis was the *ex vivo* porcine intestinal segment model. This model was applied to investigate the effects of intestinal macronutrient stimulation on GLP-1, PYY and CCK release. Additionally, this model was used to study the role of intestinal serotonin in nutrient-induced GLP-1 release. The intestinal segment model previously showed to be a suitable tool to study compound- and location-specific effects on the release of GI hormones (11, 12). The advantage of this model is that the segments mimmic the intestinal epithelium as a relatively intact tissue containing multiple cell types such as EECs, ECCs and absorptive enterocytes. The segments can be obtained from various anatomical regions of the small intestine (e.g. duodenum, jejunum, ileum and colon) making it possible to study location-specific effects. Since more segments can be obtained from one pig, effects can be studied within the same animal. Furthermore, by analyzing media that have been exposed to the segments, paracrine effects of nutrient stimulation can be studied. Although this model offers a suitable system to study cellular interactions involved in serotonin and GLP-1 release, it has also some limitations that warrant consideration. First, it should be noted that care should be taken with interpreting the findings from this *ex vivo* model, since it is unknown if all the findings resemble physiology of the human situation *in vivo*. For the research presented in this thesis the intestinal segment model was used to get an indication which macronutrients may be potent activators of the ileal brake *in vivo*. Second, this method does not distinguish between intestinal exposure to the apical or basolateral side. It is therefore also not possible to distinguish if GLP-1, PYY and CCK are released to the apical or basolateral side. A more advanced method which can be applied to study the effects of nutrient transport is the Ussing chamber (13). Third, since the segments contain multiple cells it is difficult to study which cell type is responsible for the effects on for example GLP-1 release. To study which cell types are responsible for the effects found *ex vivo,* cell-lines can be applied. For the research presented in this thesis, the secretin tumor (STC-1) cell line was applied to study if serotonin-induced GLP-1 release was stimulated from EECs and if this was receptor mediated. This mouse derived tumor cell line is widely used to study the effects of dietary compounds on CCK, GLP-1 and PYY secretion (14, 15).

Small intestinal delivery of macronutrients in healthy volunteers

To study the effects of the type of macronutrient(s) and location of their release along the small intestinal tract on energy intake and the release of satiety hormones, naso-intestinal catheters were applied to healthy human volunteers.
This technique has previously been applied to study the effects of duodenal delivery of lipids and proteins, as well as those of ileal lipid delivery (16-21). These studies have the advantage that energy intake in human volunteers can be included as primary outcome parameter. Also the effects of intestinal nutrient delivery on plasma concentrations of GLP-1, PYY and CCK can be studied. Although this technique is suitable to study effects of intestinal nutrient delivery on satiation, it comes with its own limitations that warrant consideration. First, intubation with a naso-ileal catheter for several consecutive days may induce discomfort and changes in overall well-being, or induce possible carry-over effects, thereby affecting outcomes of the study parameters. However, the contribution of the latter effect was minimized by applying cross-over study designs and random assignment of treatments. Second, in the human intubation studies presented in this thesis only acute effects on food intake were investigated. The effects of repeatedly targeting the ileum with macronutrients on food intake and on adverse effects remain to be investigated. Taken together, the naso-intestinal intubation technique offers a suitable method to study the effects of intestinal nutrient delivery on satiation, energy intake and release of satiety hormones *in vivo*.

Consistency of data derived from in vivo intubation and ex vivo intestinal segment studies

Intestinal macronutrient stimulation was performed *ex vivo* and *in vivo* to investigate underlying mechanisms behind and effects of stimulation on activation of satiety signals and satiation. The *ex vivo* intestinal segment studies and *in vivo* intubation experiments have in common that effects of undigested native macronutrient exposure to the intestinal epithelium are studied, whereas the proximal parts (e.g. mouth and stomach) of the GI tract are bypassed. By applying these methods the intestinal epithelium is directly stimulated with macronutrients.
The results of the *ex vivo* study showed that ileal simulation with all macronutrients and mixtures tested, sucrose, safflower oil and casein, resulted in an increased GLP-1 and PYY release. Ileal stimulation *in vivo* with the same preparations resulted in reduction of energy intake and increased activation of satiety signals. Although these results all point towards the direction that ileal delivery of different macronutrients results in increased release of satiety hormones and activate satiation, it should be noted that the effects on nutrient-induced GLP-1, PYY and CCK release were somewhat contradictory.
Stimulation *ex vivo* of ileal tissue segments with safflower oil, sucrose and casein increased GLP-1 and PYY release in all cases. However, *in vivo* the effects of macronutrient-induced increases in GLP-1 and PYY plasma concentrations were less pronounced. GLP-1 plasma concentrations increased after delivery of casein, whereas ileal delivery

of sucrose and safflower oil did not. In contrast, delivery of sucrose and safflower oil increased plasma concentrations of PYY, whereas casein did not. An explanation for this may be that in the *in vivo* studies concentrations of GLP-1 and PYY are analyzed in plasma, whereas in the *ex vivo* studies release from tissue segments was analyzed, which probably reflects paracrine effects. Once released from enteroendocrine L-cells, the active form of GLP-1 is very rapidly degraded by dipeptidyl peptidase IV (DPPIV). It is therefore conceivable that paracrine and neurocrine effects on GLP-1 and PYY release are missed by analyzing plasma concentrations after ileal delivery of macronutrients. The results on CCK were somewhat contradictory as well. CCK release was unaffected after exposure of ileal tissue segments to casein, sucrose and safflower oil. In contrast, plasma concentrations of CCK increased after ileal delivery of casein, sucrose and safflower oil to volunteers. Although a previous study showed increased CCK plasma concentrations after ileal delivery of safflower oil as well (19), this was somewhat surprising since CCK is present in the ileum only in very low amounts. Most CCK is present in the duodenum, which is bypassed in the ileal intubation studies (22). The differences in CCK release between *ex vivo* and *in vivo* may due to a paracrine or neurocrine feedback loop *in vivo*. Hypothetically, serotonin and/or GLP-1 released from ileal cells can activate serotonin- or GLP-1 receptors on vagal nerve endings or EECs in the more proximal parts of the small intestine resulting in CCK release from cells in the duodenum. In the *ex vivo* model such a feedback loop would not function since isolated ileal segments were exposed to the stimuli.

Taken together, the effects on satiety hormone release were not always comparable between the two different research models, which possibly may be due to the paracrine effects analyzed *ex vivo* vs. endocrine effects analyzed *in vivo*. Results show that the *ex vivo* model is a suitable tool to screen the effects of various stimuli on local release of GLP-1 and PYY, and investigate the contribution of other mediators such as serotonin. Furthermore, it was found that the macronutrients that induced GLP-1 and PYY release *ex vivo*, activated the ileal brake *in vivo*. Together these *ex vivo* and *in vivo* data support the idea of the ileal brake as a potent mechanism for weight management.

The ileal brake; a target for weight management?

The data presented in this thesis show that ileal delivery of different macronutrients activates the ileal brake and results in increased satiation and the release of satiety hormones. The ileal brake is the primary inhibitory feedback mechanism to control transit of a meal through the GI tract in order to optimize nutrient digestion and absorption (23). Under physiological circumstances this mechanism is activated when macronutrients arrive in the ileum, resulting in inhibition of gastric emptying and the transit of nutrients through the duodenum and jejunum (24). The ileal brake concept was first discovered in humans when ileal fat perfusion in man was shown to result in an inhibition of jejunal motility (25) and an increased transit time of a solid meal (26).

Although nutrients infused into the duodenum and jejunum also activate an intestinal brake (17, 18, 27), intraileal lipid infusion results in stronger inhibitory effects as compared to intraduodenal lipid infusion (28). Whether ileal delivery of macronutrients other than lipids would result in more pronounced inhibitory effects as compared to nutrient delivery to the duodenum and jejunum was still unknown. Our data showed that activation with undigested native protein has stronger effects on satiation and release of GLP-1 and CCK as compared to duodenal and jejunal activation. Stimulation of the ileum may have stronger braking effects since under physiological conditions, only small amounts of undigested nutrients reach the distal small intestine (29, 30). Once undigested nutrients reach the ileum this may be a sign of inefficient digestion and absorption by the proximal parts of the small intestine. As a consequence, the ileum generates signals such as GLP-1 to inhibit further eating, resulting in more time to digest and absorb the nutrients before new food enters the GI tract.

In the small intestine there are several mechanisms allowing to respond to fluctuations in nutrient levels (31), to make digestion and absorption as efficient as possible. One of these mechanisms is by activation of the so-called nutrient sensing receptors (32, 33). A large number of G-protein coupled receptors (GPCRs) and ion-dependent transporters have been identified as enteroendocrine nutrient sensors. Glucose sensing receptors, protein sensing receptors, fatty acid sensing receptors and sweet taste receptors all have been identified and shown to be activated by specific macronutrients and tastants such as sweet, sour, salt and umami (33-35). In addition to this nutrient receptor mediated hormone release, we found that serotonin released from ECCs contributes to (non-) nutritional stimulated GLP-1 release from EECs in the ileum. We were able to study local small intestinal effects of serotonin on GI hormone release by applying the *ex vivo* intestinal segment model. These data showed that serotonin is involved in nutrient sensing and that it stimulates GLP-1 release from the ileum via serotonin receptor-mediated processes. Additionally, when the reuptake of serotonin was inhibited by exposure to casein and safflower oil, GLP-1 release increased. These results all underline that serotonin is involved in nutrient-induced GLP-1 release. Previous studies also showed that serotonin stored in ECCs is involved in nutrient sensing. Interactions of nutrients with GPCRs on these ECCs resulted in serotonin release (36-38). Taken together, these results support the idea that there may be more than one mechanism in the ileum involved in nutrient-induced GLP-1 release which consequently may contribute to satiation *in vivo*.

Other studies already showed that serotonin is involved in food intake regulation (39). It is generally thought that increased availability of serotonin or serotonin receptor activation inhibits food intake, whereas reduced availability of serotonin or inhibition of serotonin receptors induces feeding (40). Recently, it was shown that intraduodenal infusion of tryptophan, the precursor of serotonin, increases satiation and plasma concentrations of CCK and GLP-1 (41). The authors of this paper suggested that

L-tryptophan may increase brain availability of serotonin and thereby increase satiation. However, most studies that investigate the role of serotonin do not take into account that the vast majority (95%) of our serotonin resides in the GI tract instead of the brain or blood. Of this serotonin approximately 90% is stored in the enterochromaffin cells (ECCs) and the other 10% is present in the serotonergic neurons of the enteric nervous system (42). Serotonin located in the GI tract does not reach the brain, since it cannot cross the blood brain barrier (39). It is therefore highly likely that serotonin in the ileum may have a local effect on food intake regulation as well. Although it is known that serotonin released from ECCs activates intrinsic and extrinsic primary afferent neurons resulting in activation of the peristaltic reflex (38, 43), there is a lack of knowledge about the relation between serotonin released from ECCs and food intake regulation. One reason for this may be that it is very difficult to study the role of serotonin on food intake regulation in the small intestine in humans as a separate process. Serotonin is involved in many different pathways which can lead to side-effects when these are experimentally manipulated.

Ileal nutrient sensing; efficient and safe?

Ileal delivery of all macronutrients tested decreased energy intake to the same extent, although there were some differences in the effects on GLP-1, PYY and CCK release. The issue whether dietary intake of lipid, protein and carbohydrate stimulates satiation and satiety to the same extent has received growing attention the past decade. Some investigators claim that dietary intake of all macronutrients contribute to satiety to the same extent (44), whereas others suggested that calories derived from proteins have more satiating properties as compared to calories derived from carbohydrates and lipids (45-47). Although it was shown that high protein diets effectively reduce weight (48), it has proven difficult to comply to these high protein diets for longer than 1 year (49, 50). We did not find differences in the satiating properties of lipid, protein or carbohydrate in our models. The main difference compared to studies showing more satiating properties for protein, is that we investigated the effects of small intestinal protein delivery, instead of oral protein intake. When protein is ingested under physiological circumstances, gastric acid activates the conversion of pepsinogen into the proteolytic enzyme pepsin which denatures orally ingested protein. Protein is then further digested by trypsin in the small intestine. Most of the orally ingested protein is digested into peptides or free amino acids before entering the ileum (51). By intra-ileal nutrient infusion these proximal parts of the gastrointestinal tract (e.g. mouth, stomach, duodenum and jejunum) are bypassed and undigested nutrients are delivered to the ileum. It was unknown thus far whether undigested casein delivered to the ileum results in efficient digestion and absorption as well. It was hypothesized that if ileal casein is not efficiently digested and absorbed it may enter the colon. In the colon this protein can then be fermented by microorganisms which may result in production of harmful

metabolites (52). We showed that ileal delivery of undigested native macronutrients did not result in gastrointestinal complaints in healthy subjects, and did also not result in activation of pro-inflammatory responses. Additionally, it was shown that ileal delivery of native undigested casein resulted in efficient digestion and absorption of all amino acids present in casein. Plasma concentrations of all amino acids present in casein immediately increased after intraileal protein delivery. The estimated amino acid digestion and absorption efficiency was comparable to a previous study showing that intrajejunal infusion of an amino acid mixture resulted in very rapid and efficient absorption (53). Recently it was also shown that protein digestion and absorption is accelerated after Roux-Y gastric bypass (RYGB) (54). These and other results of studies investigating the effects of RYGB support the hypothesis that the distal small intestine is able to sense and process nutrients resulting in efficient digestion and absorption of nutrients and activation of satiation.

RYGB is a surgical procedure were nutrients are shunted to the more distal parts of the small intestine. This surgery is the most effective bariatric surgery procedure, resulting in weight loss which is maintained for at least 20 years. Moreover, this intervention is effective in reducing obesity related comorbidities such as type II diabetes and overall mortality (55, 56). The exact mechanism of how RYGB exerts its beneficial effects is not completely understood. It is hypothesized that these effects are a result of increased nutrient exposure of the distal small intestine. This is supported by the findings that RYGB results in increased density of GLP-1, PYY, CCK immuno-reactive cells coupled with increased expression of encoding preproglucagon (57), and also increased postprandial plasma levels of GLP-1, PYY and CCK (58-60). Additionally, there are studies suggesting that post RYGB surgery, both taste preferences and the activity of brain areas involved in food rewarding shift towards a pattern normally seen with more healthy foods (61-66).These results, together with the results presented in this thesis all contribute to the idea that there are several mechanisms in the distal small intestine that respond to fluctuations in nutrient levels making digestion and absorption as efficient as possible, resulting in increased satiation and improved insulin sensitivity (67-69).

Taken together, the most effective strategy for weight management so far is RYGB, but this intervention is only applied to patients with severe obesity and (or) severe risk factors or comorbidities. The data presented in this thesis show that ileal nutrient delivery could be a promising strategy for future weight management strategies.

Recommendations for future research

Although ileal nutrient delivery has been shown to be a promising strategy for weight management strategies, more studies are needed to confirm its practical value and to investigate the underlying mechanisms behind the ileal brake as a target for weight management. By improving our understanding of the mechanisms by which the ileal brake operates, it may be possible to develop strategies that target its activation. However,

approaches or strategies other than intraileal infusion are needed to deliver (non) nutritional stimuli to the ileum. In this paragraph suggestions and recommendations for future research are presented focusing on the ileal brake as a potential target for weight management.

To better understand how various (non-)nutritional stimuli result in GLP-1 release it would be interesting to investigate which specific serotonin receptor(s) is(are) responsible for serotonin-induced GLP-1 release. When this is known it may offer potential targets for nutritional or pharmacological approaches to stimulate GLP-1 release. However, it should be noted that there are already several pharmacological anti-obesity approaches available but none of these have been shown to be as effective as RYGB. One such anti-obesity drug is liraglutide, a GLP-1 analogue that recently has been approved. When combined with a restriction of 500 kcal/day and with increased physical activity (≥150 min/wk), this drug results in weight loss of 6% (70). Comparable to this effect most anti-obesity drugs available so far claim to achieve 3-5% weight loss after 3 months (71) or claim to contribute to weight maintenance after a (very) low caloric diet (72). Although a loss of <5% of body weight may already improve health, safety and costs need to be considered as well. The disadvantage of using these anti-obesity drugs is that while mostly aiming to target 1 specific process involved in food intake regulation, so-called off-target effects often lead to several side-effects. It would therefore be more interesting to investigate if nutritional approaches that stay more close to normal physiology, such as ileal nutrient delivery, can result in weight loss. Indeed there is evidence that foods that are less easily digested, such as "slow foods", are more satiating and have potential to reduce the risk on obesity and type II diabetes (73).

To develop effective weight management strategies that target ileal brake activation there are still some issues to solve. First, the most optimal conditions to activate the ileal brake should be further investigated. One such condition is selection of the most optimal food compound(s) or food mixture to activate the ileal brake. So far only the effects of single nutrient stimuli on the ileal brake have been studied. Since there are various mechanisms operating in the ileum sensing different nutrients or non-nutritional stimuli (e.g. rebaudioside A), it may be possible that combinations induce stronger effects on satiation as compared to single (non-)nutrient stimuli. A previous study showed that the combination of sucralose and pea protein increases GLP-1 and CCK release more in comparison to these stimuli alone from both STC-1 cells and human duodenal biopsies (74). However, up to now there are no *in vivo* studies that compare the effects of intraileal infusion of nutrient combinations with that after single nutrient infusions. Another condition which should be taken into account is the gastric load at the time the ileal brake is activated. In our human studies it was shown that when the stomach was still partially filled just before ileal brake activation the decrease in food intake was 130 kcal. By contrast, this difference was 90 kcal if the stomach was empty just before ileal brake activation. More studies show the importance of gastric distension in the

regulation of appetite and food intake (75-77). Therefore, gastric distension and thus the timing of ileal nutrient delivery, should be taken into account in order to achieve the most optimal effects of ileal brake activation on energy intake.

Second, the data presented in this thesis show the effects of single intraileal infusions whereas data about the long term effects of ileal brake activation are still lacking. The efficacy of repeatedly targeting the ileum with undigested macronutrients or other food compounds remains to be investigated. Future studies should investigate whether the reduction of approximately 80-130 kcal after a single intraileal infusion is effectively limiting overall daily energy intake and whether this does not result in compensation of these kcal during a day. In addition, the effects on safety of repeatedly targeting the ileum should be investigated. However, based on literature describing patients undergoing RYGB it seems unlikely that repeatedly targeting the ileum results in adverse effects such as malabsorption (68).

Third, future studies should investigate whether stimulation of the ileal brake is effective in overweight and obese subjects as well. There are some contradictory results on food suppressing effects of intraduodenal nutrient infusion in lean compared to obese subjects. One study showed a less pronounced suppression of food intake after intraduodenal lipid infusion in obese subjects (78), whereas others did not find differences in appetite-suppressing effects of intraduodenal protein infusion between lean and obese subjects (16). Since there are no studies known that compare the effects of intraileal delivery of macronutrients in lean and obese subjects, future studies should investigate if ileal brake activation efficiently reduces energy intake in overweight and obese subjects as well.

Fourth, in our studies macronutrients were delivered using an naso-ileal feeding catheter. This method is to invasive to apply daily for a longer period. Hence, non-invasive techniques need to be invented to deliver food compounds to the ileum. One such approach that has been suggested to active the ileal brake is by Olibra™ (Fabuless), which is an emulsified fat included in a yoghurt. However, results of studies which investigated the effects of Olibra™ are very inconsistent and mostly without affecting energy intake or body weight (79-85). One explanation for the lack of efficiency of Olibra™ may be the absence of evidence that this fat emulsion reaches the ileum. Therefore, the feasibility of reaching the ileum with relatively large amounts of nutrients should be investigated. One such delivery technique would be microencapsulation. This technique may be applied to carry for example macronutrients that need to be protected from influences in the proximal GI tract to the ileum at which the macronutrient should be released (86). The advantage of microencapsulation is that nutrients can be incorporated in a food product, for example a drink. For ileal nutrient delivery via microencapsulation the gastric emptying rate, and intestinal transit time should be considered as well. The intestinal transit time highly varies with gender, age, BMI, feeding status (fasted or fed) and the nutrient load (e.g. liquids or solid) (87). Therefore, the time between intake

of the microencapsulated food compound and the next meal should be optimized in order to have the most optimal brake effects. There are two other research groups that successfully applied microencapsulation to deliver food components to the distal small intestine. They showed that whereas ileal delivery of 6 gram glutamine resulted in enhanced GLP-1 release, food intake inexpertly increased (88). In another study the short chain fatty acid propionate was delivered to the colon in the form of an inulin proprionate ester. This resulted in increased PYY and GLP-1 release, a reduction of energy intake and prevention of weight gain (89). Although more research is needed to investigate its efficiency, microencapsulation may be a tool to deliver macronutrients or other food compounds to the ileum.

Overall, future research should investigate the most optimal conditions to activate the ileal brake. This includes timing of ileal brake activation and activation by one single nutrient or (non-)nutritional nutrient mixtures. Furthermore, the efficacy of ileal brake activation in overweight and obese people and of repeatedly targeting the ileal brake should be investigated. Besides these aspects, non-invasive techniques, such as microencapsulation, to deliver (non-)nutritional stimuli to the ileum should be further developed.

Concluding remarks

The data presented in this thesis show that macronutrient delivery to the ileum results in activation of satiety signals and reduction of energy intake. Besides direct nutrient-receptor interaction, the ileum efficiently senses (non-)nutritional stimuli via serotonin mediated processes resulting in GLP-1 release. Together these results show that cells in the intestinal epithelium of the ileum efficiently sense undigested native (non-) nutritional stimuli resulting in efficient digestion, absorption, activation of satiety signals and finally a reduction of energy intake. Conclusively, this demonstrates that ileal delivery of (non-)nutritional stimuli has potential as a weight management strategy.

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Summary

There is a clear need to develop preventive strategies for overweight. Worldwide, in 2014, more than 1.9 billion adults were overweight of which 600 million were obese. Also in the Netherlands this number was strikingly high; in 2014, 43.2% of the adults were overweight, of which 12.3% were obese. The only effective weight loss treatment so far is bariatric surgery. This surgery does not only result in long term weight loss, but also in improved metabolic health. Studies on bariatric surgery have suggested that signals from the small intestine contribute to the beneficial effects of this treatment. However, bariatric surgery is only applied when people are extremely obese (BMI >35 kg/m^2). Although it is well known that excessive energy intake and an inactive life style result in overweight, developing preventative strategies that help people to maintain a healthy body weight have proven difficult so far. For this reason, there is a clear need for other non-surgical weight management strategies to prevent people from excessive energy intake. These strategies may be invented by addressing how targets in the small intestine activate satiety signals. The research presented in this thesis aimed to investigate small intestinal targets that may contribute to food intake regulation.

Several receptors and transporters along the gastrointestinal tract detect and sense the presence of macronutrients. As a result a number of hormones including glucagon like peptide-1 (GLP-1), peptide YY and cholecystokinin are released. These hormones activate satiation and satiety and are therefore called satiety hormones. For the research presented in this thesis it was investigated in **chapter 2** and **chapter 3** which (non-) nutritional stimuli stimulate GLP-1 and PYY release. Furthermore, we investigated if serotonin is involved in sensing these (non-)nutritional stimuli and if serotonin contributes to GLP-1 release.

Once released from the small intestine, GLP-1, PYY and CCK can act in a paracrine fashion to neighboring cells, in a neural fashion via receptors located on vagal nerve endings or act in an endocrine fashion via the blood. In **chapter 4** we investigated the contribution of neural and endocrine signaling to food intake regulation. This was done by evaluating if CCK and GLP-1 activate satiation via their receptors located at vagal nerve endings or via receptors elsewhere in the body.

When macronutrients are delivered to the distal parts of the small intestine they can activate the so-called intestinal brake mechanism. We investigated if ileal nutrient delivery resulted in the strongest effects on satiation. In **chapter 5** it was investigated if the ileal brake is stronger in activating satiation compared to the duodenal and jejunal brake, and in **chapter 6** we investigated if all macronutrients that are delivered to the ileum increase satiation. Finally, in **chapter 7** we investigated if protein that is delivered to the ileum is efficiently digested and absorbed and does not result in adverse effects such as malabsorption. The main findings, methodological considerations and interpretation of these findings are discussed in **chapter 8**, as well as implications and directions for future research.

Is serotonin involved in (non-)nutritional induced GLP-1 release?

It was found that all macronutrients, including lipid (safflower oil), protein (casein) and carbohydrate (sucrose), stimulate GLP-1 and PYY release from ileal tissue segments. On top of these macronutrients a natural non-caloric sweetener (rebaudioside A) was found to stimulate GLP-1 and PYY release as well. Although some macronutrient-receptor interactions were known to result in GLP-1 release, such an interaction was not described for all macronutrients and non-nutritional stimuli. Therefore, we investigated the hypothesis that an additional serotonin-mediated mechanism is involved in (non-) nutritional-induced GLP-1 release. We found that serotonin is released upon protein and fat stimulation. Additionally, inhibiting the reuptake of serotonin reuptake resulted in enhanced GLP-1, PYY and CCK release. Furthermore, we found that serotonin stimulates GLP-1 release from ileal tissue segments and from enteroendocrine cells via a serotonin receptor mediated process. This was the first study that showed that serotonin mediates nutrient-induced GLP-1 release from ileal tissue segments.

Do CCK and GLP-1 activate satiation in a neurocrine or an endocrine fashion?

How CCK and GLP-1 receptors present on the vagal nerve contribute to satiation was investigated by studying *ad libitum* food intake using vagotomized pigs and by blocking CCK and/or GLP-1 receptors. Food intake increased after CCK receptor blockade in both vagotomized and sham-operated pigs, suggesting that interruption of the abdominal vagal nerve does not contribute to satiation. Blocking the GLP-1 receptors did not affect food intake in both groups. Although interruption of the vagal nerve did not affect food intake, it did increase gastrointestinal transit time and affected the postprandial responses of glucose, insulin, CCK and GLP-1. These results showed that GLP-1 and CCK receptors present on the vagal nerve contribute less to satiation in comparison to GLP-1 and CCK receptors elsewhere in the body. However, these results also showed the importance of the vagal nerve for the gastrointestinal transit of food.

Ileal brake activation; a strong and safe effect on satiation?

In this thesis it has been shown that ileal delivery of small amounts of all macronutrients result in activation of satiety signals and in a reduction of food intake. Moreover, it was found that the ileal brake has stronger effects on satiation as compared to the duodenal and jejunal brake. Ileal delivery of undigested native protein resulted in decreased energy intake and increased GLP-1 plasma concentrations compared to placebo.
Bypassing the proximal parts of the gastrointestinal tract (mouth, stomach, duodenum and jejunum) did not affect the digestion and absorption of protein. Undigested casein protein, delivered to the ileum, was efficiently digested and absorbed as amino acids.

Additionally ileal protein delivery did not activate immune responses, nor gastrointestinal complains, suggesting that ileal protein delivery is safe.

Concluding remarks

Altogether the data presented in this thesis show that all macronutrients delivered to the ileum result in activation of satiety signals and reduction of energy intake. Ileal stimulation resulted in the strongest activation of satiety signals and energy intake reduction compared to duodenal or jejunal stimulation. Besides direct nutrient-receptor interaction, the ileum senses (non-)nutritional stimuli via serotonin mediated processes resulting in GLP-1 release. Conclusively, these results demonstrate that ileal delivery of (non-)nutritional stimuli has potential as a weight management strategy.

Lay summary in Dutch
(Samenvatting voor niet-ingewijden)

Overgewicht en obesitas zijn beide een wereldwijd probleem. Volgens het Centraal Bureau voor de Statistiek had in 2014 meer dan 43.8% van de Nederlandse bevolking overgewicht en van deze mensen had 12.3% obesitas. Obesitas is een groot probleem voor de samenleving omdat obesitas het risico verhoogt op allerlei ziekten zoals diabetes type II, hart- en vaatziekten en zelfs sommige vormen van kanker. Het is daarom van groot belang dat er methodes worden ontwikkeld om overgewicht en obesitas te voorkomen. De meest voorkomende oorzaken van overgewicht en obesitas zijn een inactieve leefstijl en een overmatige voedselinname. Er zijn weinig succesvolle methodes om overmatige voedselinname te voorkomen.

Wat is obesitas?
Volgens de Wereldgezondheidsorganisatie (WHO) is obesitas een aandoening waarbij vet zich zodanig overmatig in het lichaam opstapelt, dat dit aanleiding geeft tot gezondheidsrisico's.

Wanneer de 'Body Mass Index' (BMI), welke gedefinieerd is als de verhouding tussen lengte en gewicht (kg/m^2), gelijk is aan of hoger is dan 25, dan is er sprake van **overgewicht**. Er is sprake van **obesitas** wanneer iemand een BMI heeft gelijk aan of hoger dan 30.

Door te onderzoeken hoe voedselinname door het lichaam wordt gereguleerd kunnen er methodes ontwikkeld worden om overmatige voedselinname te beperken en daardoor overgewicht en obesitas te voorkomen.

Een belangrijk deel van de voedselinname wordt gereguleerd door de dunne darm. Dit blijkt, onder andere uit studies waarbij mensen een 'gastric bypass' operatie ondergaan. Op basis van dit onderzoek zijn een aantal processen in de dunne darm onderzocht die relevant zijn bij de voedselinname regulatie, zij zijn verantwoordelijk voor het stoppen met eten tijdens de maaltijd (verzadiging). Wanneer bekend is hoe deze processen werken, kunnen er voedingsmiddelen worden ontwikkeld die verzadiging activeren en daarmee overmatig eten kunnen voorkomen (**hoofdstuk 1**). De onderzochte processen zijn; het zogenaamde 'intestinal brake' mechanisme, de activatie van de nervus vagus en de rol van het signaalmolecuul serotonine.

Het maagdarmkanaal bestaat uit de slokdarm, maag, dunne darm en de dikke darm. De dunne darm bestaat uit drie delen. De twaalfvingerige darm (duodenum), de nuchtere darm (jejunum) en de kronkeldarm (ileum). Gedeeltelijk verteerde voedingsmiddelen komen in contact met de wand van de dunne darm en zo kunnen ze uiteindelijk in het lichaam worden opgenomen. De meeste voedingsstoffen worden al in het eerste en tweede deel van de dunne darm opgenomen. In het maagdarmkanaal zitten allerlei sensoren die de aanwezigheid van voedingsstoffen waarnemen. Als gevolg van deze waarneming, worden er hormonen uitgescheiden die zorgen voor een verzadigd gevoel, en dus de verdere voedselinname tijdens een maaltijd remmen. Deze hormonen zijn

onder andere glucagon like peptide-1 (GLP-1), peptide YY (PYY) en cholecystokinin (CCK).
In **hoofdstuk 2** is er onderzocht welke voedingsstoffen de afgifte van GLP-1, PYY en CCK stimuleren. Uit dit onderzoek blijkt dat wanneer het ileum in contact komt met macronutriënten zoals vet (saffloer olie), eiwit (caseïne eiwit) en koolhydraat (sucrose) of zelfs met een calorie-loze zoetstof (rebaudioside A), de afgifte van GLP-1 en PYY gestimuleerd wordt.
Verder blijkt uit het onderzoek beschreven in **hoofdstuk 3** dat serotonine, een signaalmolecuul aanwezig in het ileum, betrokken is bij de in hoofdstuk 2 bestudeerde macronutriënt-geïnduceerde afgifte van GLP-1. Uit hetzelfde onderzoek blijkt dat serotonine gestimuleerde GLP-1 afgifte gemedieerd wordt door een serotonine receptor.
Nadat GLP-1, PYY en CCK worden uitgescheiden kunnen deze hormonen op een aantal manieren een verzadigd gevoel opwekken. Dit kan door; 1. processen in naastliggende cellen te activeren (paracrien), 2. zenuwen te activeren (neuraal) of 3. processen ergens anders in het lichaam te activeren via het bloed (endocrien). In **hoofdstuk 4** wordt onderzocht in welke mate neurale en endocriene signalering bijdragen aan verzadiging in het varken. De resultaten van deze studie laten zien dat CCK verzadiging activeert via endocriene signalering, en in mindere mate door neurale signalering via CCK receptoren op de nervus vagus.

Ileal brake activatie: de maag, duodenum en het jejunum overslaan om minder te eten?

Het laatste proces dat is onderzocht is het zogenaamde 'intestinal brake' mechanisme. De 'intestinal brake' wordt geactiveerd wanneer er onverteerde voedingsstoffen in het duodenum, jejunum of ileum terecht komen. Activatie van het 'intestinal brake' mechanisme zorgt voor verhoogde verzadiging en een geremde passage en vertering van voedsel. Dit komt onder andere omdat er verzadigingshormonen zoals CCK, GLP-1 en PYY worden uitgescheiden. Wij hebben onderzocht of activatie van de 'ileal brake' sterkere effecten heeft op verzadiging vergeleken met activatie van de 'duodenal brake' of de 'jejunal brake'. Dit is onderzocht in gezonde vrijwilligers door eiwit (caseïne) of een controle behandeling (water) in het duodenum, jejunum of ileum te spuiten (infuseren) met behulp van een slangetje (sonde). Uit deze studie blijkt dat eiwitinfusie in het ileum, grotere effecten heeft op verzadiging en afgifte van het hormoon GLP-1 vergeleken met eiwit infusie in het duodenum of jejunum (**hoofdstuk 5**).
In **hoofdstuk 6** is onderzocht of de 'ileal brake' door alle soorten macronutriënten (vet, eiwit en koolhydraat) kan worden geactiveerd. Deze vraag is onderzocht door de hoeveelheid voedselinname te meten na infusie van verschillende soorten macronutriënten in het ileum. Er zijn voor deze studie gelijke hoeveelheden (51.7 kcal) vet (saffloer olie), eiwit (caseïne) of koolhydraat (sucrose) in het ileum geïnfuseerd. De resultaten van deze studie laten zien dat ileale infusie van zowel vet, eiwit als koolhydraat

zorgen voor een verminderde voedselinname, die in alle drie de gevallen even groot is. Hieruit blijkt dat ileale infusie met alle soorten macronutriënten verzadiging activeert en voedselinname verlaagd in gezonde vrijwilligers.
Wanneer macronutriënten in het ileum worden geïnfuseerd worden er belangrijke verteringsstappen overgeslagen. Daarom hebben we onderzocht of het eiwit (caseïne) dat in het ileum wordt geïnfuseerd wordt verteerd en opgenomen (**hoofdstuk 7**). Uit dit onderzoek blijkt dat ileale infusie van onverteerd eiwit efficiënt wordt verteerd en opgenomen. Verder laten de resultaten van dit onderzoek zien dat ileale infusie van onverteerd eiwit niet leidt tot een immuun respons. Samen met de bevinding dat de vrijwilligers geen klachten hadden voor, tijdens of na de infusies suggereren deze resultaten dat infusie van onverteerd eiwit in het ileum niet leidt tot nadelige effecten.

Algemene discussie

Tot slot wordt een kort overzicht gegeven van de belangrijkste bevindingen van dit proefschrift (**hoofdstuk 8**). Ook worden de voor- en nadelen van de gebruikte onderzoeksmethoden besproken, en worden de gevonden resultaten vergeleken met eerdere bevindingen in de wetenschappelijke literatuur. Ook worden in dit hoofdstuk suggesties gedaan voor vervolgonderzoek evenals suggesties over hoe de belangrijkste bevindingen uit dit proefschrift te gebruiken om overmatig eten te voorkomen.

Het onderzoek in dit proefschrift heeft laten zien dat het introduceren van kleine hoeveelheden onverteerde macronutriënten in het ileum een succesvolle strategie kan zijn om mensen minder te laten eten, omdat:

1. Macronutriënten in het ileum efficiënt worden waargenomen: alle soorten macronutriënten die worden afgeleverd in het ileum stimuleren GLP-1 afgifte. Dit gebeurt onder andere via serotonine gemedieerde processen.
2. Eenmalige toediening van kleine hoeveelheden onverteerde macronutriënten in het ileum niet leidt tot nadelige effecten.
3. Aflevering van macronutriënten in het ileum sterkere effecten op verzadiging en voedselinname heeft in vergelijking met aflevering van macronutriënten in het duodenum of in het jejunum.
4. Aflevering van relatief kleine hoeveelheden macronutriënten (51.7 kcal) resulteren in een afname in voedselinname (80 -130 kcal).

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Op naar het volgende hoofdstuk!

– Einde! –

About the author

Curriculum Vitea

Dina Ripken was born July 15, 1990 in Davos Platz, Switzerland. At the age of 2 years she and her family moved back to the Netherlands. After completing secondary school at the 'Alkwin Kollege' in Uithoorn, she started the Bachelor's program 'Life science' at the Hogeschool Utrecht. For this Bachelor's program she specialized herself in 'biomolecular research' and followed a minor in 'business engineering'. She enrolled in two internships, at the Hubrecht Institute in Utrecht and at TNO in Zeist. During her last internship she was involved in the development of a method to quantify expression levels of transporter proteins *in vivo* and *in vitro* by using Ultra performance liquid chromatography mass spectrometry.

After graduating in July 2011 she was immediately appointed as a PhD candidate to the division of Human Nutrition of Wageningen University and TNO (The Netherlands Organization for Applied Scientific Research) on the project entitled 'Gut nutrient sensing in relation to appetite control' funded by the Top Institute of Food and Nutrition (TIFN). Dina performed the research presented in this thesis under the supervision of Prof. Renger Witkamp and Dr. Henk Hendriks.
For this project she was closely collaborating with researchers from several Universities and Institutes including the Maastricht University and DLO (Dienst landbouwkundig onderzoek). Dina presented her research at international conferences and was involved in teaching.

List op publications

D Ripken, Nvd Wielen, HM Wortelboer, J Meijerink, RF Witkamp, HFJ Hendriks, Stevia glycoside rebaudioside A induces GLP-1 and PYY release in a porcine *ex vivo* intestinal model. Journal of Agricultural and Food Chemistry (2014) volume 62, p 8365 – 8370

D Ripken, Nvd Wielen, Jvd Meulen, T Schuurman, RF Witkamp, HFJ Hendriks, SJ Koopmans Cholecystokinin regulates satiation independently of the abdominal vagal nerve in a pig model of total subdiaphragmatic vagotomy. Physiology and behavior (2015) volume 139, p 167-176

D Ripken, Nvd Wielen, HM Wortelboer, J Meijerink, R Witkamp, H Hendriks, Glucagon like peptide-1 release is modulated by serotonin. (*submitted*)

D Ripken, M van Avesaat, F J Troost, A A M Masclee, HFJ Hendriks, Intraileal casein infusion increases casein amino acids plasma concentrations in humans. (*submitted*)

M v Avesaat, FJ Troost, **D Ripken**, HFJ Hendriks, AAM Masclee, Ileal brake activation: macronutrient- specific effects on eating behaviour? International Journal of Obesity (2015) volume 39, p 235–243

M v Avesaat, **D Ripken,** HFJ Hendriks, AAM Masclee, FJ Troost, Small intestinal protein infusion: evidence for a location specific gradient in braking efficacy. (*submitted*)

M v Avesaat, FJ Troost, **D Ripken,** HFJ Hendriks, AAM Masclee, Intraduodenal infusion of a combination of tastants decreases food intake in humans. American Journal of Clinical Nutrition. 2015 Oct;102(4):729-35

I C Schrieks, **D Ripken**, A Stafleu, R F Witkamp, H F J Hendriks. Effects of mood inductions by meal ambiance and moderate alcohol consumption on endocannabinoids and n-acylethanolamines in humans: A randomized crossover trial. PLOS ONE 0(5): e0126421

E vd Steeg E, R Greupink, M Schreurs , I H Nooijen , K C Verhoeckx , R Hanemaaijer , **D Ripken** , M Monshouwer M L Vlaming , J d Groot , M Verwei, F G Russel, M T Huisman, H M Wortelboer Drug-drug interactions between rosuvastatin and oral antidiabetic drugs occurring at the level of OATP1B1. Drug Metabolism and Disposition (2013) volume 41, p 592-601

Other publications

D Ripken, HJF Hendriks, Porcine ex vivo intestinal segment model*:* The impact of food bioactives on health *in vitro* and *ex vivo* models p.255-262 (2015) ISBN: 978-3-319-15791-7

Abstracts and presentations

Ripken, D.; Wielen, N.; Meulen, J. van der; Schuurman, T.; Witkamp, R.F.; Hendriks, H.; Koopmans, S.J. Is neural regulation of satiation more important than hormonal regulation of satiation? European Congress on Obesity (ECO2013), 12 May 2013, Liverpool (UK). Abstract published in: Obesity Facts 6 (Suppl. 1). *Oral presentation*

Ripken D, Wielen, HM Wortelboer, J Meijerink, RF Witkamp, HFJ Hendriks; The role of the endocannabinoid system in glucagon-like peptide release. 23rd Annual symposium of the International Cannabinoid Research Society, 21-26 June 2013, Vancouver (CA). *Poster presentation*

Ripken D, Wielen, HM Wortelboer, J Meijerink, RF Witkamp, HFJ Hendriks; Stevia Glycoside Rebaudioside A Strongly Induces GLP-1 and PYY Release in Porcine *Ex vivo* Intestinal Model . 3rd International Conference on Food Digestion Infogest 11-13 March 2014, Wageningen (NL). *Poster presentation*

Schrieks IC, **Ripken D**, Stafleu A, Witkamp RF and Hendriks HFJ. Effect of moderate alcohol consumption and ambiance during a meal on mood and plasma endocannabinoids in humans. 23rd Annual symposium of the International Cannabinoid Research Society, 21-26 June 2013, Vancouver (CA). *Poster presentation*

Avesaat M van, Troost F, **Ripken D**, Hendriks HF, Masclee A, Ileal infusion of sucrose or casein decreases food intake. Gastroenterology, Vol.146, issue 5, S-898, 3-6 May 2014, Chicago (USA). *Poster presentation*

Avesaat M van, Troost F, **Ripken D**, Hendriks HF, Masclee A, Intraduodenal infusion of a combination of tastants decreases food intake. 16-19 May 2015, Washington (USA).Abstract published in: Gastroenterology, Vol.148, issue 4, S-25. *Oral presentation*

Overview of completed training activities

Description	Organizer, Location	Year
Discipline specific activities		
Courses and training		
5B course Safe handling of radioactive materials and sources	Van Hall Larenstein, Wageningen (NL)	2011
Course on Metabolomics	NuGO/VLAG, Kuoppio (FI)	2012
Alison Douglas Summer School "Food for Thought"	Full4Health, Bavaria (DE)	2013
GCP-BROK course	CTCM, Maastricht (NL)	2015
Conferences and meetings		
International Cannabinoid Research Society conference	ICRS, Freiburg (DE)	2012
Annual meetings NWO Deurne	NWO, Deurne (NL)	2012-2014
International Cannabinoid Research Society conference	ICRS, Vancouver (CA)	2013
European conference on Obesity	ECO, Liverpool (UK)	2013
Symposium on fibers	WUR, Wageningen (NL)	2013
Infogest	INRA, Wageningen (NL)	2013
Nutritional science days	NWO, Kappelerheeze (NL)	2015
General courses		
Basic statistics	PE&RC, Wageningen (NL)	2011
Patent workshop	TIFN, Wageningen (NL)	2011
VLAG PhD week	VLAG, Baarolo (NL)	2012
Scientific writing	VLAG, Wageningen (NL)	2013
Interdisciplinary research: crucial knowledge and skills	WGS, Wageningen (NL)	2013
Philosophy and ethics of food science and technology	VLAG/WGS, Wageningen (NL)	2014

Optional courses and activities		
Preparing research proposal	WUR/TNO, Wageningen/ Zeist (NL)	2011
TIFN expert meetings	TIFN, Wageningen (NL)	2011-2015
Research presentations TNO	TNO, Zeist (NL)	2011-2015
WE days TIFN	TIFN, Wageningen/Tegelen (NL)	2012-2014
Mid term review WM001 project	TIFN, Wageningen (NL)	2013
Member of organizing committee Infogest	INRA, Wageningen (NL)	2014

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