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Identification of nutrient-specific receptors in the intestine and their role in signaling

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Cette thèse est dédiée à ma famille, pour leur amour et leur soutien

SUMMARY

Overweight, obesity and the associated diseases like diabetes, cardiovascular diseases or even certain cancers are a growing problem in all developed and developing countries. Morbidity, mortality and socio-economic costs arising from this global healthcare problem are considerable. Obesity is the expression of the imbalance between energy intake and energy expenditure. One key question is: what causes the high energy intake in humans and how does this relate to the mechanisms involved in appetite regulation and satiety signaling. The gastrointestinal tract is central in the context of satiety control. It acts as a sensor for both the quantity and quality of the foods ingested, secreting hormones that can induce hunger (orexigenic signals) and transmitting anorexigenic signals to the brain and peripheral tissues to induce satiety and prepare the organs for the incoming nutrients and energy. These hormones, secreted from endocrine cells embedded into the gastrointestinal epithelium, are now recognized to play a fundamental role in these physiological processes. *In vitro* studies with enteroendocrine cell lines and in rodent models have identified numerous nutrient sensing pathways in the gastrointestinal (GI) tract leading to hormone secretion that can also affect satiety. Clinical studies in humans have shown that GI hormone infusion (PYY, GLP-1 analogs, CCK) decreases appetite and increases the satiety feeling in volunteers, thus contributing to reduce the overall meal size and food intake. The signaling pathways underlying the gut-brain communication remain however largely elusive, and this is particularly true for the intestinal sensors involved in the first steps of this feeding regulation.

The present thesis aimed at identifying and characterizing novel nutrient-sensing pathways leading to hormone secretion in the intestine. Therefore, established enteroendocrine models were used in combination with newly adapted *ex vivo* models to assess initial processes involved in chemo-sensation in the intestinal mucosa. We identified and characterized two novel nutrient-sensing pathways in human enteroendocrine cells, using a combination of physiological and molecular tools. These original data address a new sensing pathway selective for tetrapeptides found in a human enteroendocrine cell model secreting GLP-1, and a novel bitter receptor-mediated secretion of CCK in human enteroendocrine cells also demonstrated to operate in a *ex vivo* rat intestinal model. These findings may hopefully foster additional research in the field of nutrient-sensing, particularly concerning the role of taste receptors present in the intestine that lead to hormone secretion and affect satiety.

ZUSAMMENFASSUNG

Übergewicht, Adipositas und damit assoziierte Erkrankungen wie Diabetes, Herz-Kreislauf-Erkrankungen oder bestimmte Krebsarten sind ein wachsendes Problem nicht nur in Industrieländern sondern auch in Schwellenländern. Morbidität, Mortalität und soziökonomische Kosten, die sich aus diesem globalen Gesundheitsproblem ergeben, sind beträchtlich. Übergewicht ist der Ausdruck eines Ungleichgewichts zwischen Energieaufnahme und Energieverbrauch. Eine der Hauptfragen ist: Was verursacht diese hohe Energieaufnahme in Menschen und welche Rolle spielen dabei die Mechanismen der Regulation von Hunger und Sättigung? Der Gastrointestinaltrakt spielt eine zentrale Rolle bei der Sättigungskontrolle. Er agiert als Sensor sowohl für die Qualität als auch für die Quantität der aufgenommenen Nahrung, sezerniert Hormone, die Hunger auslösen können (orexigene Signale), und leitet anorexigene Signale weiter zum Gehirn und zum peripheren Gewebe, um eine Sättigung zu induzieren und die Organe auf die ankommenden Nährstoffe und Energie vorzubereiten. Diesen Hormonen, die von enteroendokrinen Zellen sezerniert werden, wird eine grundlegende Rolle in diesen physiologischen Prozessen anerkannt. *In vitro* Studien mit enteroendokrinen Zellen sowie Studien in Nagern haben zahlreiche Nährstoffwahrnehmungswege im Gastrointestinaltrakt identifiziert, die zur Ausschüttung von solchen Sättigungshormonen führen. Klinische Studien an Menschen haben gezeigt, dass Infusionen von bestimmten Darmhormonen (PYY, GLP-1 Analoga, CCK) den Appetit zügeln und das Sättigungsgefühl in den Probanden erhöhen und so zu einer Reduktion der Gesamt-Mahlzeitengröße und Nahrungsaufnahme beitragen. Die Signalwege der Kommunikation zwischen Darm und Gehirn sind dagegen eher schwer zu fassen, was besonders für die intestinalen Sensoren zutrifft, die an den ersten Schritten dieser Nahrungsregulation beteiligt sind.

Das Ziel der vorliegenden Arbeit war es, neue Nährstoffwahrnehmungswege zu identifizieren und zu charakterisieren, die zu Hormonsekretionen im Darm führen. Dafür wurden etablierte enteroendokrine Zellmodelle in Kombination mit neu adaptierten *ex vivo* Modellen verwendet, um die initialen Schritte in der chemosensorischen Wahrnehmung in der Darmmukosa zu untersuchen. Mit Hilfe von physiologischen und molekularen Techniken wurden zwei neuartige Nährstoffwahrnehmungswege identifiziert: Die gewonnenen Daten deuten zum einen darauf hin, dass es in einem humanen enteroendokrinen Zellmodell, das zur GLP-1 Sekretion befähigt ist, einen Wahrnehmungsmechanismus gibt, der selektiv Tetrapeptide erkennt. Zum anderen wurde

erstmals sowohl in humanen enteroendokrinen Zellen als auch in einem *ex vivo* Rattendarmmodell gezeigt, dass die CCK-Sekretion durch einen Bitterrezeptor vermittelt werden kann.

Diese Ergebnisse regen hoffentlich weitere Forschung auf dem Gebiet der Nährstoffwahrnehmung an, besonders im Bereich der Geschmacksrezeptoren im Darm und ihrer Mittlerrolle bei der Sekretion von Hormonen, die die Sättigung beeinflussen können.

INTRODUCTION

1. Obesity and overweight: facts and figures

Overweight and obesity are defined by the World health organization (WHO) as abnormal or excessive fat accumulation that may impair health, resulting mainly from an energy imbalance between calorie intake and energy expenditure. Measurement of the Body mass index (BMI), a simple but crude indicator, is commonly used to assess overweight or obesity in adult individuals. Overweight is defined by a BMI equal to or more than 25, and obesity is reached for a BMI equal to or more than 30. According to the WHO, 1.6 billion adults were overweight and 400 million were obese in 2005. Among the children, at least 20 million under the age of 5 years were overweight in 2005. The latest WHO projection for year 2015 is 2.3 billion adults overweight and more than 700 million obese. The obesity problem, once considered only in high-income countries is nowadays dramatically increasing in low- and middle-income countries worldwide, particularly in urban settings, and even reaches epidemic proportions in places like remote islands (Cook islands, Micronesia, Tonga, Samoa) due to a brutal shift from a traditional diet towards a high fat / high carbohydrate diet associated with a decreased physical activity. Common health consequences of overweight and obesity include cardiovascular diseases (ranking first as cause of death worldwide), diabetes, musculoskeletal disorders and evidence exists for some cancers (endometrial, breast and colon).

2. Gastrointestinal hormones and appetite regulation

The gastrointestinal tract is the largest endocrine organ in the body, with more than 20 identified regulatory peptides secreted from the endocrine cells lining the intestinal epithelium (**Figure 1.**). Some hormones like Cholecystokinin (CCK), Glucagon-like peptide-1 (GLP-1) or peptide YY (PYY) are acting as postprandial satiety signals and are thus able to inhibit food intake through

local circuits (paracrine, endocrine) and via gut-brain communication routes (neuro-endocrine). Interactions occur between these long-term and short-term satiety factors, as interactions exist as well between orexigenic and anorexigenic peptides, building a network particularly active between the GI tract and the brain to control energy homeostasis and body weight. In that respect, the vagus nerve - which expresses most of the receptors for these regulatory peptides - appears to be a major site of interactions. Vagotomy thus abolishes the bioactivity of CCK (152), peptide YY_{3-36} (1), GLP-1 (1, 113), and pancreatic polypeptide (PP) (7). A substantial body of work in both rodent and human models has demonstrated the respective contribution of these peptides in appetite regulation. Some of the important GI-derived peptides are here briefly described according to their secretion pattern along the gastrointestinal tract.

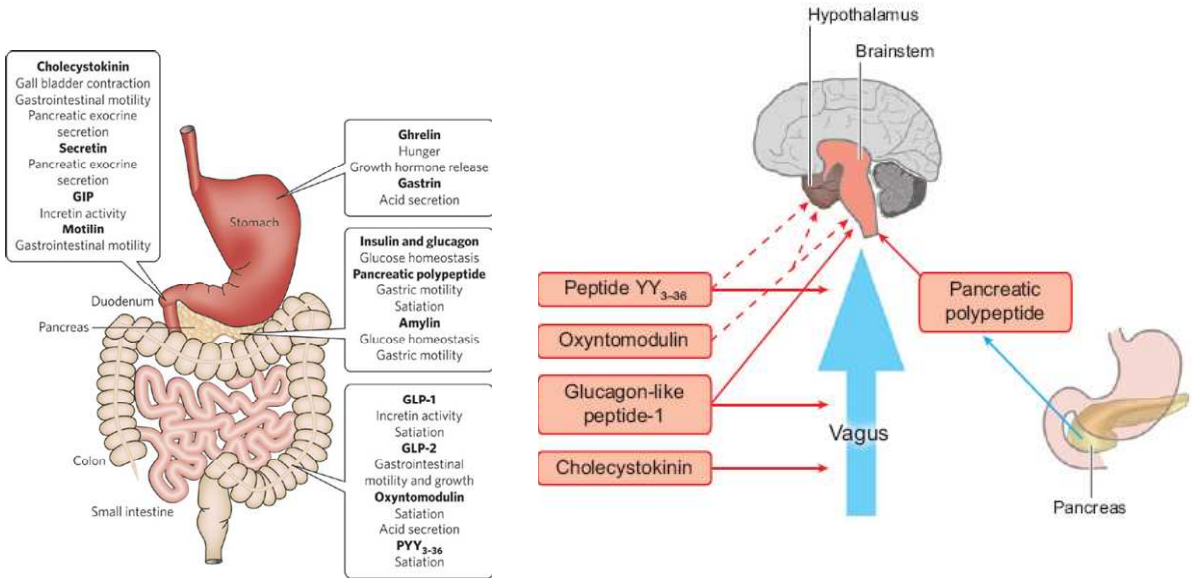

Figure 1. Important peptides secreted from the gastrointestinal tract and signaling to the brain to regulate appetite and satiety. Adapted from Chaudri, O.B., Field, B.C.T., Bloom, S.R.; Gastrointestinal satiety signals; *Int J Obes* 32; 2007

2.1. Stomach and proximal intestine

2.1.1. Ghrelin

Ghrelin is the only gut hormone known to elicit appetite (orexigenic effect). This peptide of 28 residues is produced mainly in the stomach from endocrine cells located in the gastric fundus (54). Ghrelin was first described for its binding properties towards the growth hormone secretagogue (GHS) receptor, a GPCR highly expressed in the hypothalamus, the pituitary gland and the brainsteam (10, 85). The orexigenic effect of Ghrelin was first shown in rats with stimulation of appetite and food intake after both intracerebroventricular (ICV) and intraperitoneal (IP) administration (205, 215). Chronic administration of ghrelin in rats thus leads to weight gain, partly caused by the induced hyperphagia (205, 215). Ghrelin stimulates as well appetite and food intake when administrated to both lean and obese humans (61, 214). Despite the potent orexigenic effects displayed by ghrelin, knockout mice for ghrelin do not differ from their wild-type counterparts in phenotype (196). Due to its properties, ghrelin has been proposed as a meal initiator. Thus, ghrelin plasma levels rise in the fasted state to reach a 2-fold peak just before the meal when compared to baseline levels (50). The postprandial decrease in ghrelin levels is proportional to the amount of calories ingested and circulating levels are back to the baseline within 1 hour after the meal (35). In obese subjects, fasting levels of ghrelin are lower compared to normal weight controls, and the postprandial decrease in circulating concentrations is decreased (123). An increase in circulating ghrelin levels has also been shown after diet-induced weight loss or gastric bypass surgery (51). The effects of ghrelin on food intake are thought to be mediated via the central nervous system (CNS), through the arcuate nucleus (ARC), a part of the hypothalamus now well established in the control of food intake, and through the brainstem via the vagus nerve (122, 212, 222).

2.1.2. Leptin

Leptin is one of the adipokines secreted by the adipose tissue and the fundic glands in the stomach (8). Circulating leptin levels play a pivotal role in the central regulation of food intake, energy expenditure and body weight (49) and are correlated with body fat mass (4, 70). In addition, leptin enhances the sensitivity to short-term satiety-related signals such as CCK (142). Leptin receptors (Ob-R) are co-expressed with CCK_1 receptors on vagal afferents in the gut wall, and expression of the Ob receptors is increased by fasting and decreased by refeeding within 2 hours (32, 33). All in all, this provides the possibility that leptin plays an additional role in peripheral regulation of food intake in the GI tract.

2.1.3. Glucose-dependent insulinotropic polypeptide (GIP)

GIP is secreted from the enteroendocrine K cells found at the highest density in duodenum and proximal jejunum. In response to nutrient ingestion, glucose and fat were shown to be the most potent effectors of GIP secretion. The amino acid sequence of GIP is highly conserved between species, with human, mouse and rat sharing 90% identity. In humans, the half-life of circulating GIP levels is about 7 minutes, due to its dipeptidyl-peptidase (DPP)-IV mediated inactivation (55). GIP binds to the GIP receptor (GIPR), expressed in numerous organs including pancreas, stomach, small intestine and several CNS regions. Like the GLP-1 receptor, the GIPR pertains to the heterotrimeric G-protein-coupled receptor superfamily (GPCR). Biological actions of GIP on pancreatic β-cells are similar to those of GLP-1, which is also responsible for the incretin effect along with GIP (see GLP-1). In addition, GIP exhibits additional functions in other organs (**Figure 2.**), like contributing to bone formation (23, 224), increasing the progenitor cell proliferation in the hypothalamus (161) and increasing the lipogenesis in adipose tissue (150). Knock-out mice for the

GIP receptor (GIPR -/-) are thus resistant to diet-induced obesity and exhibit reduced adipocyte mass. With a similar food intake than wild-type animals upon high fat feeding, GIPR -/- mice expend more energy and use fat as their prime energy substrate, consequently preventing an accumulation of fat in adipocytes (150).

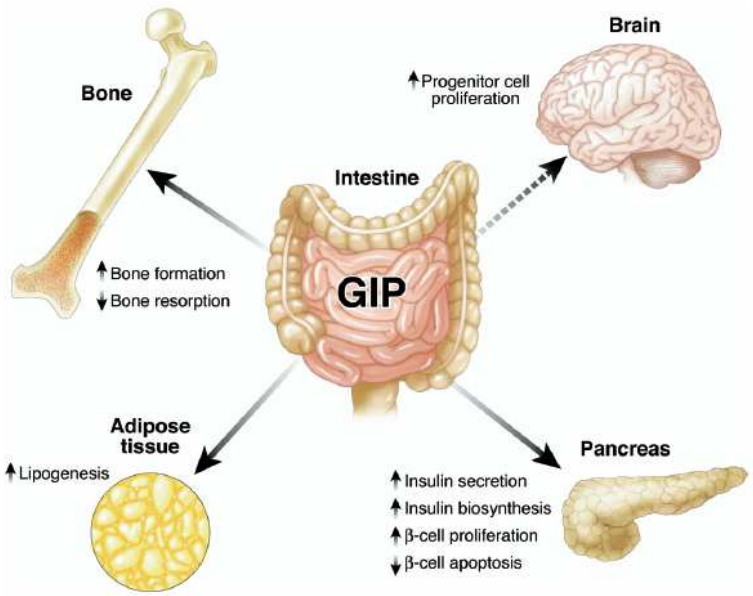

Figure 2. Physiological properties of the glucose-dependent insulinotropic polypeptide (GIP). Scheme from Baggio, L.L., Drucker, D.J.; Biology of incretins GIP and GLP-1; *Gastroenterology* 132; 2007

2.1.4. Cholecystokinin (CCK)

Cholecystokinin was the first GI hormone found to exert control on food intake both in rodents and humans (78, 112). CCK is synthesized from enteroendocrine I-cells concentrated in duodenum and proximal jejunum. CCK stimulates gallbladder contraction, pancreatic secretion and inhibits gastric emptying (31, 126) upon fat and protein stimulation. CCK is also synthesized within the CNS, mainly in the form of CCK-8. Circulating CCK levels rise within 15min after meal initiation, and have a half-life of 1-2min. CCK is therefore characterized as a signal for short-term control on

food intake (126). Several bioactive forms of Cholecystokinin exist (CCK-8, 22, 33, 58), the major circulating form in humans is CCK-33 (170). CCK binds to 2 receptor subtypes, CCK_1 and CCK_2. CCK_1R is expressed in pancreas, vagal afferent and efferent neurons, the nucleus tractus solitarius (NTS) and the area postrema (AP) (153), an area involved in the control of food intake and emesis. Vagotomy in rats has been shown to block the satiety effect induced by peripheral administration of CCK-8 (191), thus demonstrating the involvement of CCK_1 receptors expressed on the vagus nerve in mediating the CCK effect on food intake. The vagal afferent terminals found in the intestinal mucosa are in close vicinity with the basolateral membrane of enteroendocrine cells, leading to the hypothesis that CCK release could here activate vagal afferents through a paracrine mechanism. Peripheral administration of CCK in rodents and humans leads to a reduction in food intake via reduced size and duration of the meal (112), and administration of a CCK_1R antagonist (devazepide, loxiglumide) abolishes these effects. On the other hand, evidence emerges for an interaction between CCK and other anorexigenic peptides such as $PYY_{3-36,}$ at the level of vagal CCK_1 receptors. At pharmaceutical doses, CCK has been reported to induce taste aversion, nausea and anxiety in rodents (56, 155, 197). Central administration of CCK reduces as well food intake in rodents, an effect augmented by co-administration of leptin (142). The short half-life of CCK, added to the fact that a continuous administration of the peptide leads to tolerance in rats after 24h (48), constitute two major hurdles when considering CCK as a potential anti-obesity agent. Thus, CCK administered in rats more than 15 minutes before the initiation of a meal did not have any effect on the meal size (78).

2.2. Distal intestine

2.2.1. Glucagon-like peptide-1 (GLP-1)

GLP-1 production results from the tissue-specific differential cleavage of the proglucagon gene, and GLP-1 is released from the enteroendocrine L-cells located mainly in distal ileum and colon (66) but also from the alpha cells of the islets of Langerhans (66), and from neurons within the NTS of the brainstem (104). The proglucagon protein is processed by the prohormone convertase which leads to the production of glucagon, GLP-1 biologically active forms (7-37) and (7-36), GLP-2, or oxyntomodulin depending on the site of synthesis (19). GLP-1 is fully conserved between all mammalian species, thus indicating its physiological importance both in postprandial glucose homeostasis and appetite control (**Figure 3.**).

The incretin effect attributed to the peptide hormones GIP and GLP-1 explains that oral ingestion of glucose elicits a more pronounced insulin release than a similar glucose plasma level adjusted by intravenously administered glucose. The combined action of GIP and GLP-1 is thus believed to be responsible for up to 70% of the postprandial insulin secretory peak, and modulates directly the pancreatic β-cell physiology by promoting cell growth and preventing apoptosis (30, 64). GIP and GLP-1 are also responsible for reducing glucagon secretion and for slowing down gastric emptying.

Glucagon-like peptide-1 binds to its GLP-1 receptor (GLP1-R) expressed in brain regions involved in food intake regulation including the ARC regions within the hypothalamus and the NTS of the brainstem (107, 188, 206). Postprandial release of GLP-1 within 10-15 min of food ingestion is proportional to the amount of energy intake, and fatty acids and proteins are the most potent effectors for GLP-1 secretion *in vivo* (67, 88, 120). Maximum GLP-1 levels are usually reached after 40 min (96). The early rise in GLP-1 plasma levels after food ingestion has raised the

question whether a direct stimulation of the L-cells by nutrients was the stimulus, when considering their main location in the distal part of the gut that may not even be reached by nutrients. Yet, the L-cell population density in the proximal intestine has recently been recognized as sufficient to elicit this early phase in GLP-1 secretion (202). However, a proximal-distal loop involving a GIP-induced GLP-1 secretion, as well as the involvement of the vagus nerve are both considered to additionally contribute to this phenomenon. GLP-1 thus displays a biphasic secretion profile, the second peak corresponding possibly to the direct stimulation of L-cells by nutrients in distal intestine. GLP-1 injected peripherally has been shown to reduce food intake in rodents (1, 119) and to increase postprandial satiety and delay gastric emptying both in lean and obese humans (87, 158, 208). Furthermore, obese subjects are characterized by reduced basal circulating levels of GLP-1 as well as by an attenuated postprandial release when compared to lean individuals, which increases with weight loss (209). The peptide hormone is rapidly cleaved into inactive forms GLP-1 (9-37) and (9-36) within minutes by the enzyme DPP-IV in plasma. This prompted the development of GLP-1 analogs with a significantly greater plasma half-life such as exendin-4 (half-life around 30 min) (81), as well as specific DPP-IV inhibitors which are meanwhile used clinically in the treatment of type 2 diabetes.

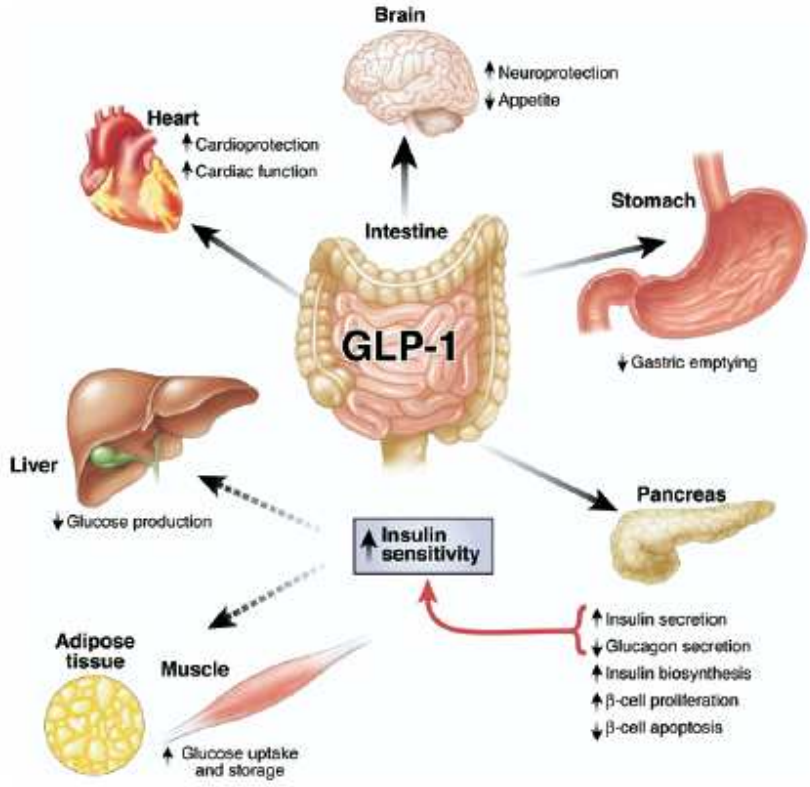

Figure 3. Physiological properties of the glucagon-like peptide-1 (GLP-1). Scheme from Baggio, L.L., Drucker, D.J.; Biology of incretins GIP and GLP-1; *Gastroenterology* 132; 2007

2.2.2. Oxyntomodulin (OXM)

Oxyntomodulin is another product of the proglucagon gene secreted along with GLP-1 and PYY_{3-36} from enteroendocrine L-cells following ingestion of a meal. Release of OXM is proportional to the ingested calories (77, 121). Administration of OXM reduces energy intake in both rodents and humans, and has been shown to reduce body weight in overweight and obese volunteers when infused sub-cutaneously and pre-prandialy during 4 weeks (53, 219). In addition, OXM administered during 4 days in human volunteers increased significantly their total energy expenditure (218). No specific receptor for oxyntomodulin has yet been identified, and OXM is thought to act via the GLP1R (9, 53). Like GLP-1, oxyntomodulin displays an incretin effect

following glucose administration (11), and is rapidly inactivated by DPP-IV in plasma. Due to its properties, oxyntomodulin is like GLP-1 considered as a potential anti-obesity agent, and efforts are made to synthesize peptide analogs resistant to DPP-IV.

2.2.3. Peptide YY (PYY)

Peptide YY_{3-36} is the main circulating form of PYY, co-secreted as PYY_{1-36} with GLP-1 and oxyntomodulin from enteroendocrine L-cells and cleaved in PYY (3-36) by DPP-IV (146). Circulating levels of PYY are low during the fasting period, peak after 2 hours following a meal and remain elevated for up to 6 hours (3). Postprandial release of PYY is proportional to the amount of energy intake, with lipids and carbohydrates being the primary nutrients eliciting its secretion. Peptide YY_{3-36} binds selectively to the Y_2 GPCR expressed on NPY neurons of the ARC (27, 111). Peripheral administration of PYY_{3-36} inhibits food intake and reduces body weight gain in several species including rodents, monkeys and humans (13, 14, 114, 133, 167, 189). Nausea has been reported as side-effect in clinical trials under infusion of pharmaceutical doses of PYY_{3-36}. A proposed mechanism for the anorectic action of PYY is the inhibition of ARC-NPY neurons with a concomitant reduction of inhibition of ARC-POMC neurons (14). There is growing evidence as well for a synergistic interaction between PYY_{3-36} and GLP-1 to inhibit food intake (198) also known as the ileal break, (130, 166) where fat sensing in the distal gut is supposed to lead to a PYY release that in turn inhibits the transit through the proximal small intestine (130, 193).

3. Receptors and transporters involved in nutrient sensing

Despite a discontinuous pattern in food intake, and thus in daily calorie intake, animals are able to maintain a very stable body weight over long periods of time. The organism achieves this through a precise match between energy intake and expenditure. This regulation of energy homeostasis on the long-term is highly dependent on the hormonal signals that reflect the status of endogenous energy stores (insulin, leptin) and the meal-related signals (Ghrelin, CCK, GLP-1, PYY). These signals are sent via endocrine and neural routes to the CNS where they are integrated and lead to the appropriate behavioral answer. Luminal sensing of the nutrients by specialized endocrine cells in the intestine represents the first event in this signaling cascade. Enteroendocrine cells possess finger-like villi equipped with chemoreceptors that project into the lumen and enable them to sense the luminal contents. This sensing process is dependent on the nutrient composition and the caloric value of the meal, involving nutrient transporters as well as sensors responding both to mechanical (gastro- intestinal volume) and chemical stimulation, like G-protein-coupled receptors (GPCRs) (**Figure 4.**). A major hurdle in this area of research is that enteroendocrine cells are difficult to study directly, and model cell lines like GLUTag, STC-1 and NCI-H716 all exhibit different nutrient sensitivities and are not fully validated as proper models for the native L-cell. Due to recent work from Reimann *et al* (172), enteroendocrine cells can now be genetically tagged, purified by FACS, cultured and finally characterized for the expression of their chemosensors. They developed therefore transgenic mice where specific enteroendocrine cell types are tagged genetically through the expression of a GFP-like fluorescent protein, under the control of a promoter for the peptide-hormone precursor proglucagon. This model represents a real breakthrough in the field of nutrient sensing, and provides exciting opportunities to explore further

the role that chemosensors play in the enteroendocrine cells. A brief review of the litterature on intestinal sensing is here compiled for each class of nutrient.

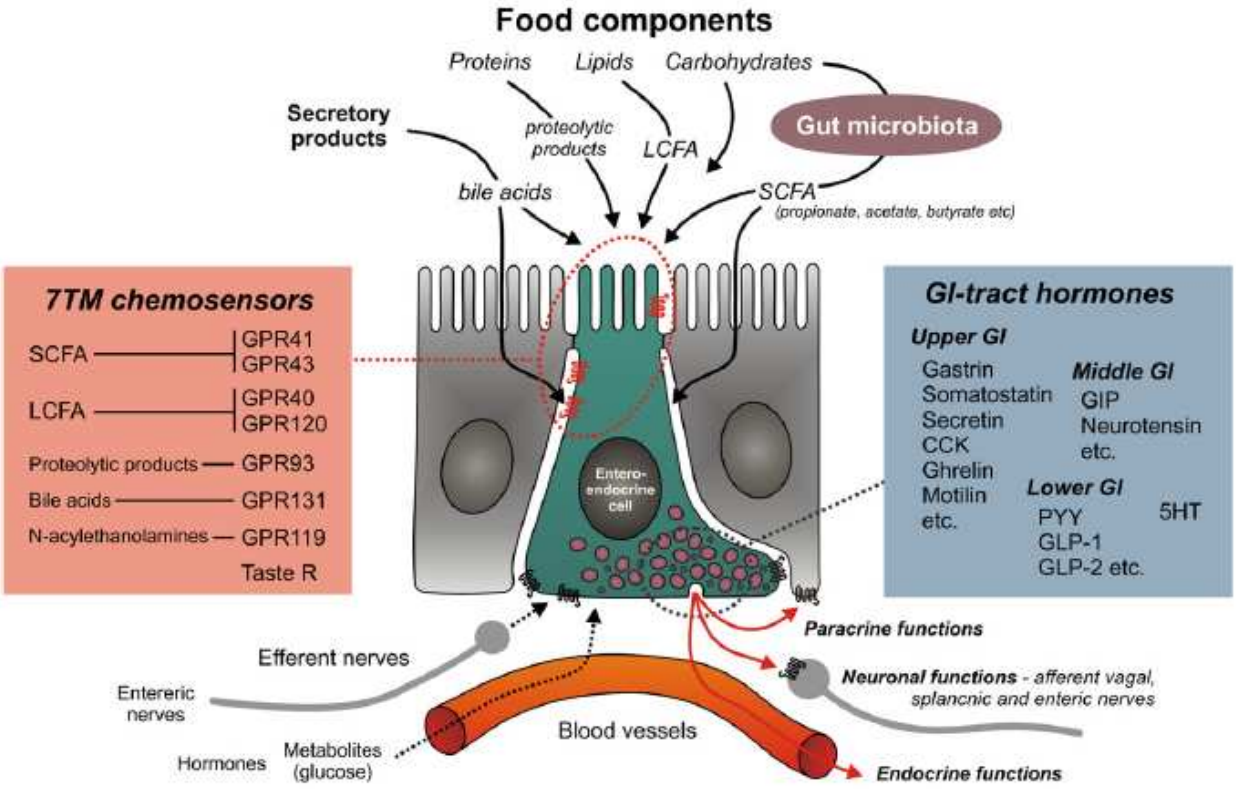

Figure 4. G-protein-coupled receptors involved in nutrient sensing from the enteroendocrine cell. Scheme from Engelstoft, M.S., Egerold, K.L., Holst, B., Schwartz, T.W.; A gut feeling for obesity: 7TM sensors on enteroendocrine cells; *Cell Metabolism*; 2008

3.1. Monosaccharide sensing

Glucose sensing in the gut regulates numerous physiological functions including glucose absorption, gastric emptying, intestinal motility and hormone secretion that affect appetite. As the major source of energy for the body is glucose, its sensing and metabolism are finely tuned, and glucose beneficiates thus of a broad and interconnected sensing system that includes ion channels,

transporters and receptors. The main structures involved in intestinal glucose sensing are here briefly described.

3.1.1. K_{ATP} channels

Perhaps the best characterized glucose sensors *in vitro*, K_{ATP} channels are found in various glucose-responsive tissues including the enteroendocrine cells, the brain and the pancreatic *β*-cells. In islet cells, glucose entry and metabolism elicit both an increase in ATP and a decrease in MgADP which induce the K_{ATP} channels closure. Consecutive decrease of the K^+ flux allows small inward currents to drive membrane depolarization and to generate action potentials, which in turn cause the entry of calcium via voltage-gated Ca^{2+} channels and stimulation of insulin secretion (179). GLUTag cells, as a model for the L-cell, are similarly electrically active as assessed by electrophysiology, and exhibit an enhanced rate of action potential firing and calcium entry upon glucose sensing that leads to GLP-1 secretion. The K_{ATP} channel inhibitor tolbutamide displays the same effect on GLUTag cells (171). Primary mouse colonic L-cells are as well comparably electrically excitable and secrete GLP-1 in response to glucose and tolbutamide partly via an increase in action potential frequency mediated via K_{ATP} channels closure (172). However, despite this functional demonstration *in vitro*, the involvement of the K_{ATP} channels in GLP-1 secretion *in vivo* is still unclear, mainly due to the fact that sulphonylureas which are used in the treatment of type 2 diabetes to stimulate insulin secretion via the inhibition of the K_{ATP} channels closure are not able to elicit a GLP-1 secretion.

3.1.2. Sodium-glucose cotransporter 1 (SGLT1) and facilitative glucose transporter 2 (GLUT2)

SGLT-1 is the main transporter responsible for D-glucose and D-galactose absorption in the small intestine. The protein uses an inwardly directed sodium gradient to drive glucose uptake into the cells - even at low luminal concentrations (12, 58). It is known that metabolizable and non-metabolizable sugars are able to elicit a GLP-1 secretion, provided that they are substrates of the intestinal glucose uptake pathway and applied in presence of sodium (92, 175). Evidence exists also in rats for a role of SGLTs in intestinal glucose sensing and hormone secretion (71). In the rodent GLUTag cell line, such a glucose-sensing pathway where sugars elicit a membrane depolarization and GLP-1 secretion was attributed to the electrogenic action of SGLT1 (82). Data obtained in transgenic mice with fluorescently labeled GIP and GLP-1-secreting cells by Reimann *et al* (164, 172) strongly suggest that SGLT-1 is of prime importance in the glucose-induced secretion of both incretins. SGLT-1 was thus found highly and homogeneously expressed in L-cells of the small intestine and colon. The low millimolar EC_{50} (0.7-4mM) associated with glucose-induced GLP-1 secretion in primary L-cells is close to the 0.3mM Km value reported for SGLT1 (58). Furthermore, GLP-1 secretion from the L-cell induced by the non-metabolizable SGLT-1 substrate α-methyl-D-glucopyranoside (MDG) is not significantly different than that of glucose at the same concentration. Taken together, these data suggest that the SGLT-1-mediated glucose sensing originates in the L-cells. Further evidence for SGLT-1 as an intestinal glucose sensor is provided *in vivo* by the work of Moriya *et al* (154), where co-administration of the SGLT-1 inhibitor phloridzin with glucose in mouse upper intestine inhibits both glucose transport and glucose-induced incretin secretion. Using the non-metabolizable SGLT-1 substrate MDG led to the same results, implying that concomitant transport of substrate and sodium via SGLT-1 elicits incretin secretion, without the need for subsequent glucose metabolism in the cell. All evidence

points currently towards SGLT-1 as the sensor responsible for glucose-induced incretin secretion in enteroendocrine cells. By contrast, whether the GLUT family members expressed in L-cells play the same role as SGLT-1 remains uncertain from the data available, although mice deficient in GLUT2 exhibit reduced intestinal GLP-1 content and plasma concentrations after an oral glucose tolerance test (36).

3.1.3. Sweet receptors (T1R2/T1R3)

The heterodimeric sweet taste receptor T1R2/T1R3 expressed in the lingual epithelium acts through a signaling pathway involving α-gustducin, phospholipase-Cβ2 and the TRPM5 channel (**Figure 5.**) to stimulate peripheral gustatory nerves and in turn brain gustatory pathways (125, 131, 139, 145, 151, 168). Integration of the sweet taste signaling in the CNS activates brain reward systems that promote appetite (20). Sweet taste receptors are as well expressed in the rodent and human intestine (62, 181, 221), where their role *in vivo* remains to be defined. Sweet taste receptors have thus been proposed as intestinal glucose sensors, firstly due to their colocalization with GLP-1 and PYY in subsets of enteroendocrine cells (181). In addition, work from Jang *et al* (102) showed that mice lacking the subunit T1R3 or α-gustducin exhibited impaired GLP-1, GIP and insulin secretion upon an oral glucose tolerance test. Stimulation of the human enteroendocrine cell line NCI-H716 with glucose or the artificial sweetener sucralose elicited GLP-1 secretion, an effect abolished by the sweet receptor antagonist lactisole or by siRNA for α-gustducin. In the same work, Margolskee *et al* (140) report that mice lacking the sweet taste machinery do not show the characteristic increase in intestinal SGLT1 expression normally induced by a high carbohydrate diet. However, the artificial sweeteners sucralose and acesulfame-K are not able to elicit a calcium answer nor GIP or GLP-1 secretion from mouse primary

enteroendocrine cells (164, 172). In addition, *in vivo* studies in humans recently failed to show an artificial sweetener-induced incretin secretion (73, 134).

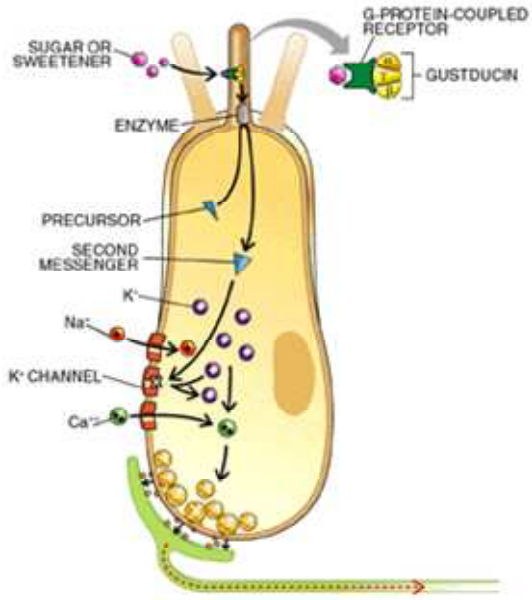

Figure 5. Signaling pathway of the heterodimeric sweet taste receptor leading to increased intracellular calcium concentration. Scheme from Smith, D.V., Margolskee, R.F.; Making sense of taste; *Scientific American*; 2001

3.2. Sensing of products of luminal proteolysis

Although protein degradation products are potent effectors of satiety hormone secretion in the gut, the sensors responsible are still largely unknown. A body of work exists on *in vitro*, *ex vivo* and *in vivo* studies demonstrating the effects of protein hydrolysates on hormone secretion and the concomitant delay of gastric emptying. The di- and tri-peptide transporter PEPT-1 expressed in intestinal epithelial cells is suspected to play a role in this sensing process, eventually through cell-to-cell communication routes with the surrounding endocrine cells. Additional potential sensing mechanisms are here briefly reviewed.

3.2.1. Umami taste receptors (T1R1/T1R3)

The heterodimeric taste receptor T1R1/T1R3, also known as umami receptor, is expressed in taste buds of the tongue and soft palate where it senses glutamate and L-amino acids (98, 125, 159). The receptor responds broadly to aliphatic amino acids but not to aromatic amino acids, and this sensing process is dramatically increased in presence of 5'-inosine-monophosphate (IMP) (159). The umami receptor is also expressed along its sweet and bitter counterparts in the GI tract (21, 62), where it regulates along with sweet taste receptors, calcium and PKC βII the trafficking of the peptide transporter PEPT1 and the facilitative glucose transporter GLUT2 in enterocytes (135). No direct relationship between umami sensing and GI hormone secretion has yet been demonstrated, but monosodium L-glutamate (MSG), a sodium salt of the amino acid L-glutamate, induces physiological changes in the gut. *In vivo* in dogs, intragastric but not intraduodenal infusion of MSG stimulates the upper gut postprandial motility and accelerates the gastric emptying, both effects are abolished by vagotomy (204). The sensory structures for MSG are here localized to the gastric corpus, as it is the case in rats (157), and the umami-induced physiological changes are mediated via the vagus nerve. *In vivo* in rats fed diets of various caloric density for 15 weeks, spontaneous ingestion of a 1% MSG solution is highly preferred to plain water, and leads to a significantly smaller weight gain, reduced abdominal fat mass and lower plasma leptin levels compared to rats consuming water alone. The changes associated to MSG consumption are likely associated to increased energy expenditure, as the animals did not show reduced energy intake or delayed development (115). In humans, MSG was shown to specifically promote gastric emptying after a liquid protein-rich meal, but not after a liquid carbohydrate-rich meal or a non-caloric water meal (223), suggesting that free glutamate in the intestine improves protein digestion.

3.2.2. Calcium-sensing receptor (CaR)

The main physiological ligand for CaR is the extracellular calcium, sensed in organs responsible to maintain calcium homeostasis in the organism such as the kidney and the parathyroid gland (29). However, CaR is also expressed in organs not involved in the calcium homeostasis regulation, including nutrient-sensing organs like the stomach, the pancreas, the liver and the GI tract (75). The putative role of CaR in protein and amino acid sensing is discussed in the section describing the identification of a novel sensing pathway for selected tetrapeptides that lead to a GLP-1 release from the human enteroendocrine cell line NCI-H716.

3.2.3. GPR93

The hypothetical role of GPR93 in sensing of proteins/peptides is also described in the results and discussion section “Identification of a novel sensing pathway in vitro”.

3.3. Dietary fat sensing

Free fatty acids play as well a role as signals. Early work in the 1960’s already demonstrated that the stimulus from dietary fat that inhibits both gastric motor and secretory functions were not triglycerides but free fatty acids (FFAs), particularly those of chain length equal to or greater than C12 (100). This CCK-mediated inhibition of gastric functions was found to be reduced by 60 to 80% following functional ablation of the vagal afferent pathway with capsaicin (97). Lipid sensing in the gut has since then been found to regulate the sensitivity to insulin (210) in addition to promote satiety and enhance insulin release through the secretion of PYY and GLP-1 (86). Long-chain fatty acids (LCFAs) are major products of dietary fat digestion, generated from the cleavage of triglycerides by gastrointestinal enzymes and providing an important energy source to the

organism. Wang *et al* found that the sensing of one LCFA metabolite (LCFA-CoA) in rat duodenum led to increased insulin sensitivity of the liver, as assessed by the pancreatic clamping method. *De novo* glucose production from the liver was reduced while glucose uptake into tissues was not affected. The sensor involved in this intestine-brain-liver neural circuit that decreases both production of glucose by the liver and food intake remains yet elusive. This rapid regulatory pathway signals to the brain via the vagus nerve, eventually through a paracrine mechanism involving hormones and vagal afferents in the proximal intestinal mucosa. However, established sensing mechanisms for free fatty acids occur mainly in the distal part of the gut through GPCR signaling, leading to satiety hormone secretion from the enteroendocrine cells in ileum and colon that promote meal termination.

3.3.1. GPR119

GPR119, a GPCR responsive to the natural ligand oleoylethanolamide (OEA) and some long-chain fatty acids was deorphanized in 2005 (192), and its expression in human pancreas and intestine demonstrated shortly thereafter (162). Stimulation of pancreatic β-cells with GPR119 agonists enhances the glucose-induced insulin secretion through the activation of adenyl cyclase and an increase in intracellular cAMP levels (45). In addition, this effect is greater when the agonists are delivered orally as compared to intravenously, suggesting the involvement of GIP and GLP-1. Accordingly, the same authors later detected the expression of GPR119 mRNA in mouse intestine as well as in the L-cell models GLUTag and STC-1, where it was colocalized with proglucagon mRNA (44). GPR119 agonists elicit GLP-1 secretion *in vitro* from GLUTag cells through an increase in cAMP and elicit both GIP and GLP-1 secretion *in vivo* in wild type mice, but not in GPR119 deficient animals (44). From work in primary mouse cell culture, GPR119 is expressed in

GIP-producing K-cells at similar levels than in colonic L-cells secreting GLP-1 (164). Due to their properties to directly stimulate insulin secretion as well as enhancing its glucose-induced production via incretin release, GPR119 agonists are currently used in clinical trials with type 2 diabetes patients.

3.3.2. GPR120

This previously orphan GPCR has been recently characterized as a receptor specific for unsaturated long-chain FFAs (94). GPR120 mRNA and protein are found both in the rodent and human intestine where they are expressed at high density in ileum, colon, caecum and rectum (149). Stimulation of GPR120 elicits the secretion of GLP-1 both *in vitro* in STC-1 cells and *in vivo* in mouse. Agonists like α-linolenic acid (ALA), which are able to induce a dose-dependent transient increase in $[Ca^{2+}]_i$ and phosphorylated extracellular signal-regulated kinase (ERK) in GPR120-expressing HEK293 cells, elicit as well a GLP-1 secretion from STC-1 cells. Use of siRNA to silence GPR120 expression in STC-1 cells inhibits both, the calcium response and GLP-1 secretion upon ALA stimulation. In addition, GPR120 and GLP1 are colocalized in human colonic neuroendocrine cells. In C57/B6 mice, plasma concentrations of GLP-1 and insulin measured in the portal vein after an acute oral administration of ALA are both significantly increased (94).

3.3.3. GPR40

Also known as free fatty acid receptor 1 (FFA1), GPR40 is activated by medium- to long-chain fatty acids and is expressed in pancreatic-β cells, intestine, brain and monocytes. GPR40 was deorphanized in 2003 by three independent teams (26, 101, 116) using human GPR40-expressing

HEK293 cells and $[Ca^{2+}]_i$ measurements in an agonist screening. GPR40 contributes significantly to the fatty acid-enhanced glucose-induced insulin secretion *in vivo*. However, whether the lack of GPR40 protects against high-fat-diet-induced metabolic diseases like type 2 diabetes remains controversial from studies in knock-out mice (110, 117, 118, 199). GPR40 has recently been found to be co-expressed with GLP-1, PYY and GIP in enteroendocrine cells of the mouse intestine. This receptor is proposed as an intestinal sensor for FFAs leading to incretin secretion, as mice lacking GPR40 display impaired incretin secretion upon an acute oral high-fat load when compared to wild-type animals (63).

3.3.4. GPR41 and GPR43

GPR41 and GPR43 are also known as FFA3 and FFA2 and were identified in function in 2003 (28) using similar *in vitro* methods as applied for GPR40. Both receptors are activated by the short-chain fatty acids (SCFAs) acetate, propionate and butyrate which are produced by anaerobic fermentation of dietary carbohydrate fibers in colon, where the gut flora reaches its highest density. In human colon, SCFAs are found in concentrations ranging from 60 to 130mM, acetate being the most prevalent whereas propionate and butyrate are found in similar amounts following roughly the ratio 60:20:20 respectively (52). The major physiological roles proposed for SCFAs in the GI tract are 1) Enhancement of the colonic motility via the release of 5-HT (74, 148) and 2) Inhibition of the upper gastrointestinal mobility along with FFAs – also known as ileal break - via PYY secretion from the enteroendocrine cells of ileum and colon (127-130). GPR43 is expressed in rat and human distal ileum and colon, both in enteroendocrine cells expressing PYY and mast cells of the lamina propria expressing 5-HT (108). GPR41 expression is found in enteroendocrine

cells expressing PYY in human colon (201). Their localization make GPR41 and GPR43 two candidate sensors for the effects attributed to SCFAs in the gut.

Another role for SCFAs sensors in the colon is the luminal monitoring of the gut microbiota. GPR43 is thus proposed to be involved in the regulation of inflammatory responses in the gut, as shown in GPR43$^{-/-}$ mice which show exacerbated inflammation in models of colitis (141). In the other hand, mice lacking GPR41 exhibit reduced circulating levels of PYY, increased intestinal transit rate and have a reduced harvest of energy (SCFAs) from the diet (185).

3.4. Bitter receptors (T2Rs)

The first role of the bitter receptors expressed in the intestine both in mammals and humans is to prevent the absorption of toxic compounds, mainly via the initiation of emesis. They thus constitute a second defense mechanism after the oral cavity, where they are as well expressed on the neuroepithelium of the tongue. The possible role of these bitter receptors in nutrient sensing and subsequent satiety hormone secretion is currently explored (**Figure 6.**) In the results and discussion section of the present project "Bitter taste sensing and satiety hormone secretion in the gut", new data reinforcing the evidence that bitter taste sensing mechanisms are coupled to hormone release from enteroendocrine cells in the intestine are presented.

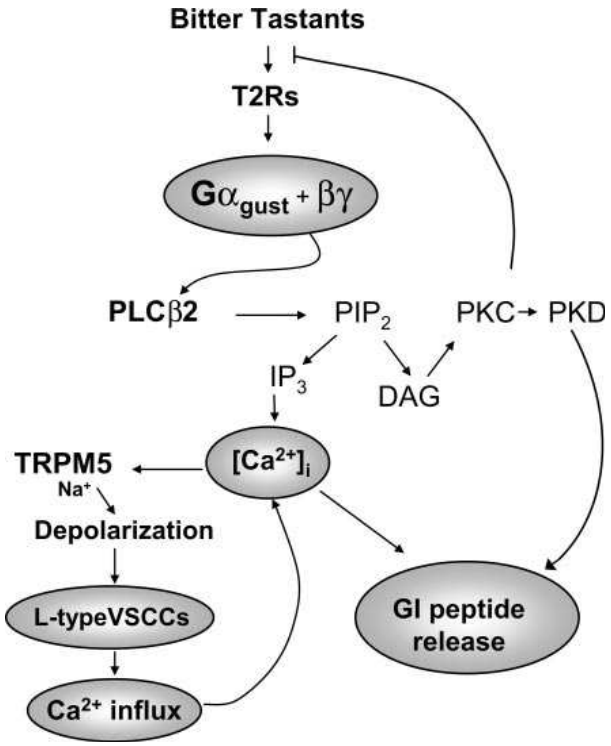

Figure 6. Putative pathways elicited by bitter tastants in enteroendocrine cells. Scheme from Chen, M.C., Wu, S.V., Reeve, J.R., Rozengurt, E.; Bitter stimuli induce Ca^{2+} signaling and CCK release in enteroendocrine STC-1 cells: role of L-type voltage-sensitive Ca^{2+} channels. *Am J Physiol Cell Physiol*; 2006.

MATERIALS AND METHODS

1. *In vitro* models

1.1. Cellular models and cell culture conditions

1.1.1. NCI-H716. Human enteroendocrine NCI-H716 cells (ATCC, CCL-251) were obtained from the American Type Culture Collection (WZ). The cells were grown in suspension in RPMI 1640 medium (Invitrogen) supplemented with 10% FBS, 2mM L-Glutamine and antibiotics (100IU/ml penicillin and 100mg/ml streptomycin). For calcium measurements, membrane potential measurements and GLP-1 secretion experiments, the cells were seeded on plates coated with Matrigel (Becton Dickinson, Bedford, MA, USA) following suppliers guidelines.

1.1.2. HuTu-80. Human enteroendocrine HuTu-80 cells (ATCC, HTB40) were obtained from the American Type Culture Collection (WZ). The cells were grown in minimum essential Eagle's medium (MEM) containing 10% FBS, antibiotics ($100U \cdot ml^{-1}$ penicillin, $100\mu g \cdot ml^{-1}$ streptomycin) and $100\mu g \cdot ml^{-1}$ L-glutamine in plastic flasks or Poly-Lysin-coated 24 wells plates. Cells were used until passage 32.

1.1.3. Human TAS2R-expressing HEK cells. HEK293 (human embryonic kidney) cells were obtained from Invitrogen (Breda, The Netherlands). For functional expression of the human bitter taste receptors, HEK cells stably expressing the chimeric G-protein α-subunit Gα16-gust44 (cloned into pcDNA4 (Invitrogen, San Diego, CA)) and one of each 25 bitter receptor genes (cloned into pcDNA5/FRT (Invitrogen, San Diego, CA)), were used. To improve receptor membrane targeting, each bitter receptor gene contained the first 45 amino acids of rat somatostatin receptor type 3 at its amino terminus. Cells were maintained in DMEM with $4.5g \cdot l^{-1}$

glucose and L-glutamine with 10% tetracycline-free FBS (Cambrex) supplemented with blasticidin (5 μg/ml), geneticin (400 μg/ml) and hygromycin (100 μg/ml) in plastic flasks or Poly-Lysin-coated 96 wells plates. Cells were used until passage 25.

1.1.4. CHO-CCK$_1$-R. A Chinese hamster ovary cell line (CHO) functionally expressing rat CCK_1R (CHO-CCK_1-R) was kindly provided by Dr. R. Smeets (University of Nijmegen, The Netherlands). CHO-CCK_1R cells were grown in DMEM and Ham's F12 medium 1:1 (DMEM-F12) with 15mM HEPES and L-glutamine supplemented with 10% FBS (Gibco, Paisley, UK), 500μg $\cdot ml^{-1}$ penicillin, 500U$\cdot ml^{-1}$ streptomycin (Cambrex, Baltimore, USA), and 500μg $\cdot ml^{-1}$ G-418 (Gibco, Paisley, UK). As negative control cells, native CHO-K1 cells (ATCC, CCL61) were used and grown in the same medium without G-418. Cells were used until passage 25. All cells were grown and maintained at 37°C / 5%CO_2.

1.2. Intracellular free calcium measurement

1.2.1. NCI-H716 cells. Changes in intracellular calcium in NCI-H716 cells were assessed by using a Varioskan plate reader (Thermo scientific). Briefly, NCI-H716 cells were seeded at 2.10^5cells/ml (0.5ml/well) in a 24-wells plate coated with Matrigel 48h previous to the experiment. Ratiometric measurement of $[Ca^{2+}]_i$ was achieved after loading the cells for 40min with the membrane-permeant fluorescent indicator FURA-2AM (Molecular probes). The dye-loading medium had the following composition: 0.25% Pluronic F-127 (Molecular Probes) and 10μM FURA-2AM in Krebs buffer pH 7.4 (All mM: 137 NaCl, 5.4 KCl, 1.2 $CaCl_2.2H_2O$; 1 $MgSO_4.7H_2O$; 0.3 $NaH_2PO_4.H_2O$; 0.3 KH_2PO_4; 10 HEPES). Effectors for testing were prepared in parallel in Krebs buffer and automatically added onto the cells during the assay. Measurements were typically

carried out for 25min with a measurement every minute. To obtain a baseline, fluorescence signals (excitation 340/380nm-emission 510nm) were measured for 5min in Krebs buffer prior to a washing step and addition of compounds to the cells. Intracellular free calcium concentration (nM) was estimated using the equation of Grynkiewicz (84). $[Ca^{2+}]_i = K_d * ß * (R-R_{min})/(R_{max}-R)$, where K_d (apparent dissociation constant) was determined at 224nM for FURA-2AM, *ß* being the fluorescent ratio (EGTA 380nM / Triton 380nM), R the fluorescent ratio measured upon effector stimulation, R_{max} provided by adding 0.1% Triton on the cells after a washing step and R_{min} by the subsequent addition of 10mM EGTA. Ionomycin (5μM) was used as a positive control in all experiments. Each effector was tested in quadruplicates.

1.2.2. Human TAS2R-expressing HEK cells. Activation of human TAS2 receptors was measured by monitoring variations in intracellular Ca^{2+} concentrations $[Ca^{2+}]_i$ using the FlexStation II 384 (Molecular Devices). TAS2R-expressing cells and non-transfected cells were seeded in supplemented DMEM into Poly-Lysin-coated 96-well plates (black wall, clear bottom, Greiner) at a density of 7.10^4cells/ml, 100μl/well and cultured overnight. The following day, transcription of the receptors was induced by adding 0.25μg·ml^{-1} doxycyclin. Cells were induced for 24h and then loaded with the calcium-sensitive fluorescent dye Fluo-4AM (Molecular Probes), following suppliers guidelines. Dye loading medium composition was the following: 0.01% Pluronic F-127 (Molecular Probes), 0.5mM Probenecide (Sigma) and 2.5μM Fluo-4AM in Tyrode's buffer pH 7.4 (140mM NaCl, 5mM KCl, 10mM Glucose, 1mM $MgCL_2.6H_2O$, 20mM Hepes). Compounds to test were prepared in parallel in Tyrode's buffer in a 96 wells plate (V96 Microwell, Nunc) and automatically pipetted onto the cells during the assay. Measurements were carried out for 90s with an interval time of about 1.6s, giving 55 data points per measurement. To obtain a baseline,

fluorescence signals (excitation 485nm/emission 520nm) were measured for 20s prior to the addition of compounds on the cells (80μl, injection speed of 100μl/s). After agonist addition, the fluorescence signals were measured for an additional 70s at 37°C. The fluorescence values (DeltaF) were calculated by subtracting the maximum fluorescence from the average fluorescence of the first 10 time points (baseline). Non-induced cells were measured in parallel as a negative control.

1.3. Membrane potential measurements with a voltage-sensitive fluorescent dye in NCI-H716 The voltage-sensitive fluorescent dye DIBAC3(5) (Molecular devices) was used to assess membrane depolarization of the NCI-H716 cells upon effector stimulation. Briefly, the cells were seeded at 2.10^5cells/ml (0.5ml/well) in a 24-wells plate coated with Matrigel and allowed to grow for 2-3 days. After a washing step, the cells were incubated 1h at room temperature in the dye loading solution (pH 7.4, All mM: 160 NaCl, 4.5 KCl, 2 $CaCl_2.2H_2O$; 1 $MgCl_2$; 10 HEPES) with 1μM DIBAC3(5) and 0.25% Pluronic F-127. The stained cells were stimulated without intermediary washing step and the fluorescence emission (616nm) recorded using the Varioskan. Each effector was tested in quadruplicates.

1.4. GLP-1 secretion studies from NCI-H716 cells

For each secretion study, NCI-H716 cells were seeded at 2.10^5cells/ml (2ml/well) in Matrigel-coated 12 wells plate. Cells were grown until confluence (2-3 days) and deprived in FCS 24h prior to the experiment. Krebs buffer as described before (see 1.2.1) supplemented with dipeptidyl peptidase (DPP)-IV inhibitor (20μl·ml^{-1}; Millipore) and 0.2% BSA was used for the secretion studies. Compounds to test were prepared in buffer and incubated on the cells (0.5ml/well) for 2h

at 37°C in a humidified incubator at 5%CO_2. Medium was then collected and centrifuged at 4°C for 10min at 1000xg to remove cell debris and the supernatants were stored at -80°C until GLP-1 determination using a commercial kit (EGLP-35K, Millipore). Stock solutions for the calcium inhibitors (nifedipine, thapsigargin, BAPTA-AM, 2-APB) were prepared in DMSO. Final concentration of DMSO never exceeded 1% on the cells. Each effector was tested in triplicates.

1.5. CCK secretion studies from HuTu-80 cells

For secretion studies, Hutu-80 cells were seeded at 2.10^5cells/ml, 0.5ml/well in Poly-Lysin-coated 24 wells plate 48h prior to the experiment. HBSS buffer (Gibco) supplemented with 10mM Hepes (Sigma) was used. Compounds to test were prepared in HBSS buffer and incubated on the cells (0.5ml/well) for 2h at 37°C in a humidified incubator at 5%CO_2. Medium (0.5ml) was collected and centrifuged at 4°C for 5min at 1000xg to remove cell debris and the supernatants were stored at -20°C until further analysis. Stock solutions for H.g.-12 and antagonist 03A3 were prepared in DMSO (respectively 100mM and 20mM). Final concentration of DMSO never exceeded 0.5% on the cells.

1.6. Determination of CCK concentrations using a CCK receptor-1 activation assay by monitoring $[Ca^{2+}]_i$.

Samples derived from secretion studies from Hutu-80 cells and rat gut rings were tested for CCK in a CCK-receptor 1 (CCK_1R) activation assay as described previously (69). This method has been shown to display higher sensitivity and lower variability than an ELISA (69). In brief, CHO-WT and CHO-CCK_1-R cells (100μl/well) were seeded into Poly-Lysin-coated 96 wells microtiter plate (black wall, clear bottom, Greiner) at a density of 3.10^5 and 4.10^5cells/ml, respectively, and

cultured overnight. Cells were loaded with Fluo-4AM as described above. Supernatants from the secretion studies were thawed from -20°C and prepared in parallel in a 96 wells plate (V96 Microwell, Nunc). The Flexstation measurement settings were identical as those used for the HEK cells expressing human bitter taste receptors. CCK_1R activation was measured by monitoring fluorescence signals in CCK_1R-expressing CHO cells after the addition of the supernatants derived from CCK-release experiments as described above. Each experiment was conducted in parallel on wild type CHO cells to assess any non-specific signal. Calibration was performed using sulphated CCK-8 as a standard.

2. *Ex vivo* models

An adapted technique of rodent everted gut sacs was used to assess intestinal hormone secretion. Everted gut sacs and rings offer the advantage of keeping the intact structure of the gastrointestinal tract as compared to cellular models. Applications for these techniques are illustrated in the results and discussion part.

2.1. Animals and materials

Everted gut sacs experiments were performed using 12-weeks-old male C57BL/6 mice (average weight 25g, Charles River Germany). Gut ring studies were performed using 8-weeks-old male Sprague Dawley rats (average weight 300g, Charles River Germany). Animals were maintained on a regular laboratory chow and fasted (6h for mice, 12h for rats) previous to the experiments. Water was displayed *ad libitum* before all experimental procedures. The studies were performed in accordance with protocols approved by the Technical University Munich. Laboratory internal guidelines for care and use of animals were followed throughout all procedures. The animals were

anesthetized with ether (Sigma) and killed by cervical dislocation. Krebs buffer used in the *ex vivo* experiments was freshly made and pregassed with 95% O_2 and 5% CO_2 for 1 hour at 37°C. Krebs buffer composition was the following (pH 6.5, all mM): 119 NaCl; 4.7 KCl; 2.5 $CaCl_2.2H_2O$; 1.2 $MgSO_4.7H_2O$; 1.2 KH_2PO_4; 25 $NaHCO_3$. In gut rings studies, 20mM MES (2-(N-morpholino)ethanesulfonic acid) was added to the buffer to prevent the increase in pH due to the lack of continous gassing during the incubation time. To prevent enzymatic degradation of the hormones of interest (GLP-1, GIP, PYY), dipeptidyl peptidase (DPP)-IV inhibitor (Millipore) was added to the samples following supplier recommendations. All steps prior to incubation were performed on ice. The incubation system used for *ex vivo* experiments was custom-made.

2.2. Mouse everted gut sacs (**Figure 7.**)

The protocol developed by Wilson and Wiseman (213) to study radiolabeled nutrient uptake was adapted to assess hormone secretion. Median laparotomy was performed and the entire small intestine removed while gently stripping out the mesentery. Duodenum was defined as a 10cm section downstream the pyloric sphincter and ileum as a 10cm section upstream the caecum. The tissue in-between was defined as jejunum and divided into two parts of equal length (referred as proximal and distal jejunum). Each part of the small intestine was then separately washed with pre-warmed and pre-oxygenated Krebs buffer before to be everted. To evert the gut, a stainless steel rod was used to push one extremity of the intestine into the gut lumen until it appeared at the end, and the eversion was completed by gently rolling the intestine on the rod. A section of 2.5cm in length from the everted intestine was used pro everted sac. A silk ligature was tied around one end of the everted section while a second ligature was placed loosely around the other end. A blunt needle attached to a 2ml syringe was then introduced into the everted section and the loose ligature

was pulled tight over it. Appropriate volume of fluid (Krebs buffer + DPP-IV inhibitor at 20 μL $\cdot mL^{-1}$) was then injected into the everted sac and the needle was withdrawn. The everted gut sac was then introduced in an Erlenmeyer filled with 10ml of the effector solution (prepared in Krebs buffer) and incubated at 37 °C, 95% O_2 and 5% CO_2 in a shaking incubator (60-100 rpm) for 1/2 hour. A paired-samples design was used, each animal being its own control. Thus, for each animal and each part of the gut (duodenum, proximal jejunum, distal jejunum and ileum), two samples were obtained: one everted gut sac was incubated in buffer whereas the other was incubated in the effector solution. Paired-t test were subsequently used to assess statistical significance of the results. After the incubation period, the everted sacs were washed in Krebs buffer and blotted dry on absorbent paper. The sacs were cut-opened and their content centrifuged at 3000xg at 4°C for 15 minutes. Volume of supernatant was measured (referred to as serosal content) and GIP (total), PYY (total) and GLP-1 (active) concentrations were measured using a 3-PLEX mouse-gut-hormone kit (MGT-78K, Millipore) read on the Luminex 100 IS system (Luminex corporation, Austin, TX, USA). Data were analysed with the Bio-Plex Manager software version 4.0 (Bio-Rad, Hercules, CA, USA), using a weighted 5-parameter logistic. The tissues were individually digested in 1ml of NaOH (1M) at 37°C for 2h, and protein content was measured using the Bradford method (25). Hormone concentrations were normalized both to serosal volume and protein content and the results expressed as normalized hormone secretion in (pg/mg protein) as Mean ± SEM.

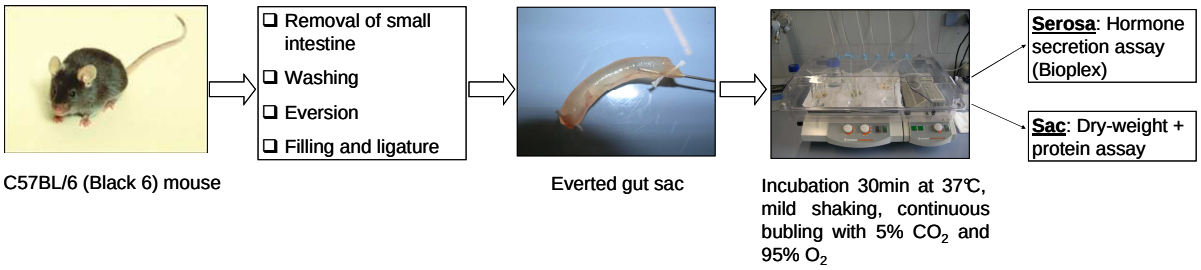

Figure 7. The mouse everted gut sac as a model to assess hormone secretion *ex vivo*.

2.3. Rat gut rings

The protocol used by Jang et al. (102) was adapted to everted rat intestine. All steps prior to incubation were performed on ice. Briefly, luminal contents were gently removed using a fresh-made Krebs buffer saturated with 95%O_2-5%CO_2 (pH 6.5, all mM: 119 NaCl; 4.7 KCl; 2.5 $CaCl_2.2H_2O$; 1.2 $MgSO_4.7H_2O$; 1.2 KH_2PO_4; 25 $NaHCO_3$; 20 MES). Duodenum and ileum segments were everted using a customized metallic rod. Rings of 0.5cm were prepared and briefly incubated in Krebs supplemented with 0.5mM DTT (dithiothreitol) in order to prevent excessive mucus production. The gut rings were then randomly transferred in 200μl of effectors (triplicates) prepared in Krebs buffer containing dipeptidyl peptidase (DPP)-IV inhibitor (20μl·ml^{-1}; Millipore) in a 96 wells plate. Stock solution for H.g.-12 was prepared in DMSO (100mM), and freshly diluted in Krebs buffer on the day of the experiment. Final concentration of DMSO never exceeded 0.5% on tissues. The gut rings were incubated 30min at 37°C in a shaking incubator (60-100rpm), after what the total content of each well was centrifuged at 4°C for 15min at 5000xg. CCK-8S levels were measured in the supernatants using the CCK_1R activation assay. PYY (total) and GLP-1 (active) levels were measured using a Milliplex MAP rat-gut-hormone kit (RGT-88K,

Millipore, Billerica, MA, USA) read on the Luminex 100 IS system (Luminex corporation, Austin, TX, USA).

3. Data processing and statistical analysis

GraphPad Prism version 4.01 for Windows (GraphPad Software, San Diego, California, USA) was used for all statistical analysis.

In the "Identification of a novel signaling pathway *in vitro*" results section, data are expressed as Mean ± SD. Differences in the potency of the effectors to elicit GLP-1 secretion from NCI-H716 cells were analyzed using one-way ANOVA followed by the appropriate multiple comparison test. Differences were considered to be significant at $P<0.05$. All experiments were performed at least in duplicate.

In the "Bitter taste sensing and satiety hormone secretion in the gut" results section**,** data are presented as Mean ± SEM. Differences in the potency of the agonists/effectors towards receptor activation/hormone secretion were analyzed using one-way ANOVA followed by the appropriate multiple comparison test. Differences were considered to be significant at $P<0.05$. Receptor activation data (TAS2Rs, CCK_1R) are expressed in DeltaF values. All i*n vitro* experiments were performed at least in duplicate. Hormone secretion data from the rat gut rings studies are expressed in fold changes of the control (Krebs buffer). For GLP-1 and PYY, data were analyzed with the Bio-Plex Manager software version 4.0 (Bio-Rad, Hercules, CA, USA), using a weighted 5-parameter logistic for calculating the hormones concentrations in samples.

RESULTS AND DISCUSSION

1. Identification of a novel sensing pathway *in vitro*: selected tetrapeptides lead to a GLP-1 release from the human enteroendocrine cell line NCI-H716

1.1. Background and aims of the study

Glucagon-like peptide-1 (GLP-1) released from enteroendocrine L-cells in the intestine has received recently considerable interest due to its ability to amplify glucose-dependent insulin secretion. Together with the glucose-dependent insulinotropic peptide (GIP), GLP-1 is responsible for the "incretin effect" observed when glucose is present in the intestine. The incretins can stimulate insulin output from ß-cells far above that obtained solely by the increase in plasma glucose levels. Dietary protein and its luminal degradation products elicit as well a GLP-1 secretion. However, whereas intestinal glucose-dependent stimulation of GLP-1 secretion can be attributed to the sodium-dependent glucose transporter SGLT1 (154, 164) expressed in epithelial but also endocrine cells in the small intestine (12, 164), the sensing mechanisms for protein-derived products remain to be identified. Models to assess intestinal GLP-1 secretion from L-cells have been established and are represented by the rodent cell lines STC-1 and GLUTag, and the human cell line NCI-H716. Derived from a caecal adenocarcinoma (164), NCI-H716 cells release GLP-1 in response to nutrient stimulation including fatty acids (174), amino acids and protein hydrolysates (173, 174), artificial sweeteners (102), bitter compounds (59) but also in response to hormones such as insulin (12, 37), leptin (6), GIP (176) and selected neurotransmitters (5, 174).

Although protein hydrolysates resp. peptones have been shown to increase proglucagon gene expression *in vitro* (47, 76) and to induce a GLP-1 release *in vitro* as well as *in vivo* in rats (79, 154) and in humans (34, 57), the signalling mechanisms underlying these processes are currently

unknown, although it has been proposed that an activation of intracellular ERK1/2 and MAPK pathways may be mandatory (173) to elicit a GLP-1 output. Commonly used protein hydrolysates for stimulation of intestinal hormone secretion are generally poorly defined in composition and usually comprise a mixture of amino acids, peptides of various length and even intact proteins. We adopted a strategy to screen for single amino acids, distinct peptides and peptidomimetics that could cause an increase in intracellular free calcium in NCI-H716 cells. Increased $[Ca^{2+}]_i$ upon nutrient stimulation is believed to prime the hormone secretion from enteroendocrine cells and provide a fast and convenient way of screening for potential effectors. Strikingly, only selective tetrapeptides were found to induce a robust and dose-dependent $[Ca^{2+}]_i$ response from NCI-H716 cells. After having shown that tetrapeptides induced as well a GLP-1 secretion from the cells, we assessed whether the specific calcium response monitored was mandatory in this secretory process and demonstrated that this tetrapeptide-induced GLP-1 release involves most likely store-operated calcium channels.

1.2. Results

1.2.1. *Tetrapeptide sensing by NCI-H716 cells is backbone-length specific and dose-dependent.*

Among all effectors from protein sources tested, only tetrapeptides were found to induce a robust and selective $[Ca^{2+}]_i$ response in NCI-H716 human enteroendocrine cells. Strikingly, a peptide backbone length of four residues appears to be required, as neither single amino-acids nor any of the dipeptides, tripeptides and pentapeptides tested for homologous series containing either glycine or alanine residues cause any calcium response (**Figure 8.*A***). Furthermore, tetra-glycine (**Figure 8.*B***) and tetra-alanine (**Figure 8.*C***) were both shown to elicit a dose-dependent calcium response

from the cells. This allowed the calculation of an EC_{50} value of around 3mM for the $[Ca^{2+}]_i$ response to tetra-glycine in NCI-H716 cells (**Figure 8.*D***).

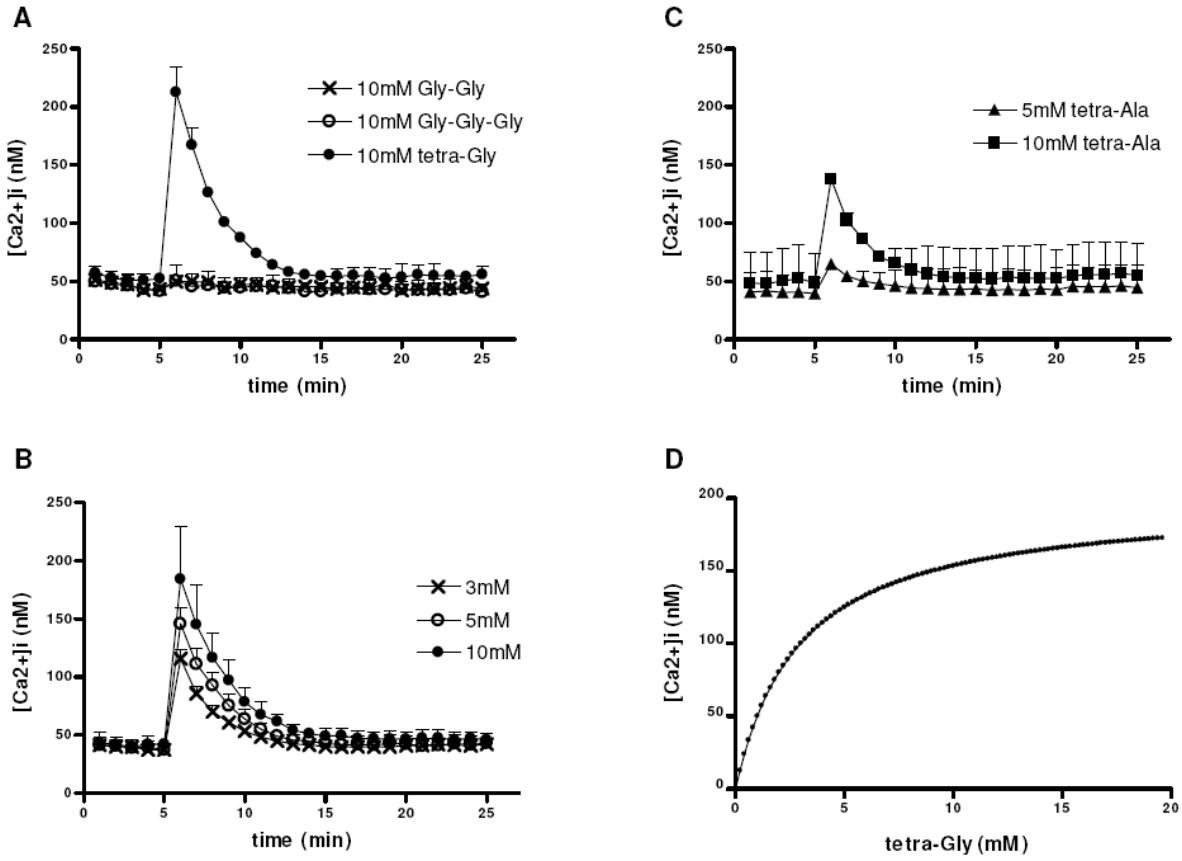

Figure 8. Selective tetrapeptides elicit changes in intracellular calcium levels from NCI-H716 enteroendocrine cells. $[Ca^{2+}]_i$ changes in FURA-2AM loaded NCI-H716 cells. **(A)** Cells exposed to 10mM of either di-glycine, tri-glycine or tetra-glycine. **(B)** Dose-dependent transient calcium increase in NCI-H716 cells exposed to tetra-glycine. **(C)** Response of $[Ca^{2+}]_i$ in cells exposed to either 5 or 10mM of tetra-alanine. **(D)** Dose-dependent elevation of $[Ca^{2+}]_i$ in NCI-H716 cells exposed to tetra-glycine for determination of the apparent EC_{50} value for activation. All data are averages of quadruplicate determinations from two independent experiments and are expressed as $[Ca^{2+}]_i$ except for **D** (trace from a representative experiment).

1.2.2. *Characteristics of the $[Ca^{2+}]_i$ response to tetra-glycine in NCI-H716 cells.*

To investigate the underlying mechanisms that allow the $[Ca^{2+}]_i$ increase in response to tetra-glycine we applied nifedipine (10μM), an established inhibitor of plasma membrane L-type Ca^{2+} channels and thapsigargin (20nM), known to cause a release of Ca^{2+} from endoplasmic reticulum (ER) stores. However, both failed to significantly inhibit the $[Ca^{2+}]_i$ response (data not shown) whereas in the same experiments, a second and third stimulation of the cells with 10mM tetra-glycine caused a 90%-reduced AUC in the $[Ca^{2+}]_i$ response compared to first stimulation (**Figure 9.*A***). Use of a buffer nominative free of calcium with or without a calcium chelating agent (0.1mM EGTA) inhibited the $[Ca^{2+}]_i$ response in cells, both in amplitude and time as shown in **Figure 9.*B*.** The most pronounced inhibition in $[Ca^{2+}]_i$ increases however was obtained using 2-Aminoethoxydiphenylborate (2-APB), a store-operated calcium channel (SOCC) blocker (24). Pre-incubation of the cells with 100μM 2-APB nearly abolished the $[Ca^{2+}]_i$ response to tetra-glycine (**Figure 9.*C***). When in addition changes in membrane potential were recorded after loading cells with the voltage-sensitive fluorescent dye DIBAC3(5), tetra-glycine application could induce a membrane depolarization. As shown in **Figure 10.**, 10mM tetra-glycine as well as 20mM KCl used as a positive control caused a pronounced membrane depolarization in NCI-H716 cells when compared to a control buffer.

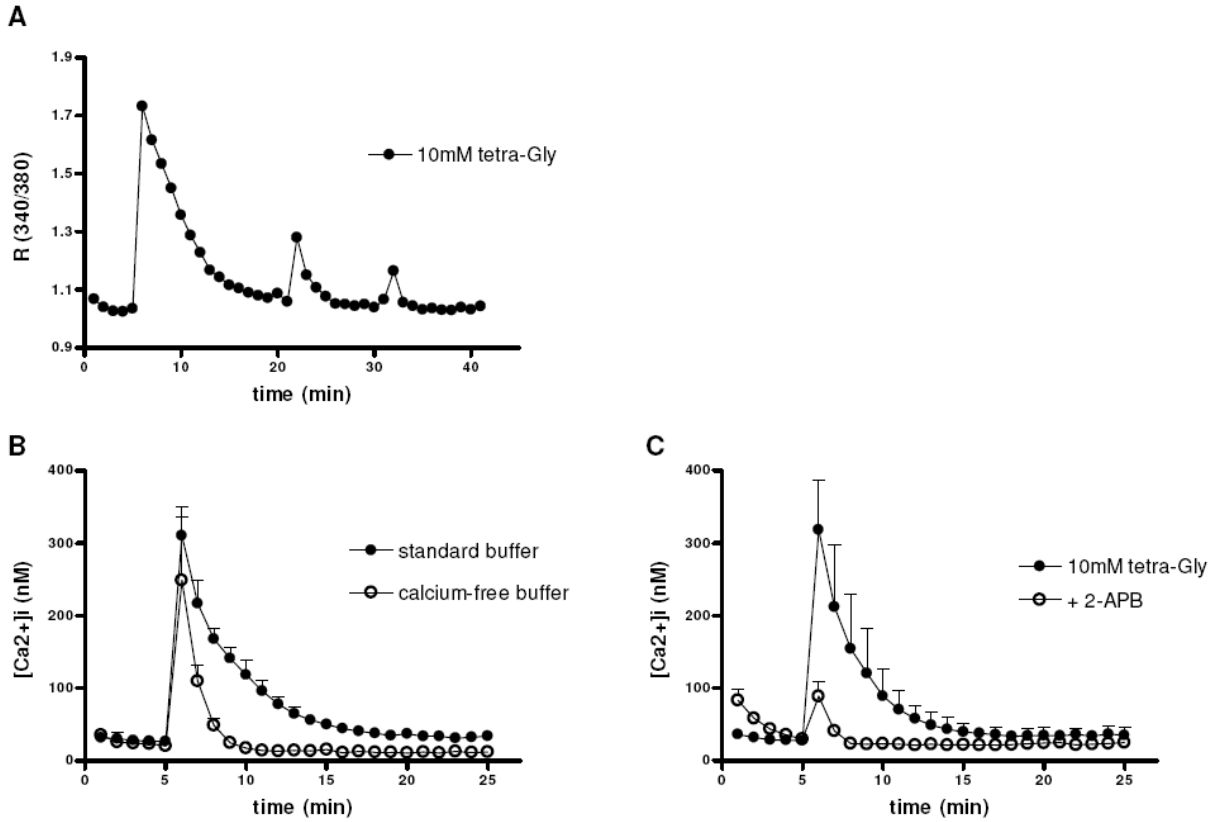

Figure 9. Characteristics of the calcium response induced by tetrapeptides in NCI-H716 cells. $[Ca^{2+}]_i$ changes in FURA-2AM loaded NCI-H716 cells. **(A)** $[Ca^{2+}]_i$ transients in response to a repeated stimulation of NCI-H716 cells with 10mM tetra-glycine. **(B)** Response of $[Ca^{2+}]_i$ in NCI-H716 cells exposed to standard buffer or a calcium-free buffer containing 0.1mM EGTA in the presence of 10mM tetra-glycine. **(C)** Response of $[Ca^{2+}]_i$ in NCI-H716 cells pre-incubated or not with 2-APB (100μM; 15min) in the presence of 10mM tetra-glycine. All data are averages of quadruplicate determinations from two independent experiments and are expressed as $[Ca^{2+}]_i$ except for **A** (trace from a representative experiment expressed as fluorescence ratio).

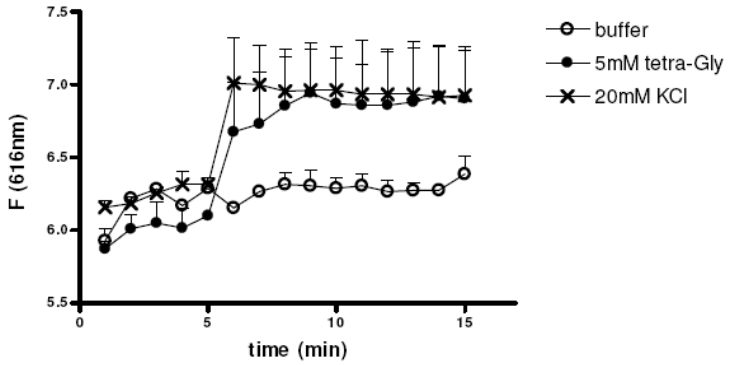

Figure 10. Tetra-glycine causes a membrane depolarization in NCI-H716 cells. Recordings of membrane potential in NCI-H716 cells preloaded with the fluorescent probe DIBAC3(5) and either exposed to buffer alone, to 20mM KCl serving as a positive control for membrane depolarization or to 5mM of tetra-glycine. Data are averages of quadruplicate determinations from two independent experiments and are expressed as fluorescence intensities (emission wavelength 616nm).

1.2.3. *Tetrapeptides elicit a GLP-1 secretion from NCI-H716 enteroendocrine cells.*

To assess whether the tetrapeptide-induced selective $[Ca^{2+}]_i$ response in NCI-H716 is coupled to hormone secretion, we determined GLP-1 concentrations in the incubation medium. Exposure of the cells for 2 hours to increasing concentrations of tetra-glycine caused an increase in GLP-1 secretion that became significant only at 20mM with an 1.5-fold increase (P<0.05) over that in control cells not exposed to peptides (**Figure 11.*A***). Other peptides such as Gly-Trp-Gly-Gly and tetra-alanine elicited also a significant GLP-1 release (**Figure 11.*B***) with a mean of 1.7-fold in total GLP-1 when compared to basal secretion from cells not exposed to peptides (P<0.05). PEP, a casein-based protein hydrolysate provided at 8% in buffer (w/v), elicited a GLP-1 release about 3-fold over that measured in control cells (P<0.01; **Figure 11.*B***).

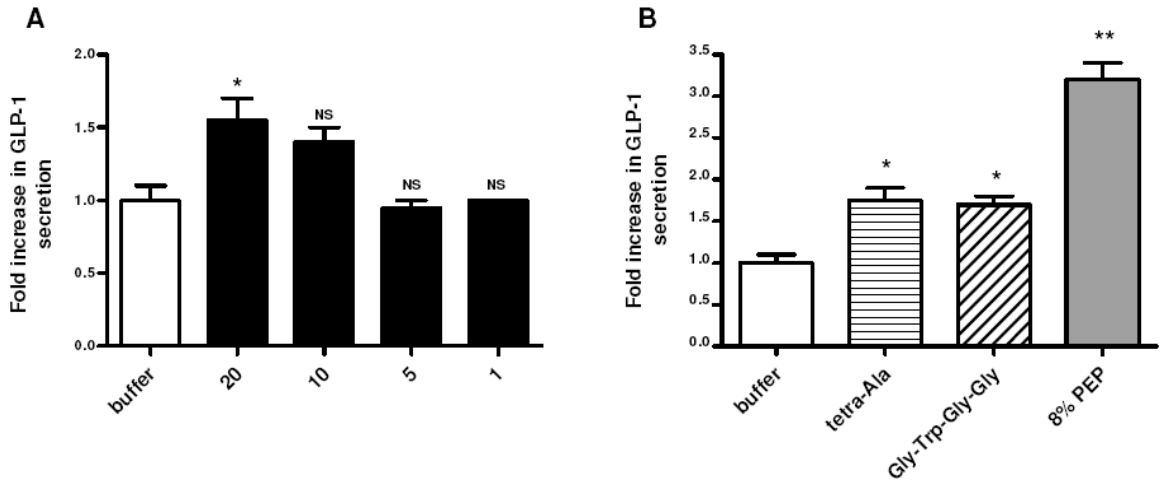

Figure 11. Selective tetrapeptides elicit a GLP-1 secretion from NCI-H716 cells. Determination of active GLP-1 levels in NCI-H716 cell supernatants using an ELISA kit. **(A)** GLP-1 secretion (expressed as fold-change) from NCI-H716 cells exposed for 2h to buffer alone (control) or to increasing concentrations of tetra-glycine (1-20mM). **(B)** GLP-1 secretion (expressed as fold-change) from NCI-H716 cells exposed for 2h to buffer alone (control), 10mM tetra-alanine, 10mM Gly-Trp-Gly-Gly or 8% PEP (w/v), a commercial protein hydrolysate. Data are averages of triplicate determinations from representative experiments; **P*<0.05; ***P*<0.01 (one way ANOVA + Dunnett's post-hoc test).

1.2.4. *Tetra-glycine-induced GLP-1 secretion in NCI-H716 cells seems to involve store-operated calcium channels (SOCCs).*

Having established a relationship between the $[Ca^{2+}]_i$ response induced by tetrapeptides and GLP-1 secretion in NCI-H716 cells, we assessed whether an inhibition of the $[Ca^{2+}]_i$ response could prevent GLP-1 secretion. Pre-incubation of the cells with the intracellular calcium chelator BAPTA-AM (100μM) for 15min abolished the GLP-1 release in NCI-H716 cells exposed for 2hours to tetra-glycine, and tended to impair as well the basal secretion in buffer (**Figure 12.*A***; *P*<0.05). Application of the SOCC blocker 2-APB (100μM) for 15min prior to tetra-glycine treatment completely abolished GLP-1 release from NCI-H716 cells (**Figure 12.*B***; *P*<0.001). Here

as well, basal GLP-1 secretion in cells not exposed to any peptide was significantly reduced (P<0.001).

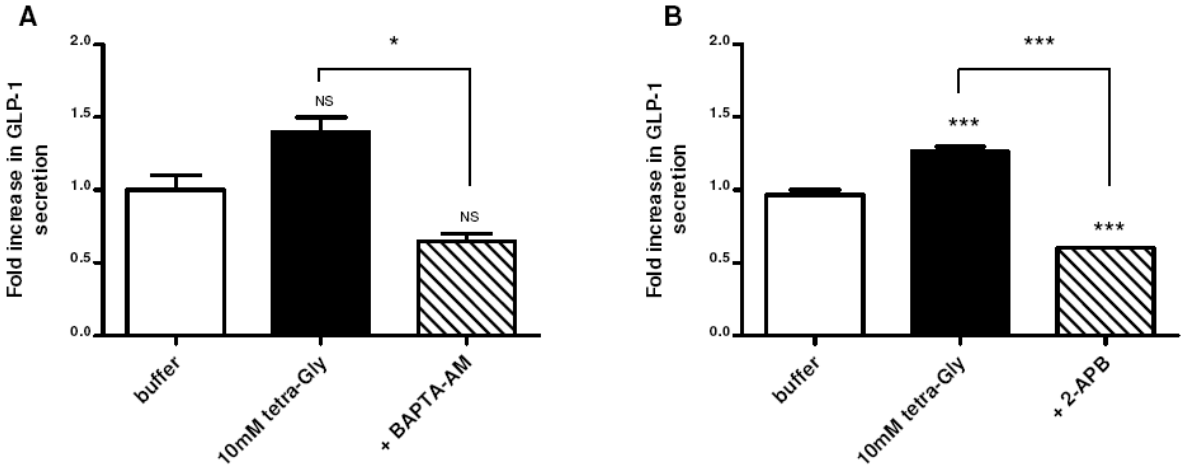

Figure 12. Effect of the intracellular calcium chelator BAPTA-AM and the SOCC blocker 2-APB on tetraglycine-induced GLP-1 release from NCI-H716 cells. (A) GLP-1 secretion (expressed as fold-change) from NCI-H716 cells exposed to buffer alone (control) or to tetra-glycine in the absence or the presence of the intracellular calcium chelator BAPTA-AM (100µM). Cells were treated for 15min with BAPTA-AM and then incubated for 2 hours with 10mM tetra-glycine. **(B)** Same experiment using cells pre-incubated 15min with 100μM of the SOCC blocker 2-APB. Data are averages of triplicate determinations from representative experiments; *P<0.05; ***P<0.001 (one way ANOVA + Bonferroni's multiple comparison test).

1.3. Discussion

We here report a novel sensing pathway that is selective for tetrapeptides and leads to a GLP-1 release from human NCI-H716 enteroendocrine cells. The tetrapeptides tested were able to dose-dependently induce a transient increase in $[Ca^{2+}]_i$ that seems to be coupled to GLP-1 output and that most likely involves store-operated calcium channels (SOCCs). Neither di-, tri- or pentapeptides comprising either glycine or alanine nor the corresponding free amino acids could elicit these responses. This emphasizes the high selectivity of the sensing mechanisms for

tetrapeptides in NCI-H716 cells and rules out that an extracellular hydrolysis of the peptides - that would release shorter peptides and/or free amino acids - contributes to the responses observed.

NCI-H716 cells display most properties of human intestinal L-cells and secrete hormones such as GLP-1 and PYY. They express several taste signalling elements like alpha-gustducin, PLCß2, TRPM5 and T1R subunits (102). NCI-H716 were shown to secrete GLP-1 upon stimulation by meat hydrolysates, essential amino acids mixtures, oleic and palmitic acid as well as non-nutrient components such as sweeteners and bitter compounds (5, 6, 102, 173, 174). However, NCI-H716 cells failed to demonstrate a significant regulation of the proglucagon gene expression (37).

Whereas in the rodent-derived cells lines STC-1 and GLUTag the roles of calcium and calcium channels have been characterised well in stimulus-secretion coupling (for reviews see (164, 203)), such studies are essentially lacking in human NCI-H716 cells. We here demonstrate a close relationship between a transient $[Ca^{2+}]_i$ increase in the cells and GLP-1 secretion upon tetrapeptide stimulation. Only those peptides that could induce a rise in $[Ca^{2+}]_i$ were able to elicit a GLP-1 release (**Table 1.**). However, PMA, alanine and the casein hydrolysate (PEP) tested increased GLP-1 release significantly despite a lack in $[Ca^{2+}]_i$ changes. In GLUTag cells, it was shown that glutamine elicited a greater GLP-1 release than alanine or glycine, although alanine and glycine induced larger $[Ca^{2+}]_i$ responses in the cells. It was later demonstrated that glutamine can potentiate GLP-1 secretion from GLUTag cells by acting downstream of the calcium signal, mainly via an increase in cAMP concentrations (164, 190). In our studies, tetra-glycine - although shown to be the most potent effector of $[Ca^{2+}]_i$ responses in NCI-H716 cells – was not the most effective for GLP-1 secretion.

Table 1. Relationship between $[Ca^{2+}]_i$ response and GLP-1 secretion in NCI-H716 cells. $[Ca^{2+}]_i$ measurements were conducted after loading the cells with FURA-2AM as described in methods. GLP-1 secretion was measured after 2hours in the incubation medium using a commercial ELISA kit for GLP-1 (Millipore). PMA: Phorbol-12-myristate-13-acetate; n.d: not determined.

Effectors (10mM unless indicated)	intracellular calcium response	Mean change in GLP-1 secretion (fold change over control)
PMA (1µM)	No	2
Ionomycine (5µM)	Yes	1
PEP (8%)	No	3.2
Tetra-glycine	Yes	1.3
Tetra-alanine	Yes	1.7
Gly-Trp-Gly-Gly	Yes	1.7
Gly-Leu-Gly-Gly	No	1
Ala-Ala-Pro-Ala	Yes	1.2
Gly-Gly-Gly-Ala	No	1
Gly-Gly-Ala-Gly	n.d	1.2
Alanine	No	2.4
Cefadroxil, Cephradine	No	1

That store-operated calcium channels might play an important role in the tetrapeptide sensing pathway was demonstrated by the ability of 2-APB to abolish both the $[Ca^{2+}]_i$ response and the GLP-1 release from NCI-H716 cells. Store-operated calcium entry (SOCE), also known as calcium release-activated calcium current (CRAC), is mandatory to refill intracellular calcium stores after stimulation, and this was shown to play an indispensable role for example in immune cells. The endoplasmic reticulum (ER) calcium sensor STIM1 is believed to activate the store-operated

calcium channels located in the plasma membrane following depletion of the intracellular calcium stores (see (194) for recent review and scheme in **Appendix 1**). Only in rat adrenocortical cells, the adrenocorticotropin (ACTH)-induced corticosterone secretion has been shown so far to involve a 2-APB sensitive calcium signalling component [Hayashi C. *et al*; 2009, accepted]. Here, the authors hypothesize that the canonical IP3-mediated ER calcium release is not required for ACTH-induced corticosterone secretion, but may rather involve via an unknown messenger the subsequent store-operated calcium entry. These findings are in accordance with our data, as pre-incubation of the cells with thapsigargin, known to deplete endoplasmic/sarcoplasmic reticulum (ER/SR) calcium stores, failed to inhibit the tetrapeptide-induced $[Ca^{2+}]_i$ response in NCI-H716 cells. Although the nature of the intracellular calcium response remains here to be determined, it might be speculated that mitochondrial calcium stores (163) or the nicotinic acid adenine dinucleotide phosphate (NAADP)-mediated calcium release from acidic stores (105, 220) are involved.

The unique finding that tetrapeptides selectively stimulate via $[Ca^{2+}]_i$ changes the GLP-1 secretion from NCI-H716 cells raises the question on the nature of the sensor. It seems plausible to assume in the first place that a G-protein-coupled receptor (GPCR) may be involved. Various GPCRs are known to be activated by peptides, and selected GPCRs were recently shown to elicit a $[Ca^{2+}]_i$ increase upon nutrient stimulation of enteroendocrine cells. Gene and protein expression of GPR43, a receptor for short chain fatty acids, was demonstrated in enteroendocrine L-cells of rat and human large intestine that also express and secrete PYY (108, 109). Free fatty acids have also been shown to induce a GLP-1 secretion from STC-1 cells and *in vivo* in mice through interaction with GPR120 (200). Although expression of the GPR93 gene in enteroendocrine cells could not

yet be demonstrated, it is expressed in enterocytes, and GPR93 was found to induce a cholecystokinin (CCK) secretion and increased transcription upon protein hydrolysate stimulation when over-expressed in STC-1 cells (42, 43). However, stimulation of the NCI-H716 cells with 5 and 10µM lysophosphatidic acid (LPA), a potent agonist for GPR93, did not elicit a $[Ca^{2+}]_i$ response (data not shown). Numerous GPCRs identified by gene sequencing are still classified as orphans (124, 211) and await the identification of ligands. It is thus plausible that new receptors that bind specifically tetrapeptides are discovered. In addition to GPCRs, the extracellular calcium sensing receptor (CaR) could also be considered here as sensor. The CaR is expressed in various regions of the human gastrointestinal tract (183, 186) and can be activated by nutrients including amino acids (see (75) for review). Whether tetrapeptides interfere with the CaR is not known, but such a role of CaR was proposed for transmitting the signal of soybean peptides leading to a CCK release from STC-1 cells very recently (156). Finally, any transporters that specifically mediate the uptake of tetrapeptides and that could be involved in GLP-1 secretion from enteroendocrine cells are not known. Intestinal epithelial cells do express the peptide transporter PEPT1, but its substrate selectivity is restricted to dipeptides and tripeptides (68, 144).

In conclusion, we demonstrate a tetrapeptide-specific elevation of intracellular free calcium in the enteroendocrine NCI-H716 cell line that causes an increase in GLP-1 secretion. Although the nature of the sensor and the intracellular mechanisms underlying stimulus-secretion coupling remain unknown, such a sensing pathway could be involved in transmitting the action of dietary proteins and hydrolysates that are known to effectively stimulate intestinal GLP-1 secretion.

2. Development of rodent intestinal *ex vivo* models to assess hormone secretion

A major hurdle in the field of nutrient sensing research is that primary enteroendocrine cells are difficult to cultivate and therefore to study. As they represent only around 1% of the total intestinal cell population, enteroendocrine cells are moreover difficult to differentiate from the surrounding cells in culture. The available cellular models like GLUTag, STC-1 and NCI-H716 (**Table 2.**) all display heterogeneous nutrient sensitivities, which make the extrapolation towards the native human L-cell difficult. This led us to develop intestinal *ex vivo* models taking into account the morphology in which the enteroendocrine cells are surrounded by enterocytes and an intact enteric nervous system *in vivo*. The data generated from these models may thus be called physiologically relevant, and offer a good combination together with the cell models towards the identification of new nutrient sensing pathways**.**

Table 2. Characteristics of available enteroendocrine cell lines.

Cell line	Origin	GI hormones secreted
STC-1	Mouse intestinal tumor	CCK
		GLP-1
		Secretin
		GIP (mRNA)
GLUTag	Mouse colonic tumor	CCK
		GLP-1
NCI-H716 (ATCC CCL-251)	Human caecal adenocarcinoma	GLP-1
HuTu-80 (ATCC HTB-40)	Human duodenal adenocarcinoma	CCK

In order to validate our rodent intestinal *ex vivo* models, we conducted preliminary studies in which we measured the hormone output in response to luminal effectors known to elicit an answer *in vitro* or *in vivo.*

2.1. GLP-1, GIP and PYY secretion patterns induced by a protein hydrolysate in mouse everted gut sacs

2.1.1. *Background and aims of the study.*

Protein is regarded as the most satiating macronutrient. Protein hydrolysates and peptones have thus been shown to elicit GLP-1 release in vitro (173, 174), in rats (79, 93) and in humans (34, 57) as well as CCK release in vitro (160) and in rats (71, 72, 169). Therefore, we aimed to assess the potency of a commercial casein hydrolysate (Peptopro®, DSM, Delft, the Netherlands; referred as PEP in the present work) to elicit hormone secretion in our everted gut sac model.

We used 12 male C57BL/6 mice for this study. For each section of the gut, the protein hydrolysate was paired with BSA (both at 4% w/v in Krebs buffer), and pH of the samples was adjusted to 6.5. Hormone secretion levels for GLP-1, GIP and PYY were here measured after a 1h incubation period.

2.1.2. *Results and discussion.*

The casein hydrolysate (PEP), containing mainly di- and tripeptides, is a potent secretagogue for GLP-1, GIP and PYY in our mouse everted gut sac model as shown in **Figure 13.** GLP-1 secretion upon 4% PEP stimulation displays a strong gradient from proximal to distal with an up to 3-fold increase towards the jejunum and ileum when compared to the samples incubated with 4% BSA (respectively 3.5-fold; $P<0.01$ and 3.4-fold; $P<0.05$; **Figure 13.*A***). GIP shows an opposite pattern,

with a secretion gradient decreasing from proximal to distal, significant in jejunum when compared to BSA-stimulated samples (3.3-fold; $P<0.01$; **Figure 13.*B***). In addition, the protein hydrolysate elicits a strong PYY release in ileum (3.5-fold of BSA control; $P<0.01$; **Figure 13.*C***). PYY concentrations were below detection levels in duodenum and jejunum.

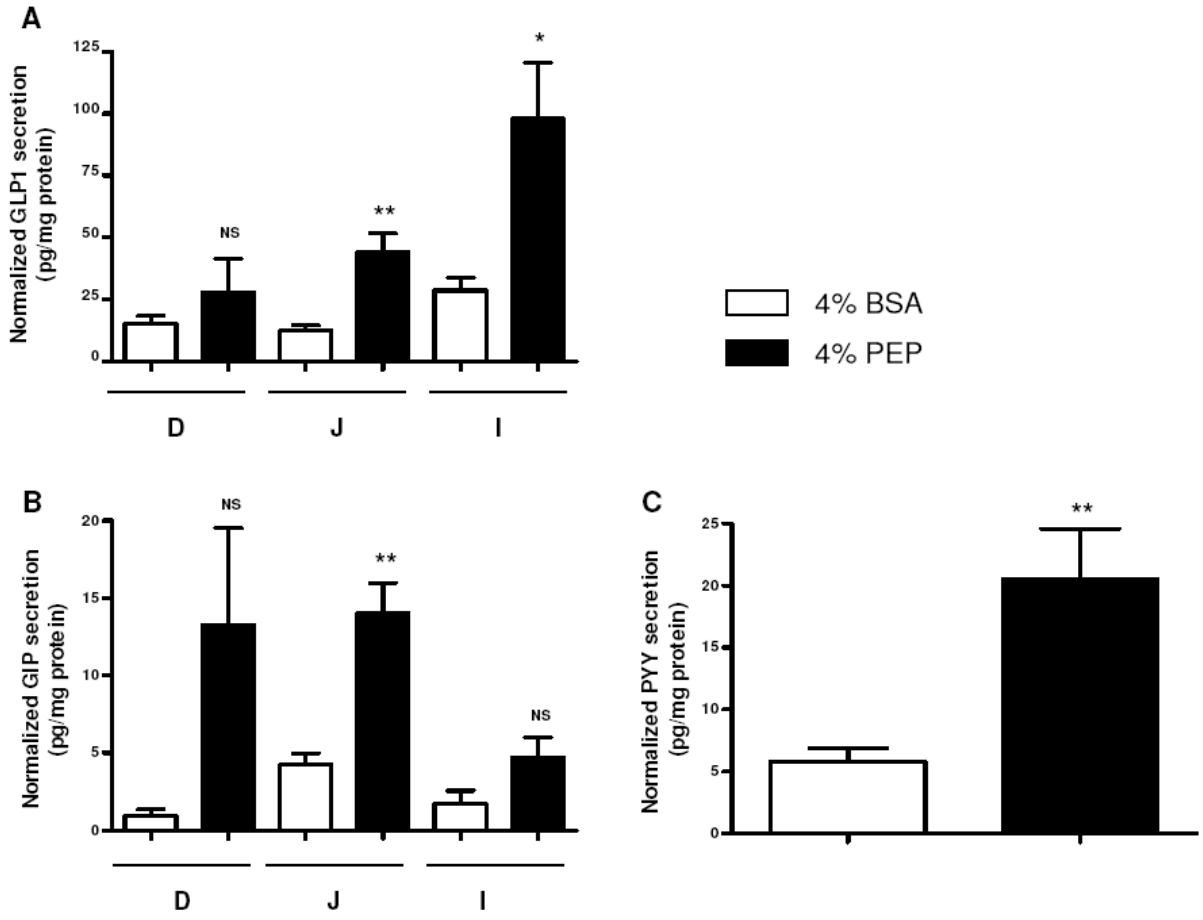

Figure 13. GLP-1, GIP and PYY release induced by a protein hydrolysate in mice everted gut sacs. Everted gut sacs from 12-weeks-old C57BL/6 male mice (n=12) were incubated for 1h at 37°C, 5% CO_2 and 95% O_2 in 4%(w/v) of either BSA or PEP. Hormone secretion in the serosal fluid was measured by Bioplex and normalized to serosal volume and protein content. Data are shown as Mean ± SEM; D / J / I = duodenum / jejunum / ileum; *****$P<0.05$; ****** $P<0.01$ (paired-t-test).

All in all, these data establish the mouse everted gut sac as a robust *ex vivo* model to assess hormone secretion in the intact intestine. In addition, the secretion patterns measured here for GLP-1, GIP and PYY fully correlate with the mRNA levels and peptide contents found in their respective secreting cells in a mouse primary L-cell study (172). Thus, GIP is secreted from K-cells found in proximal intestine (duodenum and jejunum), whereas L-cells co-secreting GLP-1 and PYY are mostly found in ileum and colon (see (39) for review).

2.2. Effect of aspartame hydrolysis inhibition on hormone secretion from mouse everted gut sacs

2.2.1. *Background and aims of the study.*

Artificial sweeteners are found in a broad range of food products. Due to their extreme sweetness (200 to 1000 times sweeter than natural sugar), the amounts used in food items are characterized by a very low caloric value. To date, few artificial sweeteners have been approved by the food and drug administration (FDA) in the US and the European food agency (EFSA) in Europe. The mostly used are aspartame (200 times sweeter as sugar), saccharin (200 to 700 times), acesulfame-K (200 times) and sucralose (600 times, sold under the name splenda®).

Safety of artificial sweeteners consumption is regularly questioned, especially in the case of aspartame for which suspicion of a carcinogenic potential has been raised. Nevertheless, in 2009, the EFSA confirmed the previously established Acceptable Daily Intake for aspartame of 40 mg/kg body weight.

Sucralose has been found to elicit GLP-1 secretion from the human enteroendocrine cell line NCI-H716 and in mice (102), through sweet receptors (T1Rs) expressed in the gut. However, whether artificial sweeteners could elicit satiety hormone secretion *in vivo* still remains controversial. Most

of the studies found in the litterature have been monitoring satiety feeling (assessed by visual analogue scales), food intake and gastric emptying but lack hormone measurements (22, 60, 132, 177, 178). Ingestion of a preload of encapsulated aspartame or its constituents phenylalanine and aspartic acid did not affect gastric emptying or satiety hormone secretion in humans (89). Recently, a human study using sucralose (134) and a study in rats using various sweeteners including sucralose (73) both reported a lack of effect on satiety hormone secretion after intragastric infusion.

We wanted to assess [1] whether the artificial sweetener aspartame could induce hormone secretion in our mouse everted gut sac model and [2] whether the dipeptide-like structure of aspartame (**Figure 14.**) played a role in the sensing process leading to hormone release. To conserve this structural feature during incubation with the tissue, we used the aminopeptidase inhibitor amastatin which has been shown to prevent up to 74% of the aspartame hydrolysis (99). The concentration of aspartame used in this study (1mM) is found in commercially available soft drinks.

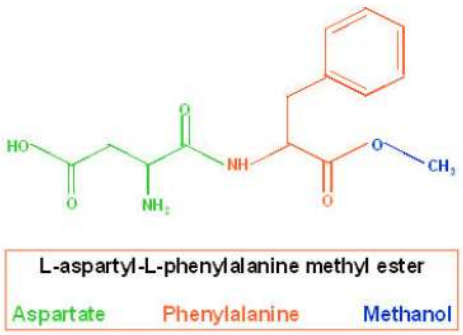

Figure 14. Structure of the artificial sweetener aspartame

2.2.2. *Results and discussion.*

Although not statistically reaching significance, a tendency of aspartame to increase hormone secretion when compared to buffer control was found in the jejunum for the 3 hormones measured (from 1.4-fold for GIP secretion up to 2.4-fold for PYY; **Figure 15.**). The co-incubation with amastatin in distal jejunum lead to a significant decrease in GLP-1 secretion (55% inhibition; $P<0.05$; **Figure 15.*A***) and PYY secretion (63% inhibition; $P<0.05$; **Figure 15.*C***) when compared to samples incubated with aspartame alone. A tendency for amastatin co-incubation to inhibit GLP-1 and PYY secretion was as well observed in the ileum. No clear pattern was found concerning GIP secretion (**Figure 15.*B***).

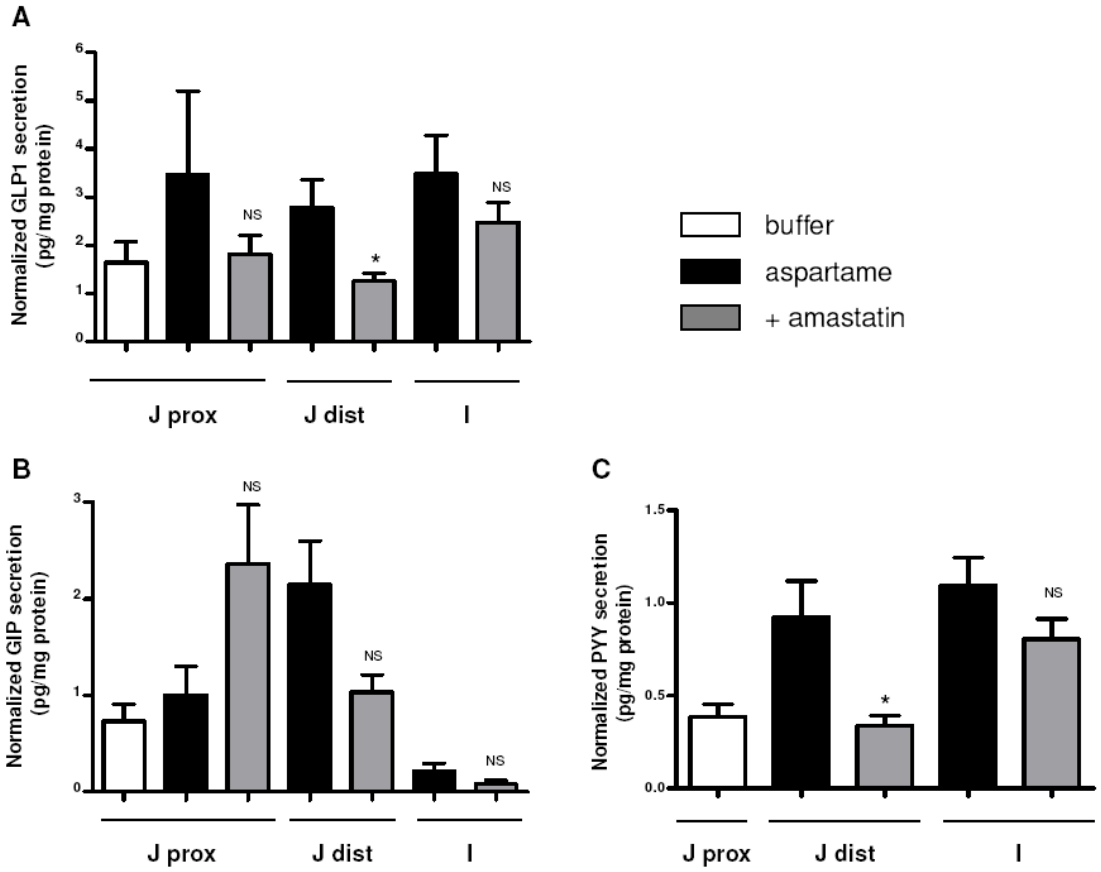

Figure 15. Effect of aspartame hydrolysis inhibition on hormone release in mice everted gut sacs. Everted gut sacs from 12-weeks-old C57BL/6 male mice (n=14) were incubated for 30min at

37°C, 5% CO_2 and 95% O_2, either in plain buffer, 1mM aspartame or co-incubated with 1mM aspartame + 10μM amastatin. Hormone secretion in the serosal fluid was measured by Bioplex and normalized to serosal volume and protein content; J prox / J dist / I = proximal jejunum / distal jejunum / ileum; Data are shown as Mean ± SEM; *****P<0.05 (paired-t-test).

Despite the fact that a high variation in response prevented significant effects of aspartame to be demonstrated, this *ex vivo* study in mouse intestine suggests that the artificial sweetener aspartame could elicit a GLP-1 and PYY secretion. However, this hormone secretion seems to require hydrolysis of the molecule into either the dipeptide or phenylalanine and aspartic acid, as co-incubation with amastatin inhibited both GLP-1 and PYY output observed in distal jejunum.

2.3. Effect of bitter tastants denatonium benzoate and phenylthiocarbamide (PTC) on GLP1 and PYY secretion from rat ileum rings.

2.3.1. *Background and aims of the study.*

G-protein-coupled receptors that signal bitter taste (T2Rs) are expressed in both rodent and human gastrointestinal tract. *In vitro*, bitter tastants like denatonium benzoate (DB) and phenylthiocarbamide (PTC) have been shown to elicit an increase in intracellular calcium followed by CCK secretion from the rodent STC-1 cell line (41). In assessing whether denatonium benzoate and PTC (**Figure 16.**) can induce hormone secretion in our rat gut rings model, the following study was carried out. The concentrations of bitter tasting compounds used (5mM) were taken from rat intragastric infusion studies (80, 90, 91).

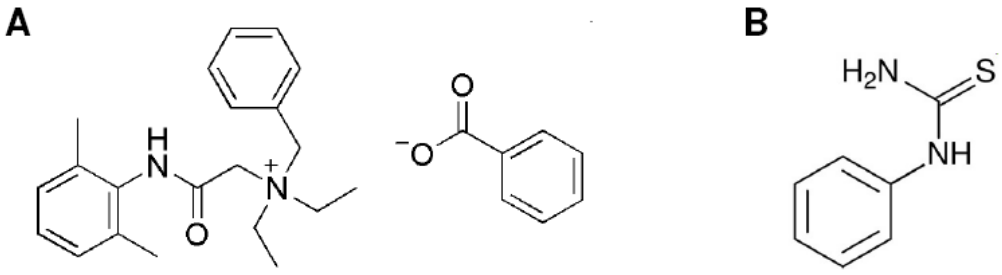

Figure 16. Bitter tastants denatonium benzoate (*A*) and phenylthiocarbamide (PTC) (*B*)

2.3.2. *Results and discussion.*

Denatonium benzoate elicited an average 2-fold, yet not significant increase in GLP-1 (**Figure 17.*A***) and PYY secretion (**Figure 17.*B***) from rat ileum rings when compared to samples incubated with buffer alone. The same concentration of PTC was also not able to significantly affect hormone secretion. The protein hydrolysate used as a positive control increased secretion of GLP-1 and PYY 4-fold (P<0.01) and 3-fold (P<0.001) respectively.

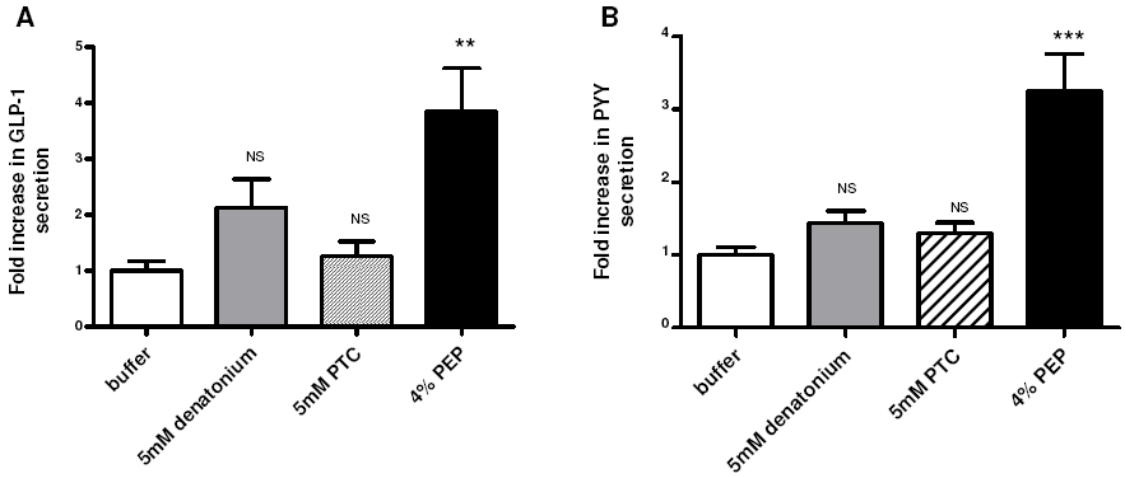

Figure 17. Effect of selected bitter tastants on hormone release in rat ileum rings. Ileum rings from 8-weeks-old male Sprague-Dawley rats (n=4) were incubated for 30min at 37°C either with plain buffer, 5mM denatonium benzoate, 5mM Phenylthiocarbamide (PTC) or 4% PEP. GLP1 (A) and PYY (B) secretion were measured by Bioplex; Data are shown as Mean ± SEM of triplicate

determinations from n=4 animals and are expressed as control fold (Krebs buffer); **P<0.01; ***P<0.001 (one way ANOVA + Bonferroni's multiple comparison test).

2.4. Conclusion

Satiety hormone secretion upon bitter stimulation in the gut remains poorly understood, and the only data currently available are derived from *in vitro* studies. Although the ultimate bitter reference compounds denatonium benzoate and phenylthiocarbamide failed to demonstrate a response in rat intestine with respect to GLP-1 or PYY secretion, rodent models could contribute to a better understanding of the physiological processes underlying bitter tasting. A combination of *in vitro* and *ex vivo* models was therefore applied to identify and characterize a novel intestinal bitter signaling pathway that is coupled to CCK secretion.

3. Bitter taste sensing and satiety hormone secretion in the gut: the steroid glycoside H.g.-12 from *Hoodia gordonii* extract induces CCK release from enteroendocrine cells via the activation of human bitter taste receptor TAS2R14

3.1. Background and aims of the study

Hoodia gordonii (Masson) Sweet ex Decne is a perennial, succulent plant specie from the Apocynacaea (previously Asclepiadaceae) family that is indigenous to arid regions of South Africa, Botswana and Namibia. Initial exploratory research with several species of *Hoodia* showed that *Hoodia gordonii* extract contained a mixture of steroid glycosides (103, 165, 207) that decreased food intake and body weight in animals (207). A 13-week feeding trial in rats with repeated administration of a purified extract of *Hoodia gordonii* containing at least 70% steroid glycosides was shown to induce a dose-dependent inhibitory effect on food intake and body weight (Nicholson *et al*. Safety profile of *Hoodia gordonii* extract: 13-week repeat administration study in the rat, in preparation). Two major steroid glycosides, H.g.-12 and H.g.-20, were purified from *Hoodia gordonii* and shown to exhibit food intake suppressive effects in rats, strongly indicating that H.g.-12 and H.g.-20 are at least two of the pharmacologically active components in *Hoodia gordonii* (207). However, the mechanisms of action by which the steroid glycosides might influence energy intake and appetite control are not fully understood. So far, only one study investigated potential mechanistic effects of one of the *Hoodia gordonii* steroid glycosides. Here it was hypothesised that H.g.-12 has a modulating effect on ATP production and its content in hypothalamic neurons after intracerebroventricular injection and after *in vitro* exposure of foetal hypothalamic neurons to the compound (136). However, it is not clear that the primary bioactivity

of *Hoodia* components is exerted post-absorptive. Furthermore, bioavailability studies must demonstrate that following ingestion, H.g.-12 has access to the brain tested regions *in vivo* and that concentrations of the active form of the molecule are likely to be achieved at the site of action.

Based on the bitter taste of purified *Hoodia gordonii* extracts, we hypothesised that the effects of *Hoodia gordonii* could in part be mediated by luminal sensing of its steroid glycosides in the small intestine. Recent scientific evidence indeed suggested functional taste-sensing mechanisms in the intestine resembling those found in the neuroepithelium of the tongue (65). Various members of the T2R family, along with components of the taste signalling pathway such as α-gustducin have been shown to be expressed in enteroendocrine cell lines, in the pancreas, as well as in the gastric and intestinal mucosa in rodents (41, 95, 106, 216, 217) and also in human colonic tissue (106, 180, 182). Data from these studies suggest that bitter taste receptors expressed in the small intestinal tract could be involved in sensing of food components. In addition, some *in vitro* and *in vivo* animal studies showed that sweet and bitter compounds known to mediate gustatory signals in the oral cavity elicit gut hormone secretion from enteroendocrine cells as well as a neural activation (90, 91, 102).

The TAS2R family contains approximately 25 receptors as identified by genomic sequencing (2, 38, 46, 143). Agonists for almost all human TAS2Rs have been identified (15, 16, 138, 147, 184); all agonists identified so far have been bitter tasting compounds (see reviews (18, 138) and tables in **Appendix 2**). Based on the presence of both bitter taste receptors and taste transduction proteins in gut enteroendocrine cells, it has been postulated that TAS2Rs may be involved in regulation of food intake via initiation of secretion of satiety hormones such as glucagon like peptide-1 (GLP-1),

peptide YY (PYY) and cholecystokinin (CCK). As described before, bitter tastants like denatonium benzoate (DB) and phenylthiocarbamide (PTC) cause an increase in intracellular calcium followed by cholecystokinin (CCK) secretion from rodent enteroendocrine STC-1 cells (41), and ofloxacin, a bitter ligand for TAS2R9, elicits GLP-1 secretion from human enteroendocrine NCI-H716 cells (59). In rats, an intragastric infusion of TAS2R agonists was reported to excite neurons in the nucleus tractus solitarius (NTS), to condition flavor aversions and to delay gastric emptying (80, 90, 91). NTS activation is followed by reflex activation of vagal efferent neurons, which itself results in changes in GI function, including inhibition of gastrointestinal motility (83). However, to date, there is only one study reporting changes in GI physiology in response to bitter compounds in rats (106), and in humans, the only study available (132) failed to show effects of an intragastric infusion of the bitter tastants quinine and naringin on gastric emptying. Direct effects of these bitter compounds on hormone secretion in enteroendocrine models have not been shown yet.

In the following section, the effect of the *Hoodia gordonii* steroid glycoside H.g.-12 on CCK secretion is demonstrated both *ex vivo* in rat intestine and *in vitro* in cultured human enteroendocrine cells. We furthermore investigated whether this hormone secretion is mediated via intestinal bitter receptors and demonstrated that H.g.-12 specifically and selectively activates TAS2R14 in Hutu-80 cells. Whether the activation of bitter receptors can elicit a hormone secretory response *in vitro* and *ex vivo* was addressed by employing a TAS2R14 inhibitor identified by screening of a chemical library. Finally, bitter receptor activation and subsequent satiety hormone secretion could be linked to some essential structural features of H.g.-12.

3.2. Additional materials and methods

The *Hoodia gordonii* steroid glycoside H.g.-12 was purified from a *Hoodia gordonii* purified extract (Russell, P.J., Swindells, C.; Chemical characterization of Hoodia gordonii extract.; Journal to be decided; 2009) and had a chemical purity of 92%. Removal of the tigloyl group from the steroid core by alkaline cleavage was used to generate detiglated H.g.-12; acid cleavage was used to generate the aglycon of H.g.-12 and combination of both processes led to the detiglated-aglycon of H.g.-12. Chemical structures are presented in **Figure 18**.

Hoodia gordonii

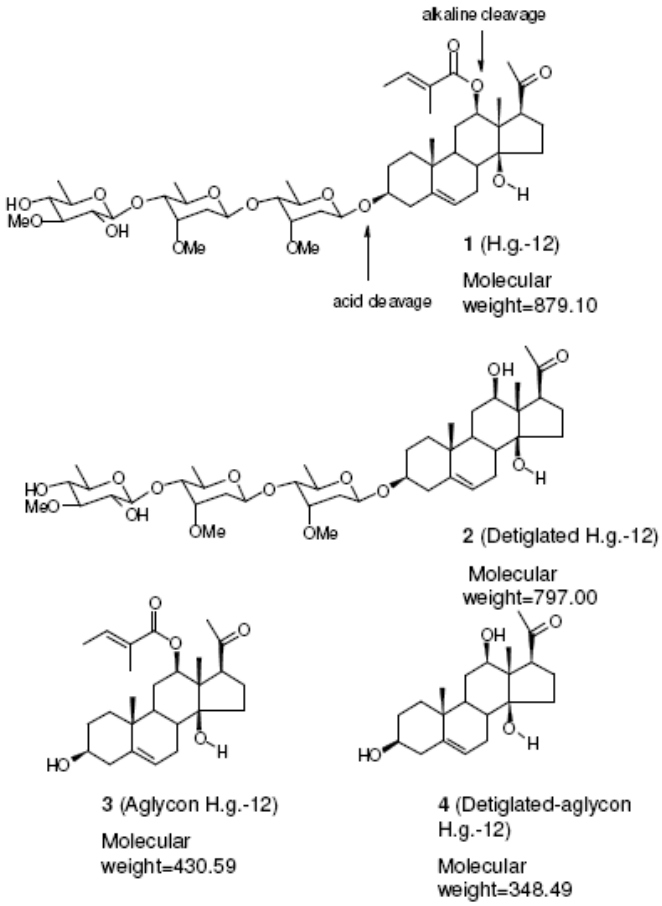

Figure 18. Chemical structures of H.g.-12, its aglycon, detiglated and detiglated aglycon. Removal of the tigloyl group from the steroid core by alkaline cleavage was used to generate detiglated H.g.-12; acid cleavage was used to generate the aglycon of H.g.-12 and combination of both processes led to the detiglated-aglycon of H.g.-12.

A commercially available library (Specs, The Netherlands) consisting of 10.000 chemical compounds (dissolved in DMSO at a 20mM stock concentration) was screened for TAS2R7 and TAS2R14 antagonists. Around 3000 compounds (final concentration 100μM) were co-incubated with 0.25mM H.g.-12 on TAS2R expressing HEK cells for identification of receptor blockers.

Polymerase chain reactions (PCR; Lightcycler, Roche Applied Science, Mannheim, Germany) were performed using Hutu-80 cells and human adult normal RNA for duodenum (R1234101-50) and jejunum (R1234230-50) purchased from BioChain (Hayward, USA) from 4 different male donors. Total RNA from Hutu-80 cells was isolated using the RNeasy mini kit (Qiagen) and cDNAs were synthesized by a ThermoScript RT-PCR system (Invitrogen). Specific PCR primers for TAS2Rs and GAPDH (to control the absence of genomic contamination in the probes) were designed using the Lightcycler Probe design software version 1.0 (Roche applied science) and synthetised by Eurofins (Eurofins MWG Operon, Ebersberg, Germany). Primers sequences were as follows: *hTAS2R7 (amplicon size = 215bp); forward primer 5'-ATTGTTCTTAGCAGTTGGAG-3'; reverse primer 5'-CTTTACCAGTGGCATAGAC-3'; hTAS2R14 (amplicon size = 344bp); forward primer 5'-AAGACTTGCAGTTCTGATT-3'; reverse primer 5'-GAGTGACATGAAGGATAAGC-3'; hGAPDH (amplicon size = 215bp); forward primer 5'-CATCGCTCAGACACCA-3'; reverse primer 5'-AGCTTCCCGTTCTCAG-3'* ; The sequence specificities of the primers were verified with NCI BLAST. In addition, RT-PCR on cDNAs from TAS2R7 and TAS2R14 expressing HEK cells were performed to test the primers reliability. PCR products were separated on agarose gel and stained with ethidium bromide. Gel images were recorded by a digital camera.

3.3. Results

3.3.1. *H.g.-12 elicits a significant CCK-8S secretion in a rat ex vivo model.*

A study with rat gut rings was conducted to assess whether H.g.-12 could elicit gastrointestinal hormone secretion. When rat duodenal tissue was exposed to 0.5mM of H.g.-12, a significant 1.4-fold increase in CCK-8S secretion was observed (**Figure 19.*A***, $P<0.001$). Here as a positive control, a protein hydrolysate (4%) was shown to significantly increase CCK secretion around 1.7-fold ($P<0.001$). In ileal tissue specimens, H.g.-12 tended to elicit a modest 1.6-fold GLP-1 secretion ($P>0.05$; **Figure 19.*B***) and a 1.5-fold increased PYY output ($P>0.05$, **Figure 19.*C***). In ileum however, the protein hydrolysate caused much more pronounced responses by increasing GLP-1 secretion up to 5-fold ($P<0.001$) and PYY secretion around 3-fold ($P<0.001$).

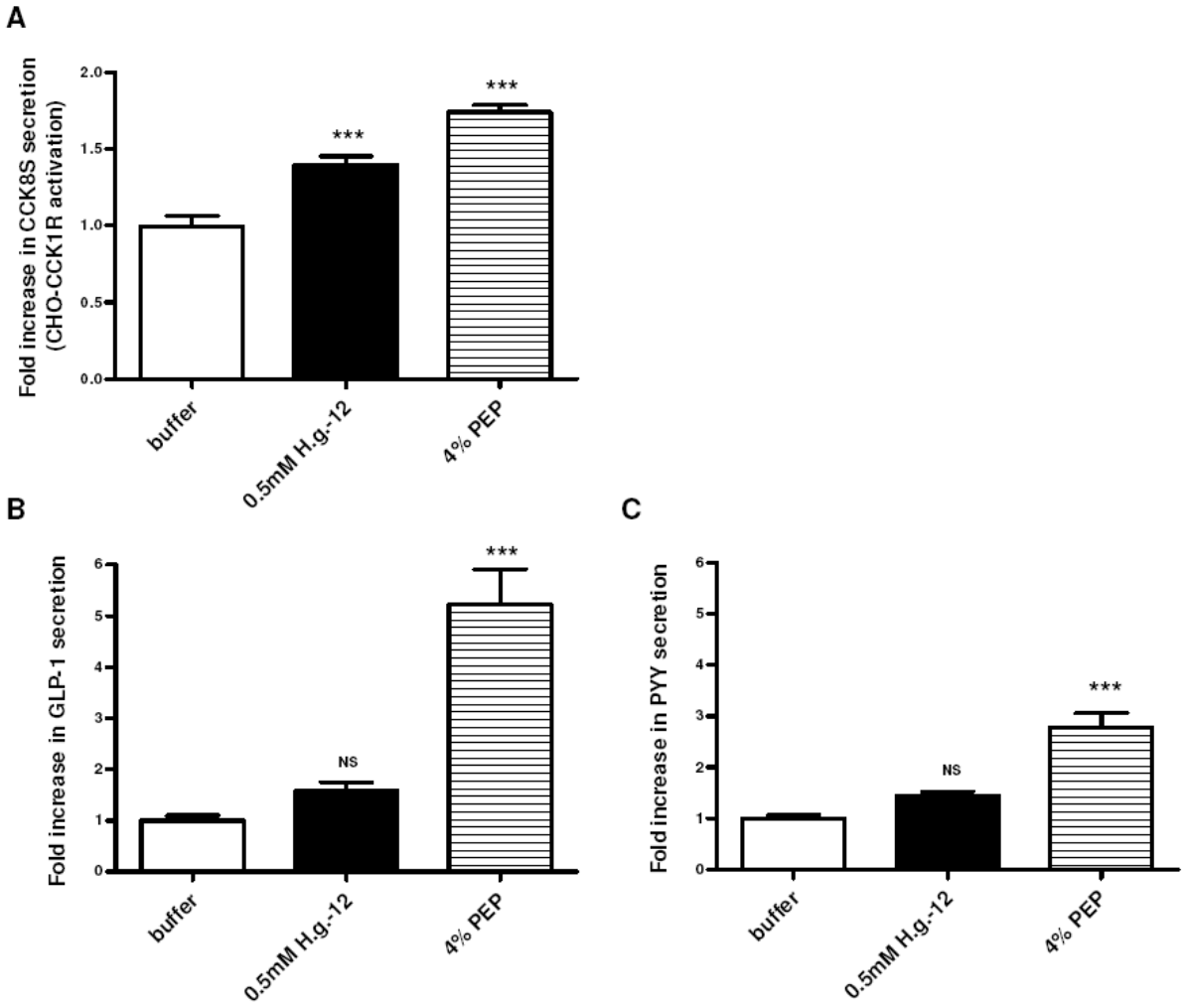

Figure 19. H.g.-12 induced hormone secretion pattern in the rat intestine *ex vivo* model. Duodenum and ileum sections (0.5cm) prepared from everted rat intestine were incubated with 0.5mM H.g.-12 and 4% PEP (positive control, protein hydrolysate) for 30 minutes at 37°C. After centrifugation (4°C) of the samples, CCK-8S levels were measured in the supernatants using the CCK_1R activation assay (***A***). Active GLP-1 (***B***) and total PYY (***C***) levels were measured using a rat-gut-hormone kit (Millipore). The data are averages of triplicate determinations from *n*=6 animals and are expressed as control fold (Krebs buffer); ****P*<0.001 (one way ANOVA + Bonferroni's multiple comparison test).

3.3.2. *H.g.-12 induces CCK release from the human enteroendocrine cell line Hutu-80.*

To investigate whether the steroid glycoside H.g.-12 could elicit hormone secretion from human enteroendocrine cells, Hutu-80 cells were exposed to increasing concentrations of H.g.-12 for 2 hours. H.g.-12-mediated CCK secretion into the medium was demonstrated by increased CCK receptor-1 activation in a receptor-expressing reporter cell line. A significant mean 3-fold increase ($P<0.001$) in CCK_1R activation as compared to the control cells was observed (**Figure 20.*A***). Whereas H.g.-12 exhibited potent effects on CCK secretion, removal of the tigloyl group completely abolished the CCK secretion from Hutu-80 cells (**Figure 20.*B***). H.g.-12 effects on CCK secretion in Hutu cells did not exhibit a clear dose-dependency. Concentrations of 30μM and below failed to elicit a significant CCK output, whereas concentrations ranging from 60μM to 500μM displayed similar effects on CCK_1R activation. In order to determine whether the effect of H.g.-12 on CCK secretion in Hutu-80 cells is specific or a more generic effect, we assessed whether bitter tastants like denatonium benzoate (DB), phenylthiocarbamide (PTC) or quinine could also elicit CCK secretion with H.g.-12 as a positive control. Whereas 0.25mM H.g.-12 in a robust manner increased 3-fold CCK release, DB, PTC and quinine at concentrations up to 10mM failed to cause a stimulation of CCK release in Hutu-80 cells (data not shown), thus demonstrating the selectivity of H.g.-12.

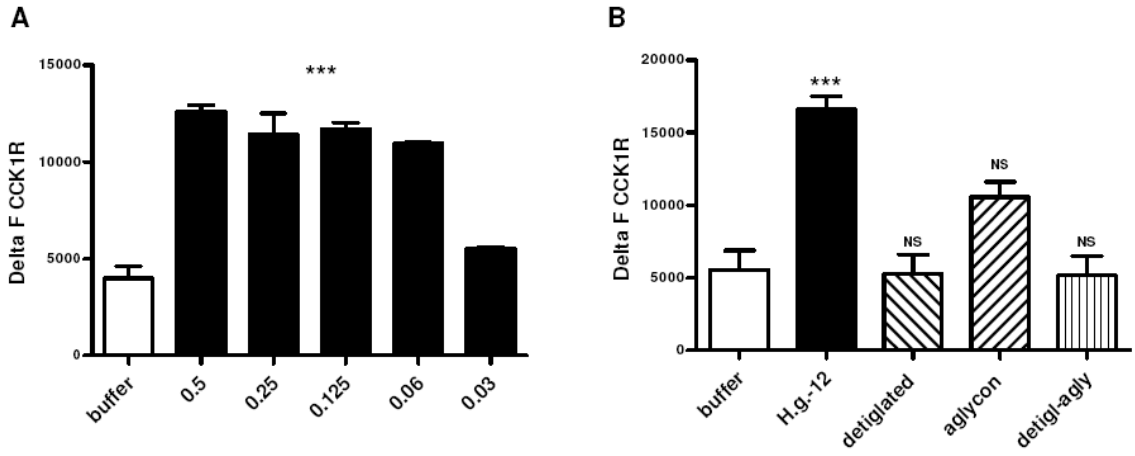

Figure 20. H.g.-12 structure-activity relationship on CCK secretion from Hutu-80 cells. Indirect determination of CCK-8S levels in Hutu-80 supernatants using the CCK_1R activation assay. ***A*:** Cells were incubated for 2 hours with increasing concentrations of H.g.-12 (0.03-0.5mM). The data are averages of triplicate determinations from three independent experiments; ****P*<0.001 (one way ANOVA + Bonferroni's multiple comparison test). ***B*:** Cells were incubated for 2 hours with 0.25mM of H.g.-12, detiglated, aglycon or detiglated-aglycon. The data are averages of triplicate determinations from three independent experiments; ****P*<0.001 (one way ANOVA + Bonferroni's multiple comparison test). All data are reported in changes of FLUO-4-AM fluorescence (Delta F).

3.3.3. *H.g.-12 selectively activates the human bitter receptors TAS2R7 and TAS2R14.*

Purified *Hoodia gordonii* extracts taste bitter. To identify whether the *Hoodia gordonii* steroid glycoside H.g.-12 selectively activates one or more human TAS2 bitter receptors, a screening of 25 human bitter receptors each expressed heterologously in HEK 293 cells was conducted (**Figure 21.**). Monitoring $[Ca^{2+}]_i$ revealed that 0.25mM H.g.-12 specifically activated hTAS2R7 (wells E4, E10) and hTAS2R14 (wells B5, B11) while receptor TAS2R43 displayed a non-specific response in induced cells (well A6). The increase in $[Ca^{2+}]_i$ was dose-dependent in case of TAS2R7 and TAS2R14 expressing cells for both H.g.-12 and its aglycon at concentrations ranging from 30μM to 250μM (**Figure 22.*A* and *B***). The absence of a plateau at high compound concentration, that

would allow calculation of an EC_{50} value, is due to the poor solubility of both H.g.-12 and its aglycon in buffer at concentrations above 250μM.

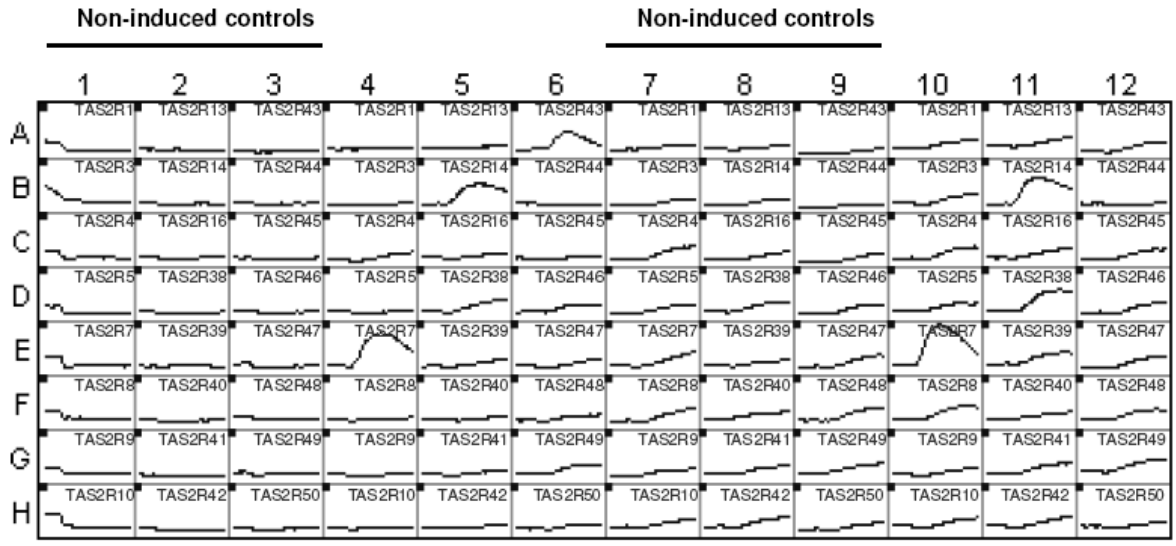

Figure 21. Screening of hTAS2Rs transfected HEK 293T cells for activation by H.g.-12. Activation of hTAS2Rs by H.g.-12 (0.25mM) was measured over 90s in TAS2R-expressing HEK cells by monitoring variations in FLUO-4-AM fluorescence (Delta F) induced by changes in $[Ca^{2+}]_i$. Non-doxycyclin-induced HEK cells were used as controls. Receptor identity is shown for each well of the 96 wells plate. Columns 1-3 and 7-9 are control cells, columns 4-6 and 9-12 are induced cells (all duplicates).

3.3.4. *Structural determinants of H.g.-12-induced activation of TAS2R7 and TAS2R14.*

The common structural elements in all *Hoodia gordonii* steroid glycosides are the steroid core, the tigloyl group and the chain of (deoxy and/or methoxy-) sugars (103). In order to understand which structures of H.g.-12 are essential for TAS2R activation, we tested H.g.-12, its aglycon, detiglated H.g.-12 and its detiglated aglycon on both TAS2R7 and TAS2R14 (**Figure 22.*C* and *D***). Whereas the removal of the tigloyl group abolished the activation of both TAS2R7 and TAS2R14, the removal of the sugar residues did not significantly alter TAS2R7 activation. The aglycon even appeared to be a better agonist for TAS2R14 when compared to H.g.-12 (**Figure 22.*D***; $P<0.001$).

Similarly to detiglated H.g.-12, the detiglated aglycon did not exhibit any effect on $[Ca^{2+}]_i$ in TAS2R7 and TAS2R14 expressing HEK cells (data not shown). All 3 molecules (H.g.-12, aglycon, detiglated) were equally soluble in buffer at 250μM concentration. To further investigate the role of the tigloyl group on TAS2R7 and TAS2R14 activation, we also tested geranyl-tiglate, citronellyl-tiglate and phenethyl-tiglate, but none of them activated the receptors significantly (data not shown).

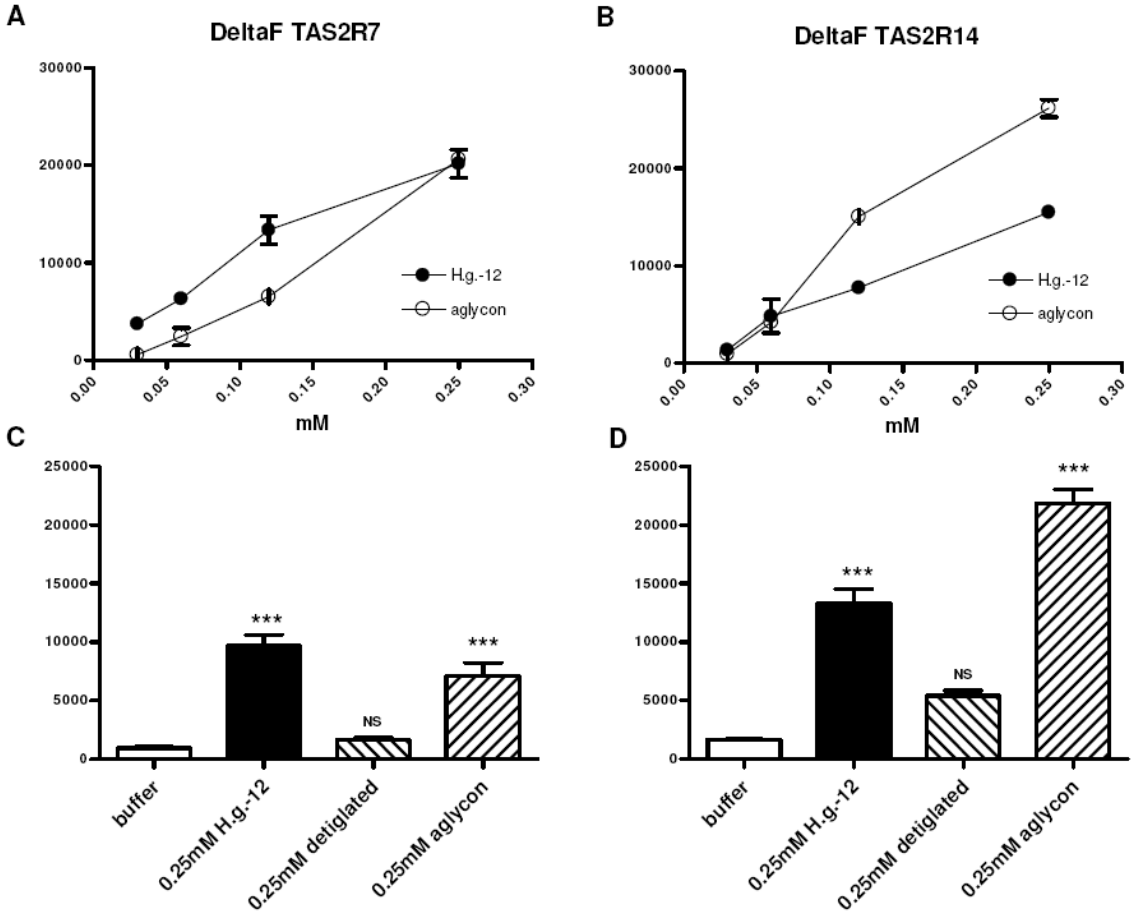

Figure 22. H.g.-12 structure-activity relationship on TAS2R7 and TAS2R14 activation.
***A* and *B*:** Changes in $[Ca^{2+}]_i$ in TAS2R7 and TAS2R14 expressing HEK cells activated by H.g.-12 and its aglycon (0.03-0.25mM). Data are averages of duplicate determinations from a representative experiment. ***C* and *D*:** $[Ca^{2+}]_i$ answer in TAS2R7 and TAS2R14 expressing HEK cells to either 0.25mM of H.g.-12, detiglated, or aglycon. The data are averages of triplicate determinations from three independent experiments; ****P*<0.001 (one way ANOVA +

Bonferroni's multiple comparison test). All data are reported in changes of FLUO-4-AM fluorescence (Delta F).

3.3.5. *TAS2R14 but not TAS2R7 is expressed in Hutu-80 cells and human proximal intestine.*

A PCR-based detection of the receptor mRNA levels was conducted in the enteroendocrine cells as well as in human proximal small intestinal tissue samples to establish the expression pattern of the two TAS2Rs. The presence of the TAS2R14 transcript was found in the Hutu-80 enteroendocrine cell line of duodenal origin as well as in human normal duodenum and jejunum (**Figure 23.**). The presence of a TAS2R7 transcript could be detected neither in Hutu-80 cells nor in human proximal intestinal tissues whereas in the positive control, TAS2R7 expressing HEK cells, the transcript was detected (data not shown).

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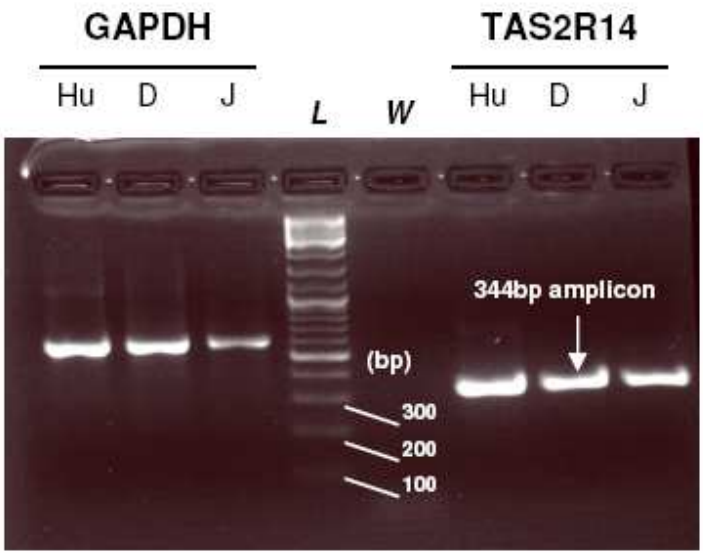


Figure 23. TAS2R14 is expressed in Hutu-80 human enteroendocrine cells and human proximal intestine. Expression of human taste receptor TAS2R14 in Hutu-80 enteroendocrine cells (*Hu*) and human normal duodenum (*D*) and jejunum (*J*). PCR was performed using the specific primers listed in methods to detect the expression of TAS2R14 (predicted amplicon size 344bp). PCR for GAPDH was performed to verify the absence of genomic contamination in the probes. *L*: DNA ladder; *W*: PCR performed without DNA as a negative control.

3.3.6. *Identification of a TAS2R14 inhibitor.*

TAS2R activation patterns by various structurally related H.g.-12 derivatives were mirrored in the CCK secretion studies in Hutu-80 cells. Thus, the removal of the tigloyl group from H.g.-12 abolished the activation of both bitter receptors and the CCK secretion in Hutu cells. This suggested that the CCK secretory effects of H.g.-12 are mediated by TAS2Rs in Hutu-80 cells. As only TAS2R14 was found to be expressed in Hutu-80 cells and human proximal intestine, we focused our efforts for the identification of an antagonist on TAS2R14. After screening of a chemical library with 10.000 compounds, a co-incubation experiment with the lead candidate called 03A3 in a concentration of 100μM in the presence of the activating ligand H.g.-12 (0.25mM) caused a 35% inhibition of TAS2R14 receptor activation (P<0.05; **Figure 24. *A***).

3.3.7. *H.g.-12 induced CCK secretion from Hutu-80 cells is mediated by TAS2R14.*

To further substantiate our hypothesis that H.g.-12 induces the secretion of CCK via TAS2Rs in Hutu-80 cells, we tested the effect of H.g.-12 on CCK secretion in the presence and the absence of the TAS2R14 inhibitor. Whereas 0.25mM H.g.-12 alone significantly increased CCK secretion 2-fold when compared to the buffer control, co-incubation with TAS2R14 inhibitor 03A3 in concentrations of 50 and 100μM completely abolished CCK release from Hutu cells (P<0.001; **Figure 24. *B***).

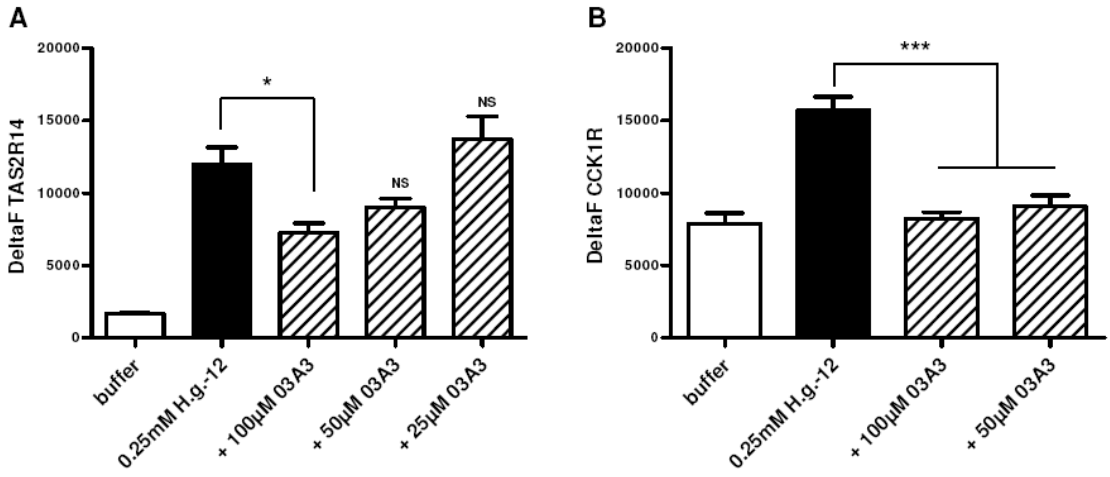


Figure 24. Inhibition of H.g.-12 induced activation of TAS2R14 and CCK secretion in Hutu-80 cells by compound 03A3. ***A*:** $[Ca^{2+}]_i$ answer in TAS2R14 expressing HEK cells to 0.25mM H.g.-12 alone (positive control) or co-incubated with compound 03A3 (25-50-100μM). $[Ca^{2+}]_i$ were determined using FLUO-4-AM and are reported in Delta F. The data are averages of triplicate determinations from two independent experiments; **P*<0.05 (one way ANOVA + Dunnett's multiple comparison test). ***B*:** Indirect determination of CCK-8S levels in Hutu-80 supernatants using the CCK_1R activation assay. Cells were incubated for 2 hours with 0.25mM H.g.-12 alone (positive control), or co-incubated with compound 03A3 (50-100μM). The data are averages of triplicate determinations from two independent experiments; ****P*<0.001 (one way ANOVA + Bonferroni's multiple comparison test).

3.3.8. *In silico search for H.g.-12 similar compounds.*

An *in silico* search for H.g.-12 structural analogues was conducted using the software *pipeline pilot* on chemical and natural compounds databases (respectively 6 Million and 200.000 compounds). Presence of a tiglate group in the candidate molecules was indicated as a mandatory criterium. The natural compounds library screening led to good candidates exhibiting up to 86% similarity with H.g.-12 (**Figure 25.**), but none of the identified compounds turned out to be commercially available.

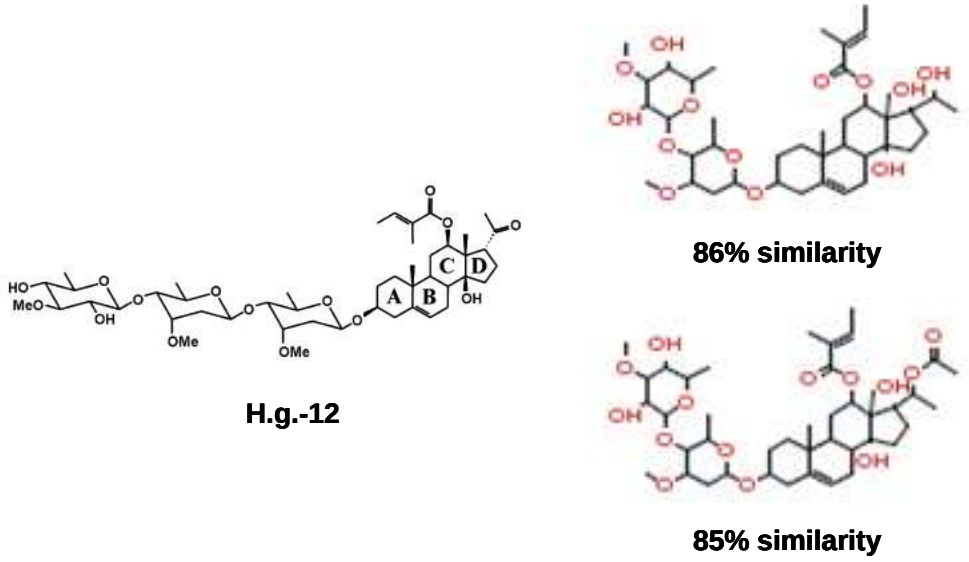


Figure 25. Natural compounds exhibiting a close chemical similarity to H.g.-12. *In silico* search with the software *pipeline pilot* done by Leo Van Buren (Unilever). Pregn-5-ene-3,12,14,17,20-pentol (*Marsdenia formosana* origin)

3.4. Discussion

We here demonstrate for the first time that the *Hoodia gordonii* steroid glycoside H.g.-12 elicits a CCK release from rat *ex vivo* tissue preparations and human enteroendocrine cells in culture. In addition, we have also established that H.g.-12 is an agonist for the human bitter receptors TAS2R7 and TAS2R14, the latter being expressed in Hutu-80 cells and human proximal intestine. The tigloyl group in the steroid molecule was found to be an essential structural feature needed for bitter receptor activation. Furthermore, by testing a novel antagonist for TAS2R14 and by employing derivatives of H.g.-12 in both TAS2R14 over-expressing cells and in human enteroendocrine cells, we have supportive but not conclusive evidence that CCK secretion from enteroendocrine cells is mediated via TAS2R14. Our experiments emphasize the notion that the steroid glycoside H.g.-12 could via bitter receptor activation in endocrine cells cause CCK-8

release for which central effects on appetite suppression are known (see (40, 195) for reviews), thereby contributing to the satiety effects as shown for *Hoodia gordonii* in a rat model (207).

Rat tissues exposed *ex vivo* to H.g.-12 responded with significantly increased CCK secretion. This tissue model takes into account the morphology in which enteroendocrine cells are surrounded by enterocytes and an intact enteric nervous system. Although the finding that H.g.-12 induced CCK secretion in this system is considered to be physiological, future studies would be required to confirm that H.g.-12 increases CCK *in vivo* to plasma concentrations which could explain a behavioural effect. Furthermore, it remains speculative whether the effect of H.g.-12 on CCK secretion in rat tissues is mediated by bitter receptor activation. It has been shown that rT2R6, the rat orthologue gene to TAS2R7 (70% of sequence identity, **Figure 26.**), is expressed in rat gastric and duodenal mucosa (216, 217), but for TAS2R14 no close orthologue has yet been found in rats. Thus, whether CCK secretion in rat duodenum depends on the TAS2R7 orthologue and whether this receptor is activated in a similar manner as the human receptor is currently not known. However, *Hoodia gordonii* extract was shown to exhibit strong food intake inhibitory effects in rats (207).

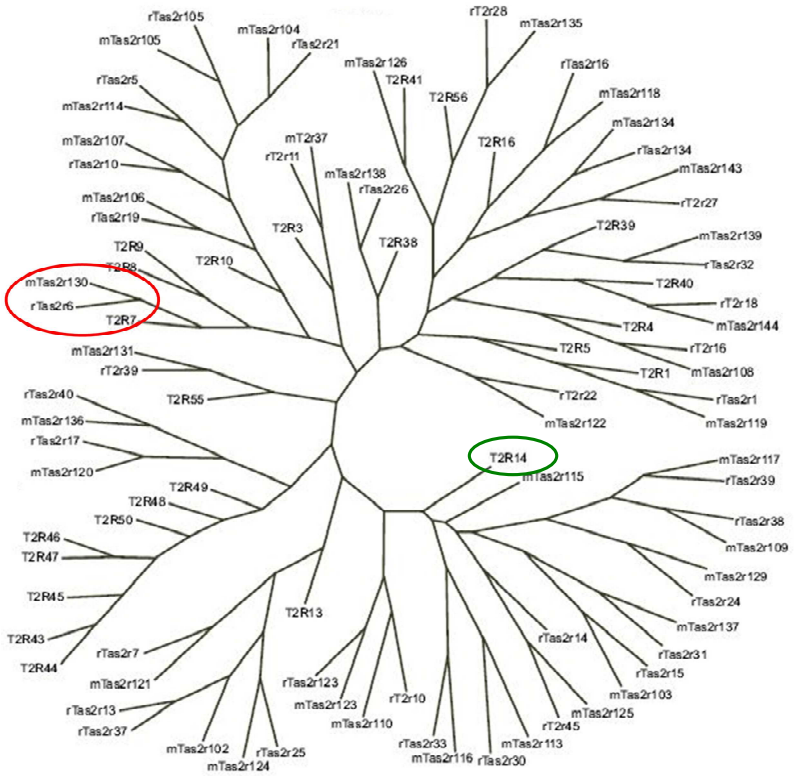


Figure 26. Phylogenetic tree of the bitter receptor family in human, rat and mouse.
Adapted from Gloriam, D., Fredriksson, R., Schioth, H.; The G-protein-coupled receptor subset of the rat genome; *BMC Genomics*; 2007.

By employing the human enteroendocrine cell line Hutu-80, we demonstrated that H.g.-12 can increase CCK secretion 2- to 3 times although no coherent dose-dependency could be observed. However, the selectivity of H.g.-12-induced stimulation in CCK secretion from Hutu-80 cells was strongly suggested by the lack of secretory effects of bitter compounds such as denatonium benzoate, PTC and quinine, even at concentrations as high as 10mM. Although PTC was shown previously to increase $[Ca^{2+}]_i$ in Hutu-80 cells (182), it did not drive CCK output in our experiments. In this respect, the capability of H.g.-12 to induce a CCK release from Hutu-80 cells

is an original feature amongst the various bitter tastants. The structure-specificity in the ligands that cause CCK release was demonstrated by the removal of the tigloyl group (detiglated and detiglated-aglycon forms) that abolished the capability for stimulation of CCK secretion.

The H.g.-12 steroid glycoside from *Hoodia Gordonii* extract is here identified as a new agonist for both TAS2R7 and TAS2R14. TAS2R7 displays a quite broad agonist spectrum with 9 ligands already described, structurally unrelated to H.g.-12 (147, 184). As for TAS2R14, it is activated by 33 compounds without an obvious common structural motif (16, 147). This makes TAS2R14 one of the broadest human bitter receptor in terms of agonist spectrum along with TAS2R10 and TAS2R46, which all 3 tend to display a preference for natural compounds (147). We have observed that removal of the tigloyl group from H.g.-12 abolishes its interaction with both tested TAS2 receptors. However, tiglic acid alone and three structurally unrelated compounds containing a tigloyl group did not activate TAS2R7 or TAS2R14. This demonstrates that the tigloyl function as such is not the sole structural feature needed for TAS2R activation. True quantitative structure-activity relationship studies were not possible due to very limited availability of H.g.-12-related structures. We observed that removal of the glycoside groups (aglycon) in H.g.-12 increased selectivity towards receptor TAS2R14 upon TAS2R7. Using a simulated gastric and small intestinal digestion model, it was recently suggested that H.g.-12 is partly deglycosylated during gastrointestinal digestion (137). In the same study, the aglycon molecule was shown to cross both the intestinal and blood brain barriers by passive diffusion. However, further studies would have to demonstrate that the aglycon is not extensively metabolised during intestinal absorption and/or first liver passage before *post*-absorptive systemic effects are proposed. This is, however,

conceptually different from the *pre*-absorptive effects of H.g.-12 at luminal-facing taste receptors in the gastrointestinal tract suggested here.

We identified the transcript for TAS2R14 as expressed in Hutu-80 cells as well as in human duodenal and jejunal tissue. In contrast, the TAS2R7 transcript was not detectable with the probes employed in cell and tissue samples. Our data confirm recent findings (182) on the presence of TAS2R14 but not TAS2R7 in Hutu-80 cells together with 13 other TAS2Rs. Although TAS2R7 and TAS2R14 are both found in the neuroepithelium of the tongue (17), they could not be detected in human colon (182) and TAS2R7 could also not be identified in human ileal NCI-H716 cells or human caecum samples (187). Concerning the proximal intestine, this is to our knowledge the first report demonstrating an expression of TAS2R14 in human duodenum and jejunum. However, detailed cellular and sub-cellular distribution of TAS2R14 proteins in intestinal tissues, combined with co-localization data for gut hormones are needed before any conclusions on the role of TAS2R14 in physiology can be made.

By screening of a chemical library, compound 03A3 was found to significantly reduce the TAS2R14 mediated $[Ca^{2+}]_i$ increase upon H.g.-12 stimulation. As a control for the antagonist specificity towards TAS2R14, a co-incubation study was performed on TAS2R7 expressing HEK cells, showing no alteration of the receptor activation (data not shown). By using the Hutu-80 model, we found that co-exposure of 0.25mM H.g.-12 with the TAS2R14 blocker completely abolished CCK secretion. These data support the selectivity and specificity of both the TAS2R14 activation by H.g.-12 and the consecutive release of CCK.

In conclusion, we provide evidence that the appetite-suppressant properties of *Hoodia gordonii* extract may originate -at least in part- from its main steroid glycoside H.g.-12. We thus proved that H.g.-12 elicits a CCK release both from rat duodenal tissues and from enteroendocrine cultured cells through the activation of 2 subtypes of human bitter receptors. CCK is known to induce satiety in rodents (78, 195) and in humans (49, 112, 132). In addition, T2R agonists administered into the stomach by oral gavage in rats were found to increase c-Fos gene expression and c-Fos positive neuron-numbers in the nucleus of the solitary tract (NTS). CCK_1 receptors located on vagal afferent terminals in the gut wall are thought to be the link in this gut-brain axis (90, 91). The vagal afferent terminals found in the lamina propria are in close vicinity to the enteroendocrine cells, suggesting that here takes place the transformation of the chemosensor input signals into neuronal signals. Combined with a possible direct interaction of CCK secreted from the intestine with the CCK_2 receptor in the brain, the inhibition of food intake associated with *Hoodia gordonii* intake in rats and putatively in humans could result, in part, from this intestine to brain communication route.

4. Future perspectives

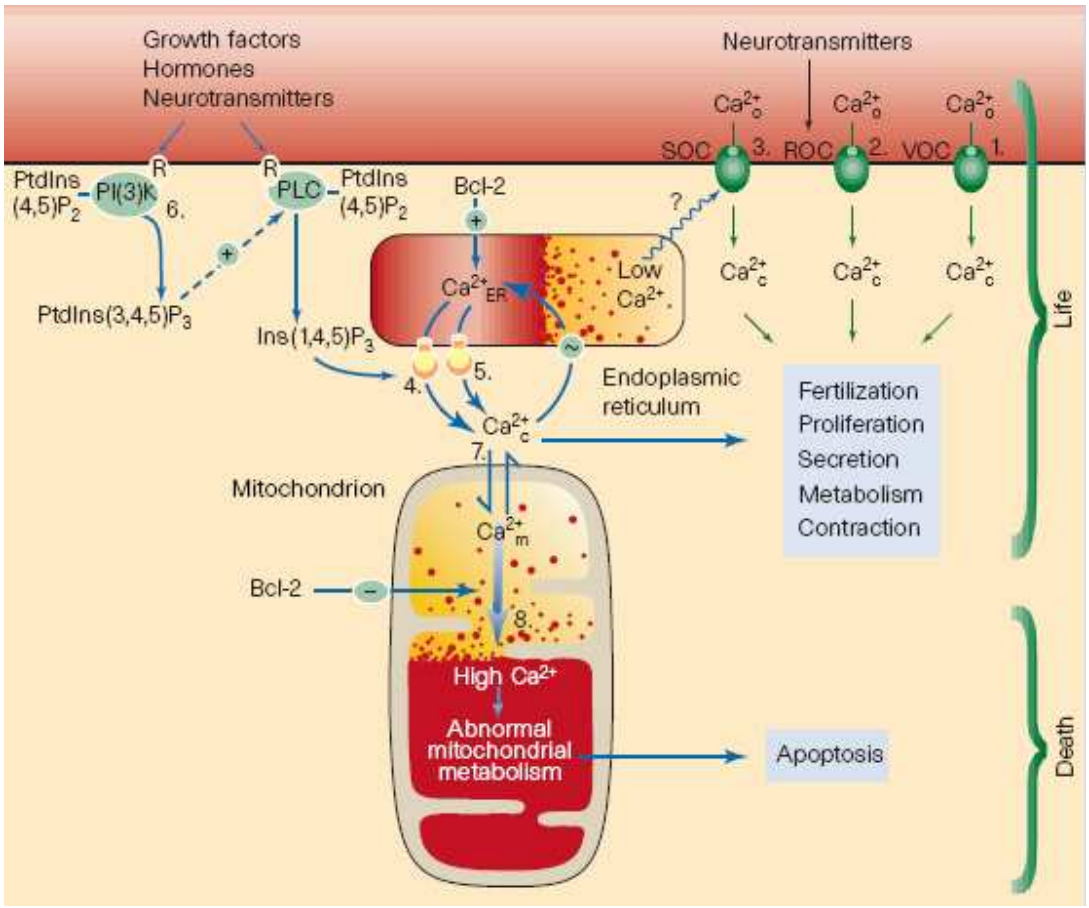
In the experiments conducted as basis of the present thesis, we identified two novel sensing pathways in human enteroendocrine cells leading to hormone secretion that in turn could also affect short- and long-term satiety (CCK, GLP-1) and stimulate glucose-induced insulin secretion from the pancreatic *β*-cells (GLP-1). This has been achieved through the combined use of available cell models and newly characterized *ex vivo* models of rodent intestine.

The beneficial role played by intestinal peptides like GLP-1 in diseases as type 2 diabetes has fostered many efforts towards the development of therapeutical strategies based on exogenous hormone injection and use of DPP-IV-resistant analogs. Knowledge of the sensors and the signaling mechanisms involved in intestinal hormone secretion in response to dietary inputs is however still limited and provided the starting points for the current project. With the focus on bitter tastants and peptide ligands for gastrointestinal hormone responses, new findings have been obtained. That bitter receptors expressed in the intestine can sense the luminal contents and elicit a subsequent CCK secretion may hopefully foster more research into these sensing processes. Previously considered as solely expressed in the lingual epithelium, bitter receptors have been shown to be expressed as well in the intestine and more particularly in enteroendocrine cells. In the time of only a few years, their role has thus evolved from bitter taste perception in the mouth towards sensors in the gut that enable physiological changes in the gastrointestinal tract.

Further exploration of the unique ability of the intestine to sense the outside world through a complex network of transporters, sensors, nerves and to then convey the information to the brain, provides exciting opportunities for new research lines in coming years.

APPENDIX

APPENDIX 1



Calcium sources and mechanisms of calcium signaling. Scheme from Berridge, M.J., Bootman, M.D., Lipp, P.; Calcium, a life and death signal; *Nature;* 1998. Store-operated calcium channels (SOC) are found on the upper right side of the picture, along with the Receptor-operated calcium channels (ROC) and the Voltage-operated calcium channels (VOC).

APPENDIX 2

hTAS2R	agonists (n)	examples agonists	EC50	References
1	**14**	chloramphenicol		Maehashi *et al*, 2008; Meyerhof *et al*, 2009
3	**1**	chloroquine	172µM	Meyerhof *et al*, 2009
4	**15**	denatonium benzoate		Chandrashekar *et al*, 2000; Meyerhof *et al*, 2009
		6-n-propyl-2-thiouracil (PROP)		
5	**1**	1,10-phenanthroline	0.1-1mM	Meyerhof *et al*, 2009
7	**9**	strychnine		Sainz *et al*, 2007; Meyerhof *et al*, 2009
		quinacrine		
		chloroquine		
		papaverine		
		quinine		
		chlorpheniramine		
		cromolyn		
		diphenidol		
		caffeine		
8	**4**	saccharin		Pronin *et al*, 2007; Meyerhof *et al*, 2009
9	**3**	ofloxacin		Dotson *et al*, 2008; Meyerhof *et al*, 2009
		procainamide		
		pirenzapine		
10	**32**	coumarin		Meyerhof *et al*, 2009
13	**2**	diphenidol		Meyerhof *et al*, 2009
		denatonium benzoate		
14	**33**	picrotoxinin	18µM	Behrens *et al*, 2004; Sainz *et al*, 2007; Meyerhof *et al*, 2009
		1-naphthoic acid	36µM	
		(-)-alpha-thujone	15µM	
		1,8-naphthalaldehydic acid		
		1-nitronaphthalene		
		picrotin		
		piperonylic acid		
		sodium benzoate		
		aristocholic acid		
16	**7**	beta-glucopyranosides		Bufe *et al*, 2002; Meyerhof *et al*, 2009
38	**21**	PROP		Bufe *et al*, 2005; Kim *et al*, 2003; Meyerhof *et al*, 2009
		PTC		
39	**11**	colchicine		Meyerhof *et al*, 2009
40	**orphan**			
41	**orphan**			
42	**orphan**			

Human bitter receptors (hTAS2Rs) and their identified agonists. Table compiled from the litterature. Humulone isomers (bitter compounds found in hops, beer) were identified as agonists for TAS2R1, TAS2R14 and TAS2R40 by Intelmann, D. *et al*., 2009, but the findings could not be reproduced by Meyerhof, W. *et al*., 2009.

APPENDIX 2 (continued)

hTAS2R	agonists (n)	examples agonists	EC50	References
43	**16**	saccharin		Kuhn *et al*, 2004; Pronin *et al*, 2004; Sainz *et al*, 2007; Meyerhof *et al*, 2009
		Acesulfame K		
		aristocholic acid		
		6-nitrosaccharin		
		IMNB		
44	**8**	saccharin		Kuhn *et al*, 2004; Pronin *et al*, 2004; Meyerhof *et al*, 2009
		Acesulfame K		
		aristocholic acid		
45	**orphan**			
46	**28**	strychnine		Brockhoff *et al*, 2007; Reichling *et al*, 2008; Meyerhof *et al*, 2009
		sesquiterpene lactones		
		absinthin		
47	**10**	6-nitrosaccharin		Pronin *et al*, 2004; Sainz *et al*, 2007; Meyerhof *et al*, 2009
		denatonium		
		denatonium derivatives		
48	**orphan**			
49	**2**	diphenidol		Meyerhof *et al;* 2009
		cromolyn	45µM	
50	**2**	andrographolide		Behrens *et al*, 2009; Meyerhof *et al*, 2009
		amarogentin		

Human bitter receptors (hTAS2Rs) and their identified agonists. Table compiled from the litterature. Humulone isomers (bitter compounds found in hops, beer) were identified as agonists for TAS2R1, TAS2R14 and TAS2R40 by Intelmann, D. *et al*., 2009, but the findings could not be reproduced by Meyerhof, W. *et al*., 2009.

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2nd Nusisco meeting, Munich, Germany, 06.2007

3d Nusisco meeting, Vlaardingen, the Netherlands, 09.2007

4th Nusisco meeting, London, UK, 01.2008

5th Nusisco meeting, Vlaardingen, the Netherlands, 09.2008

6th Nusisco meeting, London, UK, 05.2009

Poster presentations:

2007 Le Nevé, B. and Daniel, H. Selected tetrapeptides lead to a GLP-1 release from the human enteroendocrine cell line NCI-H716. Unilever R&D center, Vlaardingen, the Netherlands.

2008 Le Nevé, B. and Daniel, H. Ex vivo models to assess hormone secretion. Unilever R&D center, Vlaardingen, the Netherlands.

2009 Le Nevé, B., Foltz, M., Daniel, H. and Gouka, R. The steroid glycoside H.g.-12 from *Hoodia gordonii* extract induces CCK release from enteroendocrine cells via the activation of human bitter taste receptor TAS2R14. 4ème Congrès de la Société Française de Nutrition (SFN), Montpelliers, France.

2010 Le Nevé, B., Foltz, M., Daniel, H. and Gouka, R. The steroid glycoside H.g.-12 from *Hoodia gordonii* extract induces CCK release from enteroendocrine cells via the activation of human bitter taste receptor TAS2R14. 30th Blankenese Conference (sensory and metabolic control of energy balance), Hamburg, Germany.

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The present thesis is based on following accepted manuscripts:

I **Boris Le Nevé** and Hannelore Daniel; *Molecular Nutrition Unit, Technical University of Munich, Freising-Weihenstephan, Germany;* Selected tetrapeptides lead to a GLP-1 release from the human enteroendocrine cell line NCI-H716; ***Regulatory peptides*** (accepted and in press; November 8, 2010; doi:10.1016/j.regpep.2010.10.010)

II **Boris Le Nevé,**[1] Martin Foltz,[2,*] Hannelore Daniel,[1] and Robin Gouka[2]; [1] *Technical University of Munich* [2] *Unilever R&D;* The steroid glycoside H.g.-12 from Hoodia gordonii activates the human bitter receptor TAS2R14 and induces CCK release from HuTu-80 cells; ***Am J Physiol Gastrointest Liver Physiol*** (accepted and in press; October 7, 2010; doi:10.1152/ajpgi.00135.2010)