TECHNISCHE UNIVERSITÄT MÜNCHEN

Lehrstuhl für Ernährungsphysiologie

# Characterization of protein and peptide sensing processes in the gut coupled to gastrointestinal hormone secretion using *ex-vivo* models

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## ABSTRACT

The gut epithelium expresses a chemosensory system that allows the sensing of luminal nutrients and the release of hormones and regulatory peptides from endocrine cells. Activation of endocrine cells and the subsequent release of hormones by luminal nutrients is a key element in the regulation of digestion and absorption but also of satiety. Dietary proteins are known to be potent satietogenic food components that also release gut hormones. However, the underlying sensing mechanisms are not yet known. The aim of the present thesis was a) to establish new *ex vivo* methods for characterization of protein sensing mechanisms and b) assess in these models how individual receptors and transporters participate in peptide sensing coupled to gut hormone release. Finally, by combining *ex vivo* and *in vivo* methods it was demonstrated that a novel sensing pathway involving the peptide transporter PEPT1 in enterocytes mediates signaling into endocrine cells causing protein-dependent GLP-1 secretion. In addition, umami peptide sensing was studied and identified a G-protein coupled receptor in the intestine that is not only activated by sodium-glutamate but also by a variety of umami peptides. A network of signaling pathways and a crosstalk between different cell types appears to provide stimulus-secretion coupling in hormone secretion in response to dietary protein.

## ABBREVIATIONS

AgRP: Agouti-Related Protein

ATCC: American Type Culture Collection

BSA: Bovine Serum Albumin

CART: Cocaine- and Amphetamine-Regulated Transcript

CCK: Cholecystokinin

CCK1R: CCK receptor-1

CHO cells: Chinese Hamster Ovary cell

ChrA: Chromogranin A

DAPI: 4'-6-Diamidino-2-Phenylindole

DPP-IV inhibitor: Dipeptidyl Peptidase IV inhibitor

DTT: Dithiothreitol

ELISA: Enzyme-Linked Immunosorbent Assay

ER: Endoplasmic Reticulum

FBS: Fetal Bovin Serum

GIH: Gastrointestinal Hormones

GIP: Gastric Inhibitory Polypeptide or Glucose-dependent Insulinotropic Polypeptide

GLP-1: Glucagon-Like Peptide-1

GLUT-2: Glucose Transporter type 2

GPRc: G-Protein Coupled Receptor

hGPR93: Human G-Protein coupled Receptor 93

HEK293 cells: Human Embryonic Kidney cells

HP: High Protein load

IMP: Inosine 5′-Monophosphate

Lac: Lactisol

LCFA: Long Chain unsaturated Fatty Acids

Lectin UEA-1: Lectin Ulex Europaeus Agglutinin 1

LPA: Lysophosphatidic Acid

α-MDG: α-methyl-glucopyranoside

NPY: Neuropeptide Y

NTS: Nucleus Tractus Solitarius

OGTT: Oral Glucose Tolerance Test

OGTTPH: Oral Glucose Tolerance Test and Protein Hydrolysate

PEP: Peptorpro, protein hydrolysate

PEPT1: Peptide Transporter 1

PFA: Paraformaldehyde

PYY: Peptide YY

SCFA: Short Chain Fatty Acids

SGLT-1: Sodium-coupled Glucose Transporter-1

TEVC: Two-Electrode Voltage Clamp

T1R2/R3: T1R2/R3 sweet taste receptor

hT1R1/R3: human T1R1/R3 umami taste receptor

VDCC: Voltage-Dependent Calcium Channel

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# INTRODUCTION

## 1. Energy and food intake regulation: The gut and energy balance

### 1.1. Energy balance regulation along the gut-brain axis

Energy balance is a metabolic state when total energy expenditure equals dietary energy intake. It is well regulated both in the *short-* and *long-term*. However, in some individuals there is an imbalance between energy intake and energy expenditure resulting in weight gain and, ultimately, obesity. Adipose tissue has been found to play an important role in the *long-term* control of food intake **(Fig. 1)** by producing several endocrine and paracrine mediators, such as leptin which is released into the blood in proportion to the amount of body fat and known to influence food intake (8, 70). It is now recognized that the gastrointestinal tract is involved in the *short-term* control of food intake (108) **(Fig. 1)**. Evidence supports the existence of a system in the gut lumen that senses the presence of nutrients, leading to a release of hormones which act as postprandial satiety signals. These hormones can affect the brain through two mechanisms. Some of the secreted hormones act via the vagus nerve to stimulate ascending pathways from gut to the brain, whereas others are conveyed via the circulation directly to the brain **(Fig. 1)**. The main regions in the brain involved are the hypothalamus, in particular the arcuate nucleus, and the dorsal vagal complex in the brain stem **(Fig. 1 and 2)**. The arcuate nucleus integrates *long-term* or *short-term* signals from the periphery and from the brain stem. The arcuate nucleus contains two distinct subsets of neurons controlling food intake. One acts as a stimulus to feeding and these neurons contain neuropeptide Y (NPY) and the agouti-related peptide. The second subset of neurons acts as an inhibitor to feeding, these contain $\alpha$-MSH and cocaine- and amphetamine-regulated transcript (CART) (17, 116, 128) **(Fig. 2)**.

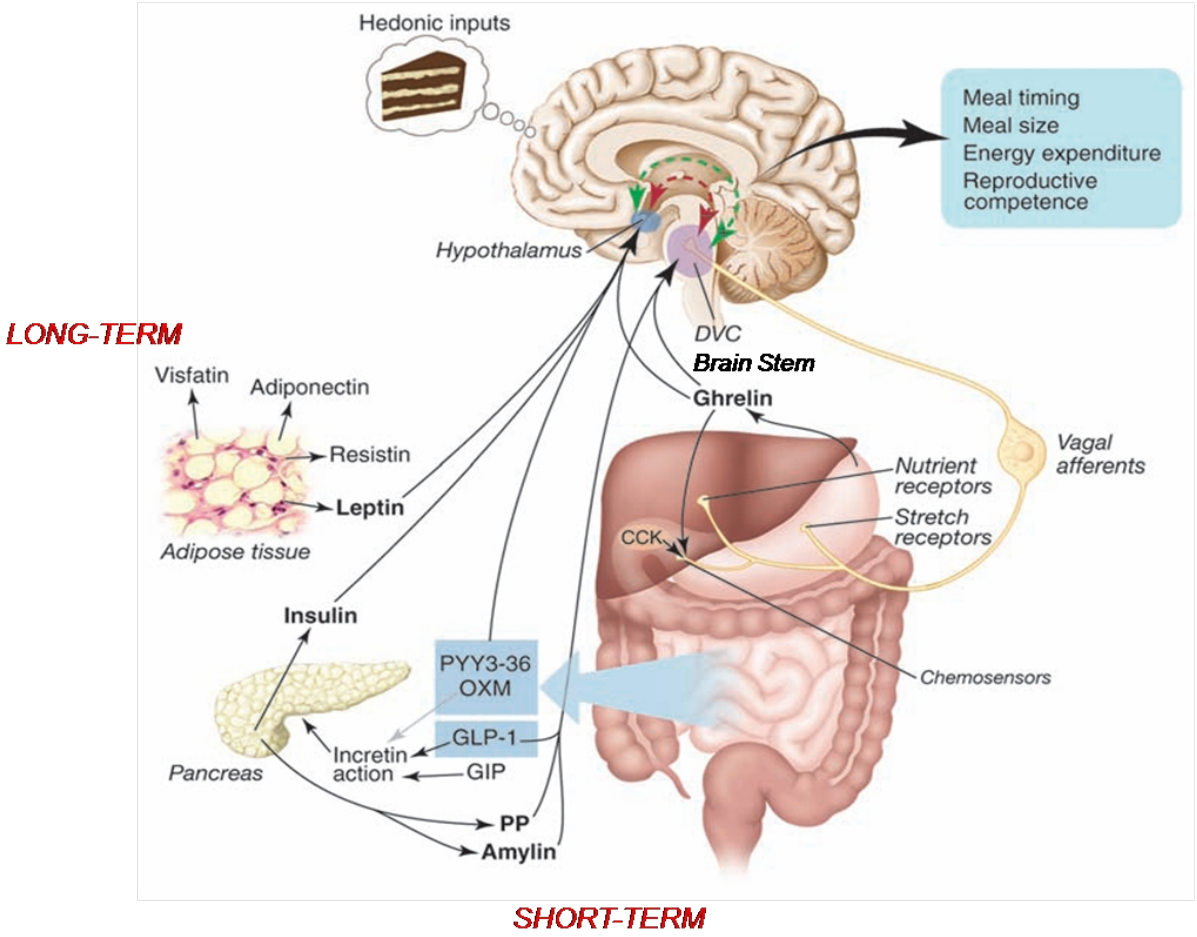


**Figure 1. Energy balance regulation along the gut-brain axis (9).** The brain integrates *long-term* and *short-term* signals to regulate energy balance. Long-term signals are produced by adipose tissue (leptin) and the pancreas (insulin). Short-term signals, such as CCK, GIP, GLP-1 and PYY are released from the gut after ingestion. These hormones can act locally to regulate gut motility or act in the brain to regulate food intake through two mechanisms: via the vagus nerve to stimulate ascending pathways from gut to the brain or by the circulation to the brain The main regions in the brain involved are the hypothalamus, in particular the arcuate nucleus, and the dorsal vagal complex in the brain stem.

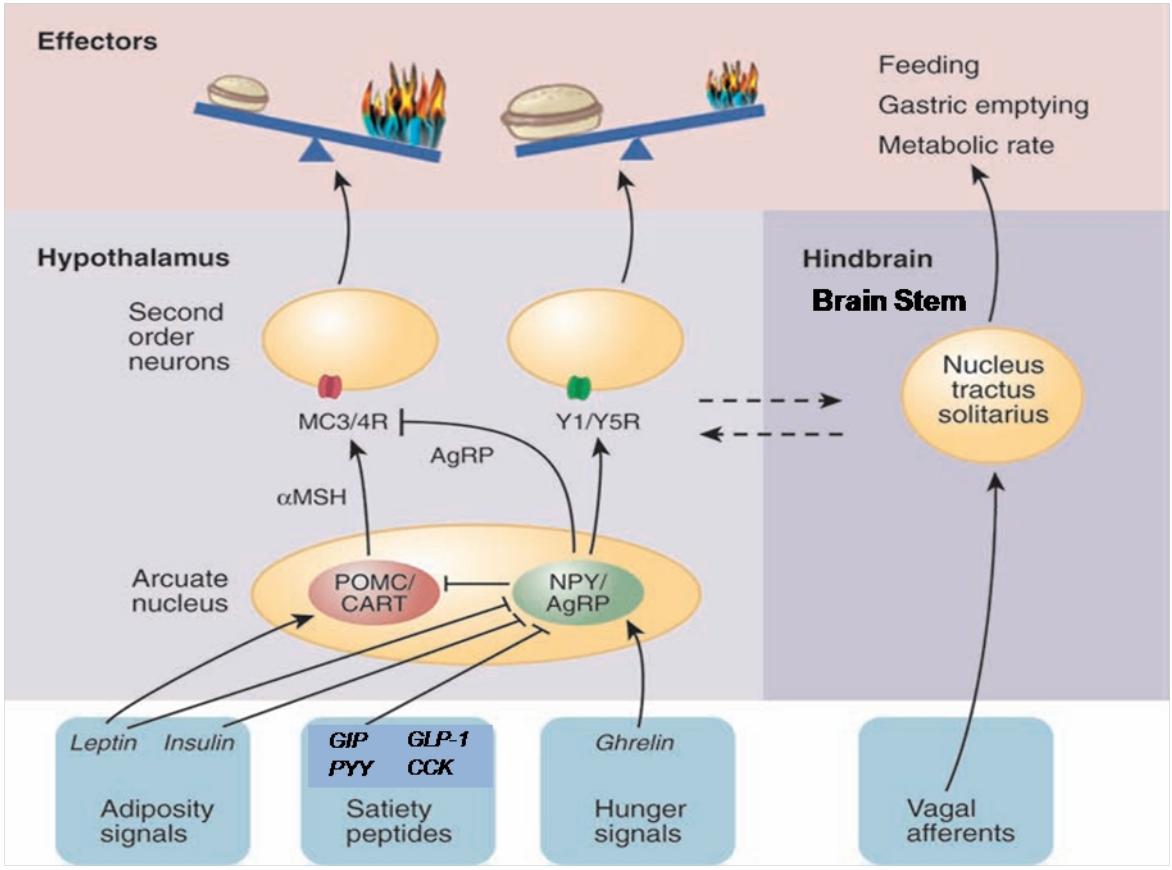


**Figure 2. Action of gut peptides on the brain (9).** The arcuate nucleus integrates *long-term* or *short-term* signals from the periphery and from the brain stem. The arcuate nucleus contains two distinct subsets of neurons controlling food intake. One acts as a stimulus to feeding, these neurons contain neuropeptide Y (NPY) and agouti-related peptide (AgRP). The second subset of neurons acts as an inhibitor to feeding, these contain α-MSH and cocaine- and amphetamine-regulated transcript (CART). Integration of peripheral signals within the brain involves the interplay between the hypothalamus and brain stem (hindbrain) structures including the NTS (Nucleus tractus solitarius), which receives vagal afferent inputs. Inputs from the brain stem and hypothalamus are integrated, acting on meal size and frequency and energy expenditure.

### 1.2. Gut hormones and their functions

The gut releases more than 20 peptide hormones from endocrine cells in response to specific nutrient stimuli (27, 35, 116). Selected gut hormones are here briefly described according to their role in the regulation of food intake and their potential as therapeutic targets **(Fig. 3)**.

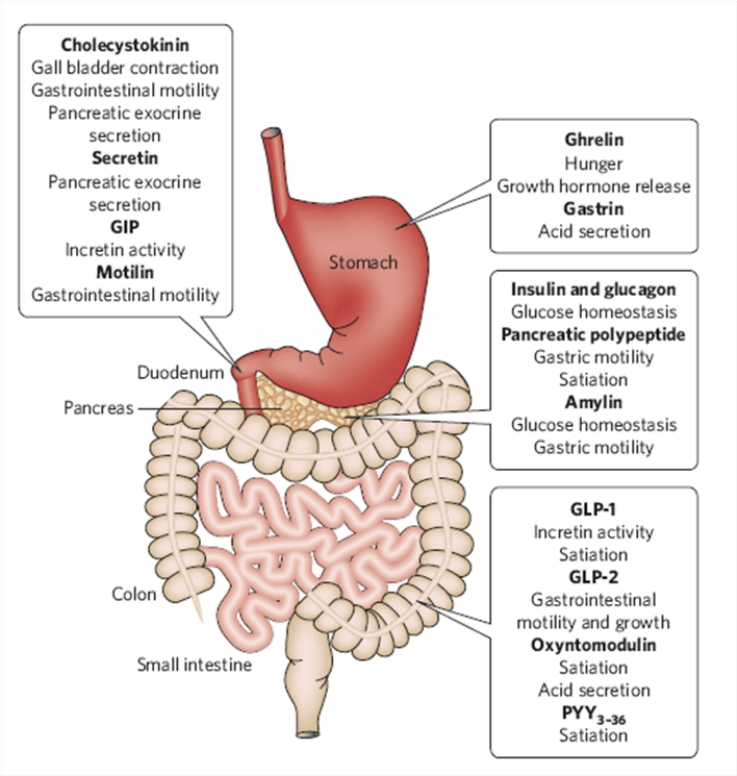


**Figure 3. Gut peptides secreted from the gastrointestinal tract (95).** Gut peptides localization and major functions.

***Ghrelin.*** Ghrelin is the only peripherally active appetite-stimulating hormone. It is produced by and secreted from the A-cells in the stomach (74) but the exact mechanisms mediating its release are unknown. Ghrelin plasma concentrations increase during fasting and fall rapidly after a meal (36). In addition to increasing appetite, ghrelin promotes the release of growth hormone (102, 119) and gastric motility through the growth hormone secretagogue-1 receptor (118). Ghrelin acts directly on the hypothalamus but also stimulates appetite via the vagus

nerve expressing the growth hormone secretagogue-1 receptor (39). Ghrelin has been shown to stimulate appetite and food intake when administered to both lean and obese humans (41). Chronic peripheral administration causes hyperphagia and obesity in rats (117, 138), however, ghrelin knockout mice exhibit normal body weight and food intake (26). Further work is needed to investigate the potential value of ghrelin-antagonists as relevant antiobesity drugs.

***GIP.*** Gastric inhibitory polypeptide, also known as glucose-dependent insulinotropic polypeptide (GIP), is synthesized in and secreted from K-cells located in the duodenum and proximal jejunum (101) GIP is rapidly degraded, with a half-life of 7 minutes, by the enzyme dipeptidyl peptidase IV (DPP-IV) (106) and its secretion is primarily regulated by nutrients, especially fats and glucose (24, 115). The primary action of GIP is the stimulation of glucose-dependent insulin secretion, GIP exhibits potent incretin activity in rodents and human subjects (42). GIP has also been shown to stimulate β-cell proliferation and exert anti-apoptotic actions *in vitro* (44, 123, 124). Furthermore, GIP was found to be involved in brain cell proliferation (100) and in bone formation (125). In addition, inhibition of the GIP signaling ameliorates obesity, obesity-related hyperglycemia and dyslipidemia in rodents, indicating that GIP can play a role in obesity and obesity-related diseases (90). In healthy humans, GIP infusion did not affect glucose and insulin at normoglycemia (5 mM glucose), but GIP co-administered with the sulfonyl urea glibenclamide increased plasma insulin levels (71). The mechanisms underlying this intriguing observation remain unclear. Whether an enhancement of GIP receptor signaling is a viable strategy for the treatment of type 2 diabetes remains to be determined. Human data demonstrating the efficacy of GIP agonists in type 2 diabetes are quite limited.

***CCK.*** Cholecystokinin was the first gut hormone found to have an effect on appetite (54). It is produced by I-cells in the duodenum and proximal jejunum, as well as in the brain and enteric nervous system (14, 93). CCK is secreted in response to luminal nutrients and dietary fat and protein are amongst the most effective stimulants (135). Circulating CCK levels increase over

10-30 minutes after meal ingestion. CCK has a mean half-life of 1-2min and exists in several molecular forms with differing chain length (75) and acts via CCK1 and CCK2 receptors. The CCK1 receptor appears to be more important in appetite regulation and is found in pancreas, pyloric sphincter, vagal afferent, as well as on the NTS (Nucleus of the Solitary Tract) of the brain stem (7, 91). CCK signals satiety effects to the hypothalamus mainly through a neural pathway to the brainstem (92). In addition, CCK stimulates gall bader contraction, pancreatic enzyme secretion and inhibits gastric emptying locally (93). Central administration in rodents has shown to decrease feeding, an effect augmented by the coadministration of leptin (86). Peripheral administration in rodents and humans led to a decrease in meals size and duration (55, 72, 92, 96). However, chronic administration in rodent leads to a compensatory increase in meal frequency and can cause nausea and taste aversion. Lower doses appear to inhibit food intake without these symptoms (136). Intravenous administration of a CCK1 receptor antagonist in humans reduces satiety and increase hunger and meals size (13). Recently, a CCK agonist given orally in human volunteers revealed a delay in gastric emptying rate (23).

***GLP-1.*** Glucagon-like peptide-1 (GLP-1) is synthesized in and secreted from intestinal endocrine L-cells of distal small intestine and large intestine in response to nutrient ingestion, and similar to GIP acts as an incretin hormone (77, 113). GLP-1 is also expressed in neurons within the NTS of the brainstem. GLP-1 is released 5-30 min after food ingestion in proportion to the energy content. Maximum circulating GLP-1 levels are usually reached after 40 min. Fatty acids and proteins have been shown to be the most potent effectors for GLP-1 secretion *in vivo*(46, 60). The biological activities of GLP-1 include stimulation of glucose-dependent insulin secretion, increased insulin sensitivity in peripheral tissues, inhibition of glucagon secretion and delay in gastric emptying and reduced food intake (21). GLP-1 receptors are found in all brain regions involved in food intake regulation including the nucleus arcuatus (ARC) regions within the hypothalamus and the NTS of the brainstem and in addition in various peripheral tissues (49, 88). Central and peripheral administration of GLP-1

to rats inhibits food intake (126, 133). Peripheral administration to humans also inhibits food intake (133) and increases postprandial satiety and delayed gastric emptying both in lean and obese humans (97). One barrier to the use of native GLP-1 in a clinical setting is its short half-life. GLP-1 is rapidly cleaved in plasma into the inactive forms GLP-1 (9-37) and (9-36) by the enzyme DPP-IV within minutes. To overcome this obstacle GLP-1 analogs with a significantly greater plasma half-life such as exendin-4 (half-life around 30 min) have been developed which are meanwhile used in the treatment of type 2 diabetes (110).

***PYY.*** Peptide YY is secreted from the endocrine L-cells of the Ileum, colon and rectum (2). Plasma levels are low in the fasted state and increase within 30 min after nutrients reach the gut (6). PYY release is particularly stimulated by fat intake as compared to carbohydrate and proteins (47). The main form of PYY is PYY 3-36, and it has a high affinity for the Y2 receptor in the hypothalamus (109). PYY has been presumed as contributing to the "ileal brake" effect, acting to inhibit further food intake once nutrients have reached the distal small intestine (32). Central and peripheral injection in rodents results in food intake reduction (11). Intravenous PYY 3-36 infusion in human reduced food intake, duration of food intake and hunger score up to 12 hours after infusion (12). Infusion of PYY 3-36 in obese volunteers resulted in a comparable reduction in caloric intake when compared with lean controls (12). This preservation of the effect of PYY 3-36 in the obese, in conjunction with apparent abnormal postpandrial release of the hormone, raises the possibility that PYY 3-36 may be involved in the pathogenesis of obesity, and is therefore an attractive therapeutic target.

## 2. Nutrient sensing in the intestine

Chemosensory information perceived during the gastric and intestinal phases of digestion is important for the regulation of gastrointestinal functions and for mediating satiety. However, the mechanisms implicated in the perception of nutrient signals, and in the signaling pathways leading to gastrointestinal hormones (GIH) release, are still unclear. A

chemical perception system allowing nutrients such as glucose, amino acids, and fatty acids to be sensed in the gastrointestinal epithelium is referred to as “gut nutrient sensing” **(Fig. 4)**. Luminal nutrients can stimulate enteroendocrine cells to release gut hormones via specific nutrient receptors or sensors acting in an endocrine or paracrine fashion to transfer nutrient information to other organs, including the brain via endocrine or vagal pathways. Various sensors expressed on enteroendocrine cells have been identify to contribute to gut nutrient-sensing **(Fig 5) –** mainly by using *in vitro* models represented by cell-lines such as GLUTag (21), STC-1 (1) and NCI-H716 (28) **(Table 1)**. However, more detailed knowledge on the physiological significance of gut nutrient sensing is necessary to understand how foods and food constituents affect gastrointestinal functions and GIH secretion that in turn can also affect brain and peripheral tissues.

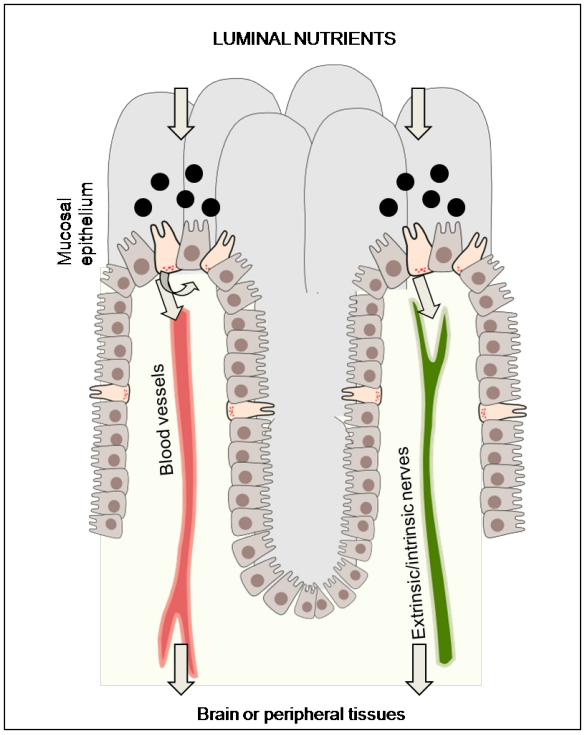


**Figure 4. Gut nutrient sensing.** When nutrients from the intestinal lumen come in contact with enteroendocrine cells hormones are released that enter blood vessels or activate extrinsic or intrinsic afferent neurons to reach the brain or peripheral tissues. Released hormones can also act directly on adjacent cells, including other enteroendocrine cells and other types of epithelial cells like enterocytes.

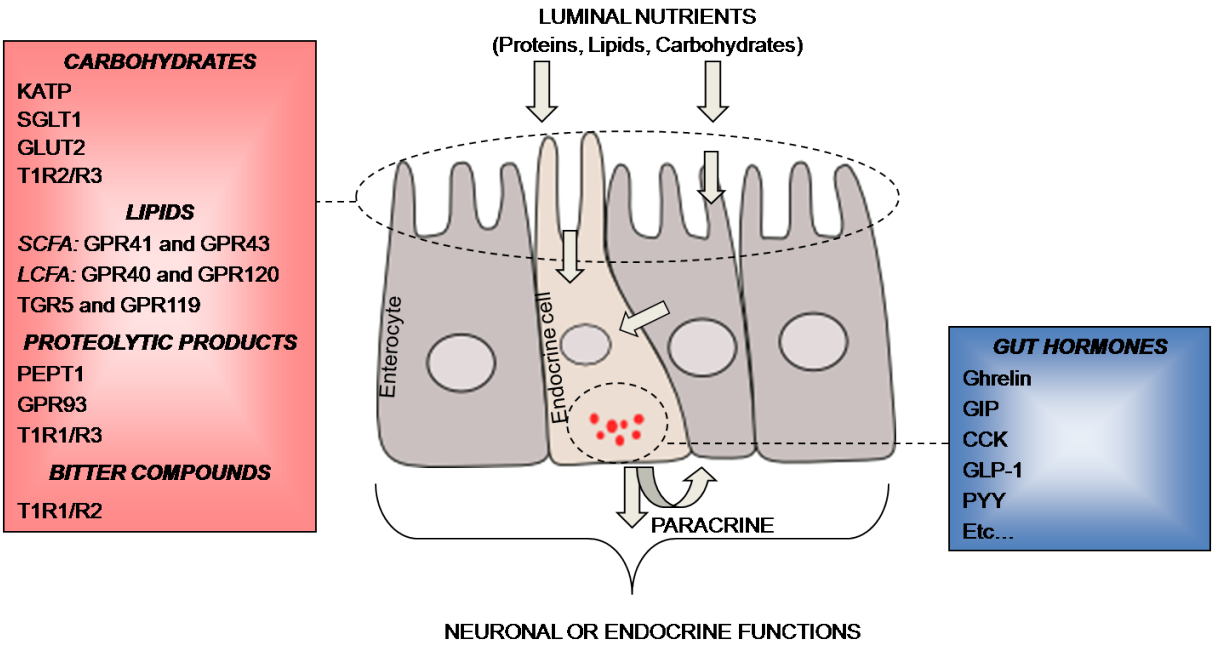


**Figure 5. The gut chemosensory system**. Luminal nutrients (Proteins, lipids and carbohydrates) are sensed by enteroendocrine cells and absorbed by enterocytes. In the red box are listed the receptor and transporter that are assumed to function as chemosensors in enteroendocrine cells whereas the blue box lists a selection of gut hormones with paracrine, neuronal or endocrine functions.

### 2.1. Carbohydrates and sweeteners

As GIP-secreting cells were found to express glucokinase, it was hypothesised that glucose-sensing in K-cells must involve a similar mechanism as employed in the pancreatic β-cell with a closure of ***$K_{ATP}$ channels*** by the increased ATP that accompanies an elevated rate of glucose metabolism. The subsequent membrane depolarization can elicit the entry of calcium via voltage gated calcium channels and the stimulation of hormone secretion (107). Despite the presence of $K_{ATP}$-channels in human (99) and mouse (101) intestinal K- and L-cells, it seems that $K_{ATP}$-channel closure does not have a prime role in incretin secretion *in vivo*. Mice lacking $K_{ATP}$-channels were found to exhibit elevated, rather than suppressed, peak GIP levels after glucose ingestion, when compared to wild type mice (95). Furthermore, studies in humans have failed to detect changes in plasma GIP or GLP-1 in response to sulphonylurea (45) administration with sulphonylurea known to affect K-ATP channels and increasing

insulin release from the beta cells in the pancreas. Moreover, patients with a mutation in glucokinase, who show from late onset of diabetes, appear to have a normal incretin secretion (95), suggesting that the molecular machinery employed for glucose sensing in the β-cell, does not seem to play a role in gut hormone secretion from enteroendocrine cells.

The sodium-coupled glucose transporter-1 (**SGLT-1**) has been shown to be expressed in the apical membrane of K-cells and murine duodenal cultures were shown to secrete GIP in response to 10 mM α-methyl-glucopyranoside (α-MDG), a non-metabolisable glucose analogue transported by SGLT-1 (101). In the GLUTag cell line, high concentrations of α-MDG (100 mM) increased action potential firing and glucose-mediated electrical activity was inhibited by the SGLT-1 inhibitor phloridzin (56). α-MDG exposure (100 mM) elicited an inward current, consistent with the typical magnitude of transporter currents, which was absent when extracellular $Na^+$ was omitted. Interestingly, primary epithelial cultures from murine small intestine secreted GLP-1 in response to concentrations as low as 10 mM α-MDG. A recent publication investigated the effects of a variety of non-metabolisable glucose analogues on glucose homeostasis in mice *in vivo* (94). The co-administration of phloridzin with glucose in mouse upper intestine inhibited both glucose transport and glucose-induced incretin secretion. Using the non-metabolizable SGLT-1 substrate α-MDG led to the same results, implying the transport of substrate and sodium via SGLT-1 elicits incretin secretion, without the need for subsequent glucose metabolism in the cell. Further insights into the role of SGLT-1 in incretin secretion in humans might also be gained by studying patients with mutations in the human SGLT-1 gene. These mutations, which either interfere with SGLT-1 transport capacity or its targeting to the brush border membrane, result in glucose/galactose malabsorption and are treated by exclusion of these sugars from the diet (139). Incretin responses in patients lacking functional SGLT-1 have not been reported to date.

The possible role of the facilitative glucose transporter **GLUT-2** in glucose sensing is still not known, however GLUT2$^{-/-}$ mice were found to display an impaired GLP-1 secretion to oral glucose (21).

A third glucose-sensing mechanism identified in enteroendocrine cells involves G-protein coupled taste receptors acting through a signaling pathway involving $\alpha$-gustducin, phospholipase-C$\beta$2 and the TRPM5 channel. The expression of the **sweet taste receptor T1R2/T1R3**, which is found in papillae cells of the tongue (25) has been reported in incretin-secreting cells (85). In addition, secretory responses to artificial sweeteners were demonstrated in the GLUTag and NCI-H716 cell lines and gustducin knock-out animals showed impaired GLP-1 secretion (64). However, studies on primary murine L- and K-cells failed to detect a significant T1R expression by quantitative PCR, and 1 mM sucralose, a saturating dose of this artificial sweetener on T1R2/3, did not significantly stimulate GIP or GLP-1 secretion from mixed intestinal epithelial cultures (101, 104). Furthermore, *in vivo* studies could not detect any significant increases in plasma incretin levels in response to artificial sweetener ingestion in rodents (51) or humans (81). The role of this pathway in glucose-mediated incretin secretion therefore is far from clear.

### 2.2. Lipids

*Long-chain unsaturated fatty acids (LCFA)* are ligands for the Gq-protein coupled receptors **GPR40** (19) and **GPR120** (63). Messenger RNAs for both of these receptors have been detected by quantitative RT-PCR in L- and K-cells (104). Knock-down of GPR120, but not GPR40, interfered with CCK-release from STC-1 cells (63) and selective GPR120 agonists, were shown to elicit a GLP-1 secretion from this cell line (59, 63).

*Short chain fatty acids* as products of bacterial fermentation in the colon, have been shown to stimulate the related G-protein coupled receptors **GPR41** and **GPR43**, which have been localized immunohistochemically on L-cells in rat and human (66, 120). In addition Karaki et al. showed the presence of GPR43 in PYY positive cells (65).

Other lipid compounds that are found in the gut lumen after a lipid rich meal, such as lysophosphatidylcholine, oleoylethanolamide or bile acids, have been shown to stimulate the Gs-protein coupled receptors **GPR119** (79) and **TGR5** (69), respectively. GPR119 agonists stimulate GIP and GLP-1 secretion *in vivo* (31, 79), while TGR5 activation has been shown to stimulate GLP-1 secretion *in vitro* and *in vivo* (69, 121). GPR119 knock-out animals showed an attenuated postprandial GLP-1 secretion, but did not reveal significantly alter glucose homeostasis (78).

### 2.3. Protein and peptides

The mechanisms by which protein mediate release of GIH remains unclear despite the fact that protein-induced satiety has been demonstrated *in vitro* (33, 34) and *in vivo* (10, 18, 20, 40, 67, 80, 130, 132). Protein hydrolysates from digested dietary protein in the upper small intestine are known to have a variety of physiological effects including the control of gastric and intestinal motility (20, 67) and food intake (10, 18, 40, 67, 80, 131, 132). Many *in vitro* studies have shown that CCK is secreted after stimulation of STC-1 cells with protein hydrolysates (33, 34, 73, 98); yet, the sensory pathway is still unknown. Most recently, the existence of a protein hydrolysate responsive G-coupled receptor, **GPR93** has been described by Choi et al. in the rat gut epithelium (29). Activation of GPR93, induced an intracellular calcium increase through the Gαq and Gαi pathway, followed by ERK1/2 phosphorylation in the rat intestinal cell culture model, hBRIE380i. Using STC-1 cells overexpressing human GPR93, Choi et al. also demonstrated that a protein hydrolysate activated GPR93 and leads to changes in CCK gene expression and secretion (30).

**PEPT1**, which is predominantly expressed in the apical membrane of intestinal epithelial cells, is a proton/oligopeptide cotransporter that selectively takes up di- and tripeptides. It has been shown *in vitro* that peptide-induced proton influx through PEPT1 triggers a membrane depolarization followed by $Ca^{2+}$ influx through voltage dependent calcium channels (VDCCs), which in turn induces hormone secretion from STC-1 cells when

cells were transfected with PEPT1 (87). Normal STC-1 cells however do not express PEPT1. Darcel et al. showed in rats that protein digests which activate vagal afferents known to induce satiety may depend on PEPT1 (38).

Reimann et al. has shown *in vitro* that the amino acid glutamine can elicit a GLP-1 secretion from GLUTag cells involving in part the sodium dependent amino acid transporter, **SLC38A2** (68, 105).

The **T1R1/R3 umami receptor** was first discovered in the oral cavity. It is a heterodimeric G-protein coupled receptor, consisting of the subunits T1R1 and T1R3. It was shown to be activated in response to L-glutamate and most other 20 proteinogenic amino acids with receptor activation potentiated by 5'-ribonucleotides such as IMP (68). The colocalization of T1R1 and T1R3 in the enterocyte apical membrane and in Paneth cells has been demonstrated in rat (83, 84). Besançon et al. detected the expression of T1R1 and T1R3 in mouse small intestine and the presence of the transcripts in human intestine (16). In addition, the vagus nerve containing sensory fibers that transmit information on food in the small intestine to the brain, specifically responds to glutamate (127). It also seems to regulate along with sweet taste receptors, calcium and PKC $\beta$II the trafficking of the peptide transporter PEPT1 and the facilitative glucose transporter GLUT2 to and from the apical membrane in enterocytes (84). These findings suggest a specific role of these receptors in digestion/absorption but also in the regulation of appetite. Umami compounds have been shown to promote digestion. When umami taste compounds, including glutamate were added to dog food, gastric secretion (129) and gut motility (122) was promoted. In addition, L-glutamate helps to activate mucosal defense mechanisms in order to maintain the mucosal integrity and physiologic responses of the upper gastrointestinal tract (3). Similarly to sweet taste, the umami taste receptor could have a role in hormone secretion.

## 3. *In vitro* models used to study nutrient sensing and gut hormone secretion

The main difficulty in establishing the sensory functions of enteroendocrine cells is the lack of a proper polarized endocrine cell preparation. All primary cell cultures available are a mixed population with a substantial proportion of non enteroendocrine cells. As the endocrine cell content of the gut changes during development, primary cultures derived from fetal tissues may not accurately reflect the physiology of adult small intestine. Although intestinal endocrine cell lines such as STC-1 (1), GLUTag (21) or NCI-H716 (28) cells are available and have been used to examine hormone secretion in response to a variety of nutrients, these cells do not have a polarized organization in culture or have dysregulated prohormone gene expression (22). A variety of assumptions have been made concerning the sensory ability of isolated enteroendocrine cells on the basis of hormone release in response to the addition of selected nutrients to endocrine cell lines (15, 28, 30, 33, 34, 43, 56, 62, 73, 98, 105). In these experiments usually a rapid increase in intracellular calcium is observed upon addition of stimulants. The kinetics of the response is usually very fast with no delay in onset as observed physiologically. In addition, it is difficult to assess whether stimulation occurs through the apical or basolateral surface of the enteroendocrine cell or through a gap junction. All three cell lines have different origins and properties **(Table 1)** and it is therefore not surprising that they are not identical in their responsiveness to nutrient stimuli. These enteroendocrine cell lines may be called artificial systems, since in the intestinal epithelium enteroendocrine cells are usually surrounded by epithelial cells, and only 1% of all cells in an epithelial layer are hormone secreting cells. The major question is whether other epithelial cells, such as the enterocytes, play a critical role in nutrient sensing.

| Cell line | Origin | Gut Hormones secreted |
|---|---|---|
| **STC-1** | mouse intestinal tumor | CCK<br>GLP-1<br>secretin<br>GIP<br>somastostatin |
| **GLUTag** | mouse colonic tumor | CCK<br>GLP-1 |
| **NCI-H716** | human cecal adenocarcinoma L cell | GLP-1 |

**Table 1. Characteristics of the current enteroendocrine cell lines.**

## 4. Aim of the study

Although enteroendocrine cell in *in vitro* cultures have become a frequently used tool for the prediction of GIH secretion in response to various stimuli they are limited when it comes to putting the findings back into physiology. Our aim was therefore by combining *in vitro* and *ex vivo* approaches to establish and characterize physiologically relevant new models representative for the small intestine. The development of these new models enabled the characterization of compounds that elicit GIH secretion and in part the characterization of underlying signaling pathways. The present work focused on the role of the di- and tripeptide transporter PEPT1 as sensor, the T1R1/R3 umami receptor and GPR93 and studied their role in peptide sensing mechanisms.

# MATERIALS AND METHODS

Dipeptides were derived from a peptide library custom synthesized and purchased from JPT Peptide Technologies GmbH (Berlin, Germany). The peptides were synthesized using solid phase Fmoc strategy. Dipeptide purity was >85% and all peptides contained <5% tripeptide contamination as determined with HPLC-UV (Waters, Atlantis Hilic Silica column, Milford MA, USA). Peptide identification was performed by means of LC-MRM-MS. Other reagents used in this study were purchased from Sigma Aldrich (Steinheim, Germany), unless indicated differently. In *ex vivo* and *in vitro* studies, a commercial casein hydrolysate, Peptopro® (DSM, Delft, The Netherlands) referred as PEP in the present work.

## 1. *In vitro* models and methods.

### 1.1. Cellular models and cell culture conditions

All cells were used until passage 25, unless indicated differently. The cells were grown and maintained in a humidified atmosphere at 37° C and 5% $CO_2$.

#### 1.1.1. Caco-2 cells.

Human enterocyte caco-2 cells were kindly provided by ATCC (50), were cultured with a culture medium consisting of MEM with Earle's salts with L-glutamine (E15-825, PAA laboratories GmbH, Pasching, Germany) 2% non essential amino acids (M11-003, PAA laboratories GmbH, Pasching, Germany), 10% FKS, 100 $\mu g \cdot ml^{-1}$ gentamycin (P11-005, PAA laboratories GmbH, Pasching, Germany). Cells were used between passages 30 and 60.

#### 1.1.2. Mode-K cells

Mouse enterocyte Mode-K cells, were kindly provided by Prof. D. Haller (Technische Universität München, Freising, Germany) (134), were cultured with a culture medium consisting of DMEM (E15-810, PAA laboratories GmbH, Pasching, Germany) supplemented

with 10% FKS (Gold, PAA, laboratories GmbH, Pasching, Germany), 4 mM L-Glutamine and 500 $\mu g \cdot ml^{-1}$ penicillin (Cambrex, Baltimore, USA), 500 $U \cdot ml^{-1}$ streptomycin (Cambrex, Baltimore, USA).

### 1.1.3. HuTu-80 cells

Human enteroendocrine HuTu-80 cells (ATCC, HTB40) (137), were obtained from the American Type Culture Collection. Cells were grown in minimum essential Eagle's medium (MEM) containing 10% FBS, 100 $U \cdot ml^{-1}$ penicillin (Cambrex, Baltimore, USA), 100 $\mu g \cdot ml^{-1}$ streptomycin (Cambrex, Baltimore, USA) and 100 $\mu g \cdot ml^{-1}$ L-glutamine.

### 1.1.4. NCI-H716 cells

Human enteroendocrine NCI-H716 cells (ATCC, CCL-251) (28), were obtained from the American Type Culture Collection. The cells were grown in suspension in RPMI 1640 medium (E15-840, PAA laboratories GmbH, Pasching, Germany) supplemented with 10% FBS, 2 mM L-glutamine, 100 $IU \cdot ml^{-1}$ penicillin, 100 $\mu g \cdot ml^{-1}$ streptomycin (P11-010 , PAA laboratories GmbH, Pasching, Germany), 2.5 $g \cdot l^{-1}$ glucose, 10 mM HEPES (S11-001, PAA laboratories GmbH, Pasching, Germany), 1 mM Na-pyruvat (S11-003, PAA laboratories GmbH, Pasching, Germany).

### 1.1.5. GLUTag cells

The mouse enteroendocrine cell line GLUTag was kindly provided by Dr. FM. Gribble (University of Cambridge, United Kingdom) (21) and used with the permission of Dr. D. Drucker (University of Toronto, Canada). Cells were maintained in DMEM (E15-806, PAA laboratories GmbH, Pasching, Germany) with 10% FKS (Gold, PAA laboratories GmbH, Pasching, Germany), 100 $U \cdot ml^{-1}$ Penicillin and 100 $\mu g \cdot ml^{-1}$ Streptomycin (P11-010, PAA laboratories GmbH, Pasching, Germany).

### 1.1.6. Coculture experiments

A mixture of Caco-2 cells (10%) and NCI-H716 cells (90%), Mode-K cells (10%) and GLUTag cells (90%) or Caco-2 cells (10%) and GLUTag cells (90%) were seeded on a coverslips at a density of 200,000 cells/ml in the respective enteroendocrine cell medium. For the coculture with Caco-2 cells, microscopy was performed after 15 days and after two days for the coculture with Mode-K cells. Whenever NCI-H716 cells were used in the coculture, the slide was precoated with matrigel (Basement Membrane Matrix 354234, BD Biosciences).

### 1.1.7. Human T1R1/R3-expressing HEK cells

HEK293 (human embryonic kidney) cells were obtained from Invitrogen (Breda, The Netherlands). For functional expression of the human umami receptor, HEK293 cells were stably transfected with the plasmids containing the G-protein α-subunit Gα15, the T1R1 and T1R3 umami receptor genes, respectively, using Lipofectamine (Invitrogen) following the manufacturer's protocol and standard procedures. Cells were maintained in DMEM with Glutamax supplemented with 10% FCS, 5 $\mu g \cdot ml^{-1}$ blasticidin, 400 $\mu g \cdot ml^{-1}$ geneticin and 100 $\mu g \cdot ml^{-1}$ hygromycin. As negative control cells, native HEK293 cells were used and grown in the same medium without blasticidin, geneticin and hygromycin .

### 1.1.8. Human GPR93-expressing HEK cells

For functional expression of the human GPR93 in HEK293 cells, the SnaBI/XbaI fragment encoding the GPR93 obtained from pCMV6-XL4-GPR93 (Origene technologies, Rockville, USA) was subcloned in the mammalian expression vector pcDNA3 (Invitrogen, Leiden, The Netherland). HEK293 cells were stably transfected with the plasmids containing the GPR93 receptor genes, using Lipofectamine (Invitrogen) following the manufacturer's protocol and standard procedures. Cells were maintained in DMEM (Cambrex, Baltimore, USA), supplemented with 10% FBS (Gibco, Paisley, UK), 500 $\mu g \cdot ml^{-1}$ penicillin (Cambrex, Baltimore, USA), 500 $U \cdot ml^{-1}$ streptomycin (Cambrex, Baltimore, USA), and 500 $\mu g \cdot ml^{-1}$ G-

418 (Gibco, Paisley, UK). As negative control cells, native HEK293 cells were used and grown in the same medium without G-418.

#### 1.1.9. CHO-CCK1-R cells

A Chinese hamster ovary cell line (CHO) functionally expressing rat CCK1R (CHO-CCK1-R) was kindly provided by Dr. R. Smeets (University of Nijmegen, The Netherlands). CHO-CCK1R 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), 500 $\mu g \cdot ml^{-1}$ penicillin (Cambrex, Baltimore, USA), 500 $U \cdot ml^{-1}$ streptomycin (Cambrex, Baltimore, USA) and 500 $\mu g \cdot ml^{-1}$ 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.

### 1.2. Immunofluorescence and confocal microscopy

#### 1.2.1. Cells

The NCI-H716 cells and Caco-2 cells coculture grown on glass coverslips was fixed in 4% paraformaldehyde (PFA) in PBS pH 7.4 for 10 min and permeabilized in 0.25% Triton X-100 in PBS for 10 min at room temperature. The cells were blocked in 10% BSA in PBS for 30 min, then washed in PBS and incubated with primary antibody, Goat anti-GLP-1 (sc-7782, Santa Cruz Biotechnology, dilution 1:50) and rabbit anti-ZO-1 (40-2200, Invitrogen, Carlsbad, Germany, dilution 1:500) in PBS containing 3% BSA/0.1% triton for 1h at room temperature in a humidified chamber. After washing in PBS (3 x 5 min), cells were stained with secondary antibody (Donkey anti goat-CY3 and donkey anti rabbit-CY5, dilution 1:400, Jackson Laboratories Immuno Research, West Grove) in PBS containing 3% BSA/0.1% triton for 1 hour at room temperature in a humidified chamber. Subsequently, cells were washed in PBS (3 × 5 min) and counterstained with DAPI (SIGMA) to stain the nucleus. Then cells were mounted on glass slides and examined by confocal microscopy.

Expression of human GPR93 was investigated using immunofluorescence as described above. An affinity purified goat polyclonal antibody raised against human GPR93 (sc-68984, Santa Cruz Biotechnology, Heidelberg, Germany, dilution 1:50) and a donkey anti goat alexa fluor 488 (1:100, Invitrogen, Leiden, The Netherland) were used as primary and secondary antibody, respectively.

**1.2.2. Tissues**

The small intestine of adult mice was fixed in 4% paraformaldehyde in PBS pH 7.4 for 15 min at 37°C followed by an incubation with wash solution (100 NaCl mM, 100 mM sucrose, HEPES 10 mM, pH 7.4) for 5 minutes at 37°C. Afterwards, the tissue was dehydrated in ascending concentrations of ethanol in water and xylene at 40°C (70% and 80% 30 min, 2 x 95% 45 min, 3 x 100% 45 min and 2x 100% xylene 45 min) and embedded in paraffin (Paraplast embedding media). Tissue sections were cut (10 µm) and dried on Superfrost Plus microslides (Menzel GmbH, Braunschweig, Germany) at 37°C overnight. Subsequently, sections were dewaxed in xylene (2 × 5 min), rehydrated in descending concentrations of ethanol in water (2 × 100% for 5 min, 1 × 100% for 2min, 2 x 96% for 2 min and, 1 x 80% for 2 min) and finally rinsed in water for 3 min. For demasking of epitopes, the sections were incubated in 10 mM citrate-buffer (in mM: 100 citric acid and 100 tri-sodium citrate-dihydrate), pH 6 for 35min at 95°C. Next, tissue sections were blocked in 3% BSA in TBS (in mM: 137 NaCl, 2.7 KCl, 24.8 Tris-Base pH 7,4) for 30 min at room temperature and incubated with the mixture of anti-chromogranin A antibody (Goat anti chromogranine A, sc-1488, 1:100) or anti-GIP antibody (Goat anti GIP, sc-23554, 1:40) or anti-GLP-1 antibody (Goat anti GLP-1, sc-7782, 1:100), and anti-PEPT1 antibody, (Rabbit anti PEPT1 produced against the synthetic peptide VGKENPYSSLEPVSQTNM, corresponding to the amino acids 693-710 of the rat PEPT1 C-terminus (Pineda, Berlin, Germany, 1:1000 (63)), in an antibody dilution solution with background reducing components (DAKO, S3022, Carpinteria, USA) overnight at room temperature and in a

humidified chamber. After washing in TBS (2 × 5 min), sections were incubated with the secondary antibodies (Donkey anti rabbit-CY5 and donkey anti goat-CY3, 1:250, Jackson Laboratories Immuno Research, West Grove) in the antibody dilution solution with background reducing component for 30 minutes in the dark at room temperature and in a humidified chamber. For L-fucosyl staining, UEA-1-FITC (1:20) was added with the secondary antibodies. Subsequently, sections were washed in TBS (3 × 5 min), and counterstained with DAPI or propidium iodide to stain the nucleus, then mounted and examined by confocal microscopy (Leica TCS SP2, spectral confocal and multiphoton system).

Human intestinal sections (abcam ab4368, Cambridge, UK) were processed as described above for mouse tissue, using an affinity purified goat polyclonal antibody raised against human GPR93 (sc-68984, Santa Cruz Biotechnology, dilution 1:100) as primary antibody. Donkey anti goat-CY3, (dilution 1:250 Jackson Laboratories Immuno Research, West Grove) used as secondary antibody.

### 1.3. Determination of CCK concentrations using a CCK receptor-1 (CCK1R) activation assay by monitoring intracellular calcium concentrations

Samples from mouse gut ring experiments were tested for CCK output in a CCK1R activation assay as described previously (73). This method shows a higher sensitivity and lower variability than an ELISA. Briefly, CHO-CCK1-R cells were seeded in a Poly-Lysin-coated 96 well microtiter plate (Greiner) at a density of $4x10^5$cells/ml. After a 24 h incubation period, the cells were 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), 0.5 µM Probenecide and 2.5 µM Fluo-4AM in Tyrode's buffer, pH 7.4 (140 mM NaCl, 5 mM KCl, 10 mM glucose, 1 mM $MgCl_2*6H_2O$, 20 mM Hepes), for 1 hour at 37°C. Samples from the gut ring secretion studies were prepared in parallel in a 96 wells plate (V96 Microwell, Nunc) and automatically

pipetted onto the cells during the assay. Activation of CCK1R was measured by monitoring variations in $[Ca^{2+}]_{in}$ using the FlexStation II 384 (Molecular Device). Measurements were carried out for 90s with an interval time of about 1.6 s. To obtain a baseline, fluorescence signals (excitation 485 nm/emission 520 nm) were measured for 20 s prior to the addition of medium on the cells (80 µl, injection speed of 100 µl/sec). After the addition of the medium derived from the gut ring secretion studies, the fluorescence signals were measured for an additional 45 s at 37°C. The fluorescence values (Delta F) were calculated by subtracting the average fluorescence of the first 20 time point (baseline) from the maximum fluorescence signal. Non-induced cells were measured in parallel as a negative control.

**1.4. Calcium Reporter assay for the determination of hGPR93 activation**

Activation of human GPR93 receptors was measured by monitoring variations in intracellular calicum concentrations $[Ca^{2+}]_{in}$ using the FlexStation II 384 (Molecular Devices). HEK cells expressing human GPR93 (HEK-hGPR93 cells) or HuTu-80 cells were seeded in supplemented DMEM into Poly-Lysin-coated 96-well plates (black wall, clear bottom, Greiner) at a density of $5x10^4$ cells/well, 100 µl/well and cultured overnight. Cells were loaded with Fluo-4AM as described above. 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. The Flexstation measurement settings were identical as those used for the CHO-CCK1R assay. Non-transfected cells (HEK cells) were measured in parallel as a negative control.

**1.5. Calcium Reporter assay for the determination of hT1R1/R3 activation**

Activation of human T1R1/R3 receptor was measured by monitoring variations in $[Ca^{2+}]_{in}$ using the FlexStation II 384 (Molecular Devices, The Netherlands). T1R1/T1R3-expressing cells and control cells expressing only Gα15 were seeded in Poly-Lysin-coated 96-well plates (black wall, clear bottom, Greiner, Alphen, The Netherlands) at a density of

$1.5x10^4$ cells/well, in a volume of 100 µl/well. Cells were allowed to grow for 48-60 h and then loaded with Fluo-4AM as described above. Before the actual measurement the dye loading medium was replaced by 150 µl of Tyrode's buffer and cells were allowed to equilibrate for 15 min at 37°C. Test compounds were prepared in Tyrode's buffer in a 96 wells plate (V96 Microwell, Greiner, Alphen, The Netherlands) and automatically pipetted onto the cells during the assay. Measurements were carried out for 120s with an interval time of about 1.5 s, giving 80 data points per measurement. To obtain a baseline, fluorescence signals (excitation 485 nm/emission 520 nm) were measured for 20 s prior to the addition of compounds on the cells (50 µl, injection speed of 100 µl/s). After compound addition, the fluorescence signals were measured for an additional 100 s at 37°C. The fluorescence values (Delta F) were calculated by subtracting the average fluorescence of the first 10 time points (baseline) from the maximum fluorescence. Cells expressing only Gα15 were measured in parallel to serve as a negative control.

### 1.6. Electrophysiology

#### 1.6.1. Expression of human PEPT1 in oocytes

*Xenopus laevis* maintenance and oocyte harvest procedures were approved by the local authority for animal care in research (Regierung von Oberbayern, approval no. 211-2531.3-9/99). Oocytes were collected under anesthesia (immersion in a solution of 0.7 $g \cdot l^{-1}$ 3-aminobenzoic acid ethyl ester) from frogs as described previously (89). At stage V/VI, oocytes were injected with 16 ng human PEPT1 cRNA and incubated for 3–4 days at 17°C in Barth solution containing (in mM) 88 NaCl, 1 KCl, 0.8 $MgSO_4$, 0.4 $CaCl_2$, 0.3 $Ca(NO_3)_2$, 2.4 $NaHCO_3$, and 10 MES (pH 6.0).

#### 1.6.2. Electrophysiology

For two-electrode voltage-clamp (TEVC) experiments, oocytes were placed in an open chamber and continuously superfused with Barth solution or with solutions containing in

addition Gly-Gln (20 mM) or Glu-Glu (2 or 20 mM) at pH 6.0. Oocytes were voltage clamped, and transport currents were measured at –60 mV using a TEC-05 amplifier and CellWorks software (npi electronic, Tamm, Germany). Current-voltage (I-V) relations were measured in the potential range of –160 to +80 mV, and the current generated by the dipeptide transport at a given membrane potential was calculated as the difference of the currents measured in the presence and absence of substrate. The kinetic parameters of Glu-Glu transport at –60 mV membrane potential and at an extracellular pH of 6.0 were calculated from least-square fits of the Michaelis-Menten equation to the data points. The current generated by 20 mM Gly-Gln is known to saturate the transporters (76).

## 2. *Ex vivo* models

### 2.1. Preparation of isolated intestinal villi for calcium imaging

The techniques applied essentially followed those previously described (111). For intracellular calcium recordings in isolated villi, the small intestine was removed, flushed with ice-cold Ringer solution (composition in mM: 147 NaCl, 4 KCl, 2.2 $CaCl_2$, pH 7.4) containing 500 µM DTT to prevent the mucus from clogging the villi, and kept in this solution throughout the preparation. The intestine was everted and cut into 0.5 cm long sections. Tissue sections were transferred to a dissecting microscope and individual villi were detached from the intestine by snapping off the gut base with fine spring scissors and sharpened microdissection tweezers (dumoxel size 5, Fine Science Tools, Heidelberg, Germany). The isolated villi were attached to a sterile glass coverslip (Pecon GmbH, Erbach, Germany) precoated with Cell-Talk adhesive (BD Biosciences, Heidelberg, Germany) and fixed with a polycarbonate membrane (25 mm diameter, pore size 3 µm, Osmonics, Minnetonka, MN, USA).

### 2.2. Fluorescence ratio imaging for assessing $[Ca^{2+}]_{in}$

The techniques applied essentially followed those previously described (112). Isolated individual villi were loaded for 45 min with 10 µM fura-2AM (F1221, Molecular Probes, Karlsruhe, Germany) at 37°C in prewarmed oxygenated Krebs-HEPES buffer (composition in mM: 119 NaCl, 4.7 KCl, 2.5 $CaCl_2$*$2H_2O$, 1.2 $MgSO_4$*$7H_2O$, 1.2 $KH_2PO_4$, 25 $NaHCO_3$ and 10 HEPES) and thereafter fixed on the coverslip and placed in a closed perfusion chamber (Lacon, Staig, Germany). This was mounted to the stage of an inverted microscope (Leica, AF6000LX, Mannheim, Germany) fitted with an incubation chamber and equilibrated to 37°C. After perfusion with prewarmed oxygenated Krebs buffer for several minutes, intact villi were selected. After reaching stable base line readings, villi were perfused with an effector solution. Changes in $[Ca^{2+}]_{in}$ in the villi loaded with fura-2 were measured ratiometrically using dual wavelength excitation, employing a designated fura-2 filter cube and a fast external filter wheel (Leica, Mannheim, Germany). This setup allowed to record fluorescence at 510 nm with excitation at 380 nm or 340 nm. Pairs of images were obtained every 5 s and a ratio image from each pair was computed. In order to localize enteroendocrine cells on the villus, a specific staining with the UEA-1 lectin (52, 53) conjugated with FITC was used.

### 2.3. Assay of GLP-1, GIP and CCK secretion from mouse intestinal rings

Methods applied followed those described by Jang et al. (64) with minor adaption. The small intestine from mouse was removed and flushed with ice-cold Ringer solution (composition in mM: 147 NaCl, 4 KCl, 2.2 $CaCl_2$, pH 7.4) containing 500 µM DTT. The intestine was everted and sliced into 0.5 cm wide rings, each gut ring was placed in Krebs-HEPES buffer (composition in mM: 119 NaCl, 4.7 KCl, 2.5 $CaCl_2$*$2H_2O$, 1.2 $MgSO_4$*$7H_2O$, 1.2 $KH_2PO_4$, 25 $NaHCO_3$ and 10 HEPES) containing 10 $\mu l \cdot ml^{-1}$ dipeptidyl peptidase IV inhibitor with or without effector in a well of a 96 well plate. Gut rings were incubated for 30 min at 37°C, the media were collected and gut hormone levels assayed by ELISA (Millipore

GmbH, Schwalbach, Germany), or by a CCK reporter assay (see below). To assess the role of gap junctions and calcium in the GIH secretion (study 1), gut rings were pre-incubated for 15 min or co-incubated with the gap junction inhibitors tamoxifen (50 μM) or 18 alpha-glycyrrhetinic acid (100 μM) (48), the L-type calcium channel blocker nifedipin (5 μM) or the endoplasmic reticulum calcium channel blocker thapsigargin (5 μM), respectively. To investigate the role of PEPT1 and T1R1/R3 in the GIH secretion (study 2), gut rings were pre-incubated for 15 min and co-incubated with the high-affinity competitive PEPT1 inhibitor Lys-(z(NO3))-Pro (500 μM), and the T1R1 inhibitor lactisol (1 mM) respectively. All experiments assessing gut hormone responses to dipeptides, tissues were co-incubated with amastatin 10 μM, a protease inhibitor.

## 3. *In vivo* models

### 3.1. Human study

#### 3.1.1. Subjects

8 healthy subjects (8 males; age: 29.2±1.7; BMI: 24.6±2.8 kg/m2) were recruited and studied twice in a randomized, cross-over design. They were selected on being in good health and not using any medication known to affect gastrointestinal function or glucose homeostasis. The study protocol was approved by the Ethical Committee at the Technische Universität München.

#### 3.1.2. Study design

All participants entered the laboratory at 8:00 am after an overnight fast on two occasions. On each test day volunteers consumed a shake consisting of 75 g of glucose or 75 g of glucose + 30 g of a protein hydrolysate (PEP) consisting mainly of di- and tripeptides (PeptoPro, DSM, Delft, The Netherlands) in 300 ml water, adjusted to 25°C. Venous blood samples were taken into EDTA tubes containing 10μL DPP4 inhibitor (Millipore Gmbh,

Schwalbach, Germany) per ml blood immediately before the challenge (t=0) and at t= 15, 30, 45, 60, 90, 120, and180 min. Blood glucose was measured immediately from whole blood using Super GL easy (HITADO Diagnostic Systems GmbH, Möhnesee, Germany). Blood samples were then centrifuged at 3000 rpm for 15 min at 20°C, and plasma was stored at -80°C until the analysis of insulin, GIP, GLP-1 and PYY by Milliplex kits (human gut hormone panel Lincoplex kit, Millipore, GmbH, Schwalbach, Germany).

### 3.2. Mouse studies

All experiments using mice were performed with wild type mouse strains C57/BL6J purchased from Charles River (Germany) or with male PEPT1$^{-/-}$ mice generated by Deltagen (San Mateo, USA; (61)). Animals were fed with standard laboratory chow *ad libitum* and had free access to water. All procedures applied throughout this study were conducted according to the German guidelines for animal care and approved by the state ethics committee under reference number 55.2-1-54-2531-140-08. The animals were anesthetized with isoflurane followed by cervical dislocation and tissue removal.

#### 3.2.1. Effects of a high protein load or glucose load given by gavage in wild-type (wt) and PEPT1-deficient (PEPT1$^{-/-}$) mice

Non fasted 18 to 28 weeks old wildtype and PEPT1$^{-/-}$ mice (n = 12-18 animals per group, 3-6 per time point) received by gastric gavage either 0.178 g protein or 2 g/kg body weight of glucose in 500 µl water with the help of a syringe attached to a feeding needle (18G, FST, Heidelberg, Germany). Blood samples (120 µl) were collected before gavage (t = 0) and 15, 30, 60 and 120 min after gavage into tubes to which a dipeptidyl peptidase IV inhibitor (10 µl/ml blood), was added. Plasma samples were stored at -80°C for determination of GLP-1, GIP (Millipore GmbH, Schwalbach, Germany), and insulin (Crystal Chem Inc. Downers Grove, USA) by ELISA, CCK was quantified by a cell based reporter assay and DPP4 activity measured by a dedicated assay obtained from Sigma. Blood glucose

concentrations were measured from tail vein samples (Accu-Chek Aviva, Roche, Germany), immediately before (t = 0), and 15, 30, 60 and 120 min after gavage.

## 4. Data processing and statistical analysis

Data in the figures are presented as mean ± SEM. GraphPad Prism version 4.01 for windows (GraphPad Software, San Diego, California, USA) was used for statistical analysis. For the *in vivo* study (human study and mice gavage study), differences in hormone secretion were analyzed using two-way ANOVA followed by the appropriate multiple comparison tests. Differences in the potency of the effectors to elicit GLP-1 and CCK secretion from mouse gut rings were analyzed using one-way ANOVA followed by the appropriate multiple comparison tests. Hormone secretion data, using ELISA and CCK reporter assay, from the gut ring studies are expressed as fold-changes over that in buffer control. Receptor activation data (hT1R1/R1 and hGPR93 receptor) are expressed in Delta F values. Differences in receptor activation were analyzed using two-way ANOVA followed by the appropriate multiple comparison tests. Differences were considered to be significant at p-value $<0.05$. All *in vitro* and *ex vivo* experiments were performed in triplicate.

# RESULTS

## 1. Development of *in vitro* and *ex vivo* models to screen for gut hormone secretion

### 1.1. *In vitro* coculture studies

The aim of this study section was to develop a new *in vitro* model to screen for compounds that can elicit a GIH secretion. Many studies have been using the enteroendocrine cell lines, however, it remains to be shown that they are indeed an appropriate model for endocrine cells in the setting of an intact epithelium. To simulate better the functional organization of the epithelium experiments have been conducted towards the establishment of cocultures of enterocytes with enteroendocrine cells. Various approaches have been made in which a total of 200,000 L-cells (NCI-H716 cells or GLUTag cells) and model enterocytes (Caco-2 cells or Mode-K cells) were mixed in different ratios and seeded onto 35-mm tissue-culture dish. Unlike the enterocyte-like Caco2 or Mode-K cells, the enteroendocrine cells grew slowly and attached extremely poor to the tissue culture support. Usually more than 80% of enteroendocrine cells were detached. In a coculture of Caco-2 cells and NCI-H716 cells, both cell types could be grown together and kept viable for 15 days, the time required for differentiation of the Caco-2 cells. However, NCI-H716 cells did not seem to differentiate under these conditions and the formation of tight junctions between Caco-2 cells and NCI-H716 cells could not be detected via immunofluorescence **(Fig. 6A)**. A coculture using Caco-2 cells and GLUTag cells revealed that GLUTag cells are not viable for more than 4 days in culture as shown in **Figure 6B** for a 15 day old coculture. Finally, coculturing Mode-K cells and GLUTag cells revealed viable GLUTag cells **(Fig. 6C)**, but Mode-K cells lacked the expression of classical epithelial nutrient transporters, such as SGLT-1, GLUT-2 and PEPT1 (data not shown)**.** Taken together, despite various attempts to establish a coculture model of epithelial and endocrine cells all attempts failed. This led to the decision to establish a suitable

intestinal *ex vivo* model that take into account the architecture of the epithelium with endocrine cells surrounded by enterocytes.

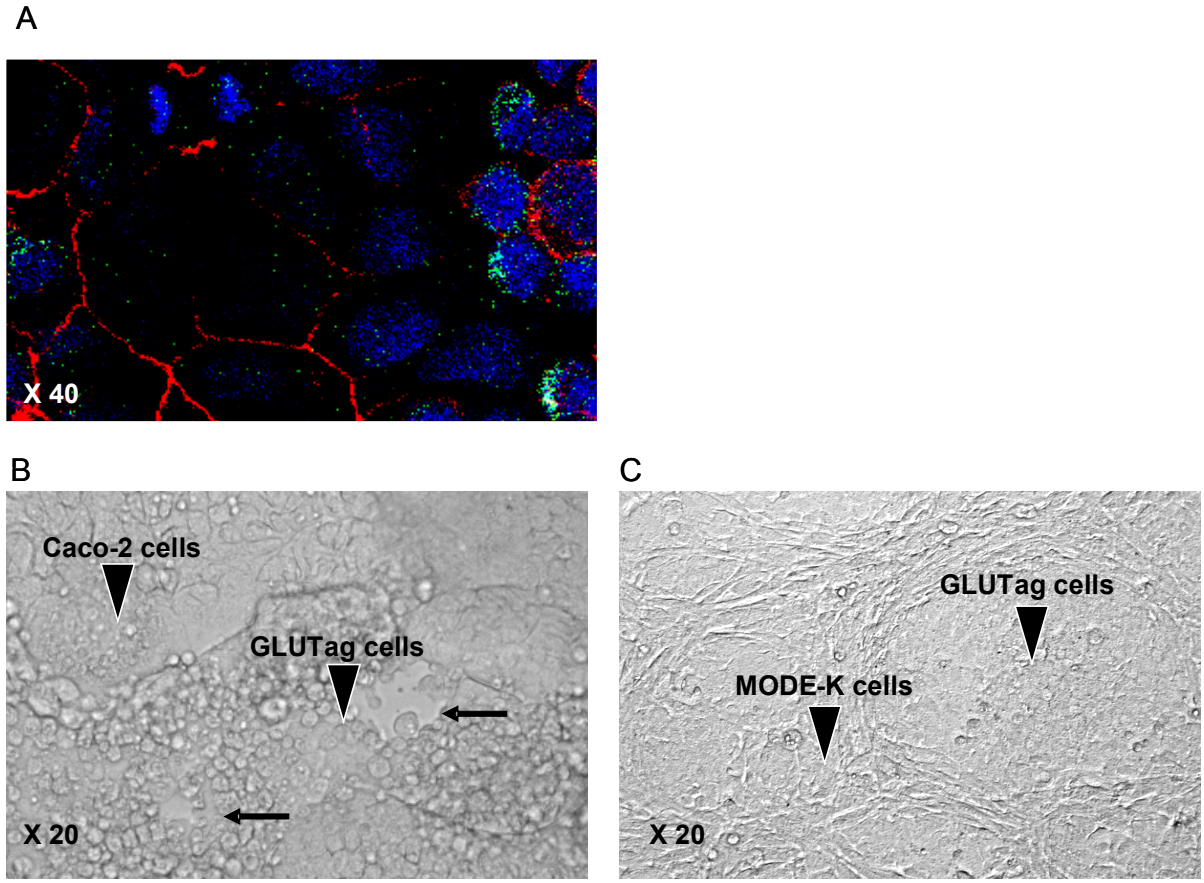


**Figure 6**. ***In vitro*** **coculture studies**. (A) Co-immunostaining of a 15 day-old coculture of Caco-2 cells and NCI-H716 cells using antibodies against GLP-1 *(green)* to localize NCI-H716 cells and against ZO1 to identify tight junctions *(red)*. (B) A 15 day-old coculture of Caco-2 cells and GLUTag cells showing the presence of non viable GLUTag cells *(arrows)*. (C) Coculture of Mode-K cells and GLUTag cells.

### 1.2. Calcium imaging in mouse isolated intestinal villi

It is established that enteroendocrine cells react to a variety of luminal stimulants mainly by increasing intracellular calcium concentrations followed by hormone secretion. The aim of the study utilizing isolated intestinal villi was to develop a physiologically relevant *ex-vivo* model to identify new nutrient compounds which can elicit a calcium increase that can be imaged via a life cell imaging system. For this purpose, freshly isolated villi from mouse duodenum, preloaded with Fura-2AM are mounted in a perfusion chamber of a life cell

imaging system. We observed that when villi were superfused with a solution of 50 mM glucose rapid changes in intracellular calcium could be detected in cells lining the tip or upper half of the villus **(Fig. 7)**.

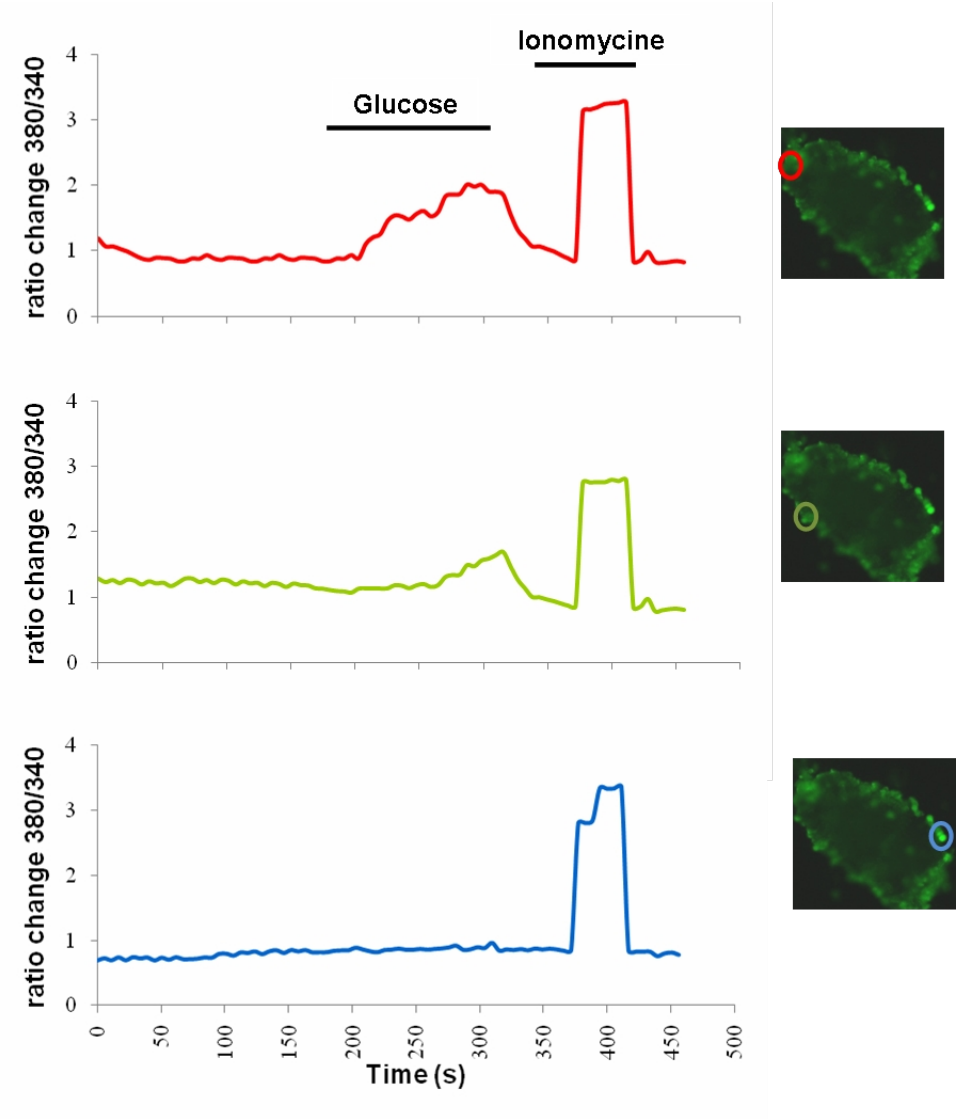


**Figure 7. Calcium imaging on isolated gut mouse villi.** Freshly isolated villi from mouse duodenum, preloaded with FURA-2AM, are mounted in a perfusion chamber of the life cell imaging system. Villi are superfused with a solution of 50 mM glucose and the emission ratio 380/340 is recorded every 5 s. As a positive control, a solution of ionomycine 10 µM is perfused. Traces from three different cells are shown.

**Figure 8** shows fluorescence ratio changes and localization of fluorescence signals representing intracellular calcium concentrations on a villus being superfused with 50 mM glucose. During the perfusion, calcium response seems to occur in many cells and not only in

isolated enteroendocrine cell. Furthermore, we observed a calcium increase from the tip of the villus progressing towards the side of the villus.

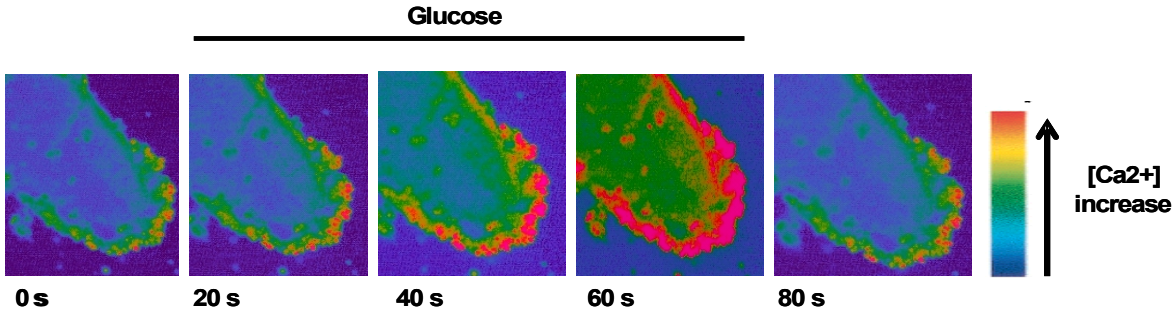


**Figure 8. Time-dependent fluorescence ratio changes.** Fluorescence ratio changes representing intracellular calcium concentrations and localization of fluorescence signals recorded by the live cell imaging system, when an isolated mouse intestinal villus was superfused with 50 mM glucose. Most of the responding cells are localized at the tip and the upper half of the villus

Using fluorescently labeled UEA-1 lectin (UEA-1) or Shigella toxin B (StBx), shown to be specific enteroendocrine cells markers (52, 118), we are able to localize enteroendocrine cells on the villus. Most of the UEA-1 or StBx positive cells are found at the tip and the upper half of the villi **(Fig.9)**. The colocalization of the two markers shows the specificity of the staining. The combination of those two methods – calcium imaging and the staining with lectin or StBx - allowed to visualize calcium responses specifically in enteroendocrine cells during the perfusion.

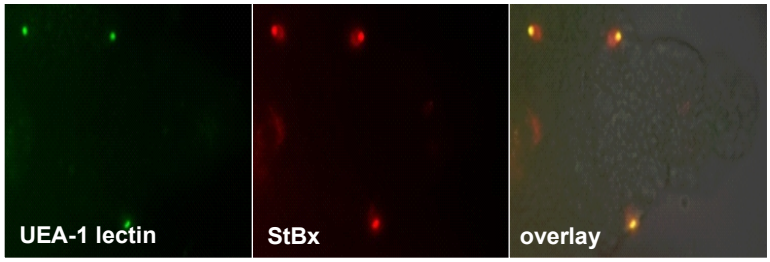


**Figure 9. Enteroendocrine cell localization on isolated villi.** UEA-1 lectin and shigella toxin B (StBx) staining with UEA-1-FITC and StxB-Cy3 localize the enteroendocrine cells on the intact villus tissue.

**Figure 10** shows a villus that was loaded with Fura-2AM and stained with UEA-1 and perfused with a solution of 20 mM Gly-Gly. We detected a calcium increase in enteroendocrine cells (UEA-1 positive cells) but also in an epithelial cell (UEA-1 negative cell). The magnitude of the signal, however, was much more pronounced in enteroendocrine cells.

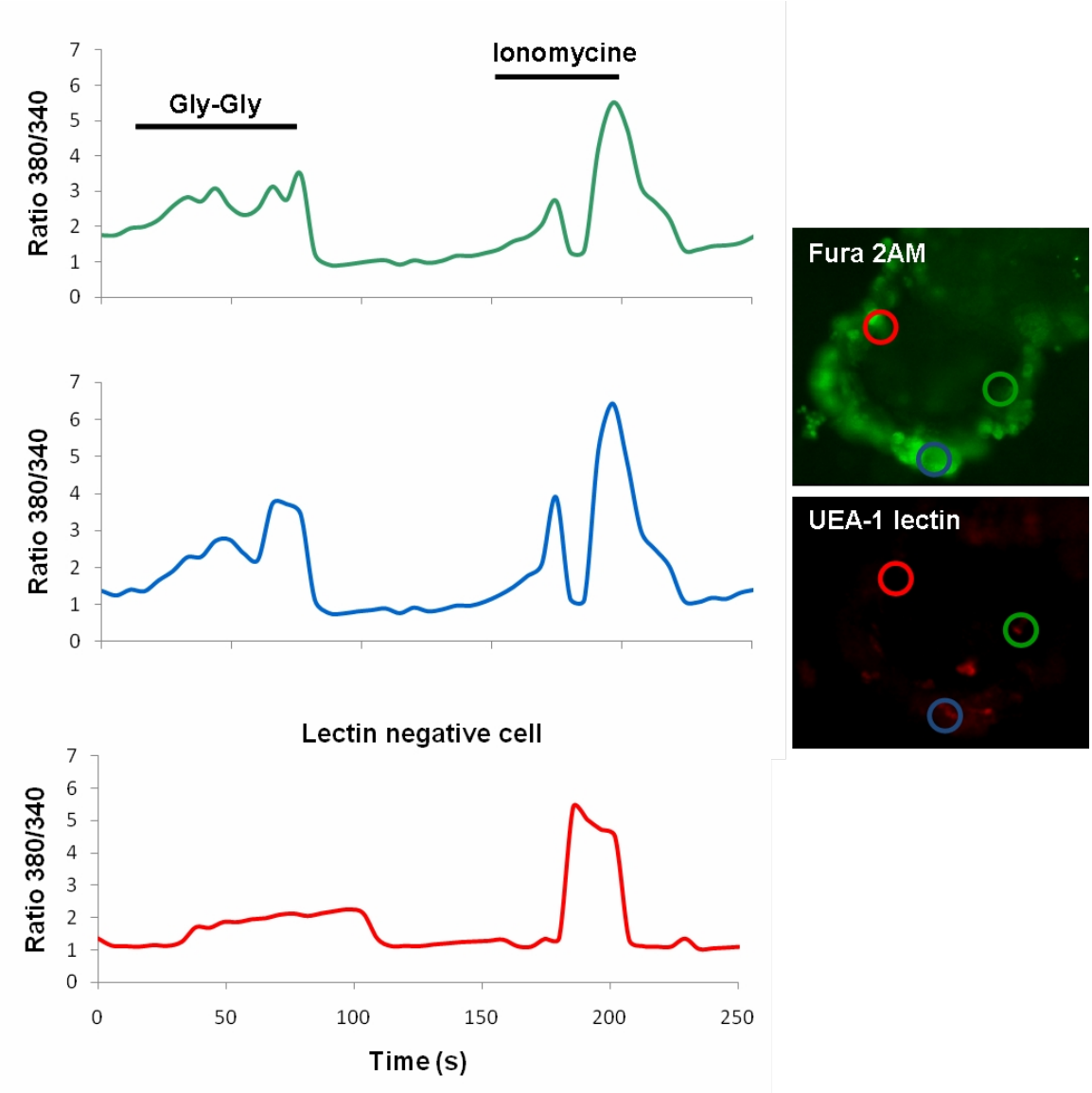


**Figure 10. Calcium changes in lectin positive and negative cells on isolated mouse gut villi.** A freshly isolated villus from mouse ileum, preloaded with FURA-2AM and labeled with UEA-lectin was perfused with a solution of 20 mM Gly-Gly. As a positive control, a solution of ionomycine (10 µM) was superfused. Traces from two lectin positive cells *(green and blue circle)* and one lectin negative cell *(red circle)* are shown.

Taken together, we have shown that calcium imaging in isolated mouse intestinal villi can be used to assess epithelial responses to luminal stimulants. The data demonstrate that calcium responses not only occur in enteroendocrine cells but also in the surrounding enterocytes. Calcium levels increase in most of the cells localized at the tip and at the upper half of the villus and seems to be transmitted from cell-to-cell in a calcium-wave. However, this method cannot be operated in high throughput and is only an indirect measure for gut hormone secretion. Therefore, a second *ex vivo* method was established.

### 1.3. The mouse gut ring model to assess gut hormone release

In order to increase the throughput for nutrient screening and to get a direct measure of GIH secretion from the intestine, the "gut ring" model was established. Briefly, freshly everted mouse small intestine is cut into 5 mm rings and is incubated in microtiter plates for 30 minutes with buffers containing the different nutrient compounds to test. Concentrations of GIH are measured in the medium after incubation and are normalized for each sample based on tissue protein content (more details in methods part, **2.3**). Proximal and distal tissues were used to investigate CCK and GLP-1 secretion respectively. In order to validate the method, we screened nutrient compounds which have already shown *in vitro* their capacity to elicit GIH secretion. A protein hydrolysate (PEP 4 %), was shown to significantly increase CCK (1.5-fold, p-value <0.05) and GLP-1 (7.8-fold, p-value <0.01) secretion **(Fig. 11A and 11B)**. Linoleic acid (1 mM) increased CCK secretion around 2.1-fold (p-value <0.01) **(Fig.11A)** compared to buffer alone. The amino acids, phenylalanine (10 mM) and leucine (10 mM) significantly increased GLP-1 secretion around 5.8-fold and 3.8-fold (p-value <0.01), respectively **(Fig. 11B)**. Glucose (20 mM) increased GLP-1 secretion approximately 7.0-fold (p-value <0.01) **(Fig. 11B)**.

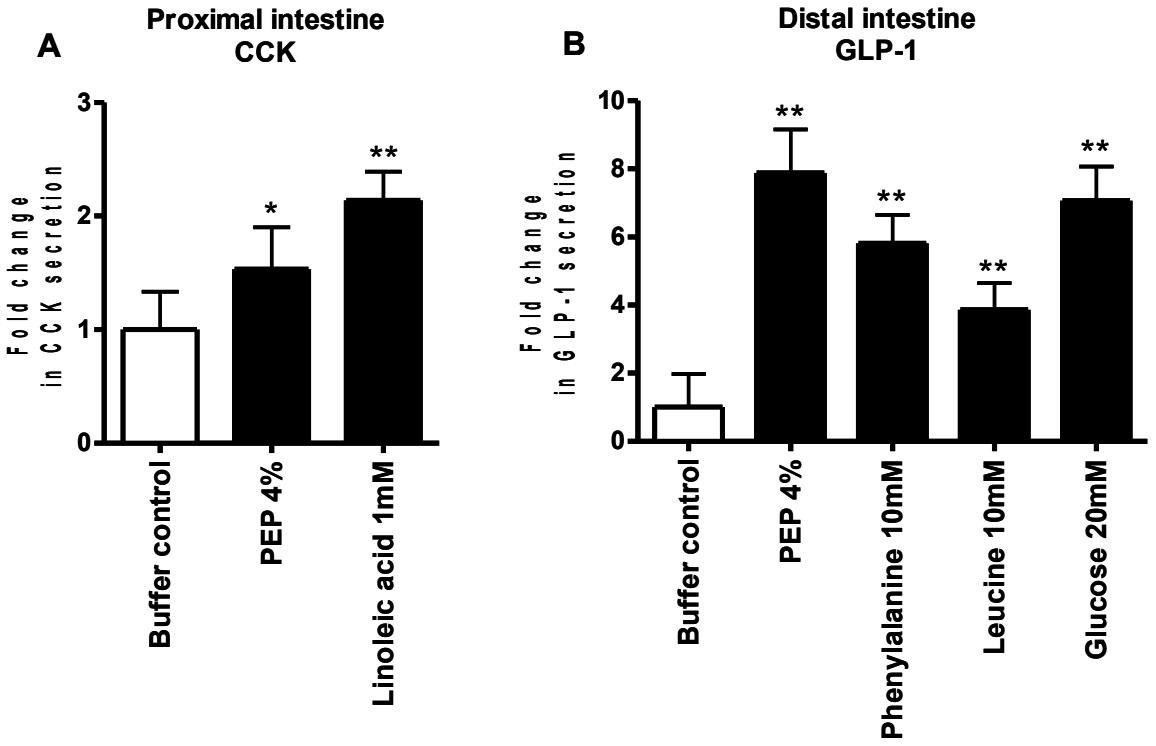


**Figure 11. Selected nutrients shown to elicit either CCK or GLP-1 release from mouse gut rings.** Freshly isolated mouse small intestinal rings were incubated in microtiter plates for 30 minutes with buffers containing the different nutrient compounds. Incubation media were assayed for CCK (A) using a calcium reporter assay with activation of the CCK1R and GLP-1 (B) by ELISA. (n=6, duplicate per mouse) Values are Mean ± SEM and are expressed as fold-change as compared to control (Buffer control). Statistical significance determined by one-way ANOVA, post test: Dunnett's; *p-value <0.05; **p-value <0.01; ***p-value <0.001)

The employed *ex vivo* models using rodent intestinal tissues provided robust changes in hormone secretion and allowed intracellular calcium responses to be recorded upon nutrient stimulation. For all further studies this combination of *ex vivo* models was used to identify transporters/receptors involved in peptide sensing and to characterize their role in GIH secretion.

## 2. Dietary protein and peptide sensing that leads to gastrointestinal hormone secretion

### 2.1. Role of PEPT1 in nutrient sensing and gut hormones secretion assessed in mouse models

PEPT1, which is predominantly expressed in the apical membrane of intestinal epithelial cells in the small intestine is a rheogenic proton-coupled cotransporter that mediates selectively the uptake of di- and tripeptides into enterocytes (37) is suspected to play a role in nutrient sensing processes and satiety control. Peptide transport via PEPT1 was shown to cause a membrane depolarization and $Ca^{2+}$ influx through voltage-dependent calcium channels (VDCCs), followed by GIH secretion from STC-1 cells. However, this could only be achieved after transfection with a PEPT1 construct (87) while normal STC-1 cells do not express the transporter and failed to secrete the hormone. In the intestinal epithelium however, enteroendocrine cells are surrounded by epithelial cells and junctional complexes with connexins provide cell-cell communication. It therefore cannot be excluded that enterocytes in concert with endocrine cells participate in nutrient sensing and GIH release. Studies in rat suggested that protein digests, which activate vagal afferents and induce satiety signals, may involve PEPT1 (38). We therefore studied whether PEPT1 is part of the protein sensing pathways.

#### 2.1.1. *In vivo* study: Responses in plasma insulin, GLP-1, CCK and GIP to a high protein and glucose gavage

To assess whether PEPT1 contributes to postprandial GIH secretion, wild-type and $PEPT1^{-/-}$ mice were gavaged with a high protein (HP) load followed by determination of plasma concentrations of GLP-1, CCK, GIP, insulin and DPP4 activity. Ten minutes after the gavage, CCK **(Fig. 12B)** and GIP **(Fig. 12C)** concentrations in plasma increased approximately 2- and 4-fold over baseline concentrations - independently of the genotype. In

wild-type mice, GLP-1 plasma levels increased approximately 3-fold over baseline and reached a maximum after 30 min whereas in PEPT1$^{-/-}$ mice no GLP-1 secretion was detected **(Fig. 12A).** As shown in **Fig. 12D** the plasma insulin levels in response to HP were not significantly different in wild-type and PEPT1$^{-/-}$ mice despite the lack of GLP-1 secretion. Although not quantified, indirect immunofluorescence revealed the presence of a normal density of GLP-1 positive cells in PEPT1$^{-/-}$ mice **(Fig.13B)** as in wild-type mice **(Fig. 13A).** There was also no difference in plasma DPP4 activity between PEPT1$^{-/-}$ and wild-type mice that could explain the differences in GLP-1 secretion **(Fig. 13C)**. The data therefore suggested that the lacking GLP-1 secretion was most likely by a functional impairment in stimulus-secretion coupling in the PEPT1$^{-/-}$ mice.

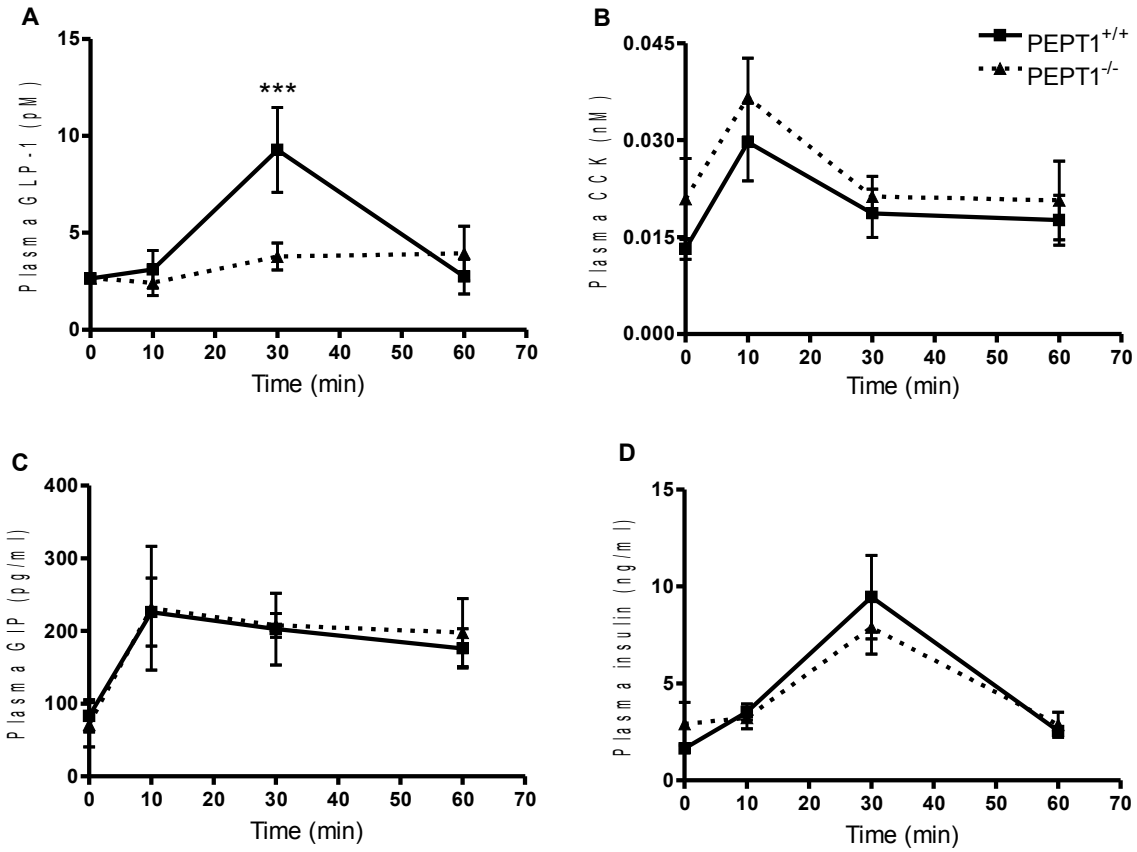


**Figure 12. Plasma hormone concentration changes in response to high protein gavage.** GLP-1 (A), CCK (B), GIP (C) and insulin (D) secretion in response to intragastric administration of protein (0.356g/ml) in wild-type (PEPT1$^{+/+}$) and PEPT1$^{-/-}$ mice ( n=36 mice per genotype, 6 per time point (A) and (B), 3 per time point (C) and (D). Statistical significance determined by two-ANOVA, values are mean ± SEM; ***, p-value <0.001.

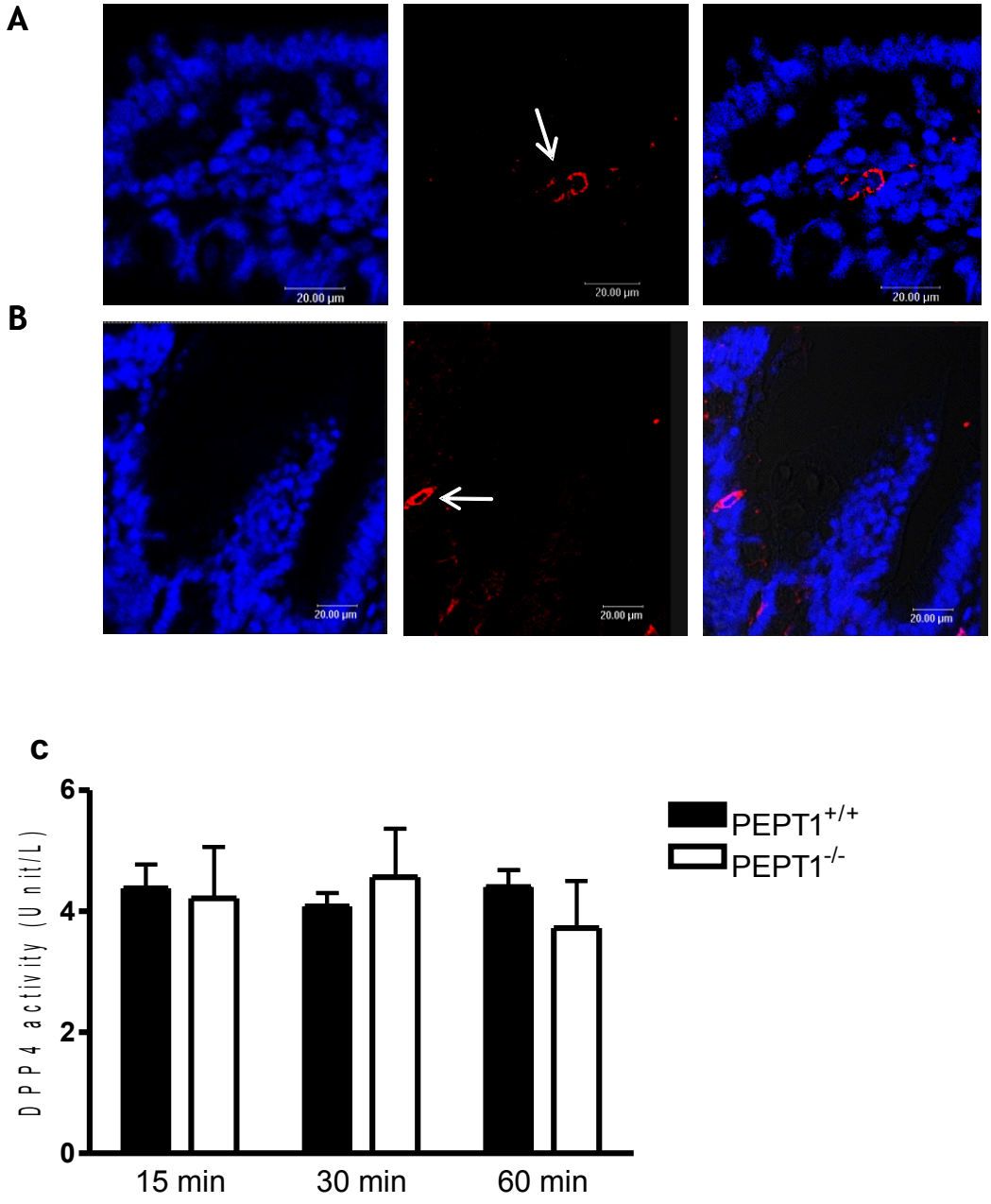


**Figure 13. GLP-1 positive cells in intestinal tissues and plasma DPP4 activity in wild-type and PEPT1$^{-/-}$ mice.** Immunofluorescence of GLP-1 positive cells (*red*) in wild-type (A) and PEPT1$^{-/-}$ mice (B). Nuclei are stained blue by DAPI-labeling. Comparison of plasma DPP4 activity after high protein gavage in PEPT1$^{-/-}$ and wild-type mice.

For assessing the specificity for sensing the protein an oral glucose tolerance test (OGTT) in wild-type and PEPT1$^{-/-}$ mice was performed. As expected, blood glucose concentrations increased and reached a maximum 15 min after the glucose load in both genotypes **(Fig. 14A)**. A rapid insulin secretion in response to glucose was observed as well

with a peak at 15 min after the gavage **(Fig. 14B)**, showing that glucose homeostasis remained unaltered in PEPT1$^{-/-}$ mice. GIP plasma levels increased in response to the glucose load and reached a maximum after 15 min **(Fig. 14C)**, with a tendency to be lower in PEPT1$^{-/-}$ mice. In both genotypes, GLP-1 plasma levels increased approximately 4-fold over baseline with a maximum after 15 min **(Fig. 14D)** demonstrating that the glucose-dependent hormone secretion was fully preserved in the PEPT1$^{-/-}$ mice.

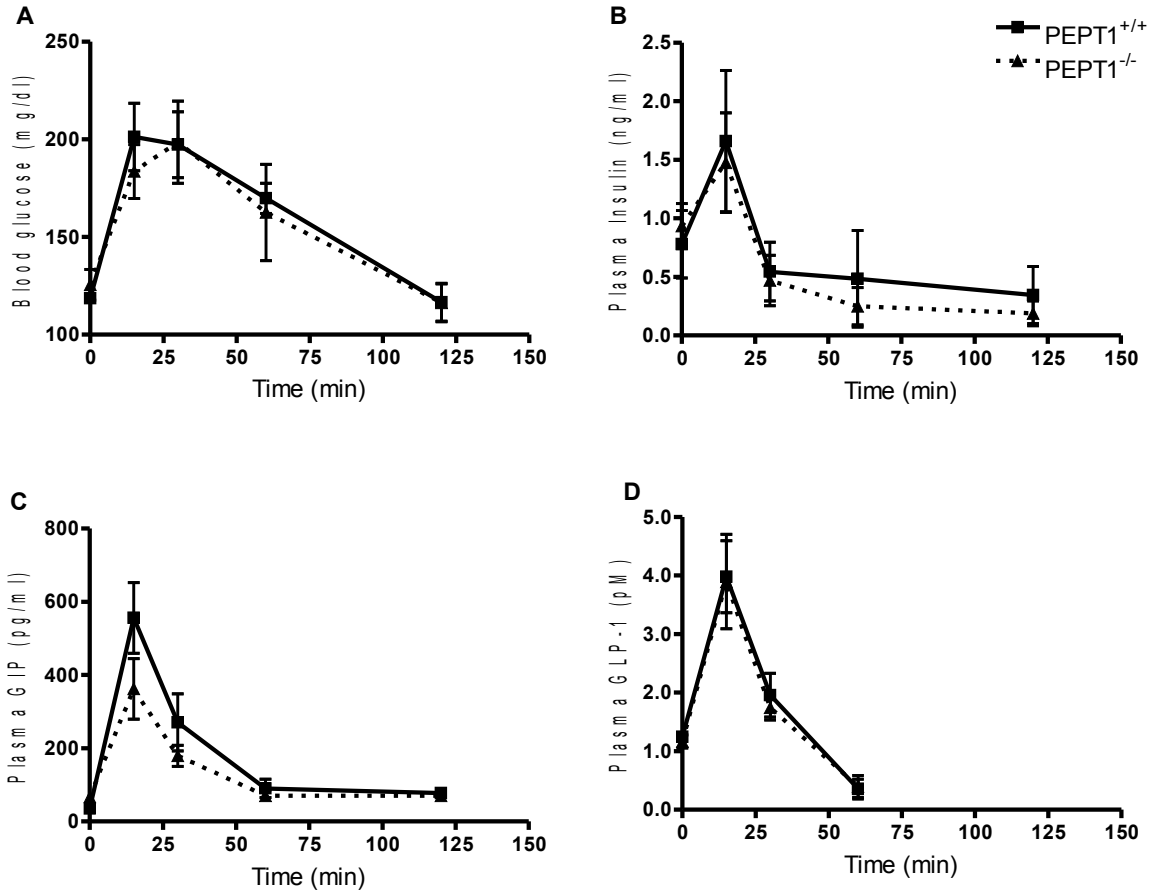


**Figure 14. Plasma glucose and hormone secretion in response to glucose gavage.** Plasma glucose (A), Insulin (B), GIP (C) and GLP-1 (D) concentrations in response to intragastric administration of glucose (2 g/kg) in wild-type and PEPT1$^{-/-}$ mice (n=16 mice per genotype, 4 per time point). Statistical significance determined by two-ANOVA, values are mean ± SEM.

### 2.1.2. Assessing the role of PEPT1 in hormone secretion *ex vivo*

To confirm the *in vivo* results, we used isolated tissue segments of proximal and distal small intestine and colon of wild-type and PEPT1$^{-/-}$ mice, exposed them to different PEPT1

substrates and measured GIH release. A significant release of GLP-1 was obtained in tissues from distal small intestinal when exposed to the PEPT1 model substrate Gly-Gly and GLP-1 secretion was drastically reduced in tissues from PEPT1$^{-/-}$ animals **(Fig. 15A)**. A GLP-1 release from gut rings of the proximal small intestine or colon could not be detected. However, other substrates known to serve as PEPT1 substrates including Gly-Sar and the ß-lactam cefadroxil also elicited a GLP-1 secretion that was blunted in animals lacking PEPT1 **(Fig. 15B)**. In addition, the protein-hydrolysate (PEP) containing mainly di- and tripeptides stimulated as well GLP-1 secretion with a significantly reduced response in tissues from PEPT1$^{-/-}$ mice.

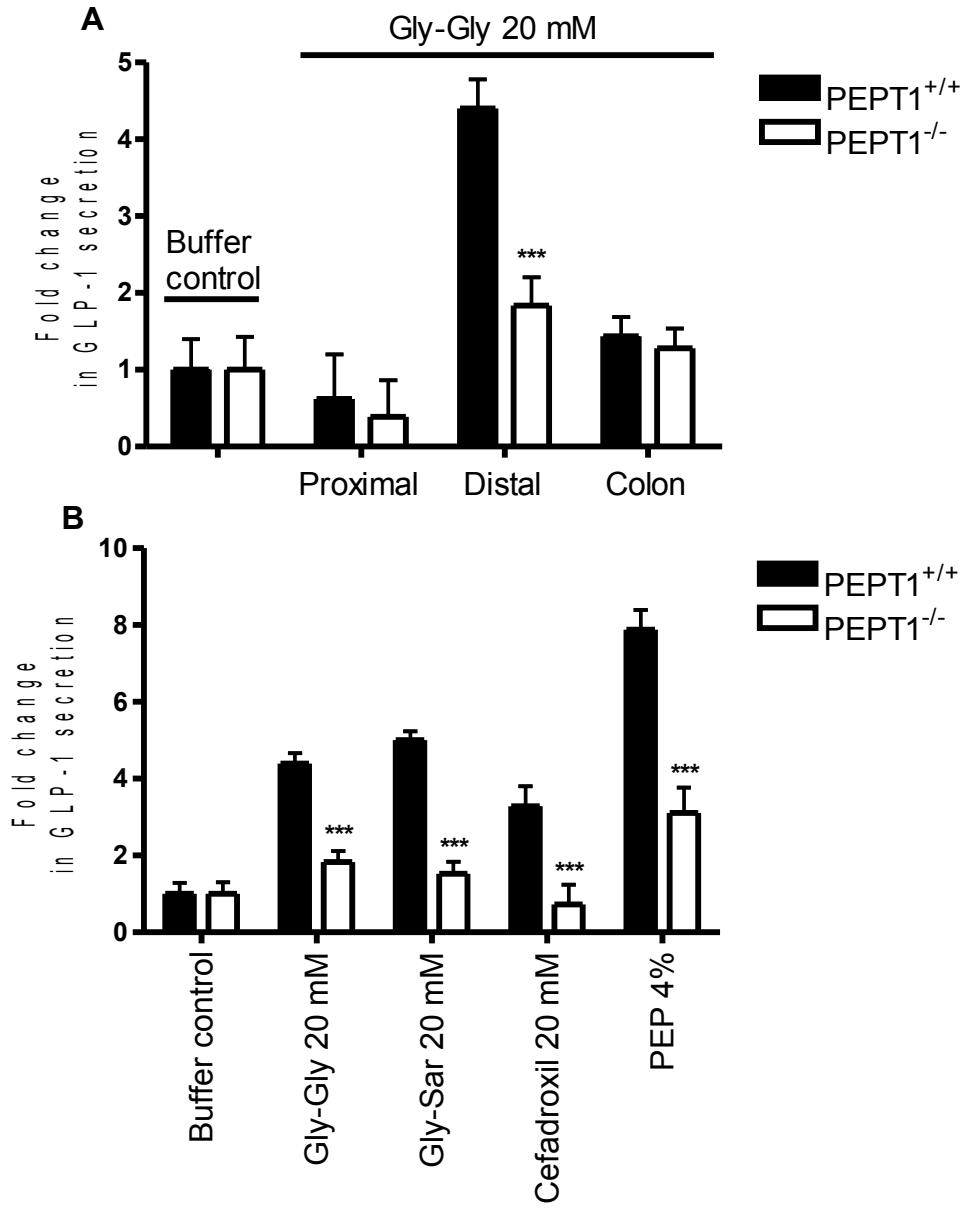

**Figure 15. GLP-1 secretory responses from mouse gut rings.** GLP-1 secretory responses from tissue of proximal and distal small intestine and colon obtained from wild-type and PEPT1$^{-/-}$ mice when exposed to 20 mM Gly-Gly (A). GLP-1 secretory response from distal tissues obtained from wild-type and PEPT1$^{-/-}$ mice when exposed to the PEPT1 substrates Gly-Gly, Gly-Sar, Cefadroxil or a 4% (PEP) solution of a protein-hydrolysate (B). (n=6 animals in duplicate). Statistical significance determined by two-way ANOVA, post test: Bonferroni; values are mean ± SEM and are expressed as fold-changes over that in control (Buffer control) with ***, p-value <0.001.

CCK and GIP secretion measured from segments of proximal intestine exposed to the same substrates did not reveal a stimulation of hormone secretion and no difference between samples from wild-type and PEPT1$^{-/-}$ mice **(Fig. 16).** However, a strong but PEPT1-independent CCK output was observed in response to 1 mM linoleic acid with a smaller effect by the protein-hydrolysate **(Fig. 16A)**. GIP secretion increased strongly in response to glucose with a slightly reduced secretion from PEPT1$^{-/-}$ tissues **(Fig. 16B)**.

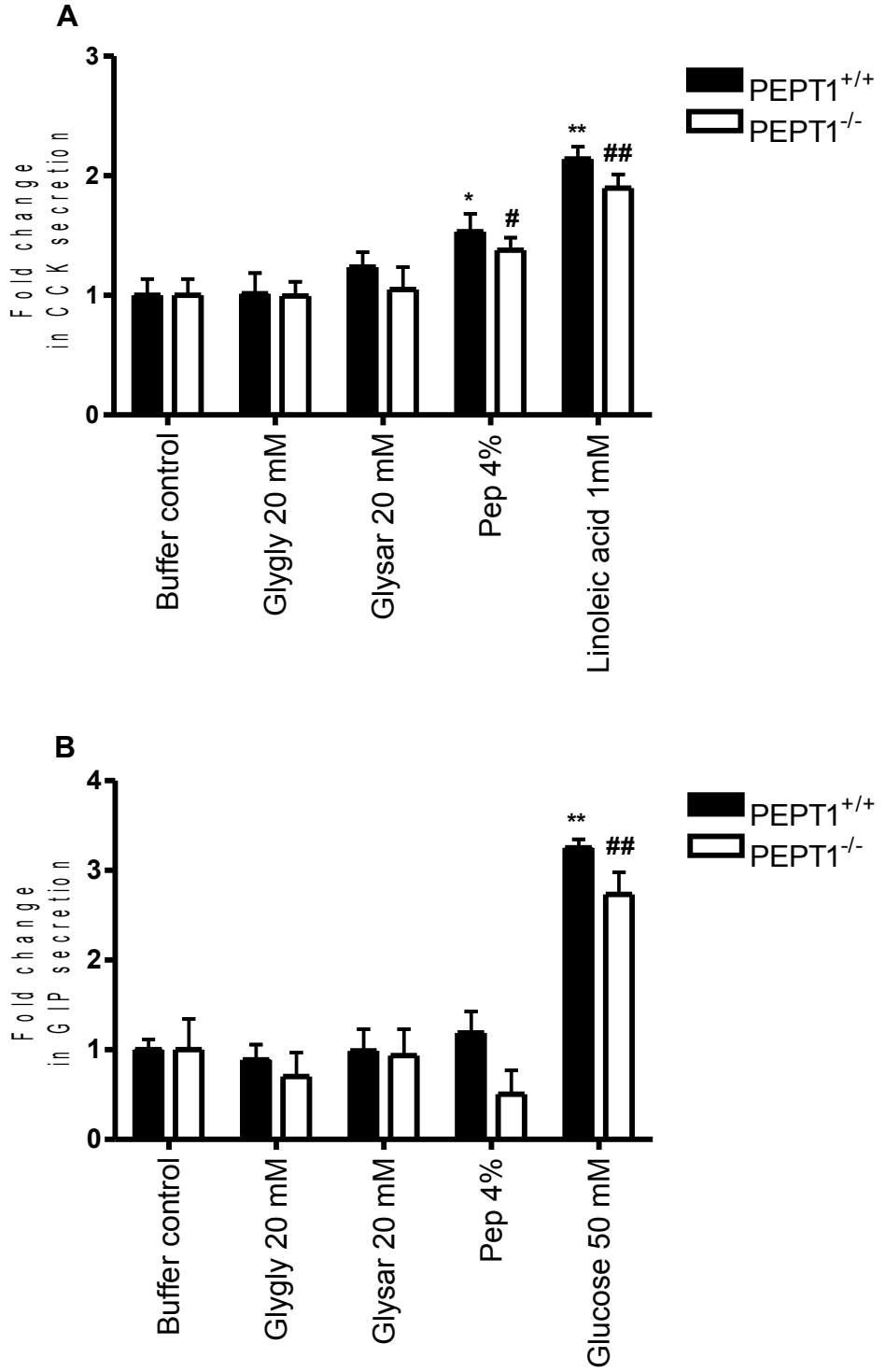


**Figure 16. CCK and GIP secretory responses from proximal gut rings.** CCK (A) and GIP (B) secretory responses from wild-type and PEPT1$^{-/-}$ mouse proximal tissues to selected nutrients (n=6 animals in duplicate per mouse). Statistical significance determined by two-way ANOVA, post test: Bonferroni; values are mean ± SEM and are expressed as fold-changes over that in control (Buffer control); * or #, $P<0.05$; ** or ##, $P<0.01$; * compared to control (Buffer control) in wild-type mice or # when compared to control in PEPT1$^{-/-}$ mice.

### 2.1.3. Role of PEPT1 in dipeptide-induced gut hormone secretion assessed by $[Ca^{2+}]_{in}$ measurements

Hormone secretion is in most cases associated with changes in intracellular $Ca^{2+}$ concentrations. PEPT1 due to the electrogenic character of its transport cycle as a proton-coupled carrier causes a membrane depolarization that could provoke the entry of extracellular $Ca^{2+}$ through voltage-gated calcium channels. Isolated mouse villi were used for fluorimetric recordings of the changes in $[Ca^{2+}]_{in}$ when tissues were exposed to selected dipeptides. Superfusion of villi with 20 mM Gly-Gly raised $[Ca^{2+}]_{in}$ in cells from wild-type mice. However, the calcium response to the dipeptide was drastically reduced in cells from PEPT1$^{-/-}$ mice **(Fig. 17)**. The response to 10 µM of the calcium ionophore ionomycine serving as a positive control to elicit a maximal increase in $[Ca^{2+}]_{in}$ was identical and independent of genotype.

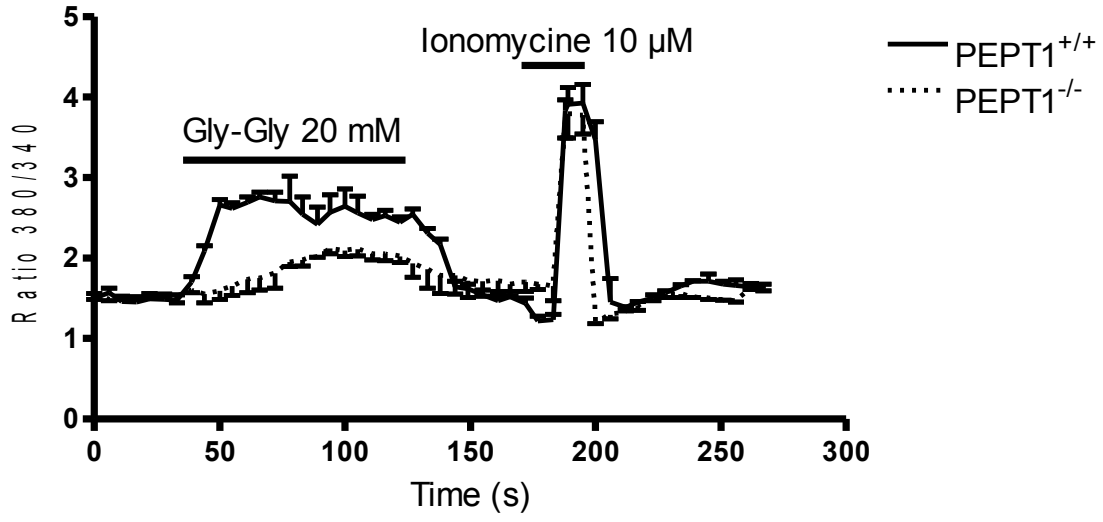


**Figure 17. Calcium imaging in isolated villi.** Recordings of changes in intracellular calcium in freshly isolated villi obtained form wild-type and PEPT1$^{-/-}$ mice in response to 20 mM Gly-Gly. Villi from mouse terminal jejunum were preloaded with FURA-2AM (10 µM) for 40 minutes and mounted in a perfusion chamber of the live cell imaging system (Leica). Villi were superfused with a solution containing 20 mM Gly-Gly and the emission ratio 340/380 was recorded every 5 s. A solution of ionomycine (10 µM) was superfused to assess maximal changes in intracellular calcium levels (n=2 animals for each genotype).

To assess the mechanism of the calcium increase intestinal tissues were stimulated by 20 mM Gly-Gly in the presence of nifedipine (5 µM), a calcium channel blocker, or thapsigargin (5 µM), which depletes ER calcium stores in an irreversible manner. In presence of nifedipine, GLP-1 secretion in response to dipeptide exposure was significantly reduced but not in presence of thapsigargin **(Fig. 18),** suggesting the calcium raise to originate from an opening of calcium channels and influx from extracellular.

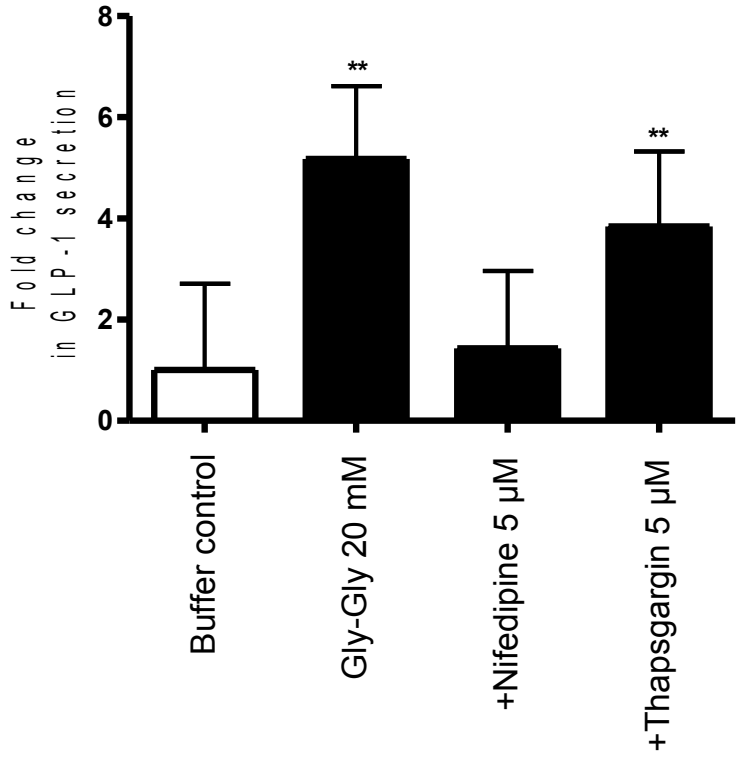


**Figure 18. Effects of a $Ca^{2+}$ channel blocker and inhibitor of endoplasmic reticulum $Ca^{2+}$ pumps on GLP-1 secretion in response to luminal dipeptide.** Gut rings from terminal ileum were exposed to 20 mM Gly-Gly in the absence or the presence of either 5 µM nifedipine or 5 µM thapsigargin (n=9 animals in duplicate per mouse). Statistical significance determined by one-way ANOVA, post test: Dunnett's; values are mean ± SEM; **, p-value <0.01.

### 2.1.4. GLP-1 secretion in response to dipeptides results from a cross talk between enterocytes expressing PEPT1 and enteroendocrine cells

PEPT1 is expressed at high levels throughout the small intestine and localized in the brush border membrane. Evidence for expression of PEPT1 in enteroendocrine cells is so far

lacking. We used immunohistochemistry in mice intestinal tissues to localize enteroendocrine cells with antibodies against GLP-1, chromogranin A (ChrA), or GIP. In addition, the apical membrane of the enteroendocrine cells was stained with L-fucosyl lectin (UEA-lectin) as a specific marker of enteroendocrine cells (52). An apical co-staining of lectin and PEPT1 could not be observed in any of the enteroendocrine cells that stained positive for GLP-1, GIP or ChrA. This strongly suggested that those cells do not express PEPT1 or only at a level not detectable via immunohistochemistry **(Fig. 19)**.

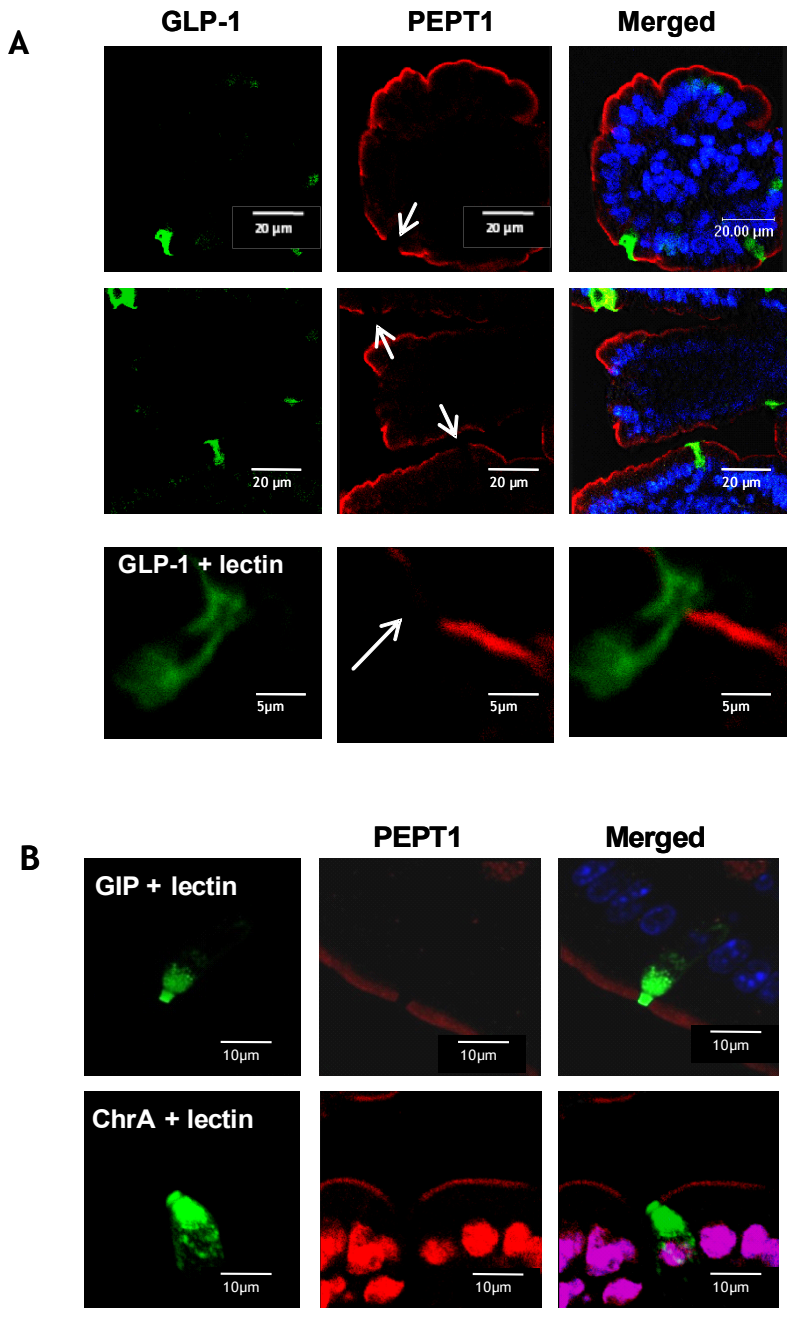

**Figure 19. PEPT1 expression in the small intestine.** Sections of the small intestine from wild-type mice immunostained using an antibody against PEPT1 (red), enteroendocrine cells (green) stained by antibodies against GLP-1 (A), GIP or ChrA (B). Brush border membranes of enteroendocrine cells are selectively stained with UEA-lectin (green). Counterstaining of nuclei with DAPI (A) or propidium iodide (B). Co-immunostaining showing in (A) *left panel*: staining with GLP-1 antibody and UEA-lectin (both green) with individual L-cells in the epithelial layer; *middle panel*: staining with PEPT1 antibody (red) shows localization of the peptide transporter in the brush border membrane of enterocytes, arrows indicate distinct gaps in PEPT1 staining; *right panel*: overlay showing that PEPT1 is not expressed in enteroendocrine cells; counterstaining of nuclei with DAPI. (B) Staining of enteroendocrine cells with GIP antibody (upper panel, green), ChrA antibody (lower panel, green) and UEA-lectin (both panels, green) and of brush border membranes with PEPT1 antibody (red) with the lack of PEPT1 staining in enteroendocrine cells.

The observation that PEPT1 seems not expressed in enteroendocrine cells raises the question of how transport of peptides via PEPT1 can elicit a GLP-1 secretion in the absence of the transporter protein in endocrine cells. Based on the known electrical coupling of cells in the intestinal lineage via connexins, it was tested whether GLP-1 release to dipeptide stimulation could result from a crosstalk between enterocytes expressing PEPT1 and enteroendocrine cells via gap junctions. For this purpose, we stimulated distal gut rings with 20 mM Gly-Gly together with one of two different gap junction inhibitors, namely tamoxifen (also used as an anti-estrogenic in treatment of human breast cancer) or 18 alpha-glycyrrhetinic acid (p43 connexin inhibitor) (48). We demonstrate that both, tamoxifen as well as 18 alpha-glycyrrhetinic acid reduced GLP-1 secretion after stimulation with 20 mM Gly-Gly from distal gut segments significantly **(Fig 20A)**. Furthermore, inhibition of GLP-1 secretion by 18 alpha-glycyrrhetinic acid revealed a dose-dependency **(Fig. 20B)**.

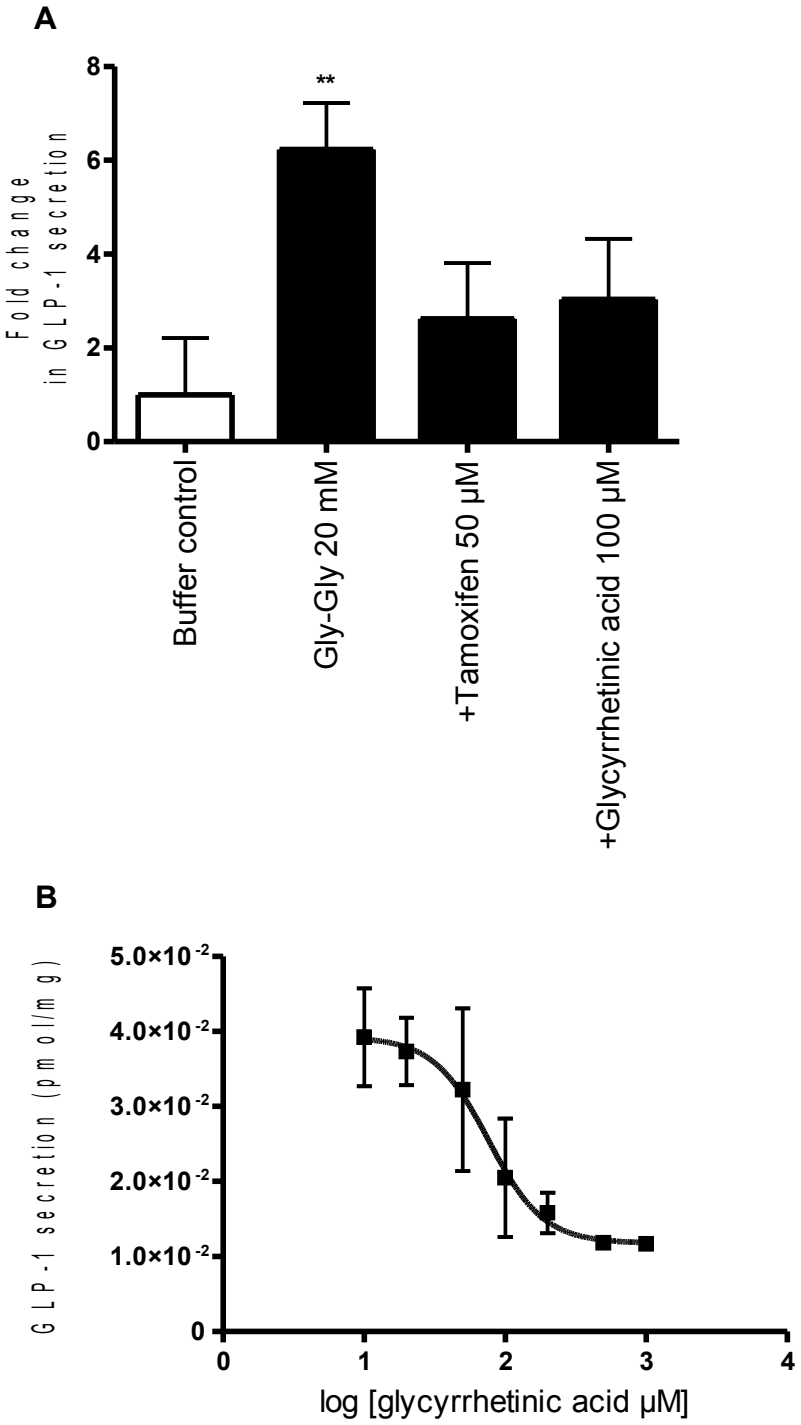


**Figure 20. Effects of gap-junction inhibitors on GLP-1 secretion.** (A) GLP-1 secretion from rings of distal small intestine when exposed to 20 mM Gly-Gly in the absence or the presence of the gap junction inhibitors tamoxifen (50 µM) and glycyrrhetinic acid (100 µM). (B) Dose response relationship for GLP-1 secretion to 20 mM Gly-Gly and glycyrrhetinic acid at different concentrations (n=6 animals in duplicate per mouse). Statistical significance determined by one-way ANOVA, post test: Dunnett's; values are mean ± SEM; **, p-value <0.01.

### 2.1.5. Assessment of the effect of a protein hydrolysate enriched in di- and tripeptides on gut hormone release in human volunteers

To verify that the human intestine responds as well to the protein-hydrolysate with GLP-1 secretion, a human study was conducted. Plasma levels of glucose, insulin, GIP, GLP-1 and PYY were measured after an oral glucose tolerance test (OGTT) with either 75 g glucose alone or 75 g of glucose and 30 g of the protein-hydrolysate (OGTTPH) in eight volunteers after overnight fasting. As shown in **Fig. 21A** lower blood glucose level were observed for the OGTTPH **(Fig.21A)** as compared to the OGTT whereas insulin plasma level were significantly higher after OGTTPH compared to OGTT (1281.8 versus 2066.038 $mg \cdot ml^{-1}$, p-value <0.01) at 45 minutes **(Fig.21B)**. GIP secretion did not differ between treatments **(Fig.21C)** whereas PYY levels did not show any responses **(Fig. 21E)**. In contrast, plasma GLP-1 concentrations in volunteers receiving the OGTTPH showed increased levels when compared to the OGTT **(Fig.21D)**.

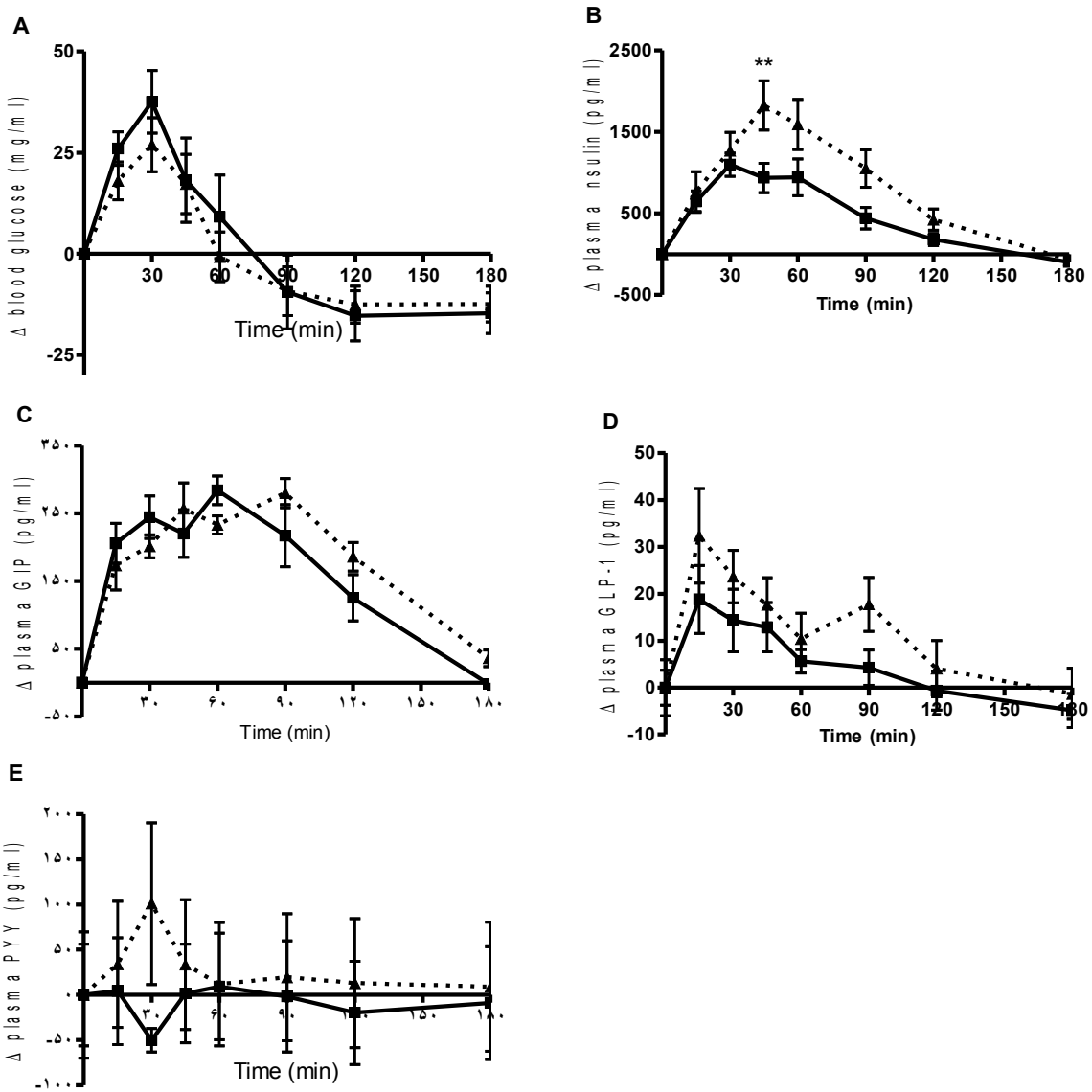


**Figure 21. Effect of glucose or a glucose/protein hydrolysate mixture on gut hormone levels and blood glucose in plasma of human volunteers.** Concentration of blood glucose (A), plasma insulin (B), GIP (C), GLP-1 (D) and PYY (E) in 8 healthy subjects in response to oral glucose (75g) or glucose (75g) + 30g protein-hydrolysate provided in 250ml water. Statistical significance determined by two-way ANOVA, post test: Bonferroni; values are mean ±SEM; **, p-value<0,01.

Taken together, the *ex vivo* and *in vivo* studies provided strong evidence that PEPT1 is required for GLP-1 secretion upon stimulation with peptides from dietary protein and PEPT1 acts as a sensor for luminal short chain peptides that via cell-cell secretion coupling and changes in $[Ca^{2+}]_{in}$ elicits specifically a GLP-1 secretion form endocrine cells in distal regions of the small intestine **(figure 33)**.

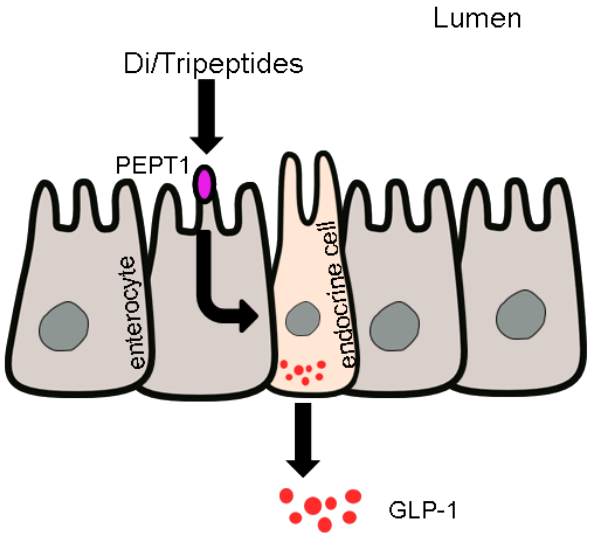


**Figure 33: A novel cross-talk of enterocytes and endocrine cells inducing GLP-1 secretion to di/tripeptides.**

## 2.2. Dietary peptides as putative ligands of T1R1/R3 umami receptors in the intestine

The aim of this study section was to investigate whether dietary peptides can also be detected by other sensors present in the gut epithelium. A recent study demonstrated the involvement of the gastrointestinal sweet taste receptor in the secretion of gut hormones (64) and in analogy therefore the T1R1/R3 umami receptor expressed in the intestine, could as well have a role in eliciting hormone secretion. The GPR93 receptor was been shown to be involved in protein sensing and GIH release (29, 30). Cloning and heterologous expression of human T1R1/R3 (hT1R1/R3) and GPR93 (hGPR93) receptors simplify the search for new ligands but also increase the number candidates to be tested with the need of a high throughput assay. The present study was designed to test a library of dipeptides on human T1R1/R3 and GPR93 receptors for identifying new food compounds that can lead to increased GIH secretion.

### 2.2.1. Expression of T1R1/R3 umami and GPR93 receptors in human tissues

The expression of T1R1/R3 umami receptor has already been shown in rat and human gut epithelium (16). Here it is demonstrated that a GPR93-immunoreactivity can be observed in the gut epithelium in human fetal tissue **(Fig.22A)** and in human adult tissue **(Fig. 22B).** GPR93 has been shown before to be expressed in rat enterocytes (29). To investigate whether the human enteroendocrine cell line HuTu-80 cells - derived from duodenum – is sensitive to a known GPR93 agonist, cells were stimulated with LPA (Lysophosphatidic Acid) in a calcium reporter assay. The stimulation with 0.1 µM, 1 µM and 10 µM LPA did not elicit a $[Ca2+]_{in}$ response **(Fig. 23)** whereas cells responded to Bombesin with a functional Bombesin receptor previously shown to be expressed in Hutu-80 cells (137).

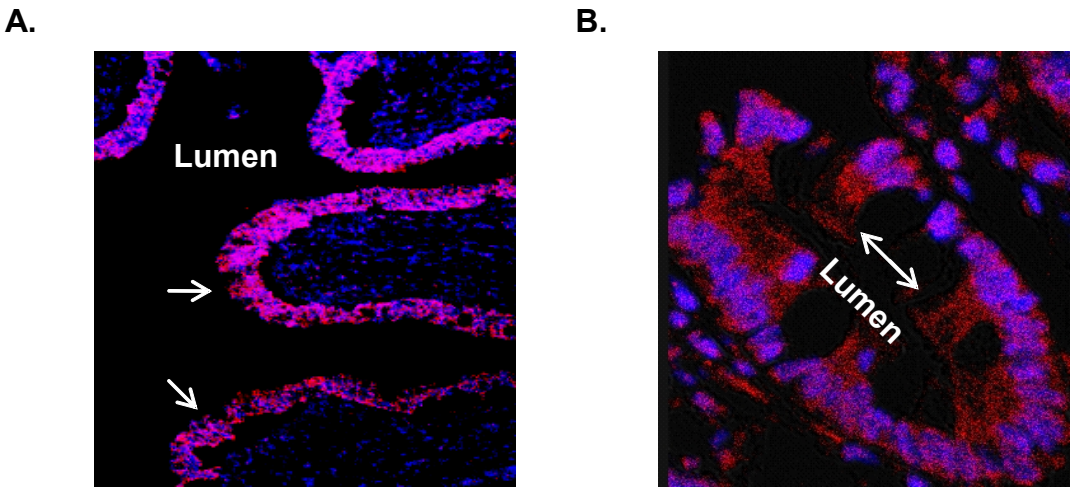


**Figure 22. GPR93 expression in the human small intestine.** Section (10 µM-thick) of human small intestine from human fetal (A) and human adult (B) tissues were stained with goat anti-GPR93 antibody and donkey anti-goat-CY3 antibody as secondary antibody and DAPI indicating nuclei (blue)

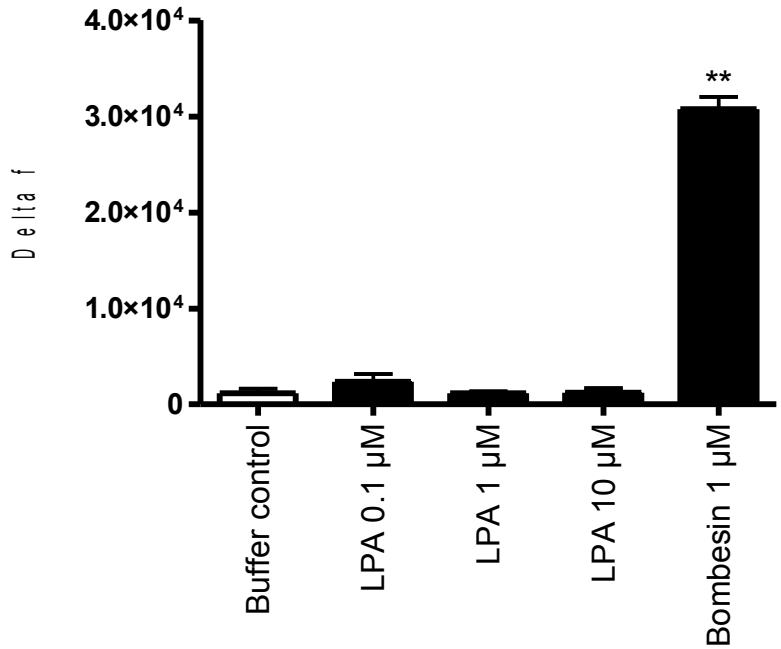


**Figure 23. Stimulation of enteroendocrine HuTu cell with LPA or Bombesin.** Intracellular calcium responses to lysophosphatidic acid (LPA, a potent GPR93 agonist) and Bombesin (positive control) in HuTu-80 cell line. (n= 3, Statistical significance determined by one-way ANOVA, post test: Dunett's, values are mean +/- SEM; **, p-value <0.01).

### 2.2.2. Dipeptide screening for activation of human T1R1/R3 umami and GPR93 receptors

To examine if dipeptides can activate the hT1R1/R3 umami receptor and hGPR93 receptor, HEK cells stably expressing hT1R1/R3 or hGPR93 were screened for receptor activation with a dipeptide library using the calcium assay. In total, 102 different dipeptides were tested at a concentration of 1 mM.

*hT1R1/R3 activation:* Ninety-nine dipeptides (1 mM) were screened for activation of hT1R1/R3 stably expressed in HEK cells using a $[Ca^{2+}]_{in}$ monitor assay. As a positive control served mono sodium glutamate (MSG, 1 mM) known to activate the receptor and wild-type HEK cells were used as a negative control. MSG increased $[Ca^{2+}]_{in}$ in HEK cells stably expressing hT1R1/R3 around 3-fold compared to HEK control cells. Some 30 dipeptides also significantly increased $[Ca^{2+}]_{in}$ in hT1R1/T1R3 expressing HEK cells, whereas 69 dipeptides exhibited no effect on $[Ca^{2+}]_{in}$ **(Figure 24 and Table 2, appendix)**. That the increases in $[Ca^{2+}]_{in}$ most likely were mediated via hT1R1/T1R3 was demonstrated by co-incubation with IMP (500 μM) that increased the slope of $[Ca^{2+}]_{in}$ changes. The dipeptide Glu-Glu also activated synergistically with IMP (500 μM) and in a dose-responsive manner the hT1R1/T1R3 with an $EC_{50}$ of 1.5 mM **(Fig. 25 A and B)**.

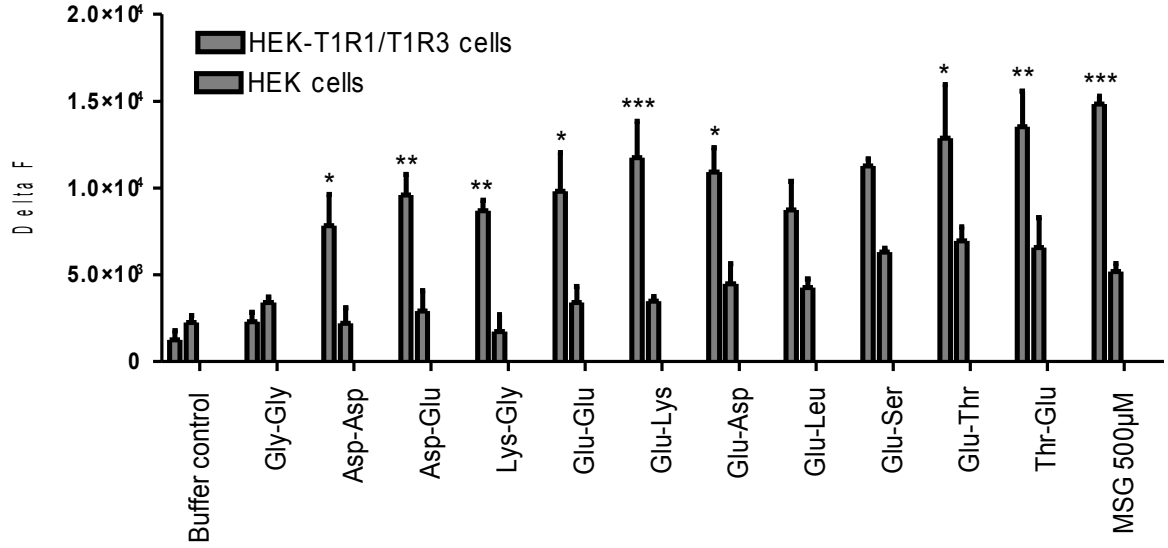

**Figure 24. Dipeptide screening for human T1R1/R3 umami receptor (hT1R1/R3) activation.** HEK293 cells stably expressing Ga15-RGαgustducin44 and hT1R1/R3 (HEK-hT1R1/R3 cells) were stimulated with 1 mM of the corresponding dipeptides. The hT1R1/R3 activation was determined using a calcium assay. The dipeptide screening has been done in parallel on wild-type (wt) HEK cells to assess any non-specific signals. MSG was used as positive control. (n= 3; Statistical significance determined by two-way ANOVA, values are mean +/-SEM; * p-value <0.05; ** p-value <0.01; *** p-value <0.001).

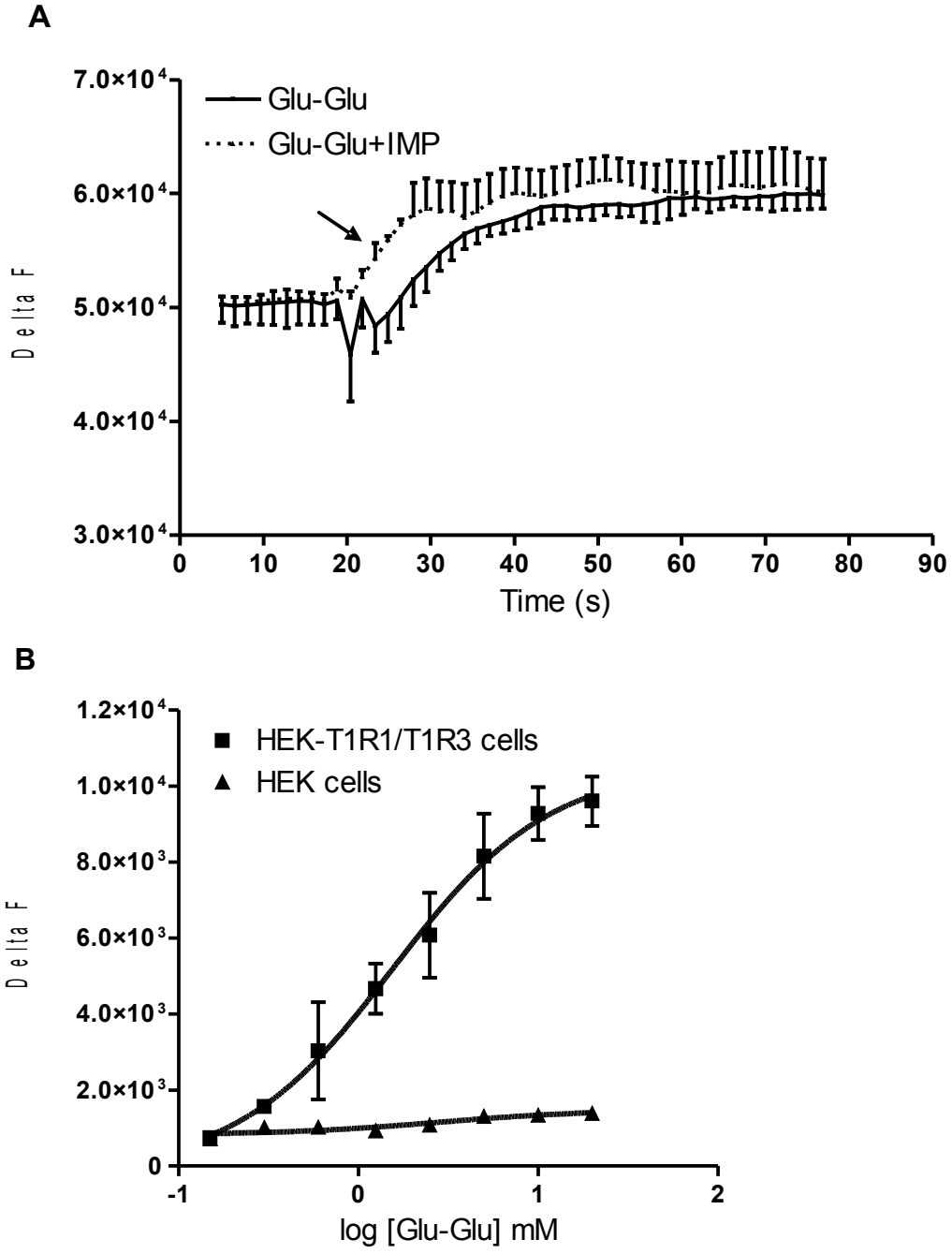


**Figure 25. Specific activation of hT1R1/R3 receptor by Glu-Glu.** (A) HEK-hT1R1/R3 cells were stimulated with 1 mM Glu-Glu or 1 mM Glu-Glu + 500 µM IMP and intracellular calcium signals were recorded over time. (B) hT1R1/R3 activation was measured using the

calcium reporter assay by stimulation with varying concentrations of Glu-Glu in HEK cells stably expressing hT1R1/R3.

For assessing the role of hGPR93, a cell line was established with stable expression of the human GPR93 protein. These HEK-hGPR93 cells responded in a dose dependant manner to stimulation with LPA **(Fig 26A)** and to a protein hydrolysate, **(Fig.26B)** as described (29). Using immunofluorescence expression of hGPR93 could be detected in cell membranes of the HEK cell **(Fig.27A)**. When the cells were treated with triton X-100, which permeabilizes the cell, we also could stain hGPR93 in the cytoplasm, indicating the presence of a cytoplasmic pool of the receptor within the cell **(Fig. 27B)**. At test concentrations of 1 mM, eight out of the 102 dipeptides showed a significant activation of the receptor **(Fig. 28 and Table 3)**. PEP (0.5%), a protein hydrolysate consisting mainly of di- and tripeptides induced as well a significant activation of hGPR93 (p-value <0.001) with positive controls such as the peptone (0.5%) and 5 µM of LPA also shown to significantly activate GPR93 (p-value <0.001) **(Fig. 28)**.

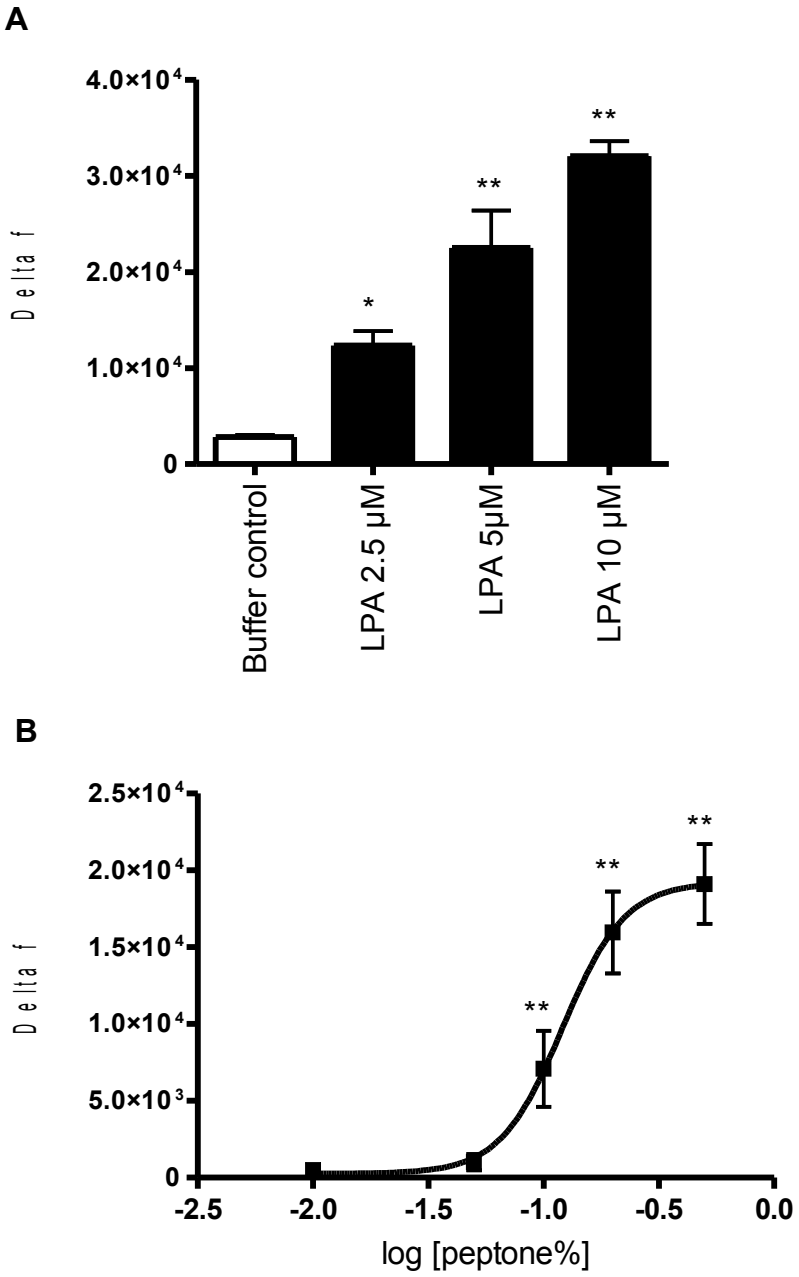


**Figure 26. Activation of hGPR93 in stably transfected HEK- cell line by LPA and peptone.** Intracellular [$Ca^{2+}$] increased dose-dependent in response to hGPR93 stimulation to oleyl-L-alpha-lysophosphatidic acid (LPA) (A) and peptone (B). (n=3, Statistical significance determined by one-way ANOVA, post test: Dunett's, values are mean ± SEM; *, p-value <0,05; **, p-value <0.01).

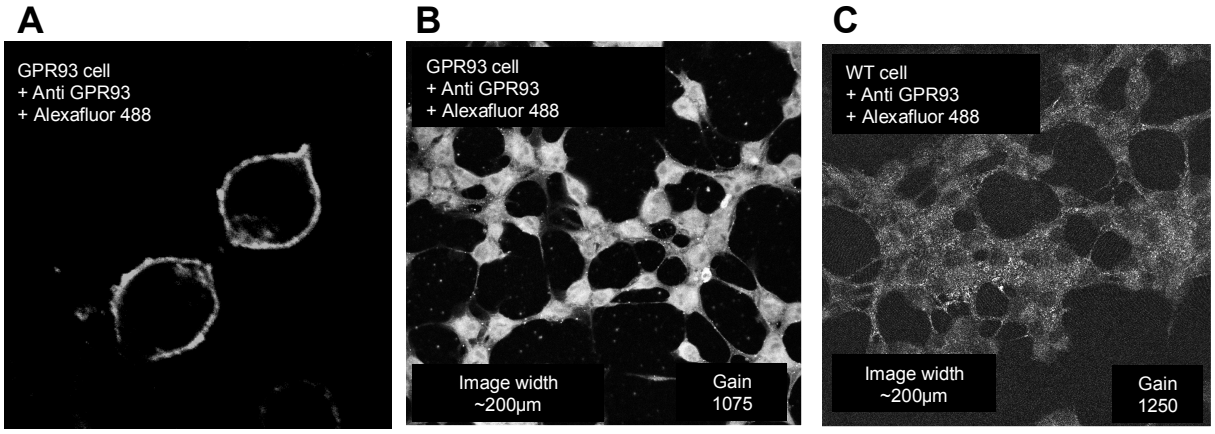


**Figure 27. Immunostaining of human GPR93 in the HEK-hGPR93 cell line.** Indirect immunofluorescence using an antibody against human GPR93 (goat anti human GPR93) on stably transfected HEK cell with hGPR93 (A), or after permeabilisation of HEK cells with with triton (B) and in non-transfected HEK cells (C).

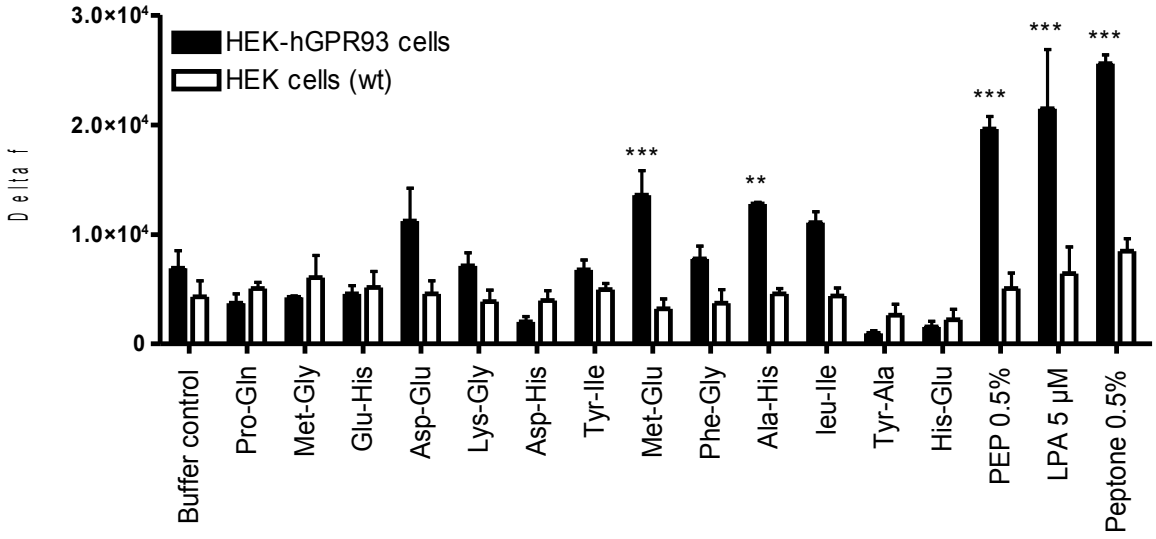


**Figure 28. Dipeptide screening for human GPR93 receptor (hGPR93) activation.** HEK293 cells stably expressing hGPR93 were stimulated with 1 mM of different dipeptides. The dipeptide screening has been carried out in parallel by using wild-type (wt) HEK cells as control for non-specific signals. Thirteen dipeptides out of 102 tested compounds are shown. PEP (0.5%) served as a surrogate mixture of di- and tripeptides. LPA 10 µM and peptone (0.5%) served as positive controls (n=3; Statistical significance determined by two-way ANOVA, values are mean ± SEM; **, p-value <0.01; ***, p-value <0.001).

### 2.2.3. Umami peptides and gastrointestinal hormone secretion

The mouse gut ring model was used to determine whether selected dipeptides that were shown to possess umami taste in sensory tests (57) can act as agonists of the

hT1R1/T1R3 receptor and elicit hormone secretion. Distal and proximal tissues were exposed to selected dipeptides at 20 mM for 30 min and secretion of GLP-1 and CCK was monitored. At pH 7.4 only the cationic dipeptide Lys-Gly increased GLP-1 secretion by 3.2-fold (p-value <0.05) compared to buffer control **(Fig. 29A)**. As a positive control, the protein hydrolysate PEP (4 %) was shown to significantly increase GLP-1 secretion around 7-fold (p-value <0.001) **(Fig. 29A)** whereas L-glutamate 20 mM as the most specific activator of hT1R1/R3, elevated GLP-1 concentrations about 3.5-fold (p-value <0.05) compared to the buffer control **(Fig. 29A)**. None of the dipeptides tested or L-glutamate did cause a release of CCK from mouse proximal intestinal tissues **(Fig. 29B)** whereas linoleic acid (1 mM) as control increased CCK secretion around 3-fold (p-value <0.001) **(Fig. 29B)**.

Most interestingly, at a buffer pH 6.0, the anionic dipeptide Glu-Glu increased GLP-1 secretion 2.2-fold (p-value <0.01) whereas no effects of Lys-Gly were found at this pH **(Fig. 30A)**. Like at buffer pH 7.4, none of the dipeptides tested caused a significant CCK secretion at pH 6.0 **(Fig. 30B)**.

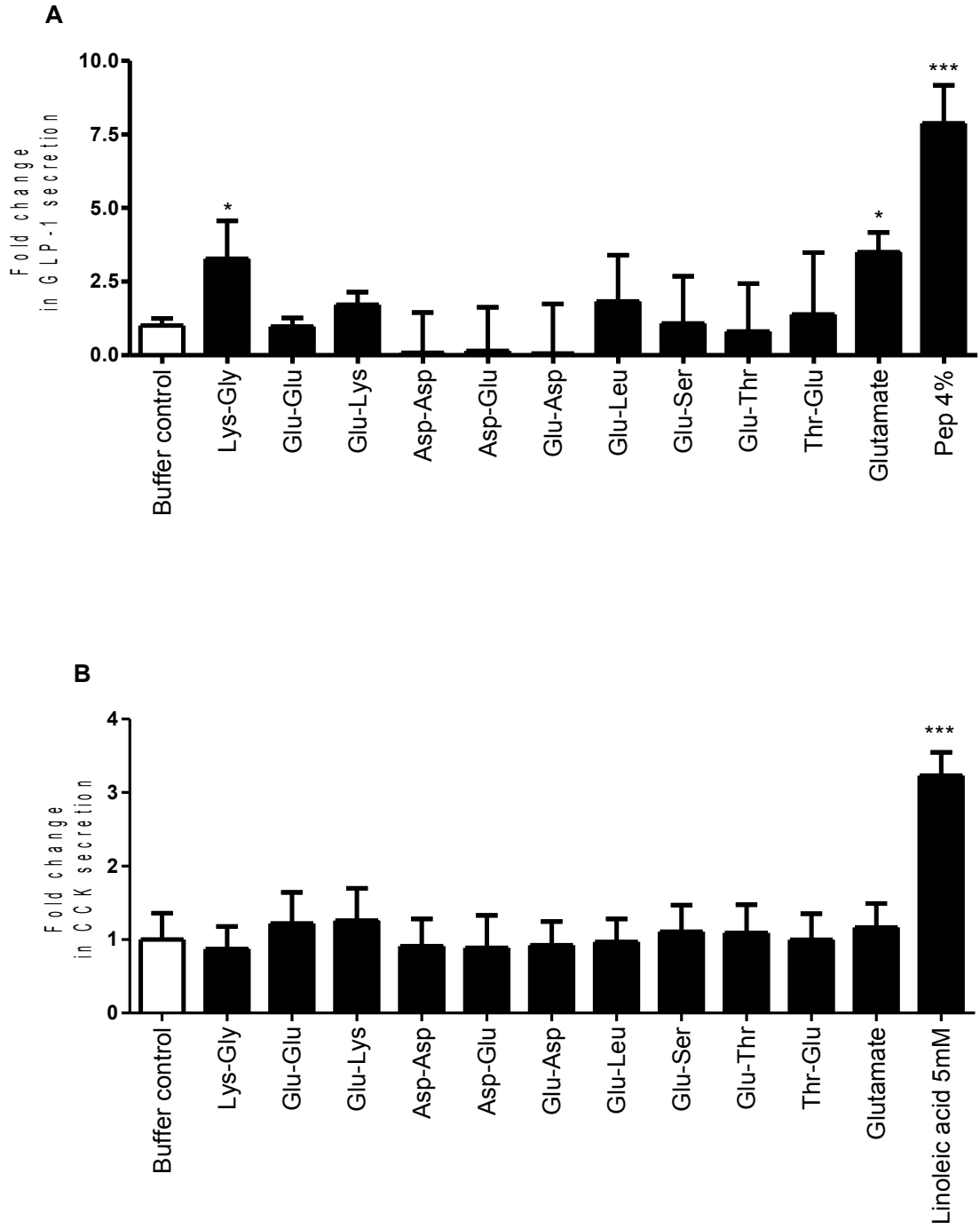


**Figure 29. GLP-1 and CCK secretory responses to umami dipeptides in mouse intestinal rings.** Tissues were stimulated with different umami dipeptides at concentrations of 20 mM at pH 7.4. Concentrations of GLP-1 (A) and CCK (B) were measured in the medium after incubation. Stimulation with 4 % PEP and with 5 mM linoleic acid served as positive control for GLP-1 and CCK secretion respectively. (n=6, Statistical significance determined by one-way ANOVA, post test: Dunett's, values are mean ± SEM and are expressed as fold change of control (Buffer control); * p-value <0.05; *** p-value <0.001).

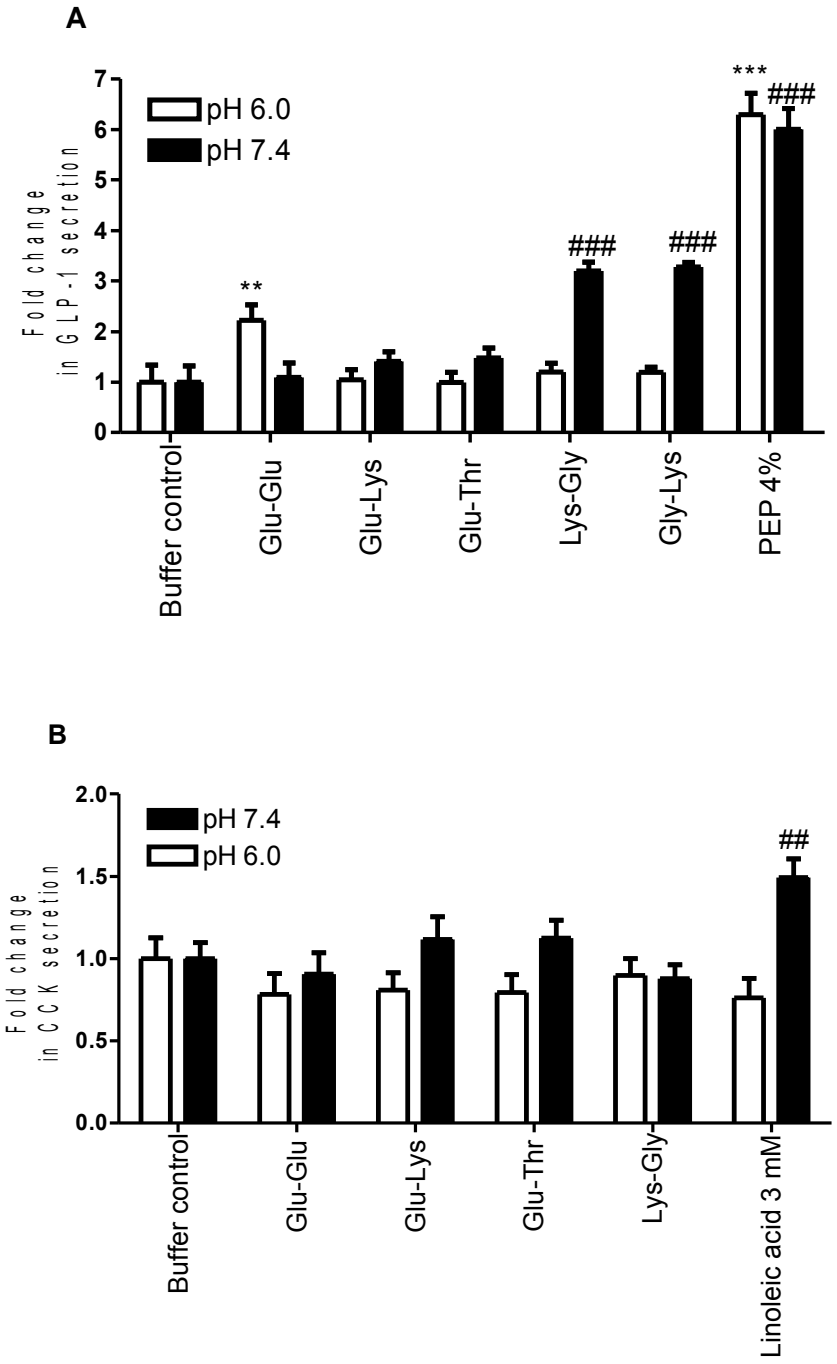


**Figure 30. Comparison of GLP-1 and CCK secretory responses to umami dipeptides at in mouse intestinal rings at buffer pH 6.0 and 7.4**. Freshly isolated mouse small intestinal rings were incubated with buffers containing the different umami dipeptides at a concentration of 20 mM at pH 6.0 or pH 7.4 and media were assayed for GLP-1 (A) by ELISA or CCK (B) using a calcium reporter assay for the determination of CCK1R activation. (n=6, Statistical significance determined by two-way ANOVA, post test: Bonferoni, values are mean ± SEM and are expressed as fold change of control (Buffer control); ** or ## p-value <0.01; ### p-value <0.001; * compared to buffer control at pH 6.0; # compared to buffer control at pH 7.4).

### 2.2.4. Sensing mechanism underlying the umami dipeptide mediated GLP-1 release

Knowing that PEPT1 is involved in sensing and that selected umami-tasting dipeptides can activate the umami receptor we tested whether GLP-1 secretion in response to the umami dipeptides involves both T1R1/T1R3 activation and PEPT1. For this mouse intestinal rings from PEPT1$^{-/-}$ mice and wild-type mice as well as specific T1R1/T1R3 and PEPT1 inhibitors were used. In tissues derived from PEPT1$^{-/-}$ mice neither Glu-Glu nor Lys-Gly (20 mM, pH 6.0 and pH 7.4) stimulated a significant GLP1 secretion when compared to buffer control **(Fig. 31)**. When in addition to Glu-Glu (20 mM, pH 6.0) the competitive high-affinity PEPT1-inhibitor Lys-(z(NO3))-Pro (PEPT1i, 500 µM) was added, Glu-Glu-induced GLP-1 secretion was abolished **(Fig. 31)**. Moreover, in gut rings derived from wild-type mice, the coactivator of the T1R1/T1R3 receptor IMP further stimulated modestly GLP1 secretion (3.5-fold) when induced by Glu-Glu (20 mM, pH 6.0) as compared to secretion observed in the absence of IMP (2.8-fold), although this was not statistically significant **(Fig 31)**. Similarly, IMP increased the L-glutamate induced GLP-1 secretion compared to glutamate alone (4.6-fold verses 3.1-fold). In tissues of PEPT1$^{-/-}$ mice, GLP-1 secretion was as high as in wild-type mice upon stimulation with L-Glutamate. The T1R1 inhibitor lactisol (1 mM) fully inhibited the GLP1 secretion induced by Glu-Glu (20 mM at pH 6.0) in both genotypes **(Fig. 31)**.

As a note: using the two-electrode voltage clamp technique in *Xenopus laevis* oocytes heterologously expressing human PEPT1, it was demonstrated that Glu-Glu is indeed substrate of PEPT1 and induces a concentration dependently increase in PEPT1 transporter activity with inward currents comparable to the known PEPT1 substrate Gly-Gln (20 mM) **(Fig. 32).**

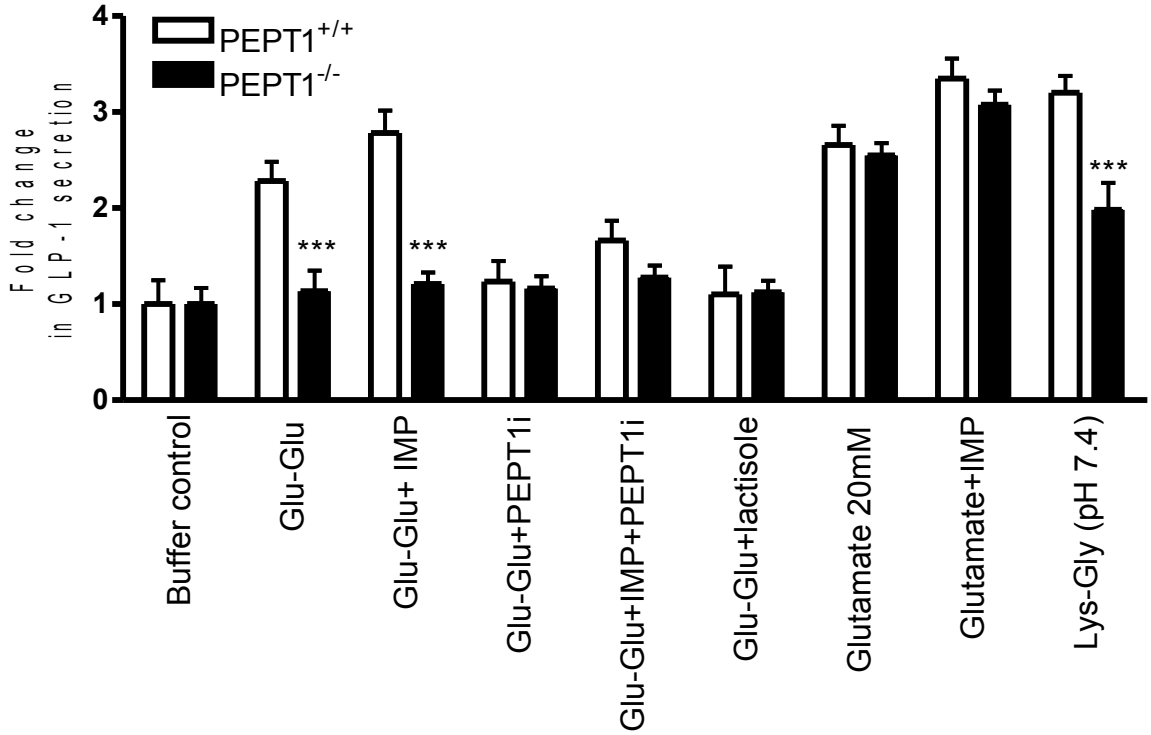


**Figure 31. Characterization of the GLP-1 secretory response to umami dipeptides in mouse intestinal rings.** Distal gut rings from wild-type mice and PEPT1$^{-/-}$ mice were stimulated with 20 mM Glu-Glu and co-incubated with or without 500 µM IMP, 500 µM Lys-(z($NO_3$))-Pro (PEPT1i) and 500 µM lactisol, 20 mM Glutamate and co-incubated with or without 500 µM IMP, 20 mM Lys-Gly. Experiment were done at pH 6.0, unless indicate differently. (n=4, Statistical significance to buffer control determined by one-way ANOVA, post test: Dunett's, values are mean ± SEM and are expressed as fold change of control (Buffer control); * p-value <0.05; ** p-value <0.01).

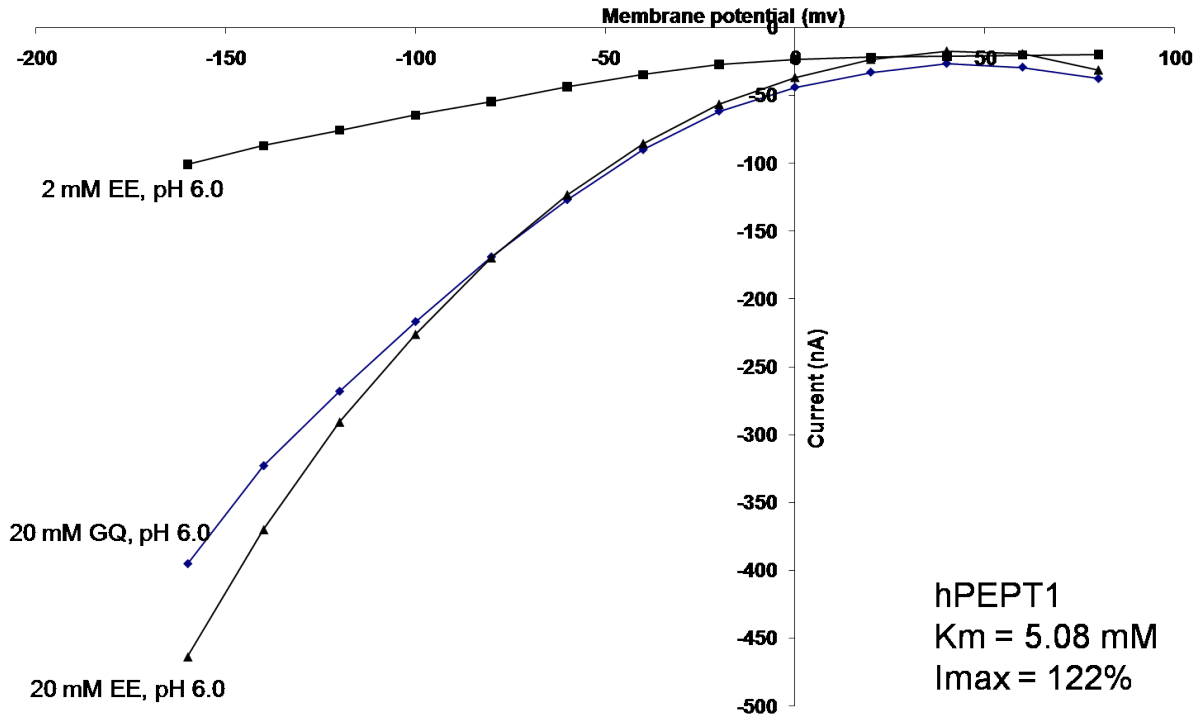


**Figure 32. Characterization of the transport of Glu-Glu by human PEPT1 in Xenopus laevis oocytes by electrophysiology.** Steady-state I–V relationships were measured by the two-electrode voltage clamp technique in oocytes expressing hPEPT1 and superfused with modified Barth solution at pH 6.0 with 2 or 20 mM Glu-Glu (EE). As a positive control 20mM Gly–Gln (GQ) was used. Current-voltage (I-V) relations were measured in the potential range of –160 to +80 mV, and the current generated by the dipeptide transport at a given membrane potential was calculated as the difference of the currents measured in the presence and absence of substrate.

In conclusion, we have shown that selected dipeptides with umami taste can activate the human T1R1/R3 umami receptor and that some of them can elicit a GLP-1 secretion from mouse intestinal rings. Using tissues from PEPT1$^{-/-}$ mice we furthermore show that GLP-1 release in response to Glu-Glu and Lys-Gly, both substrates of PEPT1, is abolished, indicating that PEPT1 plays a role in GIH release also when stimulated by umami dipeptides. The inhibition of GLP-1 release in response to Glu-Glu by lactisol and increased GLP-1 release in the presence of IMP suggest that T1R1/R3 umami receptors also contribute to the signaling that leads to GIH secretion. However, as for hGPR93, only a small number of dipeptides without a distinct structure-activity relationship were capable to activate this receptor. However, that GPR93 participates in sensing of protein and its degradation products comes obvious by its activation by peptones. Whether GPR93 prefers tripeptides and/or larger oligopeptides needs to be determined.

# DISCUSSION

Increasing the secretion of gut hormones by dietary components or individual foods seems an attractive approach for inducing satiety in weight management and for controlling metabolic responses to food intake. In particular GLP-1 is in focus by its action on enhancing glucose-dependent insulin secretion and for maintenance of β-cell function. Despite its clinical importance, the mechanisms that mediate GLP-1 secretion from intestinal endocrine L-cells in response to luminal nutrients remains to be determinated. It is known that an oral protein load is associated with increased satiety (10, 80) and altered intestinal hormone secretion (18, 40). The present work demonstrates that in human volunteers the addition of a protein hydrolysate to a standart OGTT increased plasma GLP-1 but not GIP or PYY levels and increased significantly plasma insulin concentrations. The break-down of dietary protein in the gut by pancreatic proteases and membrane-bound peptidases generates an uncharacterized mixture of all possible peptides that may be part of the protein sensing pathways that lead to hormone secretion. Previous studies provided indirect evidence that the intestinal transporter PEPT1 may be involved in protein sensing and stimulation of GLP-1 release by protein degradation products in the intestinal lumen (38, 87). By combining *in vitro* and *in vivo* studies in wild-type mice and mice lacking PEPT1, we demonstrate that the transporter appears to selectively be involved in GLP-1 but not CCK and GIP secretion in response to di- and tripeptide mixtures. However, the loss of protein-dependent GLP-1 secretion in PEPT1$^{-/-}$ mice did not show alterations in plasma insulin levels to a high protein load. Previously, Hansotia et al. showed in GIPR$^{-/-}$ or GLP-1R$^{-/-}$ mice only very modest impairments in glucose homeostasis (58). In GIPR$^{-/-}$ + GLP-1R$^{-/-}$ mice (dirko mice), Preitner et al. demonstrated an additive insulinotropic action of GIP and GLP-1 during an oral glucose load (103), suggesting the existence of compensatory mechanisms in the incretin actions of GIP and GLP-1. Imunofluorescence detected GLP-1 in endocrine cells also from PEPT1$^{-/-}$ tissues and a normal response to a glucose load suggested that GLP-1 secretion but not its

production in response to protein is impaired. Moreover, all other gastrointestinal hormones showed normal secretion *in vitro* as well as in PEPT1$^{-/-}$ mice *in vivo*. We also demonstrate that standard substrates of PEPT1 such as Gly-Gly and Gly-Sar or cefadroxil all elicit a GLP-1 secretion in wild-type mice. Stimulation with the dipeptide Gly-Gly was shown to be associated with an increase in $[Ca^{2+}]_{in}$ in isolated villi of wild-type mice with a drastically reduced increase in PEPT1$^{-/-}$ mice. In isolated tissues we also demonstrated that nifedipin reduces GLP-1 secretion to dipeptide suggesting that voltage-dependent calcium channels may mediate a calcium influx.

A variety of cell lines are in use to determine endocrine cell responses to various intestinal stimuli (1, 4, 5, 28, 30, 33, 43, 62, 73, 105, 114). In normal intestine only around 1% of all cells in the epithelial layer are hormone secreting cells and are surrounded by normal enterocytes. Both cells project microvilli into the lumen and express a variety of receptors and transporters with the capability to sense sugars, amino acids, proteins and fatty acids as well as bitter compound and artificial sweeteners. Conceptually endocrine cells are so far considered as individual sensory cells. Since PEPT1 protein could not be detected by immunofluorescence in brush borders of endocrine cells, the signal from enterocytes received by L-cells could either be of electrical nature (depolarization) and/or chemical nature by diffusion of calcium ions or protons that are cotransported by PEPT1 and that cause an intracellular acidifcation. We are currently unable to characterize the incoming signal in L-cells but demonstrate that two compounds reported to block gap-junction communication (tamoxifen and glycyrrhetinic acid) are able to block also peptide-induced GLP-1 secretion via PEPT1 in epithelial cells. The present work provides evidence for a cell-to-cell communication via gap junctions by which a signal from epithelial cells is transmitted into the L-cell for secretion of GLP-1. Such a cross-talk in stimulus secretion coupling between epithelial and endocrine cells has so far not been shown before in the context of nutrient

sensing and gut hormone secretion. In this respect the work address a new level of complexity in gut response mechanism to food intake.

Recently, Mace et al. demonstrated an interaction of various nutrient transporters and receptors and called it an “integrated network” that involved PEPT1, SGLT-1 and GLUT2 but also the taste receptors T1R1/R3 and T1R2/R3 (84). The umami receptor is proposed to regulate along with sweet taste receptors, intracellular calcium changes and PKC $\beta$II the trafficking of the PEPT1 and the facilitative glucose transporter GLUT2 from intracellular vesicles into the brush border membranes of the epithelial cell. Enterocytes expressing both PEPT1 and T1R1/R3 umami receptor in the apical membrane must therefore integrate the signals and could stimulate putative target cells located in the vicinity such as enteroendocrine cells. The current work demonstrated for the first time that selective dipeptides can also activate the umami receptor hT1R1/R3. Ten dipeptides were identified to activate hT1R1/R3 receptor expressing cells *in vitro* and those are also known to possess umami taste as identified in human sensory studies (57). They were also tested for their ability to elicit a GIH release in tissues of mice *ex vivo*. For the first time, L-glutamate was also shown to cause a GLP-1 release from mouse intestine demonstrating it could play also a role in digestion and absorption processes but also in the regulation of appetite. Two umami dipeptides were identified out of the ten tested, namely Glu-Glu and Lys-Gly, that possessed the ability to elicit a GLP-1 release at pH 6.0 or 7.4, respectively. The different pH values were used as it is known (76) from studies with PEPT1 that cationic peptides are transported more efficiently at more neutral pH (7.4) and in opposite that acidic dipeptides such as Glu-Glu have higher transport rates at lower pH (6.0). Using tissues from PEPT1$^{-/-}$ mice or the high-affinity competitive PEPT1 inhibitor Lys-(z($NO_3$))-Pro on tissues from wild-type mice it was demonstrated that GLP-1 release in response to Glu-Glu and Lys-Gly is abolished, indicating that PEPT1 plays a prominent role in hormone release when stimulated by umami dipeptides. Using the Xenopus oocyte expression system it was independently shown that Glu-Glu is a

PEPT1 substrate at pH 6.0 that generates as large currents as the zwitterionic substrate Gly-Gln. PEPT1 can transport essentially all possible di- and tripeptides with a few exceptions based on sterical constraints. PEPT1 transport in any case causes a membrane depolarization as well as intracellular acidification although the stoichiometry of substrate to protons movement varies depending on the substrates net charge at a given pH (76). In this respect PEPT1 is a versatile transporter and acts as a sensor with recognizing all possible natural di- and tripeptide substrates as derived from luminal protein hydrolysis. Its specificity however is determined by peptide chain length excluding free amino acids or peptides with > 3 residues and other structural features in substrates (73). Here it is demonstrated that depending on the pH umami dipeptides can cause GLP-1 secretion in accordance with the pH-dependent changes in substrate charge but also pH-dependent changes in PEPT1 conformation.

The experimental findings suggest that the T1R1/R3 umami receptor may also be involved in the signaling pathway that leads to GLP-1 release when umami peptides are used. This is essentially based on the inhibition of the response to Glu-Glu by lactisol and the modest enhancement by IMP. However, the exact role that the umami receptors plays in sensing of short chain peptides needs to be studied in more detail. We cannot a priori exclude that some L-glutamate as a product of cleavage of Glu-Glu by peptidases is produced that could activate the receptor although all studies were conducted in the presence of a peptidase inhibitor. T1R1/R3 is expressed throughout the gastrointestinal tract and has been shown to be activated by almost all amino acids with a potentiated response in the presence of nucleotides. Membrane-bound peptidases at the brush border membrane can release free amino acid from oligopeptides produced in the lumen during proteolytic cleavage of the dietary proteins. However, when there is a high dietary load of protein during a meal the capacity for hydrolysis may be overcome leaving a variety of short chain peptides that in addition to free amino acids can activate T1R1/R3. Upon activation of the heterodimeric T1R1/R3 in apical cell membranes the βγ subunit activates phospholipase C β2, which in turns produces inositol

triphosphate (IP3) and diacylglycerol. IP3 binds to IP3 receptors (IP3R3) that releases calcium from intracellular stores and the increase of intracellular calcium levels can activate the monovalent selective cation channel TRPM5, which depolarizes the cells. The calcium increase and membrane depolarization may then cause a GLP-1 release. It therefore seems plausible to assume that two signalling pathways are responsible for GLP-1 release by selective umami dipeptides, including PEPT1-mediated transport of the dipeptide and the sensing of dipeptide/amino acids by the umami T1R1/R3 receptor expressed in the gut epithelium. Reagrding the IMP synergism effect on GLP-1 secretion, Zhang et al, proposed a cooperative ligand-binding model involving the outer membrane N-terminal Venus flytrap domain (VFT) of T1R1 unit, where L-glutamate or ligands binds, and IMP bind to an adjacent site close to the VFT to further stabilize the conformation (140). The negatively charged phosphate group of IMP coordinates the positively charged of the T1R1 VFT to stabilize the conformation of T1R1 VFT. The positive allosteric modulator mechanism for the synergy between amino acid/peptide and IMP in activating the umami taste receptor may play a role in the enhanced GLP-1 secretion.

As a robust finding it can be concluded that PEPT1 in response to umami and other dipeptides does not contribute to a CCK release. This is interesting as several studies have shown *in vitro* by using STC-1 cells, which do not express PEPT1, that protein hydrolysates as well as peptidomimetics can elicit a CCK release (33, 34, 98). This suggests that another/other sensor systems must be responsible for the CCK release in response to dietary protein. Recently, a G-protein coupled receptor, GPR93, has been identified in the apical membrane of enterocytes and described to be activated by protein hydrolysates, and that cephaclor, a peptidometic substrate for PEPT1 failed to activate GPR93. However, cephaclor was shown to synergistically with a protein hydrolysate potentiate GPR93 activation (29) and when overexpressed in the enteroendocrine cell line STC-1, the activation by protein hydrolysates induced a CCK secretion from STC-1 cells (30). Yet, Ma et al. demonstrated that

cephaclor given orally to humans had no effect on CCK secretion and on gastric emptying in healthy volunteers (82). As cephaclor is a PEPT1 substrate, this finding supports our experiments that failed to demonstrate an involvement of PEPT1 in CCK secretion. Although we found that a small number of dipeptides were capable to activate hGPR93, they were less effective than the protein hydrolysate or peptone. Taken together, the experimental evidence argues against dipeptides as distinct entities can cause a CCK release in the upper small intestine whereas a peptone may via GPR93 able to provide stimulation. What however is recognized as a ligand by GPR93 when a peptone or protein hydrolysate is provided needs to be determined. It may be speculated that most likely larger peptides – possessing 3 or more amino acids – act as ligands. Expression of GPR93 shown in human small intestine and therefore the contribution of this receptor to nutrient signaling is intuitive. When HuTu-80 cells as a model for CCK secreting endocrine cells were stimulated with LPA, a potent agonist for GPR93, no changes in the intracellular calcium response could be recorded but, whether the receptor is expressed in HuTu cells, is currently not known. Future studies have to establish whether GPR93 is expressed in enteroendocrine cells in the upper small intestine and not only in epithelial cells as shown. This is important to know because if GPR93 would only be found in enterocytes, a paracrine signaling pathway or another example for direct cell-cell communication via a connexon has to be anticipated.

## SUMMARY AND CONCLUSIONS

The work presented here adds some relevant findings and new concepts to the intriguing question on how the intestinal mucosa can sense its luminal contents to react with proper hormone secretion to control gastrointestinal and brain functions in response to food intake. Dietary proteins are known to have pronounced effects on satiety as well as intestinal and metabolic processes; yet the underlying mechanisms by which protein ingestion is sensed are largely unknown. Experimental data in cells, mice and humans provide evidence that the intestinal peptide transporter is not only a transporter but also a sensor for di- and tripeptides as end products of the luminal protein degradation. Although not expressed in GLP-1 secreting L-cells, PEPT1 function in neighboring epithelial cells mediates a signal into endocrine cells possibly via gap-junctions that elicits the GLP-1 output. This represents a new concept in stimulus secretion coupling, based on a functional sensor system comprised of two cell types. In addition, the studies demonstrate that umami-tasting dipeptides can interact with the T1R1/R3 receptor for mono-sodium glutamate and that there is most likely a cross-talk with PEPT1. The novel G-protein coupled receptor that can be activated by linoleic acid but also by protein hydrolysates and that mediates a CCK secretion needs further characterization for identification of its ligands. Taken together, protein degradation products in the intestinal lumen seem to be able to activate a variety of signaling pathways that translate into hormone secretion.

## APPENDIX 1

| Actives | Inactives | |
|---|---|---|
| Asp-Asp | Glu-Leu | Tyr-Pro |
| Asp-glu | Glu-Ser | Ser-Arg |
| Lys-Gly | Glu-Gly | Asn-Lys |
| Glu-Glu | Tyr-Glu | Lys-Asp |
| Glu-Lys | Trp-His | Trp-Pro |
| Glu-Asp | Thr-Ala | Pro-Thr |
| Glu-Thr | Trp-Gly | Lys-lys |
| Thr-Glu | Thr-His | Ser-Pro |
| His-Glu | Ser-Ala | Thr-Gln |
| Thr-Asp | Arg-Asp | Lys-Thr |
| Asp-Gly | Ala-Gly | His-Lys |
| Ser-Phe | Val-Gly | His-Asn |
| Ile-Asn | Ser-His | Pro-Pro |
| Asp-Gln | Gln-Ala | Val-Met |
| Arg-Trp | Glu-Asp | Tyr-Asn |
| Asp-His | Ser-Gly | Lys-Gln |
| Tyr-Ile | Pro-His | Ala-Thr |
| Met-Glu | Phe-Ala | Trp-Asn |
| Phe-Gly | Ala-Gln | His-Gln |
| Ala-His | Thr-Arg | Pro-Ser |
| Leu-Ile | Tyr-Val | Asn-Tyr |
| Glu-Thr | Val-Lys | Arg-Leu |
| Asp-Asn | Asn-Asn | Ser-Asn |
| Arg-Gln | Tyr-Pro | Asn-Ser |
| Ser-Glu | Ser-Arg | Pro-Leu |
| Asn-Gln | Asn-Lys | Gln-Met |
| Gln-His | Lys-Asp | Cys-Gln |
| Asp-Lys | Trp-Pro | Gly-Ser |
| Ala-Asp | Ala-Gln | Lys-Trp |
| Pro-Gln | Thr-Arg | Asn-Leu |
| | Tyr-Val | Pro-Asn |
| | Val-Lys | Met-Gly |
| | Asn-Asn | Glu-His |
| | Ser-Gln | Tyr-Ala |
| | Gly-Gly | |

**Table 2: Screening of 99 dipeptides (1 mM) for hT1R1/R3 receptor activation.** Table shows the active and inactive dipeptides.

## APPENDIX 2

| Actives | Inactives | |
|---|---|---|
| Ser-Asn | Asp-Asp | Glu-Leu |
| Asp-Gln | Lys-Gly | Glu-Ser |
| Val-Lys | Glu-Glu | Glu-Gly |
| Gly-Arg | Glu-Lys | Tyr-Glu |
| Gly-His | Glu-Asp | Trp-His |
| Met-Glu | Glu-Thr | Thr-Ala |
| Ala-His | Thr-Glu | Trp-Gly |
| Asp-Glu | His-Glu | Thr-His |
| | Thr-Asp | Ser-Ala |
| | Asp-Gly | Arg-Asp |
| | Ser-Phe | Ala-Gly |
| | Ile-Asn | Val-Gly |
| | Arg-Trp | Ser-His |
| | Asp-His | Gln-Ala |
| | Tyr-Ile | Glu-Asp |
| | Phe-Gly | Ser-Gly |
| | Glu-Thr | Pro-His |
| | Asp-Asn | Phe-Ala |
| | Arg-Gln | Ala-Gln |
| | Ser-Glu | Thr-Arg |
| | Asn-Gln | Tyr-Val |
| | Asp-Lys | Val-Lys |
| | Ala-Asp | Asn-Asn |
| | Pro-Gln | Tyr-Pro |
| | Asn-Asn | Ser-Arg |
| | Ser-Gln | Asn-Lys |
| | Gly-Gly | Lys-Asp |
| | Thr-Arg | Trp-Pro |
| | Tyr-Val | Ala-Gln |
| | Val-Lys | Met-Gly |
| | Glu-His | Tyr-Ala |

**Table 3: Screening of 102 dipeptides (1 mM) for hGPR93 receptor activation.** Table shows the active and inactive dipeptides.

# ZUSAMMENFASSUNG

Das Darmepithel exprimiert ein chemosensorisches System zur Wahrnehmung von luminalen Nährstoffen, das die Sekretion von Hormonen und regulatorischen Peptiden aus endokrinen Zellen auslöst. Die Aktivierung von endokrinen Zellen durch luminale Nährstoffe und die darauffolgende Hormonsekretion spielt eine wichtige Rolle bei der Regulation von Verdauung und Absorption, und einigen dieser Hormone wie z. B. dem glucagon-like peptide 1 (GLP-1) werden auch eine sättigende Wirkung zugesprochen. Proteine sind bekannt dafür, sättigend wirksame Nahrungsbestandteile zu sein, die auch zur Sekretion von Darmhormonen führen. Die zugrundeliegenden Mechanismen sind allerdings noch nicht bekannt. Das Ziel der vorliegenden Arbeit war es daher a) neue *ex vivo* Methoden zur Charakterisierung von Proteinwahrnehmungsmechanismen im Darm zu etablieren und b) mit diesen Modellen zu untersuchen, in welcher Weise bestimmte Rezeptoren und Transporter an der Peptidwahrnehmung und Hormonsekretion beteiligt sind. Durch eine Kombination von *ex vivo* und *in vivo* Methoden konnte gezeigt werden, dass ein neuartiger Wahrnehmungsmechanismus, bei dem der intestinale Peptidtransporter PEPT1 in Enterozyten eine Rolle spielt, das Signal in endokrine Zellen weiterleitet, was letztendlich die proteinabhängige GLP-1 Sekretion auslöst. Des weiteren wurde die Wahrnehmung von Umamipeptiden untersucht und ein G-protein gekoppelter Rezeptor im Darm identifiziert, der nicht nur durch Natriumglutamat aktiviert wird, sondern auch durch eine Vielzahl von Umamipeptiden. Es scheint also ein Netzwerk von Signalwegen und die Kommunikation zwischen verschiedenen Zelltypen notwendig zu sein, um die Kopplung zwischen der Stimulation mit Nahrungsprotein und der Hormonsekretion zu vermitteln.

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## LIST OF SCIENTIFIC COMMUNICATIONS

### Oral communications:

1st Nusisco meeting, Paris, France, 02.2007

2nd Nusisco meeting, Munich, Germany, 06.2007

3rd 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** Fourchaud, L., Rist, M. and Daniel, H. Development of an *ex-vivo* screening system for gut sensory active ligands which elicit intestinal hormone release. Unilever R&D center, Vlaardingen, the Netherlands.

**2008** Fourchaud, L., Rist, M. and Daniel, H. Design of *in-vitro* and *ex-vivo* system to assess signalling responses and cellular cross-talk. Unilever R&D center, Vlaardingen, the Netherlands.

**2009** Fourchaud, L., Rist, M. and Daniel, H. Development of an *ex-vivo* screening system for gut sensory active ligands which elicit intestinal hormone release. Technische Universität München, Lehrstuhl für Ernährungsphysiologie und ZIEL Abteilung Biochemie, Freising, Germany.

**2009** Fourchaud, L., Rist, M. and Daniel, H. Design of *ex-vivo* model to assess signalling processes and cellular cross-talk that lead to gastrointestinal hormone secretion. Unilever R&D center, Vlaardingen, the Netherlands.

**2009** Fourchaud, L., Rist, M. and Daniel, H. Development of an *ex-vivo* screening system for gut sensory active ligands which elicit intestinal hormone release. 4th Congrès de la Société Française de Nutrition (SFN), Montpelliers, France.

## LIST OF PUBLICATIONS

**I .** Fourchaud, L., Nässl, AM., Wünsch, T., Rubio-Aliaga, I., Rist M. and Daniel, H. **The signaling function of the intestinal peptide transporter PEPT1 in GLP-1 secretion.** (in submission)

**II.** Fourchaud, L., Rauscher, B., Kottra, G., Van Buren, L., Wieland, K., Rist M., Foltz M. and Daniel, H. **Activation of T1R1/R3 umami receptor by selective dipeptides causes GLP-1 secretion.** (in submission)

# CURRICULUM VITAE

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## AUSBILDUNG

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*"Characterisation of protein and peptide sensing processes in the gut coupled to gastrointestinal hormone secretion using new ex-vivo models"*

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## PRAKTIKUM

**2008** **Unilever R&D center**, Vlaardingen, Niederlande, 6 Monate wissenschaftliche Kooperation im Rahmen der Doktorarbeit, Thema: *"Peptides as putative ligands of hT1R1/R3 umami and GPR93 receptors in the intestine"*.

**2006** **IRMES – INSEP,** Paris, Frankreich, 6 Monate, Ernährungsepidemiologie in sport.

## SONSTIGES

Sprachkenntnisse: Französisch, Englisch, Deutsch

Freizeit: Schwimmen, Tanzen, Malen, and Architektur