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

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Received 12 October 2015; received in revised form 4 March 2016; accepted 10 March 2016

Abstract

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

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

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

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

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

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

Keywords: Serotonin; GLP-1; nutrients; rebaudioside A; small intestine

1. Introduction

Gastro-intestinal (GI) hormones play major roles in the regulation of food intake, gastric emptying and GI motility. In the small intestine hormones are secreted by enteroeudocrine cells (EEC) [22], and are involved in modulating feelings of satiety and satiation using feed-back mechanisms to reduce food intake [6,22]. Well-known satiety inducing hormones are cholecystokinin (CCK), peptide YY (PYY) and glucagon like peptide-1 (GLP-1). The release of these hormones involves interaction between nutrients and G-protein coupled receptors (GPCR) or ion-dependent transporters that are expressed on EECs [22].

Recently it was found by our group that nutritional and non-nutritional stimuli such as a lipid mixture (safflower oil), carbohydrate (sucrose), protein (casein) and a non-caloric sweetener (rebaudioside A) induce GLP-1 and PYY release from ileal tissue segments [24]. Additionally, when these ‘non-predigested’ macronutrients are delivered to the ileum by a nasoileal feeding tube, they activate satiety signals, increase satiety hormone release and decrease subsequent food intake in healthy volunteers [29]. The mechanism of how these different nutrients delivered to the ileum cause satiety hormone release remains to be investigated.

It is known that lipids are sensed in the form of long and short chain fatty acids by free fatty acid receptors. Carbohydrates are predominantly sensed as glucose, possibly via the Na\textsuperscript{+} dependent sodium/glucose cotransporter member 1 (SGLT-1) or by the so-called sweet taste receptor, a G-protein coupled heterodimer (T1R2/T1R3). Proteins are mainly detected after being digested into amino acids, di- and tripeptides by the G-coupled calcium sensing receptor (CaSR), umami taste receptor dimer T1R1/T1R3, GPCR6A, and peptide transporter 1 (PEPT1) [16,22]. However, not for all nutrients stimulating GLP-1, PYY or CCK release, a receptor is known. There may be additional mechanisms allowing to respond to fluctuations in intestinal nutrient levels [9]. One such mechanism may involve paracrine cell–cell interaction via serotonin release from enterochromaffin cells (ECC).

Although it is known that serotonin is involved in food intake regulation [30], it is still unknown how serotonin stored in the small...
Serotonin contributes to food intake regulation. Serotonin is present throughout the gut in the duodenum, jejunum, and ileum and stored in dense core granules located in the basal and apical part of EECs. These cells are responsible for the production and storage of approximately 90% of the total pool of serotonin in the body [3,12]. The release of serotonin from EECs is stimulated by some dietary compounds in vitro [14] and some dietary compounds have been shown to activate the 5-HT2c receptor in vitro [19]. Several serotonin receptor subtypes are expressed in the small intestine, enteric neurons and enterocytes express 5-HT1, 5-HT2A, 5-HT2C, 5-HT3, and 5-HT4 receptors. Activation of these receptors has various effects such as neurotransmitter release, controlling motility, intestinal secretion and contraction or relaxation [8].

The present study aimed to investigate to what extent serotonin is involved in nutrient-stimulated GLP-1 release when nutrients are delivered to the ileum. It was hypothesized that nutrients stimulate serotonin release from EECs, which consequently stimulates GLP-1 secretion via a serotonin receptor at EECs. This was studied by analyzing serotonin, GLP-1, PYY and CCK release from intestinal tissue segments after exposure to a lipid mixture (saﬄower oil), protein (casein), carbohydrate (sucrose) and a non-caloric sweetener (rebaudioside A).

Next, the role of serotonin on GLP-1 release from intestinal segments was studied using the selective serotonin re-uptake inhibitor (SSRI) ﬂuoxetine. Fluoxetine inhibits serotonin re-uptake that is mediated by the membrane embedded serotonin reuptake transporter [12]. Two different models were used; an ex vivo and in vitro model. First, porcine intestinal segments were applied because these conglomerates of multiple cell types such as EECs, EGCs, and absorptive enterocytes offer a suitable system to study cellular interactions involved in serotonin and GLP-1 release. Second, serotonin-stimulated GLP-1 release was studied using the murine enterochondrocdrine STC-1 cell line [11]. STC-1 cells were exposed to serotonin and the non-specific receptor antagonist asenapine or the speciﬁc 5-HT3 receptor antagonist ondansetron.

2. Material and methods

2.1. Chemicals

Casein (food grade containing sodium, purity≥82%, batch number 15,156) was obtained from Dutch Protein Services BV (Tiel, The Netherlands). Rebaudioside A (food grade, purity≥97%, batch number 20,110,301, Chemspider ID 5294031) was obtained from Stevia Natuurlijk BV (Drachten, The Netherlands). Sucrose (food grade, purity≥99.9%) was supplied by Suiker Unie (Oude Gastel, The Netherlands). Saﬄower oil (food grade, composed of 3% C16:0, 2% C18:0, 9% C18:1 and 75%−85% C18:2) was provided by De Wit Specialty Oils (De Waal, The Netherlands). Saﬄower oil was emulsiﬁed as described previously [17,24,29] using potassium caseinate (15% w/v), xanthan gum and guar gum (both 0.1% w/v, GF supplies, Amsterdam, The Netherlands). Asenapine maleate (purity≥98%), 5-hydroxytryptamine hydrochloride (serotonin, purity≥98%), ﬂuoxetine hydrochloride, L-ascorbic acid (purity≥99%), pargyline hydrochloride and phenylmethylsulfonyl ﬂuoride (PMSF) were obtained from Sigma Aldrich (Zwijndrecht, The Netherlands). Chemicals to prepare the Krebs-Ringer bicarbonate (KRB) buffer were: D-glucose (10 mM), magnesium chloride (0.7 mM) sodium phosphate monobasic (1.3 mM), and HEPES (25 mM) were all obtained from Sigma Aldrich (Zwijndrecht, The Netherlands). Hanks’ Balanced Salt Solution (HBSS) was obtained from Life technologies Europe BV (Bleiswijk, The Netherlands) and was supplemented with 10 mM HEPES (Merck Millipore, Darmstadt, Germany). Lonza (Verviers SPRL, Belgium) provided Dulbecco’s Modiﬁed Eagle Medium (DMEM), fetal calf serum (FCS). The pH of both the buffers was adjusted to pH 7.4 and their pH was checked and adjusted to 7.4 if necessary.

2.2. Collection of porcine intestinal tissues

Intestinal tissues were obtained from 10 male pigs (Sus scrofa domesticus), considered healthy after evaluation by a veterinarian. The protocol for this study was approved by the Animal Ethics Committee Utrecht (Ethics Committee Permit Numbers 2014I.01.001 and 2014I.06.037, Utrecht, The Netherlands). The pigs had a mean ± SEM body mass of 88 ± 4 kg and a mean age of 6 months. Pigs were housed in groups, fed ad libitum with a standard pig diet (Vitaal P 003103, de Heus, Ede The Netherlands) with free access to water. The pigs were killed by a veterinarian through an intracardial injection of Euthanial (propofol 40%, 30 ml per pig) (Alfaxan, Wyoming, NL). Within approximately 5 min after the pigs were killed, the intestines were excised, and intestinal tissue of about 1 m length of different anatomical regions were collected and stored in ice-cold oxygenated (95% O2, 5% CO2) KRB buffer. Duodenal tissue was collected at 10 cm below the pylorus, jejunal tissue was collected 45 cm proximal from the ileocecal junction and ileal tissue was collected at 1 m proximal from the ileocecal junction. Tissues were transported to the laboratory and immediately used for ex vivo experiments.

The time between excision and start of the experiments did not exceed 30 min. To determine the intracellular concentrations of serotonin, CCK, GLP-1 and PYY whole tissue segments (0.5 cm3) from duodenal, jejunal and ileal tissue were immediately frozen and stored below −70 °C.

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

Concentrations of the nutrient stimuli chosen are within the physiological range and these concentrations previously showed to stimulate GLP-1 release in the same porcine intestinal segment model [24]. Incubations of porcine intestinal tissue segments with casein (1% w/v), rebaudioside A (12.5 mM), sucrose (50 mM), saﬄower oil (3.35% w/v), serotonin (30 μM) and ﬂuoxetine (0.155 μM) alone, or in the presence of ﬂuoxetine (0.155 μM) were performed as described previously [24]. In brief, upon arrival at the laboratory the intestine was rinsed with ice-cold oxygenated KRB buffer and cut open in a longitudinal direction. The outer muscle layers were carefully stripped oﬀ with the apical side upward, and circles of tissue with a diameter of 8 mm (about 0.5 cm2) were punched out using a disposable biopsy punch [Medline Industries, Inc. Mundelein, Illinois, USA]. Intestinal segments of 2 cm long of ileocecal and jejunal segments were used for incubation experiments. The intestinal segments were transferred to a 48-wells plate which was ﬁlled with 500 μl ice-cold KRB buffer per well, and kept on ice until the start of the experiment. Thereafter, the tissues were brought to room temperature within 30 min, followed by a preheating step at 37 °C for 1 h in a humidified incubator (95% O2, 5% v/v CO2). We have chosen this preheating step because all tissues exposed to the SSRI ﬂuoxetine were pre-incubated with ﬂuoxetine, 30 min before the actual incubation started, by replacing the KRB buffer with KRB buffer in which ﬂuoxetine had been dissolved. The concentrations ﬂuoxetine (0.155 μM) and serotonin (30 μM) used were selected based on pilot experiments analyzing tissue uptake of radiolabeled serotonin and in vivo release after intravenous serotonin (0.3 μg/kg/min) in pigs, and on a previous study showing an IC50 for serotonin, CCK, PYY and GLP-1 in Caco-2 cells [18]. To study the release of serotonin, CCK, PYY and GLP-1, incubations were initiated by replacing the KRB buffer solution with preheated (37 °C) isometric KRB buffer without D-glucose containing the test compounds. KRB buffer without D-glucose was used as a control since D-glucose might induce gastrointestinal hormone release. Next, the tissue was put in a humidified incubator for either 5, 10, 20 or 60 min at 37 °C (5% (v/v) CO2). After incubation (5, 10, 20 and 60 min) samples of incubation media were collected, mixed with 100 μM PMSF to inactivate dipeptidyl peptidase IV, aliquoted and stored at 4 °C for further analysis of lactate dehydrogenase (LDH) or stored below −70 °C for further analysis of serotonin, CCK, GLP-1 and PYY.

Tissue viability was checked by measuring the leakage of intracellular LDH as described before [24,31] using an LDH kit (Sigma Aldrich, Zwijndrecht, the Netherlands). LDH activity in the supernatant was expressed as a percentage of the total LDH activity present in control tissue collected before incubation. Total tissue level of lactate dehydrogenase (LDH) in the presence and intracellular serotonin, CCK, GLP-1 and PYY were determined in 0.5 cm3 tissue segments of duodenum, jejunum, and ileum, which were homogenized in ice-cold KRB buffer containing 100 μmol/L PMSF with a Potter Elvehjem type Teflon pestle tissue grinder (Braun, Melsungen, Germany) for 5 min at 200 rounds per minute. Samples were only included for further analysis when LDH leakage did not exceed 10% of the total LDH tissue content. No samples had to be excluded on the basis of this criterion.

2.4. Incubation of STC-1 cells with serotonin

STC-1 cells, a murine pluripotent enterochondrocdrine cell-line, were cultured in Dulbecco’s modiﬁed Eagle medium containing 10% fetal calf’s serum (Life Technologies Europe BV, Bleiswijk, NL) and 1% Penicillin-Streptomycin (Lonza, Wervies, BE). For the GLP-1 secretion experiments the cells were grown in a 24-wells plate. Upon reaching 80% confluency, medium was washed and replaced by Hank’s balanced salt solution (HBSS) (Life Technologies Europe BV, Bleiswijk, NL) with serotonin (0, 10, 30 and 100 μM). STC-1 cells were incubated with data [5,28]. Cells were pre-incubated with either asenapine or ondansetron for 30 min in a humidified incubator (37 °C, 95% O2, 5% v/v CO2). After pre-incubation, medium was replaced by HBSS containing test compounds and incubated for 2 h. Media samples were collected (PMSF, 100 mM) and samples were collected at −20 °C for GLP-1 analysis. Cell viability was analyzed by measuring leakage of intracellular lactate dehydrogenase (LDH) into the media, and
expressed as a percentage of total leakage induced by 1% Triton-X100 incubation. LDH leakage did not exceed 10%. Furthermore, cell viability was checked by neutral red assay [23]. None of the test conditions affected cell viability.

2.5. Gene expression analysis of STC-1 cells

STC-1 cells were grown to 80% confluence and incubated for 2 h in HBSS. RNA was isolated by using TRIzol reagent (Life technologies, Bleiswijk, Netherlands) and RNeasy micro kit. One hundred nanogram of RNA was used for Whole Transcript cDNA synthesis (Affymetrix, inc. Santa Clara, USA). Hybridization, washing and scanning of Affymetrix GeneChip Mouse Gene 1.1 ST arrays was carried out according to standard Affymetrix protocols. Arrays were normalized using the Robust Multi-array Average (RMA) method [2,13]. Probe sets were assigned to unique gene identifiers in this case Entrez IDs. The probes on the Mouse Gene 1.1 ST arrays represent 21,213 Entrez IDs [7]. Array data were analyzed using an in-house, on-line system [15].

2.6. Serotonin, CCK, GLP-1 and PYY analysis

Serotonin was analyzed according to instructions of the manufacturer by a commercially available enzyme-linked immunosorbent assay (ELISA) (BA E-5900; Labor Diagnostika Nord GmbH & Co. Germany), using a BioTek Synergy HT absorbance microplate reader (BioTek Instruments, Inc. Winooski, VT, USA). The detection range of this assay is 0.015–2.5 ng/mL. The inter-assay coefficient of variation (CV) of this kit is 18% and the intra-assay variation is 14%. If necessary, samples were diluted with assay buffer to obtain concentrations within the detection range of this kit.

Concentrations of CCK-8 (CCK 26–33) were analyzed with an optimized and validated commercial human RIA kit (EURIA-CCK, RB302, Euro-diagnostica, Malmö, Sweden). An identical sequence of CCK-8 has been found for most mammals, among them pig and man. This improved assay system has been optimized to reach a high sensitivity of 0.05 pM and does not have cross-reactivity with gastrin-17 or sulfated gastrin. The intra-assay CV was 8.9% at a concentration of 0.84 pM and 4.9% at a sensitivity of 0.05 pM and does not have cross-reactivity with gastrin-17 or sulfated gastrin. The intra-assay CV was 8.9% at a concentration of 0.84 pM and 4.9% at a sensitivity of 0.05 pM and does not have cross-reactivity with gastrin-17 or sulfated gastrin.

Concentrations of GLP-1 and PYY were measured by liquid scintillation counting using a gamma counter (EC&G, Breda, The Netherlands) and a commercially available enzyme-linked immunosorbent assay (ELISA) (BA E-5900; Bachem, Peninsula Laboratories, San Carlos, CA, USA) according to the manufacturer's instructions. This kit measures porcine PYY, and the range of this assay is approximately 4.6–150 pM. The inter-assay CV is 6% and the intra-assay CV is 3%. It was not necessary to dilute samples for this assay.

2.7. Statistical analysis

Statistical analysis was performed using the SAS statistical software package (SAS version 9; SAS Institute, Cary, NC, USA). All variables, serotonin, CCK, GLP-1 and PYY were compared with a mixed analysis of variance (ANOVA) model. Data were visually checked on normality and on constant variance of residuals by plots of residuals vs. corresponding predicted values. If data was not normally distributed, log transformation was applied for further analysis of the data as was the case for CCK, GLP-1 and serotonin. For the intestinal segment experiments the statistical model included the fixed factors time (5, 10, 20 and 60 min), location (duodenum and ileum) and treatment (control, casein, suflafower oil, sucrose, rebabaudiose A, serotonin, fluoxetine, casein and flutoxetine, suflafower oil and flutoxetine, suflrose and flutoxetine, rebabaudiose A and flutoxetine, serotonin and flutoxetine). Because all incubations were performed using intestinal tissue obtained from the same pig, intervention effects within the intestine of one pig were compared by including the random factor pig, which specifies the individual pig. For the STC-1 cell experiments the statistical model included fixed factors serotonin (0 and 30 μM) and asenapine (0, 1 and 10 μM). A post hoc test with Tukey–Kramer adjustment was used if a significant effect occurred. Data are presented as the mean ± SEM and differences are considered significant at P<.05.

3. Results

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

GLP-1 tissue concentrations were highest in ileum followed by jejunum and duodenum (0.3 ± 0.1, 6.7 ± 0.7, 10.5 ± 1.2 pmol/cm² tissue in duodenum, jejunum and ileum, respectively). PYY concentrations in tissue were highest in jejunum followed by ileum and duodenum (1.1 ± 0.4, 7.7 ± 1.0, 4.3 ± 0.8 pmol/cm² tissue, for duodenum, jejunum and ileum, respectively) and CCK intestinal tissue concentrations were highest in duodenum followed by jejunum and ileum (12.8 ± 1.6, 1.5 ± 0.3, and 0.27 ± 0.1 pmol/cm² tissue, for duodenum, jejunum and ileum, respectively).

The effects of casein, suflrose, suflafower oil and rebabaudiose A on GLP-1, PYY and CCK release from ileal and duodenal segments are presented in Fig. 1. The effects on GLP-1 release from ileal segments were the most pronounced (Fig. 1A). Casein, suflrose and rebabaudiose A all induced a marked GLP-1 increase (P<.001). The effect of suflflower oil was less pronounced (P<.05). Casein and rebabaudiose A also increased ileal GLP-1 release as compared to suflflower oil (P<.05). Nutrients and rebabaudiose A did not stimulate GLP-1 release from duodenal segments (data not shown).

None of the nutrients stimulated PYY release from ileal segments as compared to control. Casein increased ileal PYY release as compared to suflrose and suflflower oil (P<.05; Fig. 1B). Nutrients and rebabaudiose A did not stimulate PYY release from duodenal segments (data not shown).

None of the nutrients stimulated CCK release from duodenal segments as compared to control. Casein and rebabaudiose A increased CCK release (Fig. 1C) as compared to exposure to suflrose and suflflower oil at the same time point.
3.2. Serotonin stimulates GLP-1 release from ileal tissue segments and enteroendocrine cells

Serotonin increased GLP-1 release from ileal segments several fold over time (P < .001) (Fig. 2A). Incubation with serotonin did not change PYY release nor CCK released from both ileal and duodenal tissue segments (data not shown).

The role of serotonin on GLP-1 release was further explored in STC-1 cell experiments. Serotonin (30 and 100 μM) stimulated GLP-1 release from STC-1 cells as compared to control and to 10 μM serotonin (P < .001) (Fig. 3A). Serotonin-stimulated GLP-1 release was inhibited by the non-specific serotonin receptor antagonist asenapine (Fig. 3B). Effects were significant at 10 μM asenapine (P < .05) but not at 1 μM asenapine (P = .07). Exposure of STC-1 cells to asenapine alone did not affect GLP-1 release. Ondansetron (1 and 10 μM) a specific 5-HT3 receptor antagonist had no effect on serotonin-stimulated GLP-1 release (data not shown). (See Fig. 4.)

Analysis of the gene expression in STC-1 cells (N = 3) showed an average RMA intensity of 344 ± 9 for serotonin receptor 1b. Furthermore, there was a very low expression of serotonin receptor 5b, which had an average RMA intensity of 24 ± 1. The other serotonin receptors (1a, 1d, 1f, 2a, 2b, 2c, 3a, 3b, 4, 5a, 6 and 7) and SERT (SLC6a4) were expressed below the detection threshold of 20.

3.3. Serotonin intestinal tissue concentrations and induction of its release following exposure to casein and safflower oil

Tissue concentrations of serotonin showed a different distribution pattern over the small intestine compared to that of GLP-1, PYY and CCK. Tissue concentrations of serotonin were highest in duodenum, followed by ileum and jejunum (2463 ± 363, 884 ± 202, and 1371 ± 154 pmol/cm² tissue for duodenum, jejunum and ileum, respectively).

To study the effect of (non-)nutritional stimuli on serotonin release from intestinal epithelium, concentrations of serotonin secreted in the medium were analyzed after exposure of the tissues to casein, safflower oil, sucrose and rebaudioside A. Casein and safflower oil caused increased serotonin concentrations in media over time (P < .001) (Fig. 4), with media concentrations from duodenum approximately 10 times higher as compared to media concentrations from ileum (P < .001). Duodenal and
ileal serotonin concentrations in medium did not change by exposure to sucrose and rebaudioside A (Fig. 5).

3.4. SERT inhibition augments casein- and safflower-stimulated serotonin release

Blocking the cellular reuptake of serotonin with fluoxetine resulted in increased serotonin concentrations in medium from duodenal segments after 60 min ($P < .001$) (Fig. 5A). Fluoxetine induced a smaller effect on serotonin concentrations in medium from ileal tissue segments, these concentrations being increased after 20 min only ($P < .05$) (Fig. 5B).

The combination of fluoxetine and casein increased serotonin medium concentrations from duodenal and ileal tissue incubations as compared to fluoxetine alone ($P < .001$) and casein alone ($P < .05$ at 10 min) (Fig. 5). Combining fluoxetine with safflower oil increased serotonin medium concentrations from duodenum and ileum as compared to fluoxetine alone ($P < .001$) (Fig. 5).

The combination of rebaudioside A and fluoxetine increased duodenal serotonin concentrations in medium only after 10 min as compared to rebaudioside A ($P < .001$). However, serotonin concentrations in medium of ileum did not change after exposure to the combination of rebaudioside A and fluoxetine (data not shown). The combination of sucrose and fluoxetine did not change serotonin concentrations in the media of both duodenal and ileal segments (data not shown).

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

Fluoxetine alone increased GLP-1 release ($P < .001$), whereas ileal PYY and duodenal CCK release were not changed. The combination of casein and fluoxetine increased GLP-1 ($P < .01$), PYY and CCK release over time ($P < .001$) (Fig. 6). For the other combinations of nutrients and sweetener with fluoxetine no time and treatment interactions were found. However, since the release of GLP-1, PYY and CCK tended to be increased by the addition of fluoxetine as compared to nutrient exposure alone, total area under the curves (AUC) were calculated. Overall, the combination of fluoxetine with the nutrients tested and with rebaudioside A resulted in increased AUCs of GLP-1 (Fig. 7), PYY and CCK release vs nutrients alone ($P < .01$).

4. Discussion

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

All nutrients and mixtures tested as well as the non-caloric sweetener rebaudioside A stimulated GLP-1 release from intestinal ileal tissues, which confirmed our previous findings using the same intestinal segment model [24]. These data correspond to findings in vivo as well, since ileal delivery of the same ‘non-predigested’ macronutrients resulted in increased satiation and satiety hormone release [29]. For rebaudioside A, however, it is not known if this stimulates GLP-1 release in vivo as well and hence induces satiation. Another study demonstrated satiating properties of stevia in vivo [1] whereas other sweeteners including sucralose did not affect satiety hormone release in vivo [10,27].

In the experiments described here, time-dependency of satiety hormone release was studied. Compared to our previous study we
found a slightly higher PYY release from ileal segments after exposure to buffer only. It is unlikely that this effect is due to tissue damage, since LDH leakage was below 10%. CCK release from duodenal segments was analyzed for the first time using this ex vivo model. Surprisingly, rebaudioside A and casein stimulated CCK release only when compared to sucrose and safflower oil, but not when compared to control. Safflower oil was expected to increase CCK, as it was shown before that intraduodenal lipid infusions resulted in increased CCK levels in vivo [25]. In the latter study, lipid infusions of mixtures of linoleic, oleic and palmitic acid were used.

Overall, the results of the present study illustrate that the ex vivo model being a conglomerate of multiple cell types such as EECs, ECCs and absorptive enterocytes, offers a suitable system to study the cellular interactions involved in serotonin and GLP-1 release.

It is known that nutrient sensing GPCRs and ion-dependent nutrient transporters at EECs play a major role in nutrient-stimulated GLP-1, PYY and CCK release [15,21]. However, not for all nutrients stimulating GLP-1, PYY or CCK release, a receptor is known. We hypothesized that nutrient-receptor interaction results in serotonin release, and that this released serotonin enhances nutrient-stimulated GLP-1 release. A hypothetic model for this effect is depicted in Fig. 8. The proposed mechanism by which serotonin may contribute to receptor-mediated GLP-1 release is; 1) nutrients stimulate serotonin release from ECCs via interaction with GPCR receptors on ECCs [14], subsequently 2) this released serotonin stimulates the release of GLP-1 from EECs via a serotonin receptor-mediated process. This hypothesis is supported by the data found in the present study. We showed that nutrients stimulated serotonin release from ileal tissue segments and that serotonin stimulated GLP-1 release from ileal tissue segments. Also in the enteroendocrine STC-1 cell model, serotonin stimulated GLP-1 release which could be inhibited by a non-specific serotonin receptor agonist asenapine. Asenapine has binding affinities for various serotonin receptors including the 5HT1A, 5-HT1B, 5-HT2A, 5-HT2B, 5-HT2C, 5-HT5, 5-HT6 and 5-HT7 receptor [26]. It should be noted that this antagonist has binding affinities for other receptors such as adrenoreceptors and dopamine receptors as well [26]. However, because asenapine inhibited GLP-1 release after stimulation with serotonin, we believe this effect to be mediated via serotonin receptors. In contrast to asenapine, ondansetron, a specific 5-HT3 receptor antagonist did not inhibit serotonin-stimulated GLP-1 release from STC-1 cells. This suggests that receptors other than the 5-HT3 receptor mediate serotonin-stimulated GLP-1 release. A potential candidate may be the 5-HT4 receptor. Asenapine has affinity for this 5-HT4 receptor. A recent report also showed that stimulation of 5-HT3 receptors with specific 5-HT1B agonists enhanced GLP-1 plasma concentrations in mice [20]. Further studies are needed to identify the specific serotonin receptors involved in serotonin-stimulated GLP-1 release.

This study has also limitations that warrant discussion. First, the data presented support the idea that serotonin contributes to nutrient-stimulated GLP-1 release, whereas the effects on CCK and PYY were less convincing. Such an effect may relate to serotonin receptor distribution in the small intestine. Possibly, serotonin receptors on GLP-1 containing L-cells in the ileum are more abundant than those on EECs containing PYY, or CCK in the duodenum. Remarkable though was that serotonin release and tissue concentrations were highest in duodenum. Therefore, more studies are needed to investigate serotonin receptor expression on EECs and to study the relation between activation of specific serotonin receptors and GLP-1, PYY and CCK release. Additionally, it should be noted that this segment model does not distinguishes between the apical and basolateral side. It can therefore not be excluded that the apical or basolateral side reacts differentially to the nutrients.

Second, there was no control for potassium caseinate alone. Safflower oil was prepared as described previously in order to compare results between studies [17,24,29]. It has been shown that the same concentration of potassium caseinate (1% w/v) did not affect CCK, PYY concentrations nor satiety in vivo [13]. However, in our previous and current study using the same ex vivo segment model [24], we showed that another type of casein in the same concentration (1% w/v) already induced GLP-1 release. Therefore, we cannot exclude the possibility that the potassium caseinate (1% w/v) used to emulsify the safflower oil contributed to the effects found for safflower oil. Future studies should therefore always include a control for the added potassium caseinate in safflower oil.

Third, sucrose and rebaudioside A both stimulated GLP-1 release and this GLP-1 release was further stimulated after inhibition of serotonin reuptake. However, serotonin release was not stimulated by these compounds. It was expected that sucrose and rebaudioside A would result in serotonin release since other studies showed that odorants and tastants other than rebaudioside A stimulated serotonin release from enterochromaffin cells [3,14]. Possibly, the turn-over of serotonin is so high that quantities of serotonin accumulated in the incubation media were too low to be detected. It would be interesting to use alternative methods to provide more insight in the timing and sequence of the release of serotonin and satiety hormones. One interesting method is amperometrical recording [4], which has been applied to measure serotonin release from rabbit ileal mucosa and from EECs [3,21].
Fourth, the combination of nutrients and fluoxetine further enhanced nutrient-stimulated GLP-1, PYY and CCK release from intestinal segments. However, the additional effect of fluoxetine was only small in comparison to hormone release induced by the nutrients alone, possibly due to the fact that there was already a copious release of serotonin in the epithelial space. Further studies are needed to investigate the magnitude of serotonin’s contribution to nutrient-stimulated GLP-1 release.

Collectively, our data suggest an additional mechanism for nutrient-stimulated GLP-1 release via the release of serotonin by ECCs. Serotonin present in the small intestine contributes to the enhancement of GLP-1 release by responding to macronutrients, possibly via the activation of serotonin receptors on EECs. This additional mechanism contributes to the idea that nutrient sensing in the small intestine is regulated by interaction of multiple cells that respond to nutrient fluctuations in order to optimize food digestion and regulation of food intake.
Fig. 8. Proposed mechanism of serotonin modulating GLP-1 release from the ileum. This cartoon illustrates the following hypothesis; dietary compounds such as casein, safflower oil, sucrose or rebaudioside A may stimulate GLP-1 release from an EEC via interaction with a GPCR (A), simultaneously casein may stimulate extra-intestinal serotonin concentrations from ECC via interaction with a GPCR (B). Consequently this induced serotonin release may stimulate (additional) GLP-1 release via interaction with an 5-HT receptor on ECC (C).

Abbreviations Used
ANOVAmixed analysis of variance
CaSRCalcium sensing receptor
CCKcholecystokinin
ECCenterochromaffin cell
ECECongoedroendocrine cell
GLP-1glucagon-like peptide-1
GPCR G-coupled protein receptors
Glgastrointestinal
HBSSHanks' Balanced Salt Solution
KRBKrebs Ringer Bicarbonate
5-HTserotonin
LDHlactate dehydrogenase
PYPeptide YY
PMSFphenylmethanesulfonyl fluoride
SEMSstandard error of the mean
SDstandard deviation
SSRIsselective serotonin re-uptake inhibitor
TIR2taste receptor type 1 member 2
TIR3taste receptor type 1 member 3

Disclosures
The research was funded by TI Food and Nutrition, a public-private partnership on pre-competitive research in food and nutrition.

Acknowledgements
The authors thank LE. Wilms, A.E.A.M. Speulman-Saat and J.H.T. Jansen, for their technical support during the ex vivo intestinal experiments.

References