PLOS ONE

RESEARCH ARTICLE

Regulation of α-Transducin and α-Gustducin Expression by a High Protein Diet in the Pig Gastrointestinal Tract

Roberto De Giorgio[1], Maurizio Mazzoni[2]*, Claudia Vallorani[2], Rocco Latorre[2], Cristiano Bombardi[2], Maria Laura Bacci[2], Monica Forni[2], Mirella Falconi[3], Catia Sternini[4,5,6], Paolo Clavenzani[2]

1 Department of Medical and Surgical Sciences, University of Bologna, Bologna, Italy, **2** Department of Veterinary Medical Sciences, University of Bologna, Bologna, Italy, **3** Department of Biomedical and Neuromotor Sciences, University of Bologna, Bologna, Italy, **4** CURE Digestive Diseases Research Center, Digestive Diseases Division, Department of Medicine, David Geffen School of Medicine, University of California Los Angeles, Los Angeles, California, United States of America, **5** Department of Neurobiology, David Geffen School of Medicine, University of California Los Angeles, Los Angeles, California, United States of America, **6** Veterans Administration, Greater Los Angeles Health system, Los Angeles, California, United States of America

* m.mazzoni@unibo.it

Citation: De Giorgio R, Mazzoni M, Vallorani C, Latorre R, Bombardi C, Bacci ML, et al. (2016) Regulation of α-Transducin and α-Gustducin Expression by a High Protein Diet in the Pig Gastrointestinal Tract. PLoS ONE 11(2): e0148954. doi:10.1371/journal.pone.0148954

Editor: François Blachier, National Institute of Agronomic Research, FRANCE

Received: August 19, 2015

Accepted: January 25, 2016

Published: February 12, 2016

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: The present work was supported by National Institutes of Health (NIH) grants DK09 and 41301 (C.S.), grants from the Italian Ministry of Education, University and Research (MIUR; PRIN2009; R. De G.), from 'Fondazione Del Monte di Bologna e Ravenna', Italy, and funds from the University of Bologna (R. De G. and P.C.). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Abstract

Background

The expression of taste receptors (TASRs) and their signalling molecules in the gastrointestinal (GI) epithelial cells, including enteroendocrine cells (EECs), suggests they participate in chemosensing mechanisms influencing GI physiology via the release of endocrine messengers. TASRs mediate gustatory signalling by interacting with different transducers, including α-gustducin ($G_{\alpha gust}$) and α-transducin ($G_{\alpha tran}$) G protein subunits. This study tested whether $G_{\alpha tran}$ and $G_{\alpha gust}$ immunoreactive (-IR) cells are affected by a short-term (3 days) and long-term (30 days) high protein (Hp) diet in the pig GI tract.

Result

In the stomach, $G_{\alpha gust}$ and $G_{\alpha tran}$-IR cells contained serotonin (5-HT) and ghrelin (GHR), while in the small and large intestine, $G_{\alpha gust}$ and $G_{\alpha tran}$-IR colocalized with 5-HT-, cholecystokinin (CCK)- and peptide YY (PYY)-IR. There was a significant increase in the density of $G_{\alpha tran}$-IR cells in the pyloric mucosa in both short- and long-term Hp diet groups (Hp3 and Hp30) vs. the control group (Ctr) ($P<0.05$), while the increase of $G_{\alpha gust}$-IR cells in the pyloric mucosa was significant in Hp30 group vs. Ctr and vs. Hp3 ($P<0.05$); these cells included $G_{\alpha tran}$ / 5HT-IR and $G_{\alpha tran}$ / GHR-IR cells ($P<0.05$ and $P<0.001$ vs. Ctr, respectively) as well as $G_{\alpha gust}$ /5-HT-IR or $G_{\alpha gust}$ / GHR-IR cells ($P<0.05$ and $P<0.01$ vs. Ctr, respectively). In the small intestine, we recorded a significant increase in $G_{\alpha tran}$-IR cells in the duodenal crypts and a significant increase of $G_{\alpha gust}$-IR cells in the jejunal crypts in Hp3 group compared to HP30 ($P<0.05$). With regard to the number of $G_{\alpha tran}$-$G_{\alpha gust}$ IR cells colocalized with CCK or 5-HT, there was only a significant increase of $G_{\alpha tran}$ / CCK-IR cells in Hp3 group compared to Ctr ($P = 0.01$).

Competing Interests: The authors have declared that no competing interests exist.

Conclusion

This study showed an upregulation of selected subpopulations of $G_{\alpha gust}$ / $G_{\alpha tran}$-IR cells in distinct regions of the pig GI tract by short- and long-term Hp diet lending support to TASR-mediated effects in metabolic homeostasis and satiety mechanisms.

Introduction

The gastrointestinal (GI) tract has the important task of food digestion followed by absorption and metabolism of nutrients such as amino acids, sugars and fatty acids. These food-derived components are detected by a "nutrient chemosensing system" involving luminal sensors in the GI mucosa [1–6], which send information to the nervous system to initiate physiological responses regulating food intake and eating behaviour through the gut-brain axis [1–3,6]. The identification of taste receptors (TASRs) and their signalling molecules along the mammalian GI tract and the observation that TASR ligands in the gut lumen induce functional responses such as activation of vagal afferents, alteration of food intake and GI motility, aversion, release of peptides and regulation of glucose homeostasis [7–13], support a key role of these receptors in the luminal chemosensing processes. In the GI tract, TASRs are expressed by epithelial cells, mainly enteroendocrine cells (EECs). Their stimulation *in vivo* and *in vitro* initiates a signalling cascade that ultimately leads to release of chemical messengers [8,11,14]. This mechanism has been postulated to activate neural reflex pathways including intrinsic and extrinsic neurons affecting gut physiology and energy homeostasis [1–3,6].

TASRs are G-protein coupled receptors comprising two major families: the TAS1Rs family composed by three receptors (TAS1R1, TAS1R2 and TAS1R3) that function as dimers to detect umami (TAS1R1 with TAS1R3) and sweet (T1R2 with TAS1R3) [15–17], and a large family of TAS2Rs (about 25 subtypes in humans and >30 in rodents) that detect an array of diverse bitter compounds [18,19]. Upon activation, TASRs coupled to G-protein related signalling messengers, α-gustducin ($G_{\alpha gust}$), α-transducin ($G_{\alpha tran}$), and other transducers as well, lead to the intracellular Ca^{2+} increase and cellular response. $G_{\alpha gust}$ and $G_{\alpha tran}$ have been identified throughout the digestive system, from the tongue down to the distal part of the GI tract of different mammalian and non-mammalian species [13,20–29]. The involvement of different G protein subunits, such as $G_{\alpha gust}$, $G_{\alpha tran}$ and other Gi-family alpha subunits, in taste transmission has been demonstrated by several findings including the observations that not all taste cells contain $G_{\alpha gust}$, that gustatory transduction was not completely abolished in mice with deletion of $G_{\alpha gust}$ gene, and that $G_{\alpha tran}$ can partially rescue the taste response in these mice [30–32].

In our previous studies, we found that the TASR-related G protein subunits, $G_{\alpha gust}$ and $G_{\alpha tran}$ in the gut are regulated by different diet manipulation, including fasting and refeeding, high-fat diet and a low cholesterol mimicking diet in the mouse and porcine gut [27, 28]. These findings suggest plasticity in taste-related molecules in the GI tract in response to different feeding states and caloric intake. Increasing evidence support the notion that high protein diets reduce food intake, facilitate weight loss, and improve body composition in both humans and animal models [33–35]. Thus, our study was designed to test whether short- and long-term high protein (Hp) diet affected the expression of $G_{\alpha gust}$ and $G_{\alpha tran}$ immunoreactive (IR) cells throughout the pig GI tract. In addition, we characterized the phenotype of $G_{\alpha gust}$ -and $G_{\alpha tran}$-IR cells with special emphasis on chemical messengers such as peptides and biogenic amines involved in satiation and body weight regulation.

Materials and Methods

Animals

The experiments were performed at the Physiology unit of the Department of Veterinary Medical Sciences of the University of Bologna. The study was conducted according to relevant national and international guidelines on Animal Experimentations. The procedure was reviewed and approved in advance by the Scientific Ethics Committee for Animal Experimentation of the University of Bologna and by the Italian Ministry of Public Health. Twelve Large White/Duroc hybrid female pigs (12 weeks old, live weight 33.6 ± 3.05 kg) were purchased from a commercial breeder. Upon arrival, pigs were weighed, clinically examined and arranged in multiple boxes (n = 4 in each box) with slatted floor, previously cleaned and sanitized by an authorized operator. They were immediately fed with standard diet containing 14.5% protein (Big 30 Flour, Cooperativa Agricola Tre Spighe, Castel Guelfo, Bologna, Italy); tap water was freely available. The clinic exam ensured all the animals were healthy and did not show pathologies that could interfere with the experimental results. All pigs were fed with standard diet for 2 weeks in order to allow the normalization of GI function. Animals were then randomly assigned to three experimental groups; one group (n = 4) received standard diet and served as control (Ctr); one group (n = 4) was fed high protein diet (35% protein) for 3 days (Hp3); and one group (n = 4) was fed high protein diet (35% protein) for 30 days (Hp30). The component of experimental diet, energy density as well as body weight and food consumption are described in S1 and S2 Tables. Feeding behaviour was recorded every week and pigs were weighed at the beginning and at the end of the experimental design (i.e., at 0, 3 and 30 days). At the end of the experiment, animals were euthanized with i.v. bolus of Tanax (embutramide, mebenzonio iodure, tetracaine) (10 mL / head; Intervet Italia Srl, Milan, Italy) after premedication with i.m. azaperone 3 mg / kg (Stresnil; Janssen-Cilag SpA, Milan, Italy) and surgical anaesthesia, induced with 20 mg/kg ketamine i.m. (Ketavet 100; Intervet Italia Srl, Milan, Italy) and with an i.v. bolus of sodium thiopental (300 mg / animal, Pentothal Sodium; Intervet Italia Srl, Milan, Italy). We euthanized two animals for the Ctr group at 3 days and two at 30 days since in preliminary studies (data not shown) we did not see significant differences of mucosal morphology.

Samples collection

Specimens of the GI tract included stomach mucosa (cardiac, near the gastric diverticulum; pyloric, close to the pyloric sphincter), duodenum (about 10 cm from the pyloric sphincter), middle jejunum, ileum, cecum, ascending colon (near the centrifugal turns), descending colon (about 25 cm from the anus) and rectum (in the *ampulla recti*). Specimens were pinned flat on balsa wood, fixed in 4% buffered paraformaldehyde / 0.1 M phosphate buffer, pH 7.4 for 48 h at 4°C, dehydrated and embedded in paraffin [27].

Immunohistochemistry

Serial (5 μm thick) sections mounted on poly-L-lysine-coated slides were subjected to single and double immunofluorescence staining using antibodies directed to $G_{\alpha tran}$ or $G_{\alpha gust}$ and specific EEC subtype markers such as ghrelin (GHR), gastrin/cholecystokinin (GAS/CCK), 5-hydroxytryptamine (5-HT), peptide YY (PYY) shown in Table 1. Briefly, sections were deparaffinized with xylene, rehydrated with graded ethanol, and heat-treated in a microwave (2 cycles at 750 W, 5 min each) in sodium citrate buffer (pH 6.0) to retrieve the antigenicity. Sections were incubated in 10% appropriate normal serum in 0.01 M phosphate buffer saline (PBS) (1 h at room temperature) to prevent non-specific bindings, and subsequently incubated

Table 1. List and dilutions of primary and secondary antibodies.

Primary antibodies	Code	Species	Dilution	Supplier
α-Transducin	sc-390	rabbit	1:200	Santa Cruz
α-Gustducin	sc-395	rabbit	1:200	Santa Cruz
Cholecystokinin/Gastrin	CCK/GAS # 9303	mouse	1:1000	CURE/DDRC
Ghrelin	sc-10368	goat	1:400	Santa Cruz
5-hydroxitryptamine	ab16007	mouse	1:200	Abcam
Peptide YY	PAB17185	guinea pig	1:1000	Abnova
Secondary antibodies	**Code**	**Species**	**Dilution**	**Supplier**
Alexa 594 conjugated anti-mouse IgG	A11005	goat	1:600	Mol. Probes
Alexa 488 conjugated anti-rabbit IgG	A21206	donkey	1:1000	Mol. Probes
FITC conjugated anti-rabbit IgG	401314	goat	1:500	Calbiochem
TRITC conjugated anti-goat IgG	705-295-003	donkey	1:1000	Jackson
TRITC conjugated anti-guinea pig IgG	AP108R	goat	1:100	Chemicon/Millipore

CURE/DDRC (P30DK041301), UCLA, Los Angeles, CA, USA. Chemicon International, Temecula, CA, USA. Abcam, Cambridge, UK. Santa Cruz Biotecnology, Inc., CA, USA. Abnova, Jhouzih St. Neihu District. Taipei City, Taiwan. Calbiochem- Novabiochem Corporation, San Diego, CA, USA. Molecular Probes, Eugene, OR., USA. Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA.

doi:10.1371/journal.pone.0148954.t001

overnight with primary antibodies diluted in PBS and 5% of normal serum. After primary antibody incubation, a mixture of fluorescein isothiocyanate (FITC)-conjugated, tetramethyl rhodamine isothiocyanate (TRITC)-conjugated, Alexa Fluor® 594- and Alexa Fluor® 488-conjugated secondary antibodies diluted in PBS (Table 1) was added for 1 h at room temperature. Finally, the slides were washed in PBS and cover-slipped with buffered glycerol, pH 8.6.

Specificity of antibodies

Specificity of $G_{\alpha tran}$, $G_{\alpha gust}$ and CCK/GAS has been previously demonstrated by Western Blot and/or pre-adsorption test [27]. GHR, 5-HT and PYY antibody specificity was assessed by pre-adsorption with an excess of the homologous peptide (GHR, sc-10368 P, Santa Cruz, CA, USA; 5-HT, H9523, Sigma-Chemicals, St. Louis, MO, USA; and PYY, 059–06, Phoenix Pharm. Inc., Burlingame, CA, USA, respectively) (S1 Fig).

Cell counting and statistical analysis

Cell counting was performed with a 40X objective lens using a Zeiss Axioplan microscope (Carl Zeiss, Oberkochen, Germany) with appropriate filter cubes. Images were obtained with a Polaroid DMC digital photocamera (Polaroid, Cambridge, Mass., USA), and minimal adjustments to brightness and contrast were made with Corel Photo Paint and Corel Draw (Corel, Dublin, Ireland). Each specimen was evaluated and counted by two investigators in a blind fashion. For each animal, $G_{\alpha tran}$- and $G_{\alpha gust}$-IR cells were counted in 36 random microscope fields (each field 0.28 mm^2), for a total area of 10 mm^2, in the cardiac and pyloric mucosa, in 50 randomly selected villi and glands in the small intestine, and in 50 glands in the large intestine. Only villi and glands perpendicular to the *muscularis mucosae* were evaluated. The values obtained from counting $G_{\alpha tran}$- and $G_{\alpha gust}$-IR cells were grouped for each experimental group (Ctr, Hp3 and Hp30) and the means were calculated. Moreover, the mean numbers of cells showing a colocalization of $G_{\alpha tran}$ or $G_{\alpha gust}$-IRs with different EEC markers were calculated. Results were expressed as mean ± standard deviation (SD). Data were analysed by one-way ANOVA (Graph Prism 4, GraphPad Software, Inc., La Jolla, CA, USA). A $P<0.05$ was considered statistically significant.

Results

Distribution and neurochemical characterization of $G_{\alpha tran}$-IR and $G_{\alpha gust}$-IR cells in the GI tract

$G_{\alpha tran}$- and $G_{\alpha gust}$-IR cells were distributed throughout the whole pig GI tract (Fig 1A, 1C, 1E and 1G; Fig 2A and 2C), extending our previous description of $G_{\alpha tran}$-IR cell distribution [27]. Similarly to the distribution of $G_{\alpha tran}$-IR cells as reported in details in our previous publication [27] and confirmed in this study, $G_{\alpha gust}$-IR cells were observed both in the distal third and in the epithelial profile of the gastric mucosa of pyloric region, along the villus-crypt axis of the small intestine, and in the glandular epithelium of the large intestine. Most $G_{\alpha tran}$-and $G_{\alpha gust}$-IR cells had the morphological appearance of "open-type" EECs with an elongated shape, homogenous cytoplasm (Fig 2A and 2C) and two cytoplasmic prolongations, one reaching the lumen and the other the basal lamina (Fig 1G). Other $G_{\alpha tran}$-and $G_{\alpha gust}$-IR cells had the "closed-type" EEC appearance with a round shape without cytoplasmic prolongations (Fig 1A and 1C). Double labelling immunofluorescence showed that the majority of $G_{\alpha tran}$-IR and $G_{\alpha gust}$-IR cells in the cardiac and pyloric mucosa were immunopositive for 5-HT (Fig 1A and 1B). Co-expression of $G_{\alpha tran}$ / 5-HT or $G_{\alpha gust}$ / 5-HT was also observed in the villi and glandular epithelium of the duodenum (Fig 1E and 1F), where some $G_{\alpha tran}$-IR cells were immunopositive for 5-HT, while most $G_{\alpha gust}$ positive cells co-expressed 5-HT. In the cardiac and pyloric mucosa, the majority of $G_{\alpha tran}$-IR and $G_{\alpha gust}$-IR cells co-expressed GHR (Fig 1C and 1D). In the jejunum, most of the $G_{\alpha tran}$- or $G_{\alpha gust}$-IR cells distributed along the crypt-villus axis co-expressed CCK-IR (Fig 1G and 1H). In the large intestine, coexpression of $G_{\alpha tran}$ or $G_{\alpha gust}$ and PYY-IR was seen in elongated cells located in the surface epithelium as well in cells of the glandular epithelium (Fig 2A–2D). The percentages of colocalization of $G_{\alpha tran}$- or $G_{\alpha gust}$-IR cells with EEC subtypes are shown in Tables 2 and 3.

Distribution of the $G_{\alpha tran}$- and $G_{\alpha gust}$-IR cells in the three experimental groups (Ctr, Hp3 and Hp30)

In the cardiac mucosa, the density of $G_{\alpha tran}$-or $G_{\alpha gust}$-IR cells was not affected by the administration of the short or long-term Hp diet (Hp3 and Hp30, respectively; not shown). By contrast, in the pyloric mucosa, we observed a significant increase in the density of $G_{\alpha tran}$-IR cells in both Hp3 and Hp30 group compared to Ctr ($P<0.05$) as well as Hp30 vs. Hp3 ($P<0.05$); also, a similar increase was observed for $G_{\alpha gust}$-IR cells in the Hp30 vs. Ctr and Hp3 ($P<0.05$) while there were not a significant increase of $G_{\alpha gust}$-IR cells in Hp3 compared to Ctr (Fig 3A).

In the small intestine, $G_{\alpha tran}$-IR cells were significantly increased in the duodenum crypts in the Hp3 group compared to Hp30, while there was a significant increase of $G_{\alpha gust}$-IR cells in jejunal crypts in Hp3 compared to Hp30 groups ($P<0.05$) (Fig 3B and 3C). Conversely, there were not significant differences between the mean number of $G_{\alpha tran}$- or $G_{\alpha gust}$-IR cells in the duodenal and jejunal villi in the Ctr vs. the different experimental groups (data not shown). In the large intestine, we observed a progressive increase in the number of $G_{\alpha tran}$- or $G_{\alpha gust}$-IR cells from the cecum to the rectum without reaching statistically significant differences among the experimental groups (data not shown).

Expression of EEC subpopulations of cells in the three experimental groups (Ctr, Hp3 and Hp30)

In the cardiac mucosa, the number of 5-HT positive cells did not change in the three experimental groups, whereas in the pyloric mucosa, 5-HT-IR cells were significantly more numerous in Hp30 *vs*. Ctr ($P<0.01$) and *vs*. Hp3 ($P<0.01$) (Fig 4A and 4B).There were no significant

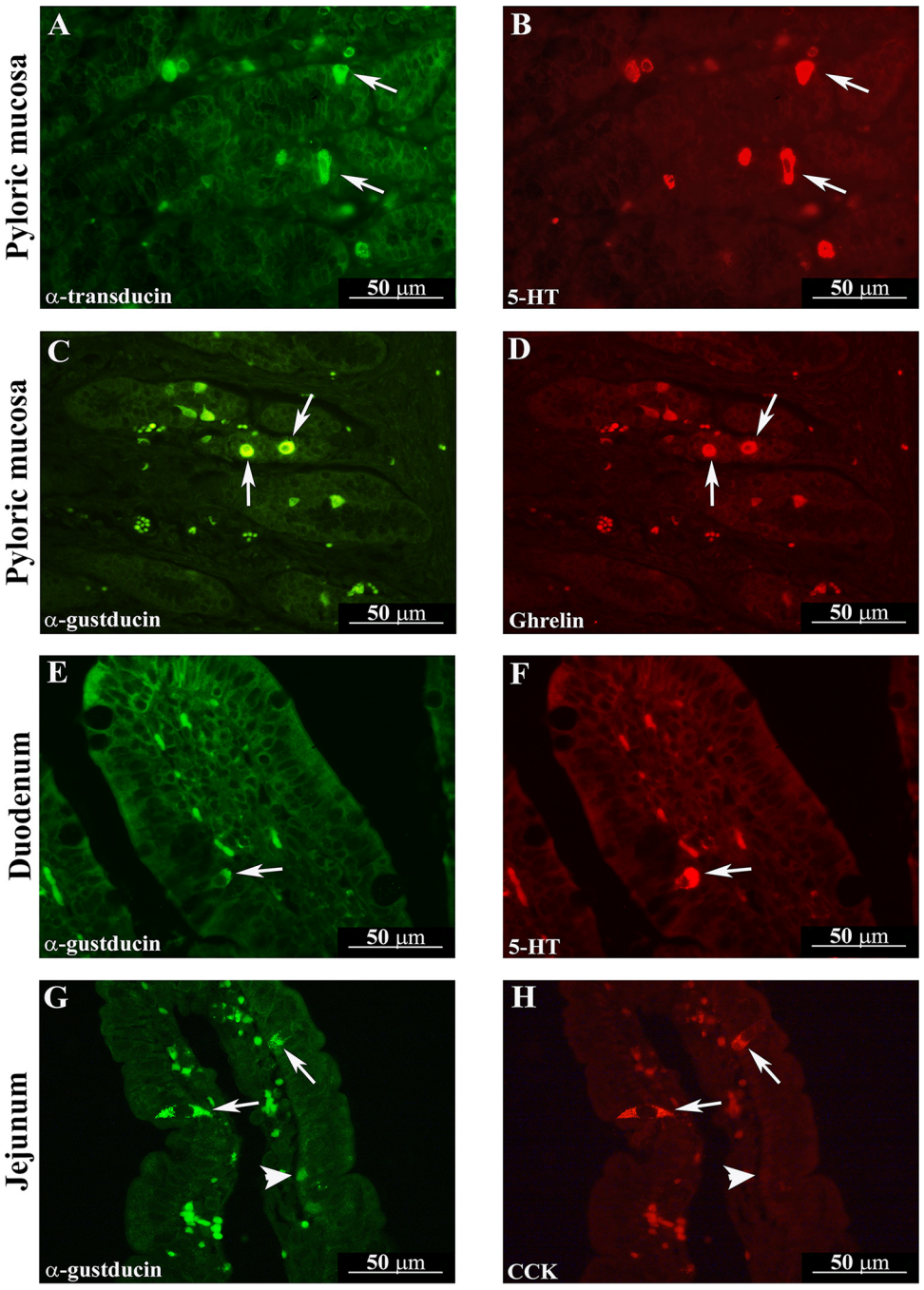

Fig 1. Colocalization of $G_{\alpha tran}$-IR with 5-hydroxytryptamine (5-HT) in the pyloric mucosa following short-term Hp diet (Hp3) (arrows in A and B). The arrows in the photomicrographs (C), (E) and (G) show $G_{\alpha gust}$-IR cells co-expressing ghrelin (D) in the pyloric mucosa of a pig fed a control diet (Ctr), 5-HT (F) in the duodenum of a pig fed long-term Hp diet (Hp30) and cholecystokinin (CCK) (H) in the jejunum of a Hp30 fed pig. The arrowheads in G and H indicate $G_{\alpha gust}$-IR cells not containing CCK-IR. Generally, the $G_{\alpha tran}$ / $G_{\alpha gust}$ labelled cells were found lying close to the basal lamina of the glands (typical closed-type morphology) (A and C, arrows). Frequently, the $G_{\alpha tran}$ / $G_{\alpha gust}$-IR cells are localized in the surface epithelium of the villi (with typical open-type morphology) (E and G, arrows).

doi:10.1371/journal.pone.0148954.g001

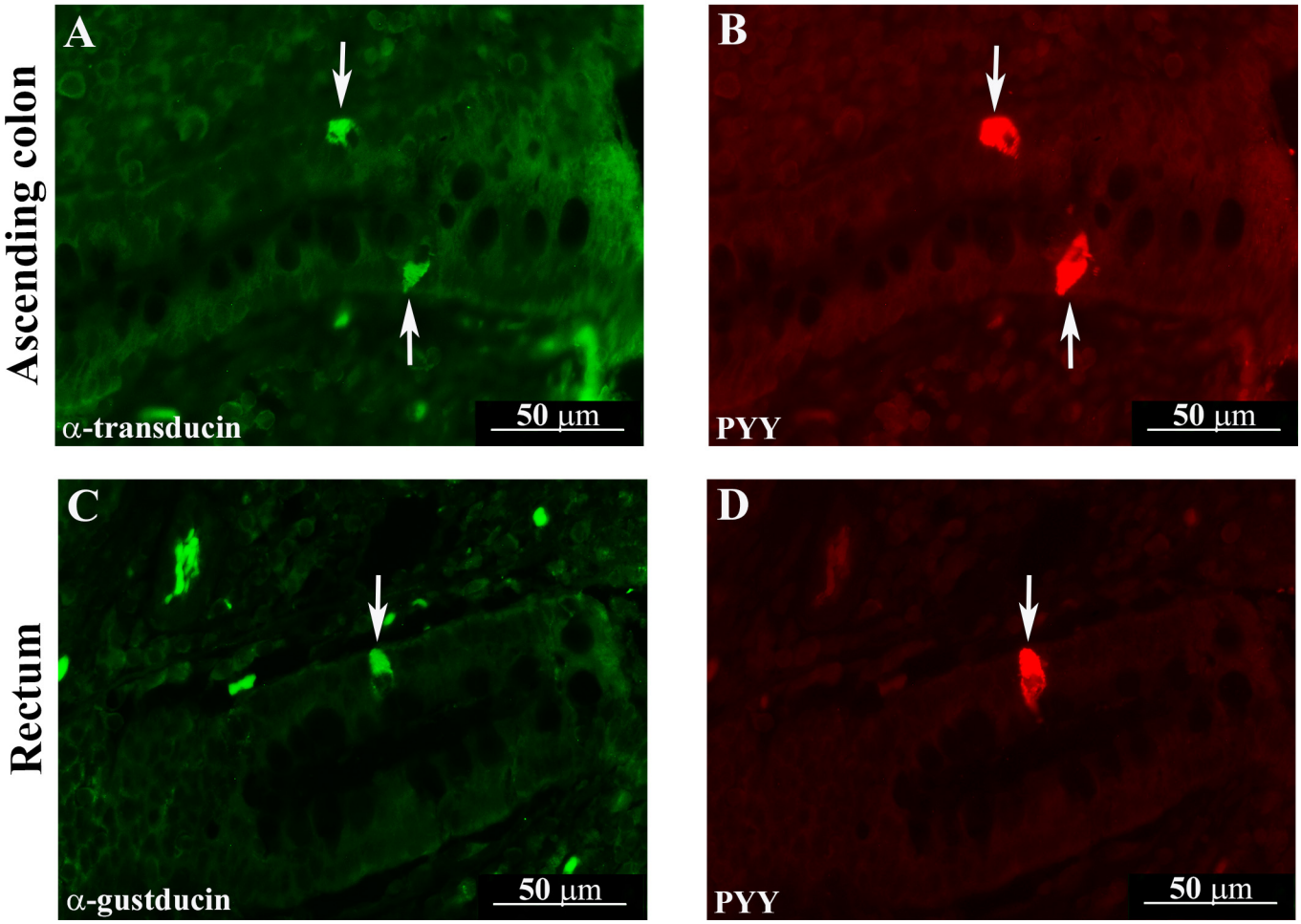

Fig 2. $G_{\alpha tran}$ and $G_{\alpha gust}$ colocalized with peptide YY (PYY) in the ascending colon of an Hp3-fed animal (A and B, arrows) and in rectum of an Hp30-fed pig (C and D, arrows), respectively. $G_{\alpha tran}$ and $G_{\alpha gust}$ positive cells containing PYY-IR located in either the glandular or surface epithelium of the ascending colon and rectum have an open-type morphology.

doi:10.1371/journal.pone.0148954.g002

differences in the expression of GHR positive cells among the groups in the cardiac mucosa, while in the pyloric mucosa we observed a significant increase in the mean value of GHR-IR cells in the Hp30 compared to the Hp3 ($P<0.01$) (Fig 4C and 4D). The density of 5-HT- and CCK-IR cells in the duodenum and jejunum (villi and crypts) did not differ significantly in the three experimental groups. PYY-IR cells were more numerous in the descending colon and rectum *vs*. cecum and ascending colon, but there were no significant differences among the different segments of the large intestine in the three experimental groups.

Table 2. Percentages of co-localization of $G_{\alpha tran}$- or $G_{\alpha gust}$-IR cells with 5-HT and GHR in the gastric and duodenal mucosa.

Cell types	Cardiac mucosa (%)	Pyloric mucosa (%)	Duodenal villi (%)	Duodenal glands (%)
$G_{\alpha tran}$ 5HT/ total $G_{\alpha tran}$	95	94	21.2	28.2
$G_{\alpha gust}$ 5HT/ total $G_{\alpha gust}$	92.2	96.2	95.2	94.6
$G_{\alpha tran}$ GHR/ total $G_{\alpha tran}$	82.8	77	- - - - - - - -	- - - - - - - -
$G_{\alpha gust}$ GHR/ total $G_{\alpha gust}$	61.3	74.6	- - - - - - - -	- - - - - - - -

doi:10.1371/journal.pone.0148954.t002

Table 3. Percentages of co-localization of $G_{\alpha tran}$- or $G_{\alpha gust}$-IR cells with CCK- and PYY-IR in the jejunum and large intestine mucosa.

Jejunum (villi) (%)		Jejunum (glands) (%)		Large intestine (%)	
$G_{\alpha tran}$CCK/ total $G_{\alpha tran}$	$G_{\alpha gust}$CCK/ total $G_{\alpha gust}$	$G_{\alpha tran}$CCK/ total $G_{\alpha tran}$	$G_{\alpha gust}$CCK/ total $G_{\alpha gust}$	$G_{\alpha tran}$PYY/ total $G_{\alpha tran}$	$G_{\alpha gust}$PYY/ total $G_{\alpha gust}$
99.8	94.4	96.2	91.1	75	70.2

doi:10.1371/journal.pone.0148954.t003

Distribution of the different subgroups of $G_{\alpha tran}$- and $G_{\alpha gust}$-IR EECs in Ctr, Hp3 and Hp30 groups

In the cardiac mucosa, the Hp diet did not produce any significant change in the mean number of $G_{\alpha tran}$ / 5-HT or $G_{\alpha gust}$ / 5-HT-IR cells (Fig 5A), whereas in the pyloric mucosa, the 30-day Hp diet led to a significant ($P<0.01$) increase of $G_{\alpha tran}$ / 5-HT-IR or $G_{\alpha gust}$ / 5-HT-IR cells compared with the others two experimental groups (Ctr and Hp3) (Fig 5B). Moreover, there were no changes in the mean number of $G_{\alpha tran}$ / 5-HT and $G_{\alpha gust}$ / 5-HT cells in Ctr, Hp3 and Hp30 groups both in villi and crypts of the duodenum (Fig 5C and 5D).

The number of cells co-expressing $G_{\alpha tran}$ / GHR or $G_{\alpha gust}$ / GHR in the cardiac mucosa did not differ in the experimental groups vs. the control (Fig 5E). By contrast, in the pyloric mucosa, the administration of the Hp diet evoked an increase of the mean number of $G_{\alpha tran}$ / GHR-IR or $G_{\alpha gust}$ / GHR cells after 3 and 30 days vs. Ctr (Fig 5F).

There were no significant differences in the number of $G_{\alpha tran}$ / CCK-IR cells in the villi in the jejunum (Fig 5G), whereas there was a significant increased number of these cells in Hp3 (18.3 ± 2.8) compared to Ctr (14.5 ± 1.3, $P<0.05$) in the crypts (Fig 5H). The number of $G_{\alpha gust}$ / CCK-IR cells in jejunal crypts was greater in Hp3 (16.8 ± 3.3) than Hp30 (10.8 ± 4.1), although this result did not reach statistical significance ($P = 0.06$) (Fig 5H).

Finally, in the large intestine, the quantitative analysis of $G_{\alpha tran}$ / or $G_{\alpha gust}$ / PYY-IR cells showed no difference in the three experimental groups (Fig 5I and 5J).

Regarding the percentages of expression of $G_{\alpha tran}$- or $G_{\alpha gust}$-IR in ECC subtypes, we observed statistical differences in the percentage of the colocalized $G_{\alpha tran}$- or $G_{\alpha gust}$- 5-HT / total 5HT-IR cells in the duodenal villi and glands (S3 Table).

Discussion

Physiological processes in the GI tract, such as secretomotor functions, digestion and absorption are coordinated and integrated events depending upon dietary intake and hormone release through constant monitoring of the luminal content by different sensory systems [1,4,6,36]. TASRs and taste-related molecules in the gut mucosa could serve as the initial molecular mechanisms underlying appropriate functional responses to luminal nutrients and non-nutrients contributing to gut chemosensitivity. This is supported by the localization of $G_{\alpha tran}$ and $G_{\alpha gust}$ in distinct populations of EECs in different mammals including rodents [13,20,23,37–39], pigs [27] and humans [24,40]. Here we showed that Hp diet affected the expression of the taste-related molecules, $G_{\alpha tran}$- or $G_{\alpha gust}$, expressed by EECs, which act as chemoreceptors in the GI tract [4]. The effects of a Hp diet on the density of $G_{\alpha tran}$- or $G_{\alpha gust}$-IR cells were more prominent at 30 days than at 3 days and particularly evident in the pyloric mucosa, compared to other regions of the gut. These findings expand previous observations on the effect of feeding and fasting and dietary factors, including low cholesterol and high fat diets, on the GI chemosensory system [27,28]. Several studies demonstrated that protein breakdown results in amino acids and protein-hydrolysates that activate sensory receptors in chemosensing EEC cells of the gastric mucosa, which modulate digestive functions including gastric emptying, acid and

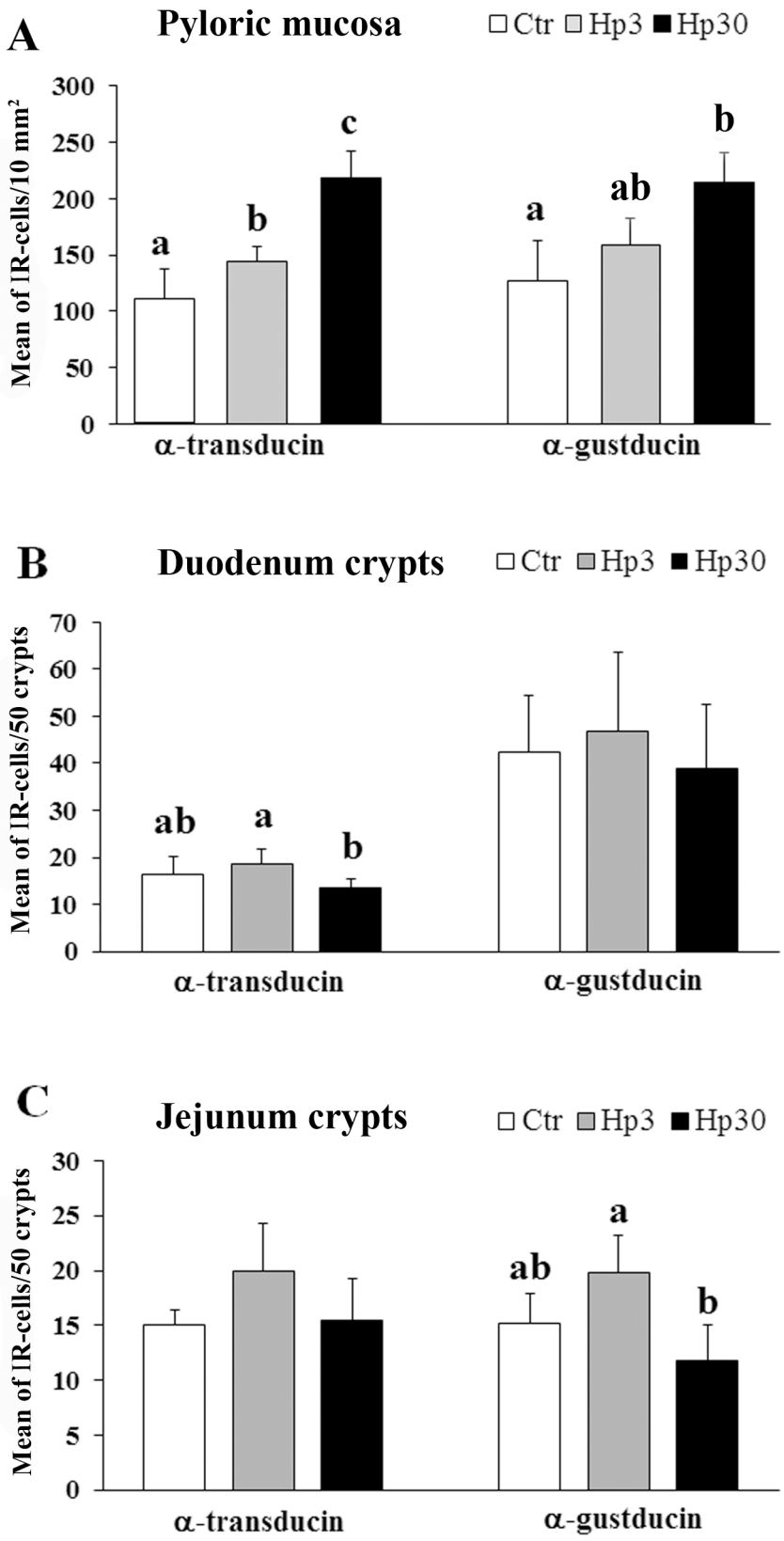

Fig 3. Quantitative assessment of the mean number of $G_{\alpha tran}$ and $G_{\alpha gust}$-IR cells in the pig pyloric mucosa (A), duodenum (B) and jejunum (C) crypts. Different letters indicate a significant ($P<0.05$) statistical difference among groups. Values are expressed as mean ± SD.

doi:10.1371/journal.pone.0148954.g003

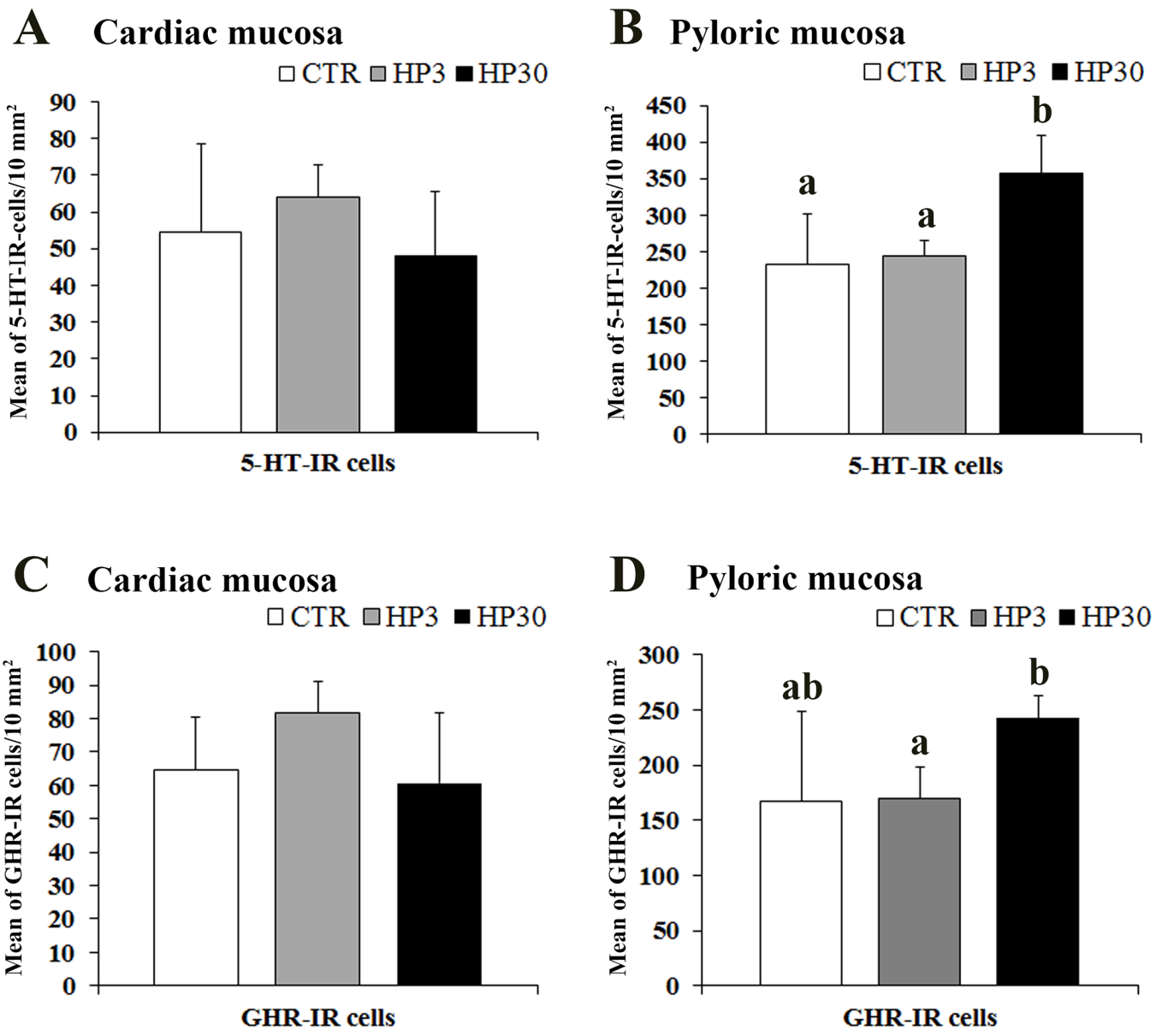

Fig 4. Quantitative assessment of the mean number of 5-HT and GHR-IR cells in the pig cardiac (A and C) and pyloric mucosa (B and D). Different letters indicate a significant ($P<0.05$) statistical difference among groups. Values are expressed as mean ± SD.

doi:10.1371/journal.pone.0148954.g004

entero-pancreatic secretion and food intake, and contribute to the maintenance of energy homeostasis, via hormone (mainly peptides) secretion [5,36,41]. Recent evidence suggests that L-amino acids may be sensed by a group of G-protein coupled receptors which include TAS1R and TAS2R families, the calcium sensing receptor (CaSR) and the G-protein coupled receptor family C group 6 member A (GPRC6A) [42]. The CaSR mainly senses aromatic amino acids and calcium (Ca^{2+}) [43–45], while the GPRC6A is a receptor that predominantly senses basic amino acids and Ca^{2+} and acts in concert with the CaSR [44,46].

Several studies have demonstrated that the G-proteins, $G_{\alpha tran}$- or $G_{\alpha gust}$, are signalling molecules transducing TAS1Rs and TAS2Rs functions [47–50], while CaSR and GPRC6A are transduced by Gαq-family proteins [51–53] or other Gαi-family proteins [54,55]. The increased

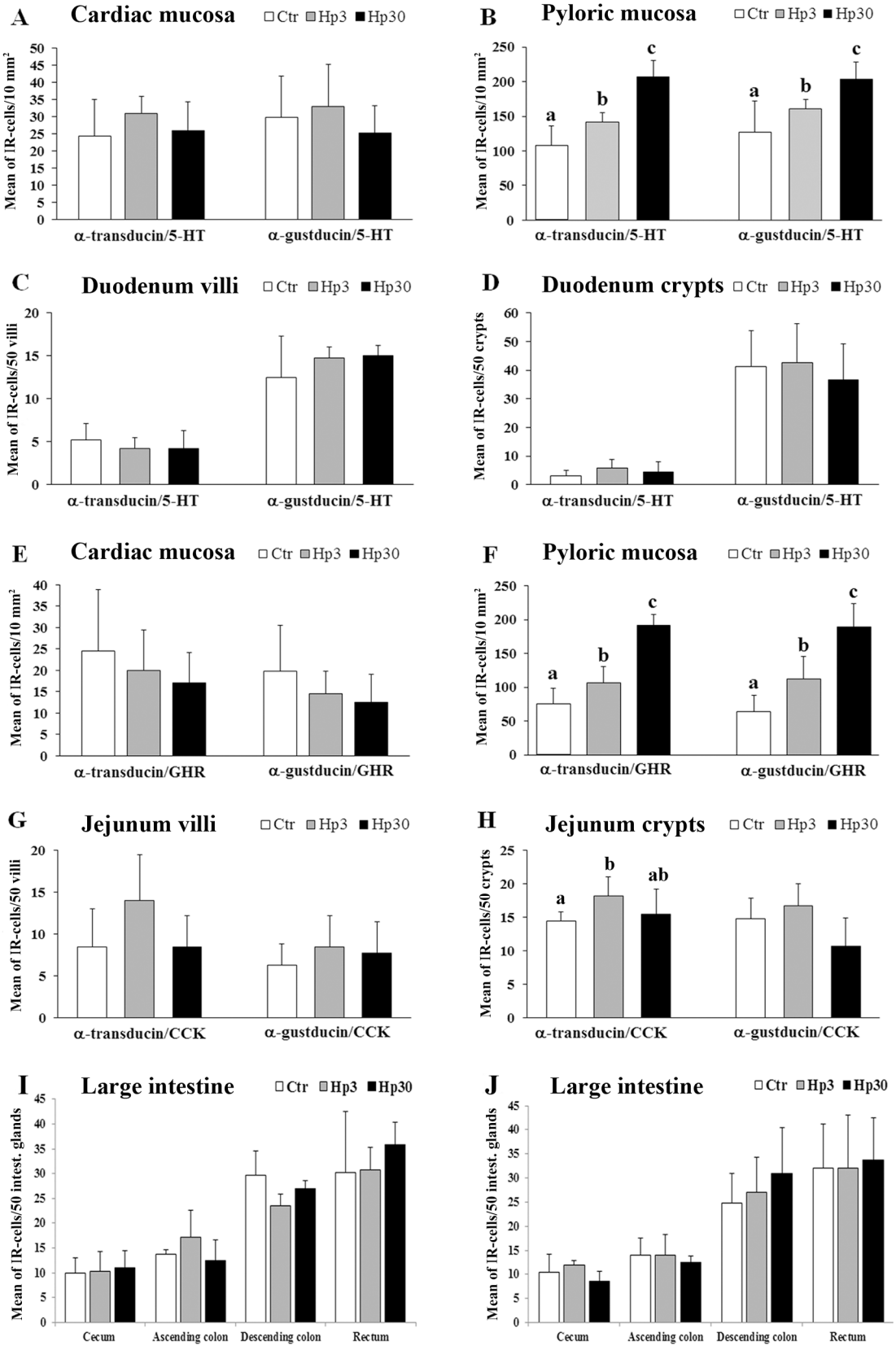
A
Cardiac mucosa
Ctr Hp3 Hp30
Mean of IR-cells/10 mm²
α-transducin/5-HT
α-gustducin/5-HT
B
Pyloric mucosa
Ctr Hp3 Hp30
Mean of IR-cells/10 mm²
a b c a b c
α-transducin/5-HT
α-gustducin/5-HT
C
Duodenum villi
Ctr Hp3 Hp30
Mean of IR-cells/50 villi
α-transducin/5-HT
α-gustducin/5-HT
D
Duodenum crypts
Ctr Hp3 Hp30
Mean of IR-cells/50 crypts
α-transducin/5-HT
α-gustducin/5-HT
E
Cardiac mucosa
Ctr Hp3 Hp30
Mean of IR-cells/10 mm²
α-transducin/GHR
α-gustducin/GHR
F
Pyloric mucosa
Ctr Hp3 Hp30
Mean of IR-cells/10 mm²
a b c a b c
α-transducin/GHR
α-gustducin/GHR
G
Jejunum villi
Ctr Hp3 Hp30
Mean of IR-cells/50 villi
α-transducin/CCK
α-gustducin/CCK
H
Jejunum crypts
Ctr Hp3 Hp30
Mean of IR-cells/50 crypts
a b ab
α-transducin/CCK
α-gustducin/CCK
I
Large intestine
Ctr Hp3 Hp30
Mean of IR-cells/50 intest. glands
Cecum
Ascending colon
Descending colon
Rectum
J
Large intestine
Ctr Hp3 Hp30
Mean of IR-cells/50 intest. glands
Cecum
Ascending colon
Descending colon
Rectum

Fig 5. Quantitative evaluation of the mean number of $G_{\alpha tran}$- or $G_{\alpha gust}$ / 5-HT-IR in the cardiac (A) and pyloric mucosa (B), in the villi (C) and crypts (D) of the duodenum. E and F show the mean number of $G_{\alpha tran}$- or $G_{\alpha gust}$ / GHR-IR cells in the pig cardiac and pyloric mucosa, respectively), and in the villi (G) and crypts (H) of the jejunum. Finally, I and J illustrate the mean number of $G_{\alpha tran}$- or $G_{\alpha gust}$ / PYY-IR in the large intestine, respectively. Different letters indicate a significant ($P<0.05$) statistical difference among groups. Values are expressed as mean ± SD.

doi:10.1371/journal.pone.0148954.g005

density of $G_{\alpha tran}$ and $G_{\alpha gust}$-IR cells during Hp diet observed in this study might reflect the upregulation of TAS1Rs that included the TAS1R1, TAS1R2 and TAS1R3 subtypes that functions as dimers. The heteromeric combination of TAS1R1-TAS1R3 has been shown to function as a broad spectrum L-amino acid sensor, responsible for mediating perception of the savory "umami", taste of monosodium glutamate [55,56], and responds to a wide variety of L-amino acids in the millimolar range [56]. On the other hand, the high content of proteins in the diet could enhance the expression of the sweet sensors, i.e. the TAS1R2 and TAS1R3, likely a compensatory effect of a decreased content of carbohydrates. This response appears to be in contrast to the TAS1R2 down-regulation induced by glucose administration reported in the mouse gut [57]. Since the degradation products of protein hydrolysis can be bitter, we cannot exclude that the Hp diets exert a modulatory role on the large TAS2Rs family [58] and therefore an increased amount of food-born bitter tastants (or, alternatively, increased amino acids per se) could result in increased TAS2R / $G_{\alpha tran}$ / $G_{\alpha gust}$ expression in the pyloric mucosa.

$G_{\alpha tran}$-IR cells density in pyloric mucosa was significantly increased after 3 days of the Hp diet administration, whereas the increase in $G_{\alpha gust}$-IR cells reached statistical significance only after 30 days of Hp diet. This suggests that the Hp diet evoked a differential regulation of the taste receptor system mainly involving $G_{\alpha tran}$ in the short-term and both $G_{\alpha tran}$ and $G_{\alpha gust}$ in the long-term. Our results indicated the occurrence of major changes in taste signaling molecules in the upper GI tract, mainly in the antrum, thus expanding previous data from our laboratory showing modulation of taste-related molecules and distinct TAS2Rs in the stomach following fasting and refeeding [27] and in the upper small intestine with low-cholesterol diet [28]. Taken together these data indicate that different dietary manipulations affect taste signaling molecules and receptors throughout GI tract segments. Based on the "intestinal sensor cell hypothesis" [59], implying that nutrients can be sensed by EECs expressing TASRs, the stomach could be thought as the "first gate" monitoring food components and activating digestive processes or aversive responses in the case of potentially harmful substances [60]. This initial response would be followed by the functional response of the upper small intestine where digestion continues and absorption initiates.

Kinsey-Jones et al. (2015) [42] reported that a Hp diet was not effective in modulating GPRC6A expression in different regions of the mouse GI tract and hypothesized the presence of multiple overlapping systems mediating the effects of dietary amino acids and proteins. Our finding of changes in the expression of G proteins transducing taste receptors in certain regions of the gut in response to Hp diets are consistent with the notion of multiple receptors involvement for amino acid sensing in the gust as reported in the lingual epithelium [61,62].

The increased density of the overall GHR-IR cells population observed in the pyloric region in the Hp30 group is in line with the reported increase of GHR levels in plasma in rats and ruminant following long-term high-protein diets (7 days to 2 weeks) [63,64]. Furthermore, our observation of an increase in $G_{\alpha tran-}$ and $G_{\alpha gust}$-IR cells containing GHR-IR in the pyloric mucosa of Hp30 group compared to Hp3 and Ctr, is in agreement with recent findings showing that amino acids and di- / tripeptides are sensed by TAS1R1-TAS1R3, which stimulate a chemosensory signalling pathway regulating ghrelin release [35]. However, we did not see any quantitative change of GHR-IR cells after Hp3 diet, whereas Lejeune et al. (2006) [65] demonstrated that a four-day administration of a Hp diet resulted in suppression of GHR plasma levels. We did not see any change in the number of PYY-IR or PYY cell coexpressing $G_{\alpha tran}$ or $G_{\alpha gust}$-IR in the colon and rectum, though PYY release is increased by Hp diet stimulation in

mammals, including humans [66–68]. These apparently discrepant results are likely due to the different measurements of GHR and PYY in tissue and blood and the different animal models. Whether an increased number of $G_{\alpha tran}$ and $G_{\alpha gust}$ / GHR-IR or $G_{\alpha tran}$ and $G_{\alpha gust}$ / PYY-IR cells is associated with increased circulating levels of these peptides was beyond the purpose of the present study and remains to be established.

Our finding that $G_{\alpha tran}$ and $G_{\alpha gust}$ cells in the small bowel co-expressed 5-HT extend previous data in the mouse [38]. Furthermore, the observation that $G_{\alpha tran}$ and $G_{\alpha gust}$ / 5-HT cells are increased in the Hp3 and Hp30 groups compared to controls, suggests that the effect of Hp diet on gut physiology (e.g. secreto-motor and nociceptive function) [69] involves the activation of the gut taste system via the release of 5-HT, a key signalling molecule in the gut.

The increase of the $G_{\alpha tran}$ / CCK cells in the jejunal crypts of Hp3 group might reflect activation of these cells by protein hydrolysates, peptides and amino acids, which have been reported to induce secretion of CCK by EECs expressing TAS1R1-TAS1R3 [70]. On the other hand, the decreased number of $G_{\alpha tran}$ / CCK cells after 30 days of high-proteins diet suggests that adaptive mechanisms come into play. CCK plays many roles in the digestive processes and has a well known inhibitory effect on food intake [71], effects that could be mediated by the activation of the taste receptor system in the gut.

In conclusion, this study shows that short- and, in particular, long-term Hp diet evoked selective changes in the expression of TASR related signalling molecules in subsets of EECs in different regions of the GI tract. Our findings further strengthen the hypothesis of a functional role of taste-related molecules in gut chemosensitivity and suggest a functional role of the gut taste system in nutrient-dependent—including proteins—gut functions.

Supporting Information

S1 Fig. Representative images of the pre-adsorption test of ghrelin (A), serotonin (5-HT, B) and peptide YY (PYY, C) primary antibodies.
(TIF)

S1 Table. A Composition of experimental diets and energy density. * vitamins, minerals and amino acids integration
(DOCX)

S2 Table. Body weight and feed consumption of experimental animals. Values are expressed as mean ± standard deviation.
(DOCX)

S3 Table. Percentages of the colocalized $G_{\alpha tran}$- $G_{\alpha gust}$ -5-HT/ total 5HT-IR cells in the duodenal villi and glands.
(DOCX)

Acknowledgments

The present work was supported by NIH grants DK09 and 41301 (C.S.), and grants from the Italian Ministry of Education, University and Research (MIUR) (PRIN2009) (R. De G.), from 'Fondazione Del Monte di Bologna e Ravenna', Italy and funds from the University of Bologna (R. De G. and P.C.).

Author Contributions

Conceived and designed the experiments: RDG MM MLB PC CS. Performed the experiments: MM CV RL CB PC. Analyzed the data: PC M. Forni M. Falconi MLB RDG CS MM.

Contributed reagents/materials/analysis tools: MLB RDG PC CS MM. Wrote the paper: CV RDG MM CS PC.

References

1. Furness JB, Kunze WA, Clerc N. Nutrient tasting and signaling mechanisms in the gut. II. The intestine as a sensory organ: neural, endocrine, and immune responses. Am J Physiol. 1999; 277: G922–928. PMID: 10564096
2. Dockray GJ. Luminal sensing in the gut: an overview. J Physiol Pharmacol. 2003; 54: 9–17.
3. Cummings DE, Overduin J. Gastrointestinal regulation of food intake. J Clin Invest. 2007; 117: 13–23. PMID: 17200702
4. Sternini C, Anselmi L, Rozengurt E. Enteroendocrine cells: a site of "taste" in gastrointestinal chemosensing. Curr Opin Endocrinol Diabetes Obes. 2008; 15: 73–78. doi: 10.1097/MED.0b013e3282f43a73 PMID: 18185066
5. Nakamura E, Hasumura M, Uneyama H, Torii K. Luminal amino acid-sensing cells in gastric mucosa. Digestion. 2011; 83: S13–18.
6. Furness JB, Rivera LR, Cho HJ, Bravo DM, Callaghan B. The gut as a sensory organ. Nat Rev Gastroenterol Hepatol. 2013; 10: 729–740. doi: 10.1038/nrgastro.2013.180 PMID: 24061204
7. Jang HJ, Kokrashvili Z, Theodorakis MJ, Carlson OD, Kim BJ, Zhou J, et al. Gut-expressed gustducin and taste receptors regulate secretion of glucagon-like peptide-1. Proc Natl Acad Sci U S A. 2007; 104: 15069–15074. PMID: 17724330
8. Margolskee RF, Dyer J, Kokrashvili Z, Salmon KS, Ilegems E, Daly K, et al. T1R3 and gustducin in gut sense sugars to regulate expression of Na+-glucose cotransporter 1. Proc Natl Acad Sci U S A 2007; 104: 15075–15080. PMID: 17724332
9. Glendinning JI, Yiin YM, Ackroff K, Sclafani A. Intragastric infusion of denatonium conditions flavor aversions and delays gastric emptying in rodents. Physiol Behav. 2008; 93: 757–765. doi: 10.1016/j.physbeh.2007.11.029 PMID: 18174110
10. Hao S, Sternini C, Raybould HE. Role of CCK1 and Y2 receptors in activation of hindbrain neurons induced by intragastric administration of bitter taste receptor ligands. Am J Physiol Regul Integr Comp Physiol. 2008; 294: R33–38. PMID: 18003792
11. Jeon TI, Zhu B, Larson JL, Osborne TF. SREBP-2 regulates gut peptide secretion through intestinal bitter taste receptor signaling in mice. J Clin Invest. 2008; 118: 3693–3700. doi: 10.1172/JCI36461 PMID: 18846256
12. Hao S, Dulake M, Espero E, Sternini C, Raybould HE, Rinaman L. Central Fos expression and conditioned flavor avoidance in rats following intragastric administration of bitter taste receptor ligands. Am J Physiol Regul Integr Comp Physiol. 2009; 296: R528–536. doi: 10.1152/ajpregu.90423.2008 PMID: 19073908
13. Janssen S, Laermans J, Verhulst PJ, Thijs T, Tack J, Depoortere I. Bitter taste receptors and α-gustducin regulate the secretion of ghrelin with functional effects on food intake and gastric emptying. Proc Natl Acad Sci USA. 2011; 108: 2094–2099. doi: 10.1073/pnas.1011508108 PMID: 21245306
14. Chen MC, Wu V, Reeve JR, Rozengurt E. Bitter stimuli induce $Ca2^+$ signaling and CCK release in enteroendocrine STC-1 cells: role of L-type voltage-sensitive $Ca2^+$ channels. Am J Physiol Cell Physiol. 2006; 291: C726–739. PMID: 16707556
15. Nelson G, Hoon MA, Chandrashekar J, Zhang Y, Ryba NJ, Zuker CS. Mammalian sweet taste receptors. Cell 2001; 106: 381–390. PMID: 11509186
16. Li X, Staszewski L, Xu H, Durick K, Zoller M, Adler E. Human receptors for sweet and umami taste. Proc Natl Acad Sci USA. 2002; 99: 4692–4696. PMID: 11917125
17. Nelson G, Chandrashekar J, Hoon MA, Feng L, Zhao G, Ryba NJ et al. An amino-acid taste receptor. Nature 2002; 416: 199–202. PMID: 11894099
18. Chandrashekar J, Hoon MA, Ryba NJ, Zuker CS. The receptors and cells for mammalian taste. Nature 2006; 444: 288–294. PMID: 17108952
19. Meyerhof W, Batram C, Kuhn C, Brockhoff A, Chudoba E, Bufe B, et al. The molecular receptive ranges of human TAS2R bitter taste receptors. Chem senses. 2010; 35: 157–170. doi: 10.1093/chemse/bjp092 PMID: 20022913
20. Höfer D, Püschel B, Drenckhahn D. Taste receptor-like cells in the rat gut identified by expression of alpha-gustducin. Proc Natl Acad Sci USA. 1996; 93: 6631–6634. PMID: 8692869
21. Höfer D, Drenckhahn D. Identification of the taste cell G-protein, alpha-gustducin, in brush cells of the rat pancreatic duct system. Histochem Cell Biol. 1998; 110: 303–309. PMID: 9749964

22. Höfer D, Asan E, Drenckhahn D. Chemosensory Perception in the Gut. News Physiol Sci. 1999; 14: 18–23. PMID: 11390812

23. Wu SV, Rozengurt N, Yang M, Young SH, Sinnett-Smith J, Rozengurt E. Expression of bitter taste receptors of the T2R family in the gastrointestinal tract and enteroendocrine STC-1 cells. Proc Natl Acad Sci USA. 2002; 99: 2392–2397. PMID: 11854532

24. Rozengurt N, Wu SV, Chen MC, Huang C, Sternini C, Rozengurt E. Colocalization of the alpha-subunit of gustducin with PYY and GLP-1 in L cells of human colon. Am J Physiol Gastrointest Liver Physiol. 2006; 291: G792–802. PMID: 16728727

25. Moran AW, Al-Rammahi MA, Arora DK, Batchelor DJ, Coulter EA, Daly K et al. Expression of Na^+/glucose co-transporter 1 (SGLT1) is enhanced by supplementation of the diet of weaning piglets with artificial sweeteners. Br J Nutr. 2010; 104: 637–646. doi: 10.1017/S0007114510000917 PMID: 20338074

26. Latorre R, Mazzoni M, De Giorgio R, Vallorani C, Bonaldo A, Gatta PP, et al. Enteroendocrine profile of α-transducin immunoreactive cells in the gastrointestinal tract of the European sea bass (*Dicentrarchus labrax*). Fish Physiol Biochem. 2013; 39: 1555–1565. doi: 10.1007/s10695-013-9808-4 PMID: 23748963

27. Mazzoni M, De Giorgio R, Latorre R, Vallorani C, Bosi P, Trevisi P, et al. Expression and regulation of α-transducin in the pig gastrointestinal tract. J Cell Mol Med. 2013; 17: 466–474. doi: 10.1111/jcmm.12026 PMID: 23414137

28. Vegezzi G, Anselmi L, Huynh J, Barocelli E, Rozengurt E, Raybould H, et al. Diet-reduced regulation of bitter taste receptor subtypes in the mouse gastrointestinal tract. PLoS One. 2014; 9: e107732. doi: 10.1371/journal.pone.0107732 PMID: 25238152

29. Mazzoni M, Bonaldo A, Gatta PP, Vallorani C, Latorre R, Canova M, et al. α-transducin and α-gustducin immunoreactive cells in the stomach of common sole (*Solea solea*) fed with mussel meal. Fish Physiol Biochem. 2015; 41: 603–12. doi: 10.1007/s10695-015-0031-3 PMID: 25673424

30. Wong GT, Gannon KS, Margolskee RF. Transduction of bitter and sweet taste by gustducin. Nature 1996; 381: 796–800. PMID: 8657284

31. He W, Danilova V, Zou S, Hellekant G, Max M, Margolskee RF, et al. Partial rescue of taste responses of alpha-gustducin null mice by transgenic expression of alpha-transducin. Chem Senses. 2002; 27: 719–727. PMID: 12379596

32. Sainz E, Cavenagh MM, LopezJimenez ND, Gutierrez JC, Battey JF, Northup JK, et al. The G-protein coupling properties of the human sweet and amino acid taste receptors. Dev Neurobiol. 2007; 67: 948–959. PMID: 17506496

33. Potier M, Darcel N, Tome D. Protein, amino acids and the control offood intake. Curr Opin Clin Nutr Metab Care. 2009; 12: 54–58. doi: 10.1097/MCO.0b013e32831b9e01 PMID: 19057188

34. Bensaid A, Tome D, Gietzen D, Evena P, Morensa C, Gausseresc N, et al. Protein is more potent than carbohydrate for reducing appetite in rats. Physiol Behav. 2002; 75: 577–582. PMID: 12062322

35. Vancleef L, Van Der Broeck T, Thijs T, Steensels S, Briand L, Tack J et al. Chemosensory signalling pathways involved in sensing of amino acids by the ghrelin cell. Sci Rep. 2015; 5:15725. doi: 10.1038/srep15725 PMID: 26510380

36. Haid D, Widmayer P, Breer H. Nutrient sensing receptors in gastric endocrine cells. J Mol Histol. 2011; 42: 355–364. doi: 10.1007/s10735-011-9339-1 PMID: 21750971

37. Wu SV, Chen MC, Rozengurt E. Genomic organization, expression, and function of bitter taste receptors (T2R) in mouse and rat. Physiol Genomics. 2005; 22: 139–149. PMID: 15886333

38. Sutherland K, Young RL, Cooper NJ, Horowitz M, Blackshaw LA. Phenotypic characterization of taste cells of the mouse small intestine. Am J Physiol Gastrointest Liver Physiol. 2007; 292: G1420–G1428. PMID: 17290008

39. Fujita Y, Wideman RD, Speck M, Asadi A, King DS, Webber TD, et al. Incretin release from gut is acutely enhanced by sugar but not by sweeteners in vivo. Am J Physiol Endocrinol Metab. 2009; 296: E473–E479. doi: 10.1152/ajpendo.90636.2008 PMID: 19106249

40. Steinert RE, Gerspach AC, Gutmann H, Asarian L, Drewe J, Beglinger C. The functional involvement of gut-expressed sweet taste receptors in glucose-stimulated secretion of glucagon-like peptide-1 (GLP-1) and peptide YY (PYY). Clin Nutr. 2011; 30: 524–532. doi: 10.1016/j.clnu.2011.01.007 PMID: 21324568

41. Haid DC, Jordan-Biegger C, Widmayer P, Breer H. Receptors responsive to protein breakdown products in G-cells and D-cells of mouse, swine and Human. Front Physiol. 2012; 3: 65. doi: 10.3389/fphys.2012.00065 PMID: 22514536

42. Kinsey-Jones JS, Alamshah A, McGavigan AK, Spreckley E, Banks K, Cereceda Monteoliva N, et al. GPRC6a is not required for the effects of a high-protein diet on body weight in mice. Obesity (Silver Spring). 2015; 23(6): 1194–200.

43. Conigrave AD, Quinn SJ, Brown EM. L-amino acid sensing by the extracellular Ca2+-sensing receptor. Proc Natl Acad Sci. USA 2000; 97:4814–4819. PMID: 10781086

44. Haid DC, Jordan-Biegger C, Widmayer P, Breer H. Receptors responsive to protein breakdown products in g-cells and d-cells of mouse, swine and human. Front Physiol. 2012; 3: 65. doi: 10.3389/fphys.2012.00065 PMID: 22514536

45. Liou AP, Sei Y, Zhao X, Feng J, Lu X, Thomas C, et al. The extracellular calcium-sensing receptor is required for cholecystokinin secretion in response to L-phenylalanine in acutely isolated intestinal I cells. Am J Physiol Gastrointest Liver Physiol. 2011; 300: G538–546. doi: 10.1152/ajpgi.00342.2010 PMID: 21252045

46. Wellendorph P, Johansen LD, Brauner-Osborne H. Molecular pharmacology of promiscuous seven transmembrane receptors sensing organic nutrients. Mol Pharmacol. 2009; 76: 453–465. doi: 10.1124/mol.109.055244 PMID: 19487246

47. Ruiz-Avila L, McLaughlin SK, Wildman D, McKinnon PJ, Robichon A, Spickofsky N, et al. Coupling of bitter receptor to phosphodiesterase through transducin in taste receptor cells. Nature 1995; 376: 80–85. PMID: 7596440

48. He W, Danilova V, Zou S, Hellekant G, Max M, Margolskee RF, et al. Partial rescue of taste receptor responses of alpha-gudtducin null mice by transgenic expression of alpha-transducin. Chem Sens 2002; 27: 719–727.

49. Margolskee RF Molecular mechanisms of bitter and sweet taste transduction. J Biol Chem 2002; 277: 1–4. PMID: 11696554

50. He W, Yasumatsu K, Varadarajan V, Yamada A, Lem J, Ninomiya Y, et al. Umami taste responses are mediated by alpha-transducin and alpha-gustducin. J Neurosci. 2004; 24: 7674–7680. PMID: 15342734

51. Buchan AM, Squires PE, Ring M, Meloche RM. Mechanism of action of the calcium-sensing receptor in human antral gastrin cells. Gastroenterology. 2001; 120: 1128–39. PMID: 11266377

52. Höfer AM, Brown EM. Extracellular calcium sensing and signalling. Nat Rev Mol Cell Biol. 2003; 4: 530–8. PMID: 12838336

53. Choi S, Lee M, Shiu AL, Yo SJ, Aponte GW. Identification of a protein hydrolysate responsive G protein-coupled receptor in enterocytes. Am J Physiol Gastrointest Liver Physiol. 2007; 292: G98–G112. PMID: 16935853

54. Ward DT. Calcium receptor-mediated intracellular signalling. Cell Calcium. 2004; 35: 217–28. PMID: 15200145

55. Li X, Staszewski L, Xu H, Durick K, Zoller M, Adler E. Human receptors for sweet and umami taste. Proc Natl Acad Sci USA. 2002; 99: 4692–4696. PMID: 11917125

56. Nelson G, Chandrasheker J, Hoon MA, Feng L, Zhao G, Ryba NJ et al. An amino-acuid taste receptor. Nature. 2002; 416:199–202. PMID: 11894099

57. Young RL, Sutherland K, Pezos N, Brierley SM, Horowitz M, Rayner CK, et al. Expression of taste molecules in the upper gastrointestinal tract in humans with and without type 2 diabetes. Gut. 2009; 58: 337–346. doi: 10.1136/gut.2008.148932 PMID: 19039089

58. Maehashi K, Huang L. Bitter peptides and bitter taste receptors. Cell Mol Life Sci. 2009; 66: 1661–1671. doi: 10.1007/s00018-009-8755-9 PMID: 19153652

59. Fujita T, Kobayashi S, Yui R. Paraneuron concept and its current implications. Adv Biochem Psychopharmacol. 1980; 25: 321–325. PMID: 6108685

60. Widmayer P, Breer H, Hass N. Candidate chemosensory cells in the porcine stomach. Histochem Cell Biol. 2011; 136: 37–45. doi: 10.1007/s00418-011-0824-0 PMID: 21667283

61. Daly K, Al-Rammahi M, Moran A, Marcello M, Ninomiya Y, Shirazi-Beechey SP. Sensing of amino acids by the gut-expressed taste receptor T1R1-T1R3 stimulates CCK secretion. Am J Physiol Gastrointest Liver Physiol. 2013; 30: G271–282.

62. Yasumatsu K, Ogiwara Y, Takai S, Yoshida R, Iwatsuki K, Torii K et al. Umami taste in mice uses multiple receptors and transduction pathways. J Physiol. 2012; 590; 1155–1170. doi: 10.1113/jphysiol.2011.211920 PMID: 22183726

63. Vallejo-Cremades MT, Gómez-García L, Chacatas-Cortesao M, Moreno C, Sánchez M, De Miguel E, et al. Enriched protein diet-modified ghrelin expression and secretion in rats. Regul Pept. 2004; 121: 113–119. PMID: 15256281

64. Takahashi T, Kobayashi Y, Haga S, Ohtani Y, Sato K, Obara Y, et al. A high-protein diet induces dissociation between plasma concentrations of growth hormone and ghrelin in wethers. J Anim Sci. 2012; 90: 4807–4813. doi: 10.2527/jas.2011-4596 PMID: 22871937

65. Lejeune MP, Westerterp KR, Adam TC, Luscombe-Marsh ND, Westerterp-Plantenga MS. Ghrelin and glucagon-like peptide 1 concentrations, 24-h satiety, and energy and substrate metabolism during a high-protein diet and measured in a respiration chamber. Am J Clin Nutr. 2006; 83: 89–94. PMID: 16400055

66. Batterham RL, Heffron H, Kapoor S, Chivers JE, Chandarana K, Herzog H, et al. Critical role for peptide YY in protein-mediated satiation and body-weight regulation. Cell Metab. 2006; 4: 223–233. PMID: 16950139

67. Lobley GE, Holtrop G, Horgan GW, Bremner DM, Fyfe C, Johnstone AM. Responses in gut hormones and hunger to diets with either high protein or a mixture of protein plus free amino acids supplied under weight-loss conditions. Br J Nutr 2015; 113: 1254–1270. doi: 10.1017/S0007114515000069 PMID: 25809236

68. Wang S, Yang L, Lu J, Mu Y. High-protein breakfast promotes weight loss by suppressing subsequent food intake and regulating appetite hormones in obese Chinese adolescents. Horm Res Paediatr. 2015; 83: 19–25. doi: 10.1159/000362168 PMID: 24923232

69. Gershon MD, Tack J. The serotonin signaling system: from basic understanding to drug development for functional GI disorders. Gastroenterology 2007; 132: 397–414. PMID: 17241888

70. Shirazi-Beechey SP, Daly K, Al-Rammahi M, Moran AW, Bravo D. Role of nutrient-sensing taste 1 receptor (T1R) family members in gastrointestinal chemosensing. Br J Nutr 2014; 2: 1–8.

71. Moran TH. Gut peptides in the control of food intake. Int J Obes (Lond). 2009; 33: S7–S10.