Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA IN

MORFOFISIOLOGIA E PATOLOGIA VETERINARIA CON APPLICAZIONI BIOTECNOLOGICHE

*Ciclo **XXV***

Settore Concorsuale di afferenza: 07/H1

Settore Scientifico disciplinare: VET/01

TITOLO TESI

TASTE RECEPTORS IN THE GUT: A CHEMOSENSITIVE MECHANISM FROM FISH TO HUMAN

Presentata da: ROCCO LATORRE

Coordinatore Dottorato: ***Chiar.mo Prof. Eraldo Seren***

Relatore: ***Chiar.mo Prof. Paolo Clavenzani***

Esame finale anno 2013

Riassunto

L'ingestione di un pasto evoca una serie di processi digestivi che consistono nelle funzioni essenziali dell'apparato digerente, trasporto degli alimenti, attività secretiva, assorbimento dei nutrienti digeriti e l'espulsione dei residui non assorbiti. La *gastrointestinal chemosensitivity* è caratterizzata da elementi cellulari endocrini della mucosa gastroenterica e da fibre nervose, soprattutto di natura vagale. Una ampia gamma di mediatori endocrini e/o paracrini possono essere rilasciati da varie cellule endocrine in risposta a nutrienti introdotti con la dieta. Tali ormoni, oltre alla loro attività diretta, agiscono attraverso recettori specifici attivando azioni di assoluta importanza nel controllo di varie funzioni tra cui l'introito calorico e l'omeostasi energetica dell'organismo. Ad integrazione di questo complesso sistema di controllo della chemosensitività gastrointestinale, recenti evidenze dimostrano la presenza di recettori del gusto (o *taste receptors*, TR) appartenenti alla famiglia dei recettori correlati alle proteine G espressi a livello della mucosa del tratto gastrointestinale di diversi mammiferi e dell'uomo.

La presente ricerca, suddivisa in diversi progetti di ricerca, è stata concepita al fine di chiarire il rapporto tra TR e nutrienti. Per definire questo rapporto sono stati usati diversi approcci scientifici, che sono andati a valutare le variazioni delle molecole segnale dei TR in particolare dell'α-transducina in condizioni di digiuno e a seguito di rialimentazione standard nel tratto gastrointestinale di suino, la mappatura della stessa molecola segnale nel tratto gastrointestinale di pesce (Dicentrarchus Labrax), il signaling pathway dei bitter TR in colture cellulari endocrine STC-1 ed infine il coinvolgimento dei bitter TR, in particolare del T2R38 in pazienti con un eccessivo introito calorico. I risultati hanno evidenziato come ci sia una stretta correlazione tra nutrienti, TR e rilascio ormonale e come questi siano coinvolti non solo nella percezione del gusto propriamente detto ma probabilmente anche in patologie croniche come l'obesità.

Abstract

The ingestion of a meal evokes a series of digestive processes, which consist of the essential functions of the digestive system: food transport, secretory activity, absorption of nutrients and the expulsion of undigested residues do not absorbed. The gastrointestinal chemosensitivity is characterized by cellular elements of the endocrine gastrointestinal mucosa and nerve fibers, in particular of vagal nature. A wide range of mediators endocrine and/or paracrine can be released from various endocrine cells in response to nutrients in the diet. These hormones, in addition to their direct activity, act through specific receptors activating some of the most important functions in the control of energy intake and energy homeostasis in the body. For integration of this complex system of control of gastrointestinal chemosensitivity, recent evidence demonstrates the presence of taste receptors (TR) belonging to the family of G proteins coupled receptor expressed in the mucosa of the gastrointestinal tract of different mammals and human. This thesis is divided into several research projects that have been conceived in order to clarify the relationship between TR and nutrients. To define this relationship I have used various scientific approaches, which have gone on to evaluate changes in signal molecules of TR, in particular of the α-transducin in the fasting state and after refeeding with standard diet in the gastrointestinal tract of the pig, the mapping of the same molecule signal in the gastrointestinal tract of fish (Dicentrarchus labrax), the signaling pathway of bitter TR in the STC-1 endocrine cell line and finally the involvement of bitter TR in particular of T2R38 in patients with an excessive caloric intake. The results showed how there is a close correlation between nutrients, TR and hormonal release and how they are useful both in taste perception but also likely to be involved in chronic diseases such as obesity.

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INTRODUCTION

THE GASTROINTESTINAL TRACT IN MAMMALS

The alimentary canal consists of the esophagus, stomach, small intestine, large intestine and the anal canal. Associated to it there are two large glands that release their secretions into the intestinal lumen: liver and pancreas.
I will consider only the gastrointestinal tract, and so I will not treat the esophagus and the associated glands. I will try to describe the generality of the gastrointestinal tract, highlighting the key parts in pigs and humans.

I) THE STOMACH

The stomach is a dilated portion of the digestive tract, which follows the esophagus at the level of cardia and it is continuous through the small intestine, in correspondence of the pylorus [1] [2] [3].
The stomach receives the insalivated boluses of food from the oral cavity through the esophagus. The bolus is temporarily stored in the stomach and soaked by gastric juice which is secreted by the gastric glands [1] [2] [3] [4]. This solution is mainly composed by pepsin, rennin and hydrochloric acid, which act on proteic substances [1]. The food, under the combined action of gastric juice and peristaltic movements is transformed into a fluid mass called chyme and is moved into the duodenum [1] [2] [3] [4]. The size and the structure of the stomach depends on the habits and on the food behavior of the singular species. Moreover the structure of the stomach depends on different feeding and lifestyle of the various species. pigs and humans are monogastric animals, but they show some differences: the human stomach (**Fig 1**) (glandular stomach) has a glandular mucosa covered with simple columnar epithelium and a capacity of 1,3 liters, while the stomach of the pig has a proventricular portion (**Fig 1**) (nonglandular mucosa; characterized by stratified squamous epithelium) and the presence of an esophageal-like mucosa, more or less extended from the cardia. The capacity of the pig's stomach is 4 liters [2] [3].

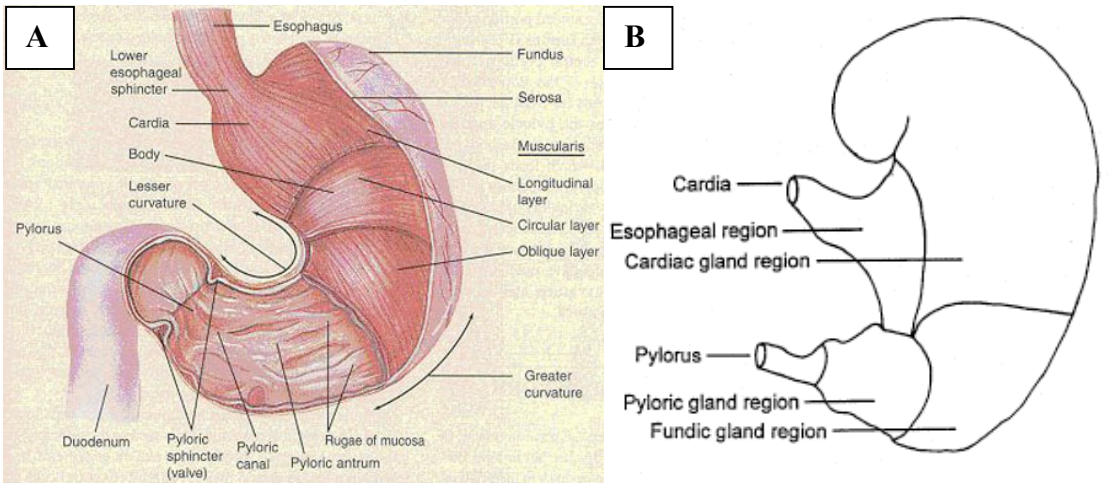

Fig 1: **A**. External and internal anatomy of the stomach of human (Tortora and Grabowski, 1996), **B**. Region of the pig stomach (http://www.thepigsite.com/articles/2749/digestive-system-anatomy-and-function)

The stomach is characterized by two curvatures: the greater curvature is convex and directed ventrally toward the left, while the lesser curvature is concave and direcetd dorsally to the right [2]. The stomach can be divided into three anatomical regions: the fundus, or blind sac, overlooking the cardia, the body, located ventrally to the fundus, which continues on to the pyloric region. The latter, which corresponds to the flexed lower portion of the stomach, consists of the pyloric antrum and pyloric canal [2].

i) Structure

The wall of the stomach consists of a mucous membrane, a muscular, and serous coat. The tunica mucosa (Mucous membrane) can be divided into layers: surface epithelium, lamina propria mucosae, lamina muscolaris mucosae, and tela submucosa [4].

Tunica Serosa

The serosa is constituted by the visceral peritoneum; it is formed by two sheets, one anterior and one posterior. In proximity of the small curvature the serosa is coated with a small amount of elastic fibers, which seem to have the task of maintaining the two ends of the stomach close together.

The tunica serosa is continuous at the level of the greater curvature with the greater omentum, near the diaphragm with the gastrophrenic ligament, and at the level of small curvature with the lesser omentum [2] [3].

Tunica Muscolaris

The musculature of the stomach is constituted by two fundamental layers of smooth muscle cells, one on the surface (longitudinal layer) and one in deep (circular layer). Depending on the considered levels we can observe a dissociation, a thickening or a change of direction of the muscle planes, in relation to the conformation and function of each region of the organ. In the fundus and in the body of the stomach, two oblique layers are added to the two fundamental layers, one of which is internal and the other external. [2] [3].

-*Longitudinal muscle layer*

The longitudinal muscle layer is incomplete and is placed immediately after the subserosa. It is reduced to two straps, one of which runs along the small curvature and the other runs along the greater curvature [2] [3].

The first one is continuous on the surface of the esophagus and opens like a fan on the faces of the stomach, reaching the gastric incisure. The other one is relatively thin and extends from the left edge of the fundus to the pyloric area, where it is reinforced by elastic fibers. In the proximity of the pylorus the longitudinal layer is complete and thickened and then continues with the longitudinal layer of the intestine [2] [3].

-*External oblique fibers*

This layer is a continuation of the longitudinal layer, which is located only in the vicinity of the fundus and the part next to it, i.e. the body. This is well developed in the pig, where it forms a relatively superficial and flat thickened layer. These external oblique fibers are poorly developed in the human stomach.

-*Circular muscle layer*

The circular layer is not present at the levelof the fundus and is thin in the adjacent part of the body of the stomach. The circular layer occupies an intermediate position between the layers descibed above and those of the internal oblique fibers; at the level of the pylorus this layer is in direct relation to the submucosa. Precisely in the pyloric part (pyloric canal), the circular layer becomes stronger and forms the pyloric sphincter, which through its contraction, completely closes the communication with the intestine [2] [3].

-*Internal oblique fibers*

This layer is present only at the level of the fundus and in the body of the stomach. It is a thickened layer that is continuous with the circular bundles of the left face of the esophagus and which is made up of two parts: cardiac loop and the oblique layer.

Tunica Submucosa

This layer is immediately beneath the mucosa; it is a layer of loose to dense connective tissue containing blood and lymphatic vessels. The submucosa also contains the submucous plexus, a critical component of the digestive tract's nervous system which provides nervous control to the mucosa [4].

Tunica Mucosa

When proventricular mucosa is present it is an esophageal-like mucosa. The rest of the stomach has a glandular mucosa, which has structural changes that allow us to divide it into three zones: fundic mucosa, cardiac mucosa and pyloric mucosa [2] [3].

-Proventricular mucosa

The proventricular mucosa (non-glandular mucosa) of the stomach is often slightly folded, it is white and dry and very similar to esophageal mucosa; in the pig the proventricular mucosa extends for 3-4 cm on the right of the small curvature, while it reaches 7-8 cm in width to the left where it leads up to the margin of the gastric diverticulum [2] [3].

The muscularis mucosa is relatively thick and has two floors of irregular bundles, which are often dissociated. The tunica propria mucosa is thick and is formed by dense connective tissue which is rich in elastic fibers [2] [3].

Like in the esophagus, the epithelium is stratified squamous non-keratinized, thick and cornified. There are no glands in the proventriculum [2] [3].

ii) Fundic mucosa

It is considered that the cardial mucosa and the pyloric mucosa derive from the fundic mucosa. The fundic mucosa occupies the fundus in humans and carnivores, whilst in pigs it is positionated fully into the body of the stomach and therefore it is not found in the fundus. The fundic mucosa is thick, soft, red to brown [2] [3].

Its overall organization is characterized by the provision of the muscularis mucosa, which is well developed and is provided with two floors of bundles, of which the exterior is longitudinal and the inner one is transverse. The transverse layer projects numerous bundles of cellular fibers between glands. They separate groups of glands forming lobules, which leads to the formation of small areas, called gastric areas, on the surface of the mucosa.

Each lobule is divided into small groups of glands, whose excretory ducts open into deep and narrow depressions called: gastric pits or crypts (**Fig 2**).

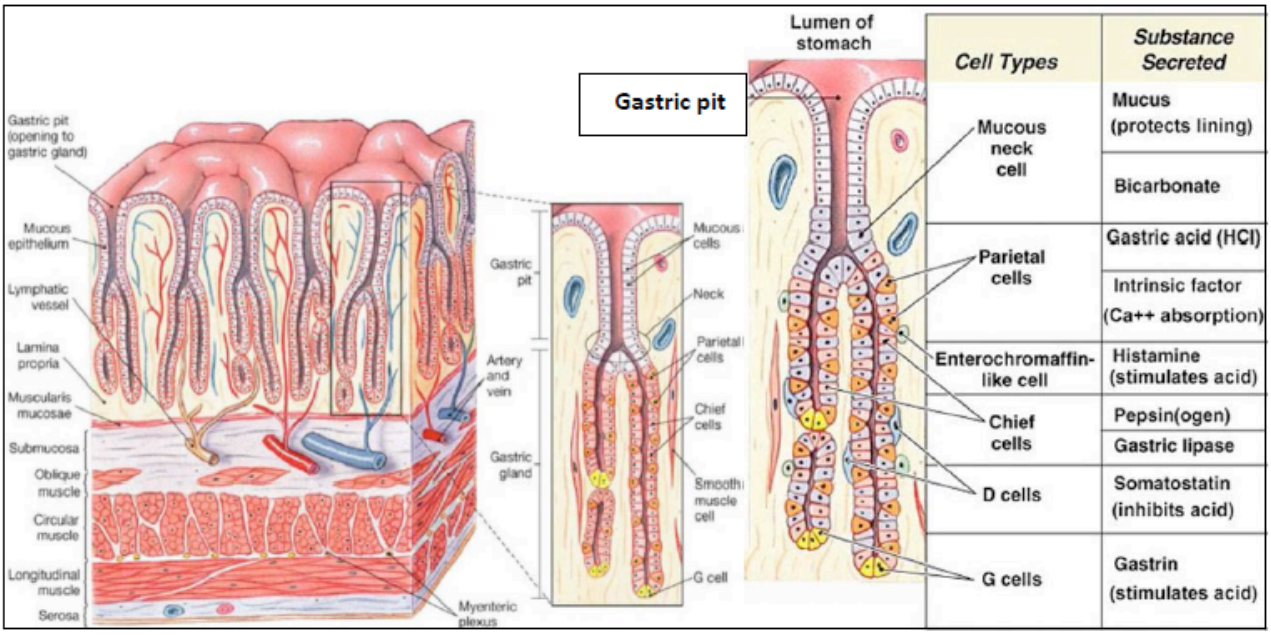

Fig 2. General cell composition and location in a gastric gland. G and D cells are mainly in pyloric glands, while paretial and chief cells in oxyntic (fundic) mucosa. (Basic and Clinical Pharmacology. Mc Graw-Hill, 2012)

-Lamina propria mucosa is the delicate connective tissue interposed between glands. It is rich in vessels with a thick subglandular layer particularly developed in carnivores. Numerous lymphocytes infiltrate the lamina propria mucosa in particular in the subglandular area, and in some cases they form lymphatic nodules, which are especially developed and evident in the pig. The epithelium is simple, formed by a single row of cells supported by a basement membrane. Many tubular glands are present in the thickness of the tunica propria, these glands occupy almost the entire thickness of the mucosa. In the adjacent part of the crypts and on the surface of the mucosa, the epithelium is formed by high and clear prismatic cells, which become lower and cubic towards the bottom of the crypts, in correspondence of the glandular orifices [2] [3].

-Region of proper gastric glands, the fundic glands or proper gastric glands are closely packed tubular glands with a rectilinear course and perpendicular to the surface they become sinuous or convoluted in the vicinity of the muscularis mucosae. Each gland is composed of a narrow and cylindrical neck, a large cylindrical and slightly flexuous body, and finally a base convoluted with trend and terminating in a blind end [2] [3].

The epithelium has 4 types of cells: 1) the mucous neck cells have a cubical shape, are positionated in the neck where they form the coating, 2) the gastric chief cells, which are

positioned in the body and at the base of the glands they are clear and their volume changes in response to their function, as well as the position of the core which can be central or baseline, 3) the parietal cells or oxyntic cells, which are localized primarily in the body of the glands, while they are very rare at the base of the glands. In the glands they are in an eccentric position, between the chief cells and the basement membrane, their cytoplasm is acidophilus. The chief and the parietal cells secrete different products; the first process the pepsinogen and chymosin or rennin; the latter are involved in the secretion of hydrochloric acid 4) the enterochromaffin cells are located between the chief cells and the basement membrane; they are equivalent to the endocrine cells of the intestine, and are so called because their cytoplasm contains granules that stain with the Ag or Cr salts. They definitely have an endocrine function [2] [3].

iii) Cardial Mucosa

The cardial mucosa gets its name because it is interposed between the esophageal mucosa and the fundic mucosa. In the majority of species including man, this takes place in the vicinity of the cardia, however in the pig it is positioned in the fundus and is well developed. Its organization is similar to that of the mucosa of the fundus, but the cardial glands are tubular branched and very tortuous. Their epithelium is composed of a single row of light-colored, cube-shaped mucous cells with basal nucleus [2] [3].

iv) Pyloric Mucosa

The pyloric mucosa is located in the pyloric part of the stomach and is also located in part of the body of the stomach. It is thinner and lighter than the fundic mucosa and is also less crinkled, with the exception that it is in the immediate vicinity of the pylorus. It has the same overall organization, but the grouping in lobules of the glands is more evident than in other parts of the stomach and the crypts are much deeper and narrower. The pyloric glands are more branched and tortuous than the proper gastric glands. It lines most of the pyloric part in the pig's stomach, except for an area near the greater curvature. Apart from some exceptions, the pyloric glands are devoid of parietal cells and their epithelium is composed only of a layer of clear cells [2] [3].

II) INTESTINE

The intestine extends from the pylorus of the stomach to the anus, with the exception of some rare cases. It is easy to recognize two major parts in the gut: the first part is narrow and relatively long and is called small intestine, the second is more voluminous and variable and is called large intestine [2] [3] (**Fig 3**).

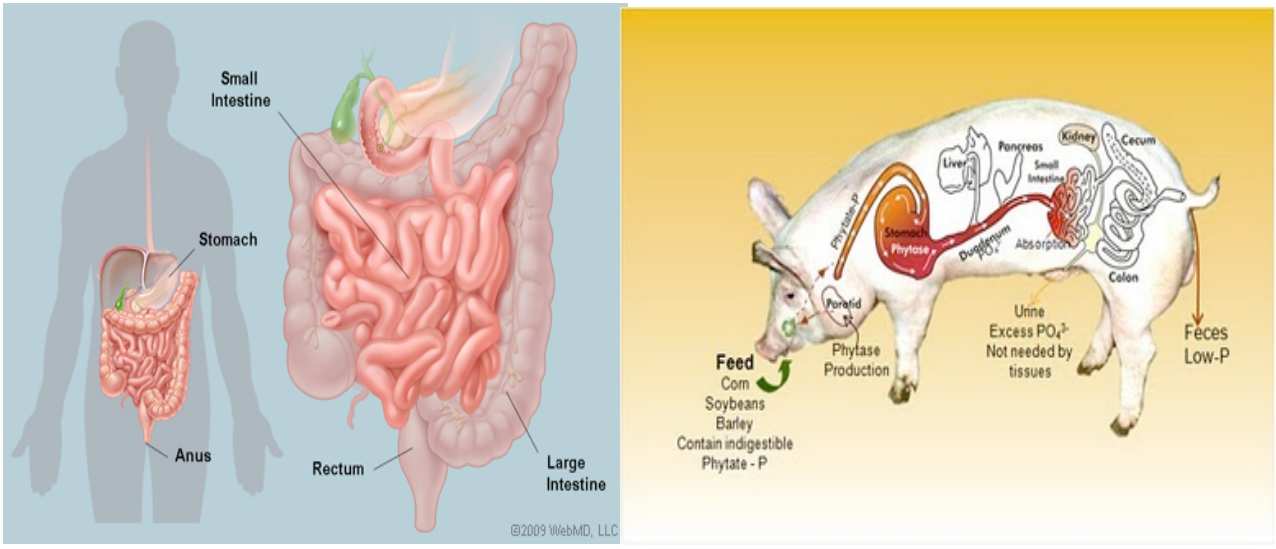

Fig 3. Schematic representation of Human and Pig gastrointestinal tract (www.webmed.com, www.vetmed.vt.edu).

i) Small Intestine

The small intestine is a long cylindrical tube of nearly uniform caliber, where the most important stages of digestion occur. The small intestine is divided into three successive and unequal segments: duodenum, jejunum and ileum. Among these, only the duodenum is clearly demarcated, whilst the boundary between the other two segments is barely visible [2] [3].

The duodenum receives the secretion from the liver and pancreas, while in the jejunum and ileum there is only the secretion from their own mucosa. These secretions continue and complete the action started by gastric juices in the stomach due to the fact that certain substances such as carbohydrates are not modified in the stomach [2] [3]. In order to perform this function the small intestine needs many specilaized structures, a large amount of digestive enzimes and a large amount of mucus, which is indispensible to preserve the mucosa from mechanical insults and irritating compounds.

The size of the small bowel depends on the habits and on the food behavior of the singular species. It is 10 times the length of the body in humans and 13 times in pigs, but may vary also from animal to animal of the same species [2] [3] [4].

a. Duodenum

Duodenum is the first part of the small intestine and extends from the pylorus to the jejunum. Its mesentery, the mesoduodenum, is relatively short, with the exception of carnivores. Two flexures divide the duodenum in 4 parts: 1) cranial part, which passes to the right along the visceral surface of the liver and ends at the cranial flexure; 2) descending part, which runs caudally from the cranial flexure towards the right kidney; this part of the duodenum is shorter in both humans and pigs; 3) transversal part, which runs towards the left and differs from animal to animal; in humans it is elongated, whilst it is shorter in carnivors and significantly shorter in ruminants; 4) ascending part, which is found in the vicinity of the left kidney; it passes cranially and as its mesoduodenum becomes longer it turns ventrally at the duodenojejunal flexure and continues into the jejunum [2] [3] [4].
The descending and ascending parts of the duodenum form a U-shaped loop around the caudal aspect of the root of the mesentery and the cranial mesentery artery [4]. The cranial part of the duodenum is closely related to the liver and pancreas; the duodenum receives the bile duct from the liver and the pancreatic duct from the pancreas [4].

b. Jejunum

The jejunum begins at the duodenojejunal flexure at the cranial end of duodenocolic fold[4]. The jejunum is a long cylindrical tube covered by peritoneum, and continues from the duodenum. In the pig it is found mainly in the ventral part of the right half of the abdominal cavity, but it extend along the floor into the left half and lie ventrally to the coiled ascending colon and cecum [4]. The internal structure of the jejunum consists of a soft epithelium with many villosities; in some areas the mucosa has a particular appearance due to the partial lack of villi and accumulation of lymphnodes. This accumulation of limphonodes forms the peyer's patch [2] [3]. Contractions called peristalsis occur-in this structure, but they never at the same time as in the ileum. Peristalsis is the contraction of

the muscle layer that helps the chyle to continue into the other parts of the gut [2] [3]. Peristalsis occur in each segmant of the gut.

c. Ileum

Ileum is the short terminal part of the small intestine and forms the link between the small and large intestine[4]. It terminates at the cecocolic junction of the large intestine forming the ileal orifice [4]. The anatomy of the terminal part of the ileum suggests that the junction of the ileum and large intestine is not only an anatomical division, but also an important functional division of the alimentary canal [4]. Similarly to the jejunum the ileum also has a cylindric shape and a soft epithelium, with many villosities and in some areas peyer's patches.

d. Structure

The duodenum, the jejunum and the ileum have a very similar structure; they only differ in some aspects. As in the rest of the digestive tract, there are four different layers: tunica serosa, tunica muscolaris, tunica submucosa, tunica mucosa [2] [3].

Tunica Serosa

The whole small intestine is covered with a thin tunica serosa, which derives from the peritoneum [2] [3]. The serosa adheres closely to the tunica muscolaris and near the mesentery it thickens and forms elastic connective tissue to facilitate the changes of caliber that the body undergoes during its functions [2] [3] [4].

Tunica Muscolaris

As in all species, the tunica muscolaris is composed of two layers: a thick circular inner layer and a longitudinal and thinner outer layer. Both are relatively thin at the level of the duodenum and thicken towards the ileum [2] [3]. Between these two layers there is a thin layer of connective tissue which welcomes a network of nerve fibers with ganglion cells, which belong to the myenteric plexus [5].

Tunica Submucosa

The submucosa can be thinner or thicker depending on the segment considered. It is formed by a layer of connective fibers and contains some elastic fibers, which allow it to form folds. Moreover, a submucosal plexus is present at this level with an extensive neuronal network mixed with ganglion cells. Furthermore, deep in the submucosa there are submucosal glands (Brünner glands). In the pig the secretion of these glands lubrificates the epithelial surface and protects it from the acidity of gastric chyme [2] [3].

Tunica Mucosa

The tunica mucosa is the most characteristic part of the bowel; it has a myriad of tiny finger-like processes called villi, which take on various shapes and positions and are specific organs that are very important for absorption [2] [3]. Each villus is coated by epithelium and presents connective tissue. Inside it is possible to note blind end lymphatic vessels surronded by an extensive network of capillaries formed by a small artery and drained by a small venule. Moreover, the villus axis presents smooth muscle cells from the muscolaris mucosae. Between the villi there are depression zones or crypts, which are considered intestinal glands [5]. The epithelium lining of the intestinal mucosa is a simple columnar epithelium, and it is formed by different types of cells (**Fig 4**).

-*Enterocytes*, these cells are prismatic or pyramidal, with the restricted part facing towards the basement membrane. In an optic microscope the apical part of these cells presents a thickened and finely striated area, with microvilli which are very important for absorption [2] [3].

-*Goblet cells*, these cells are interspersed among the enterocytes; there are fewer than enterocytes. They are glandular simple columnar epithelial cells and secrete mucin, which in the end becomes mucus; they use both apocrine and merocrine methods for secretion. Mucus mainly consists of glycoproteins and glycosaminoglycans (PAS positive) [2] [3].

-*Enteroendocrine cells*, these cells are specilized endocrine cells, which produce hormones such as serotonin, somatostatin, colechistokinin (CCK), ghrelin, glucagone peptide-1 (GLP-1), polypeptide YY (PYY) and regulate the digestive cycles. The enterochromaffin cells present in the stomach are also considered enteroendocrine cells [2] [3].

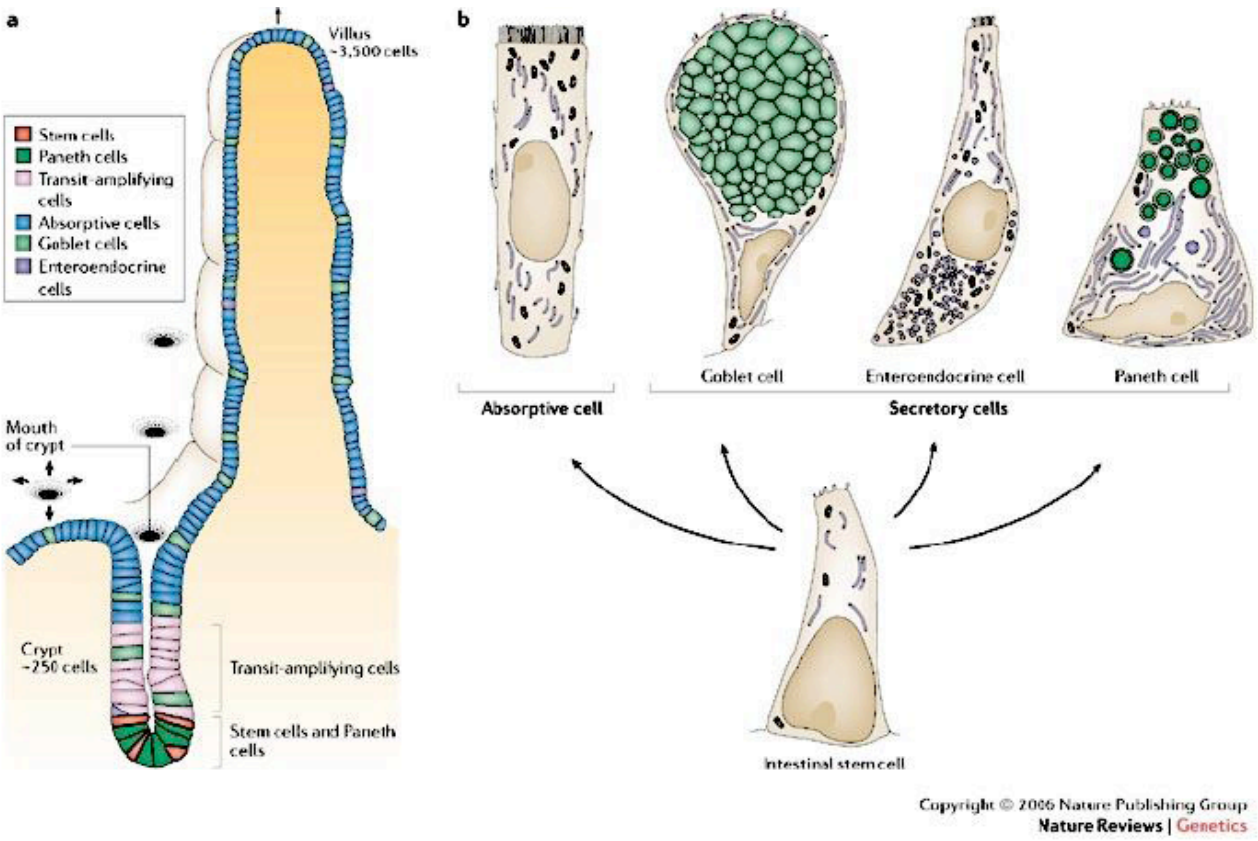

Fig 4. Different types of epithelial cells present in the intestine (Nature Reviews 2006).

ii) Large intestine

The large intestine is the part of the digestive tract that follows the small intestine and ends with the anus. It is divided into the following three segments: the cecum, the colon and the rectum plus a short anal canal. The large intestine has an almost uniform structure and retains, in all its segments, which are variable in shape and size, an anatomical and functional unit. The last part of digestion takes place in this portion of the digestive tract, and in particular the absorption of liquids, which is very high despite the lack of villi [2] [3].

a. Cecum

The cecum is the initial part of the large intestine and appears as a blind end, which is more o less voluminous and present in the gut between the ileum and colon. Depending on the species in question the cecum can be very small or well-developed, in most cases it presents sacculations and teniae [3]. In herbivores and omnivores it is quite developed, whilst it is

short and poorly developed in carnivores. It reaches maximum development in horses and rabbits. In the pig the cecum is 30-40cm long, it is positioned on the left side, and has very pronounced sacculations interrupted by longitudinal folds (tenie) [4]. Between adjacent sacculations, semilunar folds project into the interior of the gut and increase the initial surface area (the same process occurs in the colon) [4]. In humans the cecum, even though short and not voluminous, consists of two unequal segments, one proximal and one distal. The vermiform appendix is part of the distal segment [3].

b. Colon

The colon is the largest part of the large intestine, followed by the cecum, when present, and terminates with the rectum. The size and shape of the colon is related to the diet. The description of the colon in comparative anatomy is based on human nomenclature. The simple arrangement in man gave rise to the division into an ascending colon, which passes cranially on the right, a transverse colon, which passes from right to left in front of the mesenteric artery, and a descending colon, which passes caudally on the left [4].

The ascending colon in the pig has a spiral-shaped cone arrangement with apex on the left side and transverse axis disposed vertically [6], whilst the transverse colon is short and the descending colon presents a smooth appearance and is generally smaller than the ascending colon [3].

-*Ascending Colon*, this part of the colon, is very developed in ungulates and rabbits. In the pig it is from 2 to 4 meters long, wheras it is 5-10 meters long in the cow, 12-15 meters in the camel, 30-35 cm in the rabbit and 15-25 cm in humans. Due to these sizes the ascending colon is forced to bend and roll up to find a place in the abdomen [3]. In the pig it is coiled on itself and forms the spiral loop of the colon, which is between the cecum and the transverse colon and its bends are piled up to form a thick cone [4].

-*Transverse Colon* is relatively long in humans, from 50 to 60 cm, and is delimited by two angles, between which it forms a curve. The transverse colon is smooth in pigs, whereas it is bumpy in humans [3]. It passes from right to left in front of cranial mesenteric artery in the abdomen [4].

-*Discending Colon* is very long and suspended by the long descending mesocolon. The large coils of the descending colon are found in the left dorsal quadrant of the abdominal cavity [4]. It has a simple arrangement and often extends in a straight line. It is smaller than

the ascending colon and has sacculations in humans, while it is smooth in pigs [3]. As in the cecum, the colon is also provided with semilunar folds which project into the lumen of the gut and increase the initial surface area.

c. Rectum

The rectum, so called because it does not describe any convolutions, is smooth and caudal and becomes enlarged, forming the ampulla recti before ending at the short anal canal [4] [6]. Infact the rectum is a straight piece of gut which continues from the descending colon into the pelvic cavity.

d. Structure

The large intestine consists of four concentrically arranged layers proceeding from outside to the lumen: tunica serosa, tunica muscularis, tunica submucosa, tunica mucosa.

Tunica Serosa

The tunica serosa is very thin and it is derived from the peritoneum. The serosa does not cover the entire large intestine but finishes at the rectum. The rectum is covered and surronded by dense connective tissue [3].

Tunica Muscolaris

The muscular coat is composed of two layers, one circular and internal, and the other longitudinal and external. However the longitudinal layer in some places is considerably thickened and forms longitudinal bands called teniae in latin; in this part the elastic fibers are abundant (cecum and colon segments) [3] [5].

Tunica Submucosa

The submucosa in the large intestine is no different from that of the small intestine.

Tunica Mucosa

The mucosa lacks villi and the epithelium is reminiscent of the small intestine. It forms the Liberkuhn glands, which extend perpendicularly from the surface to the proximity of the *muscolaris* mucosae. These glands are more abundant and bigger than in the small intestine and are rich in mucous cells. These cells seem to be less in number in the cecum, but their number increases in proximity of the rectum [3].

THE GASTROINTESTINAL TRACT IN FISH

The gut is a tubular structure beginning at the mouth and ending at the anus (**Fig 5**). The digestive system of the fish is divided into 4 parts[7]:

- The head gut is generally divided into the oral (buccal) and gill (branchial, pharyngeal) cavities. As it is not part of the gastrointestinal tract it will not be treated here
- The foregut begins at edge of the gills and includes the esophagus, the stomach, and the pylorus.
- The midgut includes the intestine posterior to the pylorus,.and often includes a variable number of pyloric caecae (pyloric appendages) near the pylorus.
- The hindgut is marked by an increase in diameter of the gut and it ends is the anus.

Some species of fish, such as the cyprinus, lack both a stomach and pylorus, in this case the foregut consists of the esophagus and an intestine anterior to the opening of the bile duct.

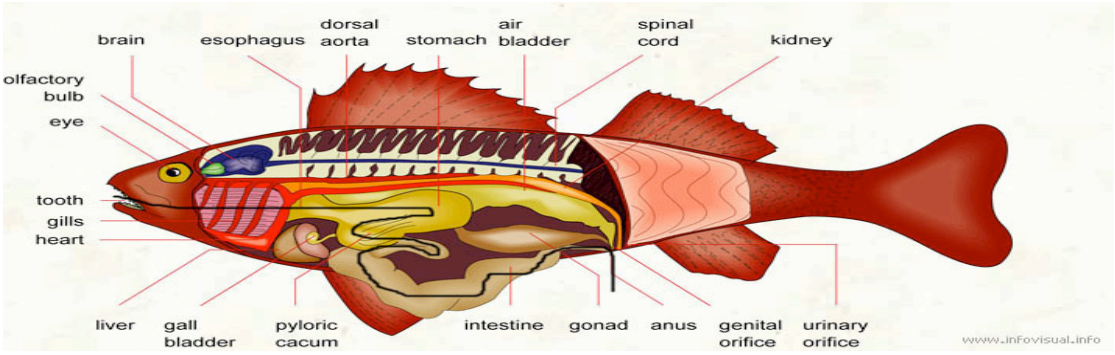

Fig 5. Overview of gastrointestinal system in the bone fish (black line is the the black line is the path of food from ingestion to expulsion) (http://www.infovisual.info/02/033_en.html).

I) THE HEAD GUT

As previously mentioned the head gut is not part of the gastrointestinal tract, but is a fundamental part of the digestive tract of the fish.

II) THE FOREGUT

The foregut includes the esophagus and the stomach. the anterior limit is given by the gills, while the caudal limit is given by the pylorus[8].

i) The esophagus

I will only briefly introduce the esophagus, because it is only the anterior limit of the stomach, thus it is not part of the gastrointestinal tract that begins with the stomach.
The esophagus is a large, short and straight tube, constituted by outside to the lumen of: tunica serosa, tunica muscolaris, tunica submucosa e tunica mucosa. There are many mucous cells that secerne mucus-like substances, which make the esophagus viscous [7].

ii) The stomach

The stomach of teleosts, when it is present, presents a variety of different shapes, and in any case represents the caudal part of the foregut [7].
The stomach can be straight, like a tube of uniform diameter with no marked anatomical differences between the esophagus and stomach as in the Northern pike (*Esox Lucius*), U-shaped or in the form of a round and muscular structure situated at the end of the esophagus and with a cardiac and pyloric region as in most teleosts, or Y-shaped with a blind sac of variable size and a cardiac and pyloric region as some teleosts and sharks [7] [8] (**Fig 6**).

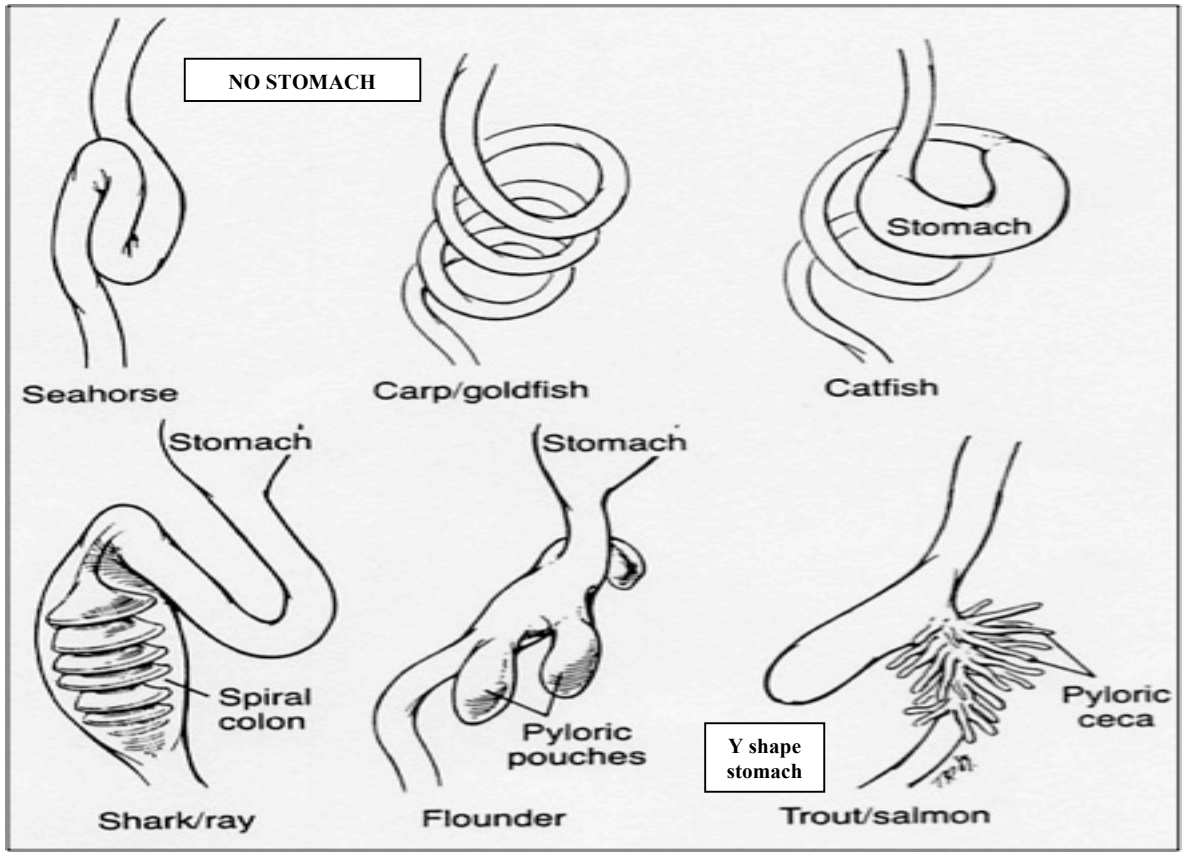

Fig 6. Different shapes of fish stomachs (Dicentrarchus labrax have a Y-shaped stomach) (http://diversityofanimalsystem.wikispaces.com/digestive+system)

The stomach is absent in different fishes, when present it has numerous gastric pits (like crypts) immersed in the mucosa and at the bottom there is the opening of the gastric glands [8]. The epithelium of the stomach and of the lining of the crypts consists of a single layer of high columnar prismatic cells; these cells in the apical part are positive to PAS reaction and so they secrete a protective mucus [8]. There are two different types of glands in the mucosa of the stomach: fundic and pyloric; the fundic glands cover most of the mucosa of the body of the stomach, while the pyloric glands are only present in the pyloric part of the stomach [8]. The fundic branched tubular glands possess more than one type of cell, this gland cell (oxyntopeptidic cell) contains acidophilic granules and produces pepsin and hydrochloric acid. The pyloric glands are less closely associated than the fundic glands; they are shorter, less frequently branched tubules [8]. Their epithelium is similar to that of the stomach, the stroma of the mucosa of the stomach contains many lymphocytes and eosinophilic granular cells, the muscularis mucosae is present and consists almost entirely of smooth, longitudinally-disposed muscle cells [7] [8]. The submucosa contains

eosinophilic granulocytes and is rich in networks of nerves, arteries and veins, while the muscolar coat is formed by a circular, longitudinal and additional inner oblique layer [8].

III) THE MIDEGUT AND THE HINDGUT

The segment of the intestine that follows the stomach is called midgut, while the terminal segment of the intestine is the hindgut. Unlike mammals, in fish there is no distinction between the small intestine and large intestine. The intestines of fish are mostly a tubular structure that can vary in size depending on their eating habits; in fact, carnivorous species often have a shorter intestine compared to herbivorous fish [7] [8]. Some species of bony fishes have an intestine with a smooth surface, others have longitudinal folds or folds which form a rather complex pattern or network. Moreover, some fish, such as higher vertebrates, have villi as their intestinal wall lining, which can be different sizes and shapes depending on the species. A villus is a finger-like process of the mucosa which is composed of an epithelial covering and a core of connective tissue containing blood and lymph capillaries. Many species have a number of protrusions extending from the midgut close to the pylorus [7] [8]. These blind-ending structures are the pyloric cecae, they possess a multi folded intestinal epithelium and their role seems to be to increase the area for the absorptive process and the duration of food retention in the intestine. The intestinal epithelium can be of a simple or pseudostratified columnar type; it is composed of cells that possess a well marked striated border called microvilli and goblet cells, which are mucus secreting cells [8]. These cells have different functions, such as absorption and secretion. In some fishes ciliated cells have been described among the ordinary prismatic cells of the intestine [8]. In the intestine of fish are present some glands similar to liberkhum glands [8]. The lamina propria and submucosa of some species contain large numbers of eosinophilic granular cells and lymphoid tissue [8]. The eosinophilic granular cells are similar to mastcells; they contain antimicrobal peptides and their release can increase the vascular permeability and promote neutrophil adhesion (innate immunity and inflamation) [8]. The muscularis mucosa is composed of a thin layer of smooth muscle, and the submucosa is generally composed of a loose connective tissue with blood vessels. In most fishes, as in mammals, the muscular coat of the intestine is very developed to ensure peristaltic contraction [8]. The rectum is the terminal part of the fish intestine [7] [8].

THE SENSE OF TASTE

Taste is the ability to respond to dissolved molecules and ions called tastants. The chemical senses (taste and smell) are the most ancient of the sensory modalities in any living species. There is no doubt that the gustatory system is essential for nutrition and survival. In fact, the discrimination between nutrient and/or potentially harmful compounds has effect on animal and human behaviour and, therefore, on their major organic and biological functions [9].

It is difficult to imagine how the many taste sensations that we perceive can be only related to four and more recently five types of taste: sweet, bitter, salty, sour and umami. These five types of taste, however, can be mixed together to produce many shades and hues of flavour.

Taste at a molecular level is very similar to the other senses. Tastants are recognized and bound by taste receptor signaling to sensory neurons in order to convey the chemosensory information to the central nervous system (CNS). However the relationship between tastants and taste is not linear connection. It would be easy to think that the five main tastants can be recognized via respective receptors, one for each type of taste. In contrast, the complex web of information generated by various tastants results from a polymodal function of taste receptors, i.e. each receptor recognizes different stimuli. The same stimuli, can be evoked by many different tastants, and each taste modality may use more than one processing mechanism. Moreover each tastant can be recognized by taste receptors only if it reaches the right threshold. For example, some compounds such as sucrose and lactose, which elicit a sweet taste in humans, activate taste receptors only at high concentration [9], while bitter substances have a nanomolar concentration threshold.

TASTE BUDS

The perception of different gustatory stimuli originates from the interaction of the molecules present in oral fluid as saliva. Found primarily on the tongue's surface, taste cells are organized in specialized structures (specialized sensory cells) referred to as "taste buds" [10] (**Fig 7**).

In mammals, the taste buds are located on the tongue, epiglottis, pharynx and in the upper part of esophagus [11]. In fish the distribution of taste cells is on the whole body surface, lips, gills, skin and barbells as well as in the mouth, pharynx and esophagus [12] [13] [14]. The distribution of taste buds in fish reflect the eating habits, hunting strategy and the different fish habitats [14] [15] [16].

Taste buds are approximately 50µm in diameter and appear to be composed of 50-150 taste cells that detect sugar (sweet taste), aminoacids (umami taste), poisons (bitter taste), acids (sour taste) and minerals (salty taste). Taste receptor cells are long and spindle shaped, with microvilli at their tips.

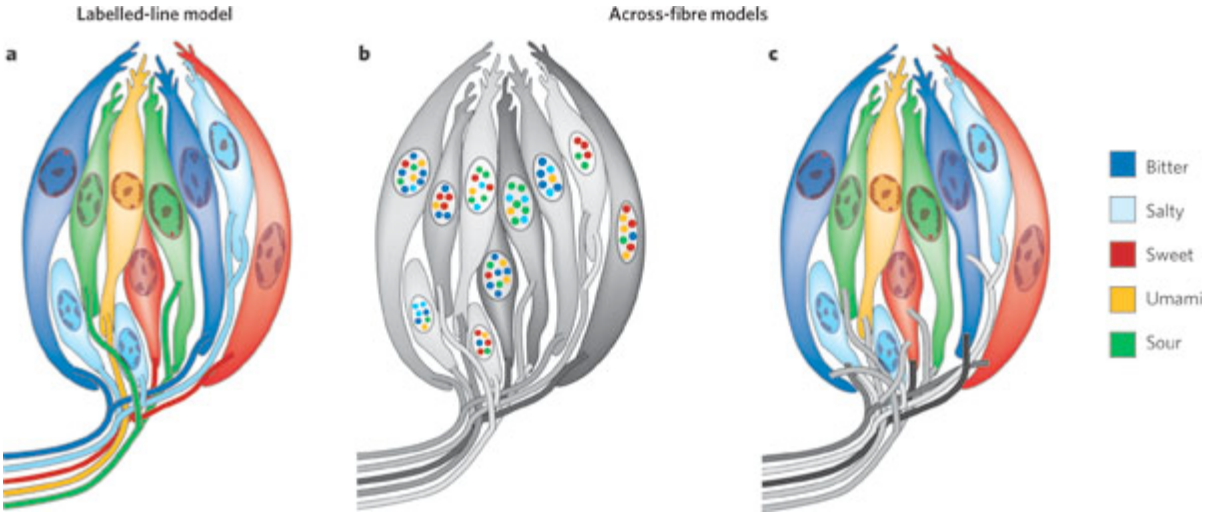

Fig 7. Taste bud organizations

The beginning of taste recognition occurs at the pore, an opening in taste buds where the microvilli of receptor cells contact the outside environment. Tastants penetrate into the pore and make contact with receptor molecules and channel within the microvillar membrane of the taste receptor cells [10][17]. Microscopic studies of taste buds, highlight four morphologically different cell types (**Fig 8**): light cells (type I cells), dark cells (type II cells), intermediate cells (type III cells) and basal cells [18].

The basal cells, small and rounded, are located at the base of taste buds and are considered to be stem cells, because it is believed that all of the other cell types derive from the basal cells. The lifespan of an individual taste cell is only from 10 days to 2 weeks, thus cells within the taste buds are continually being replaced [11] [19].

The light cells are the mature taste cells and their primary function is to support dark and intermediate cells [20], while the dark and intermediate cells are different stages of differentiations of immature taste cells [11]. Functionally all four cell types are referred to as taste cells, they are elongated cells and extend from the bottom of taste buds up to their taste pore.

The most studied cell are certainly the type II cell that expresses G-protein coupled receptors (GPCRs) for the detection of sweet, umami and bitter compounds [21]. Type III cells are thought to express sour taste receptors and detect acid taste [22]. This cell type also expresses the pan neuronal marker protein gene product 9.5 (PGP 9.5) and contains 5-hydroxytryptamine (5-HT) [23]. Type I cells express nucleoside triphosphate diphosphohydrolase-2 (NTDPase2) and the oxytocin receptor [24] [25].

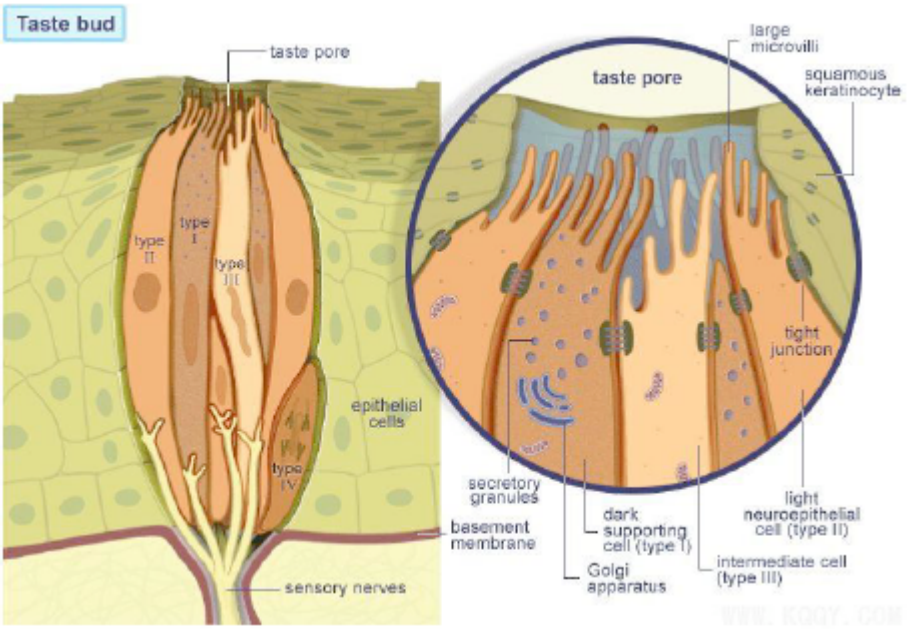

Fig 8. Cells types in taste bud: light cells (type I cells) are supporting cells, dark cells (type II cells) contain taste receptor, intermediate cells (type III cells) form synapses with afferent nerves, basal cells (type IV cells) are progenitor cells.

Mammalian taste buds are localized in structures called *papillae*, varying in number depending on the species considered. In humans, three types of papillae are present on the

tongue of domestic animals, each with morphological differences and with a different localization.

The fungiform *papillae* are numerous (in terms of hundreds) on the tip of the tongue of humans and pigs, in addition in pigs other fungiform *papillae* can be found on the anterior two-thirds of the tongue. This type of *papillae* has a simple structure generally containing a single taste bud. On the back of the tongue there are the circumvallate papillae, with a more complex structure, V-shape and many taste buds (in the pig there are only two *papillae* - one at each side of the back of the tongue, in humans they range from 8 to 12). Taste buds in circumvallate papillae line the side of the grooves with their pore facing the cleft [2] [3].

The foliate papillae are positioned on the lateral margin towards the posterior part of the tongue, they have a similar structure to the circumvallate papillae and also taste buds line the cleft of the papillae, their shape being like a leaf [2] [3]. These papillae are well developed and numerous in pigs [3].

Taste cells detect stimuli, but taste receptor cells have to convey taste information to the CNS. Nerve fibers representare the link between the taste receptor cells and the brain. Taste cells secrete neurotransmitters into the synapse, passing information from the taste receptor cells to neurons. The latter spike action potentials that signal to the brain [10] [26].

BITTER TASTE RECEPTORS

Bitter stimuli is perceived as dangerous and harmful and therefore the gustatory system induces an adverse reaction [27]. In fact the bitter stimuli has evolved as a central warning system against the ingestion of potentially toxic substances, including the alkaloid and other environmental toxins [28].

The recognition of these potentially dangerous signals by the gustatory system is associated with the development of T2R family in the oral cavity. This is known thanks to the discovery of T2Rs genes in several animal species. In the human genome about 25 T2Rs have been identified [29] [30], in birds for example only three genes, whilst in amphibians about 50 T2Rs genes. Generally in mammals the expression of T2Rs genes ranges between 15 and 36 [11] [29] [31] [32]. In general T2Rs belong to the guanine nucleotides, which are bound to the GPCRs superfamily, with a short NH_2 terminal segment and seven transmembrane α-helics, three extracellular loops, three cytoplasmatic loops and a COOH-terminal segment [10]. Specific G alpha subunits are common to all taste GPCRs called: gustducin and transducin (Gi/Go proteins) [33] (**Fig 9**).

The T2R family is composed of many receptors, only some of them, to date, are associated with a specific ligand (6% of total receptors), this is the case of phenylthiocarbamide (PTC) and denatonium benzoate (DB), which bind T2R138 (T2R38 in humans) and T2R108 (T2R4 in humans) respectively in mice.

Most T2Rs are generally known as “orphan receptors” (about 80% of total T2Rs receptors) because it is not known exactly which substance can be used as an agonist [34] [35]. It should be remembered that a single receptor can also bind different substances, this further complicates the task of researchers who seek to shed light into the great family of T2Rs.

A recent study[35] compared 25 hT2Rs with more than 100 natural and synthetic compounds, going on to establish a ranking of the most “broadly tuned” T2Rs. So today it is known that some T2Rs can respond to a wide range of bitter substances and others can have intermediate characteristics to recognize only a few bitter substances. In one of our studies, we treated the enteroendocrine STC1 culture cells with increasing doses of PTC and DB.

This was done to evaluate the activation of phosphorylation $MAPk_{p44/42}$, because PTC and DB bind T2R138 and T2R108 respectively, leading to a dose response activation of $MAPk_{p44/42}$, which confirms the involvement of these two substances with T2Rs.

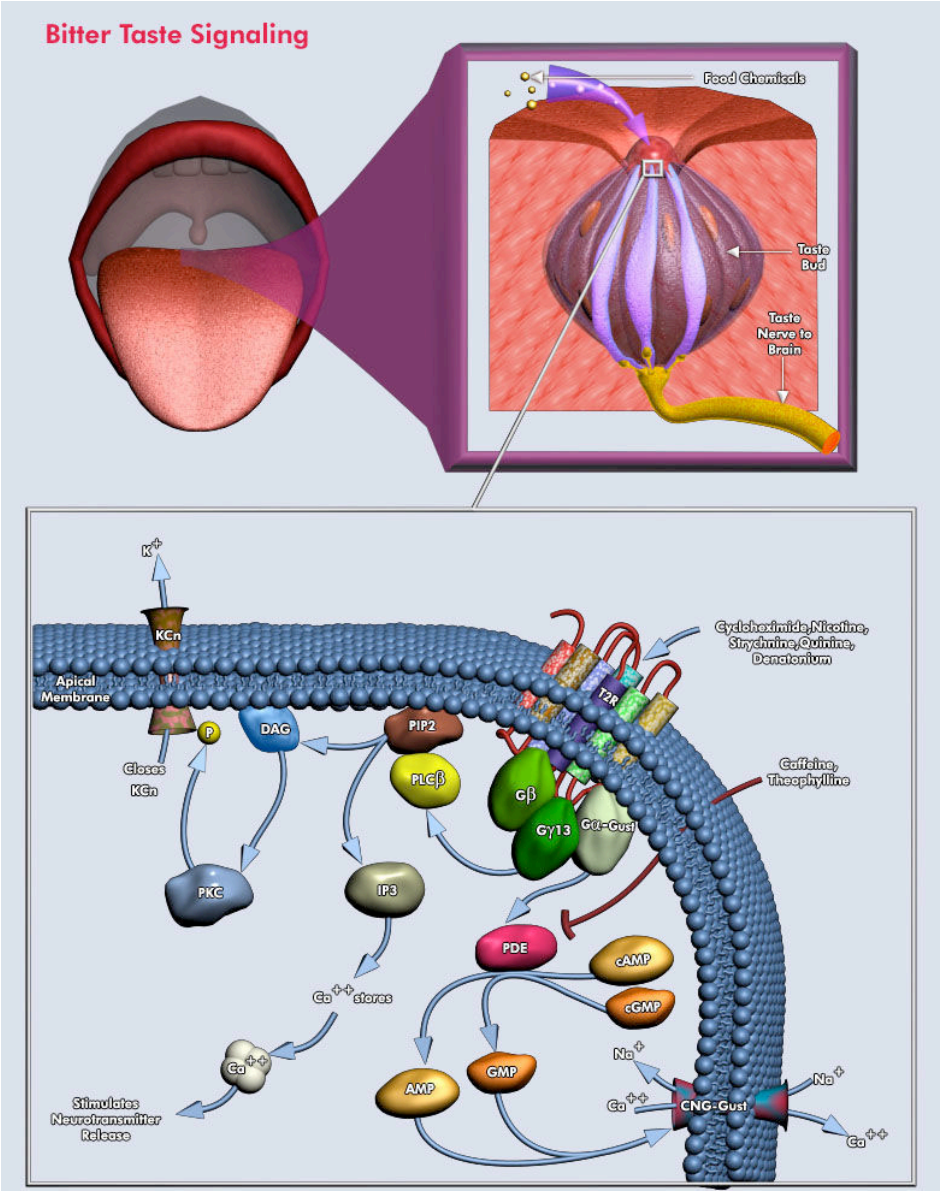

Fig 9. Bitter taste signalling pathways (http://www.qiagen.com/products/genes%20and%20pathways/Pathway%20Details.aspx?pwid=63).

α (gustducin and transducin)-SUBUNIT OF G PROTEIN COUPLED RECEPTOR SUPERFAMILY

Gustducin plays an important role in transducing bitter and sweet gustatory signals in the taste buds of the lingual epithelium. Outside of the oral cavity α-gustducin (an alpha subunit of GPCR) has been localized in the gastrointestinal tract of rats and mice [36] [37] [38] [39] and in the pancreas [40], suggesting a role for taste sensing mechanisms in the gastrointestinal tract [37] [41]. Alpha transducin was originally described in the photoreceptor cells of the retina, but it is now well established that this subunit of the G protein is present in the taste cells of the lingual epithelium and thus is implicated in taste signal transduction [42].

The presence of taste receptors in the oral cavity was confirmed through the use of immunohistochemistry, which identified the expression of α-gustducin (Gi) and α-transducin (Go) cells in the stomach and intestine mucosa of rats [43], mice [39] [44] [45], pigs [46] and man [47] [48].

In particular, both α-gustducin and α-transducin are stained in different subpopulations of enteroendocrine cells (98% of them). This has been established using immunohistochemestry to localize such as chromogranin A (an estabilished marker of endocrine cells in the GI tract), PYY, GLP-1, ghrelin, CCK, serotonin and somatostatin [46] [47] [48] [49] [50] [51].

The presence of the alpha subunit of G protein coupled receptors has been found not only in the oral cavity and gastrointestinal tract but also in the respiratory system [52]. In these systems, the alpha subunit of G protein coupled receptors has been localized in specific cells called brush cells distributed to the pancreas, stomach and intestine [53]. As described by Rozengurt and Sternini, these cells are morphologically different compared to the endocrine cells which have a “bottleneck” or “pear” shape or have an elongated pyramidal shape and the ability to secrete CCK, PYY, GLP-1 upon activation induced by taste stimuli [54] [55] [56].

Brush cells have an elongated soma with a basolateral rootlet and an apical tuft of microvilli that extends into the lumen [57], they do not contain granules and secrete neurotransmitters.

Using immunohistochemistry, α-gustducin [39] [43] and α-transducin [46] are expressed throughout the epithelium surface in the brush cells in the rat and pig gut.

In conclusion, much remains to be understood about the distribution and function of the alpha subunit of the G protein coupled receptors involved in taste perception. The α-gustducin seems to be more involved in the perception of the bitter and sweet stimuli, whilst α-transducin seems to have a secondary for scientific community. However, the study of Clavenzani *et al* [46] and the most complete study of Mazzoni *et al* [58]showed that α-transducin is localized throughout the gastrointestinal tract of pigs with exception of the esophagus. In that study, the α-transducin co-localizes with several neurotransmitters and fasting and refeeding evoke a modification in the expression of this protein in the entire gastrointestinal tract. This evidence highlights the role of α-transducin and α-gustducin in taste transduction [58].

CELLULAR SIGNAL OF TASTE TRANSDUCTION

Food intake causes a series of processes that lead the gustatory system and therefore our body to distinguish between different molecules, nutrients but also potentially hazardous or toxic substances.

In fact taste cells are able to discriminate between various substances such as ions and complex compounds as sweet and bitter. Depending on their chemical nature, the ingested substances are reception and transduction with different kinds of processes (**Fig 10**). It is also true that different molecules may also be perceived as one [11].

Once received and recognized, taste stimuli are transduced with different cellular mechanisms (e.g., membrane potential or change in the concentration of free Ca^{2+}), which lead to the release of neurotransmitters carryng information to CNS.

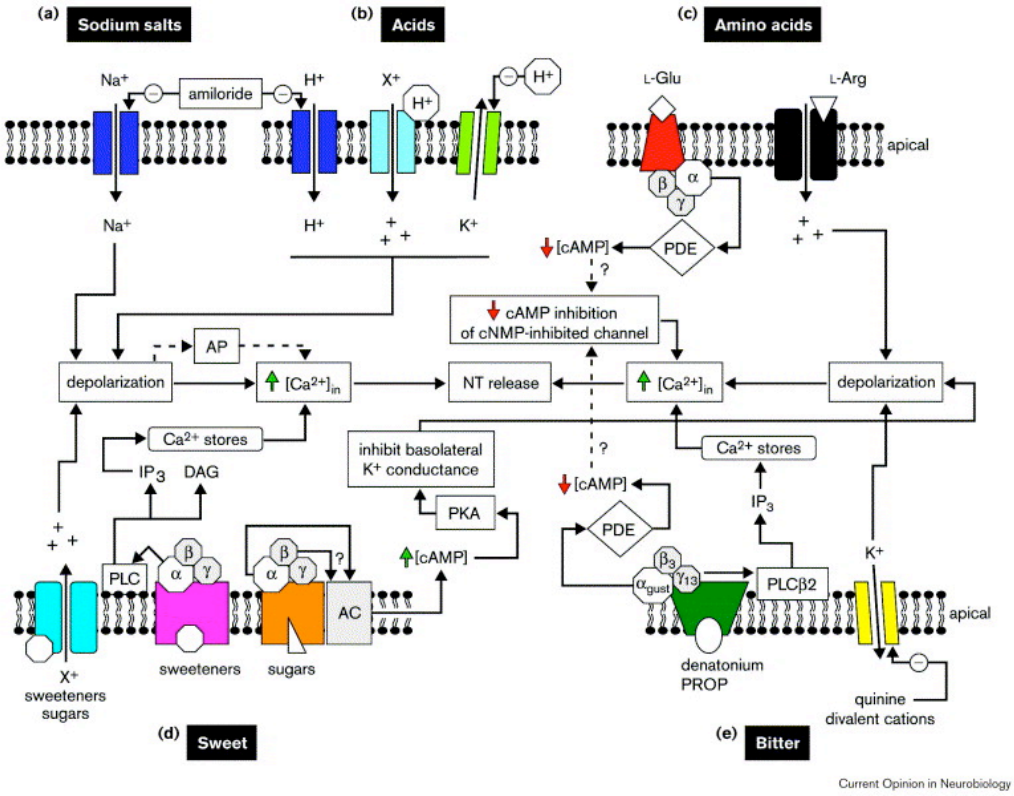

Fig 10. General transduction mechanisms in taste (publishing as Cummings B. 2006, in Cur Opin in Neurobiol)

I) BITTER

The bitter taste is often associated with toxic or harmful substances, in fact for an organism sensitivity to bitter stimuli is a protective mechanism for poison avoidance [59]. There is a

wide range of compounds that are very different chemically, but that induce the sensation of bitter.

It is well known that different substances such as caffeine, nicotine, strychnine, drugs and plants alkaloid evoke the taste of bitter [10]. For this reason the perception of bitter taste is of fundamental importance for the survival of the animal kingdom.

Bitter compounds are divided in lipophilic (kinin), which penetrate the membrane directly, hydrophilic (DB), which instead must use a mechanism as a receptor to enter in the cell. This suggests how there can be more than one intracellular signal pathway involved in bitter transduction (**fig 11**). The transduction of bitter stimuli is primarily mediated by the T2Rs, a G protein coupled receptor superfamily (a family of about 30 receptors). To date, we know two mechanisms of signal transduction for bitter taste: the activation of cell surface receptors and the following activation cascade of secondary messengers which involves phospholipase C that in turn activates the inositol tri-phosphate (IP_3), which is well known to stimulate the release of calcium ions from intracellular stores [17] [60]. The Ca^{2+} thus liberated causes the hyperpolarization of the cell via K^+ channel, but we have to remember that Ca^{2+} can also directly activate the release of neurotransmitters. The second way is identical to the first until the activation of G protein, which involves the activation of phosphodiesterase, which reduces the intracellular levels of cAMP or cGMP (cyclic nucleotides) [10] [17]. The decrease of the cAMP level activates protein kinase A, which regulates the passage of Ca^{2+} through the ion channel.

The α-gustducin subunit is definitely involved in the bitter signal transduction. This is confirmed by several in vitro and in vivo studies on laboratory animals. One study in particular confirmed the α-gustducin as an important mediator of bitter stimuli. In this study the author used KO mice for α-gustducin gene, in which the responce of Ca^{2+} to bitter compounds was measured through calcium imaging technique [61]. The result shows how the bitter stimuli are transduced mainly by α-gustducin, because in KO mice for α-gustducin gene, the response to bitter compounds was low but not zero. This evidence is very important because this means that there is a different subunit of GPCR to transduce the same bitter stimuli, maybe the α-transducin.

As proof of this, the Clavenzani et al. study on the pig GI tract shows how the same enteroendocrine cells can co-express both α-gustducin and α-transducin [46].

It should be remembered that the mechanism which implicates GPCRs is valid only for hydrophilic molecules, because lyphophilic molecules use the ions channels directly (eg. K^+ channel) to penetrate into the cell.

II) SWEET

The transduction of sweet stimuli is similar to bitter stimuli. In fact, when sweet substances bind the taste receptor the stimulatory G protein is activated inside the taste cell. The activation of the taste receptors by sweet substances such as sugar, saccharin, aspartame, and alcohol, causes a depolarization of the taste cell due to the action of cyclic nucleotides cAMP and cGMP [62] (**Fig 11**).

Avenet *et al* showed that the addition of cAMP in the taste cells of the frog, causes the activation of protein kinase, an enzyme that induces the closure of K^+ channels [63] on basolateral side of the plasma membrane blocks this in turn, the exit of the K^+ ions, causing the depolarization of taste cells and the release of neurotransmitters [19] [62].

The T1R receptors are responsible for transducing the sweet stimuli, this is a small family of receptors (T1R1, T1R2, T1R3) linked to G-protein that perform their function alone or in combinations (eg. T1R1 and T1R3 for umami taste) [64]. Likewise bitter, even the sweet taste is composed of very heterogeneous substances, in fact the gustatory signal transduction requires many processes and it seems unlikely that a single receptor can incorporate all sweet stimuli.

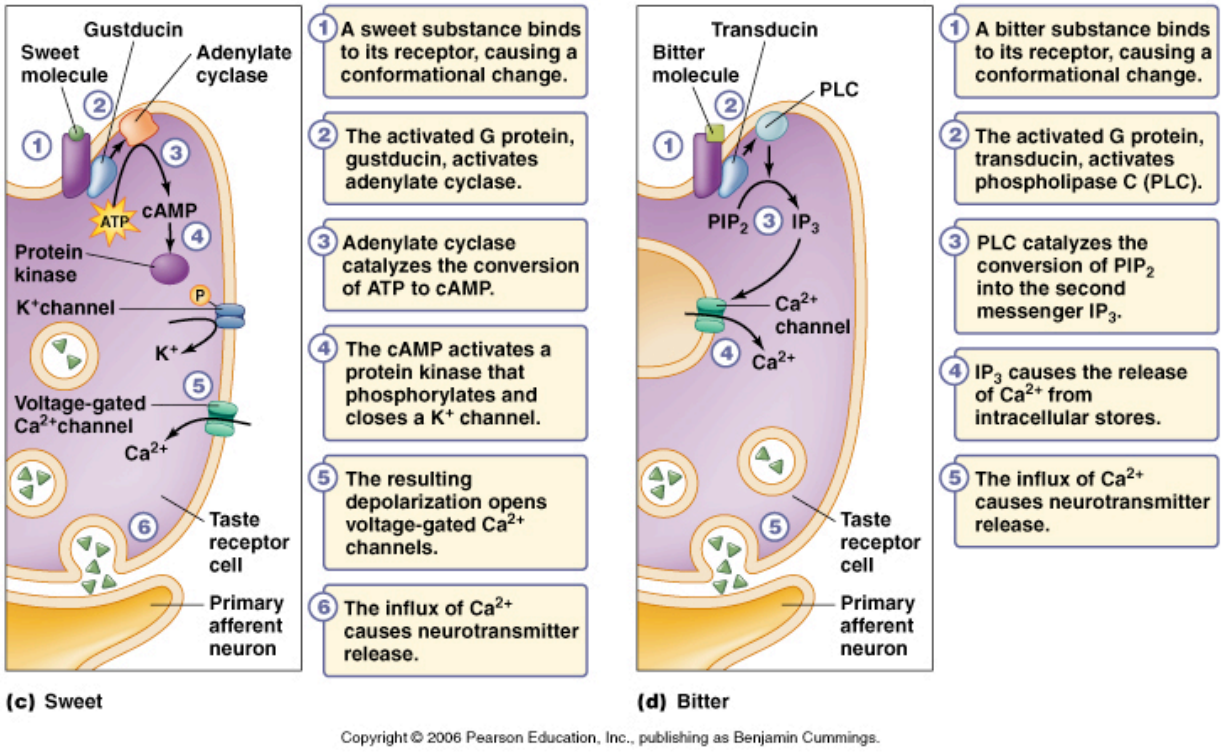

Fig 11. Sweet and bitter taste transduction

III) SALT

Salt stimuli are transduced by simple ion channels. Sodium is the most popular salty substance, it represents 90% of the inorganic ions in the extracellular fluid. About 30 yars ago, it was thought that taste cells were impermeable to ions, but different studies showed that Na^+ could be transported across the tongue ephitelial membranes in dogs [65]. In addition, it was shown that the drug amiloride inhibits the passage of Na^+ from outside to inside the taste cells, leading to a decreased perception of salt stimuli. This occurs because the amiloride blocks the Na^+ channel, as demonstrated in humans and rats [66]. The salty taste is transduced through ionotrophic mechanisms and mediated by a particular receptor, an ion channel for the amiloride-sensitive sodium, known as ENaC [10] [17]. The entry of Na^+ into the cells, depolarizes cells, leading to the release of neurotransmitters and, as a result, nerves convey sensory information to the brain (**fig 12**).

IV) SOUR

Sour stimuli are perceived when in the oral cavity there are compounds that increase the H^+ ions. Indeed sour tasting acids and substances may be inorganic, such as hydrochloric acid, or organic, such as lactic acid, both evoking H^+ release. The signal transduction occurs through the modulation of the potassium channel. The increase of H^+ block K^+ channels and this effect prevents the release of K^+ from the cells and depolarizes the cell membrane, thereby leading to neurotransmitters release [67] (**fig 12**). Other mechanisms may operate in sour stimuli transduction, such as the activation of Na^+ channel due to elevated H^+ concentrations [68].

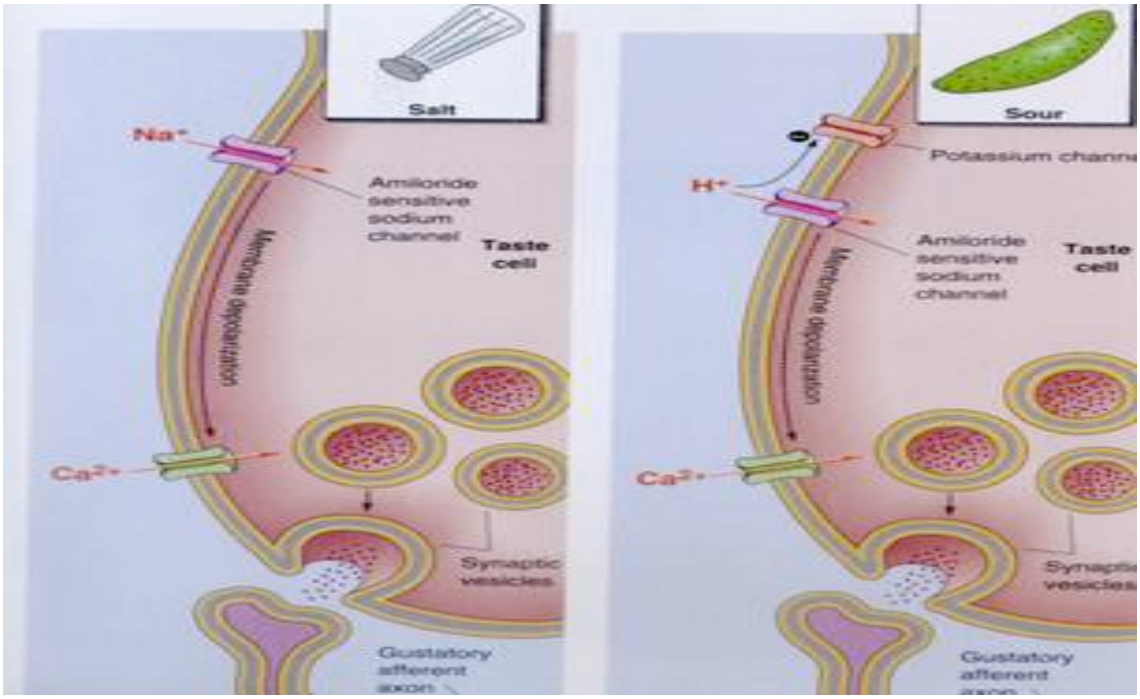

Fig 12. Salt and Sour taste transduction (Callmethedoctor.co.uk)

V) UMAMI

Umami is a recently discovered taste. It is given from substances such as L-glutamate, and 5'-ribonucleotides, including guanosine monophosphate (GMP) and inosine monophosphate (IMP) [69]. This taste was first discovered in 1908 by K. Ikeda, who coined the term "umami". Most dietary proteins contain high amounts of glutamate (e.g. protein of meat, poultry, seafood and vegetables).

Transduction for umami taste is still unclear. Biochemical studies showed that taste receptors are responsible for the sense of umami, and some receptors are taken into consideration for umami transductions, e.g. T1R1-T1R3 dimer and some modified glutamate receptors such as mGLuR4, mGLuR1. All of these receptors are found in the taste buds on the tongue [70] [71] [72]. However, to date, the specific role of these receptors in taste buds remains unclear.

TASTE RECEPTORS IN THE GASTROINTESTINAL TRACT

The digestive system is of fundamental importance in taste perception. In fact, it is through the oral cavity first and the gastrointestinal tract after that our organism is able to perceive different nutrients. Nutrient sensing influences digestive process i.e. secretory activity of the glands, absorption, bloody supply and motility of the gastrointestinal tract (GI) [73].

Tasting of various nutrients allows the body to respond to a large array of signals originated in the lumen which include nutrients, non-nutrient chemicals, mechanical factors and microorganisms. Moreover, the ability to perceive taste is important for the defensive behaviour of the GI tract generated by many harmful substances such as toxins or plants alkaloids [59]. Generally, potentially harmful substances are associated with bitter taste and induce nausea and vomiting or by impairing gastric emptying, resulting in the delayed delivery of toxins to the gut, whilst the nutrient compounds are associated with sweet taste and might stimulate saliva as well as gastric and pancreatic secretion to prepare the GI tract for digestion and absorption [45]. It is well established that the taste receptors are localized in the taste buds of lingual epithelium [32] [34] [74] as well as in the gastrointestinal tract [37] [45] in mammals and fish. In fact, recent studies performed on the mouse and rat have demonstrated that there is no difference between the taste receptors present in taste buds and those present in the epithelium of the gastrointestinal tract.

Both taste receptors belonging to T2Rs (about 30 subtypes) and a small family of T1Rs (three subtypes) have been found in the GI tract [45] [75] [76] [77]. It has also been shown that these receptors may participate in glucose homeostasis [55] [78]; in fact the oral administration of glucose can activate T1Rs in L-type endocrine cells, resulting in the release of mediators such as GLP-1 and GIP [79].

Mazzoni et al [58] showed that the α-transducin is localized in cells of the stomach, mainly in the pylorus, and in all segments of the intestine, with an increase of α-transducin cells in the pig large intestine. Other authors have shown that the α-gustducin was present in the rat, mouse and human intestne [39] [43] [44] [47] [48]. In fish (*dicentrarcus labrax*), we have shown α-transducin and α-gustducin immunoreacting in the stomach, mainly in the pyloric region and in the remaining segments of the intestine these cells were scarce.

In conclusion, taste receptors and their effector proteins, α-transducin and α-gustducin, are present throughout the digestive system from the oral cavity to the proximal part of the

colon, with the exception of the esophagus. The mechanisms of signal transduction used depend on the tastants, the downstream of secondary messengers and the following increase of intracellular calcium for the depolarization of membranes. The cell membrane depolarization elicits hormone release from enteroendocrine cells. The interplay with the nervous pathways is associated with the activation of biological responses affecting gut physiology.

GUT CHEMOSENSING AND ENTEROENDOCRINE SYSTEM

The gut responds to a wide range of substances in the lumen; these substances include nutrients, non-nutrient chemicals, mechanical factors and microorganisms [80]. Chemosensing of nutrients at mucosal level affects a variety of gastrointestinal functions, crucial for digestion and absorption. Chemosensing can also be used for detecting harmful/toxic substances, and this elicits adversive responses such as vomiting and food adverse behavior mediated by hormonal and neuronal pathways [81] (**Fig 13**). Probably the first level of the integration of information from the gut to the lumen is due to Bayliss and Starling, were the first to demostrate that the gut mucosa exhibits luminal chemosensitivity. Indeed they showed that a bioactive substance, then called secretin, was released from the gastrointestinal mucosa exposed to luminal acid [82]. There are specialized cells that respond to luminal signals, and based on these they control the digestion and the immune mechanisms.

Enteroendocrine cells are the cells which are predisposed to perceive and transduce luminal signals, releasing other molecules such as hormones or paracrine factors. The enteroendocrine cells represent only 1% of the total cells present in the gut epithelium; they are distributed along all intestinal segments and are capable of perceiving luminal signals. As a result, these cells release a series of signal molecules that activate nerve fibers or other local or distant targets [83] [84] [85].

The enteroendocrine cells represent the largest endocrine organ of the human body, and secrete a large variety of hormones or signal molecules derived from different genes, which are expressed in multiple forms [86] [87]. Enteroendocrine cells release various hormones which have different actions (see chapter “food intake mechanisms”); some of these hormones include: gastrin from enteroendocrine G cells, somatostatin from D cells, cholecystokinin from I cells, ghrelin from X/A-like cells, serotonin from enterochromaffin cells, glucagone like peptides and peptide YY from L cells, and glucose-dependent insulinotropic-peptide from K cells [88] [89]. Depending on their position in the mucosa and their morphology, enteroendocrine cells are classified as “open cells” and “closed cells”. The “open cells” have microvilli facing towards the intestinal lumen, and this characteristic means that the “open cells” are able to perceive the luminal contents and release secretory product. Moreover, “open cells” accumulate this secretory product inside

the granules in the cytoplasm and they open these granules and release the secretory product upon stimulation by exocytosis at the basolateral membrane in the intestinal space, where they can act locally or reach distant targets through the bloodstream [83] [84] [86] [88] [90].

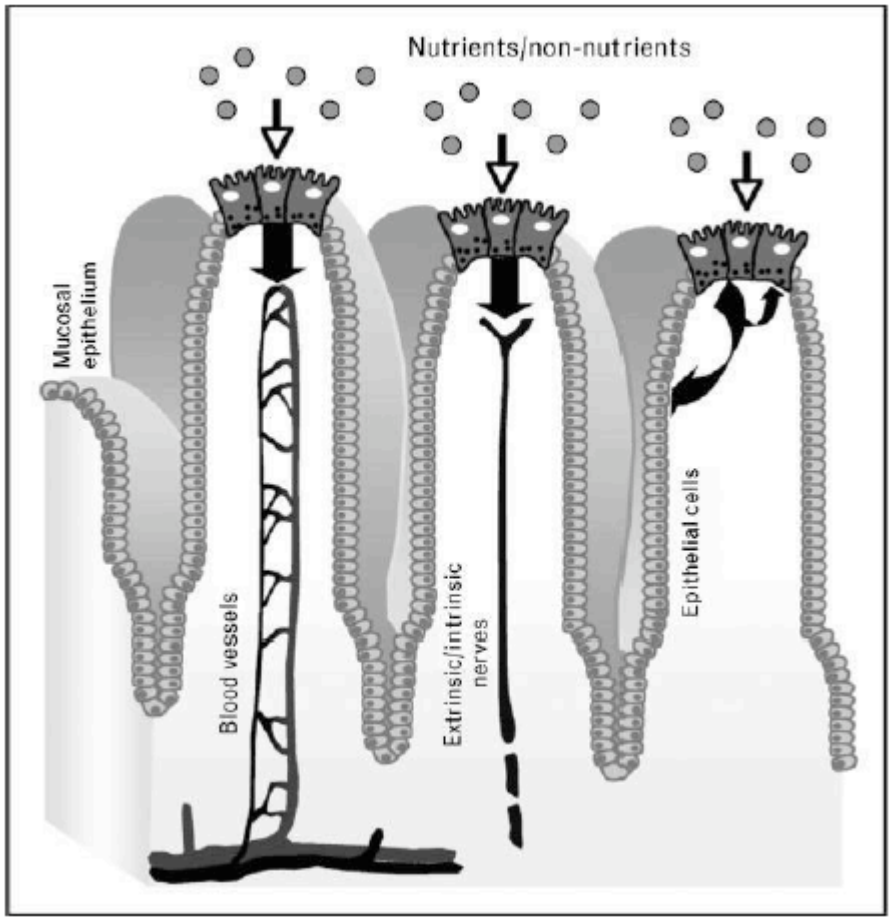

Fig 13. Possible mechanisms involved in GI chemosensing (Sternini *et al* 2006, Cur Opin Endocrinol Diabetes Obes)

This is the case, of G cells localized in the gastric antrum and pylorus, which release gastrin upon stimulation by luminal amino acids and calcium [91]; L cells of the duodenum and jejunum, produce CCK in response to saturated fats, long chain fatty acids, amino acids and small peptides from protein digestion [84] [92]. Also X/A-like cells produce Ghrelin, which is the only peptide that increases food intake [90]; or of the ileum, which produces GLP-1 or PYY from L cells in response to lipids or carbohydrates [49] [93]. GLP-1 and PYY hormones play a crucial role in the "ileal brake", which is an inhibitory feedback mechanism regulating the nutrient transit via gastrointestinal motility and secretion and reducing gastric emptying [93]. These three hormones, GLP-1, PYY and CCK, have been regarded as satiety signals [89], while PYY, GLP-1 and gastric inhibitory polypeptide

(GIP) are involved in energy homeostatis and their altered processing/ release may have a role in metabolic diseases, such as obesity and type 2 diabetes [94].

Other enteroendocrine cells include the “closed type” subgroup, which is located close to the basal membrane, although they never reach the surface and lack microvilli. Similing open, “closed cells” contain granules and under stimulation they release the contents of the granules, mainly hormonal substances.

An example of “closed cells” are the D cells of the gastric corpus that release somatostatin upon stimulation by intestinal hormones and neurotransmitters and so inhibit gastric acid secretion by acting on parietal cells and indirectly on the release of histamin [49]; enterochromaffin cells release 5-HT evoked by carbohydrates. 5-HT act in a paracrine manner on receptors located on nerve endings [95].

A second level of integration of information is represented by neurons of submucosal plexus and related nerves as well as extrinsic nerve endings [96]. These fibers do not reach the endoluminal side in fact, products secreted by enteroendocrine cells are released under stimulation by exocytosis in the basolateral membrane into the intestinal interstitium of the lamina propria, and act locally in a paracrine manner on primary afferent neurons and other cells, or through the bloodstream in an endocrine manner [97] [98]. Since virtually all gut peptides are subject to rapid liver extraction and breakdown by proteolitic enzymes, their highest concentration is found very close to the site of secretion [99] [100] [101] [102]. In this regard some gut peptides such as GLP-1 or PYY perform their anorectic effect by binding receptors near the site of secretion, or by binding receptors on vagal afferent fibers that innervate the lamina propria. The intraperitoneal administration of GLP-1 or PYY does not produce the anorectic effect if the mouse has undergone abdominal vagotomy or subdiaphragmatic vagal deafferentiation [103] [104]. Recently, researchers have shown that CCK is involved in the homeostasis of glucose. The authors propose that CCK is released in response to gut lipid sensing and binds to CCK-1 receptor on visceral vagal afferents; this carries signals to the brain through the liver and directly inhibits the production of glucose [105].

In this context, the discovery of the presence of TR in the gastrointestinal tract and their colocalization with neuropeptides, gives an important role to TR in endoluminal chemosensing in the GI tract [45] [106] [107] [108]. An example is given by Raybould *et al* [109], who showed that intragastric administration of DB (an agonist of bitter taste receptor T2R108 in mice) and PTC (an agonist of bitter taste receptor T2R138 in mice) activated the nucleus tractus solitarius (NTS), an affect that was prevented by

subdiaphragmatic vagotomy. These findings demonstrate that TRs are implicated in endoluminal chemosensing and their interplay with afferent nerves of vagal origin to transduce sensory information to the CNS [110] [111]

FOOD INTAKE MECHANISMS

It is well known that the coordination of biological activities between cells in living organisms involves several chemical messengers, mostly peptides. These polypeptides are synthesized and secreted by about 15 different enterendocrin cells, placed along the gastrointestinal tract and pancreas of mammals (gastro-entero-pancreatic complex; GEP) in response to chemical and nervous stimuli and communicate with their target cells via endocrine, autocrine and neurocrine secretion. [106] [112] [113]. Gastrointestinal regulatory peptides interact with specific receptors located on the cells surface and generate a signal which causes a series of intracellular events culminating in the synthesis and release of secondary chemical messengers that modify the secretion and motility of gastrointestinal structures, as well as the secretion of other hormones (table 1). This biological effect regulates a large number of functions, such as digestive behaviour, absorption, release of hormones, enzyme secretion, gut motility, satiety, appetite, elimination of toxins and energy homeostasis [113] [114]. Many gastrointestinal peptides are involved in delicate peripheral and central pathways that regulate appetite and satiety (**Fig 14**);

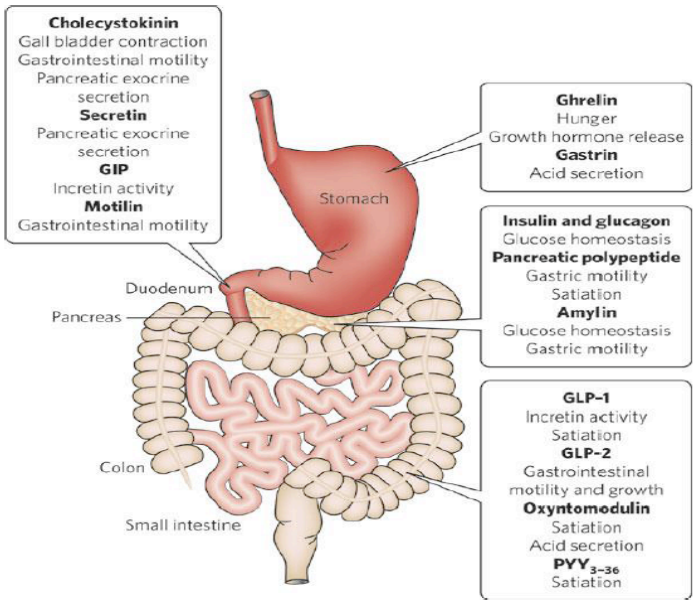

Fig 14: Different Gi hormones regulate food intake through bloodstream and vagal afferent.

the imbalance between food intake and energy consumption leads to a number of diseases such as obesity. The central regulation of body weight is linked to various peripheral signals coming from the digestive tract, pancreas and adipose tissue [115] [116]. Recent

studies in non-diabetic obese patients have highlighted the role of leptin but also other gastrointestinal peptides such as cholecystokinin (CCK), Glucagon Like Peptide-1 (GLP-1), ghrelin and peptide YY (PYY) in the control of food intake and body weight.

A change in the regulation of intestinal peptides can cause weight loss, but also an enormous sense of hunger that may persist even up to one year after the termination of that diet [117].

Peptide	Luminal Secretagogue	Cells of Origin
Gastrin	-Esp. aromatic amino acids and amines	G cells
Somatostatin	Intragastric acid	D cells
Secretin	Intraduodenal acid	S cells
CCK	Fats, proteins	I cells
GIP	Carbohydrates, triglycerides	K cells
Motilin	Poss. duodenal alkaline	M cells
GLP-1, -2	Carbohydrates (incl. non-metabolized)	L cells
Pancreatic Polypeptide (PP)	Vagal, intraluminal amino acids, glucose, fat	PP cells
Peptide YY	Intraluminal fat, protein	L cells
Oxyntomodulin	Intraluminal fat	L cells
Neurotensin	Jejunal fat	N cells

Table 1: Gastrointestinal peptide, function and localization in endocrine cells in the GI tract

I) GASTRIN

Gastrin is a linear polipeptide to 17 amino acids produced by G cells of the pyloric antral part of the stomach and by G cells from the proximal duodenum. G cells are most abundant in the medium portion of the pyloric antrum; they are equipped with microvilli towards the luminal side and thanks to which they can capture substances contained in the gastric and duodenal lumen. Gastrin is stimulated by proteins and amino acids in the lumen of the stomach or by parietal distension that causes the release of this hormone into the

bloodstream (**fig 15**). Its major function is the physiological control of gastric digestion by the regulation of acid secretion; indeed Gastrin is an important regulator of postprandial acid secretion and gastric epithelial cell proliferation and parietal cell maturation [118]. In particular, Gastrin increases the abundance of histamine secreting enterochromaffin-like cells (ECL) and plays a role in the relaxation of the ileo-cecal valve [119].

Gastrin is involved in pancreatic secretion and gallbladder emptying, and aids gastric motility [120].

Recent studies have revealed that the PTC increases the expression of the ABCB1 (ATP-Binding cassette B1) in STC1 cells through the CCK and gastrin signaling mechanism [121], suggesting that the stimulation of taste receptors and in particular of T2Rs in the gastrointestinal tract leads to the release of these incretin hormones. This evidence may indicate that the cells in the stomach that produce gastrin respond to PTC and T2Rs.

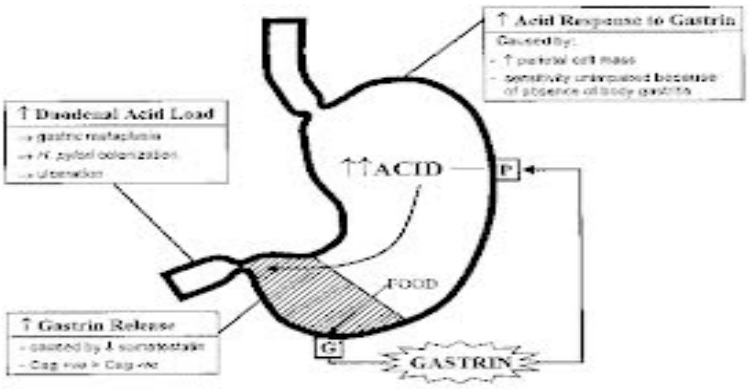

Fig 15. Biological action of Gastrin hormone (www.sciecedirect.com).

II) SOMATOSTATIN

Somatostatin was first isolated from sheep hypothalamus for its capacity to inhibit the secretion of growth hormone [122]. Subsequently, cells containing somatostatin (D cells) have been found and widely distributed in the gut mucosa and in pancreatic islets [123]. They are particularly abundant in the corpus and antral region of the stomach. Somatostatin is also widely distributed in the central and peripheral nervous system, located in the soma of neurons. Somatostatin has two active forms produced by the alternative cleavage of a single preproprotein: one with 14 (SOM-14), the other with 28 (SOM-28) amino acid residues [124].

Somatostatin released from enteroendocrine and pancreatic endocrine cells acts locally as a paracrine factor [125]. It acts on multiple receptors, there are five different somatostatin

receptors, SSTR-1 to SSTR-5, all belonging to the G protein coupled receptor superfamily [126]. In the gastrointestinal system the major somatostatin receptors are SSTR-2 and SSTR-5.

Somatostatin has a wide spectrum of biological actions in different parts of the body [126]. Its main role when secreted by hypothalamus is undoubtedly the inhibition of the secretion of pituitary hormones such as the growth hormone prolactin, and thyroid-stimulating hormone (TSH) [126] [127]. Furthermore, somatostatin inhibits the release of different hormones from all regions of the gut (Gastrin, CCK, Secretin, Motilin, Vasoactive intestinal peptide VIP, GIP), decreases the rate of gastric emptying, absorption, proliferation and reduces smooth muscle contraction and blood flow within the intestine [128].

III) CHOLECYSTOKININ

In 1906 the British physiologist Joy Simcha Cohen first described CCK as being found in the central nervous system as well as in the gut.

CCK is a linear polypeptide of 33 amino acids, it represents one of the principal endocrine regulators of digestion. We can describe CCK as a family of hormones identified by the number of amino acids (CCK58, CCK33, CCK8). CCK8 has a high structural homology with the gastrin receptor, more than 90%; in fact in our studies we used an antibody which recognized both CCK8 and gastrin. For this reason in the fast-refeeding project in the pig, we were unable to discriminate CCK8 and gastrin in the duodenum. In the brain CCK has the function of a neurotransmitter; in fact it acts as a peptide, it is released by neurons due to membrane depolarization. It is produced by various types of neurons.

In the gastrointestinal tract it is synthesized by I cells in the duodenal and jejunal mucosa and secreted in the duodenum. In enteroendocrin cells CCK has multiple biological functions, its first gastrointestinal role is the regulation of protein and fat digestion in the upper small intestine [129] (**fig16**), but in addition CCK causes the stimulation of gallbladder contraction and emptying, pancreatic enzyme secretion, intestinal motor activity and inhibition of gastric emptying. All of these functions lead CCK to send a signal to the brain, which results in satiety sensation and decrease of food intake. The ingestion of a meal moves the basal levels of CCK from ~ 1pM to 5-8 pM [130], as previously said, because the CCK is mainly induced by proteins and fat in the small intestine.

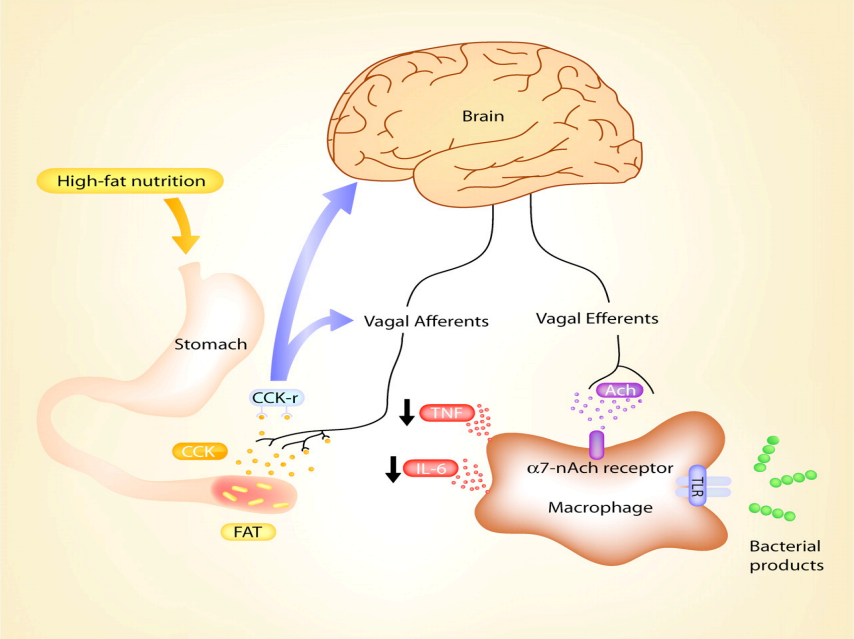

FIG 16. Nutritional stimulation of CCK (www.rupress.org).

Recent studies indicate that the stimulation of STC1 cells with PTC, an agonist for T2R138, leads to a dose-dependent increase of CCK, and this effect is mostly decreased in STC1 cells where expression is silenced by siRNA mT2R138 [131], however let us not forget that the release of CCk is increased by the stimulation of STC1 cells with denatonium benzoate, an agonist for T2R108 [54].

IV) GHRELIN

The word for the hormone ghrelin derives from the root *"ghre"*, which in Proto-Indo-European language means growth. This name is due to the ability of this peptide to stimulate the growth hormone GH. Ghrelin is a 28 –amino acid peptide, in which the serine-3 (Ser3) is n-octanoylated, this modification is essential for the activity of the hormone. It is mainly produced by X/A-like cells or G-cells [132] [133] in the pancreas. Ghrelin stimulates appetite, for this reason it is called the "Hunger Hormone", as it increases food intake and it is considered the counterpart of leptin, a hormone produced by adipose tissue that induces satiation, and its secretion increases before meals and decreases after food is eaten (**fig 17**). The growth hormone secretagogue receptor (GHSR) is a GPCR that binds ghrelin.

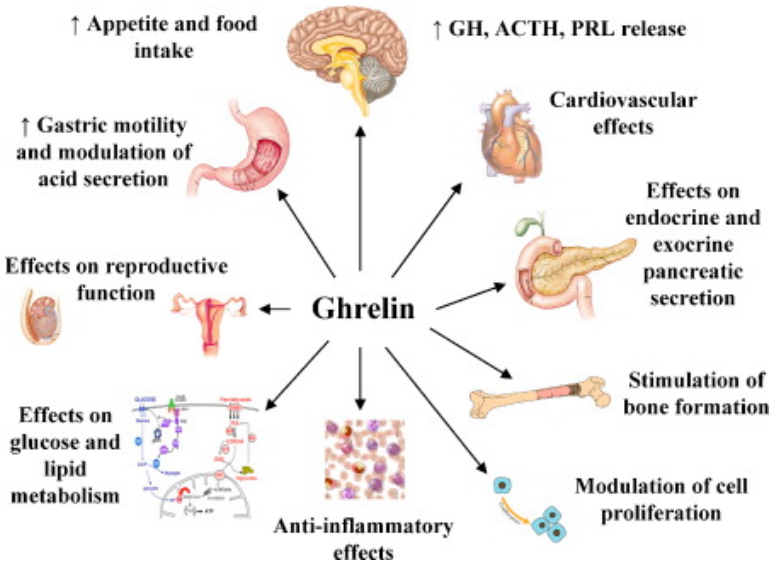

Fig 17. Biological action of ghrelin hormone.

In mammals, ghrelin homologs have been identified in humans [132], rhesus monkeys [134], rats [132], mice [135], dogs [136] and other species. The amino acid sequence of mammalian ghrelin is well conserved, especially the 10 amino acids in the NH2-terminal are identical. Therefore the NH2-terminal region is the core for the activity of peptides. Ghrelin, like many other peptide hormones, is generated by a precursor protein called pre-pro-ghrelin.

In the stomach it is more abundant in the cells of the fundus than in the pylorus [137] [138]. It is not only expressed in the stomach, but in many other sites such as the small intestine, lungs, brain, ovaries, adrenal glands, islets of langerhans and testis [139]. This wide distribution shows that ghrelin has many different actions on feeding behaviour, reproduction, sleep regulation, energy homeostasis, regulation of gastro-entero-pancreatic function and corticotrope secretion [140] [141].

Recent studies, however, demonstrate that ghrelin KO mice show normal growth, energy expenditure and food intake under normal conditions [142] [143]; this means that ghrelin plays primarily a facilitatory role in several endocrine functions. Plasma levels of ghrelin depend on several factors, mainly on the caloric content [144] and macronutrient composition of the meal [145]. Taste receptors and specifically α-Transducin, α-Gustducin and T2Rs might be involved in the mechanism of secretion of ghrelin; in fact recently it has been shown that gavage with T2R agonist increased the plasma level of octanoyl ghrelin in wild type (WT) mice and this effect is partially attenuated in Gust KO mice [51].

V) OBESTATIN

Obestatin is a peptide hormone, which is a relatively small protein, that is potentially produced in the cells lining the stomach and small intestine of several mammals including Humans [146].

It is encoded by the same gene that also encodes ghrelin; in fact, bioinformatic studies carried out by Zhang et al. in 2005 [147] have identified a 23-amino acid peptide which derived from the ghrelin peptide precursor. This was a very exciting new insight to the gut peptide field. The name that the author gave to this new peptide was obestatin, and it seems that obestatin has the ability to inhibit food intake in mice by intraperitoneal or intracerebroventricular injection. In addition, the authors described how the peripheral injection of obestatin inhibited jejunal contraction, suppression of gastric emptying and decreased body weight gain [148] [149] [150]. However, to date, the role of obestatin remains not elucidated; further studies are needed to understand the exact physiological function of this peptide.

VI) GLUCAGON LIKE PEPTIDE-1

GLP-1 is an incretin which is derived from the transcription product of the proglucagon gene. It is synthesized and secreted from endocrine L-cells in the intestine, mostly in the distal ileum and colon [151] [152] and from pancreatic alpha cells. There are two biologically active forms of GLP-1: GLP-1-(7-37) and GLP-1-(7-36)NH_2, these peptides result from the cleavage of the proglucagon molecule. GLP-1 secretion from ileal L-cells is lead on the presence of nutrients in the lumen of the small intestine and its release depends on the size of the meal [153].

Macronutrients, such as carbohydrates, proteins and lipids [154], are likely to stimulate L-cells to secrete GLP-1. Once in the bloodstream GLP-1 has a very short half-life, less than 2 minutes, due to the rapid degradation by the enzyme dipeptidyl peptidase-4 (DPP-IV). DPP-IV circulates in the plasma and is mostly located on the luminal surface of vascular endothelial cells [151] [152] [155].

GLP-1 have many biological functions such as the stimulation of insulin secretion, reduction of glucagon secretions, regulation of gut motility, gastric emptying, acid secretions and food intake (**Fig 18**).

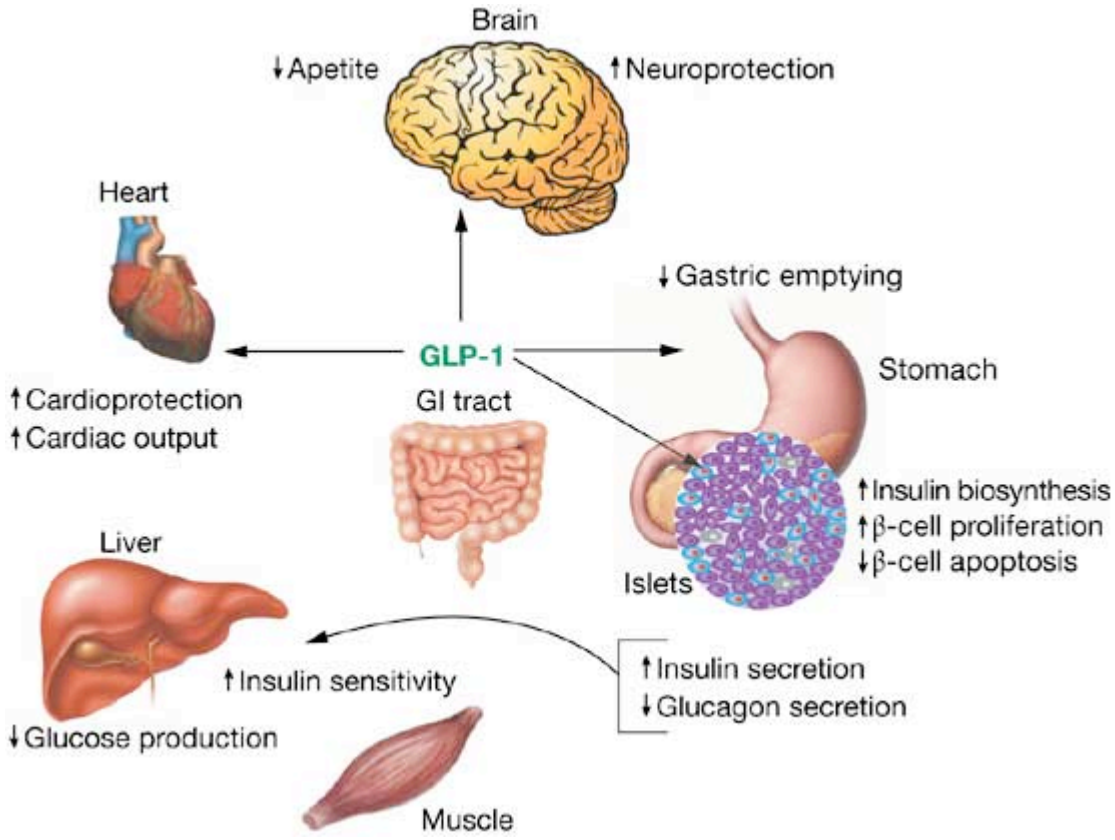

Fig 18: Biological action of GLP-1 .

GLP-1 is indicated as a major component of ileal brake mechanism in some of these biological functions. Ileal brake is a combination of effects which influence ingestive behaviour and GI functions; in particular, it is a mechanism where fat and generally the undigested nutrients at ileum level stimulate a negative feedback mechanism that leads to the secretion of hormones such as GLP-1 and PYY, which produce the inhibition of gastric emptying, intestinal motility, transit, pancreatic secretions and finally a reduction of food intake.

The secretion of GLP-1 is phase relating, depending on the macronutrient, GLP-1 is secreted in an early phase - within 15-30 min after meal onset and a late phase from 1 to 3 h postprandially [151] [152] [156] [157] [158].

The anorectic effect of GLP-1 is thought to be mediated by GLP-1 receptors (GLP-1R) [152] [159] [160].

GLP-1 is secreted in a taste receptor dependent manner by gut enteroendocrine L-cells in response to natural and artificial sweeteners [161] and, moreover, in mice Gustducin often colocalizes with L-cells producing GLP-1 [51].

VII) SEROTONIN

Serotonin is a mono-aminergic neurotransmitter, also called 5-hydroxytryptamine. Biochemically, serotonin derived from tryptophan is primarily found in the gastrointestinal tract and in the central nervous system of animals. In the human body 95% of serotonin distribution is in the enterochromaffin cells (EC) in the digestive system, the remaining 5% is synthesized in the serotoninergic neurons in the Central nervous system (CNS). It is well known that the Ecs are very common in the GI, and that cells play a crucial role in the regulation of secretion, motility and visceral pain.

Like several peptide hormones, serotonin is activated and released by a wide variety of stimuli [162], which lead to the regulation of peristalsis, gastric motility and postprandial pancreatic secretion [163] [164] [165] [166] [167] [168]. It has been proved that it is a signal molecule which participates in mucosal sensory transduction [169] [170]. In humans, but not exclusively, serotonin levels are influenced by diet, it is well knows that a diet with a high percentage of carbohydrates and a low percentage of proteins will increase serotonin through the secretion of insulin [171].

Literature shows that 27% of Gustuducin positive cells are co-localized with 5-HT [44], suggesting that the release of serotonin may be due to the activation of taste receptors by tastants present in the intestinal lumen.

CONCLUSION

Gastrointestinal chemosensitivity is essential for gastrointestinal function (e.g., digestion, absorption/secretion, motility) and as well as body homeostasis. In recent years the role of chemosensitivity has been further studied through the discovery of TR and related signaling molecules, i.e. α-gustducin and α-transducin, at gastrointestinal level. TR, α-gustducin and α-transducin play a crucial role in gut chemical sensing as they are able to perceive and distinguish many different substances such as nutrients/non-nutrients, harmful substances and toxins. Thus, because of TR, the gut represents a large chemosensory organ at the edge between the environment and the human body. Furthermore, TR have proved to be of fundamental importance in energy balance regulation and they could have a protective role in the control of caloric intake.

The general scope of my research projects performed in this PhD program was to better understand the role of TR in gastrointestinal chemosensitivity in different species. I have shown that the phylogenetic evolution of TR is almost unchanged in different species, including fish and humans. In our studies we showed that in pigs and in humans caloric intake is controlled and regulated by TR via release of gastrointestinal hormones and neuronal pathways (vagal afferents and the enteric nervous system). These findings have been confirmed by several studies on endocrine cell line (STC-1) which proved to be of strategic importance for the study of TR and in human colonic biopsy where the role of the T2R38 a bitter taste receptor could be involved in the detection of bacterial molecules (AHL) and on feeding alterations control.

Taken together, the results of these studies help to elucidate the impact of TR on gastrointestinal chemosensitivity and establish how these receptors participate in food behaviors and defense mechanisms against a variety of harmful toxins threatening the human body.

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CHAPTER 1

Enteroendocrine profile of α-transducin immunoreactive cells in the gastrointestinal tract of the European sea bass (*Dicentrarchus labrax)*

Rocco Latorre, Maurizio Mazzoni, Roberto De Giorgio, Claudia Vallorani, Alessio Bonaldo, Pier Paolo Gatta, Roberto Corinaldesi, Eugenio Ruggeri, Chiara Bernardini, Roberto Chiocchetti, Catia Sternini, Paolo Clavenzani

R. Latorre, M. Mazzoni, C. Vallorani, A. Bonaldo, P. Gatta, C. Bernardini, R. Chiocchetti, P. Clavenzani
Department of Veterinary Medical Science, University of Bologna, Italy, via Tolara di Sopra, 50 40064 - Ozzano dell'Emilia, Bologna,Italy

R. De Giorgio, R. Corinaldesi, E. Ruggeri
Department of Medical and Surgical Sciences, University of Bologna, Italy, St.Orsola-Malpighi Hospital, via Masserenti, 40138 - Bologna, Italy

C. Sternini (Corresponding author) CURE/DDRC, Division of Digestive Diseases, Departments Medicine and Neurobiology, David Geffen School of Medicine, UCLA, Los Angeles, and Veterans Administration Greater Los Angeles Health System, CA, USA
E-mail: csternin@ucla.edu

Abstract

In vertebrates, chemosensitivity of nutrients occurs through activation of taste receptors coupled with G protein subunits, including α-transducin ($G_{\alpha tran}$) and α-gustducin ($G_{\alpha gust}$). This study was aimed at characterizing the cells expressing $G_{\alpha tran}$-immunoreactivity throughout the mucosa of the sea bass gastrointestinal tract. $G_{\alpha tran}$ immunoreactive cells were mainly found in the stomach, and a lower number of immunopositive cells were detected in the intestine. Some $G_{\alpha tran}$ immunoreactive cells in the stomach contained $G_{\alpha gust}$. Gastric $G_{\alpha tran}$ cells co-expressed ghrelin, obestatin and 5-hydroxytryptamine immunoreactivity. In contrast, $G_{\alpha tran}$ cells did not contain somatostatin, gastrin/cholecystokinin, glucagon-like peptide-1, substance P, and calcitonin gene-related peptide immunoreactivity in any investigated segments of the sea bass gastrointestinal tract. Specificity of $G_{\alpha tran}$ and $G_{\alpha gust}$ antisera was determined by Western blot analysis, which identified two bands at the theoretical molecular weight of ~45 and ~40 kDa, respectively, in sea bass gut tissue as well as in positive tissue, and by immunoblocking with the respective peptide, which prevented immunostaining. The results of the present study provide a molecular and morphological basis for a role of taste related molecules in chemosensing in the sea bass gastrointestinal tract.

Keywords: chemosensory system; gut peptides; taste receptors; teleost.

Introduction

The gustatory system plays a dominant role in the detection of dietary nutrients, sodium content and the acidity of foods as well as sensing the presence of potentially harmful substances (Sternini 2007; Behrens and Meyerhof 2011). The sense of taste enables animals to adapt to specific habitats (Oike et al. 2007; Ishimaru 2009; Barreiro-Iglesias et al. 2010). In vertebrates (Chandrashekar et al. 2000; Nelson et al. 2001, 2002; Zhao et al. 2003; Behrens and Meyerhof 2011), including fish (Ishimaru et al. 2005), two families of taste receptors (TRs), T1R and T2R, which detect complex tastes, have been cloned. TRs are G-protein-coupled receptors activated by different stimuli, including sweet and bitter substances, amino acids and nucleotides, which elicit a cascade of intracellular signals (Behrens and Meyerhof 2011). In mammals, TRs are abundantly expressed in taste buds, and interact with specific G α-subunits, including α-gustducin ($G_{\alpha gust}$), which transmit gustatory signalling from the lingual epithelium to the sensory cortex in the brain (Ming et al. 1999; Margolskee 2002; Caicedo et al. 2003; Behrens and Meyerhof 2011). In addition to $G_{\alpha gust}$, several G protein subunits have been identified, which are associated with TR signaling, $G\alpha_{i-2}$, $G\alpha_{i-3}$, $G\alpha_{14}$, $G\alpha_{15}$, $G\alpha_{q}$, $G\alpha_{s}$, and α-transducin ($G_{\alpha tran}$) (Ruiz-Avila et al. 1995; Kusakabe et al. 1998). TRs and signalling molecules have been reported in the human and rodent gastrointestinal mucosa and pancreas (Höfer et al. 1996; Höfer and Drenckhahn 1998; Wu et al. 2002; Rozengurt et al. 2006). $G_{\alpha gust}$ and $G_{\alpha tran}$ immunoreactivities have been localised to epithelial, predominantly endocrine, cells of the stomach and intestine of rodents (Höfer et al. 1996; Wu et al. 2002; Hass et al. 2007; Sternini 2007; Sutherland et al. 2007), pigs (Clavenzani et al. 2009; Mazzoni et al. 2013) and humans (Rozengurt et al. 2006; Steinert et al. 2011), including ghrelin, somatostatin, cholecystokinin, glucagon-like peptide-1 and peptide YY positive cells (Rozengurt et al. 2006; Sutherland et al. 2007; Clavenzani et al. 2009; Fujita et al. 2009; Moran et al. 2010; Janssen et al. 2011; Steinert et al. 2011; Mazzoni et al. 2013). Endocrine cells, which are distributed throughout the gastrointestinal tract (GIT) mucosa and pancreas, control digestive functions and contribute to regulate caloric intake and metabolism (Holmgren 1985; Plisetskaya and Mommsen 1996; Palmer andGreenwood-Van Meerveld 2001; Nelson and Sheridan 2006). Since fish taste buds express similar receptors and downstream signalling molecules as mammals (Yasuoka and Abe 2009), the aim of this study was to test whether the TR gustatory signalling protein, $G_{\alpha tran}$ is expressed in the sea bass gut and characterize the types of $G_{\alpha tran}$ immunoreactive (-IR) cells.

Materials and Methods

Tissue preparation

Nine, non-sexed, 1 year-old European sea bass (*Dicentrarchus labrax*) were sampled from three tanks at the Laboratory of Aquaculture, Department of Veterinary Medical Science, University of Bologna, Cesenatico, Italy. The average weight and total length of the individuals were 234 ± 26 g and 26 ± 1 cm, respectively. Sea bass were sacrificed by anaesthetic overdose and segments of the GIT were harvested. The stomach and pyloric caeca were isolated from each fish and the intestine was divided into cranial, middle and caudal segments. Part of the collected tissues were frozen for Western blot assay. For immunohistochemistry, tissue samples fixed in 4% paraformaldehyde in phosphate buffer (0.1 M, pH 7.2), for 48 h at 4°C. The specimens were then dehydrated in a graded series of ethanol and embedded in paraffin. From each sample, sections of 5 μm thickness were obtained and mounted on poly-L-lysine coated slides, and then processed for immunohistochemistry.

Immunohistochemistry

Sections were processed for single and double labelling immunofluorescence. The following primary antisera (see details in Table 1) were used: $G_{\alpha tran}$, $G_{\alpha gust}$, ghrelin (GHR), 5-hydroxytryptamine (5-HT), obestatin (OB), somatostatin (SOM), gastrin/cholecystokinin (GAS/CCK), glucagon-like peptide-1 (GLP-1), calcitonin gene-related peptide (CGRP), and substance P (SP). Sections were deparaffinized, rehydrated and incubated in a humid chamber at room temperature with appropriate normal serum followed by the primary antibodies (2 days, at 4°C) and the appropriate secondary antibodies (1 hour at room temperature). For double labelling using antibodies raised in different species, sections were incubated with a mixture of primary antisera (e.g. $G_{\alpha tran}$ and SP or GAS/CCK) and immunoreactivities were visualized with secondary antibodies labelled with different fluorophores. Because the antibodies to $G_{\alpha gust}$, SOM, OB, 5-HT, GLP-1 and CGRP were produced in the same species as the $G_{\alpha tran}$ antiserum, we utilised the procedure and appropriate specificity controls previously described by Takechi et al. (2008) to visualize more than one antigen. Sections were examined using a Zeiss Axioplan microscope and the images were recorded with a Polaroid DMC digital photocamera (Polaroid, Cambridge, MA, USA).

Antibody specificity

Specificity of $G_{\alpha tran}$, $G_{\alpha gust}$, GAS/CCK, GHR, OB, and GLP-1 has been assessed by Western blot and/or immunoblocking with the corresponding peptide (see details in Table 2). Specificity of the 5-HT, CGRP and SOM antibodies was previously demonstrated in the sea bass by preadsorption test (De Girolamo et al. 1999; Visus et al., 1996). We did not perform specificity controls for the mouse monoclonal SP antibody, since the staining we obtained with this antibody was completely overlapping with the immunostaining reported by Pederzoli et al. (2004) in the sea bass with a rabbit SP antibody (Cambridge Research Biochemical, U.K.), the specificity of which was verified by immunoblocking. Specificity of the secondary antibodies was assessed by omitting the primary antibodies.

Western blot

Sea bass brain, eye and stomach and mouse brain were collected, frozen in liquid nitrogen, and stored at −80°C. Tissues were homogenized directly into a sodium dodecyl sulfate (SDS) lysis solution (Tris-HCl 62.5 mM, pH 6.8; SDS 2%, 5% glycerol) with 0.1 mM phenylmethylsulfonylfluoride. Protein content of cellular lysates was determined by a Protein Assay Kit (TP0300; Sigma-Aldrich, St. Louis, MO). For $G_{\alpha gust}$ and $G_{\alpha trans}$ antibodies specificity determination, aliquots containing 20 μg of proteins were separated on NuPage 4-12% bis-Tris Gel (Gibco-Invitrogen, Paisley, UK) for 50 minutes at 200V. Proteins were then electrophoretically transferred onto a nitrocellulose membrane.. After blocking treatment, the membranes were incubated at 4°C overnight with the respective antibodies in Tris-buffered saline-T20 (TBS-T20 20 mM Tris-HCl, pH 7.4, 500 mM NaCl, 0.1% T-20): anti-$G_{\alpha gust}$ rabbit polyclonal antibody (1:300); anti-$G_{\alpha trans}$ rabbit polyclonal antibody (1:500).

For GAS/CCK antibody specificity determination, aliquots containing 30 μg of proteins were separated on Novex 18% Tris-Glycine Gel (Gibco-Invitrogen, Paisley, UK) for 90 minutes at 125V. Proteins were then electrophoretically transferred onto a nitrocellulose membrane. After blocking treatment the membranes were incubated at 4°C overnight with anti-GAS/CCK mouse monoclonal antibody (1:1,000).

After several washings with PBS-T20, the membranes were incubated with the secondary biotin-conjugated antibody and then with a 1:1,000 dilution of an anti-biotin horseradish peroxidase linked antibody. The blots were developed using chemiluminescent substrate (Super Signal West Pico Chemiluminescent Substrate, Pierce Biotechnology, Rockford, IL) according to the manufacturer's instructions. The intensity of luminescent signal of the

resulting bands was acquired by Fluor-STM Multimager using the Quantity One Software (Bio-Rad Laboratories, Hercules, CA).

Results

Antibody Specificity

Western blot analysis showed a major band at ~45 kDa in extracts from the sea bass gastric mucosa, brain and eye with the $G_{\alpha tran}$ antibody (theoretical molecular weight in human) and a unique band at ~40 kDa in extracts from the sea bass stomach and brain and mouse brain (Fig. 1a, b) with the $G_{\alpha gust}$ antibody (theoretical molecular weight in human).

Different molecular forms of CCK have been described deriving from enzymatic cleavage of a precursor peptide of 115 aa (UNIPROT P06307) so the expected molecular weight of CCK is between 4 and 12 kDa. In our blot analysis, we identified a faint band near the theoretical molecular weight of ~15 kDa (Fig. 1c). We were unable to identify the smallest form probably because of the very low amount of each component present in the tissue.

Preadsorption of $G_{\alpha gust}$, $G_{\alpha trans}$, GLP-1, OB and GHR antisera prevented immunostaining with each antiserum (not shown) confirming tissue staining specificity.

Distribution of $G_{\alpha trans}$ cells in the sea bass gut

In the stomach, $G_{\alpha tran}$-IR cells were counted in 54 randomly selected high power microscopic fields (0.28 mm^2 each) for a total area of 15.1 mm^2. Since the intestinal mucosa differs morphologically from the stomach for the presence of folds, the number of $G_{\alpha tran}$-IR cells in the intestine were evaluated in 200 randomly selected folds for a more accurate representation of cell density in these regions of the GIT. Values were expressed as mean ± standard error mean (SEM). The GIT of the sea bass consists of a siphonal stomach, numerous pyloric caeca and a relatively short intestine. $G_{\alpha tran}$-IR cells were detected in the stomach and intestine, but not in the pyloric caeca. Intense immunolabelling was observed in the basal portion of the gastric gland and in the epithelial lining of the intestinal mucosal folds. $G_{\alpha tran}$-IR cells showed homogenously labelled cytoplasm, with an unlabelled nucleus and an elongated ("bottle-like") shape (Fig. 2a, e). These cells were characterised by two thin cytoplasmic prolongations, the first extending up to the endoluminal surface of the mucosa and the second projecting down to the basal lamina. These features indicate that these cells correspond to "open-type" enteroendocrine cells (EECs) (Höfer et al. 1999; Sternini 2008). $G_{\alpha tran}$-IR cells in the stomach (Fig. 2a, c, e, g)

were 15.7 ± 2.2, while there were 3-4 IR cells / 200 folds and 1-2 IR cells / 200 folds in the cranial and middle-caudal portions of the intestine, respectively. Furthermore, in the stomach, most $G_{\alpha tran}$-IR cells co-expressed 5-HT (Fig. 2a, b), OB (Fig. 2c, d) or GHR (Fig. 2e, f). Only a few $G_{\alpha tran}$-IR cells colocalized with 5-HT in the intestine. The colocalization between $G_{\alpha tran}$- and $G_{\alpha gust}$-IRs was observed in some cells in the gastric mucosa (Fig. 2g, h). In contrast, none of the $G_{\alpha tran}$-IR cells contained SOM (Fig. 3g, h), GAS/CCK, CGRP, SP, and GLP-1-IR in the stomach and intestine. The SOM, SP, CGRP, GAS/CCK, (Fig. 3a, b, c, d) and GLP-1 labelled cells were observed intermingled with unlabelled epithelial cells in the GIT mucosa. In addition, CGRP (Fig. 3c, e), GAS/CCK (Fig. 3f), SOM and SP antibodies labelled nerve fibers running either singly or in small fascicles in the submucosal and muscular layers, with some GAS/CCK and CGRP positive neuronal cell bodies detected only in the muscular layer (Fig. 3e, f).

Discussion

Our data provide evidence for the presence of $G_{\alpha tran}$ immunolabelled cells in the sea bass GIT and their EEC nature as indicated by the co-expression with GHR, 5-HT or OB, which are markers of distinct subpopulations of EECs. Taste transduction in vertebrates is mediated by specialised receptors organised in groups of cells, which form the taste buds (Chandrashekar et al. 2000; Nelson et al. 2001; 2002; Zhao et al. 2003; Behrens and Meyerhof 2011). The molecular mechanisms through which sweet, L-amino acid (umami), and bitter tastes signal from the tongue to the sensory cortex have been clarified by the discovery of TRs (McLaughlin et al. 1992; Hoon et al. 1999; Lindemann, 2001), which activate Gα-subunits, including $G_{\alpha tran}$ and $G_{\alpha gust}$, to transmit different tastes (Margolskee 2002; Ruiz-Avila 1995; Ming et al. 1999; Caicedo et al. 2003). $G_{\alpha gust}$ is the best characterized Gα protein associated with bitter taste transmission, however, the findings that $G_{\alpha gust}^{-/-}$ mice retain sensitivity to bitter substances, imply that other Gα-subunits, including $G_{\alpha tran}$, contribute to signalling bitter substances (Margolskee 2002; Ruiz-Avila et al. 1995; He et al. 2002). The discovery of $G_{\alpha tran}$ and $G_{\alpha gust}$ in the GIT of different species from the mouse (Hass et al. 2007; Sutherland et al. 2007; Wu et al. 2002), rat (Höfer et al. 1996) and pig (Clavenzani et al. 2009; Moran et al. 2010; Mazzoni et al. 2013) to human (Rozengurt et al. 2006; Steinert et al. 2011), supports the involvement of TRs in chemosensory mechanisms elicited by luminal contents in different species. In addition, evidence for the presence of Gα-subunit immunolabelling in the chemosensory system of

teleosts (Hansen et al. 2003, 2004; Zhang et al. 2006, Ferrando et al. 2011; Koide et al. 2009) and cartilaginous fish (Ferrando et al. 2009b; Ferrando et al. 2010) has been provided. Furthermore, Muradov et al. (2008) demonstrated $G_{\alpha tran}$ expression in long and short photoreceptors of lamprey (*Petromyzon marinus*) with the same antibody used in our paper, while Zhang et al. (2006) found $G_{\alpha gust}$ immunofluorescence in the barbells of yellow catfish (*Pelteobagrus fulvidraco*) with the same rabbit polyclonal $G_{\alpha gust}$ antibody we used in the present study. Though recent studies on the fish genome failed to detect a gene encoding an ortholog of the mammalian $G_{\alpha gust}$ gene (Ohmoto et al., 2011), Oka and Korsching (2011) showed 80% homology between $G_{\alpha gust}$ and other G proteins, and Sarwal et al. (1996) reported a high homology of Gα gene between mammals and the puffer fish *Fugu rubripes*, with an identical intron/exon structure throughout the coding regions.

The observation that only some cells showed both $G_{\alpha tran}$ and $G_{\alpha gust}$ immunoreactivities in the sea bass GIT is in agreement with previous reports in mammals showing only partial colocalization of these two Gα-proteins (unpublished, personal observation) and a differential distribution in some regions (Wu et al. 2002). $G_{\alpha tran}$ and $G_{\alpha gust}$ mediate signals initiated by tastants acting at both families of TRs, the T1Rs and the T2Rs (Wong et al. 1996; Ruiz-Avila et al. 2001; He W et al 2002; Caicedo et al, 2003). $G_{\alpha tran}$ and $G_{\alpha gust}$ could serve different chemosensitive modalities depending upon the luminal content and according to the receptor subtype being stimulated, which would be consonant with the report that different T2Rs exert their function through the activation *in vitro* of distinct Gαi related forms (Sainz et al. 2007). Our findings that $G_{\alpha tran}$ immunoreactivity was localized to distinct subsets of EECs expand previous data in other animals species (Rozengurt et al. 2006; Sutherland et al. 2007; Moran et al. 2010; Janssen et al. 2011, Steinert et al. 2011; Mazzoni et al. 2013). EECs have been reported in the stomach and intestine of several fish species (Holmgren et al. 1982; Reinecke et al. 1997; Ku et al. 2004; Bermúdez et al. 2007; Manning et al. 2008), including the sea bass, where 5-HT-, SOM- and GHR-IR EECs have been described (Visus et al. 1996; Ferrando et al. 2009a; Terova et al. 2008). Our study has shown GHR-IR cells in the gastric mucosa, many of which contain $G_{\alpha tran}$-IR. Our results are consistent with data from Terova et al. (2008), who observed high levels of GHR gene expression in the sea bass stomach. The colocalization of $G_{\alpha tran}$ and GHR-IRs is in line with data reported by Janssen et al. (2011) showing that 89% of the $G_{\alpha tran}$-IR cells co-express GHR in mouse stomach, a finding of special interest as it indicates that this cell immunophenotype is conserved through evolution. GHR might have a role in regulating food intake in response to fasting and re-feeding in sea bass (Terova et al. 2008). Moreover,

OB, an anorexigenic peptide derived from the GHR precursor, has been reported to counteract GHR effects on energy homeostasis and gastrointestinal function (Zhang et al. 2005). Furthermore, an OB encoding sequence has been recently identified in the black sea bream (Yeung et al. 2006) and in Atlantic halibut (Manning et al. 2008). Thus, our results, together with previous observations, suggest a morphological link between chemical sensing and food intake.

Our findings that the GIT mucosa of the sea bass contains GAS/CCK, OB, 5-HT, CGRP, SOM, GLP-1 and SP EECs extend previous knowledge on the distribution of bioactive messengers in the fish alimentary tract (Elbal et al. 1988; Beorlegui et al. 1992; Barrenechea et al. 1994; Groff and Youson 1997; Reinecke et al. 1997; Al-Mahrouki and Youson 1998; Domeneghini et al. 2000; Bosi et al. 2004; Ku et al. 2004; Pederzoli et al. 2004; Bosi et al. 2005a, b; Nelson and Sheridan 2006; Bermúdez et al. 2007). CGRP-, GAS/CCK-, SP- and SOM-IRs were also identified in nerve processes in the submucosal and muscular layer, and labelled neuronal cell bodies were observed in the muscular layer. Some studies have detailed peptide- and serotonin-containing innervation in fish. Bermúdez et al. (2007) have demonstrated 5-HT-, SP- and CGRP-, but not CCK-IR nerve fibers in the submucosal and muscular layers in the turbot *Scophthalmus maximus*; similar results were obtained by Bosi et al. (2005a) in chubs *Leuciscus cephalus*. Moreover, Pederzoli et al. (2004) observed SP-IR neurons in sea bass GIT. The presence of a peptidergic and serotonergic neural network, in addition to the EECs expressing the same signalling molecules, provides support for a link between chemosensory and neuronal systems, which could control GIT physiology in fish via integrated neuro-endocrine mechanisms.

In conclusion, our study demonstrates that G proteins involved in chemosensory transmission are expressed in the sea bass GIT enteroendocrine system. Nutrients may elicit the release of different bioactive messengers (mainly peptides), which directly, or via neural reflexes, contribute to the control of GIT functions and nutrient intake of this fish. Taste-related molecules might represent the initial molecular events involved in chemosensing processes. A better understanding of the mechanisms involved in luminal chemosensitivity in the fish may provide a new basis for feeding formulations to be applied in aquaculture.

Acknowledgements

This work was partially supported by grants PRIN/COFIN from the Italian Ministry of University, Research and Education 2008 and 2010 (to R. De G.); and R.F.O. funds from the University of Bologna (R. De G., R.C. and P.C.). R. De G. is the recipient of research grants from Fondazione Del Monte di Bologna e Ravenna. C.S. is supported by the National Institutes of Health, DK79155 and DK41301 (Morphology and Cell Imaging Core) and University of California Los Angeles (UCLA) Academic Senate Grant.

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Table 1 List of antibodies used in this study.

Primary antisera	**Antigens**	**Code**	**Dilution**	**Supplier**
Polyclonal rabbit anti-α-transducin	$G_{\alpha t2}$ of bovine origin	sc-390	1:50	Santa Cruz
Polyclonal rabbit anti-α-gustducin	$G_{\alpha\ gust}$ of rat origin	sc-395	1:200	Santa Cruz
Polyclonal rabbit anti-somatostatin	Synthetic somatostatin$_{15-28}$ conjugated to keyhole limpet hemocyanin	566	1:1000	INCSTAR
Monoclonal mouse anti-gastrin/cholecystokinin	Human gastrin/CCK C-terminus	GAS/CCK9303	1:1000	CURE Digestive Diseases Research Center, UCLA
Polyclonal goat anti-ghrelin	Human ghrelin	sc-10368	1:800	Santa Cruz
Polyclonal rabbit anti-obestatin	Human obestatin	----	1:1500	Prof. Rindi G.**
Polyclonal rabbit anti-5-hydroxytryptamine	Rat hypothalamus, raphe and spinal cord	20080	1:2000	INCSTAR
Polyclonal rabbit anti-glucagon-like peptide-1	Synthetic peptide: HDEFERHAEGTFTSDVSSY, corresponding to amino acids 92-110 of Human GLP 1.	Ab22625	1:1000	Abcam
Polyclonal rabbit anti-calcitonin gene-related peptide	Synthetic rat CGRP	IHC 6006	1:1000	Peninsula/Bachem
Monoclonal rat anti-substance P	Substance-P-BSA conjugate	10-S15	1:300	Fitzgerald
Secondary antisera				
FITC-conjugated goat anti-rabbit IgG			1:600	Calbiochem
TRITC-conjugated donkey anti-goat IgG			1:400	Jackson
Alexa 594-conjugated goat anti-mouse IgG			1:400	Mol. Probes
Alexa 594-conjugated donkey anti-rat IgG			1:500	Mol. Probes
Biotin-conjugated goat anti-rabbit IgG			1:400	Vector
Texas Red®-conjugated streptavidin			1:2000	Vector

**Kindly provided by Prof. Guido Rindi, Institute of Pathology, Catholic University of the Sacred Heart, Rome, Italy.

Table 2 Peptides used for absorption tests.

Peptide	**Code**	**Concentration**	**Supplier**
α-transducin	sc-390 P	20 μg peptide in 1 ml PBS	Santa Cruz Biotechnology, Santa Cruz, USA
α-gustducin	sc-395 P	20 μg peptide in 1 ml PBS	Santa Cruz Biotechnology, Santa Cruz, USA
Ghrelin*	sc-10368 P	20 μg peptide in 1 ml PBS	Santa Cruz Biotechnology, Santa Cruz, USA
Glucagon-likepeptide-1	ab50245	10^{-5} M	Abcam, Cambridge, UK

* Ghrelin peptide was used for both anti-ghrelin and anti-obestatin antibody specificity, since obestatin belongs to the ghrelin peptide family.

Figure captions

Fig. 1

Western blot analysis showing α-gustducin (a), α-transducin (b) and cholecystokinin (c) immunoreactive bands in sea bass tissue extract. (a): α-gustducin antibody detects a single immunoreactive band near the theoretical molecular weight ~40 kDa in sea bass brain and gastric mucosa (lanes 1-2 respectively) and in mouse brain (lane 3); sea bass and mouse brain served as positive controls. (b): α-transducin antibody detects a major immunoreactive band at the theoretical molecular weight ~45 kDa in sea bass gastric mucosa, brain and eye (lanes 1, 2 and 3 respectively); the brain and eye served as positive control. (c): cholecystokinin monoclonal antibody visualizes a weak, single immunoreactive band close to the theoretical molecular weight of ~15kDa in sea bass intestinal mucosa

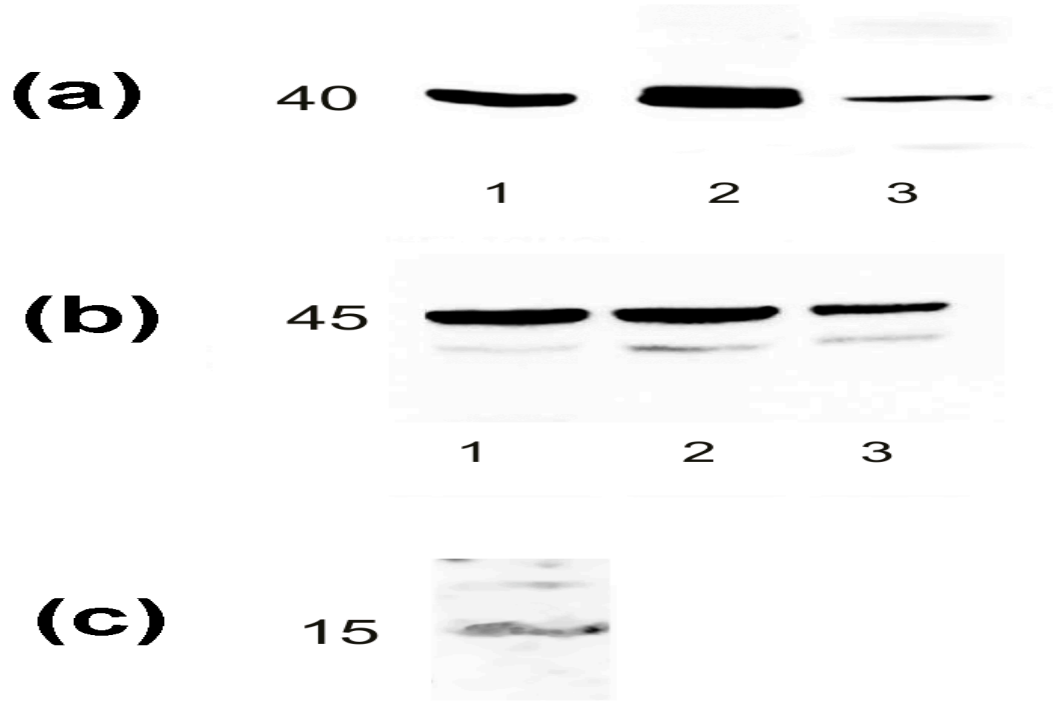


Fig. 2

Representative images of sea bass gastric mucosa. α-transducin-immunoreactivity ($G_{\alpha tran}$-IR) (a, c, e and g) colocalised with 5-hydroxytryptamine (5-HT) (b), obestatin (OB) (d), ghrelin (GHR) (f) and α-gustducin ($G_{\alpha gust}$) (h) (arrows) immunoreactivity in the basal portion of the gastric gland and in the epithelial lining of the mucosal folds. Arrowheads in a, b, c and d indicate 5-HT- and OB-IR (b and d) cells negative for $G_{\alpha tran}$ (a and c). a-f scale bars = 30μm; g- h scale bars: 20μm

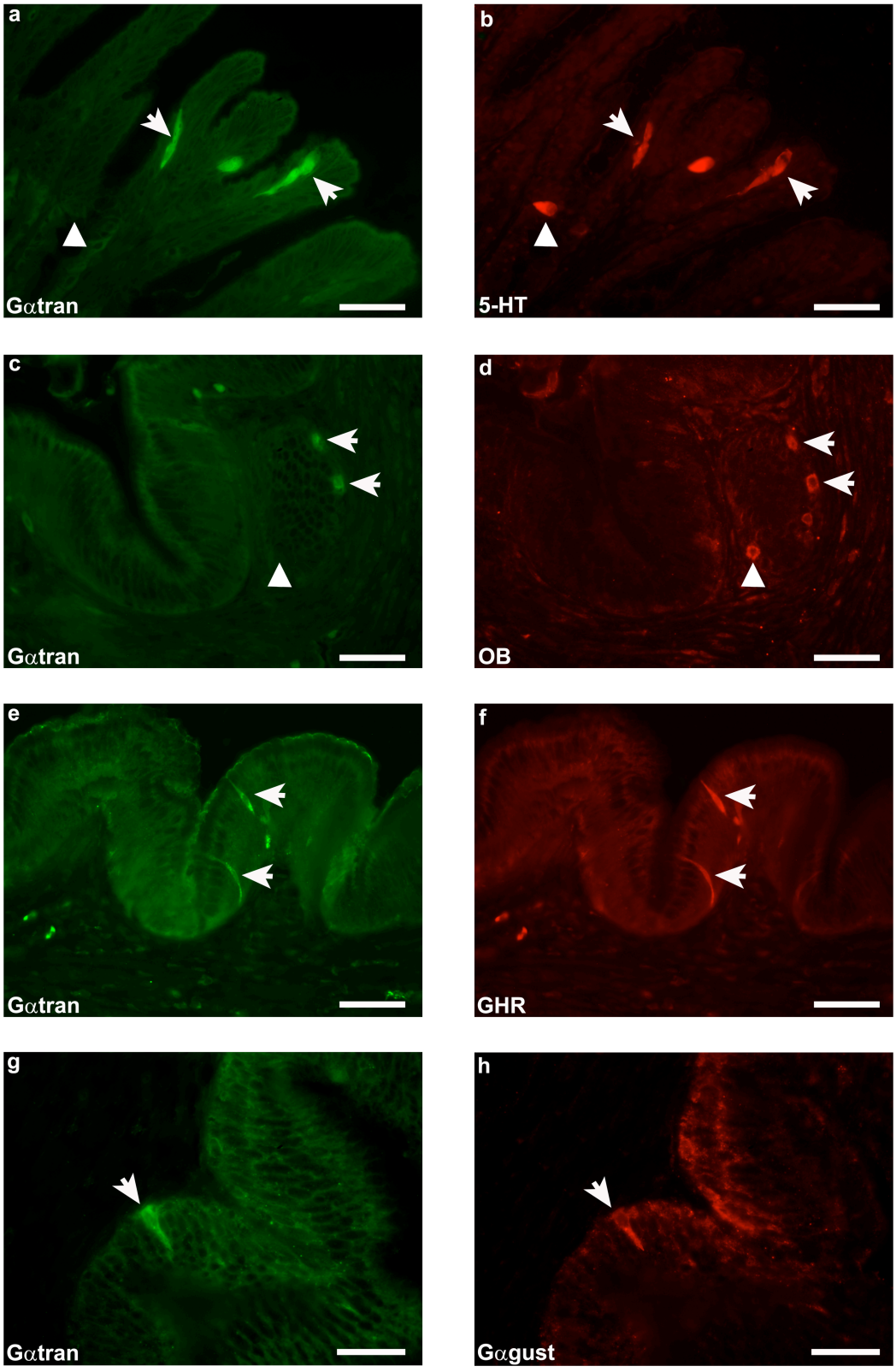
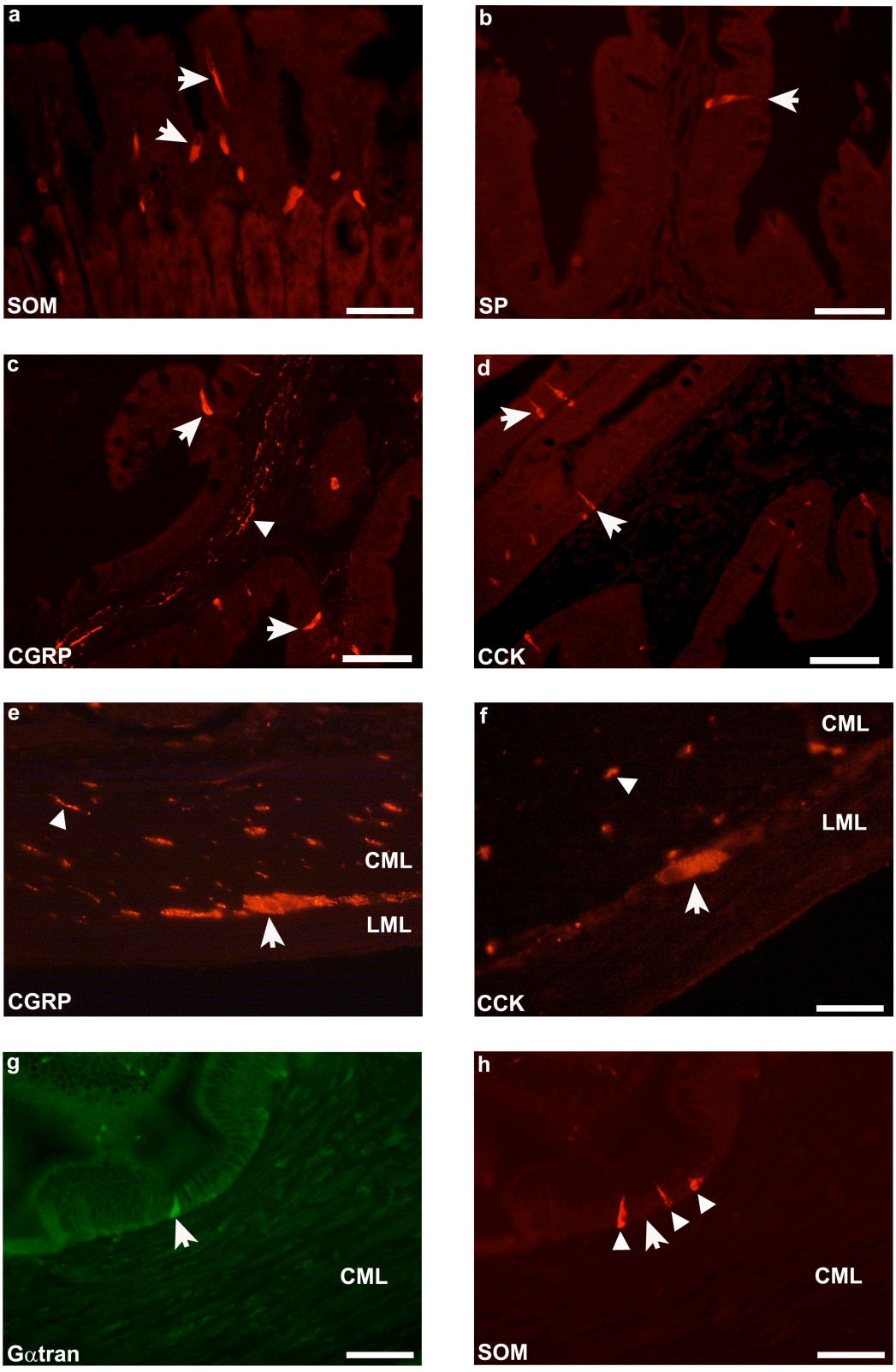

a
Gαtran
b
5-HT
c
Gαtran
d
OB
e
Gαtran
f
GHR
g
Gαtran
h
Gαgust

Fig. 3

Representative images illustrating different subpopulations of EEC cells in sea bass gastric and intestinal mucosa. Somatostatin (SOM), substance P (SP), calcitonin gene-related peptide (CGRP), cholecystokinin (CCK) and α-transducin ($G_{\alpha tran}$) (a-h) labelled cells in the stomach (a, arrows) and intestinal mucosa (b, c, d, g, arrows and h, arrowhead), respectively. CGRP and CCK immunolabelled nerve fibers (arrowheads) were observed in the stomach (e) and in the submucosal layer of the intestine (c and f). Some labelled CGRP (e) and CCK (f) neuronal cell bodies (arrows) were identified in the muscular layer. Arrow in g indicates a $G_{\alpha tran}$-IR cell negative for SOM (h) in the intestinal mucosa and arrowheads in h point to SOM immunolabelled cells. a, b, e, and h scale bars = 30 μm; c, d scale bars = 60 μm; f scale bars = 20 μm


a
SOM
b
SP
c
CGRP
d
CCK
e
CML
LML
CGRP
f
CML
LML
CCK
g
CML
Gαtran
h
CML
SOM

CHAPTER 2

J. Cell. Mol. Med. Vol XX, No X, 2013 pp. 1-9

Expression and regulation of α-transducin in the pig gastrointestinal tract

Maurizio Mazzoni [a], Roberto De Giorgio [b, f], **Rocco Latorre [a]**, Claudia Vallorani [a], Paolo Bosi [c],
Paolo Trevisi [c], Giovanni Barbara [b], Vincenzo Stanghellini [b], Roberto Corinaldesi [b], Monica Forni [a],
Maria S. Faussone-Pellegrini [d], Catia Sternini [e], *, Paolo Clavenzani [a, f]

[a] Department of Veterinary Medical Science, University of Bologna, Italy
[b] Department of Medical and Surgical Sciences, University of Bologna, Italy
[c] Department of Agri-food Protection and Improvement, University of Bologna, Italy
[d] Department of Anatomy, Histology and Forensic Medicine, University of Florence, Firenze, Italy
[e] CURE/DDRC, Division of Digestive Diseases, Departments Medicine and Neurobiology, David Geffen School of Medicine, UCLA, Los Angeles, and Veterans Administration Greater Los Angeles Health System, CA, USA
[f] Centro Interdipartimentale di Ricerche sull'Alimentazione Umana (CIRAU), University of Bologna, Italy

Received: July 4, 2012; Accepted: January 9, 2013

Abstract

Taste signalling molecules are found in the gastrointestinal (GI) tract suggesting that they participate to chemosensing. We tested whether fasting and refeeding affect the expression of the taste signalling molecule, α-transducin (G_{atran}), throughout the pig GI tract and the peptide content

of $G_{\alpha tran}$ cells. The highest density of $G_{\alpha tran}$-immunoreactive (IR) cells was in the pylorus, followed by the cardiac mucosa, duodenum, rectum, descending colon, jejunum, caecum, ascending colon and ileum. Most $G_{\alpha tran}$-IR cells contained chromogranin A. In the stomach, many $G_{\alpha tran}$-IR cells contained ghrelin, whereas in the upper small intestine many were gastrin/cholecystokinin-IR and a few somatostatin-IR. $G_{\alpha tran}$-IR and $G_{\alpha gust}$-IR colocalized in some cells. Fasting (24 h) resulted in a significant decrease in $G_{\alpha tran}$-IR cells in the cardiac mucosa (29.3 ± 0.8 versus 64.8 ± 1.3, $P < 0.05$), pylorus (98.8 ± 1.7 versus 190.8 ± 1.9, $P <$ 0.0 l), caecum (8 ± 0.01 versus 15.5 ± 0.5, $P < 0.01$), descending colon (17.8 ± 0.3 versus 23 ± 0.6, $P < 0.05$) and rectum (15.3 ± 0.3 versus 27.5 ± 0.7, $P < 0.05$). Refeeding restored the control level of $G_{\alpha tran}$-IR cells in the cardiac mucosa. In contrast, in the duodenum and jejunum, Gatran-IR cells were significantly reduced after refeeding, whereas $G_{\alpha tran}$-IR cells density in the ileum was not changed by fasting/refeeding. These findings provide further support to the concept that taste receptors contribute to luminal chemosensing in the GI tract and suggest they are involved in modulation of food intake and GI function induced by feeding and fasting.

Keywords: α-gustducin _ taste receptors _ enteroendocrine cells _ chemosensing

*Correspondence to: Catia STERNINI, MD, AGAF,
CURE/Digestive Diseases Research Center,
Veterans Administration Greater Los Angeles Health System,
Bldg 115, Room 223, 11301 Wilshire Blvd, Los Angeles,
CA 90073, USA.
Tel.: 310 312 9477
Fax: 310 825 3133
E-mail: csternin@ucla.edu

Introduction

Sensing of luminal contents by the gastrointestinal (GI) tract mucosa plays a critical role in the control of digestion, absorption, food intake and metabolism [1, 2] by triggering functional responses appropriate for beneficial or potentially harmful substances. Enteroendocrine (EEC) cells act as specialized transducers of luminal content, by releasing signalling molecules, which activate nerve fibres as well as local and distant targets to influence gut functions. EECs can be either 'open-type' or 'closed-type' depending on their microvilli reaching or not the lumen [1–3]. Both types of cells can be regulated by intraluminal content, either directly ('open cells') or indirectly ('closed cells') through neural and humoural mechanisms to release a variety of secretory products, including gastrin (G cells), ghrelin (P or X cells), somatostatin (D cells), cholecystokinin (CCK) (I cells), serotonin (enterochromaffin cells), glucose-dependent insulinotropic peptide (GIP) (K cells), glucagon-like peptides (GLPs) and peptide YY (PYY) (L cells), according to the different substances detected in the lumen [1–3]. Once released, these signalling molecules affect different functions ranging from gastrointestinal motility and secretion to feeding regulation via the brain-gut axis [1–3]. The discovery that taste receptors (TRs) and signalling molecules identified in the oral cavity are expressed in the GI mucosa, suggests that they play a role in chemosensing in the gut. TRs are G proteincoupled receptors (GPCRs) sensing bitter (T2Rs), or sweet and umami (T1Rs) tastes. T2Rs are a large family of receptors (25–36 in mammals) perceiving a multitude of tastants, whereas T1Rs comprise 3 receptors that heterodimerize to sense sweetness (T1R2 and T1R3) or umami (T1R1 and T1R3) [4–6]. T1Rs and T2Rs mediate gustatory signalling by interacting with specific Gα subunits, including α-gustducin ($G_{\alpha gust}$) and α-transducin ($G_{\alpha tran}$) [7] through the activation of different effector systems leading to intracellular Ca^{2+} increase and transmitter release. $G_{\alpha gust}$ or $G_{\alpha tran}$ immunoreactivity (IR) has been localized to epithelial EECs and non-EECs in the rodent [8–11], pig [12, 13] and human [14] GI tract and pancreatic duct [15]. The aims of this study were to characterize the cellular sites of expression of Gatran and test the hypothesis that $G_{\alpha tran}$ is modulated by fasting and refeeding in the GI tract of the pig, an animal model closer to humans compared with rodents for food intake, body size, lifespan and body proportion.

Materials and methods

Large White male pigs (n = 12), of about 45 days of age with an averageweight of 12.0 ± 0.3 kg, purchased from Suidea (Reggio Emilia, Italy), were fed with a standard balanced diet and housed individually in pens with a mesh floor in a temperature-controlled room and tap water freely available. Following 1 day adaptation, animals were divided into three groups: standard diet (control, n = 4), fasted for 24 h (fasted, n = 4) and refed for 24 h after fasting (refed, n = 4). Experimental procedures were approved by the Ethic Committee for Experimental Animals of the University of Bologna, Italy. Pigs were deeply anaesthetized with sodium thiopental (10 mg/kg body weight, Zoletil 100, Virbac) and killed by an intracardiac injection of Tanax® (0.5 ml/kg BW; Intervet Italia). Specimens of the GI tract: oesophagus (cervical, thoracic and abdominal tract), stomach (cardiac, near to the gastric diverticulum; oxyntic, in the greater curvature; and pyloric, close to the pyloric sphincter), duodenum (about 10 cm from the pyloric sphincter), middle jejunum and ileum, caecum, ascending colon (near the centrifugal turns), descending colon (about 25 cm from the anus) and rectum (in the ampulla recti) were collected, pinned flat on balsa wood, fixed in 10% buffered formalin for 24 h at room temperature (RT), dehydrated and embedded in paraffin.

Immunohistochemistry

Serial (5 μm thick) sections mounted on poly-L-lysine–coated slides were processed for single and double labelling immunofluorescence using antibodies directed to Gatran or Gagust, chromogranin A (CgA), a generalized marker for EECs, or specific markers for EEC subtypes (ghrelin, GHR, somatostatin, SOM and gastrin/cholecystokinin GAS/CCK) (Table 1). Briefly, sections were deparaffinized through graded ethanols to xylene, rehydrated and heated in sodium citrate buffer (pH 6.0) in a microwave (2 cycles at 800 W, 5 min each) for antigen unmasking. Sections were incubated in 15% normal horse serum/ 0.01 M phosphate buffer saline (PBS) (1 h at RT) to prevent non-specific staining, followed by primary antibodies in PBS (overnight) and a mixture of fluorescein isothiocyanate (FITC)-conjugated, tetramethyl rhodamine isothiocyanate (TRITC)-conjugated, Alexa Fluor® 594- and Alexa Fluor® 488-conjugated secondary antibodies all diluted in PBS (Table 1), then coverslipped with buffered glycerol, pH 8.6. As the antibodies to $G_{\alpha tran}$ and

$G_{\alpha gust}$ were generated in the same species, serial sections (3 μm thick) were used to test their colocalization.

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

Primary antibodies	Code	Species	Dilution	Supplier
α-Transducin	sc-390	rabbit	1:600	Santa Cruz
α-Gustducin	sc-395	rabbit	1:500	Santa Cruz
Chromogranin A	MON9014	mouse	1:1000	Monosan
Gastrin / Cholecystokinin	GAS/CCK#9303	mouse	1:1000	*CURE/DDC
Ghrelin	sc-10368	goat	1:800	Santa Cruz
Somatostatin	S6	mouse	1:1000	*CURE/DDC

*CURE/DDRC, UCLA, Los Angeles, CA, USA.
Chemicon International, Temecula, CA, USA; Monosan, Sanbio B.V. Frontstraat, Uden, Netherlands; Santa Cruz Biotecnology, Inc., CA, USA.)

Secondary antisera	Dilution	Supplier
Alexa 594-conjugated goat anti-mouse IgG	1:800	Mol. Probes
FITC-conjugated goat anti-rabbit IgG	1:500	Calbiochem
TRITC-conjugated donkey anti-rabbit IgG	1:500	Jackson
Alexa 488-conjugated donkey anti-goat IgG	1:800	Mol. Probes

Calbiochem-Novabiochem Corporation, San Diego, CA, USA; Molecular Probes, Eugene, Ore., USA; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA).

Specificity of antibodies

Specificity of $G_{\alpha tran}$, $G_{\alpha gust}$ and GAS/CCK antibodies has been tested by Western blot (Supplementary material) whereas specificity of CgA monoclonal antibody (clone LK2H10) has been previously reported [16]. GHR antibody specificity was assessed by pre-adsorption with an excess of the homologous peptide (sc-10368 P, Santa Cruz, CA, USA) or another ghrelin peptide (code 031-52; Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA). The pattern obtained with our S6 SOM antibody completely overlapped with that of SOM rabbit polyclonal antiserum (Monosan SANBIO B.V., Uden, The Netherlands-catalogue PS 204), whose specificity was shown by immunoblocking in the pig gut and pancreas.

Cell counting and statistical analysis

Cell counting was performed with a 40 X objective lens using a Zeiss Axioplan microscope (Carl Zeiss, Oberkochen, Germany) with appropriate filter cubes to discriminate different wave fluorescence, images were collected with a Polaroid DMC digital photocamera (Polaroid, Cambridge, Mass., USA) and minimal adjustment to brightness and contrast was performed with Corel Photo Paint and Corel Draw (Corel, Dublin, Ireland). Cell counting was performed in a blind fashion by two investigators. For each piglet, $G_{\alpha tran}$-IR cells were counted in 36 random microscope fields (each field, 0.28 mm2), for a total area of 10 mm^2, in the cardiac, oxyntic and pyloric mucosa, in 50 random villi and glands in the small intestine, and in 50 crypts in the colon. Only villi/glands/crypts located perpendicularly to the mucosal surface were counted. The values were pooled for each experimental group (control, fasted and refed respectively) and, subsequently, the mean and the percentage were calculated. Values were expressed as mean ± standard deviation (SD). Data were analysed using ANOVA One-Way (Graph Prism 4, GraphPad Software, Inc., La Jolla, CA, USA). Statistical significance was determined using the Student's t-test. A $P < 0.05$ was considered statistically significant.

Results

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

$G_{\alpha tran}$-IR cells were detected throughout the whole pig GI tract (Fig. 1A–G), except the oesophagus and oxyntic mucosa. In the pylorus, intense $G_{\alpha tran}$-IR was observed in the basal portion of the gastric gland and in the epithelial lining of the mucosal folds (Fig. 1A and F); $G_{\alpha tran}$-IR cells had elongated, 'bottle-like', morphology with homogenously labelled cytoplasm (Fig. 1E and G). In the small intestine, a subset of cells along the crypt-villus axis showed $G_{\alpha tran}$- IR (Fig. 1B, E and G), whereas in the large intestine, labelled cells were generally located in the surface and glandular epithelium (Fig. 1C and D). Most $G_{\alpha tran}$-IR cells had two thin cytoplasmic prolongations, one extending to the endoluminal mucosal surface (Fig. 1E and G) and one to the basal lamina, suggesting they are 'EEC open-type'

cells [1, 3]. In the cardiac and pyloric mucosa, some cells were confined to the basal lamina and did not reach the lumen (Fig. 1F), like 'EEC closed-type' cells [1, 3].

Distribution of the Gαtran-IR cells in different experimental groups

In the stomach, the highest density of $G_{\alpha tran}$-IR cells was in the pylorus (there was an average of about 18.9 cells/mm^2 or 5.3 cells per field); in the small intestine, the highest density of $G_{\alpha tran}$-IR cells was in the duodenum followed by the jejunum and ileum, whereas in the large intestine it was in the rectum followed by descending colon, caecum and ascending colon (Fig. 2A and B). There was a decrease in the density of $G_{\alpha tran}$-IR cells in fasted animals, which was significant in the cardiac mucosa (29.3 ± 0.8 versus 64.8 ± 1.3, $P < 0.05$ versus control), pylorus (98.8 ± 1.7 versus 190.8 ± 1.9, $P < 0.0$ l), caecum (8 ± 0.01 versus 15.5 ± 0.5, $P < 0.01$), descending colon (17.8 ± 0.3 versus 23 ± 0.6, $P < 0.05$) and rectum (15.3 ± 0.3 versus 27.5 ± 0.7, $P < 0.05$), but not in the other regions. Interestingly, refeeding restored the control level of $G_{\alpha tran}$-IR cells in the cardiac mucosa (57 ± 1 versus 29.3 ± 0.8 in fasted, $P < 0.01$), but not in the pylorus, caecum, descending colon and rectum where the number of $G_{\alpha tran}$-IR cells in refed was comparable to fasted pigs. In the jejunum, $G_{\alpha tran}$-IR cells in the refed group were less than in the fasted condition and were significantly lower than in controls (9.3 ± 0.2 in refed versus 19 ± 0.3 in control, $P < 0.01$). In the ileum and ascending colon, the number of $G_{\alpha tran}$-IR cells in fasted and refed animals was comparable to controls.

$G_{\alpha tran}$/CgA in the GI tract

The majority of $G_{\alpha tran}$-IR cells co-expressed CgA: 99% of the $G_{\alpha tran}$-IR cells in the cardiac and pyloric mucosa were immunopositive for CgA, whereas 83% and 98% of $G_{\alpha tran}$ -IR cells were immunopositive for CgA in the small and large intestine respectively. However, some cells were $G_{\alpha tran}$ -IR, but CgA negative (Fig. 1 G and H). In the stomach, Gatran-IR/CgA-IR cells were numerous in the glandular epithelium. The mean numbers of $G_{\alpha tran}$ /CgA-IR cells throughout the pig gut are reported in Table 2A. In the cardiac mucosa, the mean number of $G_{\alpha tran}$ /CgA-IR cells in control and refed groups is higher than that of fasted group ($P < 0.05$). In the pyloric mucosa, the mean number of $G_{\alpha tran}$ /CgA-IR cells in fasted and refed groups was lower than control (control versus fasted and control versus

refed, $P < 0.05$). A general decrease in $G_{\alpha tran}$ /CgA-IR cells was observed in the small and large intestine in fasted and refed compared with control. Specifically, in
the duodenum and jejunum, the $G_{\alpha tran}$ /CgA-IR cells were significantly decreased in refed compared with control ($P < 0.05$). Moreover, in the duodenum, we found a reduced number of $G_{\alpha tran}$ /CgA-IR cells in refed compared with fasted ($P < 0.05$). $G_{\alpha tran}$ /CgA-IR cells were more abundant in the caecum, descending colon and rectum of control group compared with fasted ($P < 0.05$), whereas in the caecum and in the rectum, refed showed a number of $G_{\alpha tran}$ /CgA-IR lower than control ($P < 0.05$). The percentage of the $G_{\alpha tran}$ on the total of CgAIR cells have been indicated in Table 2B. Furthermore, there were no statistically significant differences in the absolute numbers of CgA-IR cells in the gastric and intestinal mucosa among the three experimental groups.

$G_{\alpha tran}$/GHR in the gastric mucosa

$G_{\alpha tran}$/GHR-IR cells were numerous in the pylorus, from the neck to the base of the glands (Fig. 3A and B), and less abundant in cardiac glands (Fig. 3C and D). Most Gatran/GHR cells were 'closed-type', lying at the gland basal lamina. Few $G_{\alpha tran}$/GHR-IR cells in the surface epithelium were 'open-type' (Fig. 3C and D). In the cardiac and pyloric mucosa, approximately 96% and 91% of $G_{\alpha tran}$-IR cells, respectively, co-expressed GHR. $G_{\alpha tran}$ /GHR-IR cells were significantly reduced in fasted versus control pigs in both cardiac mucosa ($P < 0.01$) and pylorus ($P < 0.05$). In refed, they were partly restored in the cardiac mucosa ($P < 0.05$), but not pylorus. The mean number and percentage of the $G_{\alpha tran}$ on the total of GHR-IR cells are reported in Table 3. In the cardiac mucosa, the number of GHR-IR cells decreased in fasted versus control (114.8 ± 29.4 versus 244.5 ± 71.3, $P < 0.01$), while it increased in refed versus fasted (241.3 ± 57.5 versus 114.8 ± 29.4, $P < 0.01$). There were no statistically significant differences in the mean numbers of GHR-IR cells in the pyloric mucosa among the three experimental groups

Colocalization of $G_{\alpha tran}$ with CCK, SOM and $G_{\alpha gust}$ in the duodenum and jejunum

Co-expression of $G_{\alpha tran}$ and CCK was observed in open-type cells in the surface and glandular epithelium of the jejunum (Fig. 3E– H). As our monoclonal antibody cannot

discriminate CCK and GAS, we could not assess the actual number of GAS and CCK-IR cells in the duodenum where both cell types are present. Few $G_{\alpha tran}$ /SOM cells (about 1 positive cell/400 villi) were detected

(Fig. 4A and B). The mean number and percentage of the $G_{\alpha tran}$ compared with the total number of CCK-IR cells are reported in Table 4. In the jejunum, approximately 59% of $G_{\alpha tran}$ -IR cells coexpressed CCK. $G_{\alpha tran}$ /CCK-IR cells were reduced in fasted and refed compared with controls ($P < 0.01$) in the jejunum. $G_{\alpha tran}$ /CCKIR cells were not visualized in the pylorus and cardiac mucosa (Fig. 4C and D). Finally, occasional $G_{\alpha tran}$ / $G_{\alpha gust}$-IR cells were detected in the pylorus (Fig. 4C and D) and duodenum (Fig. 4E– H), which expressed CgA-IR (Fig. 4G and H). Furthermore, the number of CCK-IR cells decreased in fasted versus control (19.3 ± 2.5 versus 10.3 ± 1, $P < 0.01$), while no changes were observed in refed versus fasted and control groups.

Table 2A. Mean number of $G_{\alpha tran}$ / CgA-IR cells in the pig GI tract.

	* Cardiac mucosa	* Pyloric mucosa	Duodenum	Jejunum	Ileum	Caecum	Ascending colon	Descending colon	Rectum
Control	64.5 ± 1.2[a]	190.8 ± 1.9[a]	46.8 ± 0.8[a]	15.8 ± 0.3[a, b]	11.8 ± 0.3	15.3 ± 0.5[a]	13.8 ± 0.5	23 ± 0.6[a]	27.5 ± 0.7[a]
Fasted	29 ± 0.8[b]	96.8 ± 1.7[b]	36 ± 0.9[a]	12 ± 0.4[b, c]	7 ± 0.2	8 ± 0.1[b]	10.5 ± 0.2	17.5 ± 0.3[b]	15.3 ± 0.3[b]
Refed	56 ± 1[a]	111.8 ± 1.9[b]	11 ± 0.6[b]	8 ± 0.2[c]	13.3 ± 0.6	6.8 ± 0.3[b]	7.8 ± 0.2	18.5 ± 0.3[a, b]	12.8 ± 0.3[b]

*Values refer to a total area of 10 mm^2 for each group. The other values represent the percentage evaluated in 50 villi and in 50 intestinal glands for each group, respectively. Values with different superscripts within the same column differ significantly ($P < 0.05$).

Table 2B. Percentage of $G_{\alpha tran}$ / total CgA-IR cells in the pig GI tract.

	* Cardiac mucosa	* Pyloric mucosa	Duodenum	Jejunum	Ileum	Caecum	Ascending colon	Descending colon	Rectum
Control	**19%** (258/1351)	**33.7%** (763/2262)	**49%** (187/381)	**36.2%** (63/174)	**27%** (47/174)	**50.4%** (61/121)	**40.4%** (55/136)	**16.5%** (92/557)	**20.3%** (110/543)
Fasted	**7%** (116/1642)	**16%** (387/2399)	**41.4%** (144/348)	**23.9%** (48/201)	**21.9%** (28/128)	**24.4%** (32/131)	**30.7%** (42/137)	**21%** (70/333)	**17%** (61/359)
Refed	**15.7%** (224/1429)	**17.6%** (447/2546)	**23%** (44/191)	**18.4%** (32/174)	**31.2%** (53/170)	**32.5%** (27/83)	**27.7%** (31/112)	**17%** (74/434)	**12%** (51/426)

*Values refer to a total area of 10 mm^2 for each group. The other values represent the percentage evaluated in 50 villi and in 50 intestinal glands for each group, respectively

Discussion

Taste receptors are likely to represent an important mechanism for sensing nutrients and non-nutrients in the GI lumen and contribute to the initiation of appropriate physiological response of digestion/ absorption of nutrients or elimination of harmful substances via activation of neuronal and endocrine pathways. We showed that (a) $G_{\alpha tran}$ cells are distributed throughout the GI tract in the pig, a commonly used animal model for studies of human GI physiology and ingestive behaviour, with the exception of the oesophagus and the oxyntic mucosa, (b) most $G_{\alpha tran}$ cells are EEC of the 'open' type, (c) many $G_{\alpha tran}$ cells contain GHR in the stomach and CCK in the small intestine, whereas a few contain SOM in the upper bowel, (d) some $G_{\alpha tran}$ cells contained $G_{\alpha gust}$, and (e) fasting and refeeding changed the density of Gatran-IR cells, effect that was statistically significant versus controls in most, but not all gut regions. These findings support the concept that TRs participate to chemosensing processes controlling multiple GI functions, including food intake

and metabolism. Our results expand previous reports of $G_{\alpha tran}$ or $G_{\alpha gust}$ t in the rodent [3, 8–11, 17], pig [12, 13] and human [14] GI mucosa by showing a systematic analysis and characterization of mucosal cells expressing $G_{\alpha tran}$ in the pig intestine, an animal model closer to human than rodents, and providing evidence that the expression of this taste-related signalling molecule is modified by feeding and fasting. $G_{\alpha tran}$ -IR was predominantly in EECs, but the colocalization with CgA was not complete suggesting that $G_{\alpha tran}$ -IR is also in non-EECs (likely brush cells), as it has been shown for $G_{\alpha tran}$ in the mouse [10]. On the other hand, in the human colon [14] and pig small intestine [13], $G_{\alpha tran}$ has been reported exclusively in EECs. $G_{\alpha tran}$ -IR cells had a different density throughout the gut, which was high in the stomach, decreased from the duodenum to the ileum, then increased from the caecum to the rectum. These findings are consistent with species and region differences and suggest that TRs exert distinct functions according to the gut region. Like $G_{\alpha gust}$, $G_{\alpha tran}$ mediates signals initiated by tastants acting at T1Rs and the T2Rs [7, 18, 19]. Thus, $G_{\alpha tran}$ cells are likely to serve different chemosensitive modalities depending upon the luminal content and the TR stimulated [19]. The colocalization of $G_{\alpha tran}$ with GHR in the stomach, and CCK and SOM in the small intestine is in agreement with previous studies in rodents and human [8, 9, 11, 14], and in EECs lines [20]. GHR is an orexigenic peptide regulating energy balance homeostasis [21], GI motility and secretion [22], and feeding behaviour [23], in several species including pigs [24]. CCK exerts a prominent role

in satiety conveying signals elicited by nutrients (e.g. fats and proteins) via sensory nerve pathways to the brain [25]. SOM inhibits gastric acid secretion, gastric emptying and smooth muscle contraction and GI hormone release [26]. Thus, the colocalization of $G_{\alpha tran}$ with these peptides is consistent with an involvement of TRs in the control of satiety and food intake, energy balance metabolism and GI secretion and motility. Food deprivation and refeeding alter the morphology of the weaned pig GI tract mucosa with fasting inducing mucosa atrophy in the upper small intestine and refeeding partially restoring it [27]. We demonstrated that 24 h fasting and 24 h refeeding modified the number of $G_{\alpha tran}$ -IR cells in most regions of the pig gut. The number of CgA-IR cells was not modified by fasting and refeeding in most regions with the exception of the caecum and descending colon, therefore it is unlikely that the reduction in $G_{\alpha tran}$ -IR cells observed in fasted and in some regions also in refed animals is due to mucosa atrophy or lack of mucosal restoration following refeeding, although this possibility cannot be excluded. Fasting induces multiple changes in the EEC system such as increasing GHR and lowering GAS/CCK [28, 29] peptides that influence feeding behaviour and colocalize with $G_{\alpha tran}$ -IR. Our results indicated

that in the cardiac and pyloric mucosa, the number of $G_{\alpha tran}$ /GHR cells is greater in normally fed compared with 24 h fasted piglets; similarly, the overall density of GHR-IR cells was lower in fasted than fed or refed animals. However, the increased $G_{\alpha tran}$ /GHR-IR cell expression, as observed during refeeding state in our model, may not necessarily correspond to increased GHR plasma levels during fasting. A significant increase in plasma GHR was reported [30] in weaning pigs following 36 h fasting, with a decrease with 12 h fasting, indicating that the length of food deprivation affects GHR response. Animal ages might also affect hormonal responsesto fasting, as young animals possess fewer energy reserves and less body fat, while having higher energy requirements in relation to rapid body growth [31]. Our data showed a significant reduction in $G_{\alpha tran}$ /CCK-IR cells and in CCK-IR cells overall in fasted and refed pigs compared with controls. This is in agreement with previous reports of a decrease in CCK plasma concentrations and mRNA expression during fasting, while returning to pre-fasting values after either 24 h refeeding in the rat small intestine [32] and 1 h refeeding in lactating sows [33]. However, the reasons why in this study we did not detect an increase in Gatran/CCK-IR cells during refeeding remain to be elucidated. It is possible that factors such as caloric intake, type of diet and slaughter time after refeeding may contribute to explain why CCK cells do not return to prefasting values.

In summary, TRs and downstream molecules might exert a variety of functions ranging from sensing beneficial nutrients (e.g. sweet and umami), thus inducing secretion and motility to facilitate digestion, absorption and food intake, to detection of bitter, potentially harmful substances, thus inducing a defensive response. The latter could be in the form of inhibition of gastric emptying to reduce absorption, increase in intestinal secretion to facilitate elimination, vomiting or avoidance. Taste-related molecules in the distal colon and rectum could also serve as a line of defence against bacteria, which are particularly abundant in these regions. This is supported

by the findings that quorum-sensing molecules produced by Gramnegative bacteria activate a GPCR-mediated signalling cascade in EEC lines, which is likely to involve T2R (Sternini C and Rozengurt E, unpublished). Further studies are required to better understand TR functions in the GI tract in response to feeding, including their regulation with specific dietary components in relationship to peptide release in different regions of the GI tract.

Table 3. Mean number and percentage of the colocalized $G_{\alpha trans}$ / total GHR-IR cells in the cardiac and pyloric mucosa.

	Cardiac mucosa		**Pyloric mucosa**	
Control	84.8 ± 1.6^{a}	**46%** (339/735)	168.8 ± 2.6^{a}	**60.6%** (675/1113)
Fasted	26.8 ± 0.8^{b}	**23.3%** (107/459)	97.5 ± 1.7^{b}	**46.9%** (390/831)
Refed	49.8 ± 1^{a}	**23.2.7%** (199/857	$107.5 \pm 1.8^{a, b}$	**41.5%** (430/1036)

Values with different superscripts within the same column indicate statistical significance ($P < 0.05$).

Table 4. Mean number and percentage of the colocalized $G_{\alpha tran}$ / total CCK-IR cells in the jejunum.

Control	13.5 ± 0.3^{a}	**70%** (54/77)
Fasted	8.8 ± 0.2^{b}	**85.4%** (35/41)
Refed	9.5 ± 0.2^{b}	**71.7%** (38/53)

Values with different superscripts within the same column indicate statistical significance ($P < 0.05$).

Acknowledgements

Grant support: The present work was supported by NIH grants DK79155 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.). We are grateful to Dr. Chiara Bernardini, Department of Medical Veterinary Sciences, University of Bologna, for her valuable technical assistance with Western blot experiments.

FIGURES

Figure 1

Localization of $G_{\alpha tran}$-IR in the pig GI tract. A-D show images of $G_{\alpha tran}$-IR cells in the pyloric (A), jejunum (B), caecum (C) and rectum (D) mucosa (arrowheads). $G_{\alpha tran}$-IR cells have the morphology of open-type enteroendocrine cells in the top of the villi of the duodenum (E, arrowhead) and of closed-type enteroendocrine cells in the pyloric mucosa (F, arrowheads). The bottom images show a $G_{\alpha tran}$-IR enteroendocrine cell expressing chromogranin A (CgA) (G and H, respectively; arrowheads); the arrow in G and H indicates a $G_{\alpha tran}$-IR cell (G) not containing CgA-IR (H). A, C, E and F: scale bars = 50µm; B, D, G and H: scale bars = 100µm.

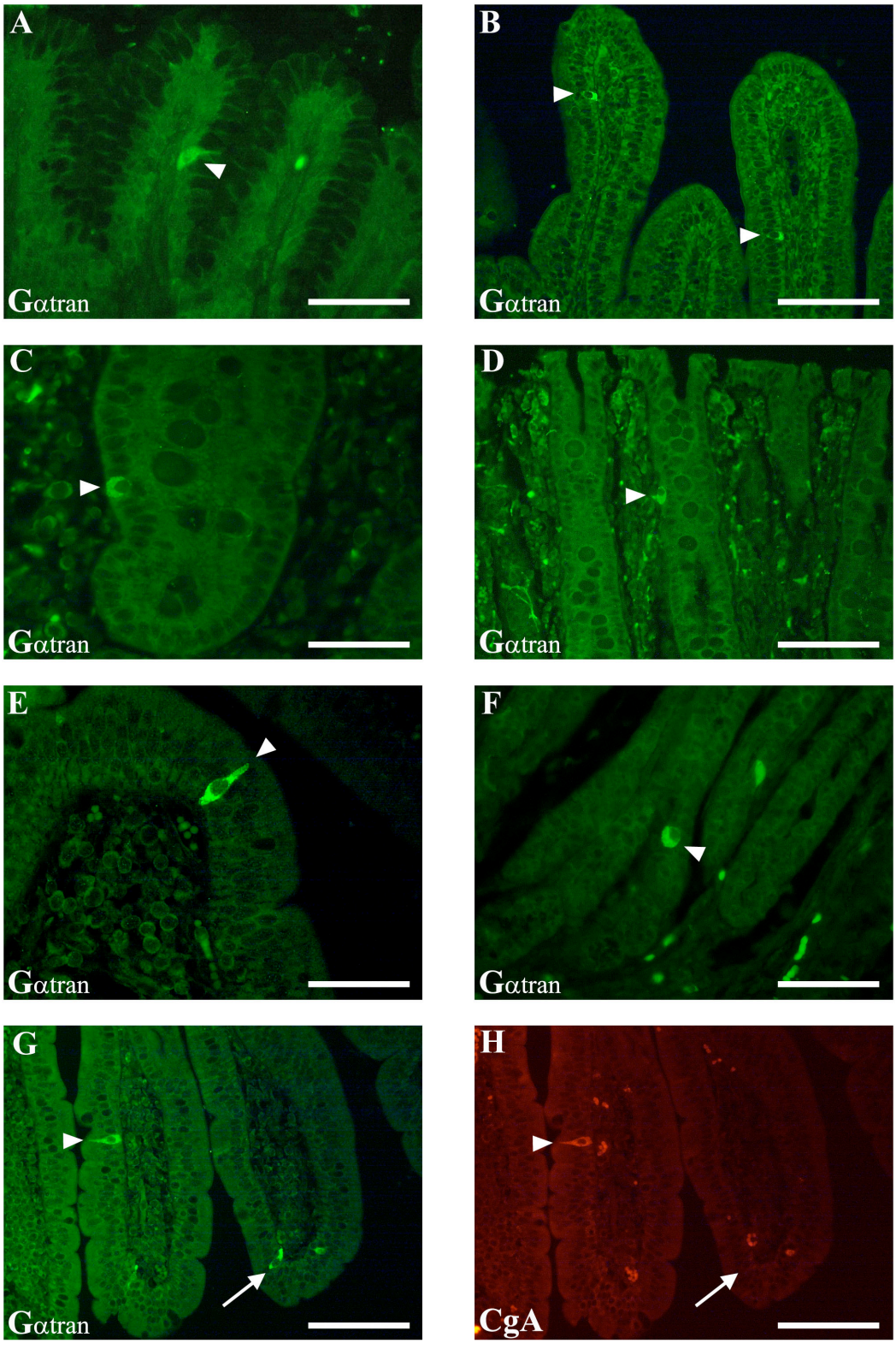

A
Gαtran
B
Gαtran
C
Gαtran
D
Gαtran
E
Gαtran
F
Gαtran
G
Gαtran
H
CgA

Figure 2

Graphs in A and B indicate the mean number of $G_{\alpha tran}$-IR cells in the different segments of the pig GI tract. Controls, fasted and refed are denoted as black, white and gray bars, respectively. Different letters indicate a significant ($P < 0.05$) statistical difference among groups. Values are expressed as mean ± SD.

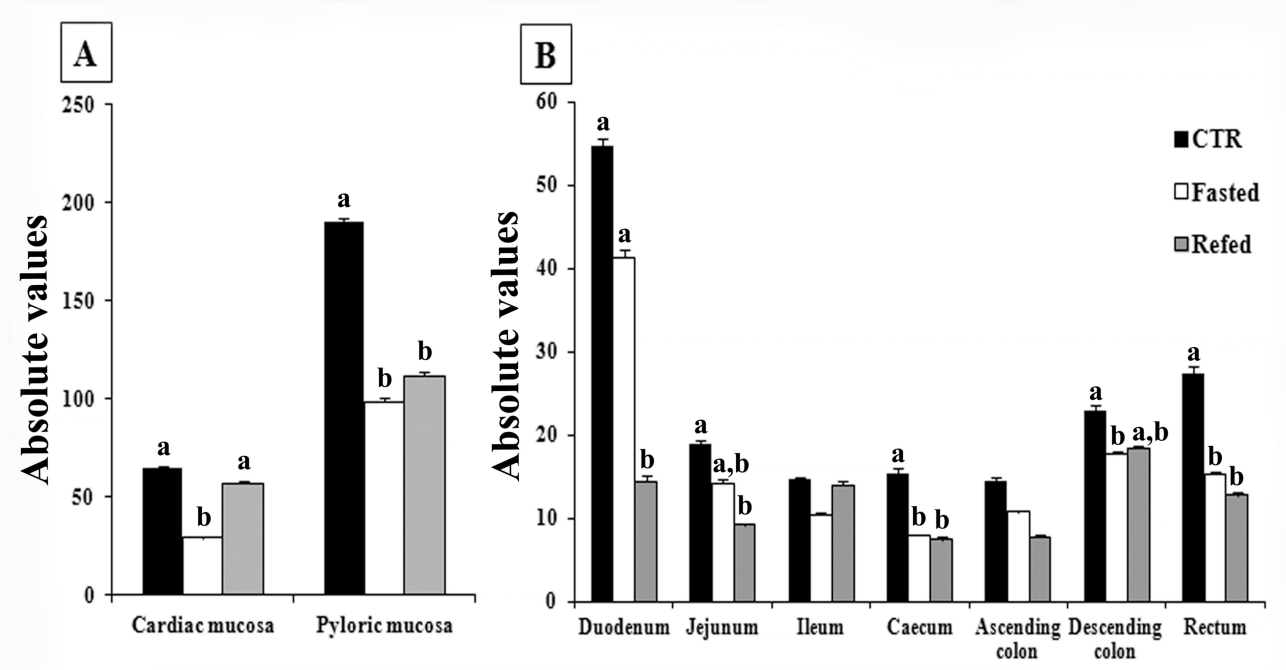
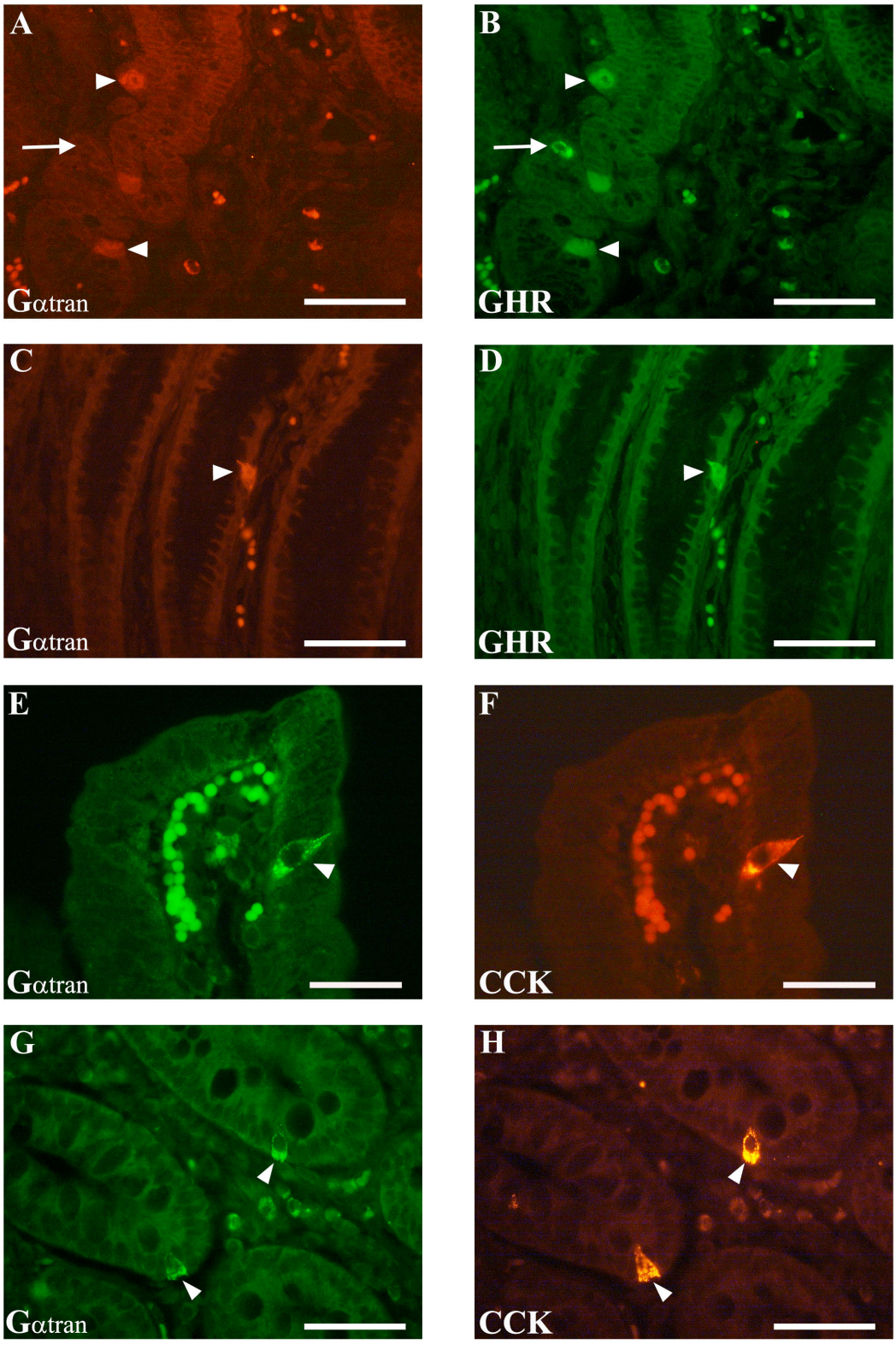


Figure 3

Colocalization of $G_{\alpha tran}$-IR (A, C, E and G, arrowheads) with ghrelin (GHR) in the pyloric mucosa (B and D, arrowheads) and cholecystokinin (CCK) in the jejunum (F and H, arrowheads). Generally the $G_{\alpha tran}$ / GHR labeled cells were found lying close to the basal lamina of the glands (typical closed-type morphology) (A and B, arrowheads); the arrows in A and B indicate a GHR-IR cell (B) not containing $G_{\alpha tran}$-IR (A). In some cases, $G_{\alpha tran}$ / GHR-IR cells were observed in the surface epithelium (typical open-type morphology) (C and D, arrowheads). The $G_{\alpha tran}$ / CCK immunopositive cells were observed in the villi (E and F, arrowheads) and in the intestinal gland of the jejunum (G and H, arrowheads). A, B, C, D, G and H: scale bars = 50µm; E and F: scale bars = 30µm.


A
Gαtran
B
GHR
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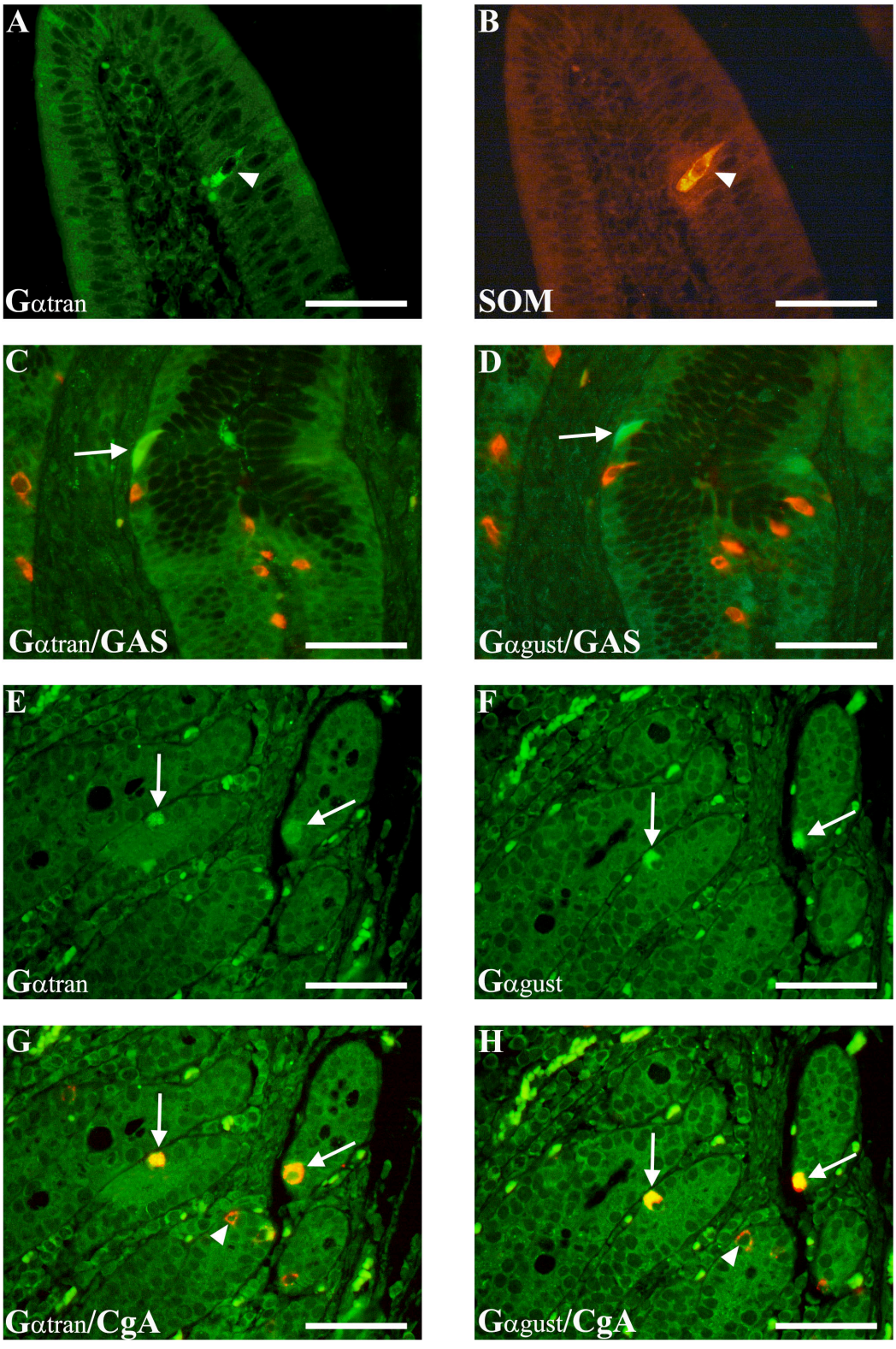
Gαtran
D
GHR
E
Gαtran
F
CCK
G
Gαtran
H
CCK

Figure 4

Enteroendocrine cells of the duodenum co-expressing $G_{\alpha tran}$ and SOM-IR (A and B, arrowheads). Some cells co-expressing $G_{\alpha tran}$ / $G_{\alpha gust}$-IRs (C and D, arrows) (in green) were observed in the pyloric mucosa; these cells were negative for gastrin (GAS-IR) (in red). Photomicrographs E and F showed co-expressing $G_{\alpha tran}$- and $G_{\alpha gust}$-IR enteroendocrine cells (in green) (arrows) in serial sections of the duodenum. The $G_{\alpha tran}$ and $G_{\alpha gust}$ colocalization is readily visible in G and H (merged images) with chromogranin A (CgA) (arrows) labeled by the red fluorochrome (arrowheads). A-H: scale bars = 50μm.


A
Gαtran
B
SOM
C
Gαtran/GAS
D
Gαgust/GAS
E
Gαtran
F
Gαgust
G
Gαtran/CgA
H
Gαgust/CgA

Supplementary data

Expression and regulation of α-transducin in the pig gastrointestinal tract

Western blot

Pig stomach (pyloric mucosa), and small intestine (duodenum, jejunum) were collected, frozen in liquid nitrogen, and stored at −80°C. Tissues were later thawed and homogenized directly into a sodium dodecyl sulfate (SDS) lysis solution (Tris-HCl 62.5 mM, pH 6.8; SDS 2%, 5% glycerol) with 0.1 mM phenylmethylsulfonylfluoride. Protein content of cellular lysates was determined by a Protein Assay Kit (TP0300; Sigma-Aldrich, St. Louis, MO).

For $G_{\alpha gust}$ and $G_{\alpha trans}$ antibodies specificity studies, aliquots containing 20 μg of proteins from tissue extracts were separated on NuPage 4-12% bis-Tris Gel (Gibco-Invitrogen, Paisley, UK) for 50 minutes at 200V. Proteins were then electrophoretically transferred onto a nitrocellulose membrane. Blots were washed in PBS and protein transfer was checked by staining the nitrocellulose membranes with 0.2% Ponceau Red. Following blocking treatment, the membranes were incubated at 4°C overnight with the respective antibodies in Tris-buffered saline-T20 (TBS-T20 20 mM Tris-HCl, pH 7.4, 500 mM NaCl, 0.1% T-20): anti-$G_{\alpha gust}$ rabbit polyclonal antibody at 1:300; anti- $G_{\alpha trans}$ rabbit polyclonal antibody at 1:500.

For GAS/CCK antibody specificity studies, aliquots containing 30 μg of proteins from tissue extracts were separated on Novex 18% Tris-Glycine Gel (Gibco-Invitrogen, Paisley, UK) for 90 minutes at 125V. Proteins were then electrophoretically transferred onto a nitrocellulose membrane. Blots were washed in PBS and protein transfer was checked by staining the nitrocellulose membranes with 0.2% Ponceau Red. Following blocking treatment, the membranes were incubated at 4°C overnight with anti-GAS/CCK mouse monoclonal antibody at1:1,000.

Following several washings with PBS-T20, the membranes were incubated with the secondary biotin-conjugated antibody and then with a 1:1,000 dilution of an anti-biotin horseradish peroxidase linked antibody. Western Blots were developed using chemiluminescent substrate (Super Signal West Pico Chemiluminescent Substrate, Pierce Biotechnology, Rockford, IL) according to the manufacturer's instructions. The intensity of

luminescent signal of the bands was acquired by Fluor-STM Multimager using the Quantity One Software (Bio-Rad Laboratories, Hercules, CA).

For $G_{\alpha trans}$ antibody, a major band of ≈45 kDa (theoretical molecular weight 40 kDa in human) was present in extracts from the stomach and intestine (Fig. 1). As positive control tissue we used mouse brain.

For $G_{\alpha gust}$ antibody, a major band of ≈40 kDa (theoretical molecular weight 40 kDa in human) was present in extracts from the stomach and intestine (Fig. 2). As positive control tissue we used mouse brain.

Different molecular forms of CCK have been described deriving from enzymatic cleavage of a precursor peptide of 115 AA (UNIPROT P06307) so the expected molecular weight of CCK is between 4 and 20 kDa. Fig. 3 shows a major band showing with a molecular weight of ≈15 Da in the intestine. The smallest form of CCK (10 different chains from 58 to 5 AA) could not be identified probably due to the very low amount of each component present in the tissue.

Fig. 1 Western Blot of $G_{\alpha trans}$ antibody shows a major band close to the theoretical molecular weight (~45 kDa). Right lane: Molecular Weight Marker; the numbers inside this lane indicate the molecular weight. The images were slightly adjusted in brightness and contrast to match background. Lane 1 Mouse brain (positive control); lane 2 pig stomach; lane 3 pig intestine).

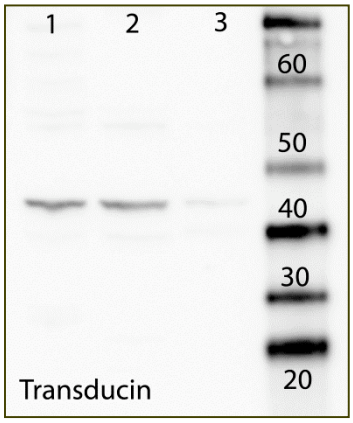


Fig. 2 Western Blot of $G_{\alpha gust}$ antibody shows a major band close to the theoretical molecular weight (~40 KDa). Right lane: Molecular Weight Marker; the numbers inside this lane indicate the molecular weight. The images were slightly adjusted in brightness and contrast to match background. Lane 1, mouse brain (positive control); lane 2 pig intestine; lane 3 pig stomach.

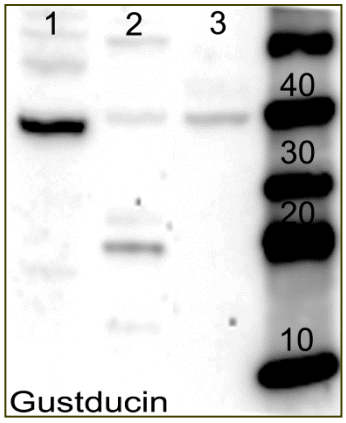
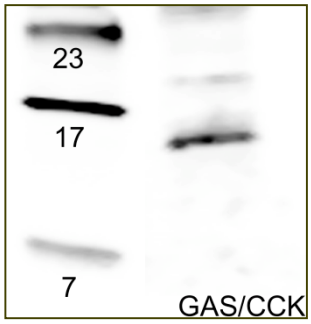


Fig. 3 Western Blot of GAS/CCK antibody shows a major band close to the theoretical molecular weight (~15) in the pig intestine. Left lane: Molecular Weight Marker; the numbers inside this lane indicate the molecular weight. The images were slightly adjusted in brightness and contrast to match background.



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CHAPTER 3

Activation of Enteroendocrine STC-1 Cell Signaling By Bitter Compounds and Bacteria Quorum Sensing Molecules (N-Acyl Homoserine Lactone)

Abstract

STC-1 enteroendocrine cell lines from the mouse small intestine are a well established model for studies of the enteroendocrine cell function. Enteroendocrine cells play a key role in the detection of luminal contents which range from nutrients to non-nutrient chemicals, including harmful substances, and perhaps bacteria, which are abundant in the gut lumen. STC-1 cells contain all the different types of hormones and peptides produced and released by eneteroendocrine cells in situ and the receptors for these signaling molecules. Recent studies have shown that STC-1 cells as eneteroendocine cells express taste receptors (TR), including bitter taste receptors and their signaling molecules, supporting the concept that TR represent the initial molecular mechanisms in the detection of luminal contents, including molecules producded by bacteria. Specifically, we were interested in exploring the possibility that N-acyl homoserine lactone (AHL), a quorum sensing (QS) molecule produced by Gram-negative bacteria activate enteroendocrine cells through the same pathway activated by bitter TR, T2Rs. The reasoning behind this idea is twofold: a T2R subtype, the T2R138 is upregulated by long-term high fat diet inducing obesity, which is known to increase the proporption of Gram-negative bacteria in the gut lumen, which has ben proposed as a mechanism responsible for the chronic inflammation developing in obesity. In addition, AHL has been reported to activate T2Rs in the respiratory system, where it has been proposed as a mechanism of defense against bacteria. In this study, we compared the effect of increasing concentrations of phenylthiocarbamide (PTC), a T2R138 agonist, denatonium benzoate (DB), a T2R108 agonist, and AHL and measured

phosphorylation of $MAPK_{p44/42}$ as outocome of cellular activation in the presence or absence of nitrendipine, a L-type voltage-sensitive Ca^{2+} channel blocker, and the bisindolylmaleimide I (GF-1), an inhibitor of protein kinase C and Probenecid an inhibitor of some T2Rs to characterize the $MAPK_{p44/42}$ signaling pathway. The study was carried out with a dose responce experiment, tracting the STC-1 cells with different bitter agonist and antagonist substances, and we measured $MAPK_{p44/42}$ phosphorylation as outcome of receptor activation using Western blotting and $MAPk_{44/42}$/$tERK_{1/2}$ antibodies. The results obtained show that PTC, DB, and AHL activate the $MAPK_{p44/42}$ cascade in a dose-dependent and time dependent manner. Nitrendipine blocked the DB-induced $MAPK_{p44/42}$ phosphorylation, wheras it did not affect the PTC and AHL-induced $MAPK_{p44/42}$ activation. By contrast, protein kinase C inhibitor markedly reduced the PTC and AHL-induced $MAPK_{p44/42}$ phosphorylation, but did not modified the DB-induced activation of $MAPK_{p44/42}$, Probenecid blocked the DB, PTC and AHL-induced $MAPK_{p44/42}$ phosphorylation. Moreover calcium imaging experiments carried out in STC-1 cells shown an increase of intracellular calcium under AHL and PTC stimulation. These studies show that T2R agonist and QS molecule activate $MAPK_{p44/42}$ signaling in STC-1 cells, through distinct pathways and that QS molecule activate the same pathway as T2R138. This suggests that STC-1 cells are activated by molecules produced by bacteria through a G-protein mediated pathway that might involve T2R138.

Introduction

a. Intestinal STC-1 cells and bitter taste compounds

The gustatory system detects nutrient compounds but also harmful and toxic compounds [1] [2]. The chemosensory information received during the phase of the gastric and intestinal digestion are critical for the regulation of various aspects of gastrointestinal functions such as the secretion of intestinal glands, nutrient absorption and digestion, motility, blood supply and satiety [3]. The enteroendocrine cells (ECCs) are specialized transducers of luminal factors, and they are very important for the control of digestion and food intake. EECs are 1% of the total number of cells in the gastrointestinal tract and represent the

largest endocrine organ of our body. These cells contain secretory granules and produce a variety of signaling molecules when they detect signals from the luminal content, which they translate to the sensory nerve terminals in the subepithelial space by releasing their secretory products [3] [4] [5]. The mediators are different according to the different types of ECCs and the same ECCs produce and release different mediators according to the stimuli [6] [7]. ECCs are present throughout the gastrointestinal wall, but they are scattered with irregular localization, and are difficult to isolate, for this reason many researchers prefer to use the endocrine cell line STC-1, which are well characterized and contain the same signaling molecules as the EECs in situ, thus representing a good model for studying chemisensing mechanisms in the gastrointestinal tract. STC1 cell lines derive from an endocrine tumor that developed in the small intestine of a double transgenic mouse expressing the rat insulin promoters linked to SV40 large T antigen and to the polyomavirus small T antiger [8]. STC-1 cells have been extensively used to study the mechanisms regulating peptide release in response to bombesin / gastrin [9], free fatty acids [10], leptin [11], orexin [12] and aminoacids [13]. Recently, Wu et all (2002) have shown that STC-1 cells express T2R family members and respond to bitter compounds. It was also demonstrated that not only the numerous T2RS genes but also taste signaling molecule α-gustducina and α-transducin are expressed in STC-1 cells [14] [15] [16]. Bitter tastants cause an increase in intracellular calcium via the activation of L-type voltage-sensitive calcium channels [16] as monitored by functional calcium imaging aproaches, indicating the presence of functional receptors in these cells. Enteroendocrine STC-1 cells release gastrointestinalpeptides, such as CCK, in response to stimulation with bitter substances (DB) [16]. Saitoh and colleagues (2007) have shown that STC-1 cells respond not only to bitter compounds, but to all five basic taste stimuli [16] [17] [18]. Recently it has been shown that the ECCs express toll-like receptors (TLR), which are molecules that recognize bacterial breakdown products, such as lipopolysaccharide (LPS), bacterial lipoproteins, double stranded DNA and flagellin. Using RT-PCR and Western Blot TRL4, 5 and 9 mRNA and proteins have been found in STC-1 cells. Moreover, the activation of these receptors by LPS induced secretion of CCK [19] [20]. These findings suggest that the ECCs are likely involved in the perception of tastants and bacterial secretions and thus might participate in the defense of intestinal mucosa. There is now increasing evidence supporting the concept that alterations of intestinal microflora and bacterial secretions may preceed the inflammatory processes that develop in obesity [22] [23] [24]. STC-1 cells are a good experimental model to test the hypothesis that bitter ligands and bacteria activate gut

ECCs. Therefore, we used STC-1 cells to explore the possibility that T2Rs might serve as a defense mechanism againt bacteria.

B Role of mitogen-activated protein kinase ($pMAPk_{p44/42}$) in bitter signaling

A large group of integral membrane receptors (G-protein coupled receptors) transmit from a diverse array of external stimuli, including neurotransmitters, hormones, phospholipids, photon, odorant and taste ligands. In response to binding ligand, the GPCRs activate diverse downstream signaling pathways, such as mitogen-activated protein kinase cascade (MAPK), including extracellular signal-related kinase (ERK), Jun N-terminal kinase (JNK), p38MAPk and big MAPK (BMK). MAPK signaling results in stimulus-dependent nuclear translocation of kinases , which in turn regulate gene expression and the cytoplasmatic acute response to mitogenic, differentiation, proliferation, stress related, apoptotic and survival stimuli [25] [26] [27] [28] [29]. MAPK pathways work through sequential phosphorylation events (known as MAPK cascade), and MAPK is the terminal enzyme in this three-kinase cascade: MAP kinase, MAP kinase kinase (MKK, MEK, or MAP2K) and MAP kinase kinase kinase (MKKK, MEKK or MAP3K), which are activated in series, as shown in (**Fig 1**).

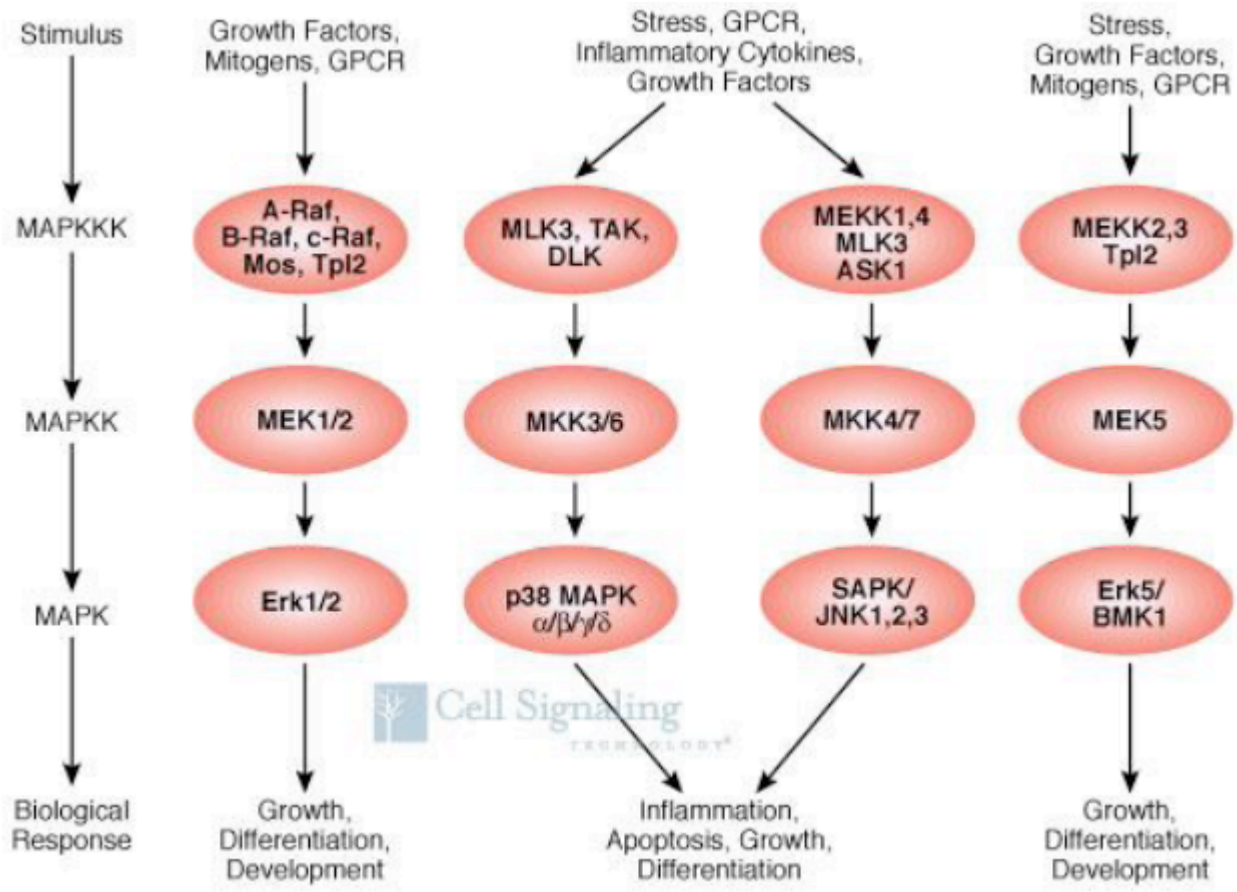

Fig 1. Different Mapk pathways, in all of them MAPKKK is activated by extracellular stimuli, and phosphorilates a MAPKK and this MAPK activate a MAP Kinase trought another phosphorilation leading to a biological responces (Cell signaling technology, http//:www.cellsignal.com/reference//pathway/MAPK_cascades.html).

The down regulation of MAPKs is via dephosphorylation by a class of proteins, such as serine / threonine phosphatases, tyrosine phosphatases, or dual-specificity phosphatases and via feedback inhibitors involving the phosphorylation of upstream molecules. In mammals, four distinct MAPK cascades are known to date (see above), but the most extensively studied are MAPK/ERK (including ERK1; ERK2; ERK3/ERK4, ERK5, ERK 7/8), SAPK/JNK (including JNK1, JNK2 and JNK3) and the p38 MAPK (including p38alpha, p38beta2, p38gamma and p38delta). ERK1 and ERK2, also known as classical $MAPK_{p44/42}$ signaling pathway, are expressed almost in every tissue and were the first of the ERK/MAPK subfamily to be cloned and they represent the best characterized pathway for MAPKs. For this reason we chose ERK1/2 as a target to measure. The ERK1/2 pathway is activated by growth factors and phorbol ester (a tumor promoter) and regulates cell proliferation and cell differentiation. Phosphorylation of $MAPK_{p44/42}$ has been used to study the activation of the intracellular pathway in response to various ligands for G protein-

coupled receptors. In this study, we evaluated $pMAPK_{p44/42}$ in relation to total expression of ERK1/2 in STC-1 cell to determine whether AHL phosphorylated $MAPK_{p44/42}$ and whether it activates the same pathway as T2R ligands.

c Quorum sensing molecule: N-Acyl-Homoserine Lactone

Cell to cell signaling with small chemical molecules is a process shared by many living organisms; mammals produce hormones, insects produce pheromones, and bacteria produce autoinducers. It conveys important information about the status of the cells and about the extracellular environment. In bacteria cell to cell communication at a specific threshold is crucial for symbiotic and pathogenic interaction with plants and animals, and host immune responses are important mechanisms to develop the infection process. Thus bacterial quorum sensing (QS) is a sophisticated prokaryotic method for coordinating their behavior with the secretion of chemical or peptide signal [30]. In fact, bacterial cells sense their population density by QS signaling and regulate the expression of some genes only when the cell density reaches a specific threshold [31] [32]. The exoproteases, siderophores, exotoxins and lipases are virulence bacterial factors controlled by QS, and QS is fundamental for the pathogen to be successful [33] [34] [35]. N-Acyl Homoserine Lactone is the most studied QS signaling system used by a large number of gram negative bacteria. The host immune response is modulated by different pathogens using different strategies and with different structural varieties between AHLs from different bacteria and even between AHLs synthesized by the same bacterium; most modifications are in the Acyl rings. AHLs regulate different genes, and have different regulatory mechanisms [35] [36] [37]. Many of these mechanisms are regulated by proteins related to LuxR and LuxI; at low cell density, the AHL is at low concentration, but at high cell density the production of autoinducer is high and it can accumulate to sufficient concentrations to activate lux genes.[38]. Probably the receptor for AHLs signal is a member of LUXR transcriptional regulator family, these family members have two domains, a C-terminal DNA-binding domain, and an N-terminal AHL-binding domain [39] (**fig 2**). Recently, Tizzano and colleagues [40] have shown that airways epithelial cells may be able to respond to AHLs produced by gram-negative bacteria with a mechanism involving taste receptors. Other studies showed how different lactones such as sesquiterpene, are natural bitter substances

that have the ability to activate bitter taste receptors, these lactones are found in vegetables and culinary herbs as well as in aromatic and medicinal plants [41].

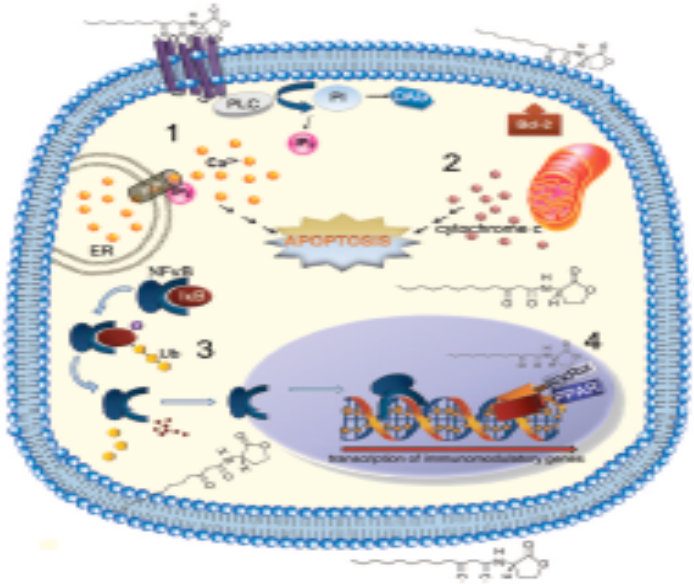

Fig 2. Mechanisms mediating AHL effects of mammalian cells. 1.AHL induced apoptosis with increasing intacellular Ca^{2+} levels. 2. Intracellular damage inhibits the protective effects of BCL-2 on mitochondria, resulting in membrane damage and apoptosis. 3. NF-*k*B has been demonstrated to be a key trascriptional factor. 4. The nuclear hormone receptor PPAR was the first eukaryotic receptor shown to directly interact with an AHL (Teplitski M. *et al* 2011 Chem Rev.).

Previous results obtained from the lab of Professor Catia Sternini showed up-regulation of mT2R138 and Gust in the colon of mice fed long-term high fat diet inducing obesity, which is known to be associated with alterations of bacteria levels in the gut lumen suggesting that T2R activation might play a role in the inflammatory processes developing in obesity as result of changes in the intestinal bacterial population. In this study we used N-(3-Oxodecanoyl)-L-homoserine lactone, which is used as an autoinducer of quorum signaling by *Pseudomonas putida, Yersinia enterocolitica* and other gram-negative bacteria, to investigate whether AHL signaling activates small intestinal enteroendocrine cells in millimolar concentrations, and whether T2Rs are involved in this process

We measured $MAPk_{p44/42}$ phosphorylation as outcome of receptor activation using western botting and $MAPK_{44/42}$/$tERK_{1/2}$ antibodies to establish whether QS produced by Gram-negative bacteria activate STC-1 cells in comparison with agonist acting at selective T2Rs. How positive control for AHL function we used Probenecid that is an antagonist for bitter taste receptor.

Probenecid is FDA approved inhibitor of the organic anion transporter Multidrug Resistence Protein 1 (MRP1) [42] [43], and is clinically used to treat gout in humans [44],

in lab probenecid is commonly used as a substances that prevent the efflux of calcium-sensitive fluorescent dyes during experiment of cellular calcium mobilization [45]. Greene and colleagues (2011) during the course of their studies of bitter taste receptor signaling, accidentally discovered that the probenecid inhibited the activation of different bitter taste receptors as hT2R16 , hT2R38 and other in response to its cognate ligand salicilin and PTC respectively [46]. The activation was rapid and was independent of probenecid's activity as a transport inhibitor, suggesting that probenecid interacts with the receptor rather than modulating downstream signaling processes [46]. Using probenecid as selective blockers of T2Rs we suppose that AHL have to be blocked by probenecid and so show the involment of T2R in AHL activation of $MAPk_{p44/42}$ in STC-1 cells.

Further study have to be done about the relationship between AHL and T2R., our next step is to use siRNA to silence T2R138 to confirm that AHL acts through this receptors

Material and Method

a. Reagents

Denatonium Benzoate (DB, D5765), Phenylthiocarbamide (PTC, P7629), N-(3-Oxodecanoyl)-L-homoserine lactone (AHL, O9014), GF-1, (B3306), nitrendipine (N144) and Probenecid (P-8761), were purchased from Sigma.

DB and PTC were dissolved at 30 mM in water and used at final concentrations of 0.5mM, 1mM, 2.5mM and 5mM for 3 minutes, AHL was dissolved at 50 mM in DMSO and used at final concentrations of 0.005mM, 0.025mM, 0.1mM and 0.25mM, each containing 0.2 % DMO, which was not toxic to cells for the time of the experiment (10 min).

GF-1 and nitrendipine were dissolved in DMSO for a stock solution of 2 mM and 0.1 mM respectively and used at a final concentration of 5µM and 1 µM in DMEM. Probenecid was dissolved at 500 mM in 1N NaOH and titrated to pH 7.0 and used at final concentration of 2.5mM in DMEM.

b. Cell lines and treatments

STC-1 mouse cell lines were a gift from Dr. Rozengurt, CURE Digestive Diseases Research Center, Division of Digestive Diseases, David Geffen School of Medicine, UCLA, Los Angeles, CA. STC-1 cell line was cultured in DMEM + GlutaMax + 10% FBS and 1xPenStrep (GIBCO 15140-122). The culture was kept at 37°C in 5% CO_2 atmosphere. Cells were starved 1h before the experiments with media without FBS and treated with bitter agonists (DB and PTC 0.5 to 5mM per 3min) or AHL (0.05 to 0.25mM per 10 min) with or without 1h pre-incubation with either Probenecid (2.5mM), GF-1 (5 μM) or nitrendipine (1 μM).

We show that probenecid specifically inhibits the cellular response mediated by the bitter taste receptor hTAS2R16 and provide molecular and pharmacological evidence for direct interaction with this GPCR using a non-competitive (allosteric) mechanism.

c. Western Blot

Cells were lysed in 2× SDS-polyacrylamide gel electrophoresis sample buffer (20 mM Tris-HCl, pH 6.8, 6% SDS, 2 mM EDTA, 4% 2-mercaptoethanol, 10% glycerol) on ice and boiled for 10 min. After SDS/PAGE, proteins were transferred on PVDF membranes. The membranes were blocked for 1h at room temperature in blocking buffer (LI-COR®), incubated at 4°C overnight with antibodies specifically recognizing pMAPK p44/42 (9106, dil 1:1000, Cell Signaling) and ERK-2 (sc-154, dil 1:500, Santa Cruz Biotechnologies). Immunoreactive bands were visualized by using infrared fluorescent secondary antibodies (IRDye 800 Goat anti Mouse, dil 1:10000, and IRDye 680 Goat anti Rabbit, dil 1:10000; LI-COR Biosciences). Images were collected using the LI-COR Odyssey infrared imaging system and analyzed with the 3.0 associated software.

Calcium Imaging

STC-1 cells were plated and cultured on 12mm coverslips in 2.0cm^2 wells in Dulbecco's Modified Eagle Medium (DMEM, Gibco®) supplemented with 10% FBS and antibiotics (100 U/ml penicillin and 100 µM/ml streptomycin). STC-1 cells were washed twice with Hank's Balanced Stock Solution (HBSS, Gibco®) (composition in table 1) containing 20 mM HEPES, adjusted to pH 7.4 with NaOH, before loading with 1.5µM fura-4AM (0.3µl of 1mM stock in DMSO, Life Technology Corporation, Invitrogen, USA) in Hibernate®A for 7 minutes at room temperature in the dark.

The coverslips were then transferred to a microscope chamber mounted onto a Zeiss Axioplan 2 upright microscope that used an eight channel gravity driven fast flow superfusion system (ALA Scientific, Farmingdale, NY, USA) to deliver the solutions. Coverslips were superfused with HBSS+HEPES for 20 minutes at room temperature before imaging. STC-1 cells were monitored at 20 second intervals for 10 minutes prior to drug application. The application of PTC (3mM and 5mM) and AHL (0.5mM and 1mM) was superfused for a duration of 25 seconds with images taken at 1 second intervals. Images were acquired on a Zeiss LSM 5 Pascal using a water-immersion Axoplan 40x (NA 0.8) objective. The excitation was provided by the 488 nm line of the argon laser, while the photomultiplier tube collected the emission through a 505 nm LP filter. Fluorescence intensity values were collected by setting regions of interest (ROIs) on the STC-1 cell bodies.

Components	Molecular Weight	Concentration (mg/L)	mM
Inorganic Salts			
Calcium Chloride ($CaCl_2$) (anhyd.)	111	140	1.26
Magnesium Chloride ($MgCl_2$-$6H_2O$)	203	100	0.493
Magnesium Sulfate ($MgSO_4$-$7H_2O$)	246	100	0.407
Potassium Chloride (KCl)	75	400	5.33
Potassium Phosphate monobasic (KH_2PO_4)	136	60	0.441
Sodium Bicarbonate ($NaHCO_3$)	84	350	4.17
Sodium Chloride (NaCl)	58	8000	137.93
Sodium Phosphate dibasic (Na_2HPO_4) anhydrous	142	48	0.338

Table 1. Molecular composition of HBSS solution.

Results

a. Bitter stimuli induce a rapid dose-dependent MAPKp44/42 phosphorylation in STC-1 cells

WB analysis showed that STC-1 cells responded to bitter ligands DB and PTC with MAPK $_{p44/42}$ phosphorylation. The activation is dose dependent. DB induced a significant phosphorylation of $MAPK_{44/42}$ at 2.5 mM (723.79% ± 149; $P \leq 0.05$) (**Fig 3a**) and PTC at 2.5mM (345.40% ± 91,65 $P \leq 0.05$) (**Fig 3b**) In a parallel experiment carried out in the lab of Professor Catia Sternini, IEC-18 cells, a mouse small intestine cell line not expressing TRs, were not activated by either PTC and DB.

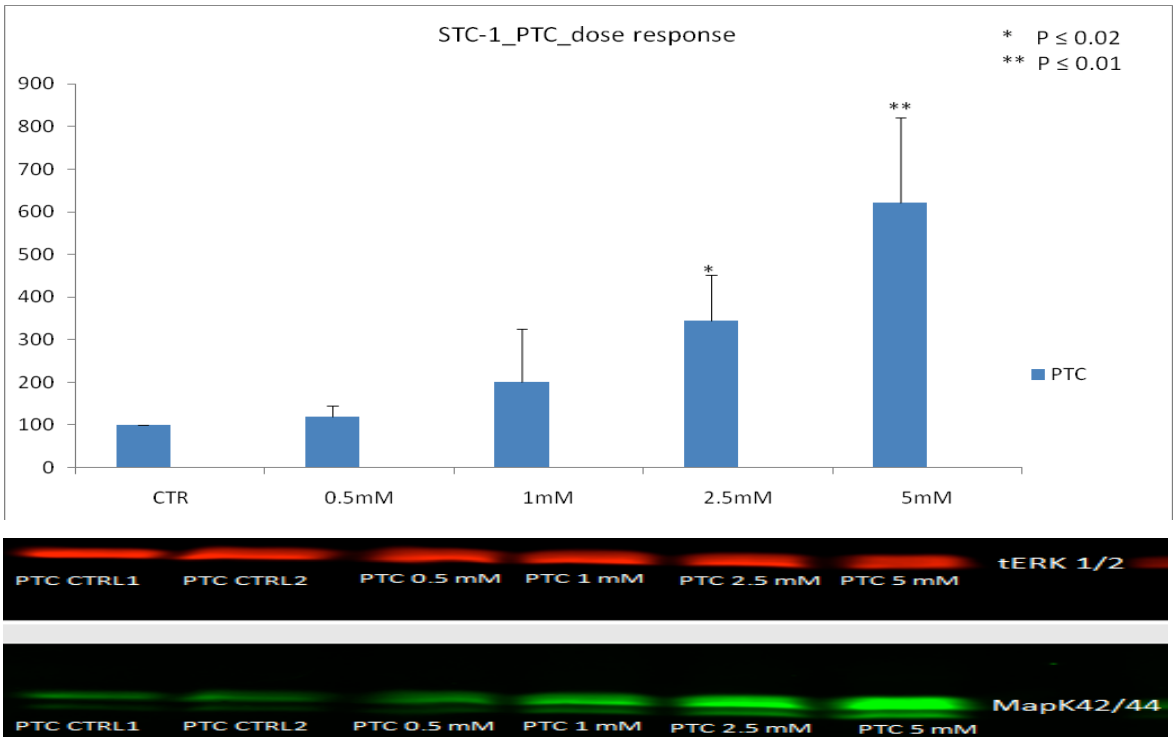

Fig 3a. WB results showing the % of phosphorylation for $MAPK_{p44/42}$ on STC-1 after 3'incubation with increasing PTC concentration. * $P \leq 0.02$ vs PTC CTR, ** $P \leq 0.01$ vs PTC CTR. Representative WB membranes showing pMAPK $_{p44/42}$ after PTC stimulation.

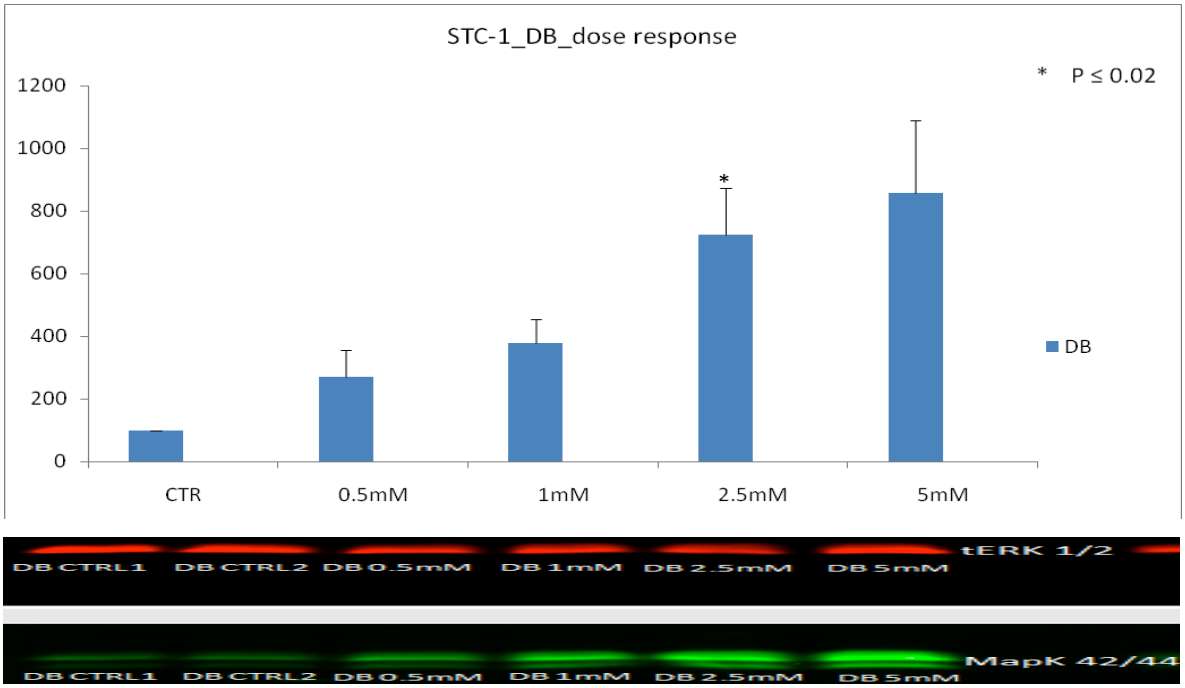

Fig 3b. WB results showing the % of phosphorylation for $MAPK_{p44/42}$ on STC-1 after 3'incubation with increasing DB concentration. * P ≤ 0.02 vs DB CTR. Representative WB membranes showing pMAPK $_{p44/42}$ after DB stimulation.

b. Different T2Rs subtypes show different pathways leading to $MAPK_{p44/42}$ activation

To further investigate the signaling pathway following T2Rs activation we used GF-1, a protein kinase C inhibitor, and nitrendipine, an L-type voltage-sensitive Ca^{2+} channels blocker, a probenecid an inhibitor of many T2Rs and measured pMAPK following PTC, DB or AHL stimulation with or without these drugs treatment. When STC-1 cells were treated with increasing concentrations of PTC or DB in the presence of GF-1, PTC induced phosphorylation of MAPK was markedly reduced (**fig 4a**) whereas DB effect was not modified (**Fig 4b**). By contrast, nitrendipine blocked DB induced MAPK phosphorylation (**Fig 4c**), but did not affect PTC activation of MAPK (**Fig 4d**).

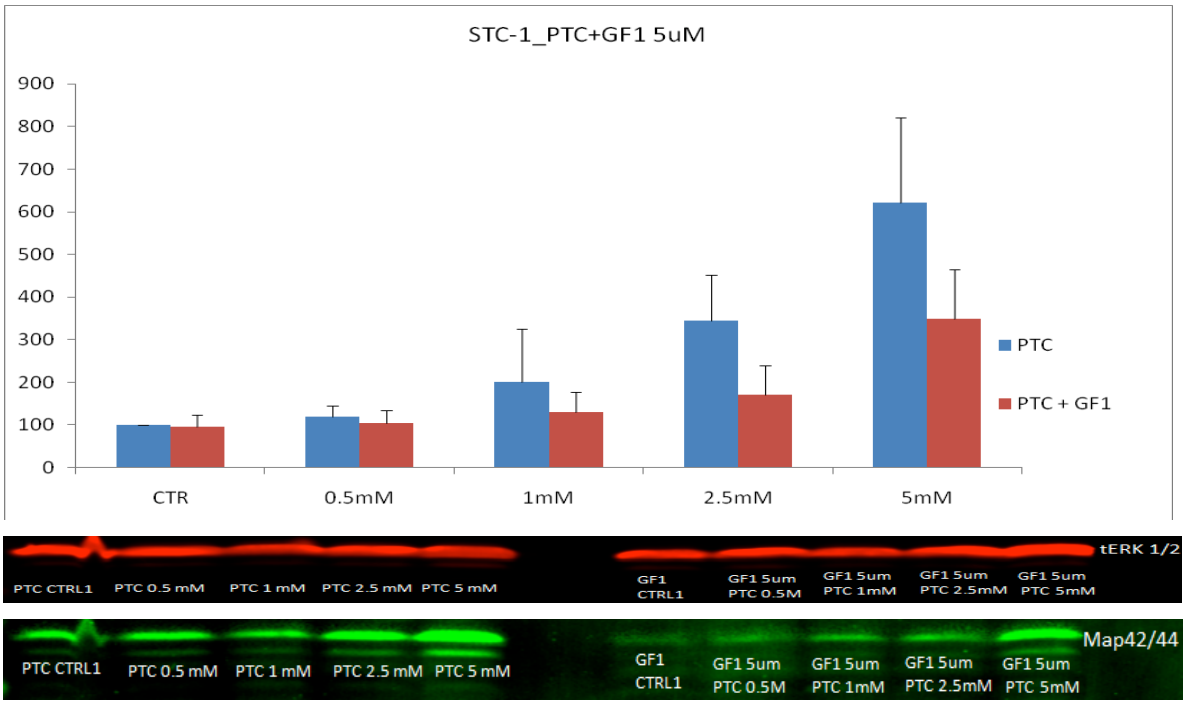

Fig 4a. PTC induces MAPk$_{p44/42}$ phosphorilation, is blocked by GF1 in a dose dependent manner in STC-1 cells. Representative WB membranes showing pMAPK$_{p44/42}$ after PTC stimulation,in presence of GF-1.

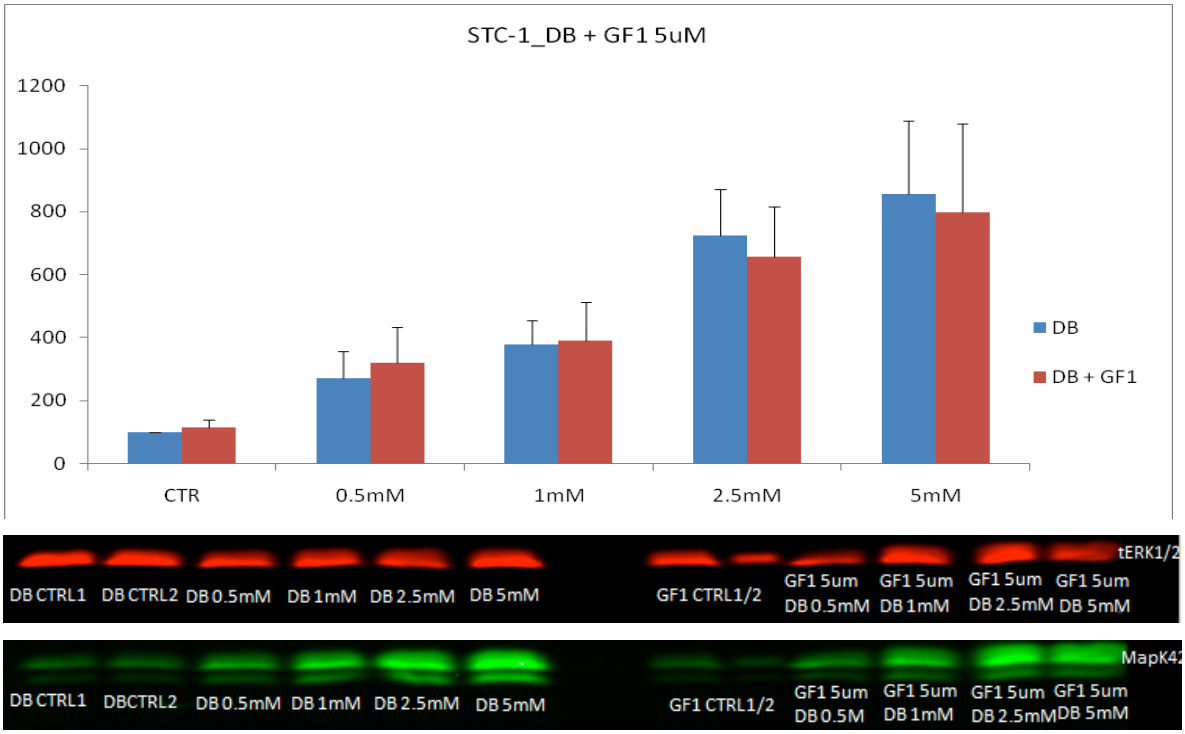

Fig 4b. DB induces MAPK$_{p44/42}$ phosphorilation, it is not blocked by GF1 in STC-1 cells. Representative WB membranes showing pMAPK $_{p44/42}$ after DB stimulation, in presence of GF-1.

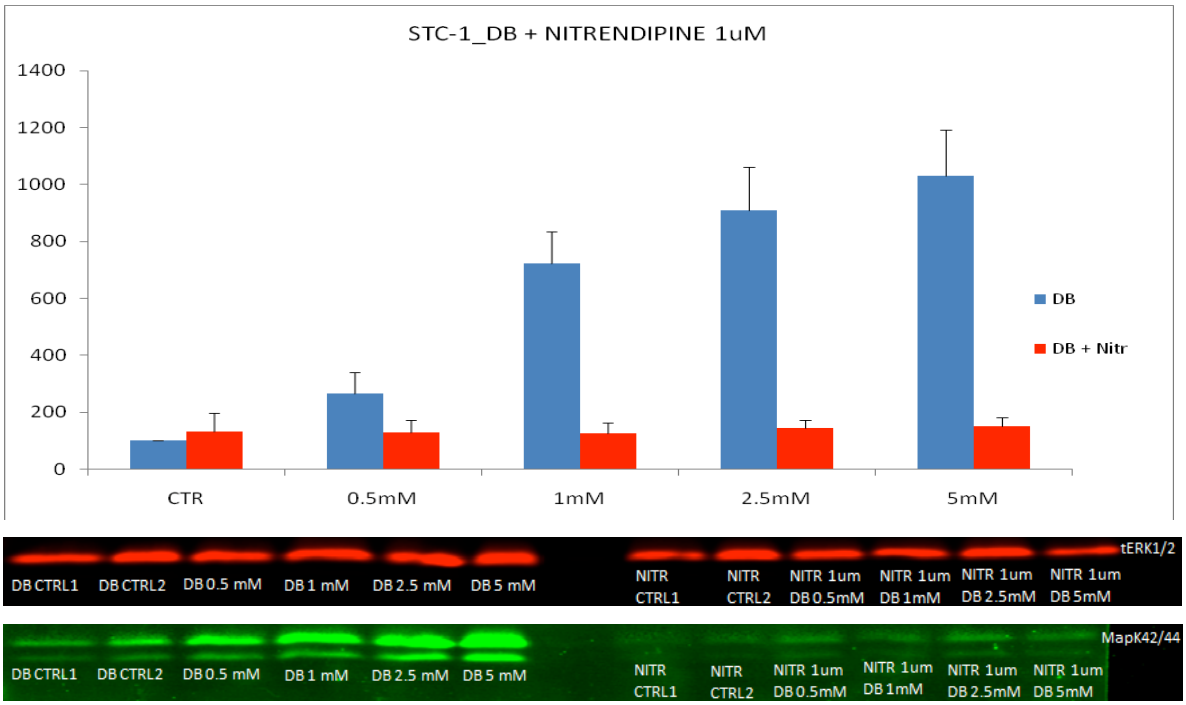

Fig 4c. DB induces $MAPK_{p44/42}$ phosphorilation, is blocked by Nitrendipine in a dose dependent manner in STC-1 cells. Representative WB membranes showing $pMAPK_{p44/42}$ after DB stimulation, in presence of nitrendipine.

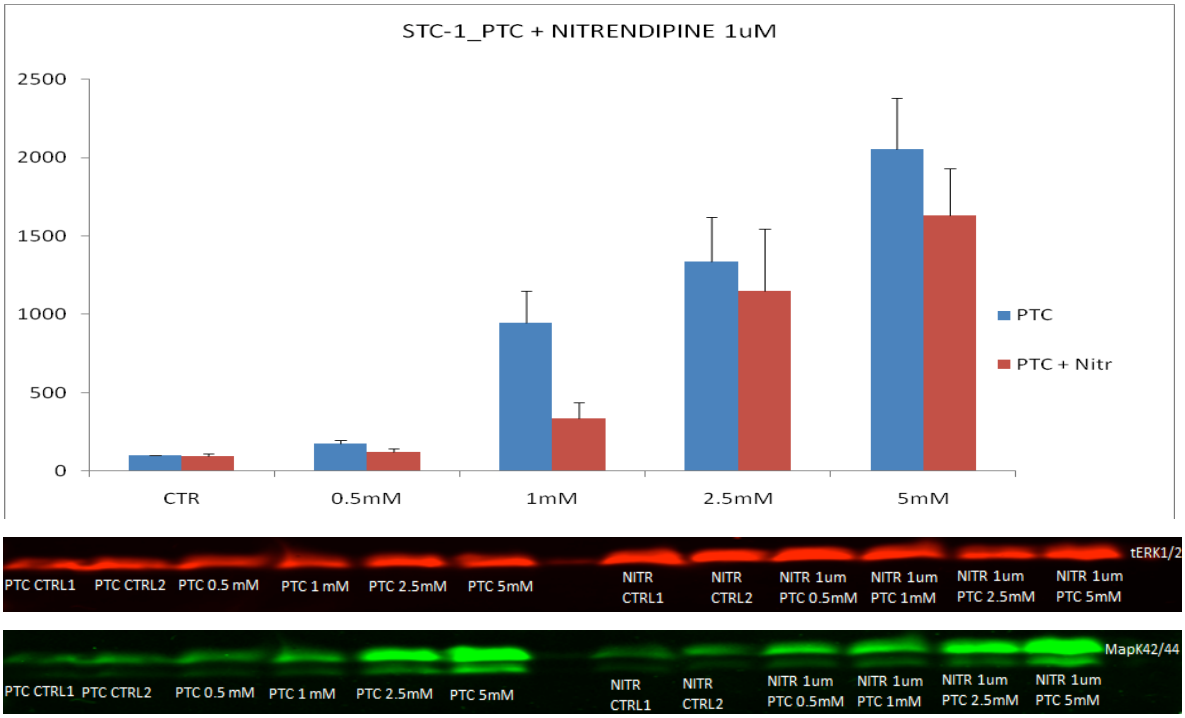

Fig 4d. PTC induces $MAPK_{p42/44}$ phosphorilation, it is not blocked by Nitrendipine in STC-1 cells. Representative WB membranes showing $MAPK_{p44/42}$ after PTC stimulation, in presence of nitrendipine.

c. STC-1 cell lines expressing T2Rs are activated by N-(3-Oxodecanoyl)-L-homoserine lactone, possibly through T2R138/T2R38?

STC-1 cell lines responded with $MAPK_{p44/42}$ phosphorylation when challenged with AHL at increasing mM concentrations (**Fig 5a**). Cells died with ≥0.25mM concentration. Preliminary data shows that both PTC and AHL induced MAPK phosphorilation, which is markedly reduced by GF-1 (**Fig 5b**) but it is not reduced by nitrendipine in STC-1 cells (**Fig 5c**), whereas DB-induced MAPK phosphorilation is blocked by nitrendipine but not by GF-1. Increasing mM concentration of AHL PTC and DB give $MAPk_{p44/42}$ phosphorylation, but when the cells are pretreated with Probenecid an inhibitor of T2Rs, the $MAPk_{p42/44}$ phosphorilation is blocked (**Fig 6a/6b)** this result demonstrate that AHL may use T2Rs for activate the transcription in STC-1 cells.

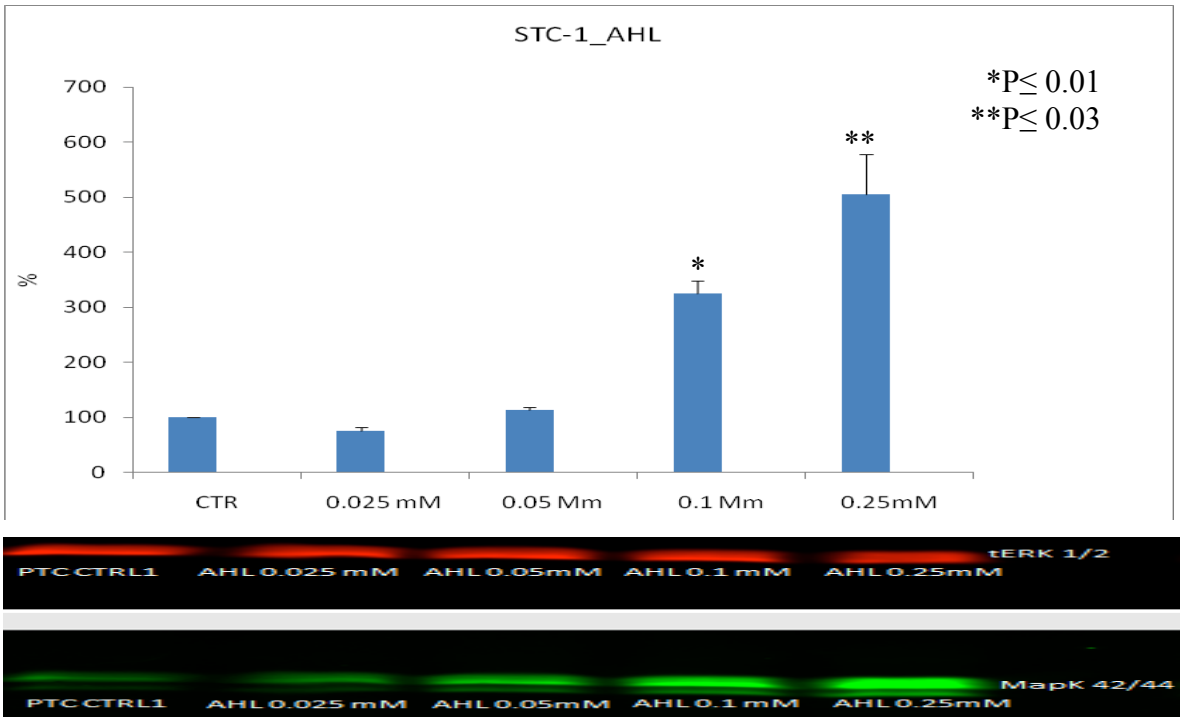

Fig 5a. WB results showing the % of phosphorylation for $MAPK_{p44/42}$ on STC-1 after 10' incubation with increasing AHL concentration. * P ≤ 0.01 vs AHL CTR, ** P≤ 0.03 vs AHL CTR. Representative WB membranes showing $MAPK_{p44/42}$ after AHL stimulation.

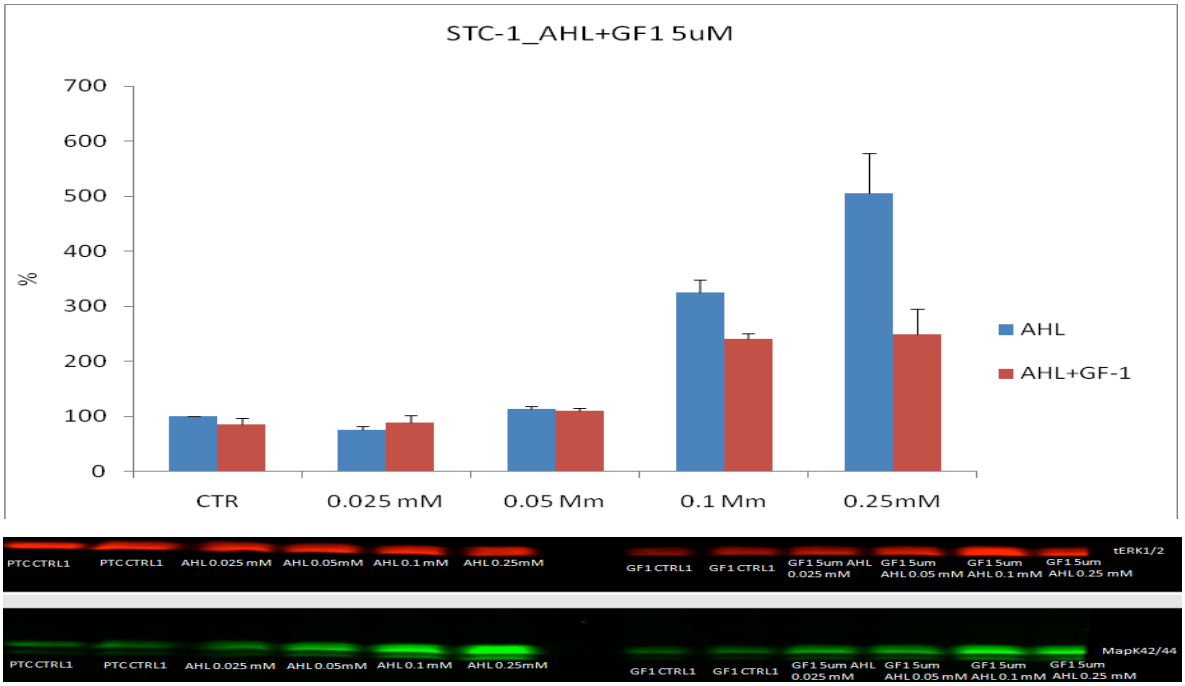

Fig 5b. AHL induces $MAPK_{p42/44}$ phosphorilation, is blocked by GF-1 in a dose dependent manner in STC-1 cells. Representative WB membranes showing $MAPK_{p44/42}$ after AHL stimulation, in presence of GF-1.

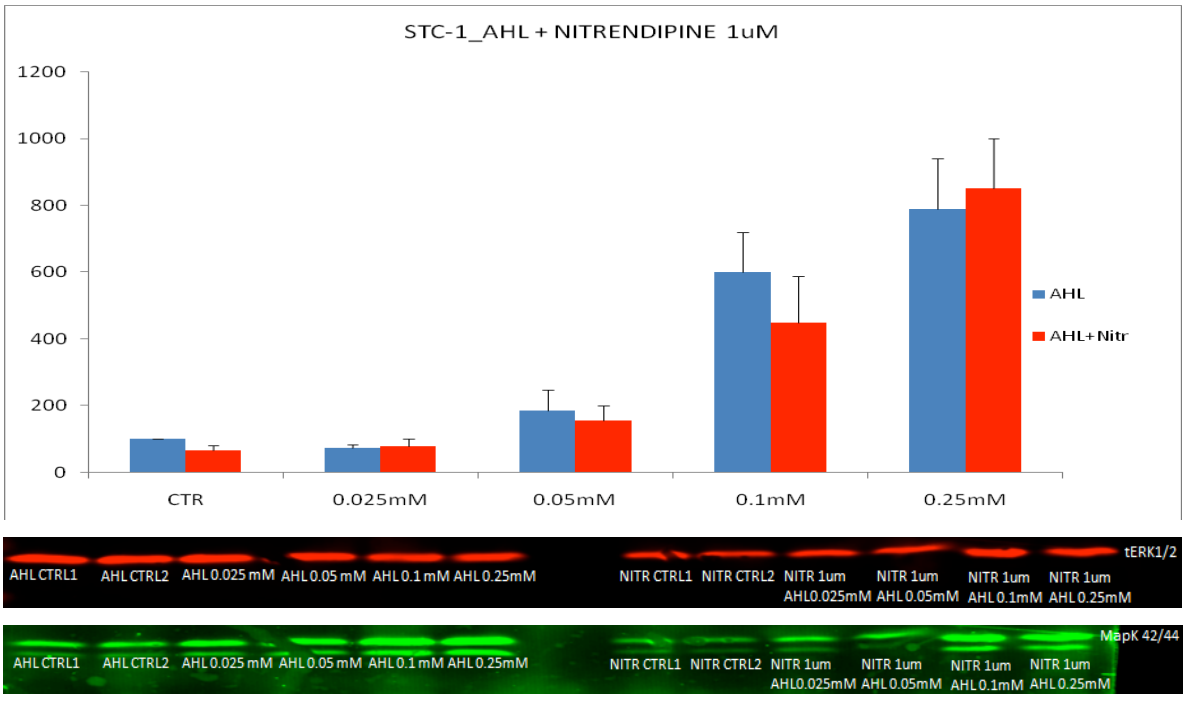

Fig5c. AHL induces $MAPK_{p44/42}$ phosphorilation, it is not blocked by Nitrendipine in STC-1 cells. Representative WB membranes showing MAPK $_{p44/42}$ after AHL stimulation, in presence of nitrendipine.

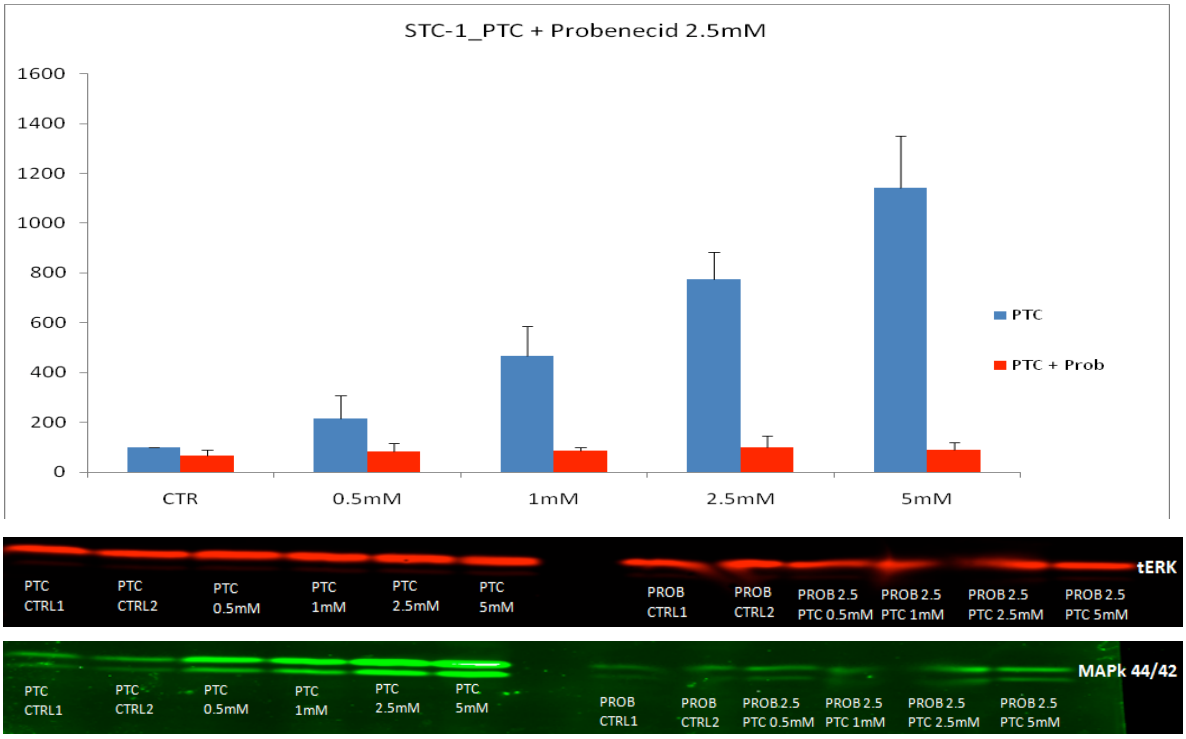

Fig 6a. PTC induces $MAPK_{p44/42}$ phosphorilation, is blocked by Probenecid in a dose dependent manner in STC-1 cells. Representative WB membranes showing $MAPK_{p44/42}$ after PTC stimulation, in presence of Probenecid.

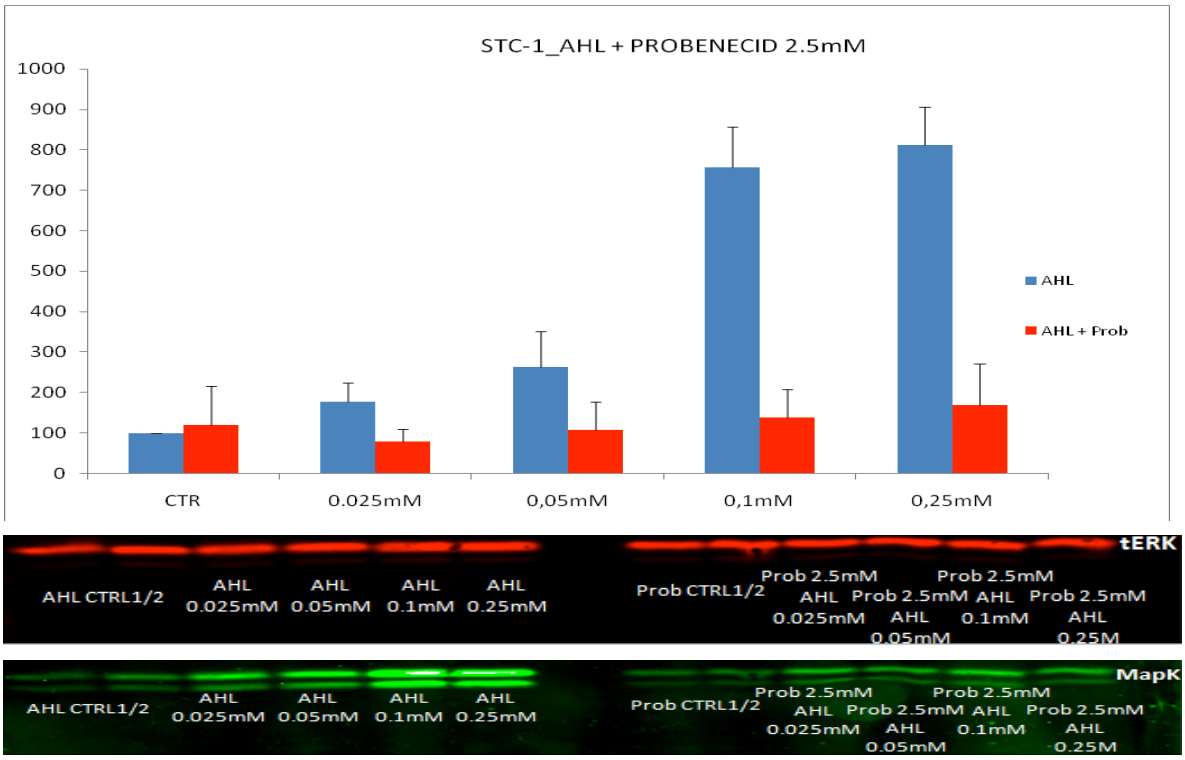

Fig 6b. AHL induces $MAPK_{p44/42}$ phosphorilation, is blocked by Probenecid in a dose dependent manner in STC-1 cells. Representative WB membranes showing $MAPK_{p44/42}$ after AHL stimulation, in presence of Probenecid.

PTC and AHL elicit rapid increase in $[ca^{2+}]_i$ in STC-1 Cells

We used a phenylthiocarbamide (PTC) a ligand that selectively binds the bitter taste receptor T2R38/138 and bacteria QS molecule (AHL) to evaluate their effect on Ca^{2+} signaling in STC-1.

Preliminary date showed that the stimulation of STC-1 cells with PTC at a concentration of 3.0mM or 5.0mM (**Fig 7**) induces a rapid increase in $[ca^{2+}]_I$. Interestingly, stimulation of STC-1 cells with a QS molecule AHL, at a concentration of 0.05 mM or 0.1mM also induces a rapid increase in $[ca^{2+}]_I$ (**Fig 7)**, supporting the data obtaining with $MAPK_{p44/42}$ as second effector system, and further supporting the concept that AHL act on effector systems activated by bitter taste receptors, including T2R38/138.

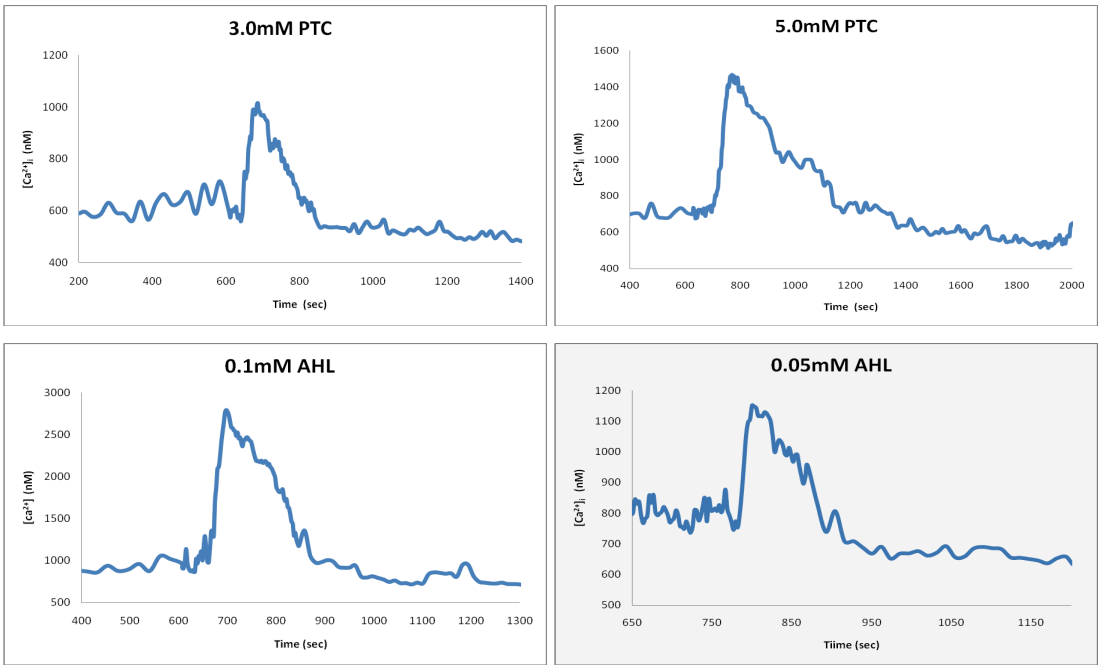

Fig 7. The bitter stimuli PTC, and the QS molecule AHL rapidly increase intracellular $[Ca^{2+}]_I$ in STC-1 cells.

Discussion and Conclusion

There is increasing evidence that taste receptors are expressed in the mice ECCs and other mammals [14] [47]. mRNAs for mT2R138, mT2R108 and their signaling molecule α-gustducin, are expressed in the endocrine cells and non-endocrine cells in the gastrointestinal tract, but also in enteroendocrin cell line STC-1. In accordance with what was previously stated, by the stimulation of STC-1 with two specific agonists of T2R108

(DB) and T2R138 (PTC) in mice, we obtain MAPK activations; this evidence suggests that STC-1 cell line has several functional T2Rs in its cellular membrane. These results were confirmed by calcium imaging, treating the cells with DB and PTC provokes an increase of intracellular levels of Ca^{2+}. We have shown that T2Rs subtypes are expressed in different species and different gastrointestinal cell types (enteroendocrine cells, brush cells), with differential distributions throughout the GI tract. To date, we know that different diets and, therefore different nutrients, are taken in by different species, which may lead to think that not only do T2Rs have a high homology among species, but they can also be modulated based on different food behavior and so this may lead to a different distribution and a different receptor type spectrum depending on the species. Indeed, throughout evolution rodents developed a significantly higher number of T2R genes compared to humans [15] and different animals show a different number and pattern of many sense receptors, including T2Rs [48]. Moreover the pathways of the T2Rs activations seem to involve $MAPK_{p44/42}$; in fact this protein was phosphorylated following dose response PTC and DB treatments as previously published by Wu et al. (2002) [14]. However, using antagonist of PKC (GF-1) and Ca^{2+} channel blocker (nitrendipine) we obtain no response of $MAPK_{p44/42}$, and so this helps us to characterize the pathways of T2Rs activations. Our data suggested that selected T2Rs ligands activate different pathways and different mechanisms of action for various types of T2R ligands. FIG.

In recent years, many researchers have been interested by the link between inflammation or obesity and gut microbiota [21] [22] [23] [24]; it is hypothesized that the gut endocrine cells detect pathogenic bacteria and more in general intestinal microflora. TRL are bacterial recognition receptors or receptors for bacterial product, such as short chain fatty acids [44]. AHL activate of $MAPK_{p44/42}$ cascade in STC-1 cell line expressing T2Rs through a PKC-depedent pathway, which is similar to the $MAPK_{p44/42}$ activation induced by PTC. This evidence is supported by the calcium imaging technique, which shows how treating STC-1 cells with AHL provokes an increased level of intracellular Ca^{2+}. We hypothesized that subpopulations of ECCs cells might detect bacterial stimuli, such as QS molecules like AHLs through T2Rs, which is in line with previous observations in the airways [40]. Overall, our findings, together with previous observations, suggest that bacteria might activate T2Rs to initiate an inflammatory process in response to pathogens such as gram-negative bacteria, and further support a functional role for T2Rs in chemosensing in the GI tract.

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CHAPTER 4

MODULATION OF THE T2R38 (BITTER TASTE RECEPTOR) IN HEALTHY HUMAN SUBJECTS BY DIET

Abstract

Taste receptors for complex tastes such as bitter taste receptors (T2Rs) or receptors for the sweet and umami tastes (T1R) are located in the taste buds of the tongue, but also in the upper respiratory tract and throughout the gastrointestinal tract. The T2Rs perceive bitter nutrients, but also potentially toxic and harmful substances, thus they are likely to play a very important role in defending the body from the external environment. T2R subtypes and signaling molecules are regulated by feeding and changes in the luminal content in animal models. The aim of this study was to test whether two distinct T2Rs, T2R4 and T2R38, which are activated by distinct tastants, are altered in the mucosa of overweight or obese compared to lean individuals. Colonic biopsies obtained from 30 healthy subjects but with different BMI (15 normal weight and 15 overweight/obese) were collected and RNA extracted for quantitative RT-PCR. Immunohistochemical analysis was also carried out in colonic specimens of 7 normal weight and 7 overweight/obese individuals to assess the distribution of hT2R38 immunoreactivity. This study showed a marked increase in the level of expression hT2R38 RNA in the mucosa of individuals with increased BMI (normal weight, 1.68 ± 0.5; overweight/obese 4.20 ± 0.9), whereas there were no changes in the expression of T2R4 mRNA. The immunohistochemical data confirm these results by showing an increased numbers of T2R38 immunoreactive cells in the overweight/obese group compared to normal weight subjects (1.9 ± 0.2 and 3.3 ± 0.1 cells in 0.42mm^2 respectively).

We can speculate that intraluminal changes induced by increased dietary consumption that is likley to be responsible for the increased BMI induce upregulation of T2R subtypes and an involvement of T2Rs in obesity or pre-obesity conditions could be suggested.

Introduction

The bitter taste receptors (T2Rs) are part of a large family of G protein-coupled receptors (GPCRs) activated by a multitude of bitter substances and they use α-gustducin and α-transducin as signalin molecules. T2Rs are found in the oral cavity on the taste buds [1] [2] [3], but also in extra oral sites such as the respiratory system [4] [5] [6], brain [7], testis [8] and throughout the gastrointestinal tract, from the stomach to the rectum [9] [10] [11]. In the tongue, T2Rs have evolved as a warning signal against toxic substances, typically bitter, by inducing avoidance or rejection. In the gut, T2Rs might function as a second tier of defense by detecting potentially dangerous or harmful substances, plant alkaloid and microorganisms [12] [13] [14], but might also be involved in physiological processes by affecting energy homeostasis, food intake, absorption and satiety. In the gastrointestinal tract (GI), taste related molecules are predominantly expressed in enteroendcorine cells and brush cells [9] [11] [15] [16]. The enteroendocrine cells contain secretory granules, which degranulate and release signaling molecules, many of which are peptides, which act as classic hormones or modulators/transmitters [13] [17] [18] [19] [20] [21]. Both anorexigenic and orexigenic peptides (Cholecystokinin, Glucagon Like Peptide-1, Pholypeptide YY_{3-36} and Ghrelin respectively) are released from the gut mucosa as a result of a fed or fasted state, and play a crucial role in regulating food intake [22]. Several studies have linked taste receptors and their signaling molecules to the release of various hormones. Indeed the stimulation of different TAS2Rs expressed in the murine cell line STC-1 with bitter ligands increased the release of the Cholecystokinin (CCK) and Glucagone like peptide-1 (GLP-1) hormones, two of the major satiety hormones [23] [24]. Since STC 1 cells are a major model system for enteroendocrine cells, this is an indirect evidence that T2Rs expressed by enteroendocrine cells are functional. Intragastric administration of bitter agonists provokes the activation of c-fos in the nucleus tractus solitaries likely acting on CCK1Rs and Y2 receptors on vagal afferents through the release of Polypeptide YY (PYY_{3-36}) or CCK from enteroendocrine cells, which have been shown to express taste receptor signaling molecules [25]. Furthermore, the intragastric administration of bitter agonists induces the secretion of the hunger hormone ghrelin, leading to a short term increase in food intake [26]. Ghrelin release leads to a temporary stimulation of appetite, but this is promptly followed by a profound decrease in food intake correlating with a decrease in gastric emptying, a possible mechanism to prevent overeating [26]. This is consistent with recent findings from Janssen P. et all (2011) that intragastric

administration of the bitter agonist Denatonium Benzoate (DB) in healthy volunteers resulted in an increase in satiation and reduced the volume of nutrients ingested [27].

All together, these observations support the concept that bitter agonists induce the release of different gastrointestinal hormones (satiety and hunger hormones) from enteroendocrine cells, thus regulating food intake and participating to the control of energy balance and obesity. Studies from our laboratories have shown that different diets induce changes in the level of taste signaling molecules [28] and T2R subtypes [29] and that long-term high fat diet resulting in obesity induced significant increase in the expression of T2R138 (the mouse T2R corresponding to hT2R38) in the mouse colon [29].

In our study, we have measured the levels of T2R38 and T2R4 mRNA in colonic biopsies obtained from healthy human subjects with different Body Max Index (BMI) to determine whether there was a correlation between body weight and hT2R38 and hT2R4 mRNA expression, which could support a role of these receptors in feeding alterations. Moreover, we characterized the types of cells expressing T2R38 in the human colon using immunohistochemistry with a Chromogranin A antibody (pan marker for endocrine cells), and markers for CCK and GLP-1

Material and Method

RT-PCR

Total RNA was isolated from colonic biopsy using Qiagen RNeasy Minikit (74104, Qiagen, Valencia, CA) and a DNase treatment was performed to eliminate genomic DNA contamination. RNA quality was estimated by absorbance at 260 nm and 280 nm ratio (OD260nm/OD280nm>1.8). Complementary DNA was generated using superscript III reverse transcriptase kit according to the manifacturer's instructions on a DNA Thermal Cycler Engine, BIO-RAD. Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) was performed using Taqman Gene expression assays hT2R38 (Applied Biosystem Hs00604294_s1,), hT2R4 (Applied Biosystem Hs00249946_s1) Standard thermal cycles (50 cycles) for Taqman Gene assays were run on a Mx3000P Real-time PCR DetectionSystem (Stratagene) and data were analyzed with Mx Pro 1000 software. 18S RNA (18S RNA, Applied Biosystem Hs03928990_g1) was used as housekeeping gene and the relative abundance of mRNA expression was calculated using

the Delta delta Ct method (User Bulletin #2, ABI Prism 7700 Sequence Detection System). Samples were run in duplicate in separate experiments and No-RT and distilled RNAse-free water controls were always included. qRT-PCR products were checked by 4% agarose gel horizontal electrophoresis and specific bands of the same base pair sizes as the expected sizes were detected.

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5µ-thick paraffin-embedded, formaldehyde-fixed specimens were cut, serially mounted on glasses and then deparaffinazed in xylene. To unmask the antigenic sites, slides were heated in 10mmol/L sodium citrate buffer (pH 6.0) for three times of 5 minutes each in a microwawe oven at 600W. After treatment, slides were allowed to cool to room temperature (RT) for 35 minutes. Sections were washed in double-distilled water and then incubated in 10% normal donkey serum for 1 hour at RT in humid chamber. Sections were then incubated for indirect double-labelling immufluorescence at 4°C overnight in a mixture/solution containing T2R38 in combination with a primary antibody direct against Chromogranin A (ChrA), Cholecystokinin (CCK), or Glucagon Like Peptide-1 (GLP-1). All of the antibodies and dilutions used in this study are listed in **table 1.**

Following several washes in phosphate buffered saline, pH 7.4 (PBS, 3 x10 min), sections were incubated with a mixture of secondary antibodies (Do anti-Rabbit Alexa 488, Do anti Goat/Mouse Alexa 594) in the dark and in a humid chamber for 1 hour at RT. After washing in PBS (3x10 min) sections were cover-slipped with aqua-poly/mount (Polysciences Inc).

Images were scanned with a confocal microscope (ZEISS 510 laser scanning confocal microscope, Carl Zeiss Inc, Thornwood, NY) running LSM5 software.

Primary antisera	Species	Code	Diluition	Supplier
hT2R38	Rabbit	ab65509	1:2000	Abcam
ChrA	Goat	Sc-18232	1:600	Santa Cruz
CCK	Mouse	9303	1:600	CURE/DDRC
GLP-1	Mouse	9369	1:1000	CURE/DDRC

Secondary antisera	Code	Diluition	Supplier
Donkey Anti-Rabbit Alexa 488	A21206	1:1000	Invitrogen
Donkey Anti-Goat Alexa 594	A11058	1:900	Invitrogen
Donkey Anti-Mouse Alexa 594	A21203	1:1000	Invitrogen

Table 1. list of primary and secondary antibodies used in this study.

CELL COUNTING AND STATISTICAL ANALYSIS

Cell counting was carried out with a 40X objective lens using a ZEISS 510 laser scanning confocal microscope equipped with appropriate filter to discriminate between FITC and Alexa 594 fluorescent. The objective 40X has a field of view of 0.028mm^2, 15 fields per sample were evaluated to obtain the number of cells positive for the hT2R38, ChrA, in an area of 0.42 mm^2. Data were analyzed using the student's *t*-test. (Graph Prism 4, GraphPad Softwere, Inc., La Jolla, CA, USA).

RESULTS

hT2R38 mRNA LEVELS IN HEALTHY LEAN AND OVERWEIGHT HUMAN SUBJECTS

The body mass index (BMI) is a measure for human body shape based on an individual's weight and height. A different BMI is likely due to a different caloric intake corresponding to increased weight. qRT-PCR analysis showed that hT2R38mRNA was of 1.68 ± 0.5 in lean subjects (N° 15 including males and females, ranging between 22-59 years old),

whereas it was 4.20 ± 0.9 in overweight/obese subjects (N° 15 males and females, ranging between 22-55 years old) (**Fig1**), which is two and a half fold for the value in lean subjects ($p< 0.05$); there was no significant different in the expression of T2R4 mRNA in lean and overweight subjects. We did not detect sex-specific difference in hT2R38 RNA levels in either normal weight or overweight/obese groups (data not show). These results suggest that hT2R38 is regulated by feeding since it was increased in overweight/obese people.

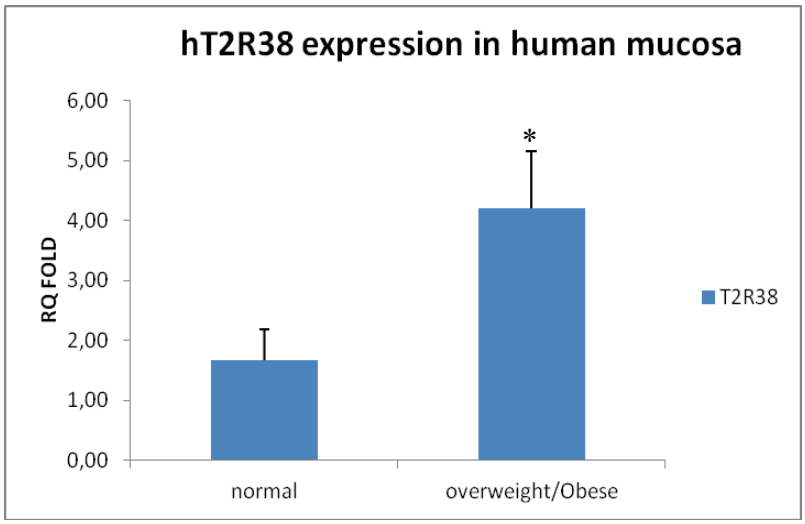

Fig 1. Effect of a different BMI on mT2R38 expression. qRT-PCR analysis shows that mT2R38 mRNA levels are significantly ($P \leq 0.05$) up-regulated in overweight/obese group compared with normal weight group.

hT2R38/ ChrA IN HUMAN COLONIC BIOPSIES

In the colon,T2R38-IR cells were generally located in the surface and glandular epithelium. T2R38-IR cells showed homogenously labelled cytoplasm, with an unlabelled nucleus and with an elongated shape. Some T2R38-IR cells were confined toward the basal lamina and few cells were characterised by two thin cytoplasmic prolongations, the first extending up to the endoluminal surface of the mucosa and the second projecting down to the basal lamina. All of the hT2R38-IR cells co-expressed ChrA (**Fig 2**), demonstrating that hT2R38 cells are enteroendocrine cells; this complete co-localization was observed in human colon of both normal weight and overweight/obese subjects. Immunohistochemistry experiments showed an increased numbers of T2R38-IR cells in the overweight/obese group compared to normal weight subjects (3.3 ± 0.1 and 1.9 ± 0.2 cells in 0.42mm^2 respectively). Some T2R38-IR cells were co-localized with GLP-1 (**Fig 3**). These cells are located in the basal part of the glandular epithelium and some GLP1-IR cells have cytoplasmatic prolongations

extending up to the endoluminal surface of the mucosa and down to the basal lamina, suggesting that these cells are “open cells type”. Many T2R38-IR cells are co-localized with CCK (**Fig4**) in the basal part of glandular epithelium; CCK cells are numerous with homogenously labelled cytoplasm and elongated shape.

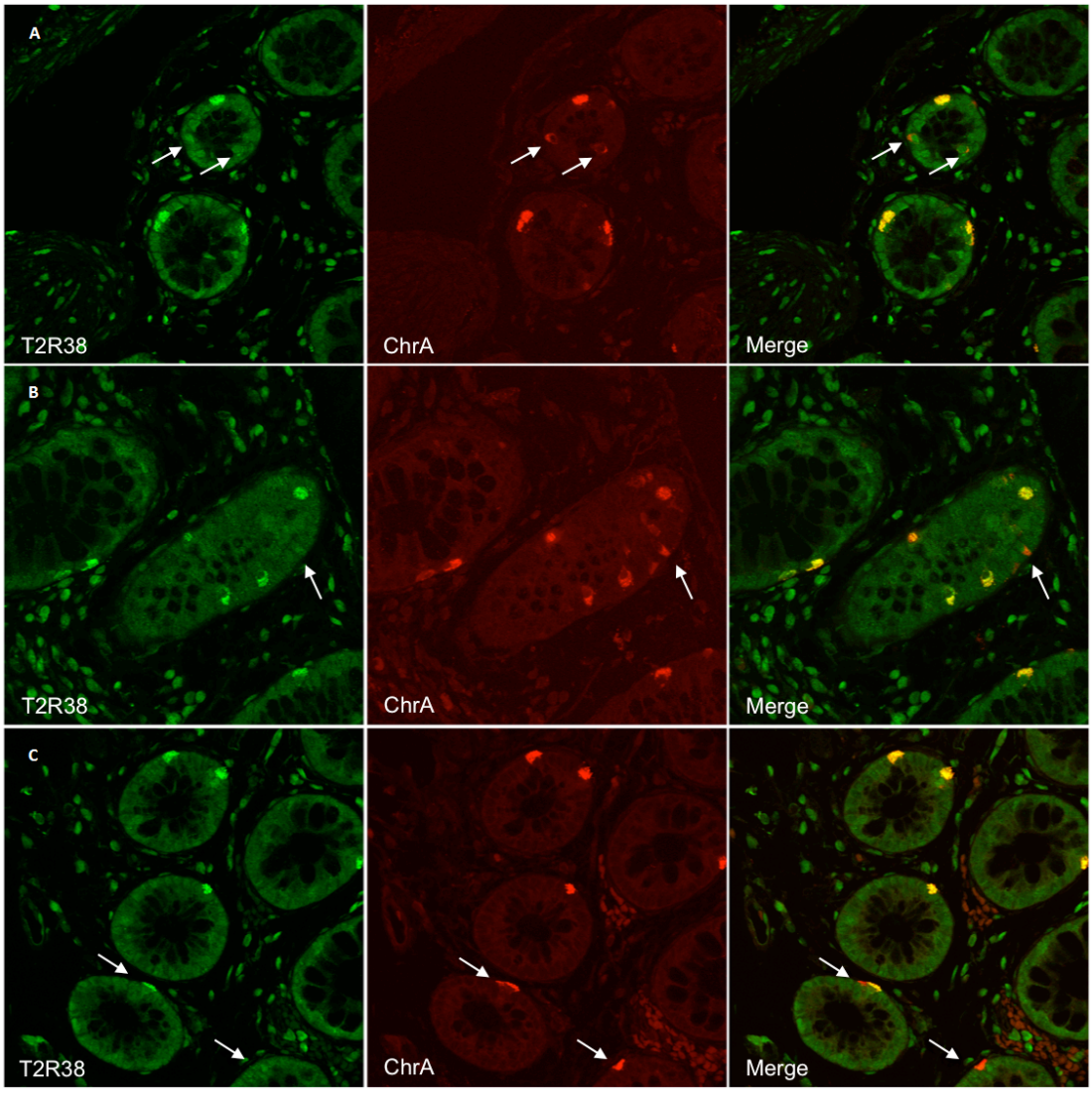

Fig 2. Representative image of human colonic mucosa. hT2R38 immunoreactivity colocalised with ChrA in normal weight (A) and overweight/obese group (B,C). The arrows indicate the T2R38-IRcells are not colocalised with ChrA

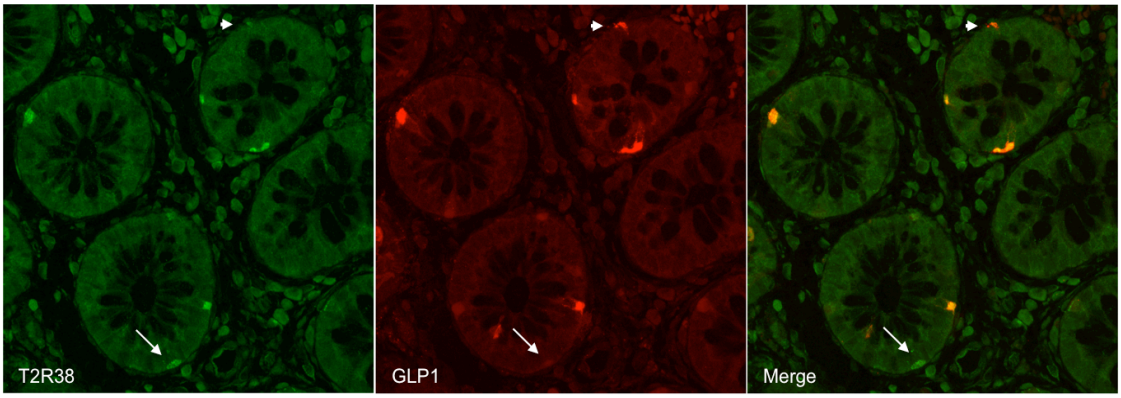

Fig 3. Representative image of human colonic mucosa. hT2R38-immunoreactivity colocalised with CCK. The arrows indicate the T2R38-IRcells are not colocalised with GLP-1

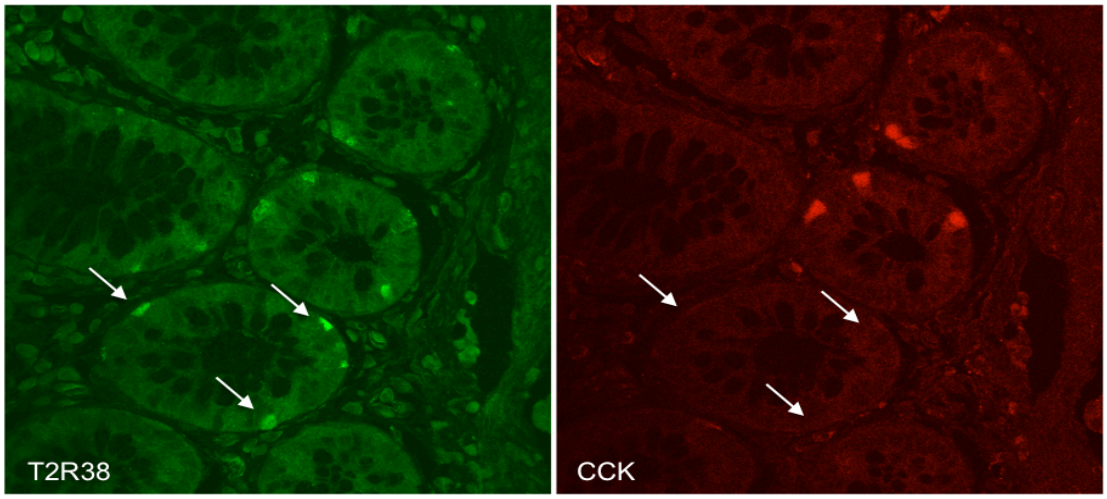

Fig 4. Rapresentative image of human colonic mucosa. hT2R38-immunoreactivity colocalised with CCK. The arrows indicate the T2R38-IRcells are not colocalised with CCK.

DISCUSSION AND CONCLUSION

Using colonic biopsies from healthy control patients with a different BMI, we have shown a positive correlation between weight and increased expression of the hT2R38 mRNA levels and T2R38-IR cells number.

Weight increase and obesity are widespread not only in populations of industrialized countries, but also among those with a low socio-economic-cultural level. They are the result of the protracted imbalance between calorie intake and expenditure which can lead to a negative effect on the health.

The underlying causes are numerous: genetic predisposition, socio-economic, environmental, cultural, psychological, behavioral, metabolic, or neuroendocrine factors.
Recently, high attention has been given to the role of gut microbiota in obesity [30] [31]; our study was performed on human distal colon, one of the gut regions with highest bacterial density of the whole body, which is estimated at 10^{11} to 10^{12} per milliliter [32]. There are several bacterial populations in the colon of mammals. The first evidence of different populations in obesity derives from studies in leptin (ob/ob) deficent mice showing a different proportion of the phyla of cecal bacteria compared to lean wilt type, (WT) (ob/+) mice. This study showed an increase in Firmicutes and a decrease in Bacteriodetes in the obese mice [33]. Similarly, in the gut of obese humans there was decrease of Bacteriodetes and an overall decrease in bacterial diversity [34] [35]. Moreover, "low grade chronic" inflammation is present in obese patients and is associated with leptin and insulin resistance [36]. This chronic inflammation in obesity is probably due to the production and release of inflammatory mediators from the adipose tissue, which can affect other tissues, such as muscle, liver, pancreas and the central nervous system. Furthermore, an increase of bacterial endotoxin, Lypopolysaccharide (LPS), has been reported in the plasma in humans fed with high fat meal [37]. LPS is released into the gut lumen when the gram negative bacteria died, and it can cross the epithelial barrier and establish an inflammatory process. One possible mechanism through which bacteria communicate with and produce damage to the host cell, is the production of substances of bacterial origin such as Homoserine Lactones (AHL), which serve as a quorum sensing molecule for Gram-negative pathogens [38].
AHL-induced $MAPK_{p44/42}$ activation was reduced by pre-treatment with probenecid, a T2R inhibitor [39]. The GPCR-mediated pathway activated by AHL in STC 1 cells is similar to the pathway utilized by ligands selectively binding the T2R138 (the mouse T2R corresponding to the hT2R38). Indeed, AHL-induced $MAPK_{p44/42}$ phosphorylation is markedly reduced by GF1, a protein kinase C inhibitor, whereas it is not inhibited by nitrendipine, a calcium blocker, as the T2R38-induced MAPK-phosphorylation. This differs from $MAPK_{p44/42}$ activation mediated by T2R108 (the mouse T2R corresponding to hT2R4), which is blocked by nitrendipine, but not by GF1. This suggests that the bitter taste receptor downstream signaling effector activated by AHL involves T2R138 or other T2Rs activating this transduction pathway. Furthermore, AHL, induces increase in intracellulal Ca^{2+} and CCK release from STC 1 similary to T2R ligands including T2R138 (Chapter 3). These data, together with the findings of increased expression of T2R38

mRNA and increased number of T2R38 immunoreactive cells, suggest that one of the fucntion of these T2Rs is detecting bacteria in the intestinal lumen. Since bacterial populations are altered by obesity-inducing diets with prevalence of Gram-negative bacteria, we can hypothesize that T2R exert a protective function against bacteria by detecting their quorumn sensing molecule and mounting an inflammatory response to reduce bacteria danage to host cells. This is consistent with data from the respiratory system, where T2Rs have been shown to be actiavted by AHL produced by Gram-negative bacteria and have been proposed as a defense mechanism against irritants invading the respiratory tract [40]. Additional studies are required to test this hypothesis, including studies in animal models with germ-free mice and antibiotic to reduce the luminal content of bacteria as well as studies in vitro silencing different T2Rs in STC 1 cells and intestinal epithelial cells following stimulation with AHL and selective T2R agonists.

In conclusion, the increased expression of hT2R38 mRNA levels and the increased number of T2R38-IR cells in overweight/obese individuals compared to lean subjects, combined with the observation that hT2R38 co-localizes with GLP-1 and CCK, signaling molecules regulating feeding behavior and the findings that T2R38 ligands trigger the release of CCK and GLP-1 in enteroendocrine cell lines support the hypothesis that T2R38 plays a role in food intake regulation, metabolic dysfunctions and homeostasis. This is further supported by studies in mice showing that mT2R138 is upregulated by different diets which varies with the gut segment investigated [29]. In addition, the findings that Gram-negative bacteria molecules, such as AHLs and bitter ligands activate the same downstream signaling effectors and that T2R138 mRNA is upregulated by long-term high fat diet inducing obesity in mice[29] and rats (data unpublished), which has been shown to be associated with increased Gram-negative bacteria in the colon lumen [41], as well as in colonic biopsies from overweight and obese subjects (this study), support the concept that T2R might function as bacteria detectors and protect from further cellular damage by bacteria.

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