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CICLO XXIV

BITTER TASTE RECEPTORS AND GASTROINTESTINAL CHEMOSENSING IN MICE

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ABSTRACT

The gastrointestinal (GI) tract is a chemosensory organ that detects nutrients in the lumen to initiate an appropriate response of digestion and absorption of nutrients or elimination of harmful substances. It has been shown that bitter taste receptors (T2Rs), a large family of GPCRs detecting bitter compounds in the mouth, and their signaling molecules, are also expressed in the GI tract mucosa. Because bitter taste has evolved as a warning mechanism in the mouth, we hypothesize that T2Rs in the GI tract might serve as a second level of defense towards harmful compounds.

In this study, we used qRT-PCR to investigate the distribution of two T2Rs subtypes (mT2R138 and mT2R108), and their signaling molecule G_{α}-Gustducin (Gust), in the mouse GI tract, and different diets to see whether they are modulated by luminal content. To test the hypothesis that T2Rs in the gut might serve as a mechanism of defense against pathogens, additional studies measured by WB the phosphorylation of mitogen activated protein kinase (MAPK) to evaluate whether T2Rs respond to Acyl Homoserine Lactone (AHL), quorum sensing molecule for Gram negative bacteria. The response on STC-1 and NCM-460 cells was compared to the ones elicited by T2Rs agonists_phenylthiocarbamide (PTC) and denatonuim benzoate (DB)_and blocked by the T2R138 antagonist Probenecid. The pathway following T2Rs activation was further characterized using GF-1, a protein kinase C inhibitor, and nitrendipine, a Ca^{++} channel blocker.

We found that mT2R138, mT2R108 and Gust are expressed throughout the entire mouse GI tract, with different levels of expression, and that different diets selectively modulate T2Rs in specific GI regions. Also, we showed that both NCM-460 human colonocytes and STC-1 mouse enteroendocrine cells express T2Rs and respond to bitter stimuli and AHL with rapid dose-dependent phosphorylation of MAPK p44/42. PTC and AHL-induced signal was blocked by Probenecid and reduced by GF-1, but not by nitrendipine, in contrast with DB-induced MAPK phosphorylation. Furthermore, exposure of NCM-460 cells to PTC or AHL for 4-24 h induced a significant increase in hT2R38 mRNA, the homologus of mT2R138.

In summary, these data suggest that T2Rs are involved in chemosensing in the GI tract and that different functions might exist depending upon receptor subtype, site of expression and molecular mechanism. We showed that different T2R subtypes are expressed in different GI cell types and that they might use different pathways. Also, we suggest that T2Rs might detect bacterial stimuli in GI cells, supporting the hypothesis that activation of these receptors might provide a second level of defense in the GI mucosa to initiate an inflammatory process in response to bacteria in the gut lumen.

ABSTRACT (italiano)

Il sistema digerente è un organo sensorio capace di riconoscere le sostanze presenti nel lume e di iniziare un' adeguata risposta, che si traduce in digestione ed assorbimento per i nutrienti o eliminazione per le sostanze nocive. E' stato dimostrato che i recettori per l'amaro (T2R), un'ampia famiglia di GPCR in grado di riconoscere composti amari nella bocca, così come le molecole coinvolte nella trasduzione del segnale, sono espressi a livello della mucosa intestinale. Poiché l'amaro si è evoluto come un meccanismo di allarme nella bocca, proponiamo che i T2R nel sistema gastrointestinale possano servire come un secondo livello di difesa verso composti nocivi.

In questo studio abbiamo usato qRT-PCR per stabilire la distribuzione di due sottotipi recettoriali dei T2R (mT2R138 e mT2R108), e della loro subunità Gα-Gustducin (Gust), nel sistema digerente del topo, e varie diete sono state usate per vedere se il contenuto del lume è in grado di modulare i recettori dell'amaro. Per testare l'ipotesi che i T2R nel sistema digerente possano servire come meccanismo di difesa nei confronti di patogeni, in ulteriori studi si è misurato tramite WB il grado di fosforilazione della proteina chinasi attivata da mitogeni (MAPK p44/42) per valutare se i T2Rs rispondano ad acil-omoserin-lattoni, molecole prodotte da batteri Gram negativi. La risposta, valutata su cellule STC-1 e NCM-460, è stata confrontata con quella evocata da agonisti per i T2R_feniltiocarbamide (PTC) e denatonio benzoato (DB)_e bloccata dall'antagonista del recettore T2R138, Probenecid. Il meccanismo che segue l' attivazione dei T2R è stato ulteriormente caratterizzato con l'uso di GF-1, un inibitore della proteina chinasi C, e di nitrendipina, un bloccante dei canali calcio.

Questo studio dimostra che mT2R138, mT2R108 e Gust sono espressi lungo tutto il sistema digerente, con diversa abbondanza, e che diverse diete sono in grado di modulare specificamente alcuni recettori, in aree selezionate lungo il sistema digerente. Abbiamo anche dimostrato che sia la linea cellulare umana di colonociti NCM-460, sia la linea di cellule enteroendocrine di topo STC-1 esprimono T2R e rispondono allo stimolo del gusto amaro e ad AHL con una rapida fosforilazione della MAPK p44/42, mostrando un profilo dose-dipendente. Il segnale indotto da PTC e AHL è bloccato dal Probenecid e ridotto dal GF-1, ma non dalla nitrendipina, al contrario del segnale indotto dal DB. Inoltre, l'esposizione delle cellule NCM-460 per 4-24 ore a PTC o AHL induce un aumento significativo dell'espressione del recettore T2R38 umano, omologo del T2R138 nel topo.

In conclusione, questo lavoro di tesi propone che i T2R siano coinvolti nell'abilità sensoria del sistema gastrointestinale e che possano esistere diverse funzioni a seconda del sottotipo recettoriale espresso, del sito di espressione e del meccanismo utilizzato. Abbiamo mostrato che diversi tipi di cellule di origine intestinale esprimono diversi sottotipi di T2R e che essi possano usare diversi meccanismi. Inoltre, suggeriamo che i T2R possano interagire con batteri nel sistema gastrointestinale, a supporto dell'ipotesi per cui i recettori dell'amaro costituiscono un secondo livello di difesa nel sistema gastrointestinale, in grado di iniziare una risposta infiammatoria per combattere i batteri patogeni presenti nel lume.

Ai miei genitori

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5-HT 5-hydroxytryptamine
ABCB1 ATP bindinc cassette B1
AHL Acyl Homoserine Lactone
BA beta actin
CCK Cholecystokinin
CgA chromogranin A
CNS Central nervous system
DAG Diacylglycerol
DB Denatonium benzoate
DMSO dimethyl sulfoxide
DPP-IV dipeptidyl-peptidase IV
EC enterocrhomaffin cells
EDTA Ethylenediaminetetraacetic acid
EECs Enteroendocrine cells
ENS Enteric nervous system
ERK extracellular signal regulated kinase
ERK Extracellular signal regulated protein kinases
GFP green fluorescence protein
GHS growth hormone secretagogue receptor
GI Gastrointestinal
GIP gastric inhibitory peptide
GLP-1 Glucagon like peptide 1
GMP guanosin monophosphate
GPCRs G protein coupled receptors
GUST G-alpha gustducine

HCl chloridric acud
HF high fat diet
IMP inosine monophosphate
IP3 Inositol 3,4,5 triphosphate
KO knock out
LPS lipopolysaccharide
MAPK mitogen activated kinase
$MgCl_2$ magnesium chloride
MSG monosodium glutamate
NTS Nucleus tractus solitaries
PB phosphate buffer
PCR Polymerase chain reaction
PDE phosphodiesterase
PFA paraformaldehyde
PKC protein kinase C
PLC Phospholipase C
PLCβ2 Phospholipase C β2 isoform
PNS peripheral nervous system
PROP 6-propylthiouracil
PTC Phenylthiolcarbamide
PYY Peptide YY
QS quorum sensing
RT-PCR real time polymerase chain reaction
S.E.M. Standard error of the mean
SDS sodium dodecyl sulphate
SGLT sodium dependent glucose cotranspoter
siRNA small interfering RNA
T2Rs Bitter activated g protein coupled receptors
TLR Toll-like receptor
TRPM5 Transient receptor potential melastatin member 5
WB Western blot
WT wild type

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Gaia Vegezzi

CHAPTER I

General Introduction

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GENERAL INTRODUCTION

a. Rationale for Proposed Research

The gastrointestinal (GI) tract is the largest interface between our body and the environment. This system is in direct contact with the outside world and it's continuously exposed to environmental factors, including food nutrients, microorganisms and toxins. Recent growing evidences indicating that the GI mucosa expresses the same transcripts for taste receptors and their signaling molecules found in the tongue [1] [2] supported the idea that the gastrointestinal tract might be able to detect luminal stimuli and to specifically respond to them through a machinery similar to the one found in the mouth. The sense of taste is important to evaluate the quality of food prior to its ingestion and among the 5 different tastes, bitter has evolved as a warning mechanism against toxic or harmful substances, which are often bitter [3]. In the GI tract the detection of intraluminal compounds is thought to activate a cascade of events culminating in the release of incretins, which can act as neurotransmitters locally and centrally, or enter the blood stream as hormones, to regulate many GI function such as absorption, pancreatic and gastric secretion, motility, food intake and metabolism [4] [5]. Strong evidence support the hypothesis that a chemosensory machinery similar to the one in the oral cavity operates in the gut. However, the molecular mechanisms underlying these biological responses to luminal molecules are still largely unknown. Also, though many taste signaling molecules are found in the gut, the pathway following T2Rs activation still needs to be fully elucidated. Understanding the location and distribution of T2Rs, the kind of molecules they can recognize in the gut and which signaling pathways are

utilized will allow for a better understanding of the role of bitter taste receptors in the GI tract and the mechanisms underlying the processes of chemosensing in gut, which regulates a variety of functions including digestion, absorption and feeding as well as initiation of defense responses against toxins or environmental hazards. This, in turn, will provide insights into understanding feeding disorders and GI pathologies that might be elicited in response to changes in intraluminal content and assist in the development of new products in the food and drug industry that could be beneficial for individuals with eating disorders, obesity and chronic inflammation.

b. The role of the gastrointestinal tract in luminal chemosensing

The GI tract is connected to every major system in the human body. It hosts the majority of the immune system and it produces 99% of the body's neurotransmitters. It is also the largest endocrine organ in the body, producing hormones that have important sensing and signaling roles in regulating body weight and energy balance. Its functions are numerous and include nutrient digestion and absorption, elimination of toxins, hormone metabolism, and energy production/expediture. A wide variety of stimuli –such as chemical, mechanical and others– occur in the GI. Once transduced, the information is used by the enteric nervous system (ENS) to generate local responses within the GI tract and to send information to the central nervous system (CNS) via the vagal and dorsal afferents.

The GI itself is as complex as the variety of stimuli affecting it. The structures forming the wall of the gastrointestinal tract from the posterior pharynx to the anus are shown in figure 1. There are some local differences, but in general the GI is composed by four layers from the lumen outward: the mucosa, the submucosa, the muscularis mucosae, and the serosa. The mucosa lining the gastrointestinal tract always consists of a

superficial layer of epithelium attached to the basement membrane, the lamina propria, containing collagen, reticular and elastic fibers and several different types of immune cells. The submucosa is a layer of connective tissue that supports the mucosa. It hosts glands and neurons (Meissner's plexus or submucosal plexus) and connects the mucosa to the underlying muscularis mucosae. The muscolaris mucosae is a layer of smooth muscle (circular and longitudinal smooth muscle) that contributes to the peristalsis which moves digested material along the gut. Between the two layers of external smooth muscle lies the myenteric plexus (or Auerbach's plexus). Lastly we find the serosa, which consists of a thin layer of loose connective tissue covered with mucus to prevents friction from the intestine against other tissues. Enclosing all these is a double layer of peritoneum, the mesenteries, which contains nerves and lymphatic and blood vessels supplying the GI tract and holds the intestine in place in the abdominal cavity.

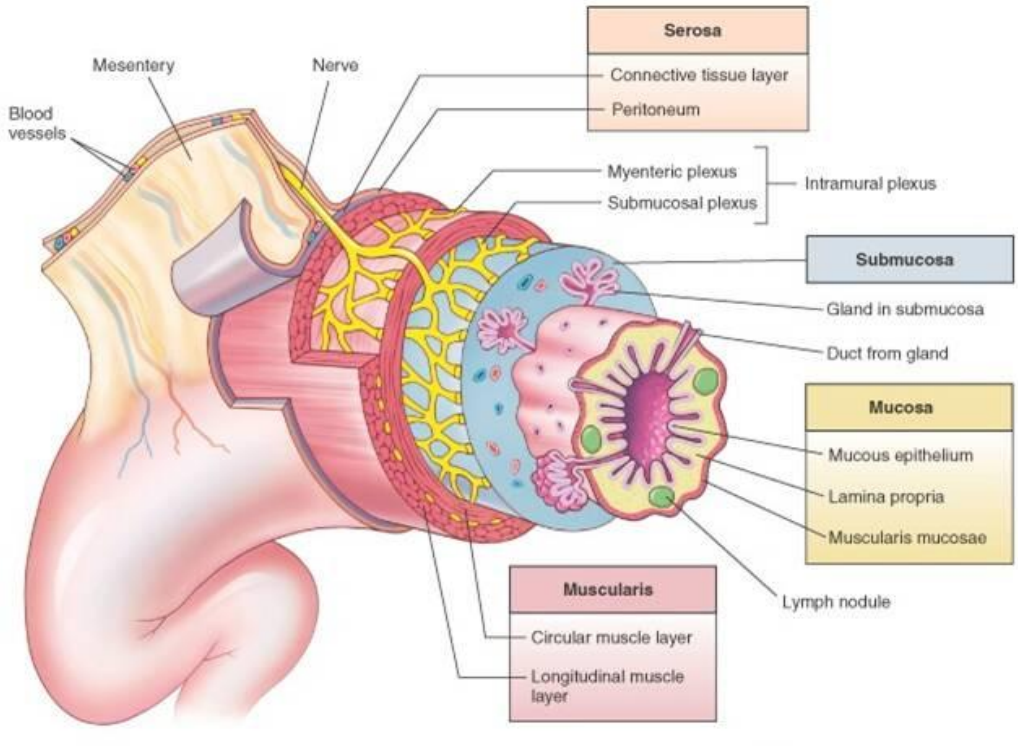


Figure 1: Representation of the layers of the wall of the intestine. [www.pharmacistworld.com]

Within the mucosa of the stomach, small intestine, and colon, the epithelial layer comprises different cell types (Fig. 2). This layer is the one in contact with luminal content and it contains specialized epithelial cells able to respond to these luminal stimuli with the release of active substances. More than 20 different types of hormone-secreting specialized cells have been identified. These cells, called enteroendocrine cells (EECs), secrete mediators that play a role in digestive functions such as gastrointestinal motility, pancreatic and gall bladder secretions, gastric emptying, energy balance and food intake [4] [5]. In the twentieth century Bayliss and Starling observed that the presence of protons in the proximal small intestine elicited a strong stimulation of pancreatic fluid secretion, mediated not by nerves innervating the gut but by a substance released in the blood stream, an hormone they called secretin [6]. This led to the recognition of the ability of gut endocrine cells to detect the presence of luminal chemicals.

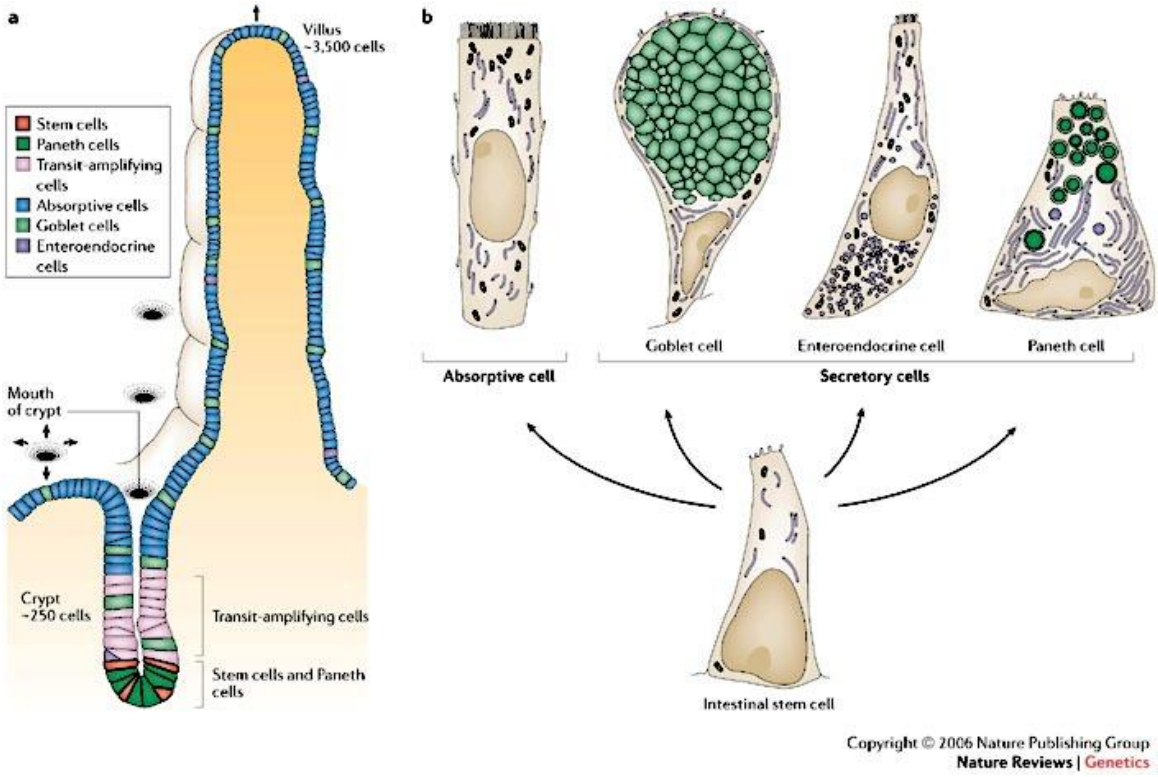


Figure 2: Representation of the different types of epithelial cells in the intestine [Nature Reviews, 2006]

Based on this discovery, enteroendocrine cells can be seen as primary chemo-receptors, able of responding to luminal content by releasing mediators that activate neuronal pathways or act in an endocrine or paracrine way through different mechanisms (Fig. 3). This model is particularly suitable for the so called “open cells” with microvilli extending to the luminal surface. “Closed cells”, however, can be regulated by luminal content indirectly through neural and humoral mechanisms [7].

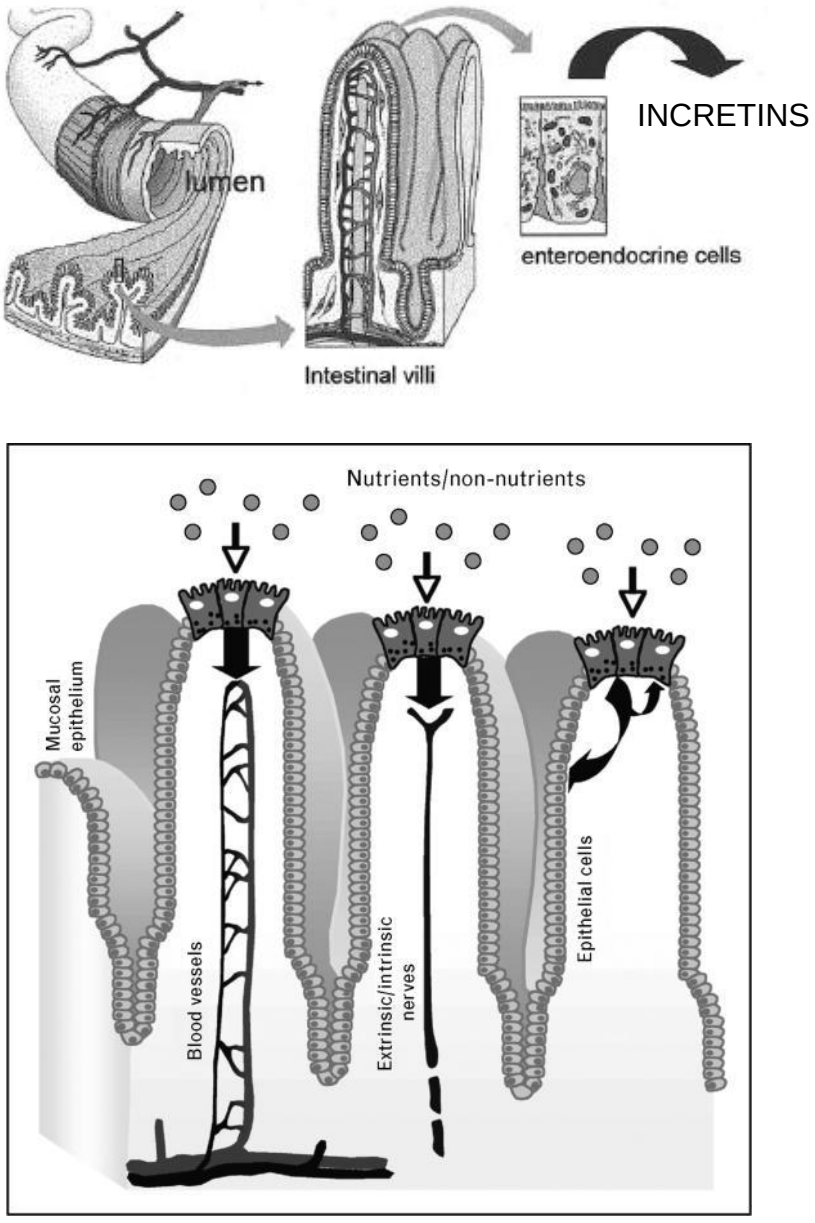


Figure 3: Possible pathways involved in GI chemosensing [from Sternini C. et al, Curr Opin Endocrinol Diabetes Obes., 2008]

The enteroendocrine cells lying within the gut epithelium secrete numerous hormones, such as gastrin (G cells), ghrelin (X\A like cells), CCK (I cells), GLP-1 (L cells), PYY (L-cells), GIP (K-cells) and 5-HT (enterochromaffin cells) in response to different nutrients. Their products are synthesized and accumulated in secretory granules and secreted, upon stimulation, by exocytosis at the basolateral membrane into the interstitial space [7] [8] [9] [10]. A short review of the most important hormones mediating chemosensing and thus regulating GI functions follows and it is summarized in Table I and Fig 4. Also, for each hormone I briefly report references about their link to T2Rs in the gut.

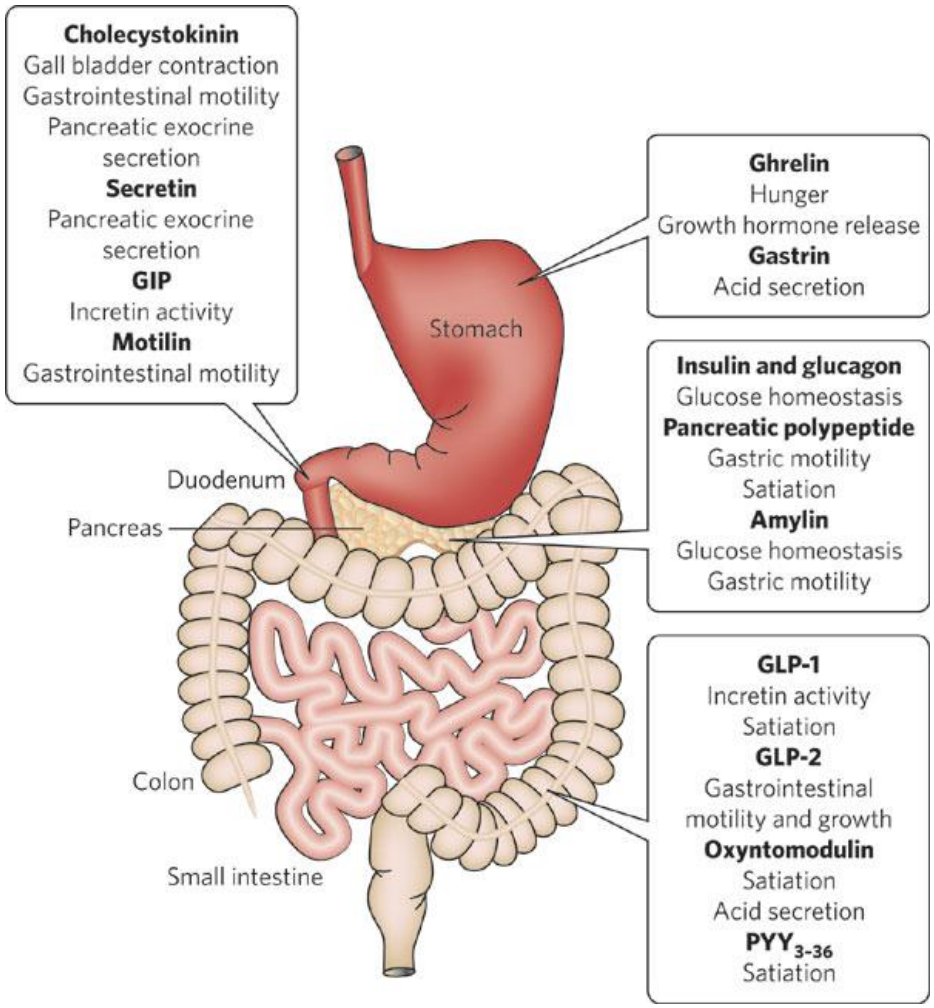


Figure 4: Gut hormones and the regulation of energy homeostasis [from Murphy and Bloom, Nature*, 2*006]

Ghrelin

Ghrelin, a 28 amino-acid peptide, is mainly produced by the “X/A-like” cells in the stomach [11] and in pancreas in response to energy need, typically before meals, and its levels are usually decreased after meals. It is also known as the “hunger hormone”, since it increases food intake, and it is considered the counterpart of leptin, an hormone produced by adipose tissue, which induces satiation instead. Ghrelin expression is not limited to the stomach, but is found in many other sites such as the small intestine, brain, lungs, skeletal muscle, islets of Langerhans, adrenal glands, ovary, and testis [12]. Therefore is clear that ghrelin has many different actions linked to feeding behavior, energy homeostasis, reproduction, sleep regulation, corticotrope secretion and regulation of gastro-entero-pancreatic functions [13] [14]. However, despite this variety of effects, ghrelin KO mice demonstrate normal growth, energy expenditure and food intake under normal conditions [15] [16], suggesting that ghrelin plays primarily a facilitatory role in several complex endocrine functions. The ghrelin receptor is known as the GHS receptor (growth hormone secretagogue receptor) and it is part of the G protein-coupled receptor family (GPCR). Plasma levels of ghrelin depend upon caloric content [17] and macronutrient composition of the meal [18], but factors involved in the chemosensory ability of ghrelin secreting cells are unknown. T2Rs might be involved in these mechanisms, since it has been recently shown that gavage with T2R agonists increases plasma octanoyl ghrelin levels in WT mice and the effect is partially blunted in Gust KO mice [19]. Also, intragastric administration of T2R agonists increases food intake during the first 30 min in WT but not in Gust and ghrelin receptor knockout mice, suggesting that T2Rs stimulation in the stomach leads to the release of ghrelin, consequently acting on food intake.

Gastrin

Gastrin is a linear peptide hormone produced by G cells of the duodenum and G cells in the stomach. Its release is stimulated by proteins and amino acids in the lumen of the stomach or by parietal distension, causing secretion into the bloodstream. It stimulates secretion of gastric acid (HCl) directly by the parietal cells of the stomach or through the activation of Enterochromaffin-Like cells secreting histamine and it plays a role in the relaxation of the ileo-cecal valve [20]. It also induces pancreatic secretions and gallbladder emptying [21] and aids in gastric motility. In the stomach, gastrin also exerts trophic effect on gastric mucosa [22]. It has recently been shown that PTC increases ABCB1 (ATP-binding cassette B1) expression in STC-1 cells through CCK and gastrin signaling mechanism [23], suggesting that T2Rs stimulation in the GI tract leads to the release of these incretins. In my study I have shown that a high protein diet (composed of casein and soy, which are degraded to bitter compounds, able to activate T2Rs) is able to increase mT2R138 mRNA expression in the stomach, effect which might found a possible explanation in reference to these previous evidences showing that gastrin producing cells, located in the stomach, respond to peptides and to PTC, a T2Rs agonist.

Cholecystokinin (CCK)

CCK was first discovered in 1905 by the British physiologist Joy Simcha Cohen. It can be found both within the brain and in the GI tract. In the brain, CCK functions as a real neurotransmitter. In fact it is found in neurons, and it's released in response to depolarization. In the gastrointestinal tract, CCK is synthesized by I-cells in the duodenal and jejunal mucosa and secreted in the duodenum. More than being a single substance,

CCK is actually a family of hormones identified by the number of amino acids (e.g., CCK58, CCK33, and CCK8. CCK58) and it assumes a helix-turn-helix configuration. CCK is very similar in structure to gastrin and has a large variety of physiological functions in the human body, such as the stimulation of gallbladder contraction and emptying, pancreatic enzyme secretion, intestinal motor activity and inhibition of gastric emptying. CCK also sends signals to the brain, which result in satiety sensations and decreased food intake. Basal plasma levels of CCK are ~ 1 pM and rise up to ~ 5 to 8 pM following meal ingestion [24] and the release of CCK is mainly induced by fat and proteins in the small intestine. Recent evidences indicate that stimulation of STC-1 cells with PTC leads to the release of CCK and this effect is reduced in cells where mT2R138 expression is silenced by siRNA [25]. CCK is also released by STC-1 cells in response to stimulation with denatonium benzoate [26].

Glucagon-like peptide 1 (GLP-1)

GLP-1 is an incretin derived from post-translational modification of the larger precursor molecule: proglucagon. Proglucagon is synthesized within the endocrine L-cells in the intestine, primarily the ileum and colon, and from pancreatic alpha cells. The release of GLP-1 is elicited by nutrients in the distal part of the small intestine, but also, to a lesser extent, from the proximal region, and the release depends on the size of the meal [27]. Macronutrients, primarily fat [28] and carbohydrates, are likely to stimulate the L-cells to secrete GLP-1. Once present in the circulation, most plasma GLP-1 is rapidly degraded (within 2 min.) by the enzyme dipeptidyl-peptidase IV (DPP-IV). Biological functions of GLP-1 are stimulation of insulin secretion, reduction of glucagon secretion, regulation of gastric emptying, motility of the gut, acid secretion and food intake. Because GLP-1 inhibits gastrointestinal motility, reduces gastrointestinal secretions and attenuates

gastric emptying, it has been indicated as a major component of the “ileal brake mechanism” (Fig. 5).

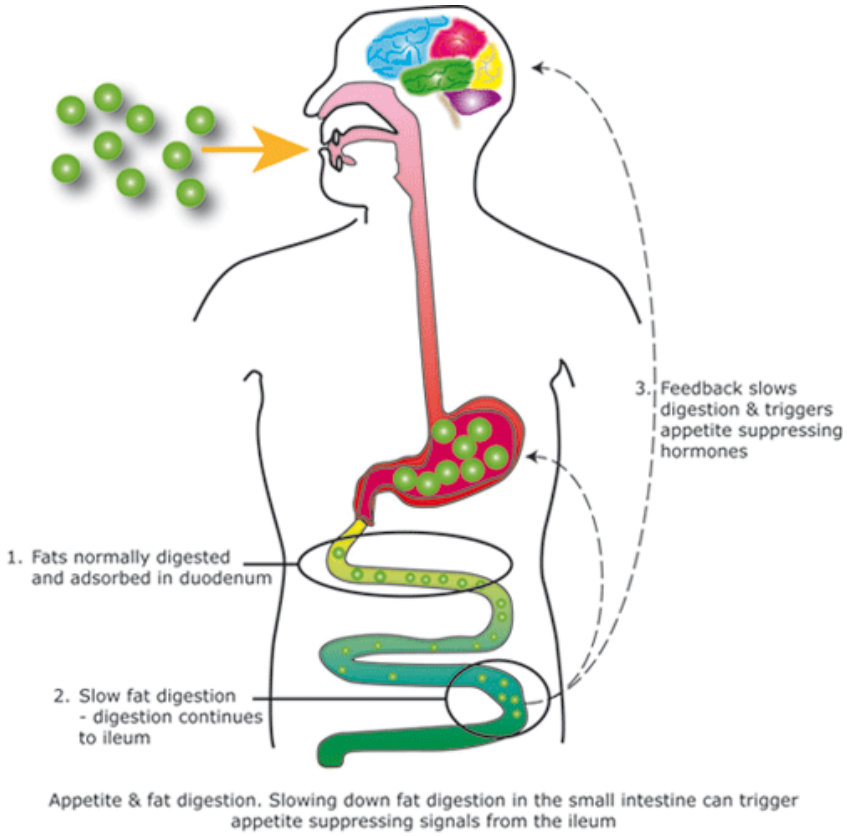


Figure 5: The ileal brake mechanism. Unabsorbed nutrients present in the ileum, especially fat, stimulate the endocrine cells to secrete gastrointestinal hormones such as GLP-1 and PYY. These hormones affect gastric emptying, intestinal motility, transit, and pancreatic secretions, and by doing so, inhibit food intake. [http://www.ifr.ac.uk/info/science/foodstructure/emulsions-interfaces.htm]

This mechanism is a combination of effects influencing ingestive behavior and GI functions. Basically it’s a negative feedback mechanism by which unabsorbed nutrients, especially fat, present in the ileum inhibit gastric emptying, decrease intestinal motility, transit and pancreatic secretions.

There is evidence that GLP-1 is secreted in a taste receptor-dependent manner by gut enteroendocrine L cells in response to natural and artificial sweeteners [29], which is

also interesting for bitter taste receptors study because there is evidence of an association between T2R and altered glucose and insulin homeostasis [30]. Moreover, in mice Gust often colocalizes with L-cells, producing GLP-1 [19].

Peptide YY (PYY)

PYY is a short (36-amino acids) protein, first isolated by Tatemoto et al. in 1980 [31] and named PYY because of the presence of a terminal amino acid tyrosine (Y) and a carboxyl terminal tyrosine amide (Y). The hormone is co-secreted with GLP-1 and released by L-cells in the distal gut (ileum and colon), but there is also a small amount of PYY, about 1-10%, in the esophagus, stomach, duodenum and jejunum [32]. PYY is also produced by a discrete population of neurons in the brainstem, localized to the gigantocellular reticular nucleus of the medulla oblongata [33] and PYY producing cells are also located in the islets of Langerhans in rats [34]. PYY concentration in the circulation is increased after meals, expecially by fat, followed by carbohydrates and proteins [35] and decreased by fasting [36]. Biological functions of PYY are vasoconstriction, inhibition of gastric acid secretion, reduction of pancreatic and intestinal secretion, inhibition of gastrointestinal motility, and food intake inhibition [37] [38]. PYY is also a mediator of the ileal brake (Fig. 5). In humans, cells expressing PYY colocalize with Gust in the colonic mucosa and colonic cells also express transcripts corresponding to members of the T2R and T1R families of bitter and sweet taste [2], suggesting that Gust-dependent signaling in open enteroendocrine cells producing PYY plays a role in sensing bitter/sweet compounds in the lumen. Also, activation of neurons in the nucleus tractus solitarii (NTS) following intragastric administration of T2R agonists involves Y_2 receptors, located on vagal afferent terminals in the gut wall [39]. Therefore,

T2Rs may regulate GI function via release of regulatory peptides including PYY and activation of the vagal reflex pathway.

Serotonin (5-HT)

Serotonin or 5-hydroxytryptamine (5-HT), a mono-aminergic neurotransmitter, is primarily found in the GI, as indicated by its original definition “enteramine” from its discoverer Prof V. Erspamer [40]. In fact, about 95% of the serotonin in the body lies in the digestive system, where it is stored in specific enteroendocrine cells, called enterochromaffin cells (EC). ECs are the predominant neuroendocrine cells in the GI tract and play a key role in the regulation of secretion, motility and visceral pain. Serotonin is released in response to a wide variety of stimuli [41] and it has been proved to be a signaling molecule participating in mucosal sensory transduction [42] [43]. It is mainly involved in the regulation of peristalsis, gastric motility, and postprandial pancreatic secretion [44] [45] [46] [47] [48] [49]. In humans, serotonin levels are affected by diet: for instance, a diet rich in carbohydrates and low in protein will increase serotonin by secreting insulin [50].

In mouse small intestine Sutherland and colleagues [51] showed that 27% of Gust positive cells co-labeled for 5-HT, suggesting that this mediator can be released in response to tastants (sweet, bitter or umami) present in the lumen.

Table I: Gut hormones, cell types and effect on food intake: summary. [from Neary MT & Batterham RL. Gut hormones: Implications for the treatment of obesity. Pharmacology & Therapeutics 2009]

Peptide	Cell type	Effect on food intake
CCK	I	↓
Amylin	P	↓
GLP-1	L	↓
PYY (3-36)	L	↓
APO-AIV	Villus epithelia	↓
Enterostatin	Exocrine pancreas	↓
Bombesin/GRP	Stomach	↓
Oxyntomodulin	L	↓
Gastric leptin	Chief	↓
Ghrelin	X/A-like	↑

In summary, based on previous evidences, reviewed here and further on in this work, we hypothesize that TRs, including T2Rs, are involved in GI chemosensing. The possible pathway involves enteroendocrine open cells, which face the lumen and are stimulated by luminal content. Intraluminal nutrient/non nutrient molecules activate receptors (possibly TRs and T2Rs) on the surface of enteroendocrine cells and lead to the release of molecules which act on neurons within the ENS or on peripheral nervous system (PNS) neurons, or enter the circulation as hormones, as shown in Fig. 3.

c. Taste

The gustatory system is essential for nutritional and survival: it allows animals to detect and discriminate between safe and dangerous food, to select the appropriate nutrients and to avoid the ingestion of harmful substances. Although we can taste a variety of chemical entities and complex flavours, it is extensively accepted that they elicit only five basic taste sensations: salty, sour, sweet, bitter and umami (meaning “savory” in Japanese). Each of these taste represent different physiological needs. **Salty** taste alerts for intake of minerals, primarily sodium ions, which play an essential role in electrolyte balance of the body, important for cell health and function. **Sour** taste detects the presence of acids, in order to maintain the acid-base balance of the body. **Sweet** signals the presence of carbohydrates, usually indicating energy rich nutrients [52]. **Umami** taste detects a few L-amino acids, mainly L-glutamate, and three main substances are considered umami taste: monosodium glutamate (MSG), guanosin monophosphate (GMP) and inosine monophosphate (IMP) [53]. This taste reflects the protein content in food. Finally, **bitter** taste evolved as a warning mechanism to prevent consuming toxins and poisons in food. Usually, tastes are not elicited by a single chemical. Also, there are different thresholds for detection of taste, and this is true both for chemicals that taste the same or for substances in different categories. For example, sucrose, 1-propyl-2 amino-4-nitrobenzene and lactose all elicit a sweet taste in humans, but the sweet sensation is prompted at different concentrations, of roughly 10 mM, 2 uM and 30 mM respectively [54].

Substances sensed as bitter typically have very low thresholds, since they alert from dangerous compounds, which are active at low (nanomolar) concentrations (Table II).

TASTE	SUBSTANCE	THRESHOLD (uM)
Salty	NaCl	0.01
Sour	HCl	0.0009
Sweet	Sucrose	0.01
Bitter	Quinine	0.00008
Umami	Glutamate	0.0007

Table II: Threshold values for different tastes. [modified from http://biology.about.com/library/organs/blpathodigest2.htm]

The sense of taste is classically referred to the mouth, where taste receptors are expressed on gustatory cells and organized in taste buds, found on the tongue. The majority of taste buds are in the lingual epithelium, on papillae. Based on the morphological structure, four types of papillae have been described on the mammalian tongue (Fig. 6).

Fungiform papillae are mostly located on the dorsal surface in the anterior two-thirds of the tongue.

Foliate papillae are on lateral margins towards the posterior part of the tongue. Circumvallate papillae, the one containing the highest number of taste buds, are few (8 to 12 in humans) and arranged in a V-shaped row at the back of the tongue.

Filiform papillae do not contain taste buds and are found all over the surface of the tongue. They are considered to have a mechanical function and to be not directly involved in taste sensation [3] [55].

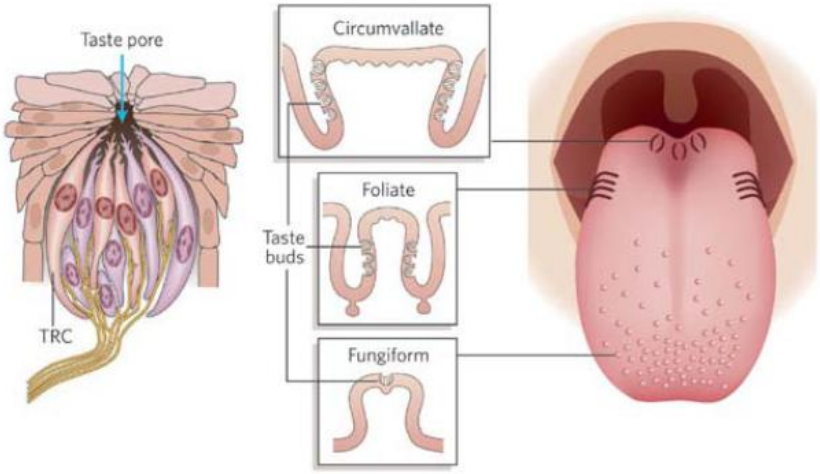


Figure 6: Taste cells in buds, organized in different kind of papillae, distributed in specific regions of the tongue.[www.nature.com/.../fig_tab/nature05401_F1.html]

Based on ultra-structural features, we can also classify different cell types in taste buds: basal cells, type I, II, and III taste cells, whose functions have not been fully established [56]; [57] (Fig. 7). Basal cells are progenitor cells, regulating taste cells turnover. Type I taste cells are the most abundant cells in taste buds. Their primary function is to support type II and III taste cells [58]. Type II taste cells are thought to be the actual taste receptor cells, containing receptors and signaling components for sweet, bitter and umami. Mammalian taste cells are not neurons and thus do not form conventional synapses onto afferent nerve fibers. Instead, they generate action potentials and release various mediators in response to taste stimuli, and this signal is transmitted by neurons that innervate taste buds. The adjacent Type III taste cells express synaptic proteins and form synapses with nerve fibers. which then transfer taste information to the central nervous system.

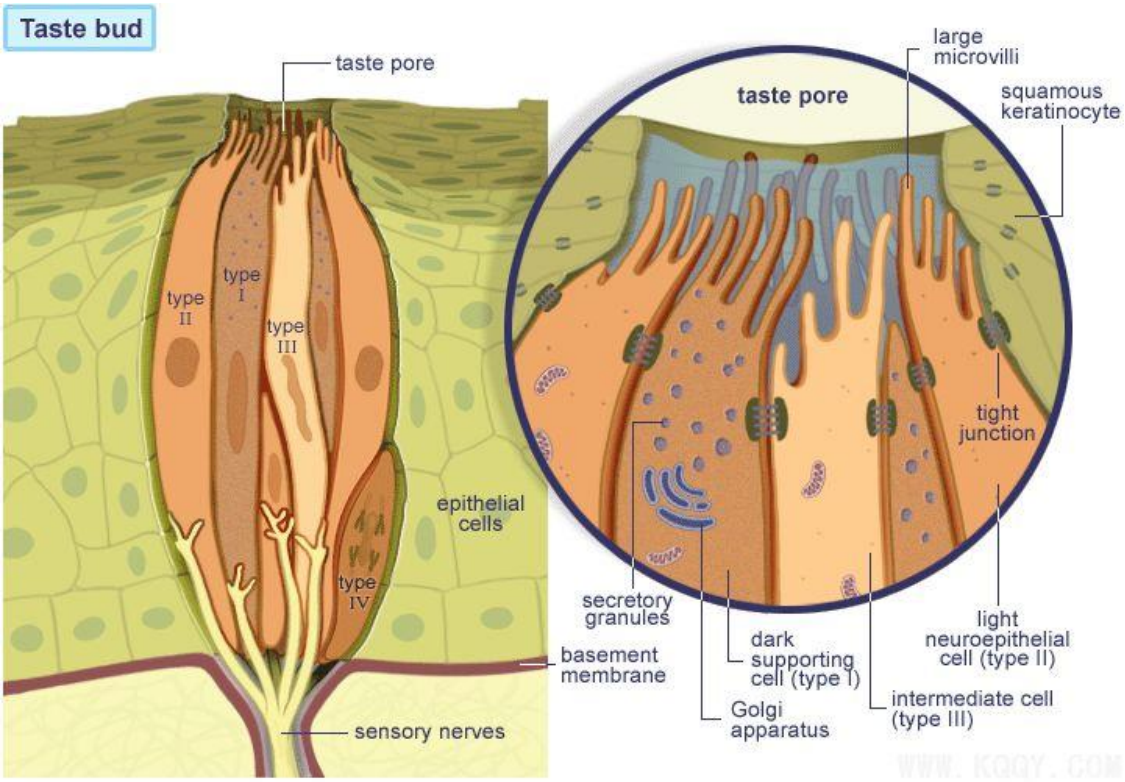


Figure 7: Different cell types in taste buds: Type I cells are supporting cells, Type II contain taste recepotrs and type III form synapses with afferent nerves, receiving information from many different taste cells.Type IV (basal) are progenitor cells appearing during the regular turnover. [http://www.kqqy.com/html/perfessional/Topics/surgery/16010.html]

Starting from the tongue, nerves relay taste information to the rostral and lateral regions of the solitary tract nucleus (NTS) of the medulla in a topographical manner. Gustatory information are then transferred from the NTS to the thalamus, and then to gustatory areas of the cortex [59] (Fig. 8).

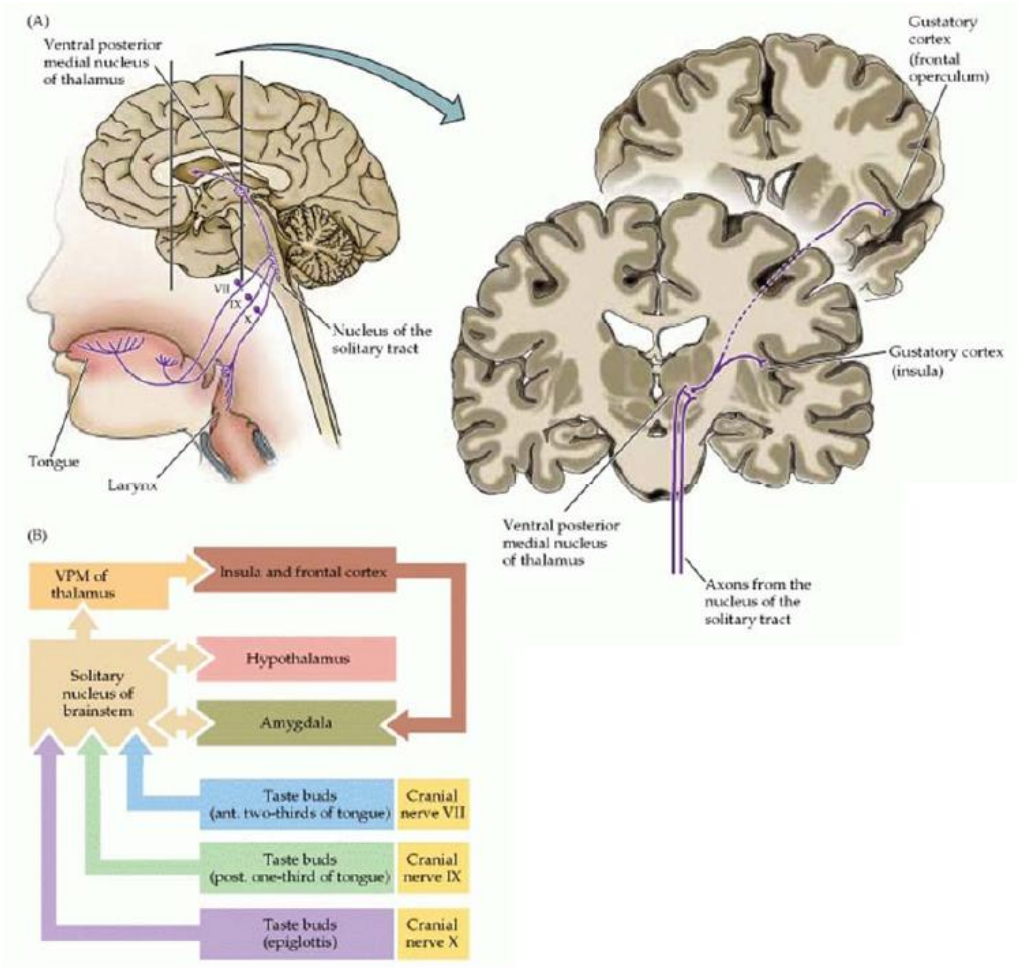


Figure 8: Organization of the taste system: (A) connection between the oral gustatory system and the nucleus of solitary tract and cortex in the brain (B) Diagram of taste information network. [from Purves D, Augustine GJ, Fitzpatrick D, et al., editors, The Organization of the Taste System, 2001]

Humans usually exhibit a strong innate aversion to strong bitter taste. However, bitter taste perception is complicated and depends on various factors, including genetic, cultural habits, age [60]. The best known example of this variation is the genetic ability to taste the synthetic compounds phenylthiocarbamide (PTC) and 6-n-propylthiouracil (PROP), agonists for a specific receptor subtype (T2R38) [61], which depends on the receptor isoform. On average, based on their phenotype, 75% of people can taste PTC, while 25% cannot.

d. Bitter Taste receptors

In all mammals bitter is one of the few innate sensations universally recognized as disgusting and it induces aversive reactions [3]. This ability to detect the presence of toxic substances is strongly associated with the development of T2Rs in the oral cavity, an evolutionary-conserved mechanism to prevent ingestion of bitter-tasting compounds, which might often be toxins (such as alkaloids, saponines, etc.) and are often contained in plants (Fig 9).

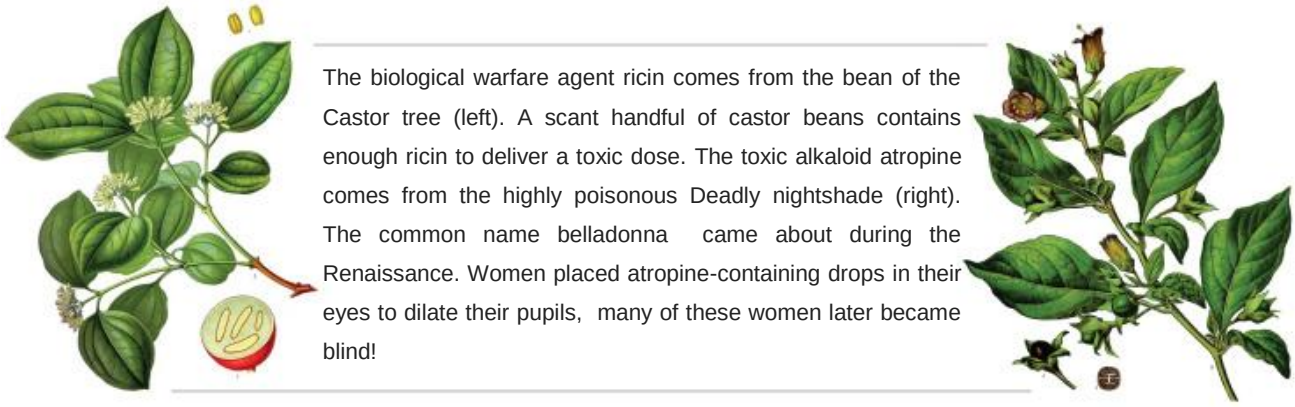


Figure 9: Bitter plants [from: http://learn.genetics.utah.edu/content/begin/traits/ptc/]

All vertebrate species investigated so far possess several bitter taste receptor genes (T2Rs). Avian and amphibian genomes represent the extremes, with 3 and ~50 T2R genes respectively, whereas mammalian species express between 15 and 36 T2R genes [62] [63] [64]. Twenty-five T2Rs were identified in the human genome [65] [66]. As a result of higher frequency of gene duplication, rodents developed a significantly higher number of T2R genes compared to humans [1]. Therefore, rodents possess about 30% more bitter taste receptors, probably corresponding to a wider spectrum of bitter sense detection. The most conserved T2Rs (e.g., hT2R1, hT2R4, and hT2R38)

between human and rodents have known bitter taste ligands, such as phenylthiocarbamide (PTC) and denatonium benzoate (DB), both used in this study. Whereas 80% (20 of 25) of the human T2Rs are deorphanized, only for 6% of mT2R genes there are known agonists [67] [68]. The situation is even more complicated because many taste receptors are “broadly tuned”, which means that they can recognize many different substances. A recent screening of all 25 hT2Rs with 104 natural and synthetic compounds [68] revealed that the most broadly tuned are T2R10 [64], T2R14 [69] and T2R46 [70]. Some others, instead, are narrowly tuned, such as T2R5-13-49 [68], meaning that they recognize one or really few compounds. Most of T2Rs, however, show intermediate characteristics: this group includes T2R138 [71] and T2R108 [67], which predominantly detect respectively PTC and PTU (T2R138/T2R38) and DB (T2R108/T2R4) and are therefore considered “specifically tuned” receptors. In this study we focused on two of these well conserved receptors: mT2R138 (which is hT2R38) and mT2R108 (which is hT2R4) and Gust, their major signaling molecule. Not only the extent of tuning but also the sensitivity differs considerably among T2Rs, ranging from EC50 values in the nanomolar range, as for the hT2R43 challenged with aristolochic acid [72], to low millimolar concentrations determined for hT2R16 stimulated with D-salicin [64]. On a qualitative level, the human T2R gene family is highly variable. As discussed before, the best example for this is the hT2R38 non-taster variant [73], which leads to a dramatic decrease in the possibility to taste the bitter substances PTC and PROP in subjects homozygous for this variant [74]. Additional examples are provided by functional polymorphisms in hT2R16 [75] as well as in hT2R43 and hT2R31 [76] or mT2R105 [67]. In general, T2Rs are seven trans-membrane receptors which couple to specific G alpha subunits, common to all taste GPCRs (sweet, bitter, umami), called Gustducin and Transducin, which are Gi/Go proteins [77]. Beta subunits are β1 β3 and gamma are γ13 subunit [78] [79]. At a molecular level, the signal transduction components after the initially activated heterotrimeric G protein are well established in

the mouth [80] [81]. Biochemical experiments [82] [83] and genetically modified mouse models [84] [85] demonstrate the involvement of phospholipase C β2 (PLCβ2). After activation by a tastant, the trimeric complex dissociates and the Gβ/Gγ heterodimer stimulates PLCβ2, resulting in the generation of the second messengers diacylglycerol (DAG) and inositol triphospate (IP_3) [78]. Next, the activation of the type III IP_3-receptor residing in the endoplasmic reticulum membrane causes calcium release from internal stores [86] [87], which, in turn leads to the opening of a transient receptor potential channel, TRPM5, located in the plasma membrane [88]. The activation of this non-selective cation channel results in cell depolarization [89] [90] and further Ca^{++} influx from voltage-gated Ca^{++} channels. This Ca^{++} increase causes an hemichannel to open and release ATP, which acts as a neurotransmitter linking taste buds to the nervous system. ATP secreted from receptor (type II) cells, in fact, excites primary sensory afferent fibers and probably also stimulate presynaptic (type III) cells to release 5-HT and norepinephrine [91] [92].

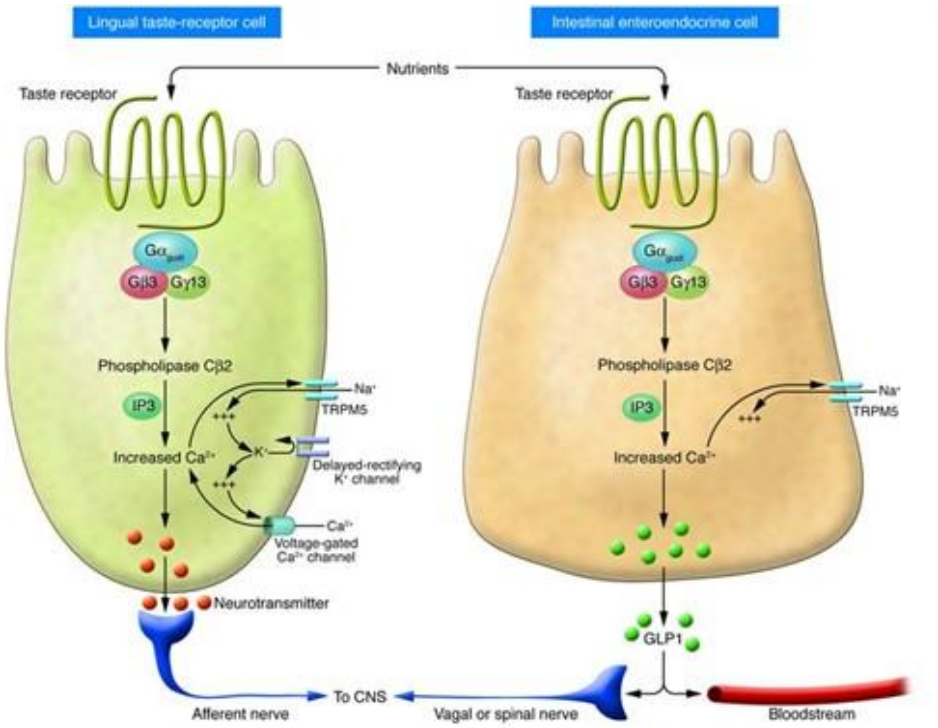


Figure 10: Taste receptors activation mechanism in the tongue and in the gut. [Cummings DE, J Clin Invest. 2007]

However, there are also observations indicating residual taste responses in various Gust KO models which do not allow to draw conclusions on the exclusiveness of Gust as initial taste transduction component for taste responsiveness in mammals [89].

Human		Mouse	
Approved symbols	Previous symbols and aliases	Approved symbols	Previous symbols and aliases
Taste receptor, type 1			
TAS1R1	T1R1, TR1, GPR70	Tas1r1	GPR70
TAS1R2	T1R2, TR2, GPR71	Tas1r2	T1R2, GPR71
TAS1R3	T1R3	Tas1r3	T1R3
Taste receptor, type 2			
TAS2R1	T2R1, TRB7	Tas2r102	T2R102, mT2R51, STC9-7
TAS2R3	T2R3	Tas2r103	T2R103, T2R10, TRB2
TAS2R4	T2R4	Tas2r104	T2R104, mT2R45
TAS2R5	T2R5	Tas2r105	T2R105, T2R5, T2R9
TAS2R7	T2R7, TRB4	Tas2r106	T2R106, mT2R44
TAS2R8	T2R8, TRB5	Tas2r107	T2R107, mT2R43, T2R4, STC5-1
TAS2R9	T2R9, TRB6	Tas2r108	T2R4, T2R8
TAS2R10	T2R10, TRB2	Tas2r109	T2R109, mT2R62
TAS2R13	T2R13, TRB3	Tas2r110	T2R110, mT2r57, STC 9-1
TAS2R14	T2R14, TRB1	Tas2r113	T2R113, mT2R58
TAS2R16	T2R16	Tas2r114	T2R114, mT2R46
TAS2R19	TAS2R48, TAS2R23, T2R19, T2R23	Tas2r115	
TAS2R20	TAS2R49, T2R20, T2R56	Tas2r116	T2R116, mT2R56, TRB1, TRB4
TAS2R30	TAS2R47, T2R30	Tas2r117	T2R117, mT2R54
TAS2R31	TAS2R44, T2R31, T2R53	Tas2r118	T2R16, T2R18, mt2r40
TAS2R38	PTC, T2R61	Tas2r119	T2R119, T2R19
TAS2R39		Tas2r120	T2R120, mT2R47
TAS2R40	GPR60	Tas2r121	T2R13, T2R121, mT2R48
TAS2R41	T2R59	Tas2r122	
TAS2R42	T2R24, T2R55, hT2R55, TAS2R55	Tas2r123	T2R123, T2R23, mT2R55, STC9-2
TAS2R43	T2R52	Tas2r124	T2R124, mT2R50
TAS2R45	ZG24P, GPR59	Tas2r125	T2R125, mT2R59
TAS2R46	T2R54	Tas2r126	T2R41, T2R12, T2R26
TAS2R50	T2R51	Tas2r129	T2R129, mT2R60
TAS2R60	T2R60	Tas2r130	T2R7, T2R6, T2R30, STC7-4, mT2R42
		Tas2r131	
		Tas2r134	T2R134, T2R34
		Tas2r135	T2R135, T2R35, mT2r38
		Tas2r136	T2R136, T2R36, mT2r52
		Tas2r137	T2R3, T2R137, T2R37, mT2r41
		Tas2r138	T2R38, T2R138, mT2R31
		Tas2r139	T2R39, mT2R34
		Tas2r140	T2R140, T2R40, T2R8, T2R13, mT2r64, mTRB3, mTRB5
		Tas2r143	T2R143, T2R43, mT2R36
		Tas2r144	T2R40, mT2R33

Table III: Complexity and variety of T2Rs. Approved gene symbols and previous symbols for human and mouse G protein-coupled taste receptors.[M. Behrens, W. Meyerhof, Physiology & Behavior, 2011]

e. Functional implications and clinical relevance of T2Rs study

T2Rs and their signaling molecules in the gut are found in cells secreting hormones that control GI functions and food intake, therefore their study is open to many clinical applications. Modulation of endogenous incretin levels by tastants may provide novel therapeutic applications for the treatment of eating and gastrointestinal motility disorders. Also, since bitter taste evolved as a warning mechanism against harmful substances, the study of bitter taste receptors in the gut might provide insights into T2Rs function as possible defense mechanism toward harmful substances. This study suggests that T2Rs might recognize bacteria in the enteroendocrine cell line STC-1 and in human NCM-460 colonocytes, as well as they do in the airways [93]. We also suggest that T2Rs can respond to a change in commensal gut microbiota, such as the one caused by a high fat diet. This change in microbiota seems to be an important component in the development of obesity and inflammation. Therefore the elucidation of T2Rs function might provide useful information that could lead to the development of new therapeutic approaches to treat obesity or to control the inflammatory response evoked by bacteria in the gut. hT2R38 variants also seem to be involved in colorectal cancer risk [94] and they might be an important factor in the development of nicotine dependence [95], expanding the clinical importance of T2Rs in these fields as well.

Furthermore, understanding the luminal chemosensory mechanisms may help to identify novel molecular targets for treating and preventing mucosal injury, metabolic diseases and abnormal visceral sensation. Moreover, further studies on bitter taste recognition in the GI might also help commercially by providing information that could lead to the creation of new products in the food and drug industry, as functional food or products controlling drug absorption.

CHAPTER II

T2R138, T2R108 and Gustducin are distributed throughout the GI tract and are specifically regulated by different diets

1. Background and aim

BACKGROUND

a. Bitter taste receptors and taste signaling molecules in the GI tract

Bitter taste receptors are expressed in the oral cavity and couple to G_α proteins, in particular G_α-gustducin and G_α-transducin [96]. These receptors were thought to have only gustatory functions, and to be limited to the oral cavity, but in the last 10 years there have been various reports of the presence of gustatory receptors and signaling molecules in extra-oral sites, with non-gustatory functions. The interest for the taste transduction machinery in the GI tract began in the late 90s. In fact, long before taste receptors expression was investigated, the presence of the taste signaling molecule G_α-gustducin in the stomach, duodenum and pancreatic ducts of rat was reported [97] [98] [99]. Later, G_α-gustducin as well as the closely related molecule G_α-transducin, which was shown to be expressed in taste cells in addition to G_α-gustducin [188], were found in other regions of the gut [100] [1] [2]. More recently, additional molecules of the taste cell signaling cascade such as PLCβ2 [83] [82] and TRPM5 [101] were localized in the GI-tract as well. However, thought almost all of the signaling components for taste signal in the mouth are found in the GI, co-localization studies performed with various taste cell markers in GI-tissues revealed that cells expressing all taste transduction components together are rare. The cell population expressing taste-related molecules is heterogeneous, including different enteroendocrine cell types as well as brush cells/solitary chemosensory cells [100] [102] [2] [99]. The apparent complexity of Gust-expressing cell types in the gut was confirmed by a recent study in mouse small intestine where three types of Gust-positive cells were identified: expressing G_α-gustducin only, G_α-gustducin and glucagon-like peptide-1 (GLP-1), or G_α-gustducin and 5-HT [51].

The first taste receptors identified in GI-tissue were the T2Rs, localized in endocrine cells in the mucosa of the gastric antrum and fundus, duodenum and gastroendocrine cells in rats stomach [102]. The same study demonstrated, using RT-PCR analysis, the presence of mouse T2R genes in the corresponding GI-tissues and in mouse STC-1 cells, a mixed population of endocrine cells, indicating a conserved role in the gut. Subsequently, several additional rat and mouse T2Rs were detected by RT-PCR in GI tissues and cell lines of gastrointestinal origin. Similar studies on human gut tissues and cell lines from GI-tissues resulted in the identification of transcripts from human T2R genes [2] [103]. It is interesting to notice that bitter taste receptors are also expressed in a variety of other extra-oral sites, such as brain [104], testis [105] and respiratory system [106] [93] [107] besides the GI tract, suggesting that there is more than a “taste” function for taste receptors.

Most studies concerning the expression of bitter taste receptors in gastrointestinal cells use RT-PCR experiments to identify T2R mRNA in human or rodent gastrointestinal tissue or model cell lines for enteroendocrine cells, but their expression in situ has not been extensively proved yet. The tools to investigate these receptors still need to be better developed. For example at present only a single cellular co-localization experiment demonstrated the presence of mT2R138 co-localizing with chromogranin A (CgA), a marker for enteroendocrine cells, in sections of mouse small intestine [25]. In this study, we show some IHC images of mT2R138 and Gust, including some examples of co-localization with different cell markers.

Functional studies, both on animals and cells, are mainly aimed to show that agonist local (GI) stimulation leads to the release of incretins by enteroendocrine cells, thus modulating GI functions, even if it’s not directly proved that these responses are T2Rs mediated. To show that T2Rs are stimulated by luminal content, Glendinning and colleagues separated oral and gastrointestinal stimuli by intragastric infusion of denatonium solutions in rodent experiments [108]. They saw that there is a robust

conditioned taste aversion in rats and that gastric emptying was slowed. Our group have also done a similar study in rats and showed aversion induced by T2R ligands stimulation [39]. Moreover, application of ligands for T2Rs, including DB and PTC to STC-1 cells, induces rapid Ca^{++} signaling [26], indicating that T2Rs in these cells are functional. The mechanism used by bitter compounds in the upper gastrointestinal tract to influence gastrointestinal function seems to involve vagal afferents and CCK and PYY receptors [39]. The pathway following receptor binding seems to resemble the one in the mouth, since many taste signaling molecules are expressed in the GI (Fig. 10). In addition, expression levels for components of the taste transduction machinery in the gut are not static but seem to respond dynamically to nutrient conditions. For example, Young and colleagues [109] have shown that mRNA expression for sweet taste receptors (T1R2 and T1R3 subunits), TRPM5 and G_{α}-gustducin were inversely related to blood glucose levels in human and mouse. Margolskee et al. [110] have shown that sweet taste receptors (T1R3 subunit) and G_{α}-gustducin are coupled to the mRNA levels of expression of Sodium-dependent glucose cotransporters (SGLT) both in mouse small intestine and in a murine enteroendocrine cell line (GLUTag). Furthermore, molecules involved in the regulation of fat metabolism have been shown to up-regulate T2Rs in EECs from mouse intestine and on the mouse small intestinal cell line STC-1 [25]. All the previous findings suggest an active role for taste receptors in chemosensing in the GI.

b. T2R138 and PTC

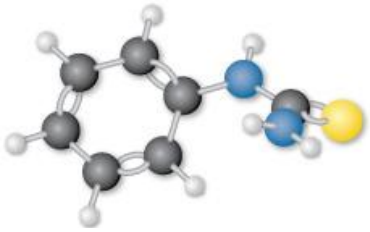


Figure 11: PTC chemical structure [http://learn.genetics.utah.edu/content/begin/traits/ptc/]

Back in 1931, a chemist named Arthur Fox was pouring some powdered PTC into a bottle. A colleague standing nearby complained that the dust tasted really bitter, but Fox tasted nothing at all. Curious how they could possibly be tasting the chemical differently, they tried to make other people taste the chemical and describe how it tasted (Fig. 12). Some people tasted nothing, some found it intensely bitter, and others thought it tasted only slightly bitter.

Figure 12: Albert Blakeslee using a voting machine to tabulate results of taste tests at the AAAS Convention, 1938.
[http://www.carolina.com/category/teacher%20resources/instructions%20and%20buying%20guides/biotech%20kit%20instruction%20manuals/using%20a%20single-nucleotide%20polymorphism%20to%20predict%20bitter%20tasting%20ability.do]

Soon after this discovery, geneticists determined that there is an hereditary component that influences how we taste bitter and PTC. The PTC gene, hT2R38, was discovered later in 2003 and today we know that the ability to taste this compound is conveyed by a single gene that codes for this taste receptor on the tongue.

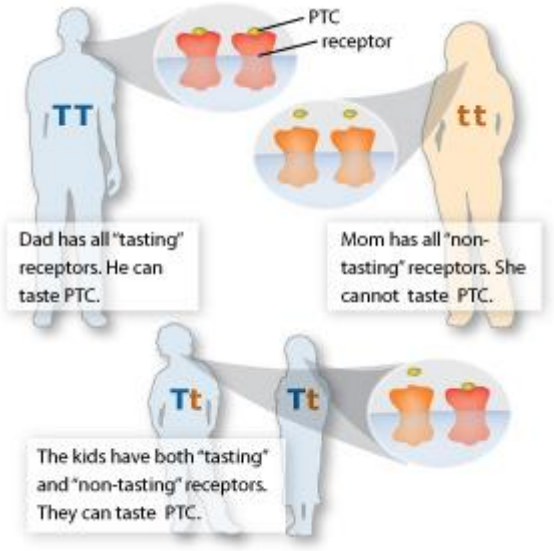


Figure 13: Allelic variants for T2R38 and phenotypes. The ability to taste PTC shows a dominant pattern of inheritance. A single copy of a tasting allele (T) conveys the ability to taste PTC. Non-tasters have two copies of a non-tasting allele [http://learn.genetics.utah.edu/content/begin/traits/ptc/]

There are two common forms of the PTC gene, and at least five rare forms. One of the common forms is a “tasting” allele, and the other is a “non-tasting” allele. Depending on the allele, the shape of the receptor protein varies and this determines how strongly it can bind to PTC, in order to sense it. Since all people have two copies of every gene, combinations of the bitter taste gene variants determine whether someone finds PTC intensely bitter, slightly bitter, or without any taste at all (Fig. 13). hT2R38 variants also seem to be involved in adiposity [111] and colorectal cancer risk [94] and the receptor might also be an important factor in the development of nicotine dependence [95].

Moreover, mT2R138 in the GI seem to participate in cholesterol metabolism [25], further expanding the possible roles for T2R138 besides its taste function.

c. T2R108 and DB

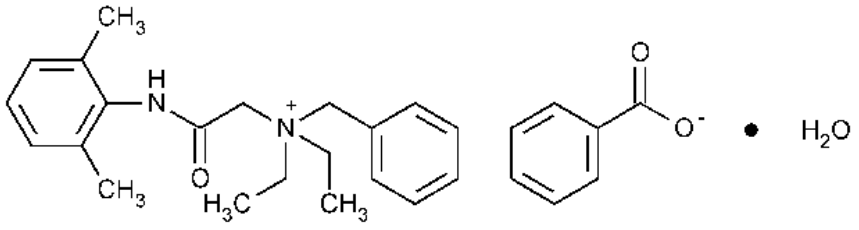


Figure 14: DB chemical structure. [http://www.lookchem.com/Denatonium-benzoate/]

Denatonium, usually available as benzoate, is one of the bitterest known substances; with a threshold of 0.05 ppm [112] and it is used in taste warning, since it is stable and inert and just a few parts per million will make a product so bitter that children and pets will not be able to swallow it. It was discovered in 1958 in Edinburgh, Scotland, during research on local anesthetics. During routine work at Macfarlan Smith, laboratory staff noticed that denatonium benzoate powdered form was extremely bitter. After a while, the company registered DB solutions under the trademark “Bitrex” (Fig. 15) and still today denatonium salts are used as aversive agents to prevent accidental ingestion of many commercially available products.

Figure 15: The Bitrex® logo, first commercial form of denatonium benzoate, used for its bitter properties. [http://www.bitrex.com/]

r/mT2R108 and its human homologous hT2R4 are found in rat brain [104], in human airway epithelial cells [113], rat duodenal mucosa [102], in mouse antrum, fundus and duodenum and in STC-1 cell line [1]. DB intragastical administration increases plasma octanoyl ghrelin levels significantly [19] and activates vagal afferent neurons in NTS [39], suggesting the presence of active hT2R4/mT2R108 in the GI.

d. Gα-gustducin

Gustducin is the GTP-binding α-subunit of a trimeric G-protein complex specific for taste GPCRs. Its sequence places it in the G inhibitory (Gi) related class of G-proteins. Its discovery dates back to 1992, when Margolskee and his colleagues syntetized degenerate oligonucleotide primers_based on the conserved sequence of known G proteins_and mixed them with a taste tissue cDNA library. The DNA products were amplified by PCR and eight positive clones were shown to encode the α subunits of G-proteins [97] interacting with GPCR. Of these eight units, two had previously been shown to encode G_{α}-transducin, a molecule involved in visual transduction and previously localized only in the retina. G_{α}-gustducin showed significant homology with G_{α}-transducin since 80% identity was found between rat G_{α}-gustducin and rat rod G_{α}-transducin and their interactions and functions are almost identical [114], indicating that G_{α}-gustducin and G_{α}-transducin are evolutionarily closely related signal molecules. When challenged with DB and quinine, both G_{α}-gustducin and G_{α}-transducin can activate taste specific PDE, indicating that both molecules are important in the signal transduction of bitter compounds. On the other hand, some studies show that G_{α}-transducin seems not involved in responses to bitter or sweet compounds but it is involved in responses to umami [89]. G_{α}-gustducin mRNA appears to be expressed in

about 40 % of the taste receptor cells and immunocytochemistry localizes the protein to rat and human taste receptor cells [115], which strongly suggests that it mediates taste signal transduction. To prove G_{α}-gustducin's role in bitter taste transduction, Margolskee's group used Gust KO mice [116]. KO mice showed reduced behavioural and electrophysiological responses to bitter compounds, which returned to normal when the G_{α}-gustducin gene was re-inserted. However, the loss of the G_{α}-gustducin gene does not completely remove the ability of the knock-out mice to taste bitter food. This indicates that G_{α}-gustducin is probably not the only mechanism for tasting bitter molecules.

Also, later on it was discovered that gustatory neurons can specifically strongly respond to a single taste stimulus (usually sweet) but can also be activated by multiple tastes [117] [118], although a neuron typically would favor one specific stimulant over others. This suggests that, while many neurons favor bitter taste stimuli, neurons that favor other stimuli such as sweet and umami may be capable of detecting bitter stimuli in the absence of bitter receptors or bitter signaling components, as in the Gust KO mice. While G_{α}-gustducin was known to be expressed in lingual taste cells, studies with rats showed that it was also present in a limited subset of cells lining the stomach and in brush cells in the intestine [98]. These cells appeared to share several features with mouth taste receptor cells, since they expressed other taste signaling molecules such as TRPM5, PDE, PLCβ2 and others. From these evidences started the idea that the same receptors and signaling molecules found in the mouth could be active and have a role in the GI tract and that they could share the same pathway operating in the mouth.

AIM

The aim of this study was to evaluate mT2R138, mT2R108 and Gust distribution along the mouse GI tract and to investigate whether these molecules can be modulated by luminal stimuli coming from different diets. This was done to assess if different T2Rs are differently distributed along the GI and to prove an involvement for T2Rs in luminal chemosensing in the GI.

2. Material and methods

a. Animals and diets

Experiments were performed on adult male C57/BL6 mice (Charles River Laboratory International, Inc, Wilmington, MA). Care and handling of the animals were in accordance with all National Institute of Health recommendations for humane use of animals. All experimental procedures were reviewed and approved by appropriate Animal Research Committees at UCLA, Los Angeles, CA, USA. For fasting and refeeding experiments, mice were fasted 18 hours and refed for 2 or 4 hours after fasting. For cholesterol lowering diet mice were fed regular chow supplemented with Lovastatin (100 mg/100 g chow) and Ezetimibe (21 mg/100g chow) for 7 days. For high fat diet, mice were fed 10% (Research Diets D12450B), 45% (D12451) or 60% (D12492) fat by calories diet for 2 or 8 weeks. For high protein diet mice were fed with 40% protein (by calories) diet, 20% from casein and 20% from soy (Harlan TD.110338) for 14 days. All experimental groups were compared to mice normally fed with regular chow. At the end of the diets, mice were sacrificed by isoflurane overdose for tissue removal. Intestinal specimens were obtained from the whole gastrointestinal tract and snap frozen for qRT-PCR or fixed for 2h in 4% PFA for immunohistochemical analysis.

b. RNA extraction and qRT-PCR

Total RNA was isolated from gastrointestinal tissue (stomach antrum and corpus, duodenum, jejunum, ileum, proximal and distal colon) using Absolutely RNA® RT-PCR Miniprep Kit (Stratagene, La Jolla, CA) and a DNase treatment was performed to eliminate genomic DNA contamination. RNA quality was estimated by the absorbance at 260 nm and 280 nm ratio (OD260nm/OD280nm>1.7). RNA integrity was verified by

presence of two distinct bands that correspond to 18S and 28S rRNA in 2% agarose gel upon electrophoresis. Complementary DNA was generated using Superscript III Reverse Transcriptase kit (Invitrogen) according to the manufacturer'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 for mT2R138 (Applied Biosystem, Mm01700131_s1), mT2R108 (Applied Biosystem Mm00498514_s1) and Gust (Applied Biosystem, Mm01165313_m1). Standard thermal cycles (50 cycles) for Taqman Gene assays were run on a Mx3000P Real-time PCR Detection System (Stratagene) and data were analyzed with Mx Pro 1000 software. Actin beta (BA) and 18S RNA were used as housekeeping genes (data shown for BA) and the relative abundance of mRNA expression was calculated using the Delta delta Ct method (User Bulletin #2, ABI Prism 7700 Sequence Detection System). Data were expressed relatively to the control group, chosen as 1, or to antrum, arbitrary chosen as the unity of measurement for each primer in distribution studies. An enteroendocrine cell line (STC-1) expressing these transcripts and a fibroblast cell line (3T3) not expressing T2Rs [102] were used respectively as a positive and negative control. Samples were run at least 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 seizes as the expected size were detected. All assays were validated for linearity of amplification efficiency and standard curves obtained using RNA samples serial dilutions.

c. Immunohistochemistry

Tissue was fixed in 4% PFA for 2 hours. Transgenic mice (with a C57/BL background) expressing GFP for G_{α}-gustducin were used to assess the expression of the signaling molecule at a protein level in the GI tract and to show some co-localization with specific cell type markers. Specimens from male C57/BL mice were used for the study on WT mouse tissue. Immunohistochemistry was performed on frozen sections, 10µm thick. Slides were washed 3 times 10' with PB 0.1 M, incubated 1h in donkey serum to block unspecific binding and immunostained overnight at 4°C with Gt anti T2R138 (Sc-34357), Rb anti Gust 1:250 (Sc-395) and Rb anti Chromogranin A 1:500 (Sc-1488), all from Santa Cruz Biotechnologies. Tissues were then incubated 2 h in secondary antibody (Dk anti Gt or Dk anti Rb ALEXA 488, dil 1:1000, for green fluorescence or Rhodamine Red X, dil 1:300, for red). Images were scanned with a confocal microscope (ZEISS 510 laser scanning confocal microscope, Carl Zeiss Inc, Thornwood, NY) running LSM5 software.

d. Data analysis and Statistics

Values were expressed as the mean ± S.E.M.. One way ANOVA followed by Bonferroni post-test for multiple comparison was used for statistical analysis when groups were more than two. Two-way ANOVA was used in groups where the results of a single treatment vs control were assessed in different GI specimens. Values $P<0.05$ were considered significant. The statistical software package Prism 5.0 (GraphPad Software, San Diego, CA) was used for these analyses.

3. Results

a. T2R138, T2R108 and Gust are expressed throughout the mouse GI tract, with different distribution

qRT-PCR and gel electrophoresis showed the presence of the amplified products generated by the Taqman Gene Expression Assay primers specific for mT2R138, mT2R108, Gust and β-actin in the entire GI tract (Fig. 16).

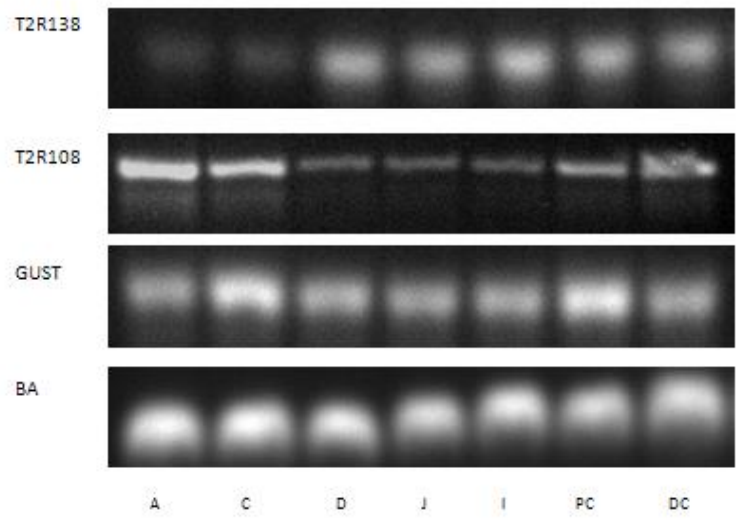


Figure 16: Agarose gels for mT2R138-mT2R108 and GUST distribution. RT-PCR products were analyzed by 4% agarose gel and single bands of the predicted size are shown for each primer, in all GI segments analyzed, confirming their specificity. (A=antrum C=corpus D=duodenum J=jejunum I=ileum PC=proximal colon DC=distal colon)

mT2R138 and mT2R108 transcripts showed a different distribution along the GI: mT2R138 mRNA (Fig. 17A) is more abundant in colon, particularly in the distal portion (distal colon=165.07±39.88, $P<0.01$ vs all other regions; proximal colon=31.57±12.25, $P<0.01$ vs jejunum, ileum and stomach) and in duodenum (24.33±5.59 $P<0.05$ vs jejunum, ileum and stomach) compared to other regions of the gut, with the lowest levels in the stomach (small intestine average=4.78±1.42, stomach average=0.79±0.21, $P<0.05$ vs duodenum; $P<0.001$ vs distal colon), while mT2R108 mRNA expression

(Fig. 17B) is most prominent in the stomach (P<0.01 for corpus vs all other GI regions). Taste receptor signaling molecule, G_{α}-gustducin transcript (Fig. 17C), is also distributed throughout the entire GI tract, with highest levels in the stomach (P< 0.01 for antrum and corpus vs small intestine; P<0.05 vs DC) and proximal colon (P<0.015 for proximal colon vs other regions, besides DC), matching the distribution of mT2R138 and mT2R108 transcripts. These receptors were not detectable in 3T3 cell line, a fibroblast cell line used as a negative control, and were highly abundant in STC-1 cells, an enteroendocrine cell line, or in the tongue, used as positive controls.

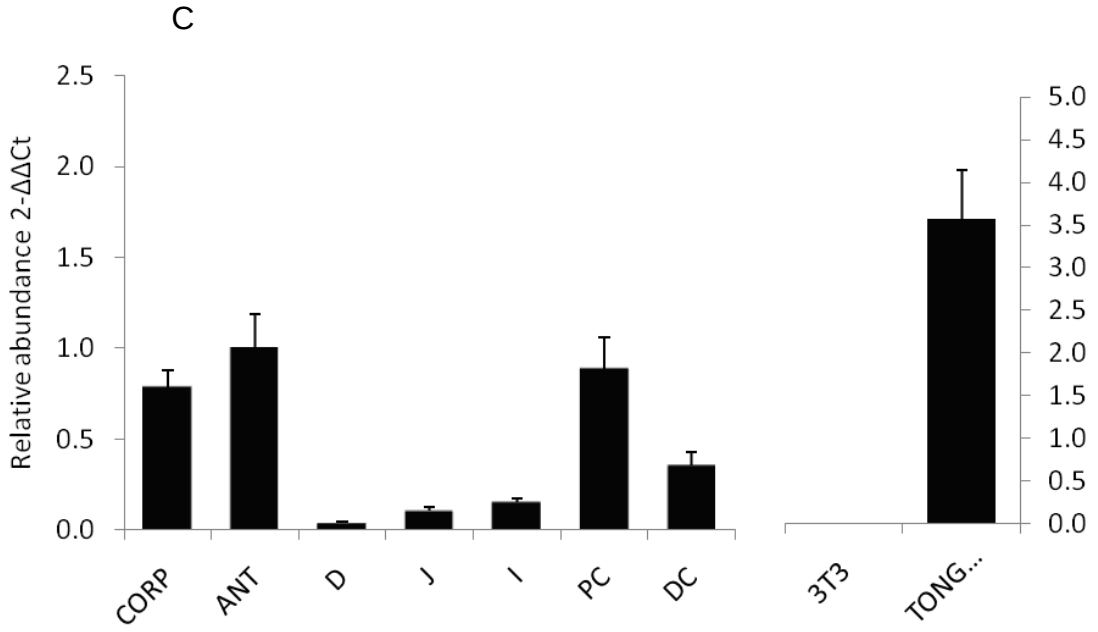


Figure 17C. Distribution of Gust, a signaling molecule common to different taste receptors, matches overall, but not completely, with the distribution of the two T2Rs. Gust is in fact abundantly expressed in stomach, as T2R108, and distal colon, as T2R138, and it's also highly expressed in proximal colon.

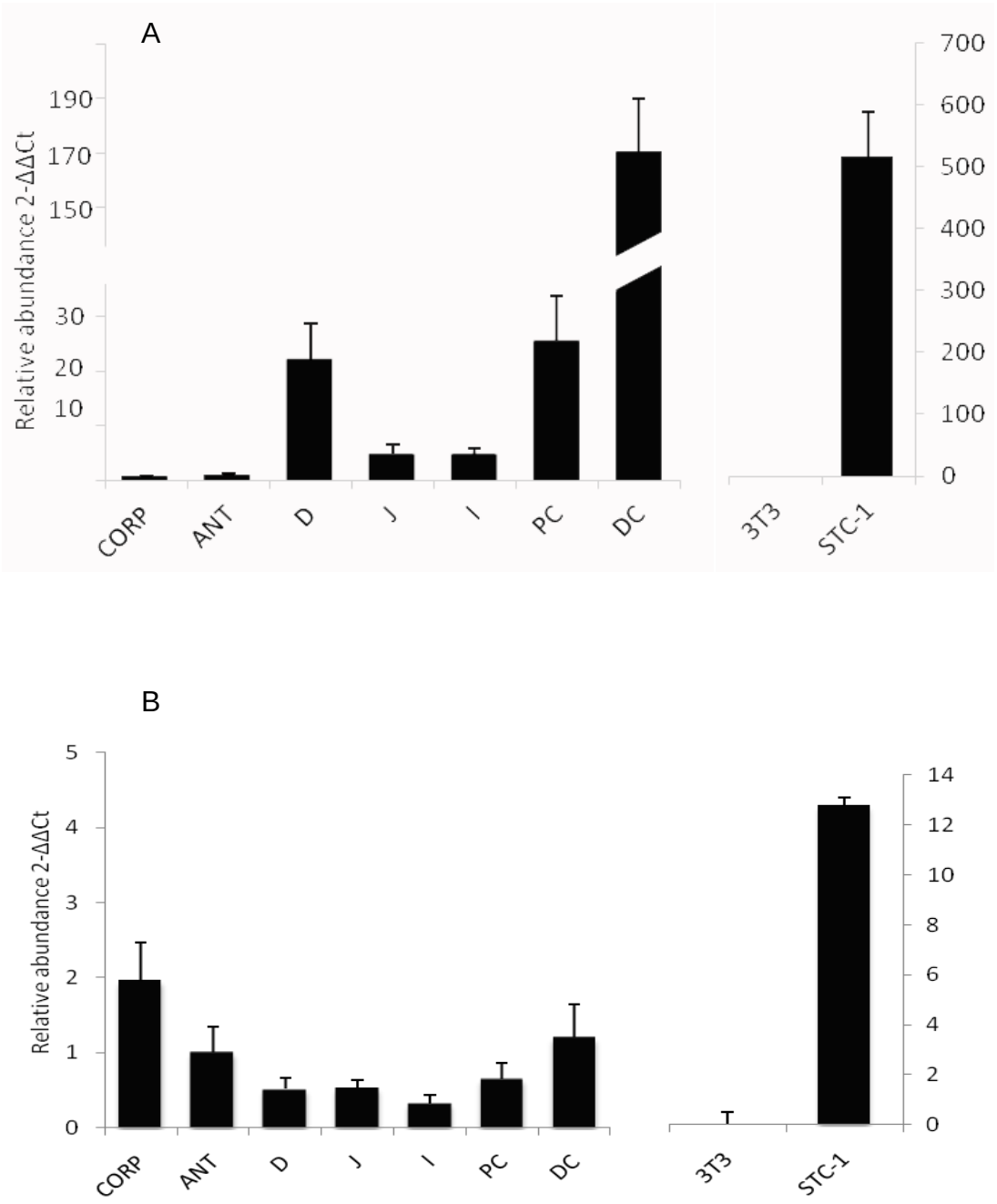


Figure 17A and B:.T2Rs and Gust distribution. mRNA levels were analyzed in the stomach (corpus and antrum), duodenum (D), jejunum (J), ileum (I), proximal (PC) and distal colon (DC) and normalized to β-actin. **A.**T2R138 distribution: this transcript is abundant in colon, expecially distal, and in duodenum. It is low expressed in stomach. **B.** T2R108 distribution: T2R108 is mostly expressed in stomach, expecially corpus (CORP), and is also abundant in colon.

Immunohistochemistry showed that mT2R138 immunoreactivity is localized to isolated epithelial cells in the villi, distributed throughout the GI tract (Fig 18a). Specificity of immunoreaction was demonstrated by the strong labeling of taste bud cells in the tongue (Fig. 18a, 1) and immune-blocking experiments showing abolition of immunostaining with pre-incubation of antibody with an excess of peptide used for the production of the antibody. Double labeling showed that in several cells mT2R138 co-localizes with Chromogranin A (Fig. 18b) and Gust (Fig. 18c), indicating that the receptor is expressed by some enteroendocrine cells and by cells expressing the main signaling molecule for taste transduction. T2R138 staining is localized at the apical membrane. mT2R138 immunoreactivity is also found in many epithelial cells exhibiting G_{α}-gustducin-driven GFP fluorescence, confirming that the same cells containing the receptor also contain its signaling protein. These G_{α}-gustducin-driven GFP fluorescent cells were distributed throughout the GI mucosa (Fig 18d) confirming previous observations of G_{α}-gustducin immunoreactivity in the mouse GI mucosa.

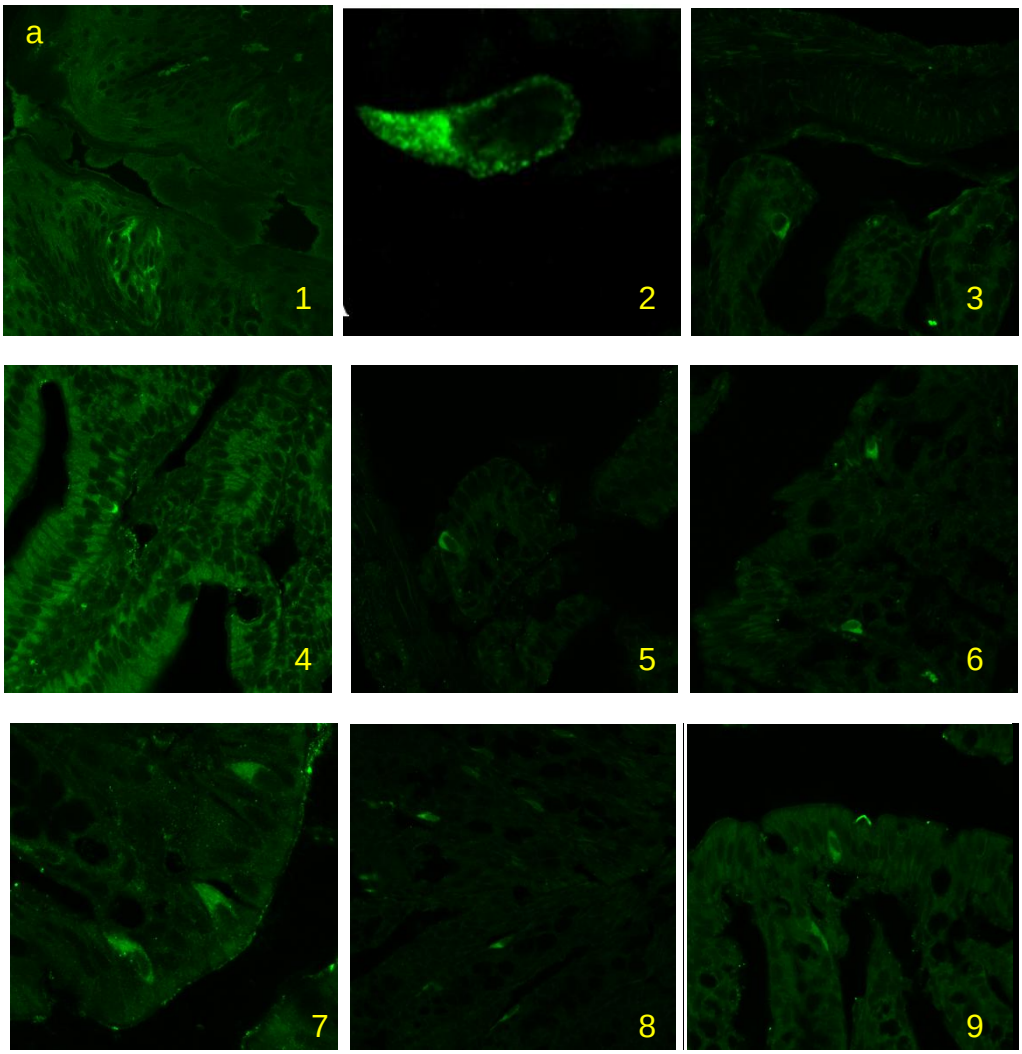
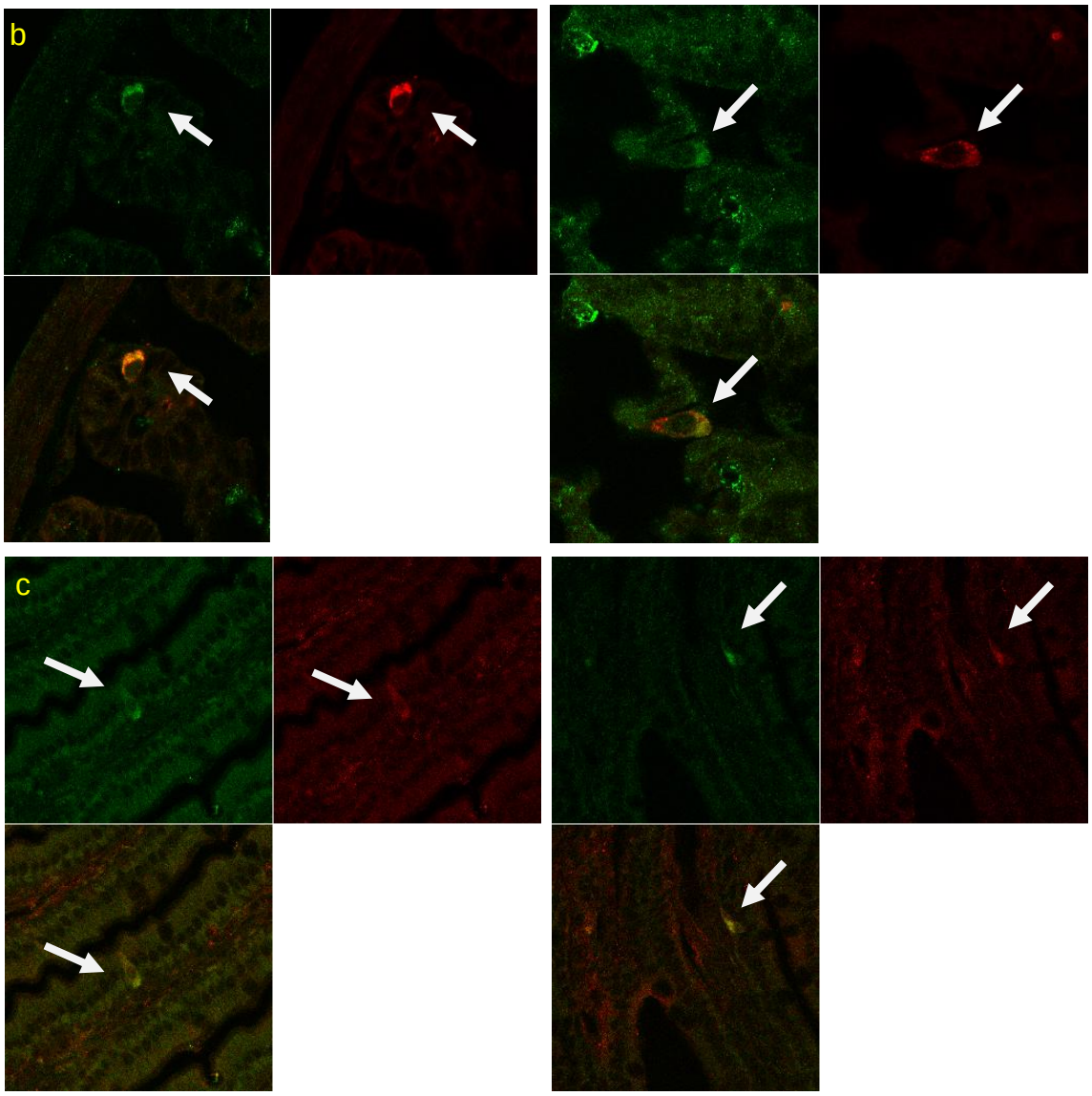
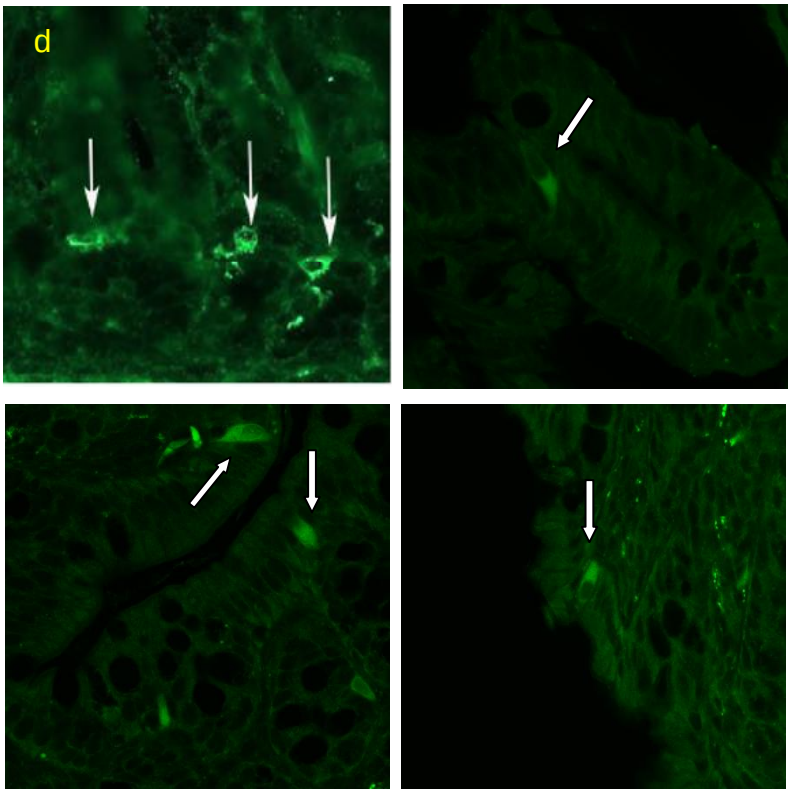


Figure 18: Immunohystochemistry **a.** Confocal pictures showing rapresentative mT2R138 immunostaining in the tongue (a1) and along the GI, proceeding from the stomach (2) to the small (3,4,5,)and the large (6,7,8,9) intestine (different magnifications)**.**



b.Double label of intestinal epithelial cell showing co-localization between mT2R138 and Chromogranin A, marker for enteroendocrine cells**. c.** Double label of small intestine (IL) epithelial cells showing co-localization between mT2R138 and Gust, main signaling molecule for TRs

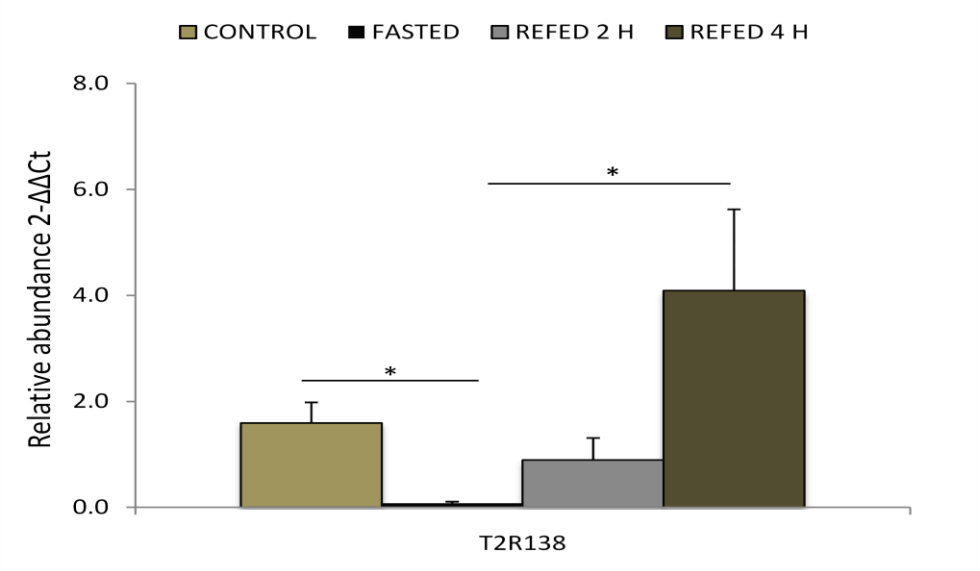


d: Image showing Gust immunostaining in Gust-GFP positive mouse stomach, small (IL, JEJ) and large (PC) intestine

b. Fasting decreases T2Rs and Gust expression

mT2R108 (control 1.15±0.20 vs 0.36±0.04 fasted, $P<0.05$) and mT2R138 (control 1.59±0.39 vs 0.07±0.03 fasted, $P<0.05$) mRNA levels were significantly decreased after 18 hours of fasting and fully restored after 4 h refeeding (Fig. 19) in the antrum, whereas changes in their level of expression were not observed in any other region of the gut. Similarly, there was a significant decrease of Gust mRNA levels in the antrum after fasting (control 1.26±0.23 vs 0.67±0.04 fasted, $P<0.05$) followed by a restoration of normal expression after 4 hrs refeeding. Gust was also significantly decreased (control

1.07±0.21 vs 0.45±0.05 fasted, P<0.05) in fasting conditions and then restored in duodenum, but not in other regions of the gut (data not shown).



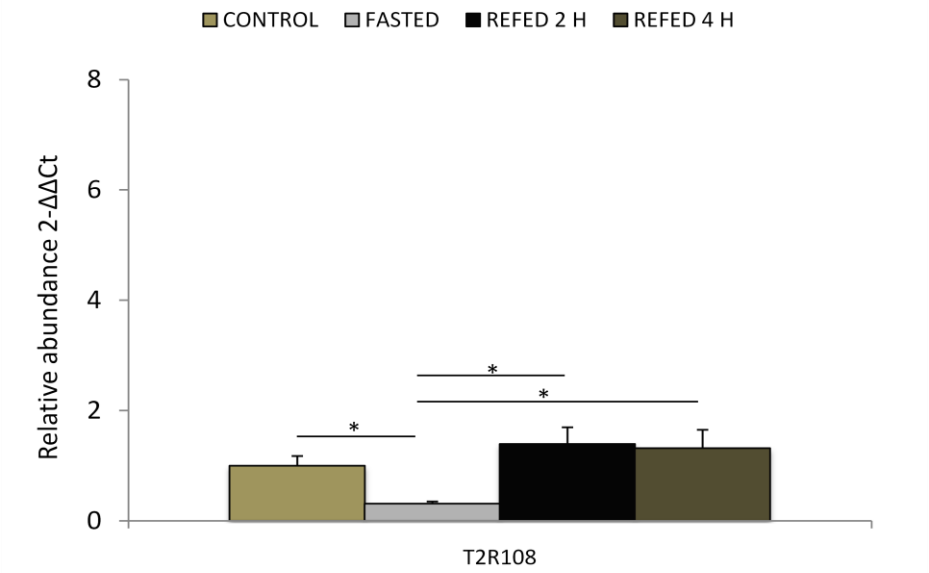


Figure 19: T2R138, T2R108 and Gust regulation by fasting/refeeding. Targets mRNA levels in the stomach were analyzed with quantitative real-time RT-PCR and normalized to β-actin in each tissue. Relative quantities were determined using the comparative ΔΔCt method. T2R138, T2R108 and Gust levels were significantly decreased (* P< 0.05) after fasting and fully restored after 4 h refeeding in stomach antrum.

c. A high fat diet increases T2R138 and Gust mRNA levels in the large intestine

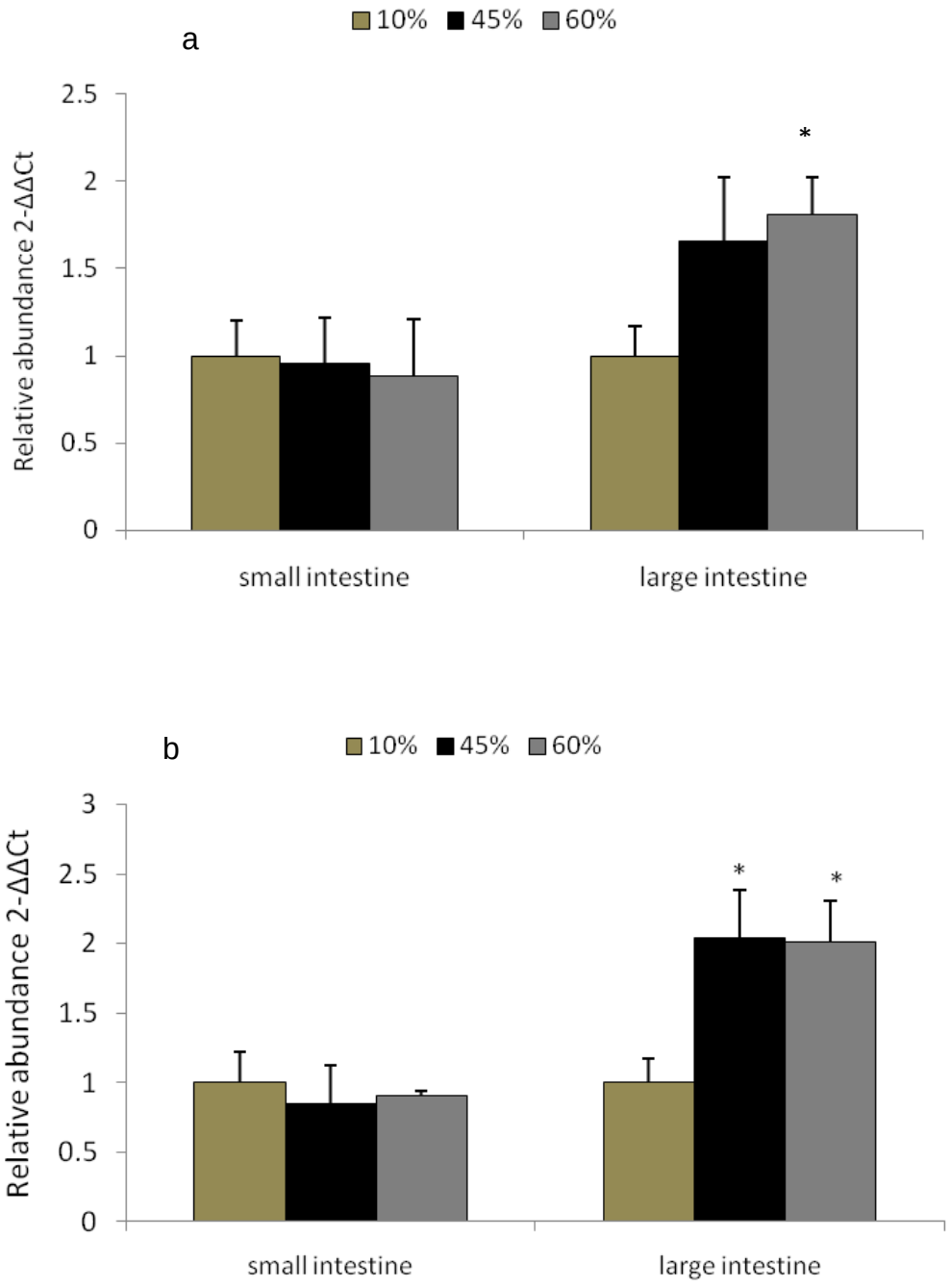


Figure 20: Effect of a high fat diet on mT2R138 and Gust expression. qRT-PCR analysis shows that mT2R138 mRNA levels (a) are significantly ($P < 0.05$ vs 10% fat) up-regulated by a long term high fat (60%) diet in the large intestine only. mT2R108 expression is not affected by this diet. Gust is also up-regulated (b) by the same fat diet and also by 45% fat diet in the large intestine, supporting the data about mT2R138.

When mice were fed 10-45 or 60% fat diet for 8 weeks, mT2R138 was selectively up-regulated by a 60% fat diet (1.80±0.21 treated vs 1.00±0.17 control, $P<0.05$) in the large intestine, but not in the small (Fig.20). No difference was found in mT2R108 expression, both in the small and large intestine. Gust was also up regulated by both 45% (2.04±0.34 treated vs 1.00±0.17 control, $P<0.05$) and 60% (2.01±0.29 treated vs 1.00±0.17 control, $P<0.05$) fat diet, matching the data obtained for mT2R138.

d. Effect of a cholesterol lowering diet on T2Rs expression

The effect of a cholesterol lowering diet on mouse T2Rs expression in the gut is illustrated in Fig. 22. First, we ensured that sterol depletion increased HMG-CoA reductase mRNA expression in treated vs control animals (Fig. 21) to ensure the cholesterol lowering drug effect [25]. Then we assessed our targets expression. Our data show that 7 days on a diet supplemented with drugs to lower cholesterol levels significantly increase mT2R138 expression in duodenum (control 1.00±0.20 vs 2.02±0.29 low cholesterol, $P<0.05$) and jejunum (control 1.00±0.28 vs 2.06±0.30 low cholesterol, $P<0.05$), but not in other regions of the small or large intestine. mRNA levels for mT2R108, instead, were not increased by the same diet in any GI region.

The signaling molecule G_{α}-gustducin was also significantly up-regulated by low cholesterol diet in duodenum (control 1.00±0.21 vs 2.80±0.65 low cholesterol, $P<0.01$).

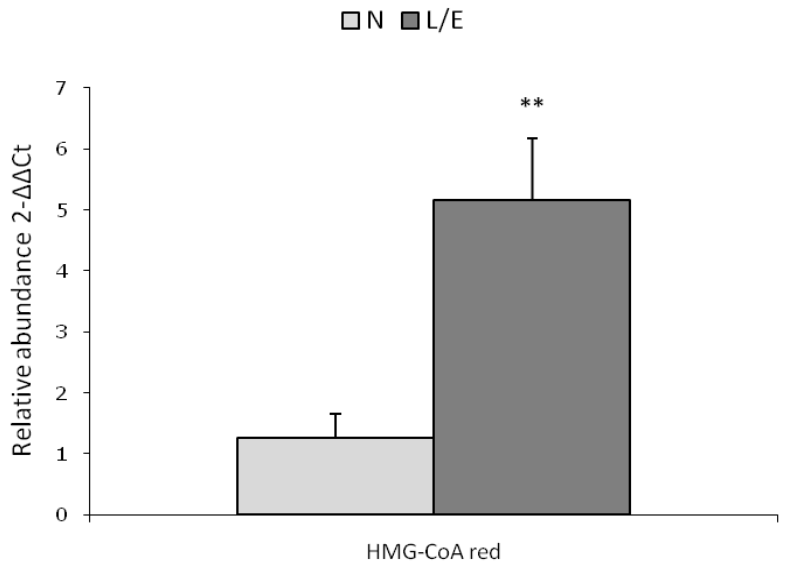


Figure 21: HMG-CoA reductase mRNA levels measured by RT-PCR in normal (N) vs treated (L/E) animals (** P< 0.01 vs normal).

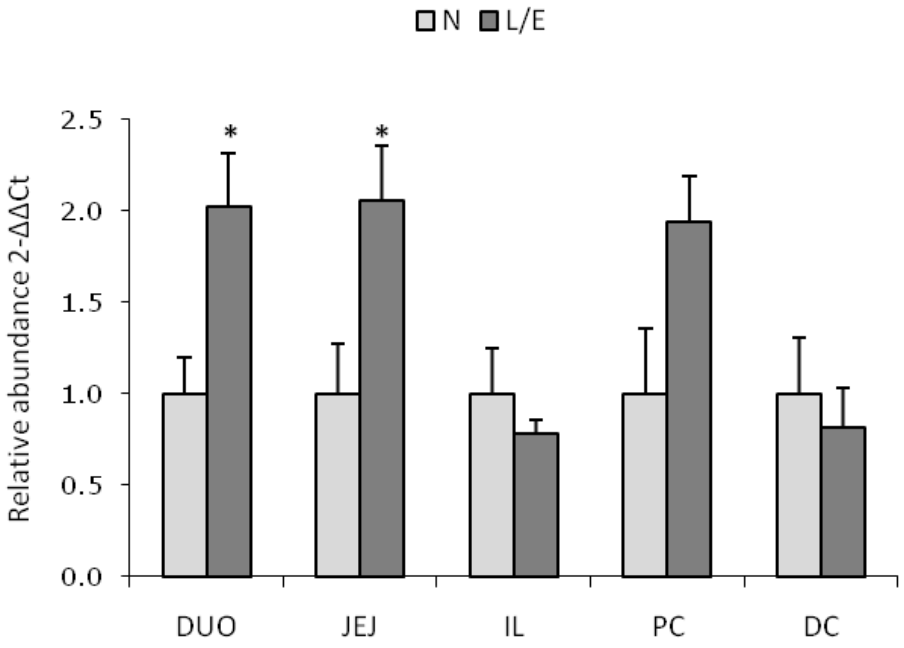


Figure 22: Effect of a low cholesterol diet on mT2R138 expression: qRT-PCR analysis shows that bitter taste receptor mT2R138 is up-regulated (* P< 0.05) in the small intestine (duodenum and jejunum), but not in the large intestine, following 7 days of a cholesterol lowering diet. Data are supported by Gust expression levels, which are also up regulated in duodenum (not shown).

e. Effect of a high protein diet on T2Rs expression in the mouse gi tract

Mice kept on a high protein diet (40%) for 14 days showed no statistically significant difference in mT2R108 and Gust mRNA levels of expression when compared to control. mT2R138 expression, instead, was significantly increased by the high protein in stomach corpus (1.00±0.10 control vs 2.05±0.27 treated, P< 0.05) and in the proximal colon (1.00±0.25 control vs 2.12 ±0.26 treated, P< 0.05), as shown in Fig. 23.

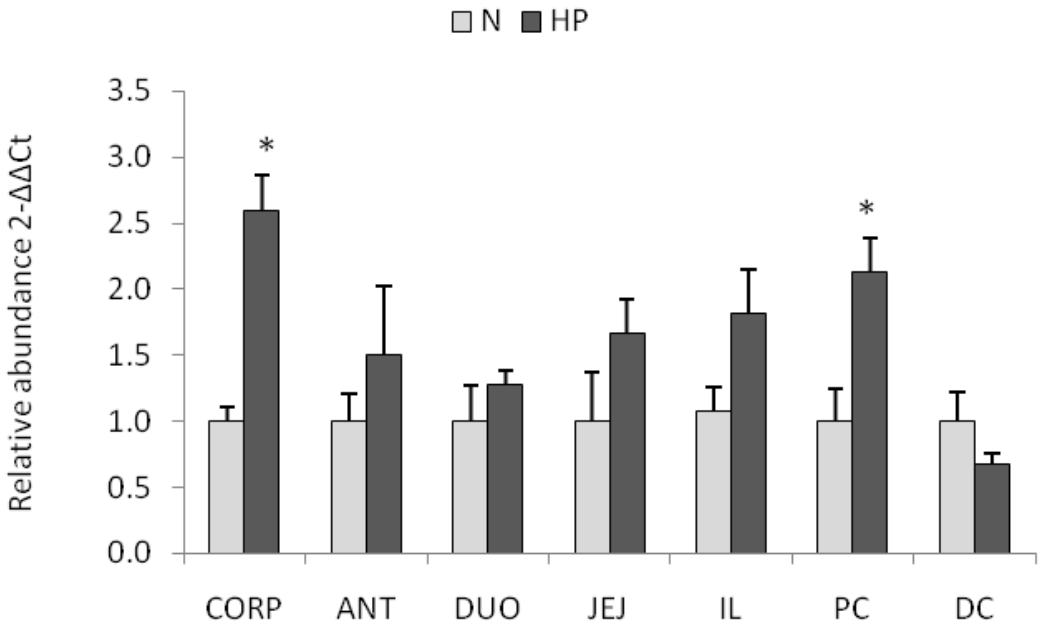


Figure 23: mT2R138 modulation after 14 days on a high protein diet (HP). A 40% protein by calories diet selectively increases mT2R138 mRNA expression in stomach corpus and in proximal colon (* P< 0.05 vs normal). Both mT2R108 and Gust were not increased by the high level of proteins in meals.

4. Discussion

Bitter taste receptors are expressed in the small [1] [102] and large [119] [2] intestine in mouse. Several GI segments and receptor subtypes have been investigated, but we are not aware of a systematic distribution study for any T2Rs in the mouse GI. The aim of this study was to investigate the physiological expression and distribution of two T2Rs subtypes (mT2R138 and mT2R108) and one of their signaling molecules (Gust) along the GI tract. We also wanted to see whether these are modulated by luminal content. We showed through immunohistochemistry and qRT-PCR that mT2R138, mT2R108 and Gust are distributed in the entire GI tract, with different levels of expression. Also, this study provides evidence that different diets modulate different mT2Rs subtypes in specific GI regions: fasting reduces mRNA expression of mT2R138, mT2R108 and Gust in stomach antrum; a high fat diet increases mT2R138 and Gust levels in the large intestine; a cholesterol lowering diet increases mT2R138 and Gust in the proximal small intestine and a high protein diet selectively increases mT2R138 in stomach and proximal colon.

a. Distribution

Our data showing expression of mT2R138, mT2R108 and Gust along the entire GI, with different levels in different regions, confirm and expand previous studies showing the presence of several T2Rs transcripts in both the upper GI [1] [102] and in colon [119] [2]. Our data are also in agreement with previously published data showing that Gust has a higher expression in stomach (antrum and fundus) vs small intestine (duodenum) in rats [102]. However, a systematic analysis and comparison of mRNA levels for these receptors in different GI segments was not published yet.

In this study we show immune-reactivity for mT2R138 and Gust along the whole GI, in control and G_{α}-gustducine-GFP mouse tissue, in single cells in the epithelial layer, and

both Gust and mT2R138 show co-localization with Chromogranin A, an enteroendocrine cell marker, and mutual co-localization. These evidences support and extend previous data [25] [120] [121] showing how T2Rs are expressed throughout the gut by epithelial cells, including EECs, and that the receptor and its signaling molecule can be found in the same cell. Immunohystochemistry also showed that Gust and mT2R138 positive cells are mainly located in the upper villi, which is in agreement with Dyer et al [122] findings about a higher expression of Gust in this area compared to the crypts or the lower villi. There is evidence that substances activating T2Rs and in general bitter tasting compounds are many and structurally diverse [68]. They belong to many chemical families, such as peptides, amino acids, fatty acids, alcohol, steroids, lactones and flavonoids [123] [124], found in food and food borne products. Our findings about the non homogeneous distribution of T2Rs along the gut, which shows different profiles for different subtypes, together with the variety of substances potentially recognized, suggests that there might be a different role for specific T2Rs in the gut depending upon receptor, site of expression and ligand.

b. Fasting and refeeding

Previous studies showed that fasting is able to modulate incretin levels in mice stomach by increasing ghrelin and somatostatin and lowering gastrin/CCK [125] [126] and physiologically the antrum, vs corpus and fundus, is particularly rich in secreting enteroendocrine cells and therefore it is particularly affected by fasting. Our data show a significant decrease for mT2Rs and Gust mRNA expression only in stomach antrum, following 18 h fasting and, by IHC, we showed that mT2Rs and Gust are expressed in stomach by enteroendocrine cells. We therefore suggest that fasting might modulate, also through bitter taste receptors expression modulation, the gastric incretine balance,

increasing somatostatin and ghrelin and decreasing gastrin secretion, in order to increase food intake, lower acid secretion and delay gastric emptying, exerting a sensory effect. This hypothesis is supported not only by the T2Rs and Gust mRNA modulation we assessed and by IHC evidence of T2Rs in EECs, but also by recent studies showing that stomach is able to directly respond to macronutrients in order to regulate gastric ghrelin release [127], and more specifically that gavage with bitter taste receptors agonists can delay gastric emptying and increase food intake by increasing plasma ghrelin levels, partially through Gust [19], consequently demonstrating the chemosensory capacity of stomach and supporting the idea that T2Rs might participate in the luminal chemosensing through incretin secretion in mouse stomach. The fact that T2Rs and Gust mRNA levels are decreased, whereas ghrelin plasma concentration increases with T2Rs stimulation, are not in contrast since mRNA levels not always match with the protein expression. An example is shown by the same ghrelin, whose mRNA expression in the stomach is increased upon 48 h fasting, but the ghrelin peptide content is decreased, and both levels are restored to normal after refeeding [126].

c. Cholesterol lowering diet

The finding of increased mT2R138 and Gust expression levels in mouse small intestine following a cholesterol lowering diet expands those by Jeon et al [25], showing the effect of this diet on the small intestine for several T2Rs. Our study investigates in detail the different regions of the small intestine and adds an analysis of the effects on the colon, assessing also Gust expression. As reported before [25], up-regulation for mT2R138 might reflect bitter taste receptors activation, since a naturally low-cholesterol diet is rich in plants, which often contain bitter potentially toxic components, compared to a high-cholesterol diet composed of significant amounts of animal meat. Thus, a low-

cholesterol diet might act on T2Rs expression and function to prevent the consumption and absorption of potentially toxic/bitter substances in plant-derived foods. Also, T2R138 is one of the most important receptors that define a “bitter tasting” phenotype [128] [129], therefore its regulation and sensitivity might be important in a diet rich in bitter potentially harmful plant-derived compounds.

d. High fat diet

Our data showed that 8 weeks on a high fat (HF) diet results in increased levels for mT2R138 and the signaling molecule Gust mRNA in mouse GI. According to our distribution data, mT2R138 (which is affected by HF diet) is highly expressed in the colon, where the microbiota is abundant. In fact the human intestine hosts about 100 trillion microorganisms, representing hundreds of species, and the colon bacterial density has been estimated at 10^{11} to 10^{12} per milliliter, which makes this GI segment probably one of the most densely populated microbial habitats on Earth [130]. Previous studies showed how a high fat diet causes changes in the gut microflora, in composition and quantity, starting after 2 weeks and becoming significant after 6-8 weeks and these changes are related to a low grade of inflammation, involved in the development of pathologies such as obesity [131] [132]. The change we detected in mT2R138 and Gust expression is developed after 8 weeks, but not after 2, and it develops in the large intestine, suggesting that the effect is more likely to be related to the change seen in the microbiota than to a direct effect of fat. Therefore, we speculate that there might be an interaction between microbes in the gut and mT2R138, which might serve as a defensive mechanism against bacteria, perhaps initiating an inflammatory response to contrast bacterial invasion.

In further support to this hypothesis, there is evidence that T2Rs in the airways can be activated by Acyl Homoserine Lactones [93], considered quorum sensing molecules for Gram negative bacteria, and we have preliminary data (shown in chapter III) showing that STC-1, an enteroendocrine cell line, and NCM-460 human colonocytes, are also activated by this same bacterial molecule.

e. High protein diet

14 days on a 40% protein diet significantly increases T2R138 mRNA levels, only in stomach and proximal colon. Diets rich in proteins are known to enhance satiety and promote weight loss acutely [133] [134] [135] and cronically [136] [137]. Mechanisms explaining protein-induced satiety mainly consist in elevated amino acid concentrations, causing the release of hormones controlling appetite and gut functions [138]. It has been published that PYY is strongly stimulated by protein meals [139], GLP-1 appears to be stimulated by a high protein diet in combination with carbohydrates [137], gastrin is strongly released by protein meals [140], whereas ghrelin does not seem to be strongly affected [141] and little is known about CCK. Also, hydrolysis of proteins produces many peptides which are major stimulants of EEC secretion of hormones influencing gastric emptying, acid secretion, pancreatic secretion and food intake [142]**.** Moreover, a wide variety of fermented proteins are bitter and some of those peptides, such as soy and casein hydrolysates, are recognized by T2Rs [143]. The exact mechanism for these effects is not clear, but there is evidence of a role for a GPCR in protein sensing in the gut in STC-1 cells [144]. Our diet used casein and soy proteins (50:50% by calories), which are hydrolyzed to several bitter compounds recognized by T2Rs [189]. Based on previous evidences, we hypothesize that the change in mT2R138 expression in proximal colon and stomach might be the result of an interaction between peptides/bitter peptides

derived from food proteins and the T2Rs. We propose that a high amount of proteins (casein and soy) and their metabolic products, which are bitter peptides, lead to release of those incretins which are mostly influenced by protein meals_ PYY, mainly located in colon, and gastrin, in stomach_ through T2Rs, which are expressed in cells releasing PYY and CCK/gastrin [2], are activated by bitter hydrolyzed proteins [189] and which levels are increased by a high protein diet in the same regions where this diet mainly causes release of incretins [139]. To further support the hypothesis that gastric peptides might be released by proteins and bitter compounds acting on T2Rs, it has recently been shown that PTC, a T2R138 agonist, acts on STC-1 leading to CCK and gastrin release [23], which is also released by proteins and amino acids in the lumen. Furthermore, intragastric administration of the bitter taste agonist DB stimulates vagal neurons via Y_2 and CCK_1 receptors in the gut wall [39] and also ghrelin release from stomach [19], supporting a connection between T2Rs stimulation by bitter compounds_such as the hydrolyzed proteins_ and a stimulation affecting stomach (releasing ghrelin, gastrin/CCK) and colon (releasing PYY).

f. Summary

In summary, mT2R138, mT2R108 and Gust are expressed along the entire mouse GI tract and show different levels of expression depending on the region. They are expressed by solitary epithelial cells, including enteroendocrine cells and cells expressing the taste signaling molecule Gust. The same receptors are selectively modulated in specific GI regions by different diets, containing different nutrients. There is an overall match between T2Rs and Gust expression modulation, which is not complete since Gust is a signaling molecule common to different TRs (sweet, bitter and umami). Also, G_α-gustducin is not the only G_α subunit related to taste, as shown by the fact that

KO mice have diminished behavioral and electrophysiological responses to many bitter and sweet compounds, but they retain residual responses to these substances [145].

This non homogeneous distribution and the region specific modulation by different nutrients for mT2R138, mT2R108 and their signaling molecule Gust support a different role for T2Rs depending upon receptor subtype, region of the gut and interaction with multiple luminal components. Overall, our data further support the idea of an involvement for T2Rs in chemosensing in the GI tract.

CHAPTER III

Evidence for a role of T2Rs in sensing bitter stimuli and bacteria in human and mouse intestinal cell lines

1. Background and aim

BACKGROUND

a. Type I Enteroendocrine Cells/STC-1 cells and bitter taste signaling

Enteroendocrine cells (EECs) contain secretory granules and release mediators into the subepithelial space –where the sensory nerve terminals are. These cells are generated in the crypts and during the differentiation process they migrate to the tip of the villus. A variety of transmitters are found in different types of EECs. Also, some transmitters are co-stored within a single type of cell. Perhaps the reason for so many types of EECs is because each type has a specific function and communicates with a specific sub-population of sensory nerves. It is clear that EECs are difficult to study directly because of the difficulty in isolating them, their sparse and irregular localization within the gut wall and their diversity. Therefore, many researchers adopt the use of cell lines as model systems for investigations of EEC functions.

The mouse enteroendocrine cell line STC-1 is a small intestinal mixed population of enteroendocrine cells. STC-1 cells have been used for studying regulation of GI hormone release in response to bombesin/gastrin [146], free fatty acids [147], leptin [148], orexin [149] and amino acids [150]. STC-1 were the first cells to be characterized for the presence of taste signaling components and their responses to taste stimulation. It was shown that numerous T2R genes, as well as the taste signaling molecules G_{α}-gustducin and G_{α}-transducin, Gβ3, Gγ13, PLCβ2 and TRPM5 are expressed in STC-1 cells [102] [1] [26]. Stimulation on STC-1 with various bitter tastants leads to an increase in cellular calcium levels through the activation of L-type voltage-sensitive calcium channels [26], as monitored by functional calcium imaging experiments, indicating the presence of functional receptors in these cells. Furthermore STC-1 cells, which produce different peptide hormones including cholecystokinin (CCK), secrete CCK following DB

stimulation in a dose-dependent fashion [26]. STC-1 cells do not only express T2R genes, but also all three T1R genes [26] [122], which should enable them to respond to sweet and umami stimuli as well. Indeed, stimuli of all 5 basic tastes activate calcium responses in this cell line [151].

Recently, it has been shown that gut EECs express Toll-like receptors (TLR). Toll-like receptors are trans-membrane molecules that recognize bacterial breakdown products, such as lipopolysaccharide (LPS), bacterial lipoproteins, double stranded DNA and flagellin. Both mRNA and protein for TLR4, 5 and 9 are found in STC-1 cells. Activation of these receptors by their ligands (LPS, flagellin and CpG-ODN) induced secretion of CCK [152] [153]. Taken together, these observations suggest that EECs may be involved in the detection of the bacterial content of the lumen and participate in mucosal defense. For all the previous, STC-1 cells are a good model to study gut enteroendocrine cell responses to bitter taste ligands, including bacteria. Therefore, we use them to study the possibility that recognition of and response to bacteria by EEC might involve T2Rs.

b. NCM-460 human colonocytes characteristics and their use in GI reaserch

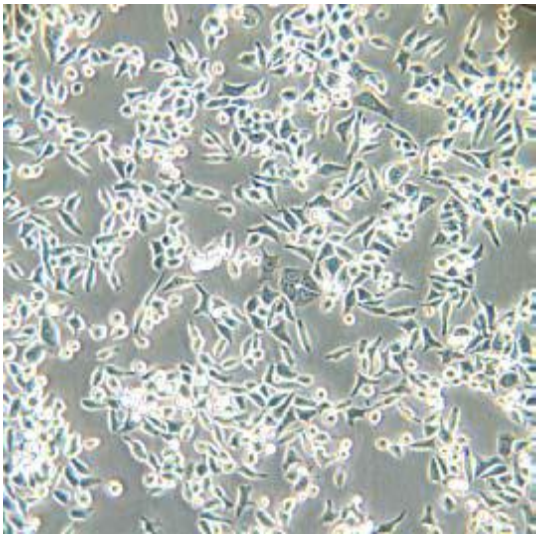


Figure 24: Phase contrast micrograph of NCM 460 cells in culture [INCELL NCM460 product information 2007,v1.0]

NCM-460 cells (Fig. 24) are normal human colonic mucosal cells, derived from the normal colon of a 68-year-old Hispanic male [154]. This cell line has extensively been used by many groups (including Rozengurt

and Pothoulakis, collaborating with us) to study multiple intestinal research areas, including infectious diseases [155], cell signaling [156], cytokine production [157] [158], vitamin transport, gene regulation [159] and protein expression and phosphorylation in multiple regulatory pathways [160] [161]. NCM-460 cells are almost exclusively epithelial cells, since they are positive to antigens against cytokeratins and villin, but are negative for antigens associated with other cell types, such as neural or endothelial cells (see table IV).

CHARACTERIZATION OF NCM460

Phenotypic Characteristics	Observations
Growth in soft agar[a]	Negative
Tumorigenicity[b]	Negative
Periodic acid–Schiff mucin staining	Positive
Immunocytochemistry assays[c]	
Cell type: Marker	
Epithelial cells: pancytokeratin	Positive (>90%)
GI epithelial cells	
Villin: cytoskeleton	Positive (>90%)
5E113: Cell surface	Positive (>90%)
Secretory epithelial cells	
Human secretory component	Positive (>90%)
Endothelial cells: Factor VIII	Negative (>90%); Positive (1–2%)
Lymphocytes (CD4; CD23: others)	Negative
Mesenchymal: other cells: Vimentin	Negative (>90%); Positive (10–15%)
Neuroendocrine cells	Positive (80–90%; suspension cells)
Chromogranin	Positive (20–40%; monolayer cells)
Neural cells	
Glial fibrillary acidic protein (GFAP)	Negative
Neurofilament (NF)	Negative
Galactosyl ceramide glycolipid (Gal-Cer)	Negative

Table IV: Immunochemical characterization of NCM-460 cell line with different markers [Moyer et al, In Vitro Cellular & Developmental Biology – Animal, 1996**]**

These cells grow in monolayer and they require a specific media (M3D™, from INCELL) to maintain an appropriate phenotype for long term culture. Also, some cells might become positive to mucin synthesis with time or might acquire some sort of transformation-associated characteristic. Therefore we always worked on new cell after passage XV.

Extensive experimental evidence suggests that differences in diets [162] [163] and variation in gut microbiota [164] might contribute to variation in susceptibility to pathologies, such as colon cancer, and NCM-460 cells have been used in this kind of studies. In this field, literature shows that long term consumption of red and processed or preserved meat, as well as other dietary products, increase colorectal cancer risk, whereas high Ca^{++}, fish, fruit and soy show cancer-inhibitory activities in experimental studies [165] [166] [167] [168]. Also, obesity_ which is associated with a mild GI inflammation_ and diabetes Type II are often associated with a higher risk of colon cancer. These evidences, together with our previous data about diets and T2Rs expression modulation and the expression of hT2R38 we detected, led us to decide to study the effect of bacteria on NCM-460 and AHL influence on T2Rs expression.

c. pMAPK p44/42

The transmission of extracellular signals into their intracellular targets is mediated by a network of interacting proteins, acting to regulate various cellular processes. One of these networks involves activation of membrane receptors followed by a sequential stimulation of several cytoplasmic protein kinases, collectively known as mitogen-activated protein kinases (MAPKs). MAPKs, also referred to as extracellular signal-regulated protein kinases (ERKs), are serine/threonine proteins that respond to a variety of extracellular stimuli and regulate many cellular activities, such as gene expression,

mitosis, differentiation, proliferation, and cell survival/apoptosis [169]. All MAPK pathways operate 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) that are activated in series, as shown in Fig 25.

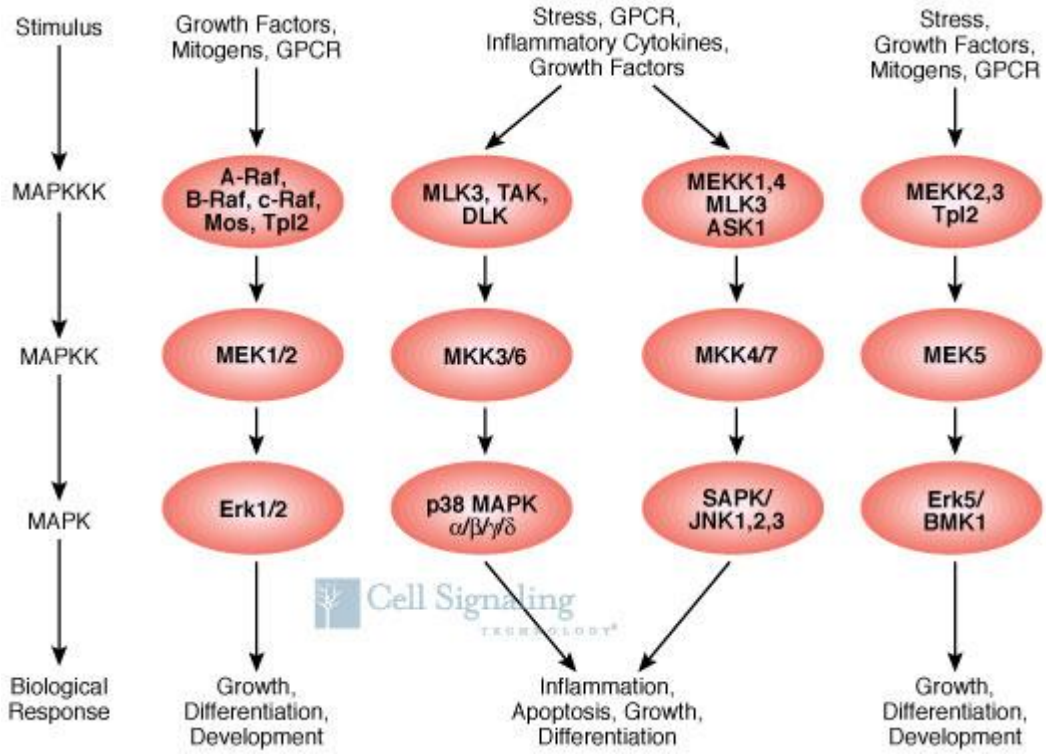


Figure 25: Different pathways using MAPKs. In all of them, a MAP3K_that is activated by extracellular stimuli_phosphorylates a MAP2K on its serine and threonine residues, and this MAP2K activates a MAP kinase through the same phosphorylation on its threonine and tyrosine residues (Tyr-185 and Thr-183 of ERK2), leading to a biological response. [Cell signaling technology; http://www.cellsignal.com/reference/pathway/MAPK_Cascades.html]

Opposite, down-regulation of MAPK pathways occurs through dephosphorylation by serine/threonine phosphatases, tyrosine phosphatases, or dual-specificity phosphatases and through feedback inhibitory mechanisms that involve the phosphorylation of upstream molecules. Several MAPK cascades have been identified in mammalian cells. So far, the three major MAPK pathways identified and 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. Therefore, they represent the best characterized pathway for MAPKs, which is the reason why we chose them as a target to measure. The ERK1/2 pathway is preferentially activated in response to growth factors and phorbol ester (a tumor promoter) and regulates cell proliferation and cell differentiation. ERK3 (MAPK6) and ERK4 (MAPK4) are structurally-related atypical MAPKs. They are primarily cytoplasmic proteins that bind, translocate and activate MK5 (PRAK, MAPKAP5). ERK5 (MAPK7), which has been discovered more recently, is activated both by growth factors and by stress stimuli, and it participates in cell proliferation. ERK7/8 (MAPK15) is the newest member of MAPKs and behaves like atypical MAPK. c-Jun N-terminal kinases (JNKs) are also known as stress-activated protein kinases (SAPKs) as they are strongly stimulated by numerous environmental stresses or genotoxic agents and modestly stimulated by mitogens, inflammatory cytokines, oncogenes or inducers of cell differentiation. p38 isoforms are responsive to stress stimuli, such as cytokines, ultraviolet irradiation, heat shock and osmotic shock, inflammatory cytokines (TNF-α and IL-1) and growth factors and are involved in cell differentiation and apoptosis.

Phosphorylation of MAPK p44/42 has been extensively used to study activation of intracellular pathway in response to various ligands for receptors on the membrane. In this study, we evaluated pMAPK p44/42 over the total expression of ERK1/2 in STC-1 and NCM-460 cells challenged with different stimuli, to establish whether T2R ligands or AHL activate different GI cells using MAPK pathway.

d. AHL and bacteria

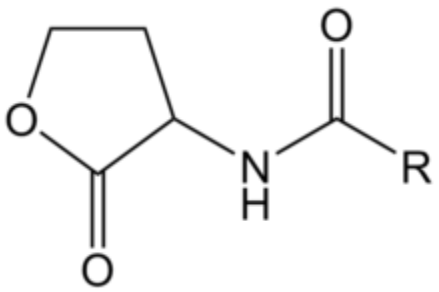


Figure 26: AHL general chemical structure.

[http://en.wikipedia.org/wiki/NAcyl_homoserine_lactone]

Species-specific cell-to-cell communication in bacteria is critical for successful pathogenic or symbiotic interactions with plant or animals, and host immune responses are important mechanisms to develop the infection process. The level and nature of these responses mainly depend upon the type of infection and its site, therefore upon both the host and the pathogen. Bacterial cells sense their population density through a sophisticated cell–to-cell communication system and regulate expression of particular genes when the cell density reaches a specific threshold. This type of gene regulation, which controls many biological functions including virulence, is known as quorum sensing (QS) [170] [171]. Virulence factors controlled by QS include exoproteases,

siderophores, exotoxins, and lipases and QS is essential for the pathogen to be successful [172] [173] [174]. One of the best-studied QS signaling system is the acyl-homoserine lactones (AHLs) system, which is used by a large number of Gram-negative bacterial species. Different pathogens use different strategies to modulate the immune response and there is considerable structural variety between AHLs from different bacteria and even between AHLs synthesized by the same bacterium, mostly in the length and substitution of the acyl chain. Also, target genes regulated by AHLs are abundant, as well as the regulatory mechanisms [174] [175] [176], as shown in some examples in table V.

Bacteria	Regulators‡	Signal	Target function
Vibrio fischeri	LuxR–LuxI AinR–AinS§	3-oxo-C6-HSL C8-HSL	Bioluminescence Bioluminescence, ?
Pseudomonas aeruginosa	LasR–LasI RhlR–RhlI	3-oxo-C12-HSL C4-HSL	Virulence and biofilm development Virulence and rhamnolipids
Agrobacterium tumefaciens	TraR–TraI	3-oxo-C8-HSL	Virulence plasmid copy number and conjugal transfer
Erwinia caratovora	CarR–CarI ExpR	3-oxo-C6-HSL	Carbapenem antibiotics and exoenzymes
Pantoea stewartii	EsaR–EsaI	3-oxo-C6-HSL	Exopolysaccharide
Rhodobacter sphaeroides	CerR–CerI	Δ7-C14-HSL‖	Aggregation
Vibrio anguillarum	VanR–VanI	3-oxo-C10-HSL	None yet identified

Table V: Examples of Homoserine Lactones (HSL) quorum sensors. [Clay Fuqua & E. Peter Greenberg, Nature Reviews Molecular Cell Biology, 2002]

However, the general mechanism of AHL-mediated quorum-sensing signaling is highly conserved: each bacterium produces a basal level of AHLs that move in and out of cell membranes through diffusion or active transportation. When AHLs reach a threshold concentration, signaling that there is a high bacterial population density, they interact with specific receptors and initiate expression of selected genes [175] [176] (Fig. 27). The specific receptors for AHLs signals are members of the LuxR family of transcriptional regulators. LuxR family members consist of two domains, a C-terminal DNA-binding domain, and an N-terminal AHL-binding domain [177]. A simple model showing AHLs quorum-sensing mechanism is shown in Fig. 27.

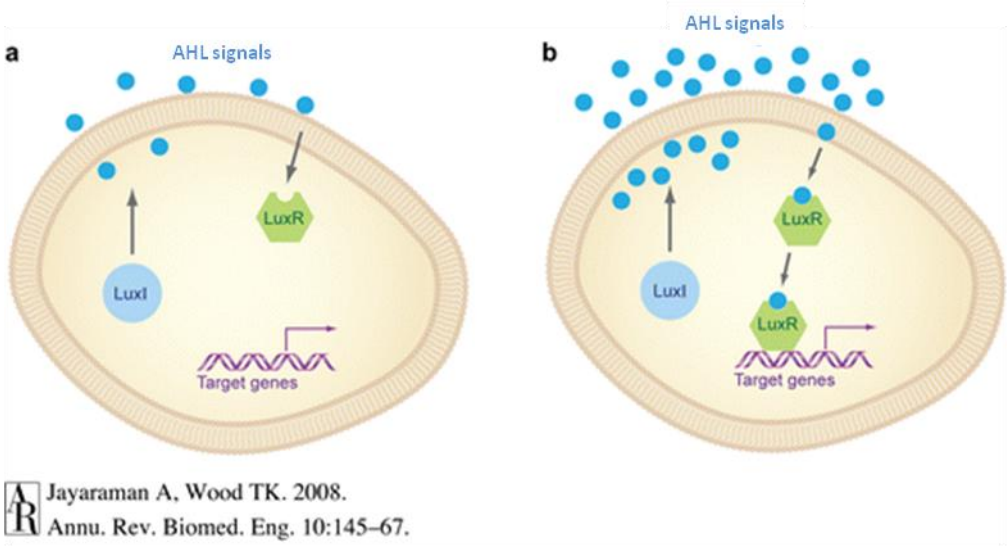


Figure 27: Generalized scheme for an AHL quorum-sensing in a bacterial cell. When AHLs concentration is enough_indicating a numerous bacteria population_ the QS molecule binds to its receptor (LuxR) leading to the expression of genes required for production of a variety of pathogenic components. [Jayaraman et al, Annu. Rev. Biomed. Eng., 2008]

Tizzano and coworkers [93] have suggested that the airways epithelial cells might be capable of responding to AHLs produced by Gram-negative bacteria with a mechanism involving bitter taste receptors. Also, many lactones _such as sesquiterpene lactones_ are natural bitter substances occurring in vegetables and culinary herbs, as well as in aromatic and medicinal plants, and previous studies have shown their ability to activate bitter taste receptors [70]. Based on these previous evidences and on our findings showing up-regulation of mT2R138 and Gust in the colon only by high fat diet, which alters the level of intestinal bacteria, we hypothesized that T2Rs in the gut might interact with bacteria and initiate an inflammatory response thus providing a defense mechanism toward bacteria invasion. 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, in order to investigate whether AHL signaling can activate small intestinal enteroendocrine cells or colonocytes at micromolar concentrations, which are likely to trigger bacterial virulence and therefore provoke an immune response.

AIM

The aim of this study was to evaluate whether different kind of GI cells express functional T2Rs and whether these are activated by Acyl Homoserine Lactone (AHL), quorum sensing molecule for Gram negative, to test the hypothesis that T2Rs in the GI tract might serve as detector system for pathogens, thus providing a mechanism of defense against infection.

2. Material and methods

a. Reagents

Phenylthiocarbamide (PTC, P7629), Denatonium Benzoate (DB, D5765), N-(3-Oxodecanoyl)-L-homoserine lactone (AHL, O9014), Probenecid (P-8761), GF-1, B3306) and nitrendipine (N144) were purchased from Sigma. Probenecid was dissolved at 500 mM in 1N NaOH and titrated to pH 7.0. AHL was dissolved at 50 mM in DMSO and used at a final concentration of 0.1 mM, containing 0.2 % DMSO, which was not toxic to cells for the time of the experiment (10 min to 24 h). GF-1 (an inhibitor of protein kinase C, PKC) and nitrendipine (an L-Type voltage-sensitive Ca^{++} channel blocker) were also dissolved in DMSO for a stock solution of respectively 2 mM and 0.1 mM and used at a final concentration of 5uM and 1 uM in DMEM for STC-1 or M3D™ media for NCM-460.

b. Cell lines and treatments

STC-1 and IEC-18 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. Both cells were cultured in DMEM + GlutaMax + 10% FBS and 1xPenStrep (GIBCO 15140-122). NCM-460 were kindly donated, by Dr. Pothoulakis labs, IBD Center, Division of Digestive Diseases, David Geffen School of Medicine, UCLA, Los Angeles, CA and were cultured in M3D™ (M3DEF-500, Incell corporation LLC) media with 10% FBS and 1x PenStrep. All cultures were kept at 37°C in 5% CO_2 atmosphere. Cells were starved 1h before experiments with media without FBS and treated with bitter agonists (DB and PTC 0.1 to 10 mM 3') or AHL (0.5 to 100 uM 10') with or without 1h pre-incubation with either Probenecid (concentrations range 0.1 to 5 mM), GF-1 (5 uM) or nitrendipine (1 uM).

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 membrane. Membranes were blocked 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 680Goat 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.

d. qRT-PCR

Total RNA was isolated from STC-1 and NCM 460 cells using Qiagen RNeasy Minikit (74104, Qiagen, Valencia, CA) and a DNase treatment was performed to eliminate genomic DNA contamination. RNA quality was estimated by the absorbance at 260 nm and 280 nm ratio (OD260nm/OD280nm>1.8) Complementary DNA was generated using Superscript III Reverse Transcriptase kit (Invitrogen) according to the manufacturer'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 for mT2R138 (Applied Biosystem, Mm01700131_s1), mT2R108 (Applied Biosystem Mm00498514_s1) and hT2R38 (Applied Biosystem_ Hs00604294-s1,) and hT2R4 (Applied Biosystem_ Hs00249946-s1). Standard thermal cycles (50 cycles) for Taqman Gene assays were run on a Mx3000P Real-time PCR Detection

System (Stratagene) and data were analyzed with Mx Pro 1000 software. 18S RNA (18S RNA, Applied Biosystem Hs03928990_g1 and Mm03928990_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 at least 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 seizes as the expected size were detected.

3. Results

a. STC-1 enteroendocrine cells and NCM-460 colonocytes express T2Rs.

qRT-PCR and gel electrophoresis showed the presence of the amplified products generated by the Taqman Gene Expression Assay primers specific for mT2R138, mT2R108 and Gust in STC-1 cells, but not in IEC-18 cells, as shown previously [102]. Also, qRT-PCR analysis of NCM-460 human colonocytes showed that this cell line expresses hT2R38, but not T2R4 (data not shown).

b. Bitter stimuli induce a rapid dose-dependent MAPK p44/42 phosphorylation in different intestinal mucosal cell populations.

WB analysis showed that STC-1 cells responded to bitter ligands DB and PTC with MAPK p44/42 phosphorylation. The activation peaked at 3 minutes and showed a dose dependent profile (Fig. 28). DB induced a significant phosphorylation of MAPK 44/42 at 2.5 mM (40.80%±10.89 treated vs 4.70%±0.33 control, $P<0.05$) and PTC at 5mM (51.18%±6.98 treated vs 4.25%±0.21 control, $P<0.05$). IEC-18 cells, a mouse small intestine cell line not expressing TRs, were not activated by either PTC and DB.

When challenged with DB or PTC and analyzed by WB for MAPK phosphorylation, results on NCM-460 human colonocytes supported qRT-PRC data, since PTC activates MAPK p44/42 in a dose dependent manner (lowest active concentration 2.5 mM: 24.82%±0.80 treated vs 17.05%±1.88 control, $P<0.05$), whereas there is no MAPK phophorylation following DB stimulation (Fig. 29).

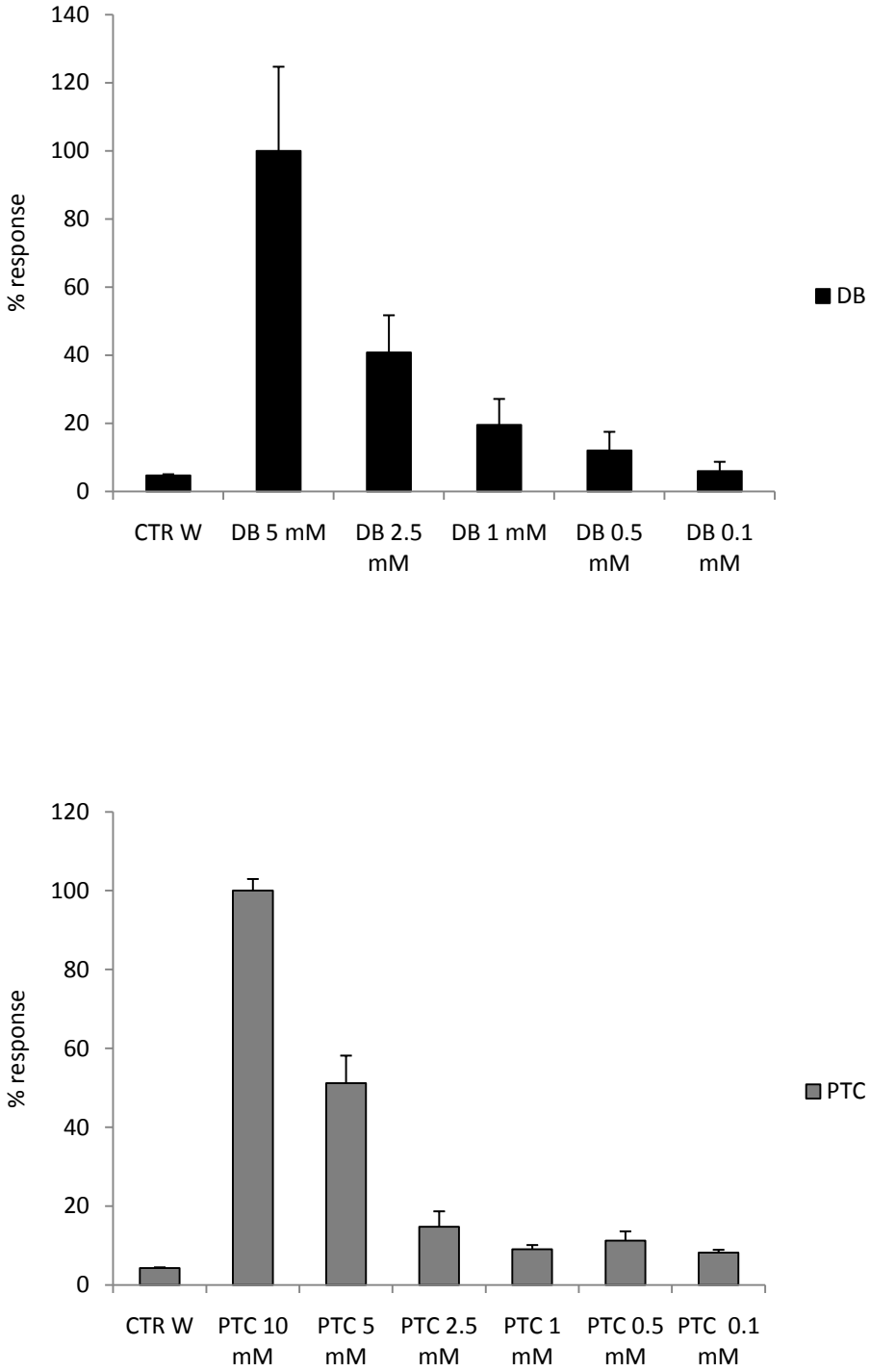


Figure 28 :WB results showing the % of phosphorylation for MAPK p44/42 in STC-1 cells after 3' incubation with increasing DB or PTC concentrations**.**

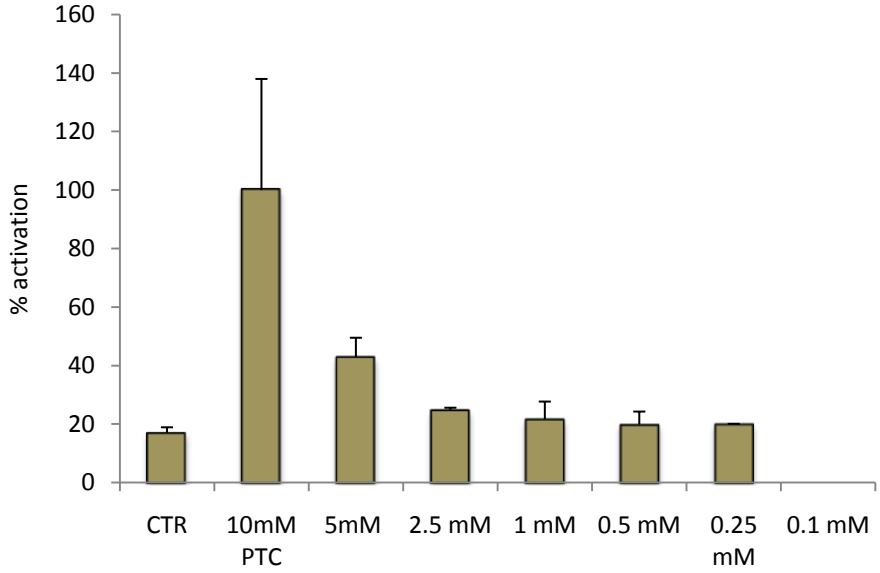


Figure 29 :WB results showing the % of phosphorylion for MAPK p44/42 on NCM 460 colonocytes after 3' incubation with increasing PTC concentrations.

c. 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^{++} channels blocker, and measured pMAPK following PTC/DB treatment in their presence. When STC-1 cells were treated with increasing concentrations of PTC or DB in presence of GF-1, PTC signal was blocked, whereas in presence of nitrendipine, DB signal was suppressed, as shown in Fig. 30.

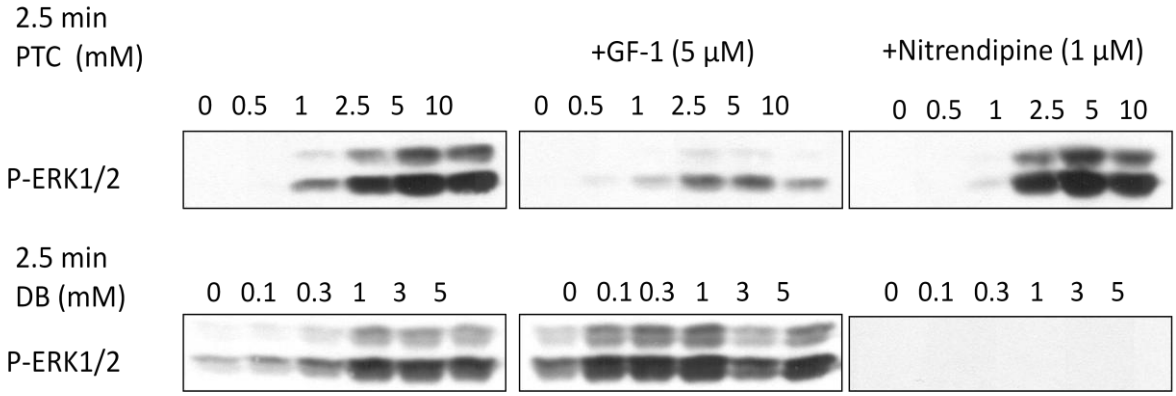


Figure 30: Representative WB membranes showing pMAPK p44/42 after PTC or DB stimulation, in absence/presence of nitrendipine/GF-1.

d. Human and mouse GI cell lines expressing T2Rs are activated by N-(3-Oxodecanoyl)-L-homoserine lactone, possibly through T2R138/T2R38

Both NCM-460 and STC-1 cell lines responded with MAPK p42/44 phosphorylation when challenged with AHL at increasing uM concentrations. Cells rapidly died with concentrations ≥250uM but survived at least 24h for concentrations ≤ 100uM. Activation by PTC and AHL was blocked by Probenecid (100.00%±14.84 PTC vs 28.38%±12.85 PTC+Probenecid 2.5mM, and 68.89%±7.66 AHL vs 19.37%±1.70 AHL+Probenecid 2.5Mm, both $P<0.05$), an antagonist for a subgroup of T2Rs including T2R138, in a dose dependent manner in STC-1 cells and preliminary data show that both PTC and AHL-induced MAPK phosphorilation is blocked by GF-1 but it is not blocked by nitrendipine in STC-1 cells (Fig. 32-33), whereas DB-induced MAPK phosphorilation was blocked by nitrendipine but not by GF-1.

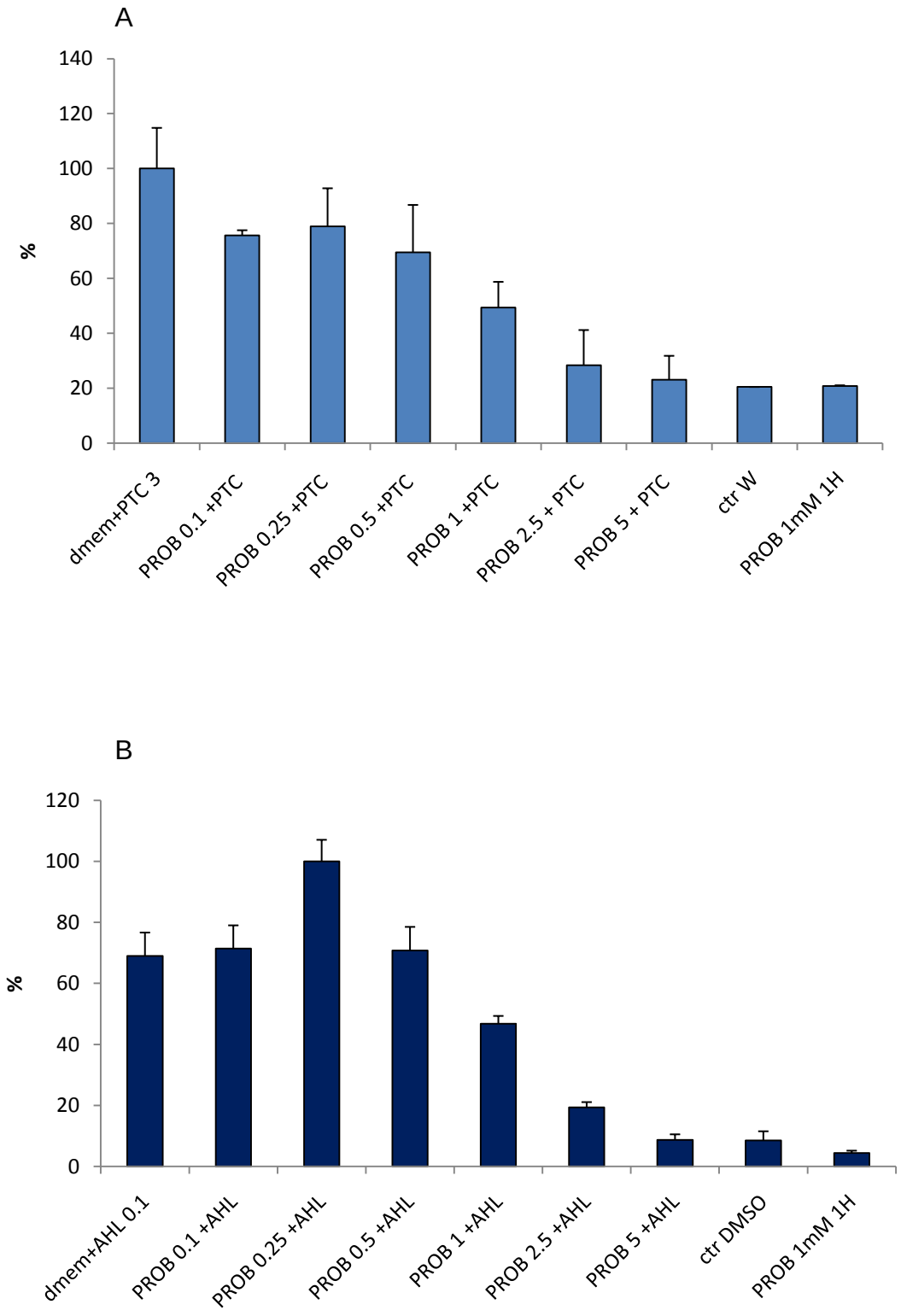


Figure 31: Graphs showing WB results for: A) 3 mM PTC-induced ERK phosphorylation, blocked by probenecid in a dose dependent manner, in STC-1 cells B) 100 uM AHL–induced ERK phosphorylation, blocked by probenecid in a dose dependent manner, inSTC-1 cells.

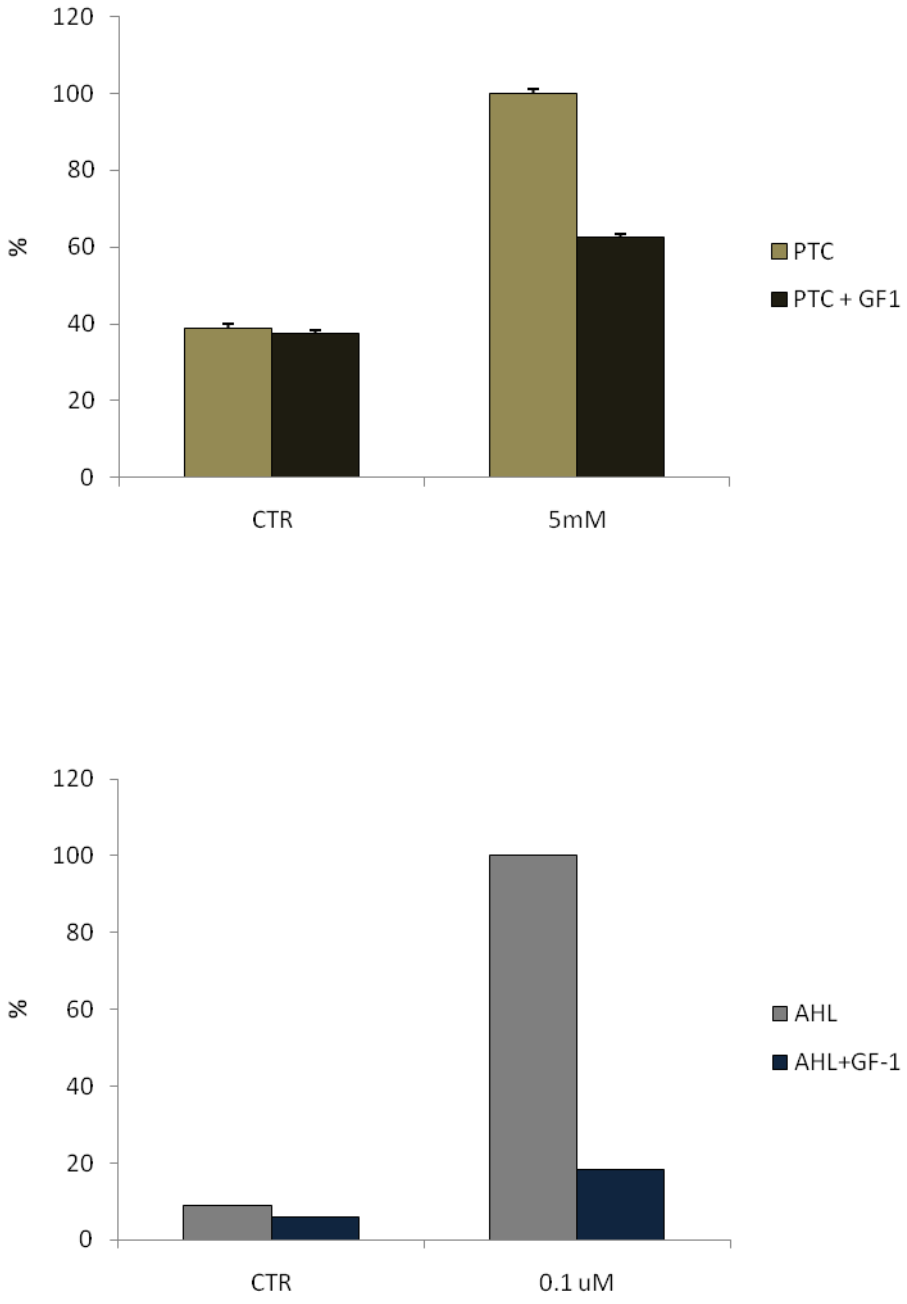


Figure 32: Graphs showing WB results in STC-1 cells for A) PTC-induced ERK phosphorylation, blocked by GF-1 B) AHL–induced ERK phosphorylation, blocked by GF-1 , similarly to PTC signal.

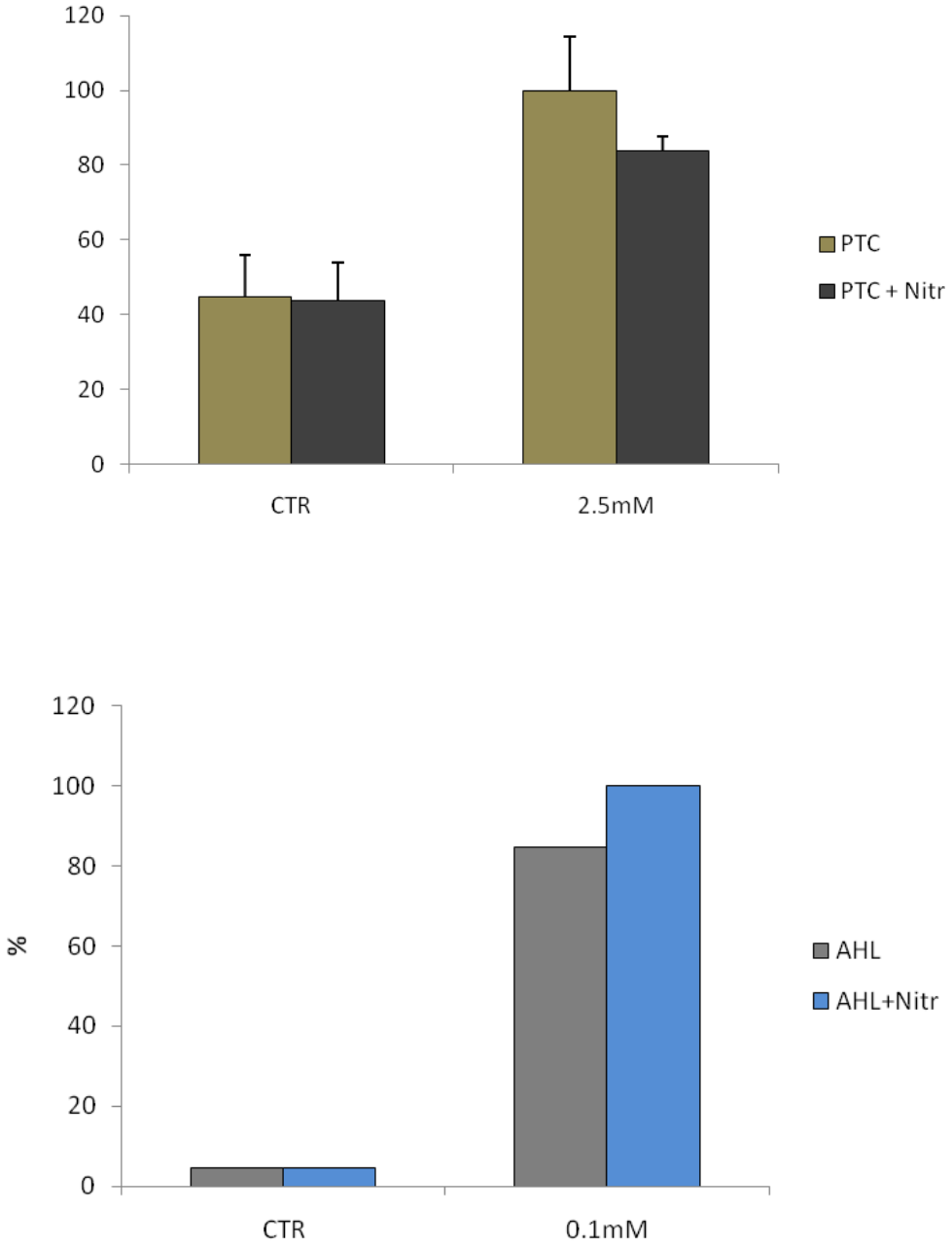


Figure 33: Graphs showing WB results in STC-1 cells for A) 2.5 mM PTC-induced ERK phosphorylation, blocked by Nitrendipine B) 100 uM AHL–induced ERK phosphorylation, blocked by nitrendipine, similarly to PTC signal.

IEC-18 cells were also activated by the lactone, but the signal was not blocked by Probenecid, if not at a high (5mM) concentration, that was above the concentration needed to block PTC response. Also, treatment with AHL 50 uM for 4 (**P<0.01) or 24h (***P<0.001) produced a significant increase in hT2R38 mRNA expression in NCM-460

cells (Fig. 34). Similarly, the same cell line challenged with PTC showed a significant (*P<0.05) increase in hT2R38 expression after 4 h.

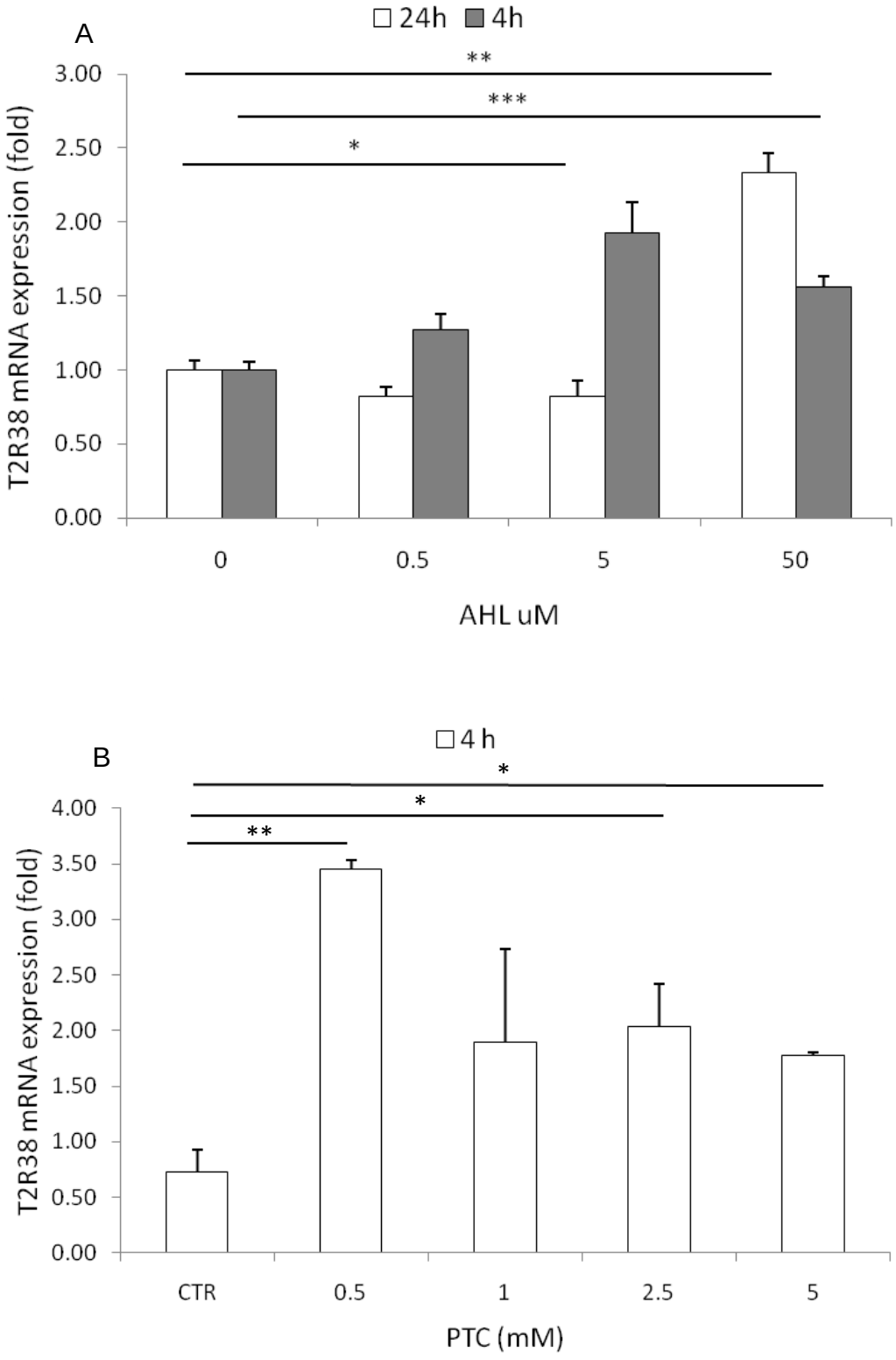


Figure 34: hT2R38 mRNA is up-regulated after 4-24h treatment with AHL (A)and PTC (B) in NCM-460 cells

4. Discussion

a. STC-1 and NCM-460 cell lines express T2Rs and are stimulated by bitter agonists through MAPK p44/42 phosphorylation

T2Rs mRNA expression in the mouse GI has been studied by different groups (e.g [1] [102] [103]) and they showed how several T2Rs, including mT2R138 and mT2R108 and their signaling molecule Gust are expressed in mouse enteroendocrine and non-enteroendocrine GI cells and in the small intestinal enteroendocrine cell line STC-1. Our study confirms that those receptors mRNA is expressed in these cells and qRT-PCR analysis of a large intestinal human cell line, NCM-460 normal colonocytes, revealed that they also express hT2R38, but they do not express hT2R4, the human homologus of mT2R108. Accordingly, when stimulated with DB or PTC_mT2R108/hT2R4 and mT2R138/hT2R38 agonists respectively_ STC-1 cells responded to both compounds with MAPK activation, whereas NCM-460 only responded to PTC, since they do not express hT2R4, the receptor for DB. These evidences confirm on cell lines that T2R subtypes are expressed in different species and different GI mucosal cell types, maybe with a specific distribution depending on the GI segment and on the specie. Since different species have evolutionary been stimulated with different food and a variety of different ingested compounds, it is possible that this might have caused a specific modulation of T2Rs expression, leading to a different distribution and a different receptor type spectrum, depending on the animal. Indeed, during evolution rodents developed a significantly higher number of T2R genes compared to humans [1] and different animals show a different number and pattern of many sense receptors, including T2Rs. [190]

Also, the pathway following T2Rs activation seems to involve MAPK p44/42, which we found to be phosphorylated following PTC or DB treatment only in cells expressing T2Rs (STC-1 and NCM-460 vs IEC-18), as previously published by Wu et al, 2002 [102]. The use of GF-1, as a PKC antagonist, and nitrendipine, as a Ca^{++} channel blocker, helped

to characterize the pathway following T2Rs activation: our data indicate that selected T2R ligands activate different pathways and suggest different mechanisms of action for different that T2R ligands.

Summarizing, based on our data, we hypothesize that T2Rs are distributed throughout the gut with different levels of expression and that selected subtypes might be expressed and have different functions in specific GI regions or different species. Their distribution and presence/absence might also depend on the receptor's function. T2Rs activation by different ligands leads to the phosphorylation of MAPK p44/42 following different pathways, depending upon the receptor, as schematized in Fig. 35.

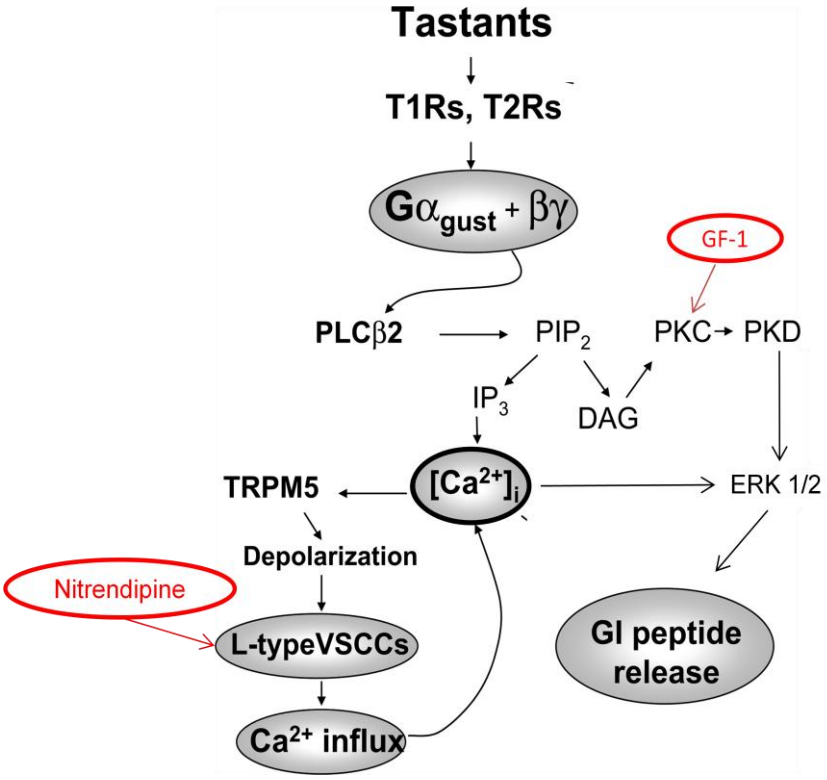


Figure 35: Possible mechanism for different T2Rs activation, involving Ca++ channels or PKC [Modified from Rozengurt E. et al, Am J Physiol Gastrointest Liver Physiol., 2006]

b. Bacteria might interact with T2Rs in the gut

It is already known that gut endocrine cells may be able to detect intestinal microbiota and pathogenic bacteria, as they express bacterial recognition receptors such as Toll-like receptors, or receptors for bacterial products, such as short chain fatty acids [178]. Since AHL activate different gut cell lines expressing T2Rs (STC-1 and NCM-460) and the signal is specifically blocked by an antagonist for T2R138 and by blocking the signaling pathway (in particular for T2R138), we hypothesized that subpopulations of mucosal cells might detect bacterial stimuli, such as QS molecules like AHLs, through T2Rs, which is in line with previous observations on epithelial cells expressing T2Rs in the airways [93]. Also, we showed that a high fat diet, which is able to unbalance in quantity and quality the gut microbiota and to initiate a mild inflammatory response, significantly affects mT2R138 expression in the colon only, and only after 8 weeks, when the bacterial change occurs, suggesting that the effect is bacteria-mediated more than directly mediated by fat. 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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