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

Rocco Latorre,
Department of Veterinary Medical Science, University of Bologna, Italy, via Tolara di Sopra, 50, 40064 - Ozzano dell'Emilia, Bologna, Italy

Maurizio Mazzoni,
Department of Veterinary Medical Science, University of Bologna, Italy, via Tolara di Sopra, 50, 40064 - Ozzano dell'Emilia, Bologna, Italy

Roberto De Giorgio,
Department of Medical and Surgical Sciences, University of Bologna, Italy, St. Orsola-Malpighi Hospital, via Massarenti, 40138 - Bologna, Italy

Claudia Vallorani,
Department of Veterinary Medical Science, University of Bologna, Italy, via Tolara di Sopra, 50, 40064 - Ozzano dell'Emilia, Bologna, Italy

Alessio Bonaldo,
Department of Veterinary Medical Science, University of Bologna, Italy, via Tolara di Sopra, 50, 40064 - Ozzano dell'Emilia, Bologna, Italy

Pier Paolo Gatta,
Department of Veterinary Medical Science, University of Bologna, Italy, via Tolara di Sopra, 50, 40064 - Ozzano dell'Emilia, Bologna, Italy

Roberto Corinaldesi,
Department of Medical and Surgical Sciences, University of Bologna, Italy, St. Orsola-Malpighi Hospital, via Massarenti, 40138 - Bologna, Italy

Eugenio Ruggeri,
Department of Medical and Surgical Sciences, University of Bologna, Italy, St. Orsola-Malpighi Hospital, via Massarenti, 40138 - Bologna, Italy

Chiara Bernardini,
Department of Veterinary Medical Science, University of Bologna, Italy, via Tolara di Sopra, 50, 40064 - Ozzano dell'Emilia, Bologna, Italy

Roberto Chiocchetti,
Department of Veterinary Medical Science, University of Bologna, Italy, via Tolara di Sopra, 50, 40064 - Ozzano dell'Emilia, Bologna, Italy

Catia Sternini, and
CURE/DDRC, Division of Digestive Diseases, Departments Medicine and Neurobiology, UCLA, Los Angeles, and Veterans Administration Greater Los Angeles Health System, Bldg 115 Room 223, VAGLAHS, 11301 Wilshire Blvd, Los Angeles, CA 90073, USA, csternin@ucla.edu, Tel: +1-310-312-9477, Fax: +1-310-825-3133

Correspondence to: Catia Sternini.
R. Latorre and M. Mazzoni contributed equally to this study

Paolo Clavenzani
Department of Veterinary Medical Science, University of Bologna, Italy, via Tolara di Sopra, 50, 40064 - Ozzano dell'Emilia, Bologna, Italy

Abstract

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

Keywords

chemosensory system; gut peptides; taste receptors; teleost

Introduction

The gustatory system plays a dominant role in the detection of dietary nutrients, sodium content and the acidity of foods as well as sensing the presence of potentially harmful substances (Sternini 2007; Behrens and Meyerhof 2011). The sense of taste enables animals to adapt to specific habitats (Oike et al. 2007; Ishimaru 2009; Barreiro-Iglesias et al. 2010). In vertebrates (Chandrashekar et al. 2000; Nelson et al. 2001, 2002; Zhao et al. 2003; Behrens and Meyerhof 2011), including fish (Ishimaru et al. 2005), two families of taste receptors (TRs), T1R and T2R, which detect complex tastes, have been cloned. TRs are G-protein-coupled receptors activated by different stimuli, including sweet and bitter substances, amino acids and nucleotides, which elicit a cascade of intracellular signals (Behrens and Meyerhof 2011). In mammals, TRs are abundantly expressed in taste buds, and interact with specific G α-subunits, including α-gustducin ($G_{\alpha gust}$), which transmit gustatory signalling from the lingual epithelium to the sensory cortex in the brain (Ming et al. 1999; Margolskee 2002; Caicedo et al. 2003; Behrens and Meyerhof 2011). In addition to $G_{\alpha gust}$, several G protein subunits have been identified, which are associated with TR signaling, $G\alpha_{i\text{-}2}$, $G\alpha_{i\text{-}3}$, $G\alpha_{14}$, $G\alpha_{15}$, $G\alpha_{q}$, $G\alpha_{s}$, and α-transducin ($G_{\alpha tran}$) (Ruiz-Avila et al. 1995; Kusakabe et al. 1998). TRs and signalling molecules have been reported in the human and rodent gastrointestinal mucosa and pancreas (Höfer et al. 1996; Höfer and Drenckhahn 1998; Wu et al. 2002; Rozengurt et al. 2006), supporting the concept that there is more than a "taste" function for these molecules and that taste receptor-related molecules exert non-gustatory functions outside the mouth (Sternini 2007; Behrens and Meyerhof 2011). $G_{\alpha gust}$ and $G_{\alpha tran}$ immunoreactivities have been localised to epithelial, predominantly endocrine, cells of the stomach and intestine of rodents (Höfer et al. 1996; Wu et al. 2002; Hass et al. 2007; Sternini 2007; Sutherland et al. 2007), pigs (Clavenzani et al. 2009; Mazzoni et al. 2013) and humans (Rozengurt et al. 2006; Steinert et al. 2011), including

ghrelin, somatostatin, cholecystokinin, glucagon-like peptide-1 and peptide YY positive cells (Rozengurt et al. 2006; Sutherland et al. 2007; Clavenzani et al. 2009; Fujita et al. 2009; Moran et al. 2010; Janssen et al. 2011; Steinert et al. 2011; Mazzoni et al. 2013). Endocrine cells, which are distributed throughout the gastrointestinal tract (GIT) mucosa and pancreas, control digestive functions and contribute to regulate caloric intake and metabolism (Holmgren 1985; Plisetskaya and Mommsen 1996; Palmer and Greenwood-Van Meerveld 2001; Nelson and Sheridan 2006). Since fish taste buds express similar receptors and downstream signalling molecules as mammals (Yasuoka and Abe 2009), the aim of this study was to test whether the TR gustatory signalling protein, $G_{\alpha tran}$ is expressed in the sea bass gut and characterize the types of $G_{\alpha tran}$ immunoreactive (-IR) cells.

Materials and Methods

Tissue preparation

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

Immunohistochemistry

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

Antibody specificity

Specificity of $G_{\alpha tran}$, $G_{\alpha gust}$, GAS/CCK, GHR, OB, and GLP-1 antibodies has been assessed by Western blot and/or immunoblocking with the corresponding peptide (see details in Table 2). Specificity of the 5-HT, CGRP and SOM antibodies was previously demonstrated in the sea bass by preadsorption test (De Girolamo et al. 1999; Visus et al., 1996). The staining we obtained with the monoclonal SP antibody was completely overlapping with the immunostaining reported by Pederzoli et al. (2004) in the sea bass with a rabbit SP antibody (Cambridge Research Biochemical, U.K.), the specificity of which was verified by

immunoblocking. Specificity of the secondary antibodies was assessed by omitting the primary antibodies.

Western blot

Sea bass brain, eye and stomach and mouse brain were collected, frozen in liquid nitrogen, and stored at −80°C. Tissues were homogenized into a sodium dodecyl sulfate (SDS) lysis solution (Tris-HCl 62.5 mM, pH 6.8; SDS 2%, 5% glycerol) with 0.1 mM phenylmethylsulfonylfluoride. Protein content of cellular lysates was determined by a Protein Assay Kit (TP0300; Sigma-Aldrich, St. Louis, MO). For Western blot using $G_{\alpha gust}$ and $G_{\alpha trans}$ antibodies, 20 μg of proteins were separated on NuPage 4–12% bis-Tris Gel (Gibco-Invitrogen, Paisley, UK) for 50 minutes at 200V, then electrophoretically transferred onto a nitrocellulose membrane. For Western blot with the GAS/CCK antibody, 30 μg of proteins were separated on Novex 18% Tris-Glycine Gel (Gibco-Invitrogen, Paisley, UK) for 90 minutes at 125V, then electrophoretically transferred onto a nitrocellulose membrane. After blocking treatment, the membranes were incubated at 4°C overnight with anti-$G_{\alpha gust}$ (1:300), anti-$G_{\alpha trans}$ (1:500) or anti-GAS/CCK antibody (1:1,000) in Tris-buffered saline-T20 (TBS-T20 20 mM Tris-HCl, pH 7.4, 500 mM NaCl, 0.1% T-20). Membranes were then washed with PBS-T20, and incubated with the secondary biotin-conjugated antibody and an anti-biotin horseradish peroxidase linked antibody (1:1,000). The blots were developed using chemiluminescent substrate (Super Signal West Pico Chemiluminescent Substrate, Pierce Biotechnology, Rockford, IL) according to the manufacturer's instructions. The intensity of luminescent signal of the resulting bands was acquired by Fluor-STM Multimager using the Quantity One Software (Bio-Rad Laboratories, Hercules, CA).

Results

Antibody Specificity

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

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

Preadsorption of $G_{\alpha gust}$, $G_{\alpha trans}$, GLP-1, OB and GHR antisera prevented immunostaining with each antiserum (not shown) confirming tissue staining specificity. The lack of immunostaining of sea bass retina incubated with the anti-$G_{\alpha gust}$ antibody (not shown) confirms that the $G_{\alpha gust}$ antibody used in this study does not recognize rod or cones transducins and provides additional support to the tissue specificity of this antibody.

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

In the stomach, $G_{\alpha tran}$-IR cells were counted in 54 randomly selected fields (0.28 mm^2 each) with a 40X objective lens, for a total area of 15.1 mm^2. Since the intestinal mucosa differs morphologically from the stomach for the presence of folds, the number of $G_{\alpha tran}$-IR cells in the intestine was evaluated in 200 randomly selected folds for a more accurate representation of cell density in these regions of the GIT. Values were expressed as mean ± standard error mean (SEM). The GIT of the sea bass consists of a siphonal stomach, numerous pyloric caeca and a relatively short intestine. $G_{\alpha tran}$-IR cells were detected in the

stomach and intestine, but not in the pyloric caeca. Intense immunolabelling was observed in the basal portion of the gastric gland and in the epithelial lining of the intestinal mucosal folds. $G_{\alpha tran}$-IR cells showed homogenously labelled cytoplasm, with an unlabelled nucleus and an elongated ("bottlelike") shape (Fig. 2a, e). These cells were characterised by two thin cytoplasmic processes, the first extending up to the endoluminal surface of the mucosa and the second projecting down to the basal lamina. These features indicate that these cells correspond to "open-type" enteroendocrine cells (EECs) (Höfer et al. 1999; Sternini 2008). The average number of $G_{\alpha tran}$-IR cells in the stomach (Fig. 2a, c, e, g) was 15.7 ± 2.2, while in the cranial and middle-caudal portions of the intestine there were 3–4 IR cells/200 folds and 1–2 IR cells/200 folds, respectively. Furthermore, in the stomach, most $G_{\alpha tran}$-IR cells co-expressed 5-HT (Fig. 2a, b), OB (Fig. 2c, d) or GHR (Fig. 2e, f). Only a few $G_{\alpha tran}$-IR cells colocalized with 5-HT in the intestine. Colocalization between $G_{\alpha tran}$- and $G_{\alpha gust}$-IRs was observed in some cells in the gastric mucosa (Fig. 2g, h). In contrast, none of the $G_{\alpha tran}$-IR cells contained SOM (Fig. 3g, h), GAS/CCK, CGRP, SP, and GLP-1-IR in the stomach and intestine. SOM, SP, CGRP, GAS/CCK, (Fig. 3a, b, c, d) and GLP-1 labelled cells were observed intermingled with unlabelled epithelial cells in the GIT mucosa. In addition, CGRP (Fig. 3c, e), GAS/CCK (Fig. 3f), SOM and SP antibodies labelled nerve fibres running either singly or in small fascicles in the submucosal and muscular layers, with some GAS/CCK and CGRP positive neuronal cell bodies detected only in the muscular layer (Fig. 3e, f).

Discussion

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

Recent studies on the fish genome failed to detect a gene encoding an ortholog of the mammalian $G_{\alpha gust}$ gene (Ohmoto et al., 2011), however, Oka and Korsching (2011) showed 80% homology between $G_{\alpha gust}$ and other G proteins, and Sarwal et al. (1996) reported a high homology of Gα gene between mammals and the puffer fish *Fugu rubripes*, with an identical intron/exon structure throughout the coding regions. Several lines of evidence support the tissue specificity of the $G_{\alpha tran}$ and $G_{\alpha gust}$ antibodies used in our study, including Western blot and immunoblocking experiments. Furthermore, the lack of $G_{\alpha gust}$ immunostaining in retina sections confirms that the $G_{\alpha gust}$ antibody does not detect transducins and provides further support of tissue staining specificity. In addition, Zhang et al. (2006) found $G_{\alpha gust}$ immunofluorescence in the barbells of yellow catfish (*Pelteobagrus fulvidraco*) with the same rabbit polyclonal $G_{\alpha gust}$ antibody we used in the present study. However, we cannot exclude the possibility that the gustducin antibody stains others G-

alpha proteins in the sea bass gut, thus the term $G_{\alpha gust}$ immunolabeling should be regarded as "$G_{\alpha gust}$ like-immunoreactivity". Finally, it should be pointed out that our transducin antibody does not differentiate between the two molecular forms of this protein, $G_{\alpha tranS}$ and $G_{\alpha tranL}$, as shown by Muradov et al. (2008), who reported the expression of both these transducins in long and short photoreceptors of lamprey (*Petromyzon marinus*) with the same rabbit polyclonal $G_{\alpha tran}$ antibody used in our study. The observation that only some cells showed both $G_{\alpha tran}$ and $G_{\alpha gust}$ immunoreactivities in the sea bass GIT is in agreement with previous reports in mammals showing only partial colocalization of these two Gα-proteins (unpublished, personal observation) and a differential distribution in some regions (Wu et al. 2002). $G_{\alpha tran}$ and $G_{\alpha gust}$ mediate signals initiated by tastants acting at both families of TRs, the T1Rs and the T2Rs (Wong et al. 1996; Ruiz-Avila et al. 2001; He W et al 2002; Caicedo et al, 2003). $G_{\alpha tran}$ and $G_{\alpha gust}$ could serve different chemosensitive modalities depending upon the luminal content and according to the receptor subtype being stimulated, which would be consonant with the report that different T2Rs exert their function through the activation *in vitro* of distinct Gαi related forms (Sainz et al. 2007). Our findings that $G_{\alpha tran}$ immunoreactivity was localized to distinct subsets of EECs expand previous data in other animals species (Rozengurt et al. 2006; Sutherland et al. 2007; Moran et al. 2010; Janssen et al. 2011, Steinert et al. 2011; Mazzoni et al. 2013). EECs have been reported in the stomach and intestine of several fish species (Holmgren et al. 1982; Reinecke et al. 1997; Ku et al. 2004; Bermúdez et al. 2007; Manning et al. 2008), including the sea bass, where 5-HT-, SOM- and GHR-IR EECs have been described (Visus et al. 1996; Ferrando et al. 2009a; Terova et al. 2008). Our study has shown GHR-IR cells in the gastric mucosa, many of which contain $G_{\alpha tran}$-IR. Our results are consistent with data from Terova et al. (2008), who observed high levels of GHR gene expression in the sea bass stomach. The colocalization of $G_{\alpha tran}$ and GHR-IRs is in line with data reported by Janssen et al. (2011) showing that 98% of the $G_{\alpha tran}$-IR cells co-express GHR in mouse stomach, a finding of special interest as it indicates that this cell immunophenotype is conserved through evolution. GHR might have a role in regulating food intake in response to fasting and re-feeding in sea bass (Terova et al. 2008). Moreover, OB, an anorexigenic peptide derived from the GHR precursor, has been reported to counteract GHR effects on energy homeostasis and gastrointestinal function (Zhang et al. 2005). Furthermore, an OB encoding sequence has been recently identified in the black sea bream (Yeung et al. 2006) and in Atlantic halibut (Manning et al. 2008). Thus, our results, together with previous observations, suggest a morphological link between chemical sensing and food intake.

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

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

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Fig. 1.

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

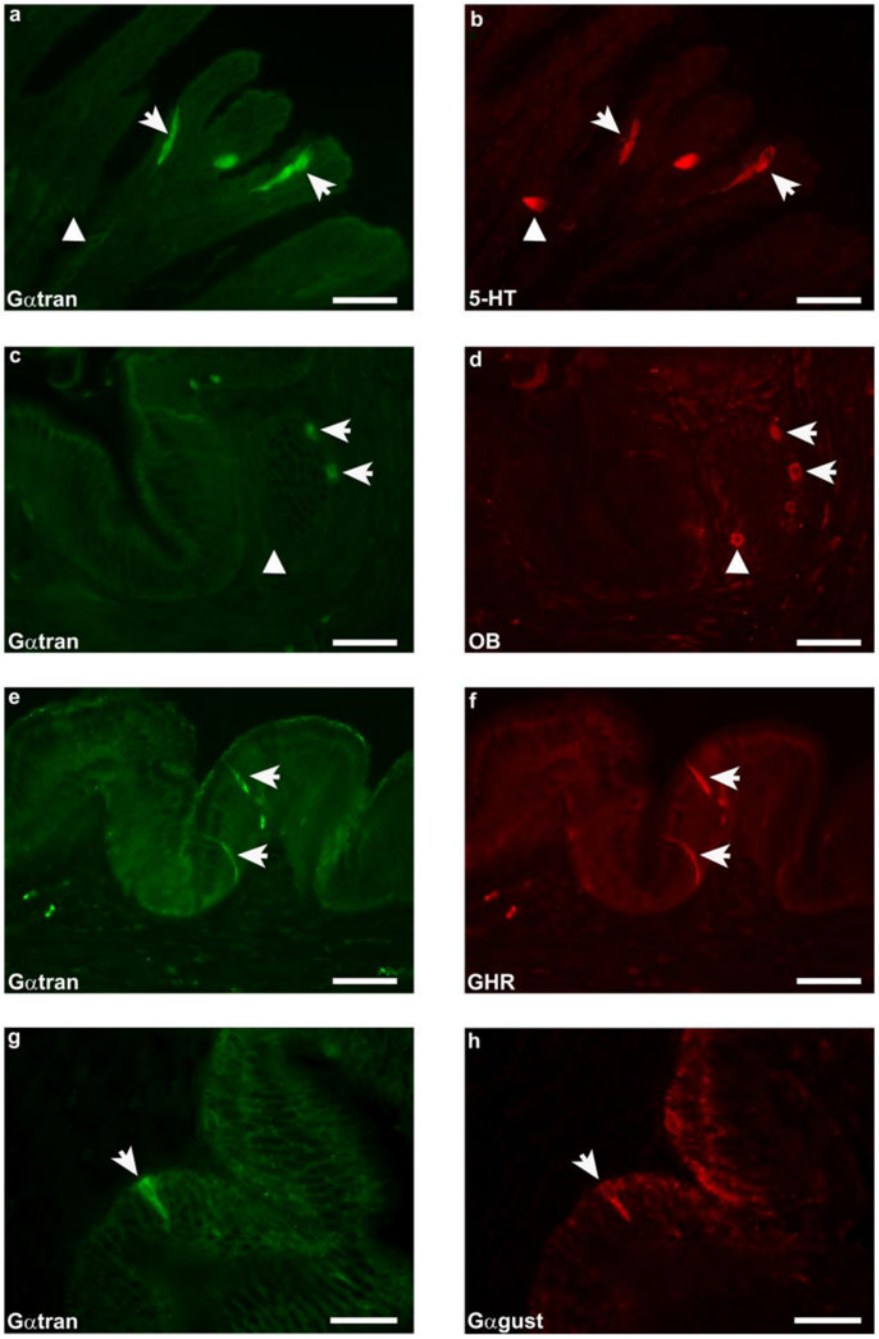

Fig. 2.

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

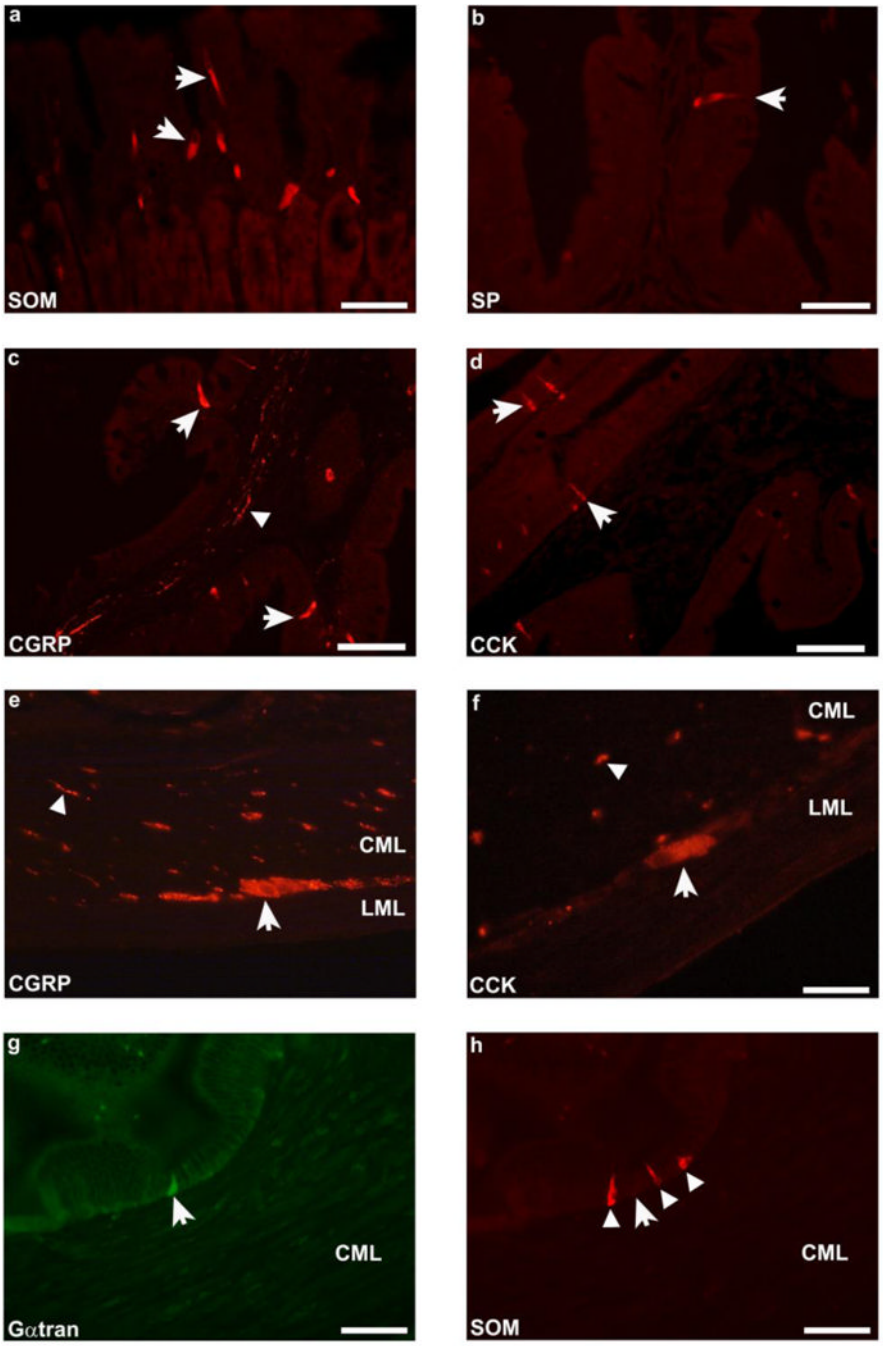

Fig. 3.

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

SOM immunolabelled cells. a, b, e, and h scale bars = 30 μm; c, d scale bars = 60 μm; f scale bars = 20 μm

Table 1

List of antibodies used in this study.

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

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

Table 2

Peptides used for absorption tests.

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

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