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REGULAR ARTICLE

Relationships of endocrine cells to each other and to other cell types in the human gastric fundus and corpus

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Abstract

Gastric endocrine cell hormones contribute to the control of the stomach and to signalling to the brain. In other gut regions, enteroendocrine cells (EECs) exhibit extensive patterns of colocalisation of hormones. In the current study, we characterise EECs in the human gastric fundus and corpus. We utilise immunohistochemistry to investigate EECs with antibodies to ghrelin, serotonin (5-HT), somatostatin, peptide YY (PYY), glucagon-like peptide 1, calbindin, gastrin and pancreastatin, the latter as a marker of enterochromaffin-like (ECL) cells. EECs were mainly located in regions of the gastric glands populated by parietal cells. Gastrin cells were absent and PYY cells were very rare. Except for about 25% of 5-HT cells being a subpopulation of ECL cells marked by pancreastatin, colocalisation of hormones in gastric EECs was infrequent. Ghrelin cells were distributed throughout the fundus and corpus; most were basally located in the glands, often very close to parietal cells and were closed cells i.e., not in contact with the lumen. A small proportion had long processes located close to the base of the mucosal epithelium. The 5-HT cells were of at least three types: small, round, closed cells; cells with multiple, often very long, processes; and a subgroup of ECL cells. Processes were in contact with their surrounding cells, including parietal cells. Mast cells had very weak or no 5-HT immunoreactivity. Somatostatin cells were a closed type with long processes. In conclusion, four major chemically defined EEC types occurred in the human oxyntic mucosa. Within each group were cells with distinct morphologies and relationships to other mucosal cells.

Keywords Oxyntic gland · Gastrointestinal hormones · Ghrelin · 5-Hydroxytryptamine · Somatostatin · Pancreastatin

Introduction

In the last several years, there has been a re-evaluation of the classification of gastrointestinal enteroendocrine cells (EECs). These cells had been thought to occur as well-defined cell types, belonging to about 12 classes that were not recognised to form subgroups. This concept has changed dramatically because recent studies, using single cell and population transcript analysis and multi-label immunohistochemistry, clearly indicate that there are multiple subtypes of EECs that express numerous, different combinations of hormones, particularly the intestinal hormones CCK, serotonin (5-HT), GIP, glucagon-like peptide 1 (GLP-1), neurotensin, peptide YY (PYY) and secretin (Egerod et al. 2012; Habib et al. 2012; Sykaras et al. 2014; Cho et al. 2015; Grunddal et al. 2015; Reynaud et al. 2016; Fothergill et al. 2017). Thus, the idea of there being a limited number of EEC types, each generally utilising a single hormone (the one cell–one hormone concept), is no longer tenable (Helander and Fändriks 2012; Fothergill and Furness 2018). While EEC subtypes in the intestines have been extensively studied, the same scrutiny has not been applied to gastric EECs.

The stomach expresses a different range of endocrine cell types than the intestines, the primary types in the oxyntic gland regions (fundus and corpus in human) being ghrelin

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cells, somatostatin cells, 5-HT (enterochromaffin) cells and enterochromaffin-like (ECL) cells that release histamine and pancreastatin. Whether gastric EECs are distinct or overlap, or whether they form chemically or morphologically identifiable subgroups, has not been investigated in a systematic way in human or other species. Nevertheless, some colocalization of gastric hormones has been reported. In the mouse stomach, 25% of gastric 5-HT cells were immunoreactive for somatostatin (Reynaud et al. 2016). In the rat and human stomach, ghrelin cells are also nesfatin-1 immunoreactive (Stengel et al. 2010, 2013).

A number of differences are observed between the commonly investigated rodent stomach and the human stomach. The rodent forestomach has a squamous epithelial lining whereas oxyntic glands occur in this region in human. Histologically, while the rodent and pig antrum have gastrin-producing glands only, the human antrum contains different types of glands, including acid-producing and mixed-type glands, not limited to a transition zone but spreading towards the pylorus (Choi et al. 2014). Another difference is that 5-HT is contained in mouse and rat mast cells (Lagunoff and Benditt 1959; Li et al. 2014) but there is little to no evidence of serotonin in mast cells of healthy humans (Parratt and West 1957). Few PYY cells were seen in cat and ferret corpus (Böttcher et al. 1993), whereas they were common in the mouse corpus (Friis-Hansen et al. 2005). In human, less than 1 pmol/g PYY was detected in the whole human stomach (Adrian et al. 1985). PYY has been reported in gastric EECs of human (Egerod et al. 2015) but, in which gastric regions, was not stated.

The distributions of gastric endocrine cells in entire human stomach specimens, using gastrin (G cells), ghrelin (A cells), somatostatin (D cells), 5-HT (EC cells) and chromogranin A (ECL cells) as discriminating cell markers, have been recently described (Choi et al. 2014), although it should be noted that antibodies against chromogranin A reveal other gastric endocrine cell types (Norlén et al. 2001), including ghrelin cells in the human stomach (Date et al. 2000). Colocalisation of hormones and the relations of EECs to each other and to other cell types were not reported. A more complete understanding of the major gastric EEC interactions with surrounding cells and with each other is important. In the current study, we study EECs of the human fundus and corpus, using dual-labelling immunohistochemistry, high-resolution confocal microscopy and 3D analysis.

Materials and methods

Stomach regions were collected from six patients who were undergoing gastric sleeve surgery for obesity at the Renown Regional Medical Center, Reno, Nevada. Resections were of the full greater curvature (from the fundus to the antrum) from male and female patients between the ages of 48 and 60 who were non-diabetic. The tissue was placed in cold fixative (2% formaldehyde plus 0.2% picric acid in 0.1 M sodium phosphate buffer, pH 7.0) kept overnight at 4 °C. Tissues were then washed three times (10 min) with dimethyl sulfoxide (DMSO) and then three times (10 min) with PBS. Tissue samples were then transferred to PBS-azide and sent to the University of Melbourne. The tissue samples were placed in 50% PBS-sucrose-azide and 50% OCT mixture (Tissue Tek, Elkhart, IN, USA) for 24 h, before being trimmed, embedded in 100% OCT and frozen in isopentane cooled with liquid nitrogen.

Immunohistochemistry

Sections of 12 μm thickness were cut, allowed to dry at room temperature for 1 h on microscope slides (SuperFrost Plus®; Grale Scientific, Victoria, Australia) and incubated with 10% normal horse serum plus 1% Triton X-100 in PBS for 30 min. Mixtures of primary antibodies (Table 1) for double staining were then placed on the sections that were left at 4 °C overnight. The tissue was washed three times in PBS and incubated with appropriate secondary antibodies labelled with Alexa Fluor dyes for 1 h at room temperature. To help reduce background fluorescence, tissue was washed with PBS for 5 min and then incubated with a quenching buffer (5 mM $CuSO_4$, 50 mM ammonium acetate, pH 5) for 30 min at room temperature. Preparations were then washed three times with PBS for 10 min. Preparations were washed twice with distilled water for 5 min then incubated for 5 min with bisbenzimide (blue), diluted 10 μg/mL in dH_2O, to stain nuclei. Sections were then washed three times with distilled water before mounting with non-fluorescent mounting medium (Dako, Carpinteria, CA, USA). An absorption test was applied to test the specificity of ghrelin antibodies binding to cells in the human stomach. The diluted anti-ghrelin antibodies were equilibrated with human ghrelin peptide (100 nM to100 μM) for 24 h at 4 °C before being used for staining of tissue sections as above. There was a concentration-related reduction in the immunohistochemical localisation. The immunoreactivity using chicken anti-ghrelin 14481 was reduced with 100 nM and 1 μM peptide and was abolished with 10 μM and 100 μM. Immunoreactivity with rabbit anti-ghrelin RY1601 was reduced with 1 μM peptide and was abolished with 10 μM and 100 μM. For all secondary antisera used, sections that were incubated without primary antibodies were used to investigate background staining and autofluorescence. There was no indication of non-specific binding of the secondary antibodies.

Image analysis

Slides were examined and imaged using an Axio Imager microscope (Zeiss, Sydney, Australia) and a LSM 800 confocal microscope with Airyscan super-resolution analysis (Zeiss).

Table 1 List of primary antibodies used and their respective dilutions

Target	Host species	Dilution	Antibody code, source and/or reference
Calbindin	Mouse	1:800	No. 18F (Swant, Bellinzona, Switzerland)
Calbindin	Rabbit	1:1000–1:2000	No. R202 (Furness et al. 1989)
Gastrin	Rabbit	1:2000	No. 8007 (gift from Dr. Jens Rehfeld)
Gastrin-CCK	Mouse	1:2000	No. 28.2 (Kovacs et al. 1997)
Ghrelin	Chicken	1:800	No. 15861 (Pustovit et al. 2017)
Ghrelin	Rabbit	1:800	No. RY1601 (Mizutani et al. 2009)
GLP-1	Rabbit	1:2000	No. 8912 (Cho et al. 2015)
H^+/K^+ ATPase	Mouse	1:300	No. 7.22 (Smolka and Weinstein 1986)
5-HT	Rabbit	1:2000	No. 20080 (ImmunoStar, Hudson, WI, USA)
5-HT	Goat	1:3000	No. 20079 (ImmunoStar; Cho et al. 2014)
Mast cell tryptase	Mouse	1:2000	MAB 1222 (Chemicon, Boronia, Australia)
PYY	Chicken	1:500	No. GW22771 (Sigma-Aldrich, Castle Hill, Australia)
PYY/NPY	Sheep	1:400	E2210 (Furness et al. 1985)
Pancreastatin	Rabbit	1:400	No. 8942 (CURE Antibody Core, Los Angeles)
Somatostatin	Mouse	1:1000	No. S895 (Buchan et al. 1985)
Somatostatin	Sheep	1:2000	No. AS01 (gift from Dr. Arthur Shulkes)

Tile scans taken with a × 10 objective were used for cell counts; for the density analysis, the number of cells was divided by the total area of the mucosal section that was counted. Tile scans were exported to be analysed off-line using ImageJ software (imagej.nih.gov/ij/). Immunoreactive cells were quantified by counting 100–500 cells in sections from each part of the stomach. This analysis was repeated in tissues from three patients. × 40 and × 63 objectives were used for high-resolution analysis. High-resolution images were exported into CorelDraw (Corel, Ottawa, Canada) for final preparation of figures. For analysis of 3D stacks, the Imaris program 8.4.1 (Bitplane, Oxford Instruments, Abington, UK) was used and image rendering was applied where appropriate. To determine whether the ghrelin cells occur in clumps, three pieces of the fundus from three different patients were analysed using the Delaunay triangulation protocol in ImageJ. Ghrelin cells were circled manually and the Delaunay triangulation for neighbour analysis was applied for the selected region of interest.

Statistical analysis

Prism 5.0 (GraphPad Software, San Diego, CA, USA) was used to analyse data and present it as mean ± SEM. Differences were evaluated using two-way ANOVA with the Bonferroni post hoc test. A P value < 0.05 was taken as significant.

Results

The localisation of endocrine cells was investigated throughout the fundus and corpus. Four immunohistochemically defined cell populations were found: ghrelin-, pancreastatin-, 5-HT- and somatostatin-immunoreactive cells. Other markers investigated were calbindin (see section below on pancreastatin), PYY and GLP-1 immunoreactivities. Few cells had PYY or GLP-1 immunoreactivity. Tissue from human colon, where cells containing these hormones are common (Martins et al. 2017), showed strongly reactive PYY and GLP-1 cells.

By position, the cells were of two types: closed cells that did not reach the lumen (the majority) and open cells that had a surface at the gastric gland lumen (a subgroup of 5-HT cells). Of the closed cells, some were small, round or ovoid cells, without discernible processes, located between other cells of the epithelium and the connective tissue of the lamina propria. Other closed cells had a similar location but had processes that ran parallel to the bases of the epithelial cells lining the glands. Some endocrine cell processes entered the connective tissue at the base of the epithelium.

Endocrine cell distribution across the mucosa of the human fundus and corpus

Endocrine cells were found in the regions occupied by parietal cells (most of the mucosal width) and regions of chief cells at the base of the mucosa (Figs. 1 and 2c). They were absent from the regions of the gastric pits, where the epithelium is composed of mucous cells.

The most common endocrine cells were those containing ghrelin and the ECL cells marked by pancreastatin immunoreactivity (Fig. 2a). The numbers of cells were counted in each region of the gastric mucosa (regions with chief cells, regions with parietal cells, regions with mucous cells) (Fig. 2c). Chief

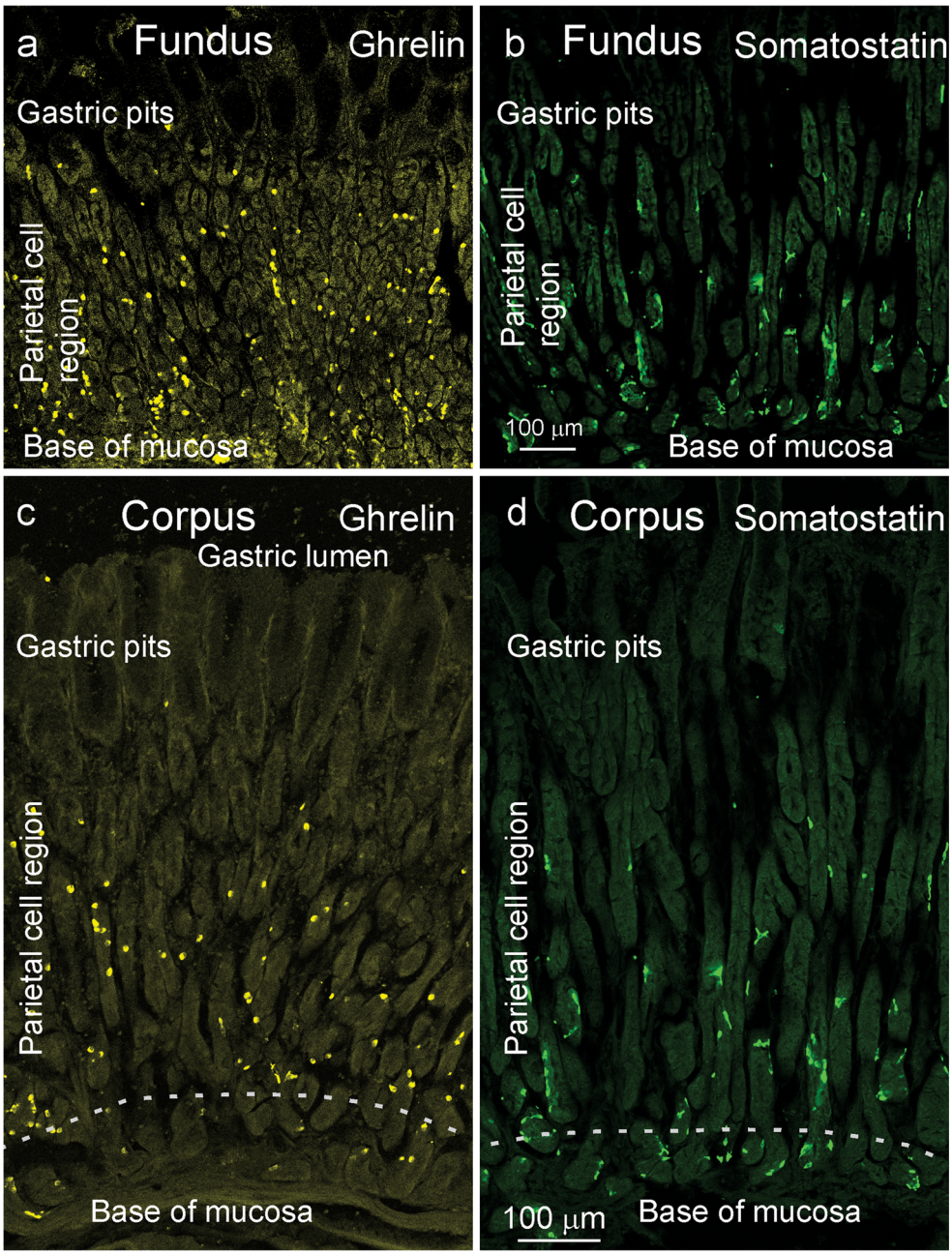

Fig. 1 Low-power views to illustrate how gastric endocrine cells are distributed across the wall of the corpus and fundus, using ghrelin (**a**, **c**) and somatostatin (**b**, **d**) as examples. Endocrine cells were scattered in the regions dominated by parietal cells and also the chief cell regions but were very rare in the regions of the gastric pits. The border between the parietal cell region (dominated by parietal cells/few chief cells) and the chief cell region (dominated by chief cell/very few parietal cells) is poorly defined. The approximate boundary is marked by the dashed lines in **c** and **d**. Quantitative data are in Fig. 2

and mucous cells were identified by shape, position and the autofluorescence that is exhibited by these cells (Fig. 1). We also used an anti-H/K ATPase antibody to mark the parietal cells. Gland regions where parietal cells were located also contained some chief and mucous cells. In the fundus and corpus, similar percentages of the mucosal width contained predominantly chief cells (6–7%), parietal cells (66–68%) and mucous cells (25–27%) (Fig. 2b). The densities of occurrence of the different EECs varied significantly (two-way ANOVA). The densities of ghrelin cells were 109 ± 24 cells/mm^2 in the fundus and 77 ± 13 cells/mm^2 in the corpus. The densities of pancreastatin cells were similar: 102 ± 12 cells/mm^2 in the fundus and 89 ± 14 cells/mm^2 in the corpus ($P > 0.05$ vs ghrelin cells for both regions, Bonferroni post hoc tests). However, 5-HT cells (45 ± 6 cells/mm^2 in the fundus and 25 ± 4 cells/mm^2 in the corpus) and somatostatin cells (44 ± 5 cells/mm^2 in the fundus and 24 ± 3 cells/mm^2 in the corpus) were less dense ($P < 0.01$), compared to both ghrelin and pancreastatin in each region.

More than 80% of endocrine cells were in gland regions dominated by parietal cells that also contained small numbers of chief or mucous cells and that occupied 66–68% of the total mucosal width (Fig. 2c). Gland regions rich in chief cells at the base of the mucosa also contained numerous EECs.

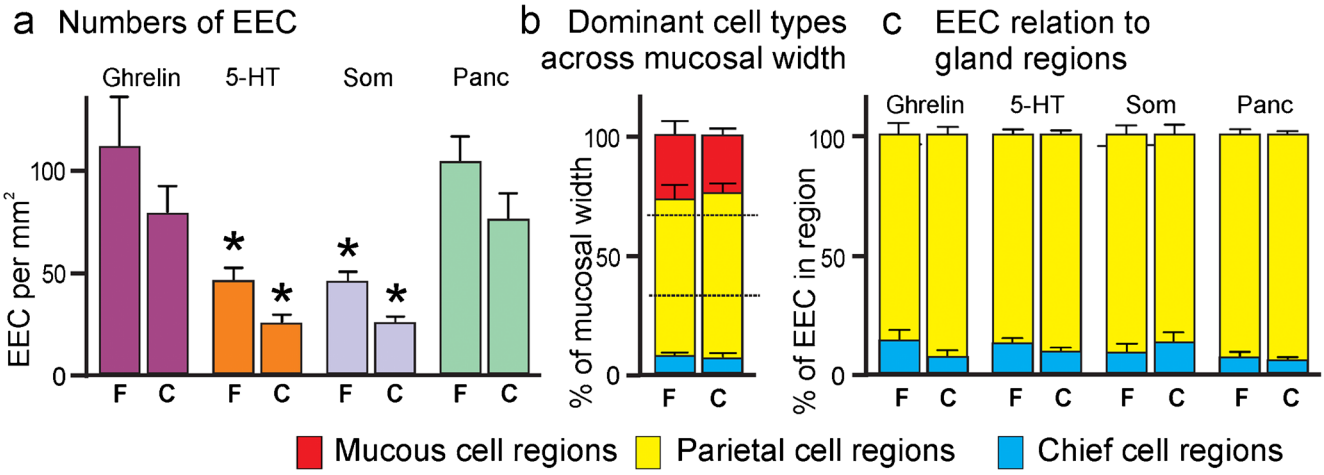

Fig. 2 Numbers of immunoreactive gastric endocrine cells and their distributions across the width of the mucosa. **a** Relative densities of the occurrence of cells in the fundus (F) and corpus (C). **b** The distributions of dominant cell types across the width of the mucosa. The dotted lines indicate the inner, middle and outer thirds. Most of the inner third, all the middle third and the bottom 20% of the outer third were dominated by parietal cells. **c** Relation of endocrine cells to mucosal regions. More than 80% of each population of endocrine cells was found in the parietal cell regions. Almost none were found in mucous cell regions. $*P < 0.01$, significantly different from both ghrelin and pancreastatin cell density in the corresponding region (two-way ANOVA with Bonferroni post hoc tests)

Around 14% of ghrelin cells were in the bases of the glands where there were chief cells but very few parietal cells in the fundus, whereas this region covers only 7 ± 1% of the mucosal width (Fig. 2b). Pits and mucous neck regions of the glands contain almost entirely mucous cells, with almost no endocrine cells (Figs. 1 and 2c).

Patterns of colocalisation

We used simultaneous labelling immunohistochemistry to investigate patterns of colocalisation in EECs (Fig. 3). Where overlap was observed, immunoreactivity of colocalised markers was generally strong (Fig. 3).

The only substantial number of cells showing co-expression contained both 5-HT and pancreastatin (Fig. 4). For 5-HT cells, 25 ± 5% were immunoreactive for pancreastatin in the fundus and 27 ± 5% in the corpus. Because of their greater numbers, a lower percentage of pancreastatin cells expressed 5-HT (12 ± 3% in the fundus and 11 ± 3% in the corpus). The pancreastatin/5-HT cells had the location and morphology of ECL cells (see below). For some of these cells, both pancreastatin and 5-HT were seen in the long processes.

For all other examples of colocalisation, the proportion of dual-labelled cells was less than 10% of the parent population (Fig. 4). Fewer than 2% of ghrelin cells and fewer than 8% of somatostatin cells showed colocalisation of another hormone (6 ± 2% of somatostatin cells were immunoreactive for ghrelin in the fundus and 7 ± 4% of somatostatin cells were immunoreactive for 5-HT in the corpus). Fewer than 8% of 5-HT cells or pancreastatin cells showed immunoreactivity for ghrelin or somatostatin (Fig. 4).

Ghrelin cell positions, shapes and relationships

Ghrelin cells were amongst the most abundant EECs in the oxyntic glands (Fig. 2) and ghrelin cells showed rare colocalisation with any other gastric hormone. The vast majority of ghrelin cells (90.3 ± 2.2%) were round, closed cells (Fig. 5c). These cells were at the base of the mucosa, commonly behind parietal cells (Fig. 5a, b). Nuclei were round or slightly oval and could occupy a high proportion of the cell's cross-sectional area. A small percentage of ghrelin cells had processes that were always directed away from the lumen and along the basal surface of the epithelium (Fig. 5c). These processes were fewer in the corpus than in the fundus. There were a small number of ghrelin cells, most of which were round and a few of which had long processes, in the connective tissue of the lamina propria, near the base of the epithelium.

As noted above, ghrelin cells were abundant in the mucosal area that was rich with parietal cells. In many cases, ghrelin cells were very close to parietal cells, even in some cases being partly surrounded by the parietal cell (Fig. 5b).

Ghrelin cells were often observed to form clumps (Fig. 6). To analyse these in an unbiased way, we used the Delaunay triangulation protocol in ImageJ. This method has been previously used to analyse relations between cells in tissue sections (e.g., Vostrikov et al. 2015). Ghrelin cells were circled manually and the program was used to determine cell radius and the distributions of centre-to-centre separations. Data were collected from three sections of the fundus from three different patients and included a total of $n = 2130$ cells. The average distance (centre to centre) to the nearest neighbouring cell was quantified and found to be 25.1 ± 0.5 μm (mean ± SEM) (Fig. 6). Assuming a random distribution, in a 2D (planar)

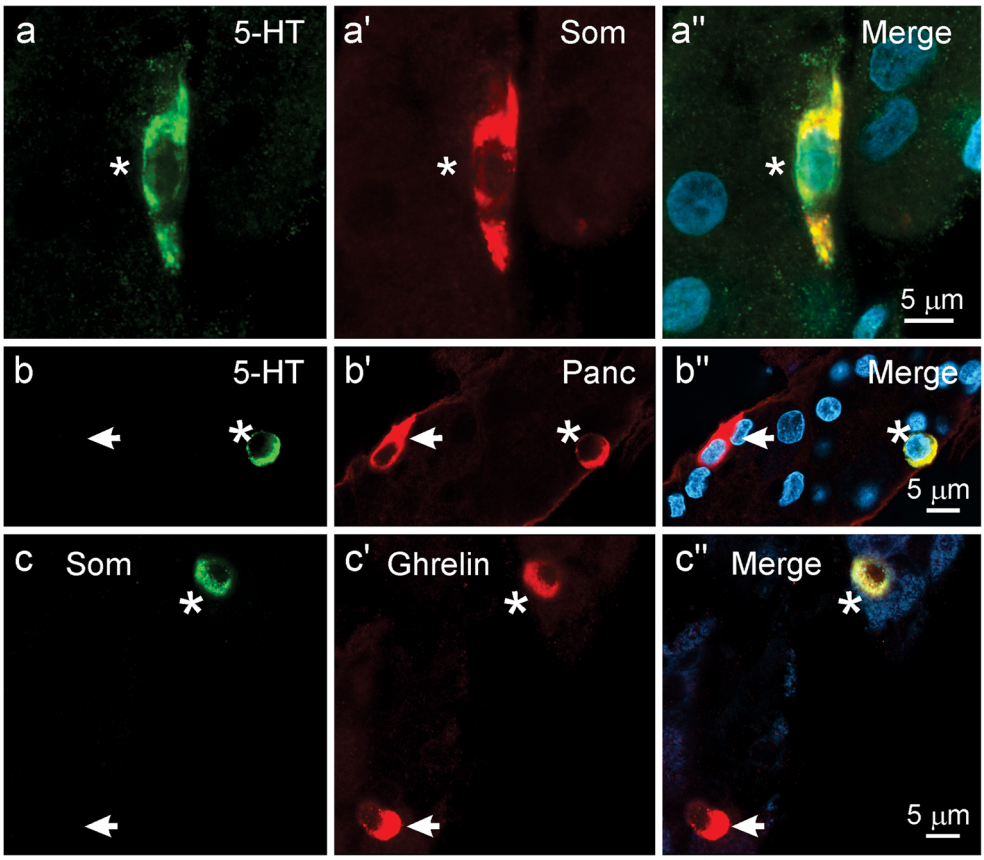

Fig. 3 Examples of colocalisation of hormones and the ECL cell marker, pancreastatin (Panc). **a**, **a'**, **a''** Colocalisation of 5-HT and somatostatin. 5-HT shows immunoreactivity in the nucleus. **b** Colocalisation of 5-HT in one of two pancreastatin-immunoreactive ECL cells. Fine processes of the pancreastatin cells can be seen. **c** Somatostatin immunoreactivity in a small round ghrelin cell. A second ghrelin cell does not have somatostatin immunoreactivity. These examples are from the fundus. Asterisk marks cells in which two markers were localised. Arrow marks the positions of cells that were immunoreactive for a single marker

section through the 3D array, the theoretical mean nearest-neighbour separation between cells is 48 μm ($\frac{\sqrt{N_a}}{2}$) where N_a is the measured density of ghrelin cells in a section of the fundus (Bansal and Ardell 1972). In our sample, N_a was 109 cells/mm^2. Since the theoretical distance, 48 μm, is well outside the 99% confidence interval (mean ± 3 × SEM) for the measured distance (25.1 ± 1.5 μm), we conclude that the cells indeed do form clumps.

A small proportion of ghrelin cells were also close to 5-HT or somatostatin cells (Fig. 7).

5-HT cell positions, shapes and relationships

5-HT cells were identified by immunoreactivity with anti-5-HT antibodies. They were distinguished from mast cells using anti-mast cell tryptase (Fig. 8). Most mast cells, revealed by anti-mast cell tryptase, were seen in the lamina propria but a small proportion of mast cell tryptase-positive cells were in the gland wall or close to the base of the gland epithelium. Mast cells showed no 5-HT immunoreactivity with the goat anti-5-HT antibody used in this study. Very faint staining was seen with the polyclonal rabbit anti-5-HT antibody. Thus, human mast cells, unlike those in some rodents, contain little or no 5-HT.

5-HT-immunoreactive EECs in the oxyntic glands were characterised by their shapes (Fig. 9). There were circular, closed cells (Fig. 9a, c), similar to the ghrelin cells that are described above; cells with a conical shape, rather typical of open-type EECs (Fig. 9a); and cells with multiple (two, three or more) processes, some with basal processes of varying lengths up to 70 μm (Fig. 9b, b', e, f). Sometimes 5-HT cells appeared to form a chain with processes of one 5-HT cell joining another (Fig. 9d). 5-HT cells with processes generally had a stronger staining than the round cells. For a small number of 5-HT cells, there was immunoreactivity within the nucleus. This immunoreactivity was only seen in cells that also had cytoplasmic 5-HT immunoreactivity. We believe that it is displacement of cytoplasmic 5-HT to the nucleus, perhaps because the nuclear pores were more open in some cells, which is possibly a tissue processing artefact.

5-HT cell processes did not have consistent relationships with other cells. They could be multiple and very long and closely approach other 5-HT or epithelial cells (including parietal cells, Fig. 9b), or run along the base of the gland or into the lamina propria without an obvious target. Some processes connected two neighbouring glands (Fig. 9f). Processes that came close to parietal cells were common and closed cells that were partially engulfed by parietal cells in a similar fashion to that seen for some ghrelin cells were also common (Fig. 9c).

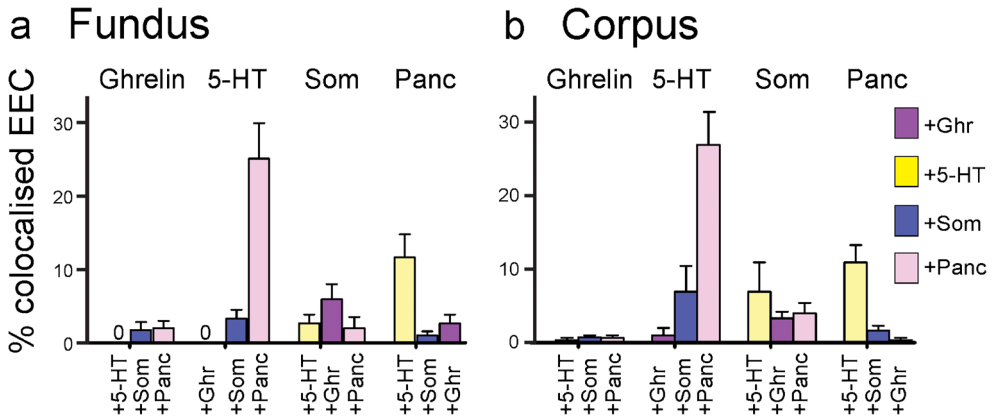

Fig. 4 Quantitation of colocalisation in the **a** fundus and **b** corpus. For each group of three columns, the cells immunoreactive for the marker indicated at the top of the columns were counted and the percentage showing colocalisation with the markers under each column was determined. 0 = no colocalisation (5-HT and ghrelin in the fundus were never colocalised)

Although 5-HT cell processes approached ghrelin cells, the majority of ghrelin and 5-HT cells did not appear to be in contact. Also, closed, round ghrelin and 5-HT cells did not appear to have close proximity to each other.

Within the gastric glands, the round cells tended to be towards the gastric lumen and 5-HT cells with processes were towards the bases of the glands, where chief cells are located. Some 5-HT cells were immunoreactive for pancreastatin (see Fig. 10). All pancreastatin cells had ECL cell morphology, whether or not they also contained 5-HT.

Somatostatin cells

Somatostatin cells were of the closed type. While the majority had processes (Figs. 1 and 7b), round somatostatin cells could also be seen (Fig. 3c). Somatostatin cells had one observable process or a thick long process and two or three other small processes. These processes commonly ended with a bulb-like shape (Fig. 7b). The majority of somatostatin cell processes were directed away from the lumen and along the base of the gland, where they came close to parietal cells or other endocrine cells. The density of somatostatin cells was close to that of 5-HT cells (Fig. 2a), with a maximum level of colocalisation of 10% with any other gastric hormone (Fig. 4).

There was no apparent preferential proximity of somatostatin cells to ghrelin cells; commonly, in sections through the tissue, they were located either in a different gland or in the same gland but without a process coming close to a ghrelin cell, although a small number of ghrelin cells were closely approached (Fig. 7b). Rare EECs had immunoreactivity for both somatostatin and ghrelin (Fig. 2).

5-HT and somatostatin showed a small degree of colocalisation in the stomach (Fig. 3). 5-HT and somatostatin cells could be very close to each other especially in the bottom third of the gland where each had a high density of occurrence. Closer to the gastric lumen, somatostatin cells occurred at a lower density and were less close to each other.

Pancreastatin cells

Pancreastatin immunoreactivity was confined to a population of numerous cells that were located at the base of the mucosal epithelium, as described elsewhere for ECL cells (Gustafsson et al. 2011). In some cases, as previously described (Lönroth

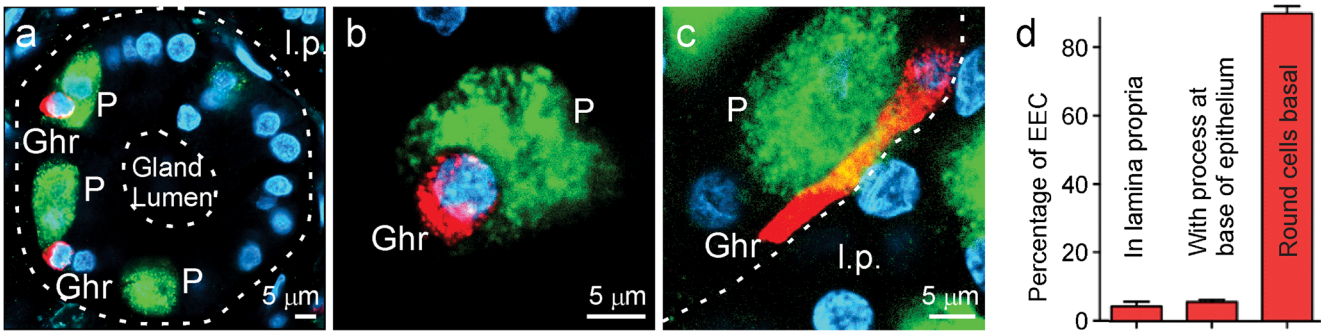

Fig. 5 Examples of ghrelin cells showing their shapes and positions in the glands. Dotted lines show the basal and luminal surfaces of the glandular epithelium in **a** and the basal surface in **c**. Blue is DAPI staining of cell nuclei. Ghr, ghrelin cell, cytoplasm red; P, parietal cell (H/K ATPase staining), cytoplasm green; l.p., lamina propria. **a** Cross-section of a gastric gland showing ghrelin cells at the base of the epithelium (Ghr). The cytoplasm of parietal cells is stained by H/K ATPase. This antibody does not recognise H/K ATPase in the surface membrane so the regions of the canaliculi are not revealed. **b** Close association between closed-type ghrelin EEC and a parietal cell. **c** Ghrelin cell with a process parallel to the base of the mucosa, very close to a parietal cell. **d** Relative numbers of the different morphological types of ghrelin cells

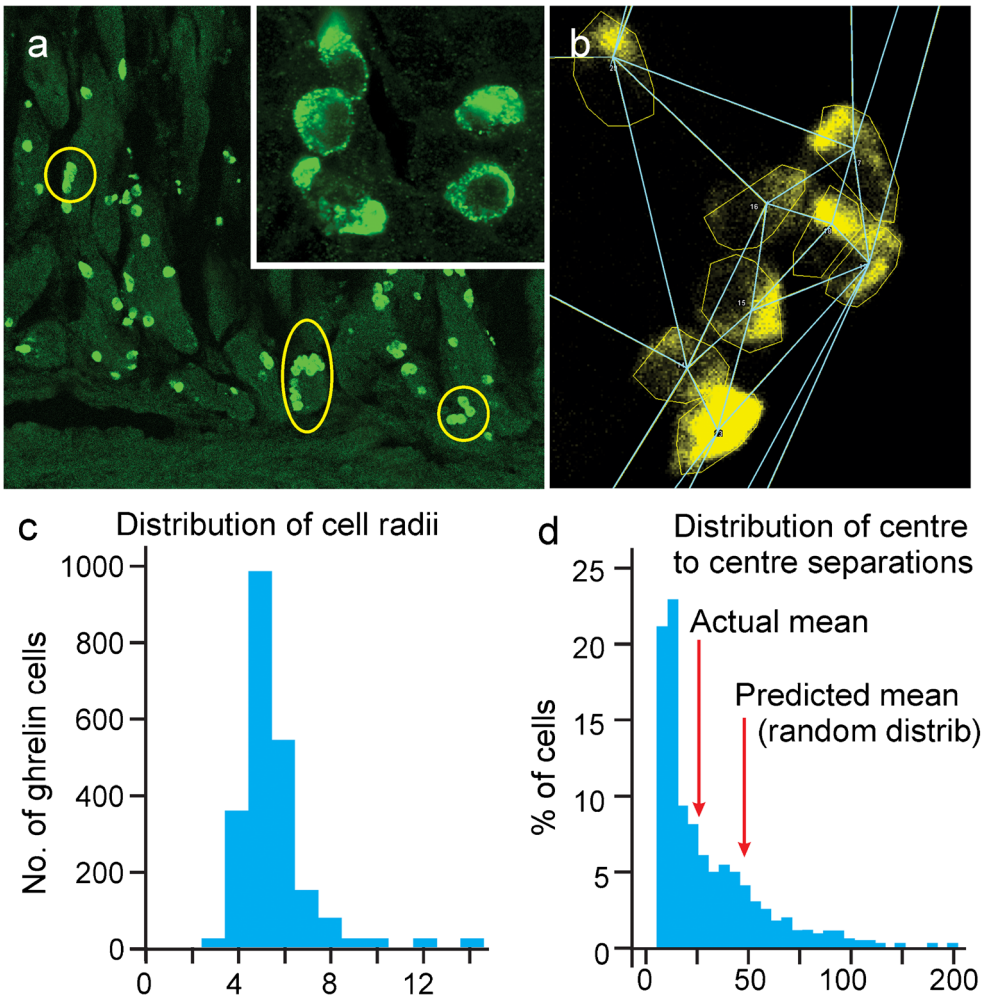

Fig. 6 Clumping of ghrelin cells. **a** Micrograph showing ghrelin cells in the gastric mucosa. The circles in yellow surround examples of clumps of ghrelin cells. Inset shows a clump at greater magnification. **b** Determination of distances between cell centres using ImageJ. The lines show the distances between cell centres of mass determined by the ImageJ program. Each line is a computer-determined centre-to-centre vector. **c** Distribution of ghrelin cell maximum radii. **d** Distribution of centre-to-centre distances to the nearest neighbouring cell for 2130 ghrelin cells in three fundus sections from three different patients, with the actual mean and the mean predicted if the cells were randomly distributed indicated. Distances in microns.

et al. 1990; Gustafsson et al. 2011), cells had discernible long fine processes that came close to parietal cells. In other cases, processes were not recognisable. Some instances were seen where 5-HT cells appeared to be interposed between an ECL cell and a parietal cell. Ninety percent of the pancreastatin-immunoreactive ECL cells were in the parietal cell-dominated regions of the mucosa.

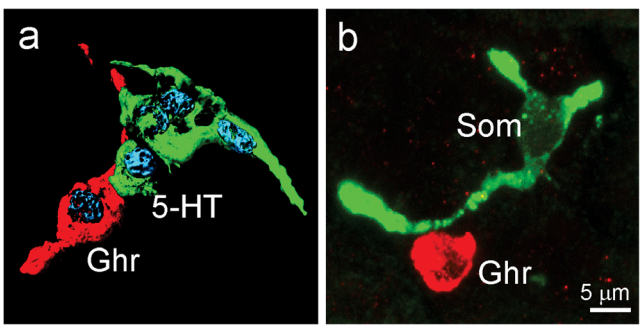

Fig. 7 Ghrelin (in red) relations with a group of four 5-HT cells (**a**) and a somatostatin cell (**b**). **a** This ghrelin cell has a process that comes close to the 5-HT cells. Imaris-rendered image. **b** Close approach by the process of a somatostatin cell to a ghrelin cell

Although pancreastatin is an effective ECL cell marker (Norlén et al. 1997; Andersson et al. 1998), calbindin has also been reported in human gastric ECL cells (Furness et al. 1989; Bordi et al. 2000), so we investigated calbindin. Only a small number of calbindin-immunoreactive cells dispersed within the mucosa were observed. As reported above, about 12% of pancreastatin cells were 5-HT immunoreactive (Fig. 10b, b'). Histamine or histidine decarboxylase is not good markers of ECL cells, as 80% of histamine cells in the human stomach are mast cells, some of which are similarly positioned to ECL cells (Håkanson and Sundler 1991).

Discussion

The four major types of chemically defined endocrine cells in the oxyntic gland regions (fundus and corpus) of the human stomach were largely distinct, not showing the extensive and rather complex patterns of colocalisation of hormones that are seen in the small and large intestines. The only substantial group of cells with colocalised markers was a population of

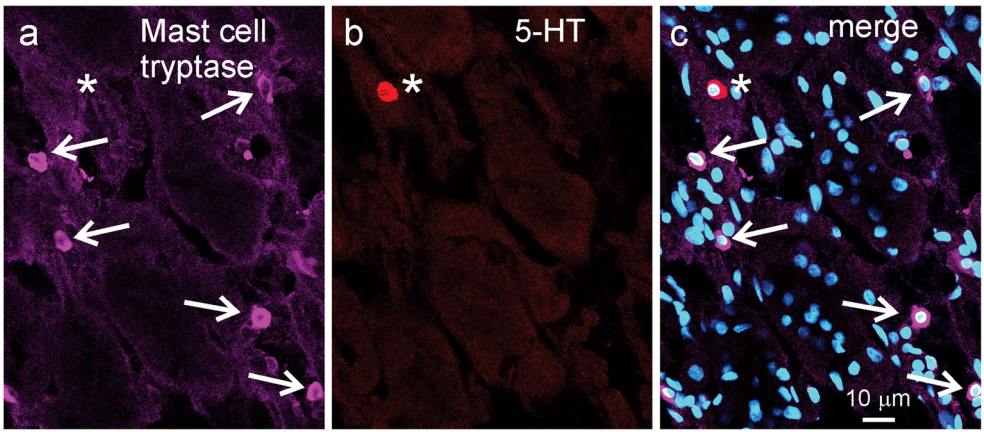

Fig. 8 Double labelling of 5-HT (using the goat anti-5-HT antibody) with mast cell tryptase in the human stomach. Mast cells in the human stomach (**a**; arrowed) were not immunoreactive for 5-HT (**b**, asterisk). A 5-HT cell, not immunoreactive for mast cell tryptase, is seen in **b**. **c** Merged image, showing the nuclei of the mast cell tryptase- and 5-HT-immunoreactive cells and other cells in the field (DAPI stain)

5-HT/pancreastatin cells. These had ECL cell morphology, being elongated cells with long processes at the base of the mucosal epithelium that appear to be a subpopulation of ECL cells, representing about 12% of the ECL cells. Little or no 5-HT occurred in mast cells in these human samples, although human mast cells have the ability to synthesise 5-HT and, in disease states, can contain significant amounts (Kushnir-Sukhov et al. 2007). There were two morphological types of

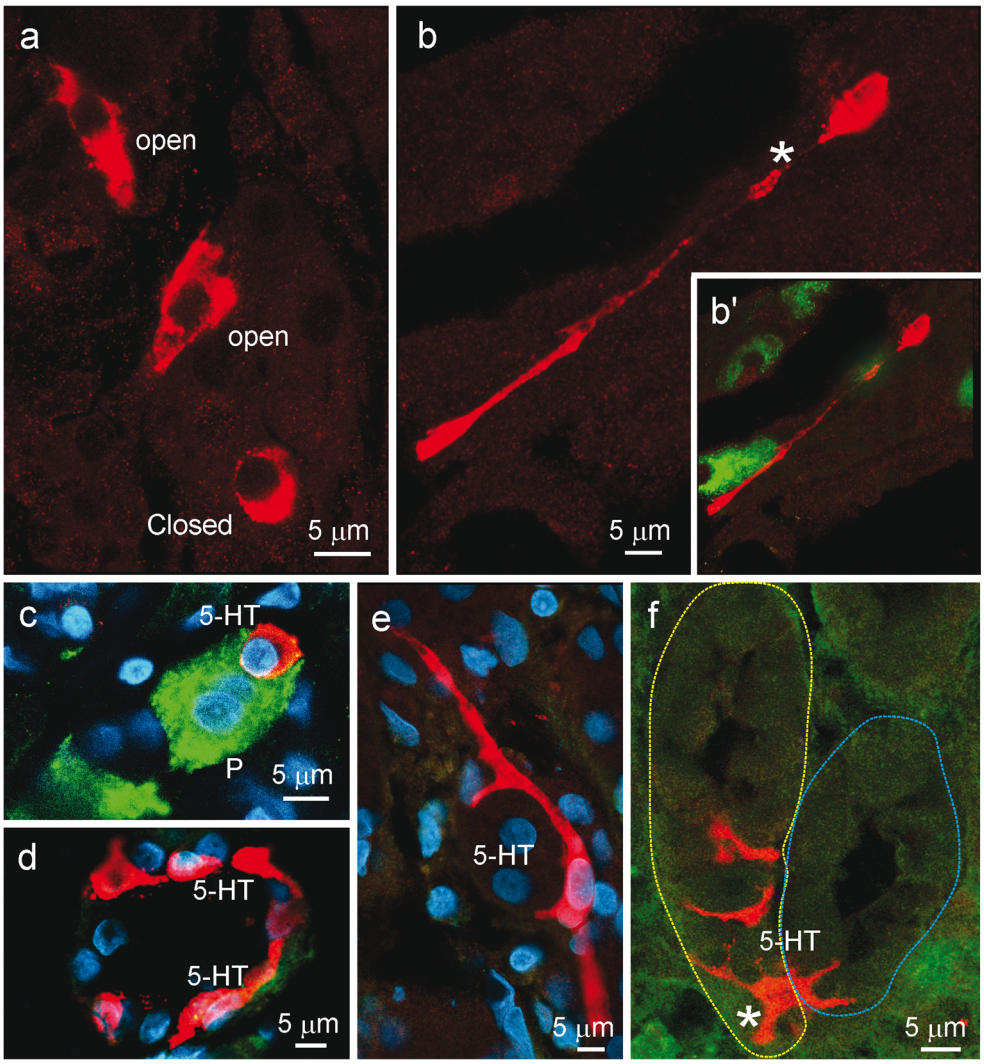

Fig. 9 Different types of 5-HT cell shapes (red) seen in the gastric mucosa. **a** Round, closed cell and two open-type cells with conical shapes. **b** Cell with a single long process of about 70 μm, running along the gland membrane with its end close to a parietal cell (green in the inset, **b'**). **c** A 5-HT cell in close proximity to a parietal cell (P), whose surface has indents. **d** A chain of 5-HT cells. **e** A 5-HT cell with processes oriented in different directions. **f** 5-HT cells with multiple processes. The cell that is marked (asterisk) has processes related to two gastric glands

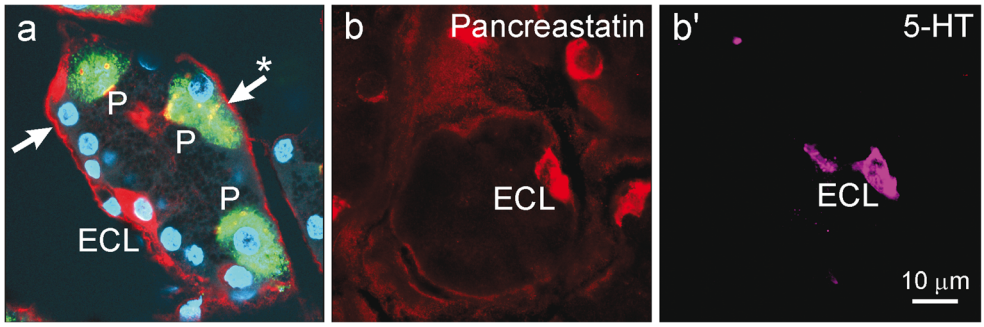

Fig. 10 Identification of ECL cells by their immunoreactivity for pancreastatin. **a** Anti-pancreastatin revealed elongated cells at the base of the epithelium of the glands. The ECL cell processes (arrows) were at the bases of the epithelial cells and came close to parietal cells (arrow with asterisk). **b**, **b'** Double labelling for pancreastatin (**b**) and 5-HT (**b'**). About 12% of pancreastatin cells were 5-HT positive. Pancreastatin-positive cells had typical ECL cell morphology and were distinguishable by shapes and positions from 5-HT-immunoreactive non-ECL cells. P parietal cell, revealed by H^+/K^+ ATPase

5-HT cell, in addition to the 5-HT-containing ECL cells. These were round, closed-type EECs and cells with multiple processes adjacent to the base of the mucosa.

Ghrelin cells

Gastric endocrine cells are the major source of circulating ghrelin (Kojima and Kangawa 2005). Most ghrelin cells in the human stomach were small and round, without contact with the lumen, a similar morphology to that described previously in the human and rat gastric corpus (Date et al. 2000; Dornonville De La Cour et al. 2001; Rindi et al. 2002). Many of these cells were between parietal cells and the basement membrane of the epithelium, in some cases indenting parietal cells with which they were in very close contact (Fig. 5b). Despite this close proximity of ghrelin cells to parietal cells, ghrelin has no direct effect on acid secretion (Dornonville de la Cour et al. 2004). High doses of ghrelin can increase acid secretion but this effect is indirect, through vagally mediated release of histamine, an effect that is prevented by vagotomy or histamine receptor block (Yakabi et al. 2006). Thus, there is doubt that the close association of ghrelin and parietal cells has functional meaning. As discussed below, the reason for the cell position may be to react to circulating factors but not to the gastric content.

Ghrelin levels in the circulation rise before a meal and decline after a meal. The decline is caused by food components, including glucose, fats and amino acids, reaching the small intestine (Williams et al. 2003; Overduin et al. 2005). Communication from the small intestine to the stomach could be mediated by intestinal hormones that are released by nutrients (Lippl et al. 2004). This is likely for glucose, because intraduodenal glucose but neither glucose in the stomach nor intravenous glucose, reduces ghrelin release (Williams et al. 2003; Steensels et al. 2016). In addition, nutrient receptors on ghrelin cells could be stimulated by nutrients that are absorbed in the intestine and pass via the circulation to the stomach. Consistent with this, ghrelin cells express fatty acid receptors (FFAR2 and FFAR4) whose activation inhibits ghrelin secretion (Engelstoft et al. 2013; Gong et al. 2013). On the other hand, a vagovagal reflex does not appear to be involved in the post-prandial nutrient-mediated suppression of ghrelin release (Veedfald et al. 2018). Thus, the regulation of ghrelin release by circulating factors is consistent with the round ghrelin cells being adjacent to the small blood vessels of the lamina propria but not in contact with the lumen.

Serotonin cells

5-HT-containing endocrine cells in the rat stomach are open cells, connecting to the lumen (Yu et al. 2001), whereas we found most cells to be of the closed type in the human stomach; being a mixture of closed round or ovoid cells, closed cells with processes and conical open cells. Kusumoto et al. (1988) also reported a mixture of open- and closed-type 5-HT cells in the oxyntic gland regions of the human stomach. Round or ovoid cells were commonly close to parietal cells and were seen in invaginations of the parietal cells, similar to the close relation of closed ghrelin cells and parietal cells. Processes of closed 5-HT cells also came close to parietal cells, as previously reported for human (Kusumoto et al. 1988). Some 5-HT cells had processes that spanned between glands; these might perhaps contribute to the coordination of gland activity. 5-HT inhibits acid secretion by a direct action in the stomach that is not nerve mediated (Canfield and Spencer 1983; Lepard et al. 1996). This could feasibly be exerted at the close connections between the 5-HT cells and parietal cells. Gastric 5-HT is released by acidification (Yu et al. 2001), suggesting that 5-HT may be involved in a negative feedback control of acid secretion.

However, numerous roles of gastrointestinal 5-HT have been demonstrated or proposed (Diwakarla et al. 2017; Martin et al. 2017) that could be either exerted in the stomach, or at a distance (5-HT is found in the venous drainage of the

stomach). One possible role is in the initiation of nausea and vomiting (Andrews et al. 1998). In the minutes and hours after their ingestion, cytotoxic drugs, such as cisplatin, cause nausea and vomiting that is prevented by 5-HT_3 receptor antagonists in human and in laboratory animals. The 5-HT_3 antagonist-sensitive responses are prevented or greatly reduced by bilateral vagotomy, leading to the theory that toxic compounds that are ingested trigger the release of 5-HT from the stomach and/or proximal intestine and that the 5-HT acts on 5-HT_3 receptors on vagal afferent endings (Andrews et al. 1990). It has been shown in the upper small intestine but has not been investigated in the stomach that nerve endings expressing 5-HT_3 receptors come close to the basal surfaces of 5-HT-containing EECs (Bellono et al. 2017). Thus, the open-type 5-HT cells in the stomach may recognise toxins and elicit expulsion of the toxic material through vagal reflexes.

Another type of gastric 5-HT cell is the subgroup of ECL cells that contains 5-HT (see below).

ECL cells

ECL cells were identified by immunoreactivity for pancreastatin, a secreted product of these cells (Håkanson et al. 1995). The cells were basally located and had fine cytoplasmic extensions, as reported for the rat stomach (Chen et al. 1998; Gustafsson et al. 2011). Pancreastatin cells with immunoreactivity for ghrelin or somatostatin have been reported in the rat stomach (Andersson et al. 1998). However, we found fewer than 3% of pancreastatin cells had ghrelin immunoreactivity and fewer than 2% were somatostatin immunoreactive. On the other hand, about 12% of pancreastatin cells were 5-HT immunoreactive. The ECL cells have amine-handling properties, including expression of aromatic amino acid decarboxylase (AADC) and the vesicular monoamine transporter 2 (Chen et al. 1998) and it is possible that they accumulate 5-HT or take up and convert 5-hydroxytryptophan from the environment. A physiological role of 5-HT as a hormone released from ECL cells seems unlikely, as the primary hormone of ECL cells, histamine, has a physiologically important role in stimulating acid production by parietal cells, whereas 5-HT inhibits release from activated parietal cells.

Somatostatin cells

It has been previously reported that somatostatin cells of the oxyntic mucosa in rat are of the closed type. However, they do have up to three processes and sometimes are in groups (Hauso et al. 2007), as we also found in human. Overall, somatostatin appears to be an inhibitor of other cell types. Somatostatin is inhibitory to the release of ghrelin from the stomach (Lippl et al. 2004), although processes of somatostatin cells only rarely come close to ghrelin cells. Also, somatostatin cells had processes close to parietal cells, as previously reported for rat (Larsson et al. 1979) and somatostatin released in proximity to the parietal cells inhibits acid production (Schubert et al. 1988). Moreover, somatostatin inhibits and anti-somatostatin antibodies augment histamine release from the fundus, indicating that it has an inhibitory role on ECL cells (Chuang et al. 1993; Vuyyuru et al. 1995, 1997). Thus, somatostatin cells are inhibitory to both acid and ghrelin release but whether separate groups of somatostatin cells exert these different functions is not known.

Conclusions: EEC relationships in the corpus and fundus

The chemically distinct ghrelin, 5-HT and somatostatin gastric endocrine cells and the ECL cells form distinct groups, in contrast to the extensive colocalisation of hormones in the EECs of the small intestine. The only sizeable overlap is that about 12% of ECL cells contain detectable 5-HT. All the cells, except a small number of 5-HT cells, were closed cells. Thus, the signals to these cells come from the circulation, the local extracellular environment, closely located EECs and nerve endings. They can signal to each other, to parietal and possibly chief cells and to afferent nerve endings.

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