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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 control of the stomach and to signalling to the brain. In other gut regions, enteroendocrine cells (EEC) exhibit extensive patterns of colocalisation of hormones. In the current study, we characterised EEC in the human gastric fundus and corpus. We utilised immunohistochemistry to investigate EEC with antibodies to ghrelin, 5-HT, somatostatin, PYY, GLP-1, calbindin, gastrin and pancreastatin, the latter as a marker of enterochromaffin like cells (ECL). EEC 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 EEC 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, that is, 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 3 types; small round closed cells, cells with multiple, often very long, processes, and a sub-group 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 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 (EEC). 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 EEC that express numerous different combinations of hormones, particularly the intestinal hormones CCK, 5-HT, GIP, GLP-1, neurotensin, 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 EEC.

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 cells, somatostatin cells, 5-HT (enterochromaffin) cells and enterochromaffin-like (ECL) cells that release histamine and pancreastatin. Whether gastric EEC 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 mouse stomach, 25% of gastric 5-HT cells were immunoreactive for somatostatin (Reynaud et al. 2016). In rat and human stomach, ghrelin cells are also nesfatin-1 immunoreactive (Stengel et al. 2010; Stengel et al. 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 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 toward the pylorus (Choi et al. 2014). Another difference is that serotonin (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 EEC 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 has 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 human stomach (Date et al. 2000). Colocalisation of hormones and the relations of EEC 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 have studied EEC 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 6 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 fundus to 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 3 times (10 min) with dimethyl sulfoxide (DMSO) and then 3 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 (SuperFrostPlus®; Gracle Scientific, Vic, 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 (5mM CuSO₄, 50mM 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 Bisbenzimid-Blue, diluted 10 µg/mL in dH₂O, to stain nuclei. Sections were then washed 3 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 to 100 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 and 100 µM. Immunoreactivity with rabbit anti-ghrelin RY1601 was reduced with 1 µM peptide and was abolished with 10 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 AxioImager microscope (Zeiss, Sydney, Australia) and a LSM 800 confocal microscope with Airyscan super-resolution analysis

(Zeiss). Tile scans taken with a 10x 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 3 patients. 40x and 63x 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, U.K.) was used and image rendering was applied where appropriate. To determine whether the ghrelin cells occur in clumps, three pieces of fundus from three different patients were analysed using the Delaunay triangulation protocol in ImageJ. Ghrelin cells were circled manually, and 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 $\pm$ SEM. Differences were evaluated using two-way ANOVA with Bonferroni post hoc test. $P < 0.05$ 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 which had a surface at the gastric gland lumen (a sub-group 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, 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 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 EEC cells varied significantly (2 way ANOVA). The densities of ghrelin cells were 109 ± 24 cells/mm² in fundus and 77 ± 13 cells/mm² in corpus. The densities of pancreastatin cells were similar; 102 ± 12 cells/mm² in fundus and 89 ± 14 cells/mm² in corpus ($P > 0.05$ vs ghrelin cells for both regions, Bonferroni post-hoc tests). However, 5HT cells (45 ± 6 cells/mm² in fundus and 25 ± 4 cells/mm² in corpus) and somatostatin cells (44 ± 5 cells/mm² in fundus, 24 ± 3 cells/mm² in 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 EEC. 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 $7 \pm 1\%$ 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, 2C).

Patterns of colocalisation

We used simultaneous labelling immunohistochemistry to investigate patterns of colocalisation in EEC (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, $25 \pm 5\%$ were immunoreactive for pancreastatin in the fundus, and $27 \pm 5\%$ in the corpus. Because of their greater numbers, a lower percentage of pancreastatin cells expressed 5HT ($12 \pm 3\%$ in fundus and $11 \pm 3\%$ in 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 co-localisation, 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 ($6 \pm 2\%$ of somatostatin cells were immunoreactive for ghrelin in the fundus, and $7 \pm 4\%$ of somatostatin 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 EEC in the oxyntic glands (Fig. 2) and ghrelin cells showed rare colocalisation with any other gastric hormone. The vast majority of ghrelin cells ($90.3 \pm 2.2\%$) 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 was collected from three sections of 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 $25.1 \pm 0.5 \mu\text{m}$ (mean $\pm$ SEM), (Fig. 6). Assuming a random distribution, in a two-dimensional (planar) section through the three-dimensional array, the theoretical mean nearest neighbour separation between cells is $48 \mu\text{m} \left(\frac{\sqrt{N_a}}{2} \right)$ with N_a = the measured density of ghrelin cells in a section of fundus (Bansal and Ardell, 1972). In our sample, N_a was 109 cells/mm^2 . Since the theoretical distance, $48 \mu\text{m}$, is well outside the 99% confidence interval (mean $\pm 3 \times \text{SEM}$) for the measured distance ($25.1 \pm 1.5 \mu\text{m}$), 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 EEC 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 EEC (Fig. 9A); and cells with multiple (2, 3 or more) processes, some with basal processes of varying length up to $70 \mu\text{m}$ (Fig. 9B, B', E, F). Sometimes 5-HT cells appeared to form a chain with processes of one 5HT 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 which 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, 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 2 neighbouring glands (Fig. 9F). Processes that came close to parietal cells were common, and closed cells that were partially engulfed by parietal cells in similar fashion to that seen for some ghrelin cells were also common (Fig. 9C). 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 morphology, whether or not they also contained 5-HT.

Somatostatin cells

Somatostatin cells were of the closed type. While the majority had processes (Fig. 1, 7B), round somatostatin cells could also be seen (Fig. 3C). Somatostatin cells had one observable process or a thick long process and 2 or 3 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. Somatostatin cells density 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 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 EEC 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 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.

Although pancreastatin is an effective ECL 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 are not good markers of ECL, 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 co-localisation of hormones that are seen in the small and large intestines. The only substantial group of cells with colocalised markers was a population of 5-HT/pancreastatin cells. These had ECL morphology, being elongated cells with long processes at the base of the mucosal epithelium, that appear to be a sub-population of ECL, 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 5-HT cell, in addition to the 5-HT containing ECL cells. These were round, closed type EEC 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 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 or 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 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 vago-vagal 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 have found most cells to be of the closed type in human stomach, there 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 co-ordination 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 feed-back 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-HT₃ receptor antagonists in human and in laboratory animals. The 5-HT₃ 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-HT₃ 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-HT₃ receptors come close to the basal surfaces of 5-HT containing EEC (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 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 seems unlikely, as the primary hormone of ECL, 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 did have up to three processes and sometimes were 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; Vuyyuru et al. 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 EEC of the small intestine. The only sizeable overlap is that about 12% of ECL 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 EEC and nerve endings. They can signal to each other, to parietal and possibly chief cells and to afferent nerve endings.

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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 panels C and D. Quantitative data is in Fig 2.

Fig. 2.

Numbers of immunoreactive gastric endocrine cells and their distributions across the width of the mucosa. A: Relative densities of occurrence of cells in fundus (F) and corpus (C). B: The distributions of dominant cells 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. * significantly different from both ghrelin and pancreastatin cell density in corresponding region (2-way ANOVA with Bonferroni post-hoc tests, $P < 0.01$).

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.

Fig. 4.

Quantitation of colocalisation in the fundus and corpus. For each group of 3 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).

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 recognize H/K ATPase in the surface membrane so the regions of the canaliculi are not revealed. B: Close association between a 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 Image J. The lines show the distances between cell centres of mass determined by the Image J 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 3 fundus sections from 3 different patients, with the actual mean and the mean predicted if the cells were randomly distributed indicated.

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.

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).

Fig. 9.

Different types of 5HT 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 it 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 (*) has processes related to 2 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 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.

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	#18F (Swant, Bellizona, Switzerland)
Calbindin	rabbit	1:1000 – 1:2000	#R202 (Furness et al, 1989)
Gastrin	Rabbit	1:2000	#8007 (gift from Dr Jens Rehfeld)
Gastrin-CCK	Mouse	1:2000	#28.2 (Kovacs et al. 1997)
Ghrelin	Chicken	1:800	#15861 (Pustovit et al. 2017)
Ghrelin	Rabbit	1:800	#RY1601 (Mizutani et al. 2009)
GLP-1	Rabbit	1:2000	#8912 (Cho et al. 2015)
H+/K+ ATPase	Mouse	1:300	#7.22 (Smolka and Weinstein 1986)
5-HT	Rabbit	1: 2000	#20080 Immunostar, Hudson, WI, USA
5-HT	Goat	1: 3000	#20079, Immunostar. (Cho et al. 2014)
Mast cell tryptase	Mouse	1:2000	MAB 1222, Chemicon, Boronia, Australia
PYY	Chicken	1:500	#GW22771, Sigma-Aldrich, Castle Hill, Australia
PYY/NPY	Sheep	1:400	E2210 (Furness et al. 1985)
Pancreastatin	Rabbit	1:400	#8942 CURE Antibody core, Los Angeles
Somatostatin	Mouse	1:1000	#S895 (Buchan et al. 1985)
Somatostatin	Sheep	1:2000	#AS01 (gift from Dr Arthur Shulkes)