

Distribution and characterisation of CCK containing enteroendocrine cells of the mouse small and large intestine

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## Abstract

There is general consensus that enteroendocrine cells, EEC, that contain the enteric hormone cholecystokinin (CCK) are confined to the small intestine and predominate in the duodenum and jejunum. Contrary to this, EEC that express the gene for CCK have been isolated from the large intestine of the mouse and there is evidence for EEC that contain CCK-like immunoreactivity in the mouse colon. However, the human and rat colons do not contain CCK cells. In the current study, we have used immunohistochemistry to investigate CCK peptide presence in endocrine cells, PCR to identify *cck* transcripts and chromatography to identify CCK peptide forms in the mouse small and large intestine. The colocalisation of CCK and 5-HT, hormones that have been hypothesized to derive from cells of different lineages, was also investigated. CCK immunoreactivity was found in EEC throughout the mouse small and large intestine, but positive cells were rare in the rectum. Immunoreactive EEC were as common in the cecum and proximal colon as they were in the duodenum and jejunum. CCK gene transcripts were found in the mucosa throughout the intestine, but mRNA for gastrin, a hormone that can bind some anti-CCK antibodies, was only found in the stomach and duodenum. Characterisation of CCK peptides of the colon by extraction, chromatographic separation and radioimmunoassay revealed bioactive amidated and sulphated forms, including CCK-8 and CCK-33. Moreover, CCK-containing EEC in the large intestine bound antibodies that target the biologically active sulphated form. Colocalisation of CCK and 5-HT occurred in a proportion of EEC throughout the small intestine and in the cecum, but these hormones were not colocalised in the colon, where there was CCK and PYY colocalisation. It is concluded that authentic, biologically active, CCK occurs in EEC of the mouse large intestine.

Keywords: cholecystokinin gastrointestinal hormones 5-hydroxytryptamine colon peptide  
YY

## Introduction

It has been widely reported, and is supported in textbooks and reviews, that CCK containing enteroendocrine cells (EEC) are restricted to the duodenum, jejunum and proximal ileum in the rat, human and other species (Larsson and Rehfeld 1978; Chao and Hellmich 2012; Gribble and Reimann 2015). In rat, the **C-terminal** tetrapeptide amide that is common to gastrin and CCK

occurs in the ileum, but no CCK immunoreactive EEC were found in the proximal part of the colon (Larsson and Rehfeld 1978). Moreover, biologically active CCK is absent from the rat distal colon, although small amounts of pro-CCK are detectable (Lüttichau et al. 1993). In human, CCK is prominent in the duodenum and jejunum, but is absent from the colon (Rehfeld 1978; Maton et al. 1984; Martins et al. 2017). A need for further investigation in mouse is highlighted by observations of Egerod et al (2012), who used an eGFP reporter coupled to the *Cck* promoter to locate sites of *Cck* expression and found an unexpectedly large population of *Cck*-eGFP-positive EEC throughout the intestine including the colon. In a similar study, glucagon gene expressing EEC from the mouse colon were shown to express *Cck* transcripts (Habib et al 2012). However, these gene expression studies have not identified whether the cells that were identified produce a CCK peptide product. Earlier, using immunohistochemistry, Roth et al (1992) reported CCK-like immunoreactivity in numerous EEC of the mouse proximal colon, but the immunoreactive product was not identified. The *Cck* gene is translated to pre-pro-CCK. The post-translational processing of this precursor produces a range of peptides, including CC-58, 39, 33, 25, 18, 8, 7 and 5, which may be sulphated or non-sulphated, or amidated or not amidated (Rehfeld 1978; Reeve 1986; Agersnap and Rehfeld 2015), depending on the post-translational machinery possessed by the cell (Rehfeld et al. 2008). Amidated CCK-8 sulphate is generally considered the biologically active form, the bioactive sequence being the heptapeptide sequence, -Tyr(SO<sub>4</sub>)-Met-Gly-Trp-Met-Asp-Phe-NH<sub>2</sub> (Rehfeld et al. 2007).

The well-established roles of CCK released from EEC are to facilitate digestion by stimulation of gallbladder contractions and pancreatic exocrine secretion, and to signal satiety by activating vagal afferent fibres (Owyang 1996; Beglinger and Degen 2004). Each of these functions is associated with CCK released from EEC of the small intestine in response to nutrients, particularly fats and proteins. CCK is described in most text books as the only hormone expressed by I cells in duodenum and jejunum. It is now clear that this is not so and that *Cck* can be expressed with the genes for many other gut hormones, including secretin, glucose-dependent insulintropic peptide (GIP), EEC peptides derived from preproglucagon, i.e., glucagon-like peptide-1 (GLP-1), GLP-2 and oxyntomodulin, neurotensin and peptide YY (PYY). In most cases colocalisation of CCK and other gut hormones has been confirmed by double labelling immunohistochemistry (Egerod et al. 2012; Habib et al. 2012; Sykaras et al. 2014).

These studies suggest that there is a major developmental lineage for enteroendocrine cells in the mouse that leads to cells that express CCK, GIP, GLP-1 and 2, PYY, neurotensin and secretin.

It has been proposed that other, separate, lineages lead to (i) EEC that express 5-HT and substance P, (ii) EEC that express ghrelin and in some species motilin, and (iii) EEC that express somatostatin (Roth et al. 1992). However, 5-HT and CCK, that are hypothesised to be in cells of different lineages, have been reported to be colocalised in the mouse small intestine (Cho et al. 2014; Reynaud et al. 2016). About 60% of the CCK cells were also 5-HT immunoreactive (Cho et al. 2014). In the mouse proximal colon, Roth et al (1992) reported that fewer than 1% of 5-HT immunopositive cells contained CCK.

CCK is sulphated in its C-terminal, which is necessary for its biological activity in modifying digestive function and satiety, but it also occurs as the non-sulphated peptide (Rehfeld et al. 2007; Agersnap and Rehfeld 2014). It also has similarity to gastrin, with which it shares a C-terminal pentapeptide amide (Larsson and Rehfeld 1977). Thus, depending on the antibodies used, CCK-like immunoreactivity could be due to the presence of non-sulphated CCK, gastrin or gastrin gene derived peptides. In the present work we have used antibodies specific to the sulphated and non-sulphated forms of CCK to determine the localisation of CCK in EEC throughout the mouse gut. We have investigated the expression of CCK and gastrin genes in the mucosa throughout the digestive tract and have isolated and characterised immunoreactive CCK. We have also investigated patterns of co-localisation of CCK with 5-HT throughout the intestines, and with PYY in the colon.

## **Materials and methods**

### **Animals and tissue preparation**

Experiments were conducted according to the National Health and Medical Research Council of Australia guidelines and approved by the University of Melbourne Animal Experimentation Ethics Committee. Adult C57BL/6 male mice were housed in the Biomedical Animal Facility at the University of Melbourne and were provided standard chow and water ad libitum. Mice (8-10 weeks, 20-25 g) were anesthetized with isoflurane and then killed by cervical dislocation.

Segments of gastric corpus and antrum, the descending part of the duodenum, first 4 cm of the jejunum starting from the root of the mesentery, 4 cm of proximal ileum 4 cm away from jejunum, 4 cm of distal ileum 2 cm away from the cecum, cecum, middle of the proximal colon, first and second half of the distal colon (called distal colon A and B respectively) between the colonic flexure and the pelvic brim, and the rectum within the pelvis were removed. For

immunohistochemistry, the segments were opened along the mesenteric attachment, cleaned of contents and pinned unstretched, with mucosa up, to balsa wood sheets in ice-cold phosphate-buffered saline (PBS: 0.15 M NaCl in 0.01 M sodium phosphate buffer, pH 7.2). The tissue was then placed in fixative (2 % formaldehyde plus 0.2 % picric acid in 0.1 M sodium phosphate buffer, pH 7.2) overnight at 4 °C. The following day, tissues were washed 3 times (10 min) with dimethyl sulfoxide (DMSO) and then 3 times (10 min) with PBS then transferred to PBS-sucrose-azide (PBS containing 0.1 % sodium azide and 30 % sucrose as a cryoprotectant) and stored at 4 °C. The next day, the tissue samples were placed in 50% PBS-sucrose-azide and 50% OCT mixture (Tissue Tek, Elkhart, IN, USA) for a further 24 h, before being trimmed and 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®; Menzel-Glaser; from Thermo Fisher, Scoresby, Vic, Australia) and incubated with 10 % normal horse serum for 30 min. Mixtures of primary antibodies (Table 1) for double staining were then added, and sections were left at 4 °C overnight. The tissue was washed three times in PBS and incubated in secondary antibody (Table 2) for 1 h at room temperature. To stain the nuclei with Hoechst 33258 solution (Bisbenzimidazole-Blue, diluted to 10 µg/mL in dH<sub>2</sub>O), preparations were washed once with PBS then twice with distilled water and then incubated for 5 min with the stain. Sections were then washed 3 times with distilled water before mounting with non-fluorescent mounting medium (Dako, Carpinteria, CA, USA).

### **Image analysis**

After staining, slides were kept overnight then examined using an AxioImager microscope (Zeiss, Sydney, Australia). For quantitation purposes, both a V-slide fluorescent slide scanner (Zeiss) and an LSM 800 confocal microscope with Airyscan (Zeiss) were used to capture images. These images were exported to be analysed off-line using ImageJ ([imagej.nih.gov/ij/](http://imagej.nih.gov/ij/)). Immunoreactive cells were quantified by counting around a hundred cells from each part of the gut. A higher number was counted when needed statistically. This analysis was repeated in 3 animals. The Airyscan microscope was used to capture high resolution images that were exported into CorelDraw (Corel, Ottawa, Canada) for final preparation of figures.

## RNA isolation and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from freshly dissected mucosal scrapings of the mouse stomach, duodenum, jejunum, ileum, proximal colon, distal colon A, distal colon B, and rectum from 3 mice using the RNeasy Mini Kit (Qiagen, Melbourne, Australia). 1 µg of total RNA from each sample was reverse transcribed using iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Gladesville, NSW, Australia) according to the manufacturer guidelines. cDNA was amplified by PCR using 5PRIME MasterMix (5PRIME, Hamburg, Germany) with appropriate intron-spanning primers. Forward and reverse PCR primer sequences were respectively: for CCK, 5'-AGCGGCGTATGTCTGTGCGT-3' and 5'-CACTGCGCCGGCCAAAATCC-3' to produce a 316-bp product (Gonzalez-Coto et al. 2014); for gastrin, 5'-AGCTGACCCAGCGCCACAAC-3' and 5'-ACTGCGGCGGCCAAAGTCCA-3' to produce a 338-bp product (Gonzalez-Coto et al. 2014); and for β-actin (which was used as a control for cDNA quality), 5'-GCTCGTCGTCGACAACGGCTC-3' and 5'-CAAACATGATCTGGGTCATCTTCTC-3' to produce a 353-bp product. The CCK and gastrin primers were from Invitrogen (Australia) and the β-actin primers were provided with the Superscript III First-Strand Synthesis System for RT-PCR (Invitrogen, Australia). All amplifications were performed with an initial step at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 61°C for 30 s, extension at 72°C, with a final extension at 72°C for 5 min. Reactions were also conducted without reverse transcriptase to confirm the absence of genomic DNA. Amplification products were visualised by electrophoresis on a 1.5% agarose gel containing SYBR Safe DNA Gel Stain (Thermo-Fisher Scientific, Australia). The gels were imaged with the Gel Doc EZ Gel Documentation System (Bio-Rad Laboratories, Gladesville, NSW, Australia). The PCR gene product for CCK was sequenced at the Centre for Translational Pathology (Department of Pathology, The University of Melbourne, Australia).

## Extraction and chromatographic characterisation of CCK

The jejunum and distal colon were taken from six male C57BL/6 mice for extraction and characterisation of CCK immunoreactive peptides. **The distal colon was chosen, rather than the proximal colon, because feces are more readily removed from the distal colon.** The mucosa was immediately stripped free from the intestinal wall and specimens were frozen on dry ice and stored at -80°C. Samples were immersed in liquid nitrogen and pulverized by a cryoPREP impactor. Pulverized samples were boiled for 20 min in 10 mL/g distilled water and homogenized. The extracts were centrifuged at 10,000 rpm and the supernatant was collected as the “neutral extract”. The pellet was then resuspended in 0.5 M acetic acid (10 mL/g), homogenized, and incubated for 20

min at room temperature. It was then centrifuged at 10,000 rpm for 30 min at 4 °C and the supernatant was collected as the “acid extract”.

The CCK peptides in the extracts were measured using two sequence-specific assays as previously detailed (Agersnap and Rehfeld 2015). CCK-antiserum # 92128 (Table 1) binds the  $\alpha$ -amidated C-terminus of only sulphated CCK peptides. This antiserum does not react with either sulphated or nonsulphated gastrins. CCK-antiserum # 94179 was used to detect nonsulphated CCK. It binds the N-terminal pentapeptide sequence of nonsulphated CCK-8.

The tissue extracts were applied to Sephadex G50 superfine columns to separate the molecular forms of CCK according to size. The samples were applied to calibrated columns and eluted with 0.02 mol/L barbital buffer, pH 8.4, containing 1 g/L bovine albumin and 0.1 M NaCl at a flow rate of 4 mL/h. The fractions were collected and the concentration of CCK measured by radioimmunoassay. The total concentration of sulphated and nonsulphated CCK in each tissue was measured by RIA in each of the neutral and acidic extracts separately. AUC calculations were performed on chromatograms for sulphated and nonsulphated CCKs, respectively, based on the known elution of the individual CCK forms in the calibrated columns. The estimation of the concentration of each molecular form was performed for the neutral and acidic extracts separately.

## 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 with 2-tailed Student's t tests and by ANOVA.

## Results

The immunohistochemical localisation of CCK was investigated throughout the intestinal tract from the duodenum to the rectum (Fig. 1), using an anti-CCK antibody, R183B, that recognises both CCK and gastrin (Cho et al. 2014). We also used two peptide specific antibodies, #92128 (Rehfeld 1998) that recognises sulphated CCK, but not gastrin, and # 94179 (Agersnap and Rehfeld 2014) that recognises the non-sulphated form of CCK. The distributions of CCK-immunoreactive cells observed with anti-CCK R183B and anti-sulphated CCK antibodies (#92128) were virtually the same; this is shown quantitatively for the large intestine in Fig. 2. An antibody against the non-sulphated form of CCK (# 94179) did not reveal EEC in the large intestine. Scattered cells that



were immunoreactive for non-sulphated CCK cells were observed in the duodenum and jejunum.

In the small intestine, the numbers of EEC that expressed CCK (revealed by antibody R183B) increased from  $19.9 \pm 3.1$  cells/mm<sup>2</sup> in the duodenum to  $39.1 \pm 5.7$  cells/mm<sup>2</sup> in the proximal ileum, n=3 mice) then decreased starting in the distal ileum ( $26.5 \pm 4.3$  cells/mm<sup>2</sup>, n=3 mice). **The cell numbers in both the duodenum and jejunum were significantly different to the numbers in the proximal ileum ( $P<0.05$ ).** The decrease continued gradually in the large intestine to reach extremely rare cells in the rectum ( $14.1 \pm 2.3$  cells/mm<sup>2</sup> in proximal colon to  $3.1 \pm 0.6$  cells/mm<sup>2</sup> in distal colon A, n=3 mice). **The decrease along the large intestine was significant (1 way ANOVA,  $P<0.05$ ).**

### **Colocalisation of CCK and 5-HT in the small and large intestine**

Investigation of the colocalisation of CCK and 5-HT showed colocalisation throughout the small intestine and in the cecum. The greatest proportion of CCK cells that also contained 5-HT was in the cecum (75% of CCK cells in the cecum coexpressed 5-HT), while in the proximal ileum, where the greatest number of CCK immunoreactive EEC was found, 40% of CCK cells expressed 5-HT. EEC that contained both CCK and 5-HT were not found in the colon, although cells with each hormone alone were found (Figs 3, 4).

### **Colocalisation of CCK and PYY in the large intestine**

As described above, in contrast to the small intestine and cecum, CCK and 5-HT are in separate EEC in the colon. **The most numerous EEC type (other than 5-HT cells) in the mouse colon are PYY/GLP-1 cells (Cho et al. 2015).** We therefore investigated the overlap between CCK and PYY immunoreactivity, which we chose to do in the proximal colon where CCK cells are numerous. In double-stained sections from the proximal colon, analysed in 3.2  $\mu$ m Z-stacks from each of the 3 mice, about 65% of PYY cells were also immunoreactive for CCK (Fig. 5).

### **CCK and gastrin mRNA expression, *Cck* and *Gast*, detected by RT-PCR**

CCK mRNA was detected by RT-PCR using intron-spanning primers in mucosal extracts from the stomach, duodenum, jejunum, ileum, proximal colon, proximal region of the distal colon (distal



colon part A), distal region of the distal colon (distal colon part B), and rectum (Fig 6). Bands for *Cck* transcripts had the predicted molecular sizes of 316 bp as previously reported (Gonzalez-Coto et al. 2014). *Cck* expression was very weakly detected in the mucosal extract from one mouse stomach and was not detected in the other 2 mouse stomach samples. Bands were not observed in control reactions with the omission of reverse transcriptase and in the absence of template cDNA. Quality of RNA extracted was validated with the detection of  $\beta$ -actin in all mouse mucosal samples. RT-PCR *Cck* products were confirmed to have the correct structure by sequencing samples from the duodenum and distal colon part B.

RT-PCR for the gastrin transcript was strong in the stomach and weak in the duodenum, but was not detected in any other region, indicating that the EEC located in all regions, with the possible exception of the duodenum, expressed *Cck*, not *Gast*.

### Characterisation of CCK forms

Bioactive (amidated and sulphated) CCK was found in both the colon and jejunal extracts, but at lower concentrations in colon extracts,  $2.9 \pm 0.9$  pmol/g, than in jejunal extracts,  $55.9 \pm 11.2$  (n = 6; Table 3). This difference in concentration can be related in part to differences in cell numbers, 3.5 per mm<sup>2</sup> in the mucosa of the distal colon compared to 20 per mm<sup>2</sup> in the jejunal mucosa (Fig. 1). Non-amidated (gly-extended) CCK and pro-CCK were also found in both regions. Acid extracts from the colon revealed a major extended CCK, CCK-33, plus peaks corresponding to CCK-58 and CCK-12. Neutral extraction revealed that the dominant sulphated form was CCK-8 (Fig. 7). Chromatographic characterization of jejunal CCK revealed three major peaks, corresponding to CCK-33, CCK-22 and CCK-8, and a small amount of CCK-58.

### Discussion

This study shows that CCK, revealed by two antisera, one of which is specific for sulphated CCK and the other having a broader binding characteristics, occurs in enteroendocrine cells throughout the mouse intestine, including the colon, where the literature suggests that CCK does not occur in mammals. Gene transcripts for CCK, but not for gastrin, were found in the mucosa of the colon. Gastrin transcripts were only detectable in the stomach and, weakly, in the duodenum. Peptide extraction from the colon revealed the presence of CCK, primarily its sulphated forms. This extracted CCK is deduced to come from EEC, as these are the only CCK-immunoreactive cell

type in the colon, CCK-immunoreactive neurons and nerve fibres being absent in mature mouse small intestine and colon (Lay et al. 1999; Rehfeld et al. 2008, present study). The presence of CCK in colonic EEC in the mouse is consistent with studies using a *Cck*-reporter mouse (Egerod et al. 2012), and contrasts with human, where CCK cells could not be found in the sigmoid colon (Martins et al. 2017) and adult rat, where CCK is also absent (Larsson and Rehfeld 1978; Lüttichau et al. 1993). There were no differences in the distributions in the mouse colon determined by the two antibodies that recognise sulphated CCK. Cells were not observed in the large intestine with an anti-non-sulphated CCK antibody (which did show a small number of EEC in the duodenum and jejunum). The results indicate that biologically active sulphated CCK occurs in **mouse colonic EEC**.

As noted in the introduction, the well-established roles of CCK released from EEC are to facilitate nutrient digestion by contracting the gallbladder to release bile into the intestine, to stimulate the release of digestive enzymes from the pancreas, and to signal satiety by activating vagal afferent fibres (Owyang 1996; Beglinger and Degen 2004). All of these signals arise from the stimulation of receptors on EEC by nutrients in the luminal contents. The nutrient receptors on CCK-secreting cells in the small intestine include receptors for fats, amino acids and bitter compounds (Raybould 1999; Chen et al. 2006; Edfalk et al. 2008; Wang et al. 2011; Daly et al. 2013). The highest abundance of intestinal CCK cells was in the mouse ileum (Fig. 1). CCK released from this part of the intestine may signal satiety in response to nutrients that are not digested and absorbed in the duodenum or jejunum.

The colocalisation of 5-HT in a proportion of CCK cells in the small intestine suggests that **the two hormones** may interact in signalling from this region. 5-HT and CCK have synergistic effects to increase pancreatic enzyme secretion, a function that is linked to release of CCK from the proximal small intestine (Owyang 1996). In conscious rats, nutrient infusion into the small intestine elicited increases in pancreatic enzyme secretion that were reduced by 54% when CCK receptors were blocked and 92% when both CCK and 5-HT receptors were blocked (Li et al. 2000). CCK and 5-HT also act synergistically to suppress food intake (Voigt et al. 1995; Hayes and Covasa 2005). The CCK-mediated satiety effect is caused by nutrients stimulating CCK release from EEC in the proximal small intestine and CCK activating vagal afferent nerve endings (Raybould 2007). Thus it is relevant that combined CCK and 5-HT receptor block causes a greater reversal of suppression of eating in response to intraduodenal nutrient than either antagonist alone (Savastano and Covasa 2007). **5-HT and CCK may also have synergistic actions in the control of segmentation**

in the small intestine (Ellis et al. 2013). These authors found that segmentation that was elicited by fat in the lumen fat was mimicked by inhibiting 5-HT uptake (thus increasing 5-HT levels) and also by CCK. Furthermore, the increase in segmentation caused by fat was inhibited by both CCK and 5-HT receptor antagonism.

In the large intestine, we found that a high proportion of PYY cells also contained CCK, whereas 5-HT cells were not CCK immunoreactive, consistent with earlier observations (Roth et al. 1992). There may be a synergism between CCK and PYY released from the colon, both being satiety factors. PYY is released when short chain fatty acids, oleic acid, glucose or an amino acid mix are instilled into the colon of the rat (Fu-Cheng et al. 1995). In humans, intrarectal administration of sodium acetate, but not intravenous acetate, caused a significant increase in plasma PYY (Freeland and Wolever 2010) and in mouse, intracolonic propionate also caused PYY release (Psichas et al. 2015). A link between release of PYY from colonic EEC and satiety has been demonstrated in humans (Chambers et al. 2015). These authors utilised an inulin-propionate ester to deliver propionate to the colon of human volunteers. Studies of inulin-propionate ester metabolism and plasma propionate after a meal indicated effective delivery to the colon. Inulin-propionate released PYY, whose concentration in plasma was increased 6 fold, 60 min after the propionate was estimated to reach the colon. Acute administration of inulin-propionate reduced appetite and inclusion of 10 g/day in food for 24 days significantly reduced weight gain (Chambers et al. 2015). Thus the literature suggests that CCK and PYY released from colonic EEC in mouse may have synergistic effects on food intake, but this has yet to be tested experimentally. It is also possible they interact to affect local colonic functions, such as contractions of smooth muscle or epithelial water and electrolyte transport. For example, CCK affects smooth muscle of the colon, contracting the circular muscle in the cat (Mangel 1984) and dog (Huizinga et al. 1984) and relaxing it in the rat (Kishimoto et al. 1994). PYY decreases water and electrolyte transport in the mouse and human colon (Cox and Tough 2002; Tough et al. 2011), but the effects of CCK do not appear to have been investigated. Actions of CCK released from colonic EEC to control the gall-bladder or pancreatic function do not have apparent physiological rationales. Clearly further research is required to deduce the range of possible roles of CCK produced by EEC in the large intestine of mouse.

This study adds to the evidence that the distributions and chemical attributes of EEC differ considerably between species. Other examples are the presence of motilin in EEC and its physiological roles in human, rabbit, dog and pig, and its absence from rat, mouse and guinea-pig

(Smith et al. 1981; Itoh 1997; Sanger and Furness 2016) and the prominence of PYY containing EEC in the duodenum of pig and its absence or rarity in the duodenum of other species (Cho et al. 2015).

In conclusion, we have demonstrated that a subpopulation of colonic EEC in mouse synthesise and store CCK in a biologically active form. In the small intestine, there is colocalisation of 5-HT in a proportion of CCK cells, but this does not occur in the colon. In the proximal colon, about 65% of PYY cells contain CCK. It is concluded that CCK cells of different regions may have different functions, and that in the colon PYY and CCK may synergise to regulate food intake.

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### Figure descriptions

**Fig. 1** Distribution of CCK immunoreactive enteroendocrine cells, per mm<sup>2</sup> of mucosa, along the mouse small and large intestines. Duod = duodenum; Jej = jejunum; P il = proximal ileum; D il = distal ileum; Cec = cecum; PC = proximal colon; DC(A) = the first half of the distal colon; DC(B) = the second half of the distal colon; Rect = rectum.

**Fig. 2** Comparison of numbers of EEC in the colon and rectum revealed by antibodies R183B (pan CCK-gastrin antibody) and 92128 (anti-sulphated CCK-8). Zeros indicate that EEC were not seen with either antibody in the rectum.

**Fig. 3** Double staining for CCK and 5-HT. Examples of EEC in which CCK and 5-HT are separately expressed (EEC with arrows) and in which both hormones are expressed (EEC with asterisks). Each set of panels (**a, a', a''** etc) **show the same field** with CCK, 5-HT and the merged images illustrated. Scale bar: 10 µm.

**Fig. 4.** Patterns of colocalisation of CCK with 5-HT in EEC of the small and large intestine. There was no colocalisation of CCK with 5-HT in the large intestine and there were no CCK cells in the rectum. **Data is the mean ± SEM, from 3 mice, with > 100 cells per mouse for each region.**

**Fig. 5:** Colocalisation of CCK in PYY cells of the mouse proximal colon. Examples of cells in which PYY and CCK were colocalised (**a, a', a''**) and in which only PYY was located (**b, b', b''**) are illustrated. Scale bar: 10 µm. About 65% of PYY containing EEC were also CCK

immunoreactive (c). Data is mean  $\pm$  SEM, cells analysed from 3 mice.

**Fig 6:** Reverse transcriptase-polymerase chain reaction (RT-PCR) gel showing the predicted product size of 316 base-pairs (bp) of CCK in mucosal extracts from the mouse stomach, duodenum, jejunum, proximal colon, proximal region of the distal colon (distal colon part A), distal region of the distal colon (distal colon part B), and rectum (upper panel). The predicted product size of 338 base-pairs (bp) of gastrin occurred in extracts from stomach and duodenum, but not other regions (lower panel). Results in the presence (+) and in the absence (-) of reverse transcriptase (RT) are shown. Duod, duodenum; Jejun, jejunum; P colon, proximal colon; D col A, distal colon part A; D col B, distal colon part B; NTC no template control.

**Fig 7:** Elution profile of CCK immunoreactive peptides (amidated and sulphated), extracted from the distal colon, after gel chromatographic separation, measured with the specific assay for amidated, sulphated CCK (using antibody #92128). The upper panel (A) is CCK in the acetic acid extracts. The first two peaks correspond to CCK-58 and -33 (the major peak). The third peak includes CCK-12. The lower panel is CCK in the neutral tissue extract, which is mainly CCK-8, and perhaps a little CCK-12.

## Tables

**Table 1** List of primary antibodies used and their respective dilutions.

Target	Host species	Dilution	Antibody code, source and/or reference
CCK	Rabbit	1:2000 to 1:1000	R183B(Cho et al. 2014)
CCK, sulphated	Rabbit	1 : 5000	#92128 (Rehfeld 1998)
CCK, non-sulphated	Rabbit	1 : 3000	# 94179 (Agersnap and Rehfeld 2014)
5-HT	Goat	1 : 10000	#20079; Incstar, Hudson, WI, USA (Cho et al. 2014)
PYY	Chicken	1:500	#GW22771, Sigma (Cho et al. 2015)

**Table 2** List of secondary antibodies, their dilutions and sources

Antibody or stain	Associated fluorochrome	Dilution	Source
Donkey anti-rabbit	Alexa Fluor® 594	1:1000	Molecular Probes, Mulgrave, Vic, Australia
Donkey anti-chicken	Alexa Fluor® 488	1:500	Molecular Probes
Donkey anti-sheep	Alexa Fluor® 488	1:500	Molecular probes
Donkey anti-rabbit	Alexa Fluor® 568	1:1000	Molecular Probes

**Table 3** CCK and its precursors in the mouse intestine (pmol/g tissue, n=6).

Region	Amidated + sulphated CCK	Gly-extended CCK	Pro-CCK
Jejunum	55.9 ± 11.2	4.9 ± 1.1	2.6 ± 1.8
Colon	2.9 ± 0.9	1.1 ± 0.3	2.1 ± 0.9

## References

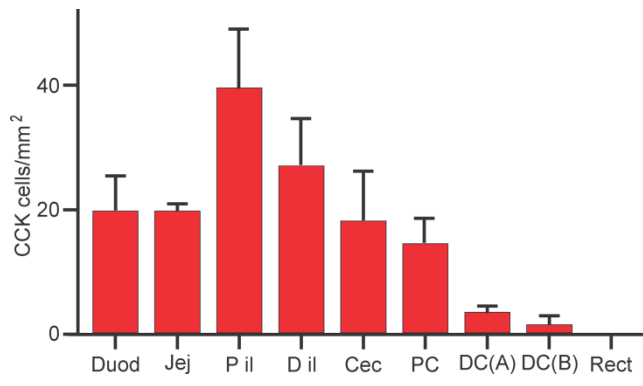
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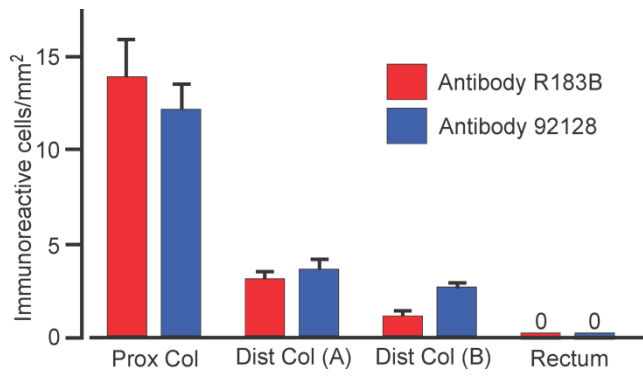
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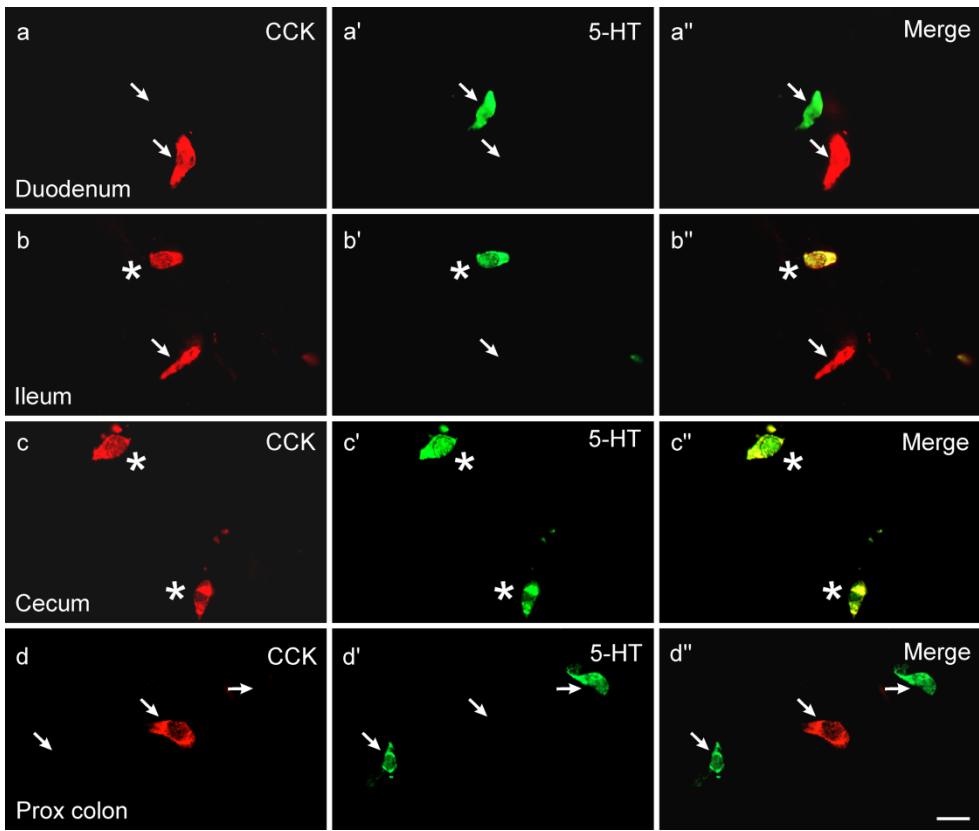


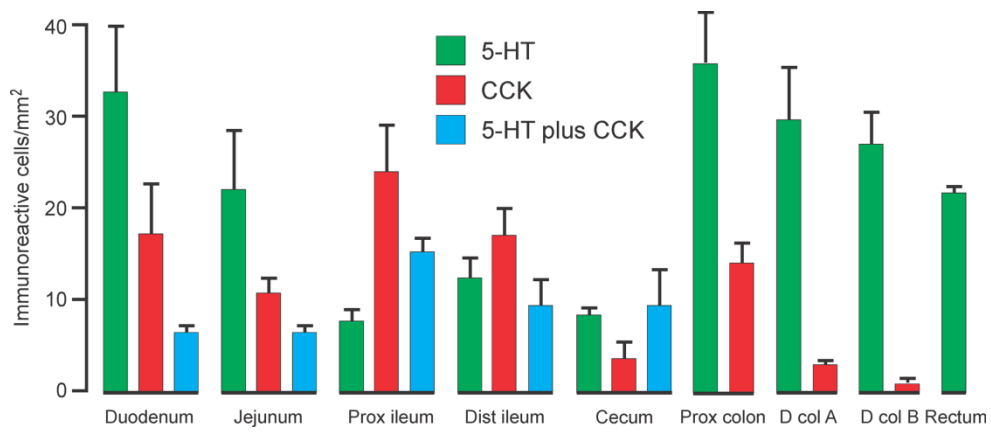
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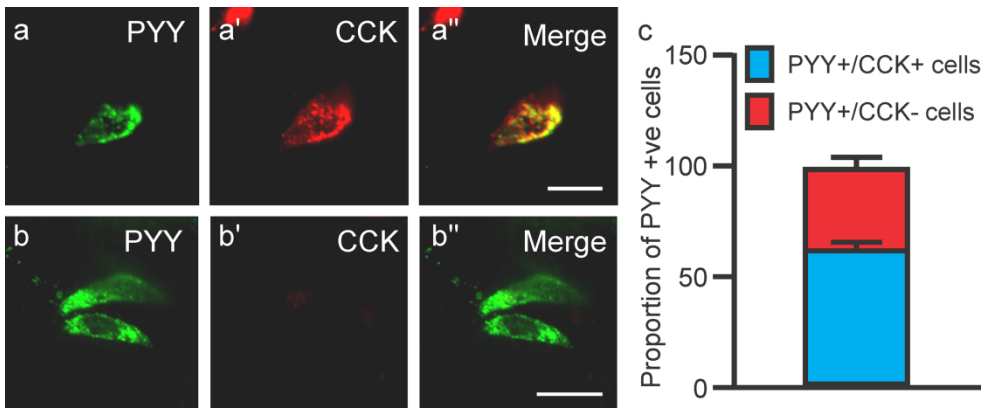
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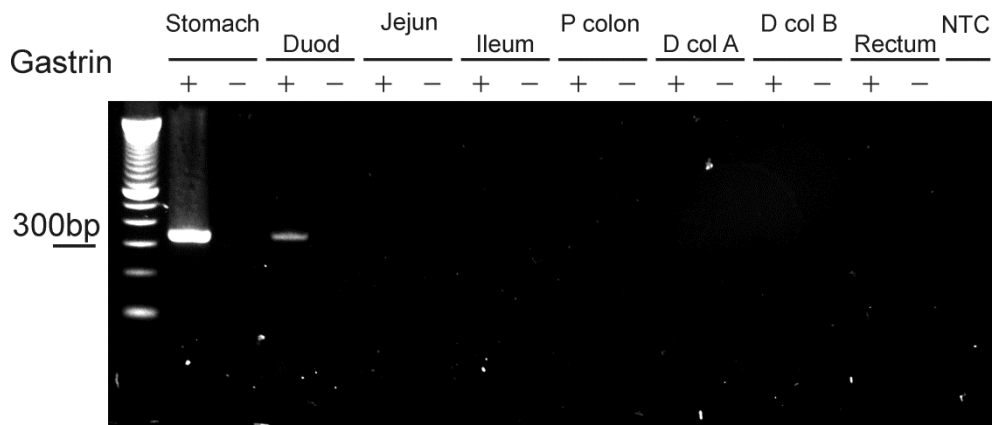
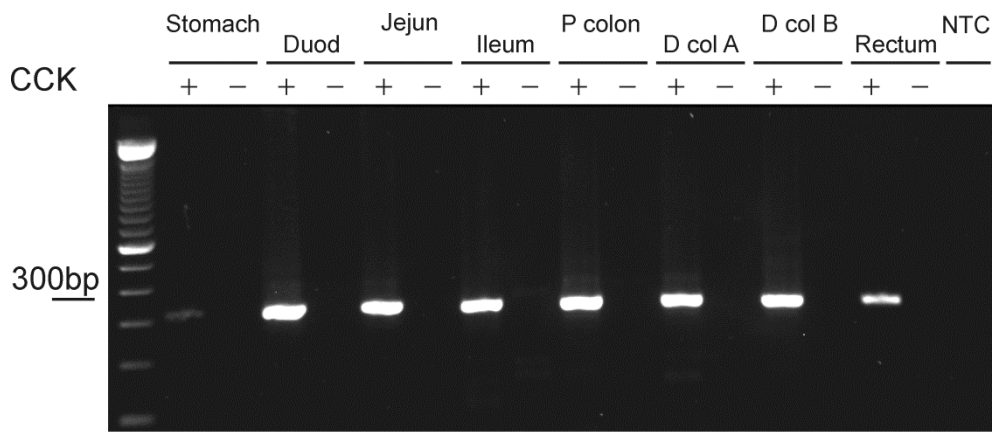


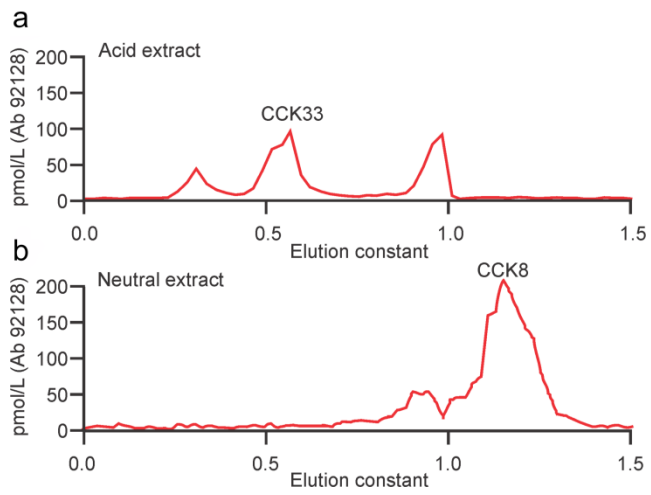














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